Bloodstream infections and antimicrobial resistance in Zanzibar, Tanzania, with special focus on typhoid fever and malaria

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Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

This research was performed in a collaboration between Mnazi Mmoja Hospital (MMH), Zanzibar, Tanzania, the University of Bergen, and the Norwegian National Centre for Tropical Infectious Diseases, Department of Medicine, Haukeland University Hospital. In addition, Pål Arne Jenum, Vestre Viken Hospital Trust (VVHT) and University of Oslo, and several colleagues from Vestre Viken Hospital Trust, and Fredrik Müller, University of Oslo, contributed.

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Nimeshikwa na ndwele ya homa, leo nenda na ulele-ngoma.

(I've a fever, I'll dance with the dead.)

Kiswahili proverb.

Summary

Background: Infectious diseases contribute significantly to the global burden of diseases, with low-income countries disproportionately affected. In life-threatening infections such as bacterial bloodstream infections (BSIs), rapid treatment with appropriate drugs can save lives. Use, overuse and misuse of antibiotics have led to a worrying global increase of antimicrobial resistance (AMR), threatening the successful treatment of serious infections globally, particularly in low-resource areas. The insufficient information available on both etiology of BSIs and prevalence of AMR in Sub-Saharan Africa, including Zanzibar, puts patients at risk of inadequate treatment and death.

Aims: This study aimed at investigating the etiology of BSIs in Zanzibar, Tanzania, and the AMR patterns of the bacteria detected, to guide clinical management.

Methods: In two prospective observational cross-sectional studies of hospitalized patients suspected to have BSIs (2012-13 and 2015-16), clinical data were collected, and blood obtained for culture, identification and susceptibility testing. *Salmonella* Typhi isolates were whole genome sequenced. Malaria rapid diagnostic test (RDT), microscopy, and polymerase chain reaction (PCR) was done in 2015-16.

Results: In 2012/2013 (469 patients, paper I), we found *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter* species and *Staphylococcus aureus* to be the most frequent bacteria causing BSIs. For the first time, extended-spectrum beta-lactamase (ESBL) producing bacteria were detected in BSI in Zanzibar, both in community- and in hospital-acquired infections. In 2015/2016, *Salmonella* Typhi was the leading cause of bacterial BSIs (61 of 1037 patients, paper II), with a high prevalence of *S.* Typhi with multidrug resistance and low-level ciprofloxacin resistance, and all isolates belonging to the 4.3.1 genotype which dominates in East Africa. Malaria was found in 9% (63/731) of the patients (paper III). In comparison to PCR, the sensitivity of the malaria RDT and microscopy was 64% (36/56) and 50% (18/36), and the specificity 98% (561/575) and 99% (251/254), respectively.

Conclusions: The high proportion of multidrug-resistant (MDR) *S.* Typhi with low-level ciprofloxacin-resistance causing BSI in Zanzibar limits treatment options. The detection ESBL-producing bacteria in community-acquired BSI is worrying, as these difficult-to-treat microbes can be practically incurable in the local situation. RDTs and microscopy are useful tools in the routine diagnostic of malaria, however, negative results must be evaluated with caution.

The study highlights the need of further surveillance of BSIs and AMR and of establishing strategies to fight the further spread of AMR. The study results can inform local treatment guidelines and imply the need of prevention measures against typhoid fever including vaccination, and for maintenance and strengthening of the malaria control program in Zanzibar.

Sammendrag

Bakgrunn: Globalt bidrar infeksjonssykdommer signifikant til sykdom og død, og lavinntektsland er uforholdsmessig rammet. Ved livstruende infeksjoner som blodbaneinfeksjoner kan rask behandling med adekvate legemidler redde liv. Bruk, overforbruk og feil bruk av antibiotika har ført til en bekymringsfull global økning av antibiotikaresistens som truer behandlingsmulighetene for alvorlige infeksjoner globalt, særlig i ressurssvake områder. Informasjonen som er tilgjengelig om både etiologi av blodbaneinfeksjoner og antibiotikaresistens i Afrika sør for Sahara, inkludert Zanzibar, er utilstrekkelig og setter pasienter i fare for mangelfull behandling og død.

Mål: Denne studien tok sikte på å undersøke både etiologi ved blodbaneinfeksjoner i Zanzibar, Tanzania, og resistensforholdene til de påviste bakteriene for å bidra til den kliniske håndteringen av pasientene.

Metoder: Kliniske og mikrobiologiske data ble samlet inn i prospektive observasjonelle tversnittsstudier av hospitaliserte pasienter mistenkt for blodbaneinfeksjoner. Dette inkluderte blod for dyrkning, identifikasjon og resistensbestemmelse av de isolerte mikrobene. *Salmonella* Typhi stammer ble helgenomsekvensert i to perioder (2012/2013 og 2015/2016). Malaria ble diagnostisert ved hjelp av hurtigtest (rapid diagnostic test, RDT), mikroskopi og polymerasekjedereaksjon (PCR) i 2015/2016.

Resultater: I 2012/2013 (469 pasienter, artikkel I), fant vi at *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter* species og *Staphylococcus aureus* var de bakteriene som oftest forårsaket blodbaneinfeksjoner. For første gang ble betalaktamase med utvidet spektrum (extended-spectrum beta-lactamase, ESBL) produserende bakterier oppdaget i Zanzibar, både ved infeksjoner som har oppstått utenfor sykehuset og i sykehuservervete infeksjoner. I 2015/2016 var *Salmonella* Typhi den hyppigste årsaken til bakterielle blodbaneinfeksjoner (61 av 1037 pasienter, artikkel II), med høy prevalens av multiresistente *S.* Typhi med lavgradig resistens for ciprofloxacin, og alle isolater tilhørende 4.3.1 genotypen som dominerer i Øst-Afrika. Malaria ble funnet hos 9% (63/731) av pasientene (artikkel III). Sammenlignet med PCR var sensitiviteten av

malaria hurtigtest og mikroskopi henholdsvis 64% (36/56) og 50% (18/36), og spesifisiteten 98% (561/575) og 99% (251/254).

Konklusjon: Den høye andelen av multiresistente *S.* Typhi med lavgradig ciprofloksacinresistens som forårsaker blodbaneinfeksjoner i Zanzibar medfører at det er få behandlingsalternativer igjen. Funn av ESBL-produserende bakterier i blodbaneinfeksjoner ervervet utenfor sykehus er bekymringsfullt fordi det betyr at disse mikrobene allerede har spredd seg i samfunnet. Infeksjoner forårsaket av disse mikrobene er vanskelig å behandle og kan i praksis være umulig å kurere i den lokale situasjonen. Hurtigtester og mikroskopi er nyttige verktøy i rutine diagnostikk av malaria, men negative resultater må tolkes med forsiktighet.

Funnene understreker behovet for videre overvåkning av etiologi av blodbaneinfeksjoner og forekomst av antibiotikaresistens, og for å etablere strategier for å bekjempe den videre spredningen av antibiotikaresistens. Studien kan bidra til å etablere lokale behandlingsretningslinjer og til å peke på behovet for forebyggende tiltak mot enterisk feber inkludert vaksinering, og på behovet for å vedlikeholde og styrke malaria kontroll programmet på Zanzibar.

Abbreviations

AMR Antimicrobial resistance

BSI Bloodstream infection

cipR low-level ciprofloxacin resistant

CLSI Clinical and Laboratory Standards Institute

DNA Deoxyribonucleic acid

ESBL Extended-spectrum beta-lactamase

ESKAPE ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and ***E****nterobacter* species

EUCAST European Committee on Antimicrobial Susceptibility Testing

HGT Horizontal gene transfer

HIV Human immunodeficiency virus

ICE Integrative conjugative elements

IQR Interquartale range

LAMP Loop-mediated isothermal amplification

LMIC Low- and middle-income countries

MALDI-TOF Matrix assisted laser desorption/ionization time-of-flight

MDR Multidrug resistant

MDR/cipR 4.3.1.1 Multidrug resistant/low-level ciprofloxacin resistant sub-lineage of the 4.3.1.1 genotype

MIC Minimum inhibitory concentration

MMH Mnazi Mmoja Hospital

MRSA Methicillin resistant *Staphylococcus aureus*

NordicAST Nordic Committee on Antimicrobial Susceptibility Testing

PCR Polymerase chain reaction

QRDR Quinolone-resistance-determining regions

RDT Rapid diagnostic test

SNP Single nucleotide polymorphism

WGS Whole genome sequencing

WHO World Health Organization

XDR Extensively drug resistant

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List of Publications

Paper I

Annette Onken, Abdulrahman K. Said, Melissa Jørstad, Pål A. Jenum, Bjørn Blomberg. **Prevalence and antimicrobial resistance of microbes causing bloodstream infections in Unguja, Zanzibar.**

PLoS One. 2015 Dec 23;10(12)

Paper II – submitted to the journal "PLOS Neglected Tropical Diseases"

Annette Onken, Sabrina Moyo, Mohammed Khamis Miraji, Jon Bohlin, Msafiri Marijani, Joel Manyahi, Kibwana Omar Kibwana, Fredrik Müller, Pål Arne Jenum, Khamis Ali Abeid, Marianne Reimers, Nina Langeland, Kristine Mørch, Bjørn Blomberg. **Predominance of multidrug-resistant *Salmonella* Typhi genotype 4.3.1 with low-level ciprofloxacin resistance in Zanzibar.**

Paper III

Annette Onken, Christel Gill Haanshuus, Mohammed Khamis Miraji, Msafiri Marijani, Kibwana Omar Kibwana, Khamis Ali Abeid, Kristine Mørch, Marianne Reimers, Nina Langeland, Fredrik Müller, Pål Arne Jenum, Bjørn Blomberg. **Malaria prevalence and performance of diagnostic tests among patients hospitalized with acute undifferentiated fever in Zanzibar.**

Malar J. 2022 Feb 19;21(1):54

1 Background

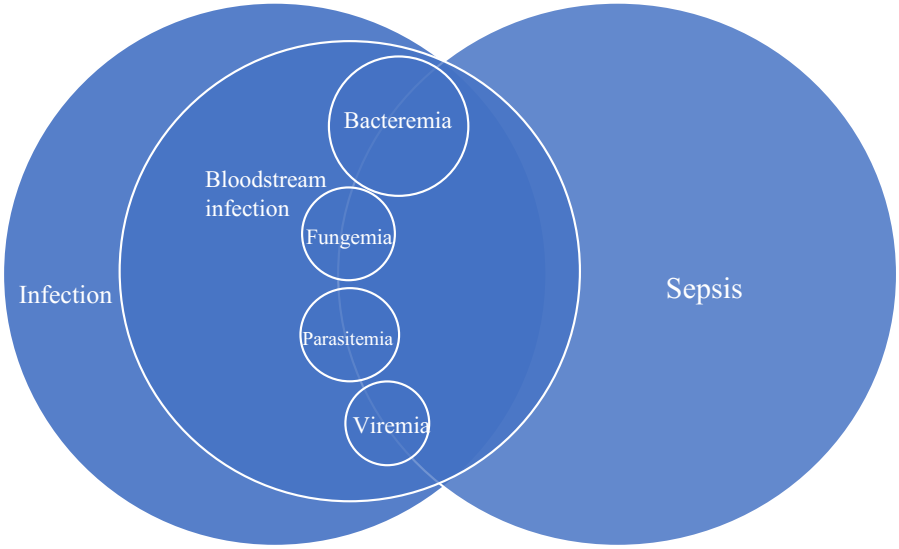
1.1 Definitions

The term bloodstream infection (BSI) describes a condition where the presence of a viable pathogen in the patient's blood is associated with symptoms of overt infection (1-3). In a laboratory confirmed BSI, a laboratory analysis has provided evidence for the presence of a pathogen in the patient's blood. The evidence for bacterial and fungal BSIs is usually based on growth of the pathogen in blood culture (2, 4), while in parasitic BSIs, such as malaria, it is based on detecting the parasite by microscopy or malaria rapid diagnostic test (RDT) (5). For the clinical assessment of patients suspicious for BSIs, nonspecific symptoms such as fever, hypothermia, hypotension, and chills are used (3). BSIs are not congruent with one specific clinical syndrome (6), they occur in a whole range of diseases including serious, potentially life-threatening generalized infections and sepsis. By uncovering the microbiological etiology and, mostly, also the antimicrobial resistance pattern, the "entity" BSI is valuable both from a clinical and an epidemiological point of view (6). The expression BSI is mostly used for infections caused by bacteria and fungi (1-3), but can, in principle, be understood to include infections caused by parasites such as malaria (7) and human African trypanosomiasis (8), as well as viral infections (9). In this thesis, it is used for bacterial and fungal BSIs unless otherwise specified. The terms bacteremia, fungemia, parasitemia and viremia are commonly used to describe the presence of these different groups of microorganisms in the blood (10, 11). In contrast to a secondary BSI, in a primary BSI no other source of infection can be identified (3).

Whereas the term "bloodstream infection" is mainly determined by the pathogen detected, "sepsis" describes foremost a clinical "entity" (6). The expression stems from the ancient Greek word *sepsis* (σῆψις), meaning putrefaction or decay of organic matter (12). The definition of sepsis has been changed over time, to include new insights and to increase its applicability in clinical work. Whereas in 1991 it was defined as a systemic inflammatory response to infection (11), the latest review from 2016 redefined sepsis as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" (13). The patient groups covered by the terms

bloodstream infection and sepsis are only partially overlapping (figure 1) (1, 6, 14). Sepsis, by the current definition, encompasses only patients with severe infections associated with organ dysfunction, and while an infectious cause is suspected, in a substantial part of sepsis cases pathogens cannot be identified from the blood. Conversely, BSI assumes that a pathogen is detected in the blood, but encompasses a wide spectrum of patients from mild febrile infections up to severe cases of sepsis. Bacteremia is defined by detection of bacteria in the blood, but notably includes even asymptomatic persons with bacteria in the blood, in addition to the full spectrum of symptomatic cases. The definitions of bloodstream infection, bacteremia, fungemia, viremia, and sepsis are shown in table 1, including the references they are based on. See figure 1.

Figure 1. Relationship between infection, sepsis, bloodstream infection, bacteremia, fungemia, parasitemia, and viremia



In contrary to this thesis, some publications do not distinguish between BSI and bacteremia (not considering the presence of clinical symptoms) (15).

Table 1. Definitions of bloodstream infection, bacteremia, fungemia, viremia, parasitemia, and sepsis

Term	Definition
Bloodstream infection	Presence of a viable pathogen in the patient's blood + systemic signs of infection (1)
Bacteremia	Presence of viable bacteria in the patient's blood (11)
Fungemia	Presence of viable fungi in the patient's blood (11)
Viremia	Presence of viable viruses in the patient's blood (11)
Parasitemia	Presence of viable parasites in the patient's blood (11)
Sepsis	Life-threatening organ dysfunction caused by a dysregulated host response to infection (13)

The term "antibiotic" means literally "against life". Originally, it has been used to describe compounds produced by microorganisms to kill or stop the growth of competing microbes. The terms "antimicrobial" and "antibiotic" are now synonymously applied to drugs used for both treatment and prevention of bacterial infections in humans, animals and plants, and they cover both natural and artificially synthesized substances (16, 17). Though most frequently used in connection with bacterial infections, the expression "antimicrobial" also encompasses medicines used against fungal, parasitic or viral infections. The terms "antibacterial", "antifungal" and "antiviral" are used for drugs against the respective type of infections.

The term **"**antimicrobial resistance" describes the ability of microorganisms to survive or multiply in the presence of specific antimicrobials which are designed to inhibit or to kill them. In this context, the terms "sensitivity" and "susceptibility" both describe

the opposite of the term “resistance” (18). In the laboratory, the susceptibility of a microbe to a certain antimicrobial is mostly tested by standardized methods measuring the minimum inhibitory concentration (MIC) value (19), or the growth inhibition zone around disks containing specific antibiotics. In the latter case, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has calibrated disk diffusion zone diameters to MIC values (20). In 2019, EUCAST introduced the following new definitions for sensitivity testing categories to better guide the clinician in choosing an effective antimicrobial treatment (21, 22):

- “A microorganism is categorized as S –‘Susceptible, standard dosing regimen’, when there is a high likelihood of therapeutic success using a standard dosing regimen of the agent.”
- “A microorganism is categorized as I – ‘Susceptible, Increased exposure’ when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection.”
- “A microorganism is categorized as R – ‘Resistant’ when there is a high likelihood of therapeutic failure even when there is increased exposure” (21).

In this paper, we follow these definitions of EUCAST.

In medicine, the terms “multidrug-resistant” (MDR), “extensively drug-resistant” (XDR) and “pandrug-resistant” are applied to classify different patterns of resistance in healthcare-related bacteria. Multidrug-resistance is well-defined for certain microbes such as *Mycobacterium tuberculosis*, where it means resistance to the two most important anti-tuberculous drugs, rifampicin and isoniazid (https://www.cdc.gov/tb/topic/drtb/default.htm), and *Salmonella* Typhi, where it refers to resistance to the three historically most important anti-typhoid antibiotics, chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole (23, 24). For *S.* Typhi, this definition of MDR will be used in this thesis. Still, there are no generally approved definitions of the term multidrug-resistance applicable to all bacteria. In this thesis, the definitions which an expert group initiated by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and

Prevention (CDC) has proposed, are used: multidrug resistance is defined as “acquired non-susceptibility to at least one agent in three or more antimicrobial categories”, XDR as “non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories)” and pandrug-resistance as “non-susceptibility to all agents in all antimicrobial categories” (25).

In this study, a bloodstream infection is defined as community-acquired, if a pathogen has been recovered from a blood culture taken within the first 48 hours after the admission of the patient. If the blood culture yielding the pathogen has been taken more than 48 hours after the patient’s admission, the BSI is categorized as hospital-acquired.

In this thesis, a bacterial “clone” is defined as “any bacteria propagated from a single colony and isolated at a specific time and place that show common phylogenetic origin” (26).

1.2 Burden of infectious diseases

Infections contribute significantly to the global burden of diseases, although the numbers have been declining in the recent years. Still, for 2019 a large study estimated 13.7 million deaths worldwide to be related to infections (27). According to the World Health Organization (WHO), 14.5% of all deaths in 2019 were caused by infections, with lower respiratory infections responsible for 4.7% of all deaths (rank 4), diarrheal diseases for 2.7%, tuberculosis for 2.2%, human immunodeficiency virus (HIV) infections for 1.2%, malaria for 0.7% and neonatal sepsis and infections for 0.6% (28). Low- and middle-income countries, especially sub-Saharan Africa, are affected to a disproportionally large extent (27, 29), because, at least partly, malnutrition, lack of access to clean drinking water and appropriate health care and of functioning sanitation and hygiene are adding to the load of infections (30). Especially concerning is the impact on the youngest, as infectious diseases still are a leading cause of death in under five-year-old children, even though the number of cases is decreasing (30, 31). The burden of infectious diseases in low-resource areas is difficult to assess and may be considerably higher than estimated as many diseased are not in contact with health care systems (32). The WHO underlines the importance of communicable diseases for

the well-being of the world's population, as they are targeting the global burden of infections in their "Sustainable Development Goals" (33).

1.2.1 Bloodstream infections

Globally, bacterial BSIs add significantly to morbidity and mortality (7, 15, 34), with infants and older people especially at risk (35). Primary bacterial BSIs are estimated to be responsible for 2,9 million deaths in 2019 (27). Secondary BSIs may be categorized by the original focus of an infection, for example the urinary tract, and not reported as BSI, leading to an underestimation of the burden (27). In high-income countries in Europe and North America, an annual incidence of BSIs between 113 and 204 per 100,000 population is reported (34), with numbers increasing in recent years (2), and rated among the seven most frequent causes of death (34). For low-resource regions, including Africa, data on the burden are limited (6, 7), however, the load is presumably higher compared to high-income countries (7), due to factors such as malnutrition, inadequate hygiene, lack of access to clean drinking water, and the higher prevalence of HIV infections (27, 36, 37). While children with bacterial BSIs and severe malaria frequently have similar clinical presentation, the treatment is completely different, and both conditions require immediate adequate therapy (38).

Etiology of bloodstream infections

Prevalence and etiology of the most frequent pathogens causing bacterial BSIs are varying significantly in different geographic regions (37, 39), over time (39), in different age groups (7, 39) and regarding the way of acquisition (in the community or in a hospital) (39). In a broad study on the etiology of bacteremia including 45 countries in the years 1997-2016, *S. aureus* (21%), and *E. coli* (21%) were the predominant microbes overall followed by *Klebsiella pneumoniae* (8%), *Pseudomonas aeruginosa* (5%) and *E. faecalis* (5%). In the 1-5 years old patients, *Streptococcus pneumoniae* was the second most frequent microbe (11%) after *S. aureus* (16%). Both in community-acquired and in hospital-acquired BSIs, *S. aureus* and *E. coli* were on top of the list of the most numerous pathogens, yet in community-acquired infections they were much more dominant (58%) compared to 31% in hospital-acquired infections. In the latter, a higher amount of the non-fermenting gram-negative rods *P. aeruginosa* and *Acinetobacter* spp. was detected (39). However, in this large study no

African countries were included. A comprehensive review and meta-analysis on community-acquired bloodstream infections in Africa, including studies from 1984 to 2006, found that *Salmonella enterica* was the most frequent pathogen causing bacteremia in 29% overall and in 42% of adults, followed by *S. pneumoniae* (18% overall), *S. aureus* (10%) and *E. coli* (7%). Different to adult patients, gram-positive bacteria were the dominating pathogens in children, with *S. pneumoniae* leading (7). Yet, as the studies included have been performed many years back in time, the situation may be different now. In another large review on the etiology of community-acquired BSIs in Africa (data from 1984 to 2014) and Asia (data from 1991 to 2015), *Salmonella enterica* (35%, with 29% typhoidal *Salmonella*), *S. pneumoniae* (13%) and *E. coli* (9%) were the overall leading pathogens. Looking at Africa only, non-typhoidal *Salmonella* (30%), *S. pneumoniae* (15%) and *E. coli* (7%) were most frequently detected (40). In children, a meta-analysis of studies on BSIs published from 1990 to 2019 found that *S. aureus* (18%), *S. pneumoniae* (17%) and *E. coli* (11%) were the most common microbes in Africa (41).

BSIs caused by non-typhoidal *Salmonella* are frequent in sub-Saharan Africa, accounting for up to 39% of BSIs acquired in the community (42). They are associated with HIV-infection (42, 43), and, in young children, with anemia, malnutrition and malaria (42). Moreover, in HIV-infected patients *Mycobacterium tuberculosis* and fungi are more frequently isolated (7) including *Cryptococcus* and *Histoplasma* (44). *M. tuberculosis* has accounted for about 16% of bloodstream infections in HIV infected adults (45), and as much as 39% during the period when antiretroviral treatment was not available (43).

A large review and meta-analysis on the etiology of bacteremia or sepsis in neonates included data from 1980 to 2018, with focus on the years 2008 to 2018, and found *S. aureus* to be the most frequent pathogen overall (25%), and in eastern Africa (20%), followed by *Klebsiella* spp (21%), and *E.coli* (10%) (46).

According to a meta-analysis, the average in-hospital case fatality rate of bacterial BSIs in Africa is 18% (7). Yet, a fatality rate as high as 35% and 38% was reported in studies from Tanzania (47) and Malawi (37), respectively.

Antimicrobial resistance in bacterial bloodstream infections

A comprehensive international study on BSIs covering a 20-years period, detected an increase of AMR in gram-negative rods including global dissemination of extended-spectrum beta-lactamase and carbapenemase producing strains. The proportion of *Enterobacterales* causing BSI that are multidrug-resistant has risen from 6% in 1997 to 2000 to 16% in 2013 to 2016, according to the SENTRY report including global data, but notably excluding the African continent (39). The highest frequency of multidrug resistance was found in non-fermentative gram-negative rods (26% of *P. aeruginosa*, 71% of the *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex), including pan-resistant microbes (39).

In a meta-analysis on BSIs in children in low- and middle-income countries (LMIC) including studies from 1990 to 2019, thirty percent of the *S. aureus* reported from Africa were methicillin-resistant *Staphylococcus aureus* (MRSA), and 21% of the *E. coli* were resistant to third generation cephalosporins (41).

In a retrospective study on 740 pathogens isolated from blood culture samples in 2011-2012 from adults at a tertiary South African Hospital both community- and healthcare-acquired infections were included. Over 92% of the community-acquired *Enterobacterales* were sensitive to common antimicrobials (gentamicin, 3. generation cephalosporins and ciprofloxacin), in contrast to healthcare-acquired infections where 65%, 59% and 70% were susceptible, respectively. Only methicillin-susceptible *S. aureus*, and no MRSA was detected in community-acquired infections, but nearly half of the healthcare-acquired infections were MRSA (48). This difference may be due to a higher exposition of bacteria to antimicrobials in a healthcare environment compared to the community, leading to a higher selection of resistant microbes.

A review on AMR in bacteremia in Africa included 27 studies published from 2008 to 2019. For *E. coli* they found a pooled median resistance of 42% to cefotaxime, of 75% to co-trimoxazole, 87% to ampicillin, and 44% to fluoroquinolones. Deduced from resistance to third generation cephalosporins, the percentage of ESBL-producing *E. coli* varied between 19% and 42%. In *S. aureus*, deduced from resistance to oxacillin, a median MRSA rate of 18% was reported. According to this paper, only 23% of the

African Union member states had surveillance data on AMR in bacteremia, including four studies from Tanzania (49).

Data on etiology and AMR patterns of BSIs in Africa are limited due to factors such as lack of laboratory facilities and trained personnel, surveillance and research studies, and limited finances (7, 50). General empiric treatment guidelines for children for low-resource areas, as elaborated by the WHO (51, 52), may not cover for the local situation both regarding etiology and AMR patterns (47, 50). More research and updated regional data are needed both for improving the treatment of the patients as well as for the prevention of BSIs (6, 40).

Typhoid fever

Within the bacterial bloodstream infections, typhoid fever is a severe, potentially life-threatening systemic disease.

History of typhoid fever

In 1829, the French doctor Pierre Charles Alexandre Louis recognized a distinct disease associated with persistent fever, similar to, yet different from epidemic typhus. He labeled it typhoid fever, with "typhoid" meaning "typhus-like" (53). The term "typhus" originates from the Greek *typhos*, meaning heavy stupor, and is also related to *typhein*, meaning "to smoke" (54), which may illustrate the mental state of patients with serious typhoid fever. The English physician and epidemiologist William Budd realized, in 1839, that the illness is transferred by the fecal-oral route (55). In 1880, the German pathologist and bacteriologist Karl Joseph Eberth discovered the agent causing typhoid fever (56) which is now known as *Salmonella* Typhi.

Classification and genetics

Based on taxonomical studies this gram-negative, rod-shaped, flagellated bacterium has been named *Salmonella enterica* subspecies *enterica* serovar Typhi (shortened to *S.* Typhi), as it represents an own serovar of the *Salmonella enterica* species subspecies *enterica* within the *Salmonella* genus in the *Enterobacterales* order (23, 57). *Salmonella* Typhi, as well as *Salmonella* Paratyphi A, B and C are also categorized as typhoidal *Salmonella.* This classification is due to the distinct and similar clinical diseases which they can cause, typhoid and paratyphoid fever, also

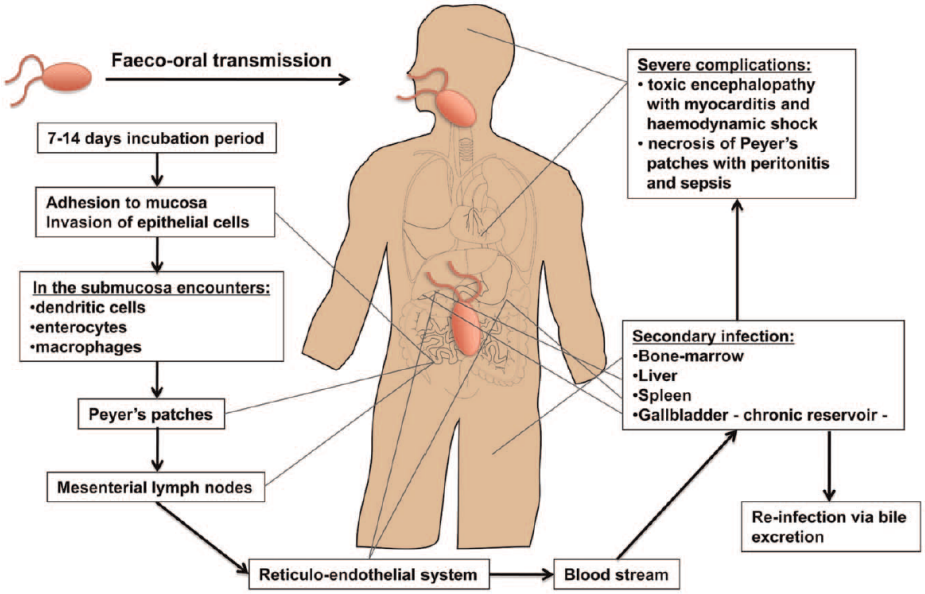
described with the umbrella term enteric fever. All of them, except for *Salmonella* Paratyphi C, infect humans only (58). *Salmonella* Paratyphi A can cause equally severe disease as *Salmonella* Typhi. The remaining over 2500 different *Salmonella enterica* serovars are called nontyphoidal *Salmonella*. They are primarily causing diarrhea, but may also lead to invasive illness, especially in young children and in patients with an impaired immune system (59, 60).

The different serotypes of *Salmonella enterica* can be classified by the Kauffmann and White scheme or modifications of it, according to the presence of different lipopolysaccharide (O) and flagellar (H) antigens. *S.* Typhi has several antigens in common with other *Salmonella enterica* serotypes. The somatic lipopolysaccharide antigens O9 and O12, and the protein flagellar antigen Hd are more specific for *S.* Typhi *Salmonella*. In addition, *S.* Typhi and *S.* Paratyphi C have the virulent polysaccharide capsular antigen Vi (59, 61, 62).

Molecular biological analyses including whole genome sequencing have extended the knowledge on *S.* Typhi tremendously. In 2001, Parkhill et al. published the entire genome (63). Studies of the deoxyribonucleic acid (DNA) sequence including the frequency of single-nucleotide polymorphisms (SNPs) suggest that *S.* Typhi is a relatively new clone with the last common ancestor assumed about 50,000 years ago (64). Interestingly, *S.* Typhi is monomorphic (65), meaning it shows little genetic variety, and it harbors a large number of pseudogenes (seemingly functionally inactive genes). This divergent gene expression of *S.* Typhi compared to non-typhoidal *Salmonella* may lead to differences in the identification of this pathogen by the immune system of the infected, and consequently induce a dissimilar clinical course. Or these genes may have become redundant due to the unique interactivity of *S.* Typhi with its hosts (62).

Figure 2. Dissemination of *S.* Typhi during systemic infection

(by courtesy of the authors de Jong et al 2012 (62))



Typhoid fever is usually transmitted by the fecal-oral route via water or food contaminated with infectious human feces of recovering patients or chronic carriers. Trials with volunteers reported an infective dose of up to 10^7 microbes to generate clinical illness in the participants, although as little 10^3 microbes may be sufficient (23, 66). The amount of bacteria ingested and the virulence of the microbe can affect the incubation time (67). Notably, after oral ingestion and adhesion to the epithelium of the small intestine, the pathway of *S.* Typhi differs significantly from that of the vast majority of non-typhoidal *Salmonella*. The bacterium traverses through the epithelium of the small bowel to the submucosa and is internalized by macrophages in the local lymphatic tissue. Highly important, both in this stage and in the further course of the infection, is *Salmonella* Typhi's ability to survive and replicate intracellularly within macrophages, mononuclear phagocytic cells, and other cells. In this initial phase, the

pathogen proliferates, and some of the descendants can find their way to the lymphatic system and to the blood stream and then spread systemically, most frequently to the reticuloendothelial system of liver, spleen, and bone marrow, and to the gallbladder and the Peyer's patches of the terminal ileum, potentially also to other organs (23, 62, 68). The majority of *S.* Typhi in the blood circulation is located intracellularly in phagocytic cells (23), probably aiding the transition to the reticuloendothelial system. *S.* Typhi possesses a set of potent virulence factors, including the Vi-antigen and the typhoid toxin (68), which, besides others, help the bacterium to survive intracellularly and evade the immune system of the host (68).

Clinical manifestations, morbidity and mortality

Typhoid fever has no specific symptoms. The clinical manifestations and the severity of the disease differ substantially in various populations, including different age groups (23). In hospital-based studies, most of the patients are children from five years of age and young adults. However, it is suspected that, in endemic regions, a considerable amount of outpatients with typhoid, especially children under five years of age, may not be detected (23). The incubation time is commonly 7 to 14 days (23, 60). Fever, usually rising during the first week, is the predominant sign, and may, in uncomplicated disease, be the only one perceptible. Other relatively common symptoms include headaches, cough, nausea, vomiting, obstipation and diarrhea. Although so called rose spots (erythematous maculopapular skin lesions) are historically described as typical for typhoid fever, in many patients they are not found. The large range of possible complications of typhoid fever include serious events such as bowel perforation, gastrointestinal bleeding and encephalopathy. These are reported to occur in about 10% of the hospitalized patients, up to 27% in some reports, and particularly with longstanding illness of at least two weeks duration (23, 60, 69).

Untreated, patients with typhoid fever experience an often serious, three to four weeks lasting illness (60). In the pre-antibiotic era, according to a publication from 1913, the case fatality ratio varied significantly in different reports, from 5% up to 20% or even higher (70). However, these numbers may have been imprecise and must be interpreted with care. More recent investigations indicate that most of the cases have

an uncomplicated course, not needing treatment in hospital, and that presumably about 1 to 2 % of patients die (23, 71-73). In areas with limited resources, the case fatality ratio of hospital-treated patients with typhoid fever varies significantly from less than 2% to 30% to 50%, with children under one year of age and the oldest patients especially at risk. Late start of appropriate antimicrobial therapy increases the risk for an adverse outcome (23, 72). About 5 to 10% of untreated cases experience a relapse (23, 60). One to four percent of the survivors become chronic carriers (74, 75), defined by excretion of the pathogen in stool or urine for over a year (23). The gallbladder is a preferential location for chronic carriage (74). Carriers are mostly asymptomatic and can spread the pathogen (23).

Diagnosis

As the symptoms are unspecific, the clinical diagnosis is difficult. The list of endemic acute and subacute febrile diseases representing differential diagnoses to typhoid fever is long. It includes bacterial infections such as tuberculosis, deep abscesses, rickettsial infections, endocarditis, leptospirosis and brucellosis, parasitic illnesses such as malaria, visceral leishmaniasis, and toxoplasmosis, and viral diseases including dengue, infectious mononucleosis and influenza, as well as encephalitis and noninfectious illnesses such as lymphoproliferative and connective-tissue diseases (23, 60). Hence, the diagnosis depends on laboratory analyses. Detection of *S.* Typhi in normally sterile body sites confirms the diagnosis (59). However, in low-resource areas diagnostic tools such as bacterial culture are often not available or affordable (76). If available, blood culture is commonly used, yet the result will only be available after one to several days. Bone marrow culture may be defined as a “true gold standard” (77), as, in acute disease, the amount of *S.* Typhi found in bone marrow is about tenfold higher than in blood. Despite its sensitivity exceeding 80%, bone marrow culture is rarely performed as it is a more invasive procedure (59, 78). The sensitivity of blood culture depends on factors such as point in time when taken (most sensitive in the first week) and sufficient blood volume, due to a low number of bacteria circulating in the blood (60, 78). A larger review estimates a sensitivity of 61% (79), but it may vary from 40 % to 80% (23, 59, 60). Even with automated blood

culture systems, sensitivity does not exceed 80% using bone marrow culture as gold standard (77). Culturing enables antimicrobial susceptibility tests, an important advantage of this method. Other material including rose spots biopsy, stool, and urine samples may also be culture positive (59). However, positive culture of stool or urine must be judged carefully, as it may be caused by coincidental chronic carriage (60).

The Widal test, a serological method, has been used for over 100 years. It detects antibodies against the pathogen's O- and H-antigens. As it is relatively easy to use and cheap, this test or variants of it are still much in use, yet controversial and most often not recommended, mainly due to low sensitivity and specificity (77, 80, 81). The poor specificity may, at least partly, be explained by cross reactivity (23, 82), and, in endemic areas, by persistent antibodies after previous episodes of typhoid fever. A review of 37 studies evaluated the performance of newer rapid tests based on the detection of antibodies (mainly TUBEX, Typhidot, and Test-It Typhoid including variations) and concluded that these rapid diagnostic tests (RDTs) have so far not shown a groundbreaking improvement. However, the authors emphasized that studies of a better quality are needed (83). The performance of RDTs should also be evaluated in different geographic settings (84). More recent approaches, including tests based on IgA detection, may be promising, but such tests are not yet commercially available (82, 85).

Nucleic amplification methods for detecting the pathogen, such as polymerase chain reaction (PCR) and nested PCR, have been developed, but they are so far little used (59, 77, 84, 86). Also, studies on the sensitivity have shown diverging results (60, 84). Compared to blood culture, a commercially available multiplex-PCR showed a sensitivity of only 14% (1/7) for *Salmonella* including *Salmonella* Typhi (87).The fact that only a small amount of *S.* Typhi is circulating freely in the blood (<1 microbe per ml) may reduce the sensitivity of PCR blood tests (59, 77, 86). Costs and the need for laboratory equipment and expertise are presumably hampering the introduction in low-resource areas (77).

In summary, reliable, affordable and "easy to use" rapid tests for the diagnosis of typhoid fever are lacking and should be developed (77, 84, 88).

Treatment and antimicrobial resistance

Appropriate antibiotic therapy can reduce mortality and complication rates, shorten the period of illness, and may eliminate fecal carriage and diminish spread of the disease (60).

At the end of the 1940ies, chloramphenicol was the first antibiotic successfully used to treat typhoid fever, followed by ampicillin and trimethoprim-sulfamethoxazole in the 1960ies. However, *S.* Typhi has in many cases developed multidrug resistance. This is defined by resistance against all of these three former first-line antibiotics, and has widely spread since the 1970s and is associated with higher mortality (24, 89). Subsequently, fluoroquinolones were used extensively, and, not surprisingly, in the beginning of the 1990s, fluoroquinolone resistant *S.* Typhi appeared (65, 90, 91). Third generation cephalosporin and azithromycin are effective against *S.* Typhi, and so far only sporadic resistance to these drugs is reported (92). Yet, in 2016, the first outbreak of extensive drug-resistant (XDR) *S.* Typhi was observed in Pakistan, the isolates found to be resistant to both ciprofloxacin and third-generation cephalosporins in addition to multidrug resistance (24, 93).

Fluoroquinolones, azithromycin, and third-generation cephalosporins are now the antibiotics commonly used to treat typhoid fever (82). As resistance to the former used antimicrobial drugs chloramphenicol and trimethoprim-sulfamethoxazole has decreased in some regions, their reintroduction is discussed (92). Due to the significant geographical differences of AMR, knowledge on local resistance patterns and national guidelines should lead the decision on which drug to use (92).

For the treatment of typhoid fever, third-generation cephalosporins are mostly administered parenterally and usually require that the patient is hospitalized, leading to higher costs. Azithromycin is quite expensive (82). Hence, the treatment choices for MDR *S.* Typhi with additional resistance to fluoroquinolones are limited in low-resource regions. The WHO has classified fluoroquinolone resistant *Salmonella* as pathogens for which new antimicrobials are highly needed (94) and emphasizes that AMR patterns must be surveyed.

For minimizing complications and for the best outcome, early diagnosis and adequate treatment is crucial, especially in children (95).

Molecular mechanisms of antimicrobial resistance

Molecular biological investigations based mainly on PCR and whole genome sequencing (WGS) have added significant insights into AMR mechanisms in *S*. Typhi (96, 97).

Genetically, *S*. Typhi's multidrug resistance relates to the presence of a composite transposon. It was initially introduced through IncHI1 plasmids, in the 4.3.1 genotype the IncHI1-PST6 plasmid (98-100). On this transposon, genes conveying resistance to penicillins (*bla*-TEM1), trimethoprim (*dfrA7*), sulfonamides (*sul1* and *sul2*), chloramphenicol (*catA1)* and streptomycin (*strA, strB)* (101, 102) are located. Subsequent investigations detected this composite transposon incorporated into the bacteria's chromosome (97, 101-103).

Fluoroquinolone resistance in *S.* Typhi can evolve in two ways: firstly, by mutations, so-called single nucleotide polymorphisms (SNPs), in the quinolone-resistance-determining regions (QRDRs) of the chromosomal *gyr* and *par* genes (24, 96, 104), secondly by plasmid-mediated quinolone resistance. In the latter case, the plasmids are carrying *qnr* genes (96, 105-107). Mutations in the QRDR regions, especially *gyrA* genes, are the most common ones in *S*. Typhi (96, 102).

Acquisition of two or three genes coding for fluoroquinolone resistance is associated with a corresponding gradual increase of the minimum inhibitory concentration to these antibiotics. Isolates with three mutations, so-called "triple-mutants", are found high-grade resistant to fluoroquinolones (96, 107).

S. Typhi resistance to third generation cephalosporins is associated with the acquisition of extended-spectrum beta-lactamases (ESBLs) which are located on plasmids (96, 108). Azithromycin resistance is conferred by mutations in the gene encoding the efflux-associated transporter AcrB (108).

Epidemiology

Typhoid fever occurs mostly in areas lacking both supply with clean drinking water and sewage systems (60). Historically, in the 19th century, Europe and the USA suffered high burdens of typhoid fever due to overpopulated living conditions and the lack of sewage disposal, until access to safe water and the introduction of functioning wastewater systems led to a drastic reduction of its occurrence (23).

To a large degree due to the same causes as earlier observed, it is today predominantly a disease of regions with limited resources (23). It is endemic in a large number of countries, particularly in Asia and sub-Saharan Africa (23, 60), including epidemics (109) and outbreaks (110, 111). Children in urban informal settlements are especially at risk (112). In high-resource countries only sporadic cases occur, mostly in connection with travel or chronic carriers (60).

Globally, over nine million typhoid cases per year and about 100,000 to 200,000 deaths are estimated (113-115). Because of lack of laboratory diagnostic and provision of health care these numbers are uncertain (23). Incidence differs significantly in various geographic areas, with numbers of over 800 cases per 100 000 persons per year in some regions in sub-Saharan Africa (112, 116), and incidence in specific urban settings exceeding that in the countryside (112).

Molecular biological methods including whole genome sequencing (WGS) have added important information on the spread of *S.* Typhi. The relatively new implementation of a phylogenetic genotyping scheme, GenoTyphi, has made the interpretation of WGS data for *S.* Typhi easier (117, 118). Analyses of worldwide collected *S.* Typhi isolates with GenoTyphi demonstrated that the *S.* Typhi population encompasses several subclades which are distinct in different geographical areas (97, 102, 117). Globally, the main part of MDR *S.* Typhi infections are caused by the genotype 4.3.1, earlier referred to as haplotype H58 (102), which disseminated from South Asia to East Africa, among other regions (97, 99, 102, 117). This genotype which can also be low-grade resistant to fluoroquinolones, has spread regionally and has led to outbreaks in various areas of the world (102).

Blood culture diagnostic and WGS analyses are highly valuable tools for monitoring the spread of resistant *S.* Typhi strains, including their AMR mechanisms, in the various global regions. With these data, the local clinicians can be informed on adequate treatment options in the local setting. Also, the findings may help to establish adequate prevention measures. Yet, in many African regions, routine blood culture diagnostic and further analyses such as WGS are often not available due to limited resources (119). From Zanzibar, as from many other regions in Africa, the data are sparse. A study performed in 2009/2010 in Pemba, the second largest island of the archipelago, found *S.* Typhi to be the most frequently detected pathogen in bacterial BSIs (80, 120).

Prevention

Typhoid fever is transmitted via fecal contamination of food and water. Hence, preventive actions must be aimed at providing safe food and drinking water which includes functioning sanitation systems and education on hygiene (23, 55, 113).

Vaccination is an important pillar in the prevention of typhoid fever. Of the over twenty vaccines which have received authorization, the World Health Organization (WHO) gives preference to the use of typhoid conjugate vaccines due to its better immunological properties, suitability for young children and expected longer duration of protection. Age dependent alternatives are unconjugated Vi polysaccharide vaccine and live attenuated Ty21a vaccine. For regions with highly endemic typhoid fever or a high amount of antimicrobial resistance in *S.* Typhi, the WHO especially recommends the introduction of typhoid vaccine as part of the national vaccine plan, adapted to the local epidemiology of the disease. The effect of vaccination programs should be evaluated together with other measures aimed at access to safe water, hygiene education and functioning sanitation (121, 122).

1.2.2 Malaria

Malaria is a severe, potentially life-threatening systemic infection caused by a parasite which is vector-transmitted to humans by the *Anopheles* mosquito. Endemic in many tropical and subtropical regions, it has significant importance (123-125), with a high number of the global population at risk of being infected (126). It is a crucial

differential diagnosis to bacterial bloodstream infections (7, 38, 127), as it requires a completely different therapy.

History and epidemiology

The disease malaria was likely recorded around 2700 BC in the region of today's China, and, over time, in different cultures including Mesopotamia, Egypt, Hindu, and Greece (128), with the occurrence of the illness associated with swamps (128, 129). The expression "malaria" originates from the Italian language with "mal'aria", meaning bad air, and illustrates probably the previous understanding that the illness was caused by malicious fumes or miasmas (129). In 1880 in Algeria, the French physician Alphonse Laveran was the first to detect the parasites causing malaria when he microscopically examined a blood drop taken from a soldier. This discovery constituted a milestone in the understanding of the pathogenesis (129, 130). In 1897, the British medical doctor Ronald Ross was the first to show that mosquitoes feeding on malaria-infected humans would later display the parasite in their guts (131). In 1898, he described the transmission of the parasites by mosquitoes to birds (132), and, in the same year, the Italian physician Giovanni Battista Grassi and colleagues demonstrated that humans develop malaria when bitten by infected mosquitoes (128, 133). Since that time, there have been intense efforts worldwide to reduce the malaria burden and even eliminate the disease, with varying success (134).

Historically, the quinine containing cinchona bark (135) or "Peruvian bark" (129) has been used for the treatment of malaria (135, 136). Intravenous quinine (129, 136), and, from the mid-20th century, chloroquine have been used as specific anti-malaria therapy (136).

A major milestone in the fight against malaria was the introduction of the Global Malaria Eradication Program of the World Health Organization in 1955. However, it was discarded in 1969 because of organizational, economical and technical problems (134). In the following decades, the number of malaria cases increased, to a high degree due to the development of resistance both to antimalarial drugs and to insecticides, the breakdown of programs working for the control of the vector, and the lack of dedication and economical support (134, 137).

For treatment, in the 20th century, further antimalarial drugs have been introduced, as sulfadoxine, pyrimethamine, mefloquine, amodiaquine, and artemisinin. Historically, quinine and chloroquine were regarded as the most important antimalarial drugs. Yet, in the recent decades, artemisinin has taken this role (124, 138). The rapid acting intravenous artesunate has become first-line treatment of severe malaria, and artemisinin-based combination therapy has become the standard in uncomplicated malaria (138, 139). However, the development of antimalarial drug resistance of the *Plasmodium* parasite has become a major challenge. In *P. falciparum*, similar to chloroquine resistance, resistance to sulfadoxine-pyrimethamine and later to artemisinin has first been detected in certain regions of Thailand, Myanmar and Cambodia before emerging in Africa (124).

With a major starting point in the year 2000, new global campaigns against malaria have been launched, including a special focus on children under five years of age (140). Subsequently, the number of people dying of malaria in endemic regions in Africa has been cut in half within the first fifteen years of the 21st century, and the incidence decreased by 40 percent. The number of countries with endemic malaria has decreased from 178 in the first half of the last century (134) to 85 countries in 2022 (141). Supported by an increase of the global economic support by about factor twenty, interventions have been based on two main elements. One is the control of the vector and is mainly aimed at hindering the mosquito in biting humans by using insecticide-treated bednets, and indoor residual spraying (142-145). The other element consists of diagnosis including RDTs and immediate treatment of clinical disease with artemisinin-combination therapy (142, 143, 145).

After several decades of research, vaccination is now a further element and a landmark in the fight against malaria, as the first vaccine against *Plasmodium falciparum* malaria, called RTS,S, was approved, and is recommended by the WHO since 2021 to children at risk (146), and for implementation in malaria programs (http://www.who.int/initiatives/malaria-vaccine-implementation-programme). RTS,S is a protein-subunit vaccine which aims at creating immunity to the pre-erythrocytic stage of the parasite, stopping the sporozoites from entering the liver cells (147). The

vaccine does not protect against the infection, but reduces the severity of the disease and the risk of a fatal outcome (146, 148). The use of other protective measures such as sleeping under a mosquito net and indoor residual spraying remains necessary (146). In October 2023, the World Health Organization has recommended a second malaria vaccine, R21/Matrix-M (WHO recommends R21/Matrix-M vaccine for malaria prevention in updated advice on immunization). Both vaccines employ antigens from circumsporozoite protein of the malaria parasite combined with hepatitis B surface antigen. The adjuvants used are different, AS01 for RTS,S and Matrix- M™ for R21/Matrix-M. The 1-year efficacy of 3 doses of R21/Matrix-M is reported at 77% against clinical malaria in 5-17 months old children (149). The efficacy of 3 doses of RTS,S vaccine in a similar age group was reported at 50% (150). Yet, the two vaccines have not been tested head-to-head. An advantage of the R1/Matrix-M vaccine is that production is taking place in malaria-endemic African countries. Further vaccines are in the pipeline (146, 147).

However, the malaria burden is still high, for the year 2022 the WHO still estimated 249 million malaria cases and 608,000 deaths worldwide. Africa is carrying the highest load accounting for 94% of the cases and 95% of the global deaths, with children under five years especially at risk for a fatal outcome (141) due to a lack of immunity (137). Compared to other infectious illnesses, the worldwide control of malaria is more challenging, as it is a vector-transmitted, complex disease, caused by different species of the parasite *Plasmodium*. Resistance development of both the *Anopheles* mosquito to insecticides and of the parasite to antimalarial drugs, especially to artemisinin, threatens the fight against malaria (124, 142, 151).

Malaria has not only high impact on morbidity and mortality, but leads also to high economic costs and may have huge impact on the economic development (152).

Epidemiology Zanzibar

Historically, Zanzibar has been a high transmission area for malaria, with *P. falciparum* representing the most frequent species (140, 153). However, since 2003, a successful comprehensive malaria elimination campaign has been run (154). A PCR based mass screening performed in rural areas of Unguja in 2012 detected a

prevalence of *P. falciparum* of about 2% (153). Likewise, a PCR-based study from the year 2015, performed in rural regions in Unguja and Pemba, the two main islands of the archipelago, found a prevalence of 2%, of *P. falciparum* and *P. malariae*. Evaluated by microscopy or rapid diagnostic test (RDT), the prevalence decreased up to 96% compared to 2003 (154). Since 2009, the efforts are directed towards elimination of malaria. However, this aim is hard-to-reach (155). With its tropical climate it is offering optimal living conditions for the vector, and the incidence can easily rise, if control efforts weaken.

Although Zanzibar has been a low transmission region the last 20 years, malaria still causes febrile illness there, and outbreaks have become more common (156). After many years with low incidence, most people, especially children who may not at all have been infected, will now presumably have a lower immunity and therefore, potentially, be more susceptible to the infection and at a higher risk for a fatal outcome (137, 139).

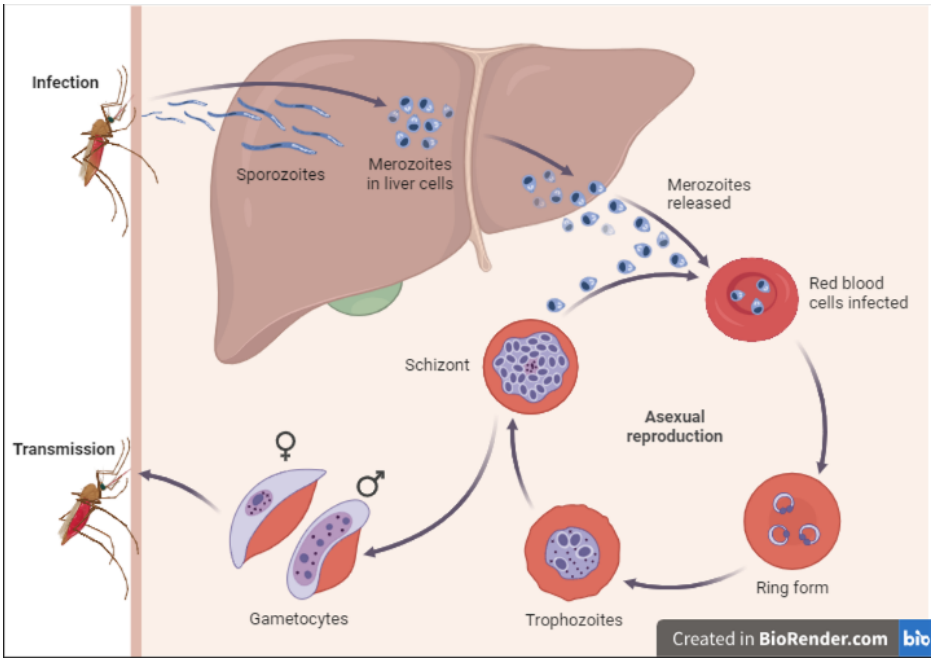
Pathogenesis, clinical manifestations and immunity

Pathogenesis

Malaria is caused by six species of the parasite *Plasmodium,* and is predominantly vector-transmitted. *P. falciparum* is the most deadly, and also the most frequent in sub-Saharan Africa, whereas *P. vivax* is most frequent in most countries outside sub-Saharan Africa. Besides them, *P. malariae*, *P. ovale curtisi, P. ovale wallikeri,* and *P. knowlesi* can cause malaria in humans, with the latter a mostly zoonotic disease (monkey malaria) (124, 138, 148, 157).

Malaria has a complicated life cycle with stages both in humans and in mosquitoes as demonstrated in figure 3 and 4.

Figure 3. Life cycle of the *Plasmodium* parasite in humans



The vector, the female *Anopheles* mosquito, has been infected by a blood meal on an infected human and transmits the *Plasmodium* sporozoites to humans by a bite (figure 3). In the human, the sporozoites get via the blood vessels to the liver and into hepatocytes where they, in this symptom-free pre-erythrocytic phase, multiply asexually to 10,000 to over 30,000 daughter cells (merozoites) within about six to eight days (124, 138, 147). The hepatocytes rupture and release the merozoites which make their way to the blood and invade erythrocytes. In the erythrocytic or blood stage, the merozoites multiply asexually within the red blood cells into six to 30 merozoites each (schizogony). When the merozoites are released, the erythrocytes are destroyed (124, 138). The merozoites find new erythrocytes for a new cycle which takes 24 to 72 hours depending on the *Plasmodium* species (124). The number of *Plasmodium* parasites in the blood then increases exponentially (158). With a parasite density of about 50/µl blood, clinical symptoms manifest and the parasite is commonly traceable by microscopy and by rapid diagnostic tests (124). The incubation time is varies depending on the *Plasmodium* species. In *P. falciparum*, *P. vivax* and *P. ovale*, symptoms often start 10 to 14 days after the infecting bite of the mosquito, however

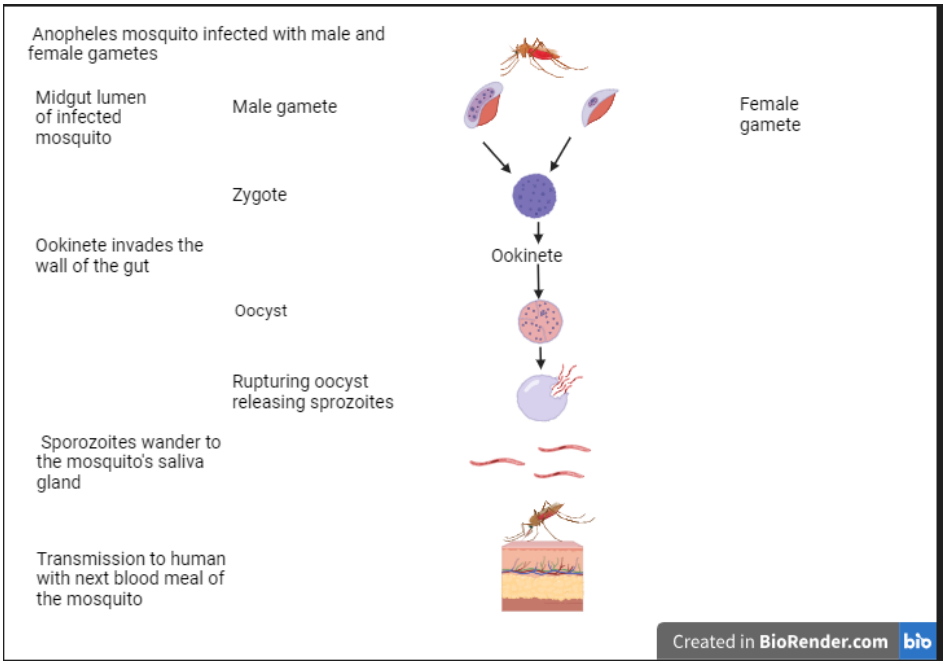
there is great variation (124, 159). The incubation time may be much longer, including when the infection is partially suppressed by chemoprophylaxis (160). The severity of the disease depends on the *Plasmodium* species and the susceptibility and immune response of the host (161). The blood stage of the infection is often cleared quickly, but can also last months, or lead to overwhelming disease and death of the patient (137, 161). Importantly, *P. vivax, P. ovale* and *P. malariae* attack only certain stages of the erythrocytes, hence leading commonly to low-level parasitemia, only. In contrast, *P. falciparum* and *P. knowlesii* have a much broader attack rate of the different stages of the erythrocytes, potentially leading to very high levels of parasitemia (124). In *P. falciparum* malaria, the parasite causes a surface change of the infected erythrocytes, leading to their adherence by specific binding by antigens to the endothelium of the blood vessels. By this “cytoadhesion”, a sequestration of the infected red cells, which are containing mature parasites, can occur in crucial organs such as the cerebrum, damaging endothelium and obstructing the local microcirculation (124). Sequestration has been described as the “key pathological event” in severe malaria (136). In addition, the plasticity of uninfected erythrocytes can be affected and reduce the flow in the blood vessels as well as their life span. The spleen of the host reacts to the infection, besides others, by eliminating both infected and uninfected red blood cells (124). The immune reaction of the infected human can add to the pathogenesis (124).

The reproduction cycles of the parasite in the blood can continue and lead to a chronic infection with the host becoming anemic (124, 138).

P. vivax and *P. ovale* are able to go into a sleeping state in the liver, so-called hypnozoites, and may, unless eradicated by treatment with primaquine, lead to a relapse from weeks up to over a year later (124).

During the cycles of multiplication in the bloodstream, some parasites develop to gametocytes (male microgametes or female macrogametes). These gametocytes can be internalized by a female *Anopheles* mosquito in a bite/ blood meal (figure 4).

Figure 4. Life cycle of the *Plasmodium* parasite in the vector *Anopheles*



In the midgut of the mosquito, a macrogamete and a microgamete can fuse to a zygote, which develops into a mobile ookinete, invades the wall of the gut and develops into an oocyst. The oocyst grows and releases sporozoites which wander to the saliva gland of the vector, ready to be transmitted to a human with the next blood meal of the mosquito (161). Within the mosquito, the whole development period of the *Plasmodium* lasts 7-10 days (146).

Clinical manifestations

The clinical severity of malaria ranges from asymptomatic and mild disease to life-threatening illness. In uncomplicated malaria, the symptoms are unspecific, such as fever, headache, malaise, nausea, vomiting, diarrhea, and muscle pain (123, 146, 158), and the mortality is below 1% (123). Mild, uncomplicated disease can quickly develop to severe illness and death, especially in patients without immunity (139, 146). The symptoms are mainly caused by the breakdown of the erythrocytes and the inhibition

of the bone marrow together with hypoxia, low blood sugar levels and lactic acidosis, also reduced blood circulation due to low blood pressure and, in *P. falciparum* infections, adhesion of the erythrocytes to the walls of the blood vessels (139, 158).

Symptoms of severe malaria are different in children and adults (138) and can, in addition to symptoms mentioned above, include reduced consciousness, cerebral malaria (defined as "coma caused by *P. falciparum*" (138)), prostration, seizures, anemia, utmost fatigue, breathing problems, hypoglycemia, dark or bloody urine, icterus, and severe bleeding (139, 148), with coma, impaired kidney function and acidosis prognostic unfavorable signs (139). Especially at risk for developing severe malaria are infants, children under five years of age, pregnant women, non-immune individuals, and patients infected with HIV, patients suffering of malnutrition and patients with invasive bacterial infection (148, 162).

Severe malaria has a mortality of 10% to 20%, if treated appropriately, and up to 100% if untreated (139, 146). Hence, when malaria symptoms occur, early diagnosis and adequate treatment within 24 to 48 hours is crucial (139, 146). Most cases of severe malaria are due to *P. falciparum*, but it can also be caused by *P. vivax* and *P. knowlesii* (139).

The degree of parasitemia is usually an indication of the severity of the disease (136, 139). Yet, some patients with a high amount of parasites in the blood may have little symptoms, and some patients with a low-density parasitemia may be severely ill, as shown in a study on Tanzanian children (163). This indicates that, in addition to the density of parasites, other influences have an impact on the severity of the infection, such as virulence of the *Plasmodium* and inflammatory reaction of the infected human (163).

P. falciparum infections can become chronic reported lasting up to three years (158). Double infections with malaria and bacterial BSI occur (164, 165). In children, in the long run recurrent and persistent *P. falciparum* and *P. vivax* infections have unfavorable consequences on development and growth (124).

Immunity

Human immunity to malaria means the ability of the host to kill the parasite or to stop the reproduction of it (137). It is sophisticated, and specific to both species and phase of *Plasmodium* (158), and only partly understood (124). It consists of innate and acquired immunity (137, 158), with innate meaning inherited or natural immunity that the host possesses without previous contact with the parasite (137). Certain genetic traits can contribute to the innate immunity, as, for example, the sickle-cell mutations which are found in the genome of many people from sub-Saharan Africa and which lead to sickle-shaped erythrocytes. Humans who are heterozygote carriers of these mutations, are protected against severe malaria and thereby have an evolutionary advantage in malaria endemic regions. However, the homozygotes suffer from serious sickle-cell disease (166).

The acquired immunity can be gained passively, such as in neonates receiving it from their mothers or by administering protective agents, or actively by infection (137) or vaccination. Acquired or adaptive immunity can be divided into the impact on risk and degree of clinical disease in a given amount of parasitemia, the impact on the amount of parasitemia, and in the development of premunition (137). The latter describes a state of subclinical low-grade parasitemia which contributes to protection against new infections (137, 139, 154).

The degree of acquired immunity depends on repeated exposition to the parasite, more precise on the number of bites by infected mosquitoes over time (137, 138). The development and preservation of an effective immunity needs recurrent infections throughout life (158). It is lost within months, for example when leaving endemic areas or when there is no longer transmission in a region (137). In high-endemic regions, young children have the highest risk of a severe disease, whereas most of the older children and young adults will experience a mild disease. The majority of adults will have a subclinical infection due to immunity acquired in earlier infections (138). Reduction of malaria transmission in Africa means that fewer will reach the immunity state of premunition. Consequently, not only the youngest, but individuals of all ages will face a significant risk of developing severe malaria (139).

The immune system may reduce the peak of the parasitemia and may avoid severe illness, however, they frequently are not able to completely get rid of the parasite. This may result in an on-going low-level parasitemia which can last even years (158).

The amount of malaria transmission in a region is commonly estimated by the incidence or the prevalence of malaria infections (146). The number as well as properties of the vector including survival capability and biting behavior have a crucial influence on the transmission (124). Transmission has an impact on the development of protective immunity in the local population. It can be stable, meaning continuous and high during the whole year, which will, over time, frequently lead to protective immunity (124). Here, severe illness is uncommon in those who are over five years old (137). If the transmission is unstable, as in seasonal transmission usually related to the rainy season, effective immunity may not be achieved (124). In many regions in sub-Saharan Africa, including Zanzibar, the transmission pattern has changed from stable high transmission (140) to unstable seasonal low transmission due to effective malaria control programs (124). Here, changes in conditions including climatic variations and weakening of malaria control efforts, can lead to substantial increases of malaria cases and significant death rates in the whole population due to little immunity (124).

Diagnosis

Clinical diagnosis

Clinically the main symptom is fever (124, 167), and it is not possible to differentiate between malaria and febrile illnesses of different etiology such as bacterial and fungal BSIs and viral infections, with the latter both transient or potentially dangerous such as dengue fever (32, 44, 168-172).

Diagnostic tests

Reliable tests for malaria are important for the correct treatment and best outcome. The use of malaria laboratory tests has also been propagated to reduce overtreatment, as probably one third of the children with the clinical diagnosis of severe malaria actually suffer from a different illness, commonly sepsis (136).

False positive test results lead to a misuse of antimalarial medication and may contribute to the development of AMR, whereas false negative results cause higher morbidity and an increase of the death rate (167). In addition, malaria tests are also used for surveillance purposes, especially in connection with malaria control programs (167). In this thesis, the main focus is the use of malaria tests for the diagnosis in symptomatic patients.

Microscopy

Microscopy of a Giemsa stained blood smear, both thick and thin film, is the traditional and standard laboratory method for diagnosing malaria (5, 139, 145, 167, 173). Microscopy needs a microscope and a trained staff (145), however it is relatively cheap and quick (145, 167). Further, it makes species and stage identification and quantification possible and thereby helps to assess the severity of the infection and to evaluate treatment efficacy (167).

Yet, both sensitivity and specificity depend on training and experience. In a very experienced technician, the sensitivity may be as low as 50 parasites per µl (corresponding to 0,001% of the erythrocytes infected) (5, 174), in a well-trained microscopist it is commonly 100-200 parasites per µl (5), whereas a less trained staff may have a tenfold higher threshold compared to a very experienced one (173) leading to considerable risk of false negative results. Sensitivity is assumed to be usually up to 75%, only (145).

Microscopy is also time and labor consuming, and it may need an hour to prepare the slides (173). It does not uncover sequestration of the parasite (167). Mixed infection may be overlooked, and false positive occur due to misinterpretation (167).

Rapid diagnostic tests (RDTs)

Of several methods based on immunodiagnostic, immunochromatographic point-of-care rapid diagnostic tests (RDTs) for malaria are most common and increasingly in use (151, 167), also as part of the worldwide malaria control programs (151). The main advantages of the rapid diagnostic tests are that they are fast and easy to perform which can make them an important tool in low-resource areas with limited access to laboratory facilities and trained technicians (5).

These malaria RDTs are lateral flow devices based on the identification of one or more of certain malaria antigens such as the histidin-rich protein 2 (HRP2) of *P. falciparum*, the human *Plasmodium* lactate dehydrogenase (pan-pLDH), the LDH specific for *P. falciparum* or *P. vivax*, as well as aldolase (145, 167, 175). Dependent on the specific test kit, RDTs may be able to detect one or several *Plasmodium* species (145, 167). Commonly, the reported threshold for detection is around 100 to 200 parasites per µl (174), and not under 50 parasites per µl (176). A substantial number of different RDTs from different manufacturers is available, but the performance of the various kits differs significantly concerning sensitivity and specificity (145, 175). The WHO has published their comprehensive in vitro performance tests of a large number of the commercially available RDTs for *P. falciparum* and *P. vivax* (151, 175). This overview of the quality of the various RDTs has also encouraged several manufacturers to improve their tests (175).

Besides limitations in sensitivity and specificity, a disadvantage of RDTs is the lack of quantitation (151, 167). Also, due to remaining antigen, RDTs can remain positive after treatment and should not be applied for evaluating the effect of therapy (145, 151). Data on the performance of RDTs on *Plasmodium* species other than *P. falciparum* and *P. vivax* are limited (151).

In *P. falciparum*, certain parasites variants lacking the HRP2 antigen can lead to false negative RDTs. These variants have been detected in different regions of the world including mainland Tanzania (125, 177).

Molecular biological methods

Within molecular biological technology for malaria detection, several PCR methods have been developed including conventional PCR, the quicker real-time PCR, nested PCR, quantitative PCR and multiplex PCR (167, 178, 179). PCR has very high sensitivity (178, 180) under five parasites per µl (167), with some variations dependent on the method used (178), yet reported hundred times higher compared to microscopy and RDT (5). In endemic areas, PCR detects twice as many malaria cases compared to microscopy (180). Likewise, the specificity is high (173, 174, 179, 181), and, depending on the specific method applied, both quantitation (178), species

identification (173), and the detection of double or triple infections (182) are possible. Assays are commercially available (182).

Yet, so far performing PCR needs considerably longer time compared to microscopy and RDT. In addition, both an educated staff and expensive equipment is needed. Hence, in the diagnostic of clinical malaria, especially in low-resource areas, it has until now not replaced these methods (5, 180).

Another disadvantage is that PCR can remain positive several weeks after infection has been successfully treated (183). The detected DNA may represent remains of the parasite. Also, there may be a risk of over-diagnosis in clinical settings by detection of low-grade parasitemia which may not be the cause of the acute illness (184).

PCR is regarded especially useful in malaria surveillance in pre-elimination settings (5, 174, 180) and in research (5, 180). Another method based on molecular biological analyses, the loop-mediated isothermal amplification (LAMP), is quicker and less expensive than PCR (127, 185) and may therefore become an alternative to microscopy and RDT (5). Newer approaches of point-of-care use of molecular biological methods in low-resource-settings are under evaluation (186).

Treatment, chemoprophylaxis, vaccine

Treatment

As uncomplicated malaria can rapidly develop into severe life-threatening illness, appropriate therapy should be started as soon as possible, preferably within 24 to 48 hours after the onset of symptoms. If possible, the diagnosis should be confirmed by a diagnostic test to avoid unnecessary treatment (146). The WHO has elaborated detailed treatment guidelines for malaria caused by the different *Plasmodium* species, including recommendations for important at-risk groups such as pregnant women and young children (146). An oral artemisinin-based combination therapy is advocated for uncomplicated *P. falciparum* malaria. Combination therapy with a partner drug in addition to artemisinin is necessary to prevent development of resistance (146). For severe malaria, parenteral artesunate, an artemisinin derivative, is the recommended first-line therapy (124, 139).

Treatment recommendations depend also on the AMR patterns of the parasite in a specific region (139).

Knowledge of the *Plasmodium* species causing the infection is important for choosing the adequate treatment. Primaquine is used against the gametocytes in *P. falciparum* malaria to fight transmission and against the hypnozoites to hinder relapse in *P. vivax* and *P. ovale* infections (139).

Chemoprophylaxis

The WHO recommends chemoprophylaxis to certain vulnerable subgroups in endemic areas, including non-immune travelers (124, 146), and intermittent preventive treatment of pregnant women and young children (146).

1.3 Antimicrobials and bacterial antimicrobial resistance

1.3.1 Historical background and global status

Since millions of years, microbes have produced substances with antibiotic properties as for example the beta-lactams. Likewise, they have gained certain capabilities to resist antimicrobials, for example by the production of certain enzymes as beta-lactamases. In accordance with Darwin's theory of natural selection, these capacities enable them to survive and thrive in the presence of antibiotic substances, both naturally existing and man-made ones (187, 188).

The era of anti-infective treatment started in the first half of the twentieth century, however was soon followed by the development of antimicrobial resistance (figure 5).

Figure 5. New antimicrobials and development AMR over time

From Browne et al. 2020 (189), by courtesy of the authors.

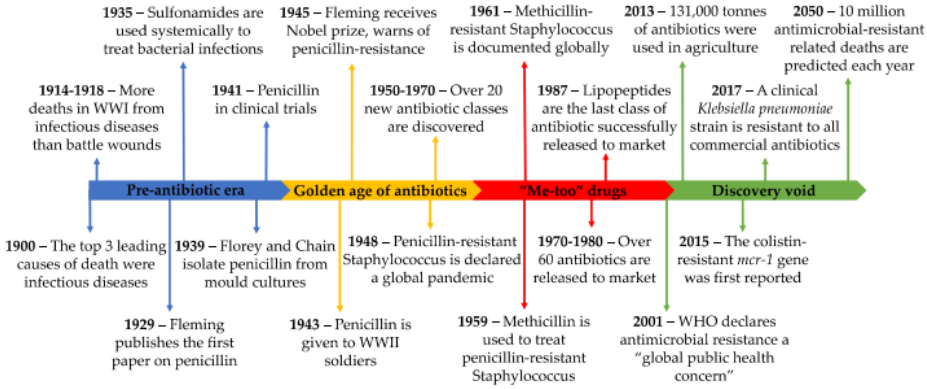


Figure 1. A summary of events in the antibiotic-resistance timeline. WHO, World Health Organization; WWI/II, World War I/II.

On the 17th International Congress in Medicine in 1913, Paul Ehrlich set a major milestone, when he presented his idea to develop "magic bullets", small made-to-measure compounds with antimicrobial activity (190). In 1928, Alexander Fleming observed that the mold *Penicillium* produces a substance "penicillin" with antibacterial property (191), and in the 1930ies Gerhardt Domagk and his group discovered the antimicrobial properties of synthetic sulfonamides (192). It was not before the 1940ies that the enormous clinical effect of penicillin in the treatment of potentially life-threatening infections was proven, and, funded by the government of the United States of America, the pharmaceutical industry developed mass production of penicillin for treating infected soldiers in World War 2 (16, 193). However, already before it was used in patients, Abraham described antimicrobial resistance to penicillin. He discovered that microbes could produce the enzyme penicillinase, a beta-lactamase, which can destroy penicillin by hydrolytically cleaving its beta-lactam ring (17, 194). In 1950, half of *Staphylococcus aureus* strains were resistant to penicillin (195). Penicillinase-stable antimicrobials, including methicillin, were developed, but in 1961 the first methicillin-resistant *S. aureus* (MRSA) was detected (195). Furthermore, already in 1959 and in 1960 different Japanese research groups reported that antimicrobial resistance can be transferred between different bacterial species (196).

However, at that time neither the impact on the spread of antimicrobial resistance nor the global consequences of resistance development were widely acknowledged (17, 196). The time from the mid-1940ies to the mid-1960ies is regarded the “golden age” for detection of antimicrobials. The development of antimicrobial resistance to the existing drugs was met by new antibiotics (197). Compounds with antimicrobial properties were mostly discovered in nature, such as the beta-lactams, and tetracycline. To a minor degree, purely synthetic drugs were manufactured in the laboratory, such as the sulfonamides, and the quinolones. Several new classes of antimicrobials were detected including aminoglycosides, chloramphenicol, tetracycline, macrolides, lincosamides, streptogramin and glycopeptides. In addition, existing classes were chemically altered (198, 199).

Several milestones are worth mentioning regarding the beta-lactam antibiotics, which are our most important antibiotic group with excellent antibacterial activity and relatively mild adverse effects, and belonging to the most frequently used antibiotics (188, 200). In 1961, ampicillin, a broad-spectrum penicillin with effect also on many gram-negative bacteria, was introduced, followed, in 1962, by the cephalosporins, a new subclass of beta-lactams, and, subsequently, by second generation and third generation cephalosporins. The latter are hugely important in the treatment of severe infections, as they are very effective against a broad spectrum of bacteria including an excellent activity against important gram-negative pathogens within the *Enterobacterales*, such as *E. coli* and *Klebsiella* species. In 1985, the carbapenems were introduced into the market, a new beta-lactam subclass possessing outstanding broad-spectrum activity against most gram-positive and gram-negative bacteria (197, 198).

Since the 1940ies, treatment with antimicrobials has reduced mortality of serious infections significantly (198), including a first breakthrough for the treatment of tuberculosis with the development of streptomycin (201). Antibiotics are tremendously important for human health (17), and essential for modern medicine, including neonatal intensive care, organ transplantation, and cancer treatment (199, 202).

However, the development and spread of antimicrobial resistance may endanger some achievements of modern medicine (202).

In gram-negative bacteria, the production of beta-lactamases is considered the most important resistance mechanism directed at beta-lactam antibiotics (200). Earlier, overproduction of inducible chromosomal beta-lactamases in certain gram-negative rods such as *Enterobacter* sp. and *Pseudomonas aeruginosa* was considered worrisome (188). However, transferable beta-lactamases have, over time, proven of even greater significance. In 1965, with TEM-1 the first plasmid-mediated beta-lactamase in gram-negative rods was described. The location on plasmids means that the genes coding for the enzyme can be transferred between different bacterial species, a particularly important and potentially harmful property, as it can lead to significant dissemination of resistance. The enzyme TEM-1, able to hydrolyze ampicillin, was initially isolated in Greece in an *E. coli* growing in the blood culture of a patient with the name Temoniera, but has disseminated worldwide to numerous bacterial species (200, 203). In the 1980ies, the so-called extended-spectrum beta-lactamases (ESBLs) were described for the first time (204, 205). Though there is no unanimity on the exact definition of ESBLs (206), "classical" ESBLs are commonly regarded as certain plasmid-mediated beta-lactamases found within gram-negative bacteria, mostly belonging to the order *Enterobacterales.* They are able to destroy both first, second and third generation cephalosporins as well as monobactams, and can be disabled by beta-lactam inhibitors such as clavulanic acid (207). According to their structure, the several hundred ESBLs can be divided into subgroups such as TEM, SHV, and CTX-M. Previously, TEM-, and SHV-variants were most frequent, but in the later years CTX-M has been globally dominating, worryingly often in combination with multidrug-resistance with co-resistance against other antibiotic classes (188, 200, 208). Another group of concern are plasmid-mediated AmpC enzymes, such as CMY-variants. First described in 1989, they are also broad-spectrum beta-lactamases with activity against 3. generation cephalosporins in *Klebsiella* species and *E. coli* (209). Even more worrying is the spread of plasmid-mediated carbapenemases, especially in important pathogens within the *Enterobacterales* order (including *Klebsiella* sp. and *E. coli*), as well as in *P. aeruginosa*, and in *Acinetobacter* species. These enzymes are

inactivating the carbapenems, and many can, in addition, hydrolyze most of the other beta-lactams (210). Due to a higher occurrence of ESBLs, carbapenems have been increasingly administrated, thereby favoring a selection of resistant strains (208). Since 2001, intercontinental spread, major epidemics and outbreaks of several acquirable carbapenemases have been reported, such as *Klebsiella pneumoniae* carbapenemases (KPC), metallo-beta-lactamases such as the New Delhi metallo-beta-lactamases (NDM), and OXA-carbapenemases, in the *Enterobacterales,* including *Klebsiella pneumoniae* and *E. coli*, as well as in *P. aeruginosa* and *Acinetobacter baumannii* (188, 200, 211).

Further important microbes, problematic because of their AMR, include the gram-positive methicillin-resistant and vancomycin-resistant *S. aureus* (94, 212), and vancomycin-resistant *E. faecium* (94), and MDR and extensive drug-resistant *Mycobacterium tuberculosis* (212-216). MRSA, third generation cephalosporin resistant *E. coli* and *Klebsiella pneumoniae*, and carbapenem-resistant *A. baumanii* and *K. pneumoniae* are leading causes for deaths attributable to AMR (217).

Presumably selected by use of antimicrobials (26), resistant bacterial clones with the ability to cause severe infections have developed, such as extensively drug resistant *S.* Typhi belonging to the 4.3.1 genotype (93). Several of these clones with acquired multidrug-resistance offer little treatment options and have disseminated worldwide, causing epidemics and outbreaks and sometimes life-threatening infections (200). They are defined as high-risk clones due to their antimicrobial resistance patterns and their ability to spread such as certain *E. coli,* and carbapenemase-producing *K. pneumoniae* clones (26, 218).

The lack of therapeutic alternatives in the treatment of MDR gram-negative rods has led to the re-introduction of last-resort antimicrobials such as the polymyxins (colistin and polymyxin B), earlier abandoned because of potentially serious adverse effects (219). Not surprising, plasmid-related colistin resistance has been detected subsequently (220), and geographic overlap of transferable resistance of *E. coli* and *Klebsiella* to carbapenems and polymyxins has been reported, also from African

countries (221). This underlines the threat of potentially untreatable infections caused by highly resistant microbes.

While AMR has increased significantly and spread globally in the recent decades, only a small number of new antimicrobials have been introduced, most of them modifications of known drugs, and very few new classes, such as oxazolidonones, and cyclic lipopeptides (94, 201). Especially for gram-negative bacteria, new effective drugs are lacking (197, 198). Reasons are high development costs and limited profit expectations of the pharmaceutical industry, regulatory challenges and the lack of promising candidates (222). In 2017, the World Health Organization (WHO), highly concerned about a shortage of new antibiotics (223), has published a priority list of critical pathogens, based on resistance patterns and clinical importance, in order both to elaborate plans for combatting their spread, as well as to push research and development of new antimicrobials (94, 224). In this list, the WHO highlighted the so-called ESKAPE germs (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (225), which are particularly important in hospital settings (226).

Antimicrobials have saved numerous lives (198), and their detection is regarded tremendously important for human health (17), but development of AMR may put an end to the success. Some argue that we have already reached a post-antibiotic era (227, 228).

Both in the health sector and in food production the use and partly misuse and overuse of antibiotics has risen hugely in the recent decades, not foreseeing its tremendous impact on microbial selection and the consequential growth of AMR (196). In a multifactorial and interdependent development, the number of resistant microbes has increased dramatically both in the environment, but also in humans (187). In the later years, the global importance of the development and spread of AMR has been globally recognized, and it is now widely regarded a threat to global health (229-231). The WHO ranges AMR within the ten most important health dangers (231). It is a foremost cause of death, a comprehensive analysis estimated that AMR caused 1,27 million deaths globally in the year 2019. Countries with low resources are especially burdened

(217). An expert analysis ordered by the government of the United Kingdom estimated 700,000 deaths per year in 2016 and up to 10 million deaths annually, including over 4 million deaths per year on the African continent, by the year 2050, unless effective steps are taken (232, 233).

The rate of AMR differs significantly in different regions of the world (234). Particularly from low-and middle-income countries data on AMR and its consequences including attributable mortality are limited (234), partly due to little testing and limited laboratory capacities (50), and more studies are needed (234-237) for improving the prevention and treatment of infections (238) and preventing the AMR development (234).

1.3.2 Resistance mechanisms

Biochemical mechanisms of antimicrobial resistance

The biochemical mechanisms of antimicrobial resistance can be divided into three groups (187):

1. Production of enzymes which change or inactivate the antimicrobial. Examples are the hydrolysis of beta-lactams by beta-lactamases, and the alteration of aminoglycosides by aminoglycoside-modifying enzymes.
2. Inhibiting the antimicrobial from entering the microbe or by removing it from the microbe. The former can happen by mutations affecting porins which are the common entrance paths for hydrophilic antibiotics (such as beta-lactams, fluoroquinolones, tetracyclines) through the outer membrane of the bacterium. By contrast, efflux pumps can actively transport antimicrobials such as tetracyclines out of the cell. Even multidrug efflux pumps exist.
3. Alteration of the target of the antimicrobial. Examples are the change of penicillin-binding proteins in MRSA and penicillin-resistant *Streptococcus pneumoniae*, affecting beta-lactam antibiotics, and the change of the glycopeptide target of action in vancomycin-resistant enterococci by acquiring the *van* genes. (239)

In figure 6, the various sites of attack of the antimicrobials and the different biochemical mechanisms of antimicrobial resistance including are demonstrated.

Figure 6. Biochemical mechanisms of antimicrobial resistance showing both the different sites of attack of the antimicrobials as well as the various mechanism of antimicrobial resistance

From Uluseker et al. 2021 (240), by courtesy of the authors. The figure is modified from Wright 2020 (241).

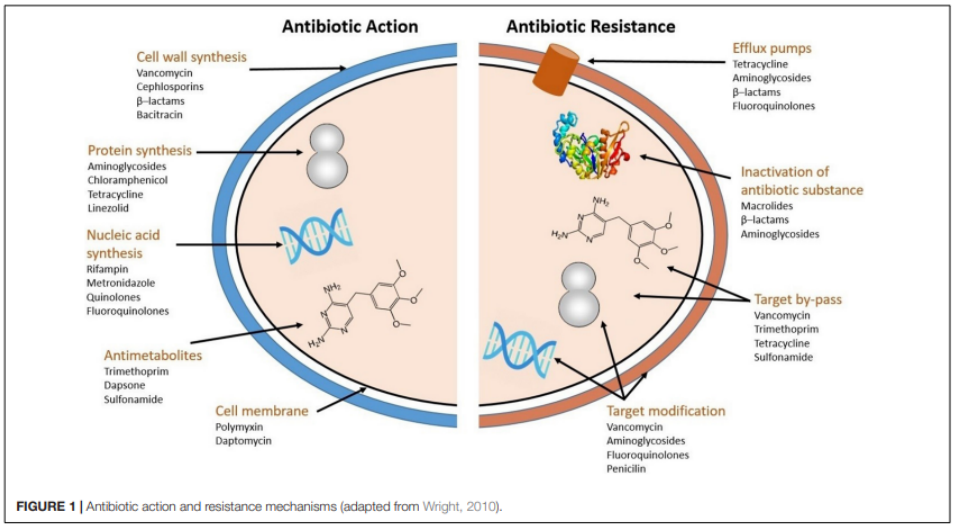


FIGURE 1 | Antibiotic action and resistance mechanisms (adapted from Wright, 2010).

Molecular genetics of antimicrobial resistance

Resistance determinants of microbes are located on genes which again can be localized either on the chromosomes or on transferable elements. Resistance can emerge by single point mutations of one nucleotide base par (as for example in beta-lactamase such as the TEM- and SHV-type of ESBL (203, 242)), by alterations in the expression of existing genes (such as in inducible beta-lactamases in *Enterobacter* spp. (200)) or by gaining preexisting genes via horizontal gene transfer (HGT). In HGT, genes are conveyed from one cell to another by different mechanisms, mostly by conjugation meaning transfer via mobile genetic elements such as plasmids and integrative conjugative elements (ICEs). The latter are also called conjugative transposons. Yet, mobile genetic elements can move genes not only between different microbes, but also within a microbe (226, 243). Furthermore, free DNA can be transferred directly between microbes by a process called transformation. Bacteriophages, a certain type of

virus, can transport genetic material by so-called transduction (243). Use of antimicrobials is assumed to push HGT (243). Together with the vertical transmission of chromosomal mutations via cellular division, HGT is regarded hugely important for AMR development. Even more, HGT is considered especially harmful, since the resistance determinants are effectively spread from one microbe to another, even between different species of microbes (244). Furthermore, HGT is considered to play a highly significant role in the dissemination of multidrug resistance, especially in gram-negative bacteria (245).

1.3.3 Emergence and spread of antimicrobial resistance

If a microbe has intrinsic resistance to an antimicrobial, this property is inherent. For instance, gram-negative bacteria are intrinsically resistant to vancomycin, because their structure includes an outer membrane which vancomycin cannot penetrate. In contrast, acquired resistance means that a formerly sensitive microorganisms becomes resistant. This can happen either by a mutation or by gaining genes coding for resistance via horizontal gene transfer (239).

Any exposure of microbes to antimicrobials will lead to a Darwinian selection process. Organisms possessing or gaining appropriate resistance properties will have better chances to survive, hence their population will increase compared to those lacking these properties (187, 244).

Two main factors are crucial for promoting antimicrobial resistance: the use of antimicrobials and the dissemination of resistance (244).

Use of antimicrobials

Any use of antimicrobials whether in medicine or in food production, both adequate use as well as misuse, will put a Darwinian selection pressure on a microbial population and thereby push the occurrence and spread of antimicrobial resistance (187).

Use of antimicrobials within the human health sector

Antimicrobial treatment has an impact both on the pathogen, but also on the patient's microbial normal flora, notably the gut flora. This increases the risk of AMR development and spread to other people as well as the environment (200).

In a European cross-national study on *E. coli* infections in outpatients, the use of a higher amount of antimicrobials was associated with a higher rate of AMR (246). According to a global study including 76 countries, antimicrobial consumption, measured by defined daily doses, has risen with 65% in the period between the years 2000 and 2015, and the antimicrobial consumption rate (defined daily doses per 1,000 inhabitants per day) has risen with 39%, especially due to increases in low and middle income countries (LMIC) (247). These numbers are deeply worrying as they imply an increased pressure for the development of AMR, especially in LMIC, yet, due to a lack of resources, data from low-resource regions on both consumption and AMR are patchy and needed (235, 247, 248). However, the increasing consumption in LMIC may partly reflect that access to antimicrobials had been inadequately low compared to the high burden of infections (247).

Sub-therapeutic dosage of antimicrobials only suppresses, but does not kill the pathogens, and enhances the development of AMR (187, 249). Poor quality of drugs (202), and counterfeit drugs (250), problems especially present in LMIC (250), may lead to underdosing. In low-resource areas, limited availability of health services may increase self-prescription of antibiotics. Self-prescription and over-the-counter sale of antibiotics can lead to misuse of antimicrobials (wrong indication, dosage, and duration of antimicrobial therapy) and push the development of AMR, as most patients lack professional knowledge on correct indication and use. However, there is no evidence that doctors prescribe more appropriate (250, 251). Over-prescription (202), as found in viral airway infections in primary health care (212), is contributing to overuse. Lack of stewardship programs leads to inadequate use of antimicrobials and increase the risk of AMR development (248).

In poor countries, diagnostic tests may be unavailable (7, 50, 250), or unreliable (as the Widal test for typhoid fever), leading to wrong diagnoses and treatment and thereby to misuse of antimicrobials.

Use of antimicrobials outside of the human health sector

Many antimicrobials are used in animals, thus enhancing the risk of resistance development (244).

Dissemination of AMR

Wherever resistant microbial populations are selected by the exposure to antibiotics, whether in health institutions, in private homes, in food production, or in the pharmaceutical industry, they can be disseminated directly by humans or animals. In addition, the environment such as soil and water can be polluted with drugs via waste containing antimicrobials including pharmaceutical waste, waste from farms, sewage, poor quality of sanitation both in the community and in health-care institutions (187, 244, 252). In hospitals, insufficient control of infections can lead to colonization of patients and health workers, and secondary to infections by resistant microbes (244). In LMIC, the risk of AMR spread is presumably significantly higher due to the lack of access to hand disinfection/washing, and sanitation and to overcrowding both in the community and in health care institutions (250, 251). Factors such as migration and travel, trade and international connectedness contribute substantially to the spread of resistant clones of microorganisms (187, 229, 253, 254).

1.3.4 Consequences of AMR

Already in the beginning of the 20th century, Paul Ehrlich emphasized the importance of "frapper fort et frapper vite", translated "to hit strong and hit quickly", to combat serious diseases (190). In serious infections, early and adequate treatment may save lives as shown in sepsis, where quick appropriate empirical antimicrobial therapy can reduce mortality and is therefore recommended (255-258). However, in infections caused by resistant microbes, standard empirical antibiotic therapy may be inadequate, thereby leading to a delay or even lack of appropriate treatment. The required second line antimicrobial may be less effective, and/or have more side effects (259). As a consequence, the infections may last longer, become more serious, and even have a

higher death rate, as highlighted by the WHO (229). Longer illness including a longer hospital stay, and possibly more expensive drugs can lead to higher costs (229). Furthermore, although indicated in the treatment of certain infections with resistant pathogens, broad-spectrum antimicrobials may be applied too easily and too widely, thereby increasing the risk of selecting resistant strains and further development of antimicrobial resistance (260). Acquiring AMR may also change the virulence of the pathogen (261). The infections may as well spread to a greater extent as the patients may remain infectious for a longer period of time.

In LMIC several factors may add to the consequences of antimicrobial resistance, however, data are limited (235, 248). Microbiological diagnostic and local surveillance data are often lacking, hence clinicians have to rely on empirical treatment solely, which may lead to inadequate treatment (202) and to prolonged disease, increased mortality and costs, and potentially further development of AMR (248). If local surveillance data suggest a high prevalence of AMR, this may lead to empirical treatment with broad-spectrum antimicrobials. Then, if routine laboratory diagnostics, including blood culture and antimicrobial susceptibility testing are not available, adjustments and narrowing of the antimicrobial coverage cannot be based on microbiological data, potentially leading to an unnecessarily long duration of broad-spectrum antibiotic use. This will put an additional selective pressure on the microbial flora of the patient and, secondarily, the environment.
The large number of infections in LMIC may add to both the clinical and the financial impact of antimicrobial resistance. Furthermore, appropriate antimicrobials (second-line drugs) may not be available or too expensive (202).

Consequences of AMR in bloodstream infections

Rapid empirical treatment of bloodstream infections with a suitable antibiotic is presumably important for an optimal outcome of the patient (262). However, earlier publications showed diverging results, though mainly due to diversity of the study populations and weaknesses of the study protocols (263). Two recent large studies and an extensive meta-analysis of patients with bloodstream infections found higher mortality in patients receiving inappropriate empirical antimicrobial therapy compared to those receiving appropriate therapy, with appropriate empirical therapy defined by

in vitro susceptibility of the causative microbe (263-265). One of these publications reported a nine times higher risk of inappropriate treatment in patients with resistant microbes in blood culture compared to those with sensitive pathogens (264), hence underlining the negative impact of antimicrobial resistance.

Correlation AMR and mortality, morbidity and costs

The impact of antimicrobial resistance on the outcome is usually analyzed by observational studies, which, by their nature, are vulnerable to confounding factors. Differences between the patients regarding age, underlying diseases and comorbidities including impairment of the immune system, severity and type of infection, geographical location, hospital- or community-acquired illness as well as different virulence of the various pathogens causing the infection can have an important impact and should be adjusted for. A fatal outcome can, for example, be caused by an underlying disease or by antimicrobial resistance or by the combination of both. Control groups must be defined with caution (259, 261, 265-267). Meta-analyses have the advantage of including large numbers of cases and hence facilitate statistically significant results. However, the included publications must be comparable or must be revised for significant confounding factors (259).

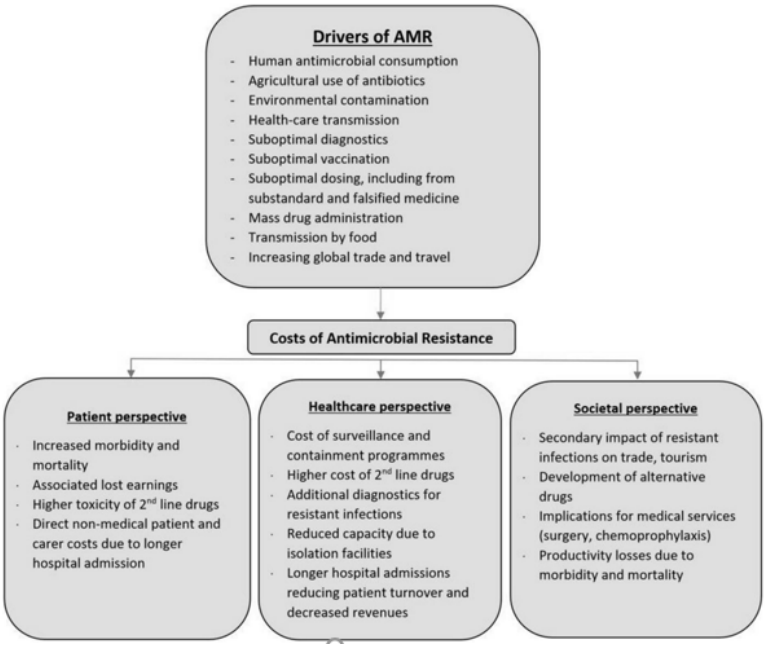
The design of studies on the impact of antimicrobial resistance is very heterogeneous. The results are diverging including varying significance and risk rates, presumably largely due to confounding factors which are not adjusted for correctly (259, 261), or too few cases included in the studies (268). However, a large number of publications have reported a significant association between antimicrobial resistance and an increase of mortality, morbidity (defined as length of hospital stay) and financial expenses (47, 236, 266, 269-273), both in hospital-acquired and in community-associated infections (274). This includes a higher mortality in infections caused by resistant microbes of the above mentioned ESKAPE group in LMIC (235, 238), in infections by carbapenamase-producing bacteria (275-278), as well as in bloodstream infections by ESBL-producing Gram-negative rods (259, 270, 279), including a study from Tanzania (280), and in MRSA bacteremia (268, 270, 281).

1.3.5 Complexity of both forces leading to the development of AMR and of impacts of AMR

In figure 7, the complexity of forces leading to the development of AMR as well as the impact of AMR both on the individual patient, the health care system and the society is illustrated (282).

Figure 7. Drivers and costs associated with antimicrobial resistance

From Shrestha et al. 2018 (282), by courtesy of the authors. Adapted from Holmes et al. (187) and McGowan (283).



As the problem of AMR development and spread is multifactorial and different areas as the health sector, food production as well as the environment are interconnected, a "One Health" approach is advocated where experts from various areas are collaborating on elaborating the best solutions (244).

2 Rationale

Bloodstream infections caused by bacteria, fungi and by *Plasmodium* spp. contribute significantly to morbidity and mortality in LMIC (7, 44, 125, 284). For successful therapy of patients with severe systemic infection, it is essential to establish the etiology, and, in bacterial BSIs, the antibiotic susceptibility of the causative pathogen (47), and, as far as possible, exclude bacterial and fungal BSIs or malaria when the illness is caused by viral or non-infectious causes. In serious infections such as sepsis and bloodstream infections, quick and adequate antimicrobial treatment can be

lifesaving (256-258, 263-265). Without appropriate treatment, a larger proportion of patients with bloodstream infections dies, as demonstrated in patients with bloodstream infections caused by ESBL-producing pathogens (47, 280, 285). Geographically, the etiology of bloodstream infections and the antimicrobial susceptibility patterns of the pathogenic bacteria differ significantly (6, 7, 37). Hence, knowledge on the local etiology and, in bacteria, the antimicrobial susceptibility patterns of the causal pathogens, is important for adequate treatment and for elaborating treatment guidelines (6, 286). In Zanzibar, as in most African countries, data on the etiology of BSIs and, in bacteria, of the AMR patterns of the pathogens causing the infection are lacking (6, 7).

Worldwide spread of AMR threatens effective therapy (217). The situation is especially challenging in low-resource areas as second line drugs are often more expensive or not available (202, 217). Adequate application of antimicrobials is necessary for preventing further development of AMR (202, 217).

Clinical symptoms and signs do not reliably differentiate between bacterial and fungal BSIs, malaria, other BSIs, various zoonoses and viral illnesses. In practice, clinicians are forced to treat patients blindly without any microbiological information.

There is great need for improved information on etiology and drug susceptibility in severe infections in Zanzibar and in LMICs in general. Blood culture is the main tool to investigate etiology in bacterial BSIs (10, 287). For the diagnosis of malaria, microscopy and/or RDTs are mainly used (5, 145, 151, 167), however, the reliability of these tests varies (5, 145, 151). Therefore, we set out to assess the etiology of bacterial and fungal bloodstream infections and the proportion of illness attributable to malaria in patients hospitalized with suspect systemic infection in Zanzibar. Furthermore, analysis of antimicrobial susceptibility patterns can help both the clinician in treating the individual patient appropriately and the responsible authorities in elaborating rational guidelines. An evaluation of malaria RDT and microscopy performance in clinical setting can add important information for the clinician.

3 Hypothesis

1. Among microbes causing bacterial BSI in Zanzibar, there are bacteria carrying clinically important AMR properties such as ESBL-production.

2. Multidrug resistant *S.* Typhi, which are isolated from hospitalized patients with suspected systemic infection in Zanzibar, belong to the 4.3.1 clone prevailing in East Africa.

3. Malaria is still a common cause of illness in patients hospitalized with suspected systemic infection in Zanzibar.

4. Routine malaria tests, RDT and microscopy, have acceptable sensitivity and specificity for the diagnosis of malaria in patients hospitalized with suspected systemic infection in Zanzibar.

4 Aims of the study

The general aim of the PhD project was to assess the etiology of bloodstream infections and the proportions caused by bacteria, fungi, and malaria parasites in patients hospitalized at the pediatric and the medical department of Mnazi Mmoja Hospital (MMH), Zanzibar, and to characterize the antimicrobial resistance of the detected bacteria.

Moreover, this study aimed at investigating the performance of RDT and microscopy in the diagnosis of malaria in hospitalized patients with fever.

The results of the study may help support evidence-based guidelines for rational use of antimicrobials in patients suspicious of having a bacterial BSI. They may also help to plan strategies against the development of AMR. Furthermore, the results may aid to assess how frequent malaria is as a differential diagnosis in hospitalized patients with symptoms of severe systemic infection. Moreover, they may help to choose the most suitable diagnostic tests and treatment of these patients.

The specific aims of the study were

1. To identify the prevalence of the various bacteria, fungi, and malaria parasites in hospitalized patients at MMH, Zanzibar, presenting with fever, or low temperature, or who are otherwise suspected of having a systemic infection.
2. To describe the antimicrobial susceptibility patterns in bacterial isolates causing bloodstream infections in the study population.
3. To describe the sensitivity and specificity of routine malaria tests (rapid diagnostic tests and microscopy) compared with polymerase chain reaction (PCR) in hospitalized patients at MMH presenting with fever, or low temperature, or who are otherwise suspected of having a systemic infection.
4. To describe genetic characteristics of resistant bacteria from Zanzibar and compare them to strains described in studies from other parts of the world.

5 Study populations and methods

5.1 Study settings

The studies were performed at MMH, Zanzibar, Tanzania. Microbiological analyses were conducted both at the Pathology Laboratory Department, MMH, at the Department of Microbiology, Vestre Viken Hospital Trust, Drammen, Norway, and at the Department of Clinical Science, University of Bergen, Bergen, Norway.

5.1.1 Zanzibar

The archipelago of Zanzibar is located in the Indian Ocean close to the equator, about 30 km off the coast of East Africa, and has a size of 2654 square km. It has a tropical climate and two rainy seasons, from March to May and from October to November. The two largest islands, Unguja and Pemba, constitute the greatest part of the archipelago with about two third of the population living on Unguja and about one third on Pemba. In addition, Zanzibar consists of more than 50 small islands. The capital and largest town, Zanzibar city, is located on Unguja. Together with Tanganyika on the mainland, Zanzibar is forming the United Republic of Tanzania. However, Zanzibar is semi-autonomous, with its own president, and an own administrative structure with judicial and legislative power including a Ministry of Health and Social Welfare (288). With an annual per capita income of 1099 USD in the year 2021, Tanzania is economically classified as a lower-middle income country

by the World Bank (289). The United Nations Development Program (UNDP) currently rates Tanzania as number 160 of 192 countries on the human development index (290), (2023 data, https://hdr.undp.org/data-center/documentation-and-downloads).

Within a decade, the population of Zanzibar has increased by about 45% from 1.3 million in 2012 to nearly 1.9 million in 2022. The archipelago accounts for about 3% of Tanzania's population, with over 900,000 Zanzibaris living in urban settings (291, 292). Based on household consumption, the World Bank reports a poverty decrease of 9% for Zanzibar in the period from 2009 to 2019. However, with the distinct population increase at the same time, the number of poor people decreased only slightly (293). The percentage of young people is high, with about 43% under 14 years and 34% between 15 and 34 years in 2014 (294). Tourism is a major source of income (292) and contributes, together with other services, to more than 50% of the gross domestic product, whereas manufacturing, construction and mining add 20% and agriculture together with fishing 18% (288).

In the period from 2012 to 2021, life expectancy at birth in Zanzibar has risen from 60 years (295) to 68 years (66 years for men and 71 years for women) (291). From 2010 to 2015/16, the under-five mortality decreased by 23% to 56 deaths per 1,000 live births. However, the neonatal death rate remained almost unchanged in this period, with 28 deaths per 1,000 live births in 2015/16 compared to 29 in 2010. For comparison, in 2016, Norway had approximately 30-fold lower both under-five mortality (2 per 1000 live births) and neonatal mortality (1 per 1000 live births) (https://data.worldbank.org/indicator/SH.DYN.MORT?locations=NO). In 2016, stunting was found in 24% and underweight in 14% of under five-year-old children in Zanzibar, both indicators for malnutrition. In the same year, infant vaccination coverage by age 24 months was about 81%. Maternal mortality has declined in the last twenty years, but is still high with 267 deaths per 100,000 live births in 2017 (296), which is 100-fold higher than in Norway (297).

Presumably, Zanzibar has a high burden of communicable diseases, although exact data are scarce. As an indicator, Tanzania has a high burden of tuberculosis (298), a

survey performed in 2012 found 293 cases of pulmonary tuberculosis in adults per 100,000 adult population for the whole country, and 124/100,000 for Zanzibar (299). Furthermore, the archipelago has repeatedly suffered from cholera outbreaks (300). According to a survey performed in 2016-2017, HIV prevalence is low in Zanzibar with under 1% compared to 4% in the whole country (291).

5.1.2 Mnazi Mmoja Hospital, Zanzibar

The studies were performed at the main campus of MMH which is located in Zanzibar city on the island Unguja. In the year 2020, MMH reported in total 776 beds distributed over three locations, with the main campus comprising 630 beds (544 beds in the year 2012), divided into 20 clinical departments including internal medicine, pediatrics, obstetrics and gynecology, surgery, and orthopedics. In addition, MMH offers service to outpatients in various specialty clinics. Annually, it serves in average 27,185 inpatients and 74,975 outpatients and performs 12,658 deliveries. It is the only tertiary referral hospital of the Zanzibar archipelago, and offers also primary and secondary care to the local population. It is teaching hospital for the State University of Zanzibar (301, 302).

5.2 Study populations and study periods

In paper I (pilot study) 469 patients (242 males, one patient no information on gender), including 113 neonates (≤1 month, 57 males, one patient no information on gender), 148 younger children (> 1month <5 years, 84 males), 40 older children (≥5 years < 15 years, 25 males), and 168 adults (76 male), admitted to the medical department, the pediatric department and the neonatal department of MMH, were consecutively included over a period of, in total, about eight months (26 March to 22 June 2012, 26 October to 21 December 2012, and 4 February to 22 April 2013), if they, either on admission or during their hospital stay, had fever (>38.2°C in adults, >38.4°C in children) or hypothermia (<36.0°C), or were otherwise suspected to have systemic bacterial infection as judged by the clinician.

For the pilot study, we originally planned a three-month study phase. However, fewer patients than expected were included in the initial study period, so we failed by far to reach the projected number of study participants. In addition, we had repeated

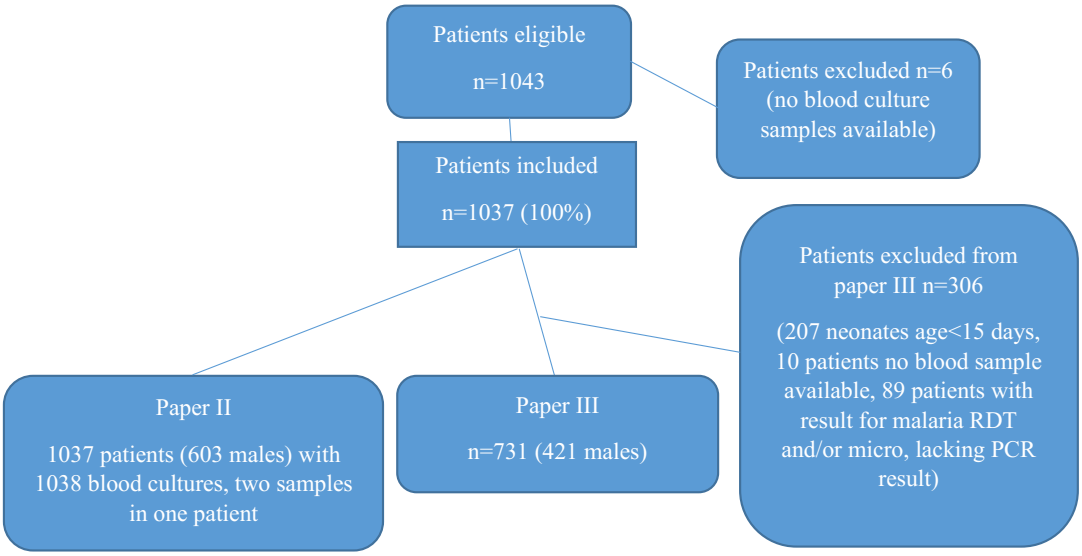
problems with the supply including the blood culture bottles. Therefore, we had to extend the study period twice.

In the papers II and III (main study) 1043 patients were eligible. The patients were consecutively included over a period of 18 months (17 March 2015 to 4 October 2016). The same inclusion criteria as in the pilot study were used. However, in paper III neonates under 15 days of age were excluded.

In paper II the study population consisted of 1037 patients including 220 neonates (≤1 month, 134 males), 285 younger children (> 1 month <5 years, 152 males), 143 older children (≥5 years ≤15 years, 79 males), 386 adults (>15 years, 237 males), and 3 unknown age (1 male) (figure 8).

In paper III, the study population consisted of 731 patients, including 10 neonates (15 days ≤1 month, 6 males), 250 younger children (> 1month <5 years, 135 males), 130 older children (≥5 years ≤ 15 years, 72 males), 338 (>15 years, 207 males), and 3 unknown age (1 male) (figure 8).

Figure 8. Patients included in the main study (paper II and paper III)



5.3 Study designs

Both the pilot study (paper I) and the main study (papers II and III) were prospective observational cross-sectional studies with consecutive inclusion of patients suspected of systemic infection. Both clinical and microbiological information was acquired.

5.4 Methods

5.4.1 Data and sample collection

Both for the pilot study and for the main study, the treating physicians selected the patients according to the inclusion criteria. In each patient, a case report form (questionnaire) (Appendix A, Appendix B) was filled by the physician or nurse. The filled questionnaires were kept in a locked cupboard on the ward. They were then collected by the study investigators (Annette Onken, Melissa Jørstad, Mohammed Khamis Miraji, Marianne Reimers) and kept locked at the Pathology laboratory, MMH. At the same place, the laboratory data were kept locked. A.O. and M. J. transferred the anonymized data to password protected excel files for further data analysis.

The samples (a blood culture sample in the pilot study and the main study, an additional EDTA blood sample in the main study) were obtained by the physician or nurse and sent to the Pathology laboratory at MMH. In the main study, the physician or nurse also performed a malaria RDT and noted the result on the case report form.

Storage and transport of isolated microbes and of blood samples

Study isolates of bacteria and fungi considered pathogens were frozen and stored at minus 20˚C at the microbiological laboratory at MMH in Zanzibar. These isolates were shipped to Norway by a courier company, in ambient temperature (pilot study, in a transport medium for aerobe, anaerobe and fastidious bacteria), or on dry ice (main study). The isolates were then stored at minus 80˚C in the biobank of Haukeland University Hospital, Bergen, Norway (Biobank number 12032, REK Vest number 165.04.). However, some isolates (pilot study 17 of 79 isolates, 22%, and main study 33 of 174 isolates, 19%) are lacking, as they did not survive storage in Zanzibar or the transport to Norway.

The EDTA-blood samples were stored at minus 20°C at MMH and shipped on dry ice to Haukeland University Hospital, Bergen, Norway for malaria PCR to be performed later.

5.4.2 Diagnostic laboratory procedures

Blood culturing and phenotypic identification of blood culture isolates

Both in the pilot study and in the main study, blood was inoculated in BACTEC Myco/F lytic blood culturing vials (one bottle per patient per event) and incubated for seven days at 37°C, and inspected daily (Monday to Friday) and once on either Saturday or Sunday by manual reading as described by Archibald LK, et al (303). In absence of an automated blood culture system, as it was the case in our studies, the vials can be inspected manually for microbial growth using a hand-held ultraviolet lamp (303).

At MMH, positive blood culture samples were analyzed by microscopy of Gram-stained preparations and subcultivated for two days on blood and chocolate agar in 5% CO_2 and on MacConkey agar in aerobe atmosphere. For further identification at MMH, standard biochemical tests (304) including the commercial tests API 20E, API 20NE (bioMerieux, Marcyl'Etoile, France) were used. At VVHT, standard biochemical tests including the commercial tests VITEK 2 (bioMerieux), and, in the main study, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF, Bruker Corporation, Billerica, MA, USA) were used. Both at MMH and VVHT serogrouping of *Salmonella* was performed by omnivalent A-67 and Vi-antigen (sifin diagnostics gmbh, Berlin, Germany).
If the phenotypic identification of the recovered bacteria failed at VVHT, identification by MALDI-TOF mass spectrometry and/or 16SrDNA PCR sequencing was done at Oslo University Hospital and/or the Norwegian Institute of Public Health, Oslo, Norway (paper I).

Phenotypic susceptibility testing of blood culture isolates

Phenotypical susceptibility testing was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (https://www.eucast.org/ast_of_bacteria). Disc diffusion technique (OxoidTM, Basingstoke, United Kingdom) and minimal inhibitory concentration (MIC) gradient

test (BioMerieux in the pilot study, and Liofilchem®, Roseto degli Abruzzi, Italy in the main study) were used both at the laboratories of MMH in Zanzibar and at VVHT in Norway. At VVHT, in addition commercial susceptibility testing with Vitek 2 (BioMerieux) was performed. For the interpretation of the results of the susceptibility testing, the S-I-R system was applied in accordance with the EUCAST guidelines (https://www.eucast.org/ast_of_bacteria).

Molecular biological analyses of blood culture isolates

Polymerase chain reaction (PCR)

Enterobacterales resistant to cefotaxime and/or ceftazidime were analyzed with ESBL CTX-M in-house PCR (305) and AmpC in-house PCR (306), performed at VVHT (Paper I).

Whole genome sequencing of *Salmonella* Typhi isolates (Paper II)

DNA extraction and library preparation

For the *Salmonella* Typhi isolates of the main study, DNA for sequencing was extracted from single colonies using the WizardR genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA library preparation was carried out by using TruSeq™ Nano reagents (Illumina, San Diego, CA, USA).

On the three S. Typhi isolates of the pilot study, genomic DNA was isolated using Invitrogen™ PureLink™ DNA kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. DNA library preparation was performed using the standard protocol of the Nextera XT DNA Library Preparation Kit (Illumina) up to the library amplification.

Short read sequencing

Short read sequencing was carried out on two runs of a MiSeq instrument (Illumina) with 300 bp paired end reads, according to the manufacturer´s instructions.

Long read sequencing

Long-read nanopore sequencing was carried out on six selected *S.* Typhi isolates from the main study (including isolates with various antimicrobial resistance pattern and from both the year 2015 and 2016) as well as the three available *S.* Typhi isolates from

the pilot study (performed in 2012-2013), using the rapid barcoding kit (SQK-RBK004) and MinION sequencer (Oxford Nanopore Technologies Ltd., Oxford, United Kingdom).

Whole genome sequencing was outsourced: for the *S.* Typhi isolates of the main study, the Illumina short read sequencing service was provided by the Norwegian Sequencing Centre (https://www.sequencing.uio.no), a national technology platform hosted by Oslo University Hospital and the University of Oslo, supported by the Research Council of Norway and the Southeastern Regional Health Authorities. The Illumina short read sequencing of three strains of the pilot study as well as the MinION sequencing of the nine isolates (six strains of the main study and three strains of the pilot study) was provided by Akershus University Hospital, Lørenskog, Norway.

Assemblies

The Pathogenwatch online platform (https://pathogenwatch.com) was applied for *de novo* assembling all short-read sequences. In addition, the 58 short-read sequences from the main study were assembled using Bowtie v. 2.3.4.2 (307), Samtools v.1.7 (308) and BCFTools v. 1.9 (308, 309). Here, the largest closed genome representative for one of the two lineages was used as reference.

Unicycler v. 0.4.8 (310) was applied for hybrid assembly, running with Pilon v. 1.23 (311) and Racon v. 1.4.3 (312) for error correction and sequence polishing on 'normal' settings. Of the six isolates of the main study, five were entirely assembled eventuating in one contig, while isolate 50-M123 was returned with 14 contigs. Together with one closed assembly from the different lineages, Medusa v. 1.6 (313) was applied to sort the 14 contigs so that they matched the other *Salmonella* isolates as closely as possible. To align sequence start with the other genomes of the study, manual editing of contigs was subsequently performed on the 50-M123 isolate.

Analysis of antimicrobial resistance determinants, sequence types, and plasmids

For the analysis of antimicrobial resistance determinants and sequence types, we applied ResFinder (v. 4.1) with 90% threshold and 60% coverage, and MLST (v. 2.), both services from the Centre for Genomic Epidemiology server (http://www.genomicepidemiology.org/), respectively. Antimicrobial resistance

determinants and sequence types were also analysed on the Pathogenwatch platform. We used PlasmidFinder (Danish Technical University, Denmark: http://cge.cbs.dtu.dk/services/PlasmidFinder/) to investigate for the presence of plasmids. In the software tools, we applied the threshold default settings.

Genotypic identification

We used the online platform Pathogenwatch (https://pathogen.watch) (314) to assign *S.* Typhi genotypes. The Pathogenwatch tool uses GenoTyphi (code available at https://github.com/katholt/genotyphi) (314). By the GenoTyphi typing scheme the *S.* Typhi population is divided into four major lineages and over 75 clades and sub-clades (117) with the globally disseminated 4.3.1 (H58) genotype further subdivided into lineages I and II (4.3.1.1 and 4.3.1.2) (102).

Phylogenetic analysis

We used Conserved Signature Inserts Phylogeny Server (v. 1.4) (https://cge.food.dtu.dk/services/CSIPhylogeny/) for creating two single nucleotide polymorphism (SNP)-based phylogenetic trees, applying all default values of the software including minimum depth at SNP positions 10x, minimum relative depth at SNP positions 10%, minimum SNP quality 30. For the phylogenetic tree which compared the relatedness of our *S.* Typhi strains, we used the *S.* Typhi strain with accession number ERL12960 of genotype 4.3.1.1, isolated in India in 2012 (315), as a reference genome. In the second tree, we compared the *S.* Typhi sequences of the study isolates with those of 38 published *S.* Typhi WGS sequences from other parts of the world, all belonging to the 4.3.1. genotype (the same genotype as the isolates of our study). For annotating both phylogenetic trees we used the Interactive Tree of Life (v. 5.6.3) (316).

Analysis of the genetic environment of the MDR *S.* Typhi isolates

Here, we annotated the two representative *S.* Typhi from our study (ZNZ13L78 and ZNZ57M188) manually applying a combination of RAST (317), Basic Local Alignment Search Tool (BLAST) (v. 2.11.0) (318), ResFinder (v. 4.1) (319) and MobileElementFinder (v. 1.0.3) from the Centre for Genomic Epidemiology server and in SnapGene (v. 3.3.4) from GSL Biotech (available at snapgene.com). On the chromosomal gene segment of about 25kb of both our two study strains and of the

strain ERL12960, all carrying the multidrug resistance determinants, we performed a comparative analysis using genoPlotr (320) .

Malaria analyses (Paper III)

In the main study, blood for on-site malaria RDT and microscopy as well as blood in EDTA tubes was obtained. PCR and RDT were done on all patients except for neonates under 15 days of age. Microscopy was only performed on clinicians' requests.

Microscopy

Staining of the thick and thin blood film with a 10% Giemsa solution and microscopy were performed according to the procedures of MMH. Microscopy was performed by a trained laboratory technician.

Rapid diagnostic test for malaria

For the major part of the study, we used the RDT "First Response Malaria Ag. LDH/HRP2 Combo Card Test". However, due to stock-out, the alternative RDT, "CareStartTM Malaria HRP2/pLDH (Pf/PAN) Combo Test" was used during the last six weeks of the study. RDT testing was performed according to the instructions of the manufacturers.

PCR

Malaria PCR was performed at Haukeland University Hospital, Bergen, Norway. For DNA extraction from 500 µl whole blood, MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used. For the detection of Plasmodium DNA, a genus-specific PCR with the target *cytochrome b (cytb)* on the mitochondrial genome, and, for quantitation, q-PCR with a customized plasmid, as earlier described, were applied (178). The amount of parasitemia by PCR was measured in copies/µl blood.

In samples positive in genus-specific PCR, species-specific real-time PCRs were performed using the 18 S gene of *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* as

target as earlier described (321, 322). Positive samples, in which the species could not be identified by species-specific PCR, were sequenced as previously described (321).

Quality control

Quality control was performed both internally at the microbiological laboratory of MMH in Zanzibar and at the two collaborating microbiological laboratories in Norway at Vestre Viken Hospital Trust, Drammen, and at Haukeland University Hospital, Bergen. In Zanzibar, the reference strains *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, *S. pneumoniae* ATCC 49619, *Haemophilus influenzae* CCUG 23946 and *Klebsiella pneumoniae* ATCC 700603 ($ESBL_A$-positive) were used for internal quality control routines of both identification and susceptibility testing. All study isolates of bacteria and fungi considered pathogens which survived the transport to Norway were subjected to new identification and susceptibility testing, mainly at Vestre Viken Hospital Trust. Three isolates were re-analyzed at Haukeland University Hospital.

Whole genome sequencing: QIAxpert® (QIAGEN, Valencia, CA, USA) was used for purity control of the DNA extraction of the 58 *S.* Typhi isolates of the main study.

For quality control of the genus-specific malaria PCR, in results with low amplification, defined by cycle threshold values ≥30, re-analysis in triplicates was performed. In disaccord between PCR, RDT or microscopy result, the samples were re-analyzed by PCR including DNA extraction using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Statistical methods

Paper I: To compare differences between proportions, Fisher's exact test was used with cutoff for statistical significance at $p = 0.05$.

Paper II: Descriptive statistical methods (proportions) were applied.

Paper III: Dichotomous variables were evaluated using Chi-square test, and factors with multiple levels (i.e. age groups) were evaluated by binomial logistic regression. Continuous variables such as age and level of parasitemia, were evaluated by pairwise Wilcoxon rank-sum test for two groups and by Kruskal Wallis test for multiple groups.

Analyses were carried out in R version 4.1.2, Rstudio version 2021.09.1 (R Core Team, Vienna, Austria, https:// www.r-project. org/).

Size of study population

The size of the study populations is based on the following sample size calculations: For the pilot study, we had the goal of getting an approximate percentage of bacterial bloodstream infections in febrile patients. In an earlier study in nearby Dar es Salaam, Tanzania, bacterial or fungal pathogens were found in the blood cultures of 14% of children with fever (47). We assumed a similar percentage in the pilot study and had the goal of being able to detect a 50% deviation from this value. For a power of 90% and significance level of 0.05, we determined a sample size of 295 persons. We planned to include 300 patients within a three-month study phase. With growth of pathogens in presumably 10-20% of the blood cultures, we would get 30 to 60 bacterial isolates. We assumed this number could give a reasonable estimate of the prevalence of the most frequent bacteria. As the assumptions for the sample size calculations contain uncertainties, we increased the sample size to n=450.

For the sample size calculations of the main study, we also assumed that we would detect bacterial pathogens in about 14 % of the febrile patients based on the findings of the study from Dar es Salaam as well as our pilot study. We had the goal of being able to detect a 25% deviation from this value (i.e. >17.5% or <10.5%). For a power of 80% and significance level of 0.05, we determined a sample size of 817 persons for giving an approximation of the most frequent bacteria. In addition, we wanted to investigate whether infections caused by bacteria with antimicrobial resistance had a higher mortality. The results of these investigations will be published in a paper not part of this thesis, but is mentioned here as it was part of the rationale for the sample size. With an estimate of 14% of the patients having growth of bacterial pathogens in blood culture, we estimated that in 20-50% of these patients the pathogens may be resistant to empirical treatment based on previous data (47). In this earlier study in Dar es Salaam, the case fatality rates in BSI were 30% and 48% in BSI caused by microbes sensitive or resistant to empirical therapy, respectively. For evaluating whether BSIs with resistant microbes are associated with a higher case-fatality number, we calculated that we needed minimum n=54 patients per group, in total a minimum of

108 patients with bacterial BSIs (aiming at a power of 80% and significance levels of 0.05). Assuming 14% of patients with BSIs, we would need to include n=772 patients to get 108 patients with bacterial bloodstream infections. As the assumptions for the sample size calculations contain uncertainties, we increased the sample size to n=1000.

5.5 Ethical considerations

Informed written consent was obtained from the patient or a responsible family member (for children below 18 years of age), before taking blood for microbiological investigations. Kiswahili, the national language of Tanzania, was used for obtaining consent. An information letter and consent form were developed for children and adults and translated to Kiswahili (Appendix C, Appendix D). In critically ill patients with suspected sepsis or meningitis, a blood specimen was taken without consent, as these investigations are strongly recommended as routine tests in such situations, and since it may be unethical and inappropriate to waste time on formal procedures in such situations. The patient, respectively the patient's responsible family member, were then approached in retrospect for written consent to use the specimen and information for the study. If a patient did not want to participate in the study, he or she did still get the offer of blood culture and (in the main study) of a malaria RDT.

Patients were excluded, if they, or, in the case of children, their responsible family member, declined consent for participation in the study.

The research protocol of paper I was approved by the Zanzibar Medical Research and Ethical Committee (ZAMREC), record no ZAMREC /0004/JAN/012, and by the Regional Committee for Medical Research Ethics Health Region West (REK III), Norway, record no 201124397/2011/2439/REK vest. The research protocol of paper II and III was approved by the Zanzibar Medical Research and Ethical Committee (record no ZAMREC/0002/November/2014, renewal no ZAHREC/02/June/2019/41), and by the Regional Committee for Medical Research Ethics Health Region South East Norway (record no 2014/1940/REK South-East).

6 Main results of the studies

Paper I The pilot study

At MMH, Zanzibar, Tanzania, in 2012/2013, four hundred sixty-nine patients with fever and/or other signs of systemic infection including 113 neonates, 188 children and 168 adults were consecutively enrolled. Clinical information and blood for culture were collected and antimicrobial susceptibility testing of the detected pathogens was performed. Fourteen percent of the patients (66/469) had growth of pathogens from the blood, and 79 pathogenic microbes were isolated. Of those, 75% were gram-negative bacteria, 23% gram-positive and 2% yeast, with *Klebsiella pneumoniae* (14%, 11 isolates), *Escherichia coli* (13%, 10 isolates), *Acinetobacter* spp. (13%, 10 isolates, including six in neonates (data not shown in paper)), and *Staphylococcus aureus* (11%, nine isolates) being the most frequent pathogens. Eighty-five percent (56 patients) of the patients had acquired the infection in the community, 12% (eight patients) in the hospital, in two patients, these data were lacking. Fifty percent (33/66) of all blood cultures with growth of pathogens were from neonates up to one month of age (data not shown in paper). Of the pathogens, 54% (43/79) were from patients belonging to this age group.

In five patients, extended-spectrum beta-lactamase (ESBL) producing *Enterobacterales* (*E. coli*, *K. pneumoniae*) were detected. Three of them were infected in the community, two in the hospital. Three of these five patients died. Of the seven *Salmonella* Typhi, six were multidrug resistant. *Streptococcus pneumoniae* was isolated only once.

Paper II Typhoid fever in Zanzibar (submitted to the journal PLOS Neglected Tropical Diseases)

From March 2015 to October 2016, in a period of 1.5 years, 1037 patients of all ages admitted to MMH, Zanzibar, Tanzania with fever and/or otherwise suspected of severe systemic infection were consecutively taken up into the study, and clinical information and blood for culture were obtained.

Sixteen percent of the patients (161/1037) had growth of pathogens from the blood, and 174 pathogens were isolated, including 153 (88%) Gram-negative bacteria.

The paper includes only data on *Salmonella* Typhi, which was found in 61 patients, accounting for 38% of the pathogens.

However, other frequent findings were *Klebsiella* spp. (n=24, 14% of the pathogens), *E. coli* (n=21, 12%), *Acinetobacter* spp. (n=13, 7%) and *S. aureus* (n=10, 6%). Nine percent (63/731) had malaria. Thirteen percent (138/1038) of the blood culture samples were regarded to be probably contaminated, with coagulase-negative staphylococci most frequent (92%, n=127).

Antimicrobial susceptibility testing was performed on the 61 *S.* Typhi isolates and on additional three *S.* Typhi isolates from the pilot study in 2012/2013. On 58 of the 61 *S.* Typhi isolates of the 2015/2016 study period and on the three *S.* Typhi isolates from the pilot study short read whole genome sequencing was performed, and six selected isolates from 2015/2016 and the three isolates from 2012/2013 were, in addition, analyzed with long read whole genome sequencing.

In 63 of the 64 isolates (98%), multidrug resistance was detected. In MDR isolates, $bla_{TEM\text{-}1B}$, *sul1* and *sul2*, *dfrA7* and *catA1* genes were found. In 69% (43/62), low-level ciprofloxacin resistance was detected, with 41 isolates containing a single gyrase mutation *gyrA*-D*87G*, and with the only non-MDR isolate comprising a single gyrA-*S83F* mutation. All strains were sensitive to ceftriaxone and azithromycin. With all isolates belonging to the 4.3.1 genotype, all MDR strains belong to the 4.3.1 lineage I (4.3.1.1). The multidrug resistance determinants were detected on a composite transposon integrated into the chromosome. In the phylogenetic analysis, the subgroup with both multidrug resistance and low-level ciprofloxacin resistance clustered together with two external strains.

Paper III The malaria study

In a period of 1.5 years from March 2015 to October 2016, seven hundred thirty-one patients admitted to MMH, Zanzibar, Tanzania with fever and/or otherwise suspected of severe systemic infection were consecutively enrolled, including 362 children from

15 days to 14 years of age and 369 adults. Clinical information and blood for malaria testing was collected. The malaria prevalence was analyzed by PCR. Applying PCR as gold standard, the performance of routine diagnostic tests, RDT and microscopy, was studied.

A malaria prevalence of 9% (67/731) was detected. In children under five years of age, the malaria prevalence was significantly lower with 5% (14/260) compared to older children (15%, 20/131, p = 0.001) and patients from 16 to 30 years of age (13%, 15/119, p = 0.02), but did not differ significantly from patients over 30 years of age (6%, 14/217, p = 0.7). *Plasmodium falciparum* was detected in all malaria positive patients, except for one patient with a *Plasmodium ovale* infection. Ten of the malaria cases had no history of recent travel outside Zanzibar. In comparison with PCR, the sensitivity of malaria RDT was 64% (36/56), the specificity 98% (561/575), and the sensitivity of microscopy was 50% (18/36), the specificity 99% (251/254). In patients with false negative RDT and microscopy, the parasitemia was significantly lower (median of 7×10^3 copies/µL, interquartile range (IQR) 2×10^3 – 8×10^4, p = 0.002 in RDT, and of 9×10^3 copies/µL, IQR 8×10^2 – 7×10^4, p = 0.006 in microscopy) in comparison to the parasitemia of patients with true positive results in RDT (median of 2×10^5 copies/µL, IQR 3×10^4 – 5×10^5) and in microscopy (median of 2×10^5 copies/µL, IQR 6×10^4 – 5×10^5).

7 Discussion

7.1 Discussion of the main results

7.1.1 Prevalence and etiology of bloodstream infections at Mnazi Mmoja Hospital

Prevalence of bacterial and fungal bloodstream infections

The pilot study performed in 2012/2013 (paper I) resulted in the first published data on the prevalence of bacteremia and fungemia in Unguja, Zanzibar. We found that bacterial and fungal bloodstream infections were the cause of 14% (pilot study) and 16% (main study) of cases of suspected acute severe infection admitted to hospital.

Previously, only one other study from Zanzibar has been published on bacterial BSI. It was performed in 2009/2010 on the neighbor island Pemba, reporting a prevalence of

4% (166/2209) (120). This lower prevalence compared to our studies may be explained by differences in the study population. First, the Pemba study included both inpatients and outpatients, with outpatients representing by far the majority (80%, 1689/2209) (80). Possibly, fewer of the outpatients suffered from a severe illness such as BSI. Secondly, in Pemba only patients older than two months of age were included. Our studies included neonates, and in the pilot study, fifty percent of the patients with pathogens in blood culture were neonates, and the prevalence of BSI in this subgroup was high (29%) (Table 2).

Table 2: Age distribution of the study population (pilot study and main study) of all included patients and of those with growth of pathogens in blood culture (including unpublished data from the main study)

	Neonate (≤ 1 month)	>1 month <5 years	5 to 15 years	adult	No info on age	Total
Pilot study	113	148	40	168		469
Pilot study with pathogen in blood culture	33 (29%)*	9 (6%)*	8 (20%)*	16 (10%)*		66 (14%)*
Main study	220	285	143	386	3	1037
Main study pathogen in blood culture	41 (19%)*	26 (9%)*	29 (20%)*	64 (17%)*	1	161 (16%)*

* in brackets: percentage of age group with growth of pathogen

At a tertiary hospital in mainland Tanzania in the nearby town of Dar es Salaam, a retrospective study was performed on blood culture findings in the year 2018 including all age groups. Pathogens were detected in 11% of the patients (323). Two prospective studies on younger children admitted to hospital in Dar es Salaam reported a prevalence of bacteremia/fungemia of 14% (255/1828) in 2001/2002 (47) and of 11% (236/2226) in 2017/2018 (324).

In a meta-analysis of community-onset bacterial and fungal BSIs in hospitalized patients in Africa the median prevalence was 15% (range 3.4-38.2%) (40). Another meta-analysis on hospital-based community acquired bacterial or fungal bloodstream infection in Africa reported a total prevalence of 10% (range 4.2-38.2%) with 14% in adults and 8% in children (7). A review on pediatric bacteremia in LMIC detected an overall mean prevalence of 19% (range 12.0-27.5%), and of 16% (range 8.4-24.4%) in Africa (41). All three reviews found a considerable range of numbers. Hence, the prevalence rates found in our two study periods are within the expected span. Possible explanations for the variations may include geographical differences of the burden of disease, different care-seeking behavior of the patients and access to healthcare, varieties in use of antimicrobials prior to blood culture sampling, and various sensitivity of the blood culture methods used.

In the pilot study, the neonates had the highest prevalence of bacterial or fungal BSI (29%), and half of the blood cultures with growth of pathogens were from patients in this age group (data not mentioned in paper) (table 2). In a study on neonates performed in 2009 in Mwanza on mainland Tanzania, the prevalence of positive blood cultures in patients clinically suspected of sepsis was even higher (50%, 149/300, including both early and late onset sepsis) (285). The high prevalence of BSI in this age group may be because neonates are especially vulnerable as their immune system is immature (325). In low-resource areas, insanitary conditions during the delivery and a higher number of home deliveries may add to the risk of infection (325). Also, a higher rate of premature birth in sub-Saharan Africa may contribute, as premature infants are more at risk for severe infections (326). In a global study on sepsis in neonates and young infants (<60 days of age) in LMIC, pathogens were detected in 18% (564/3195) of the patients (327). A review and meta-analysis based on data from sub-Saharan Africa for the period 1980 to 2018, found a neonatal BSI prevalence of 25% (7856/31564) (46). Possible explanations of the variations between the different studies may include differences in the study populations and care-seeking behavior, and geographical variations.

We found pathogens in the blood culture of 6% of children under five years of age, in 20% of older children, and in 10% of adults (table 2). The low number in the younger children may be explained by a high number of febrile illnesses with a different etiology, such as viral infections. However, we have not performed tests for viruses, so this remains speculative.

Prevalence of malaria

Paper III is both the first report on the prevalence of malaria in hospitalized patients in Unguja and the first PCR based study on malaria prevalence in hospitalized patients in Zanzibar. It is based on investigations of the main study in 2015/2016. With PCR we used a highly sensitive method for detecting malaria and discovered a malaria prevalence of 9%.

A study including both in- and outpatients with fever at three hospitals of the neighbor island Pemba in the years 2009-2010, used microscopy and/or RDT and reported a much lower malaria prevalence (1%) (120). The prevalence difference between the study in Pemba and our study may be explained by different sensitivities of the malaria tests, as PCR is a very sensitive method compared to RDT and microscopy (5, 167, 178, 180), and, particularly, by differences in the study populations with inclusion of outpatients, by seasonal variation, and by epidemiological differences between Pemba and Unguja.

Between 2010 and 2015, health facility based studies in both Pemba and Unguja including PCR diagnostic found a prevalence of malaria in febrile patients of up to 3% (154, 328, 329), and of under 2% in febrile outpatients in Unguja (154).

A meta-analysis including 13 studies on community acquired BSIs and malaria in Africa in the years from 1984 to 2006, detected a malaria prevalence of 56% (by microscopy) with a wide range from six to 62 (7). However, the epidemiology of malaria has since changed substantially in the wake of comprehensive antimalarial campaigns.

In the last decades, Zanzibar has changed from a high- to a low-transmission region for malaria (154), with dissemination now mainly related to certain times of the year

and specific areas (153). Considering the low prevalence in febrile outpatients in Unguja as well, our finding of a 9% prevalence in inpatients is higher than one may have expected. Consequently, our data are demonstrating that clinicians have to consider malaria as a relevant differential diagnosis in febrile patients. They also emphasize that constant surveillance and prevention is needed in the local setting, as stressed by the considerable rise of malaria incidence, and of hospitalizations and deaths due to malaria in Zanzibar from 2015 to 2020 (330). In 2021, after intensified efforts, the numbers decreased (331).

In all malaria positive patients of our study, *Plasmodium falciparum* infection was detected, except for one with *Plasmodium ovale* malaria. This finding is in line with community-based studies from Zanzibar from 2012 and 2015, where *P. falciparum* was the dominant species (153, 154).

In our study, older children (15%) and younger adults (13%) had a significantly higher prevalence compared to younger children (5%). Similar data are reported in a study including outpatients with uncomplicated febrile illness in the two largest islands of Zanzibar, Unguja and Pemba (328), and in a mass screening performed in Unguja in 2012 (153). These findings may be partly explained by the lower immunity in the population due to a lower malaria transmission in Zanzibar in the later years (154). The lower malaria prevalence in the youngest could be explained by the use of bed-nets (154, 328).
In 2017 to 2018, in the nearby city Dar es Salaam on mainland Tanzania, in a study on BSIs in febrile hospitalized children under five years of age, *P. falciparum* malaria was detected in 10% of the patients using PCR (324). The difference in prevalence may be explained by geographical variations and differences in the study population of the different hospitals.

Since our study employed PCR, a highly sensitive method, patients with low-grade parasitemia were also detected. Low-grade parasitemia may in some patients represent premunition and may be an accidental finding not causing the acute symptoms (154, 184). In low-transmission areas as Zanzibar, subclinical infections with low parasitemia is common (154, 176). Hence, in some of the study patients with positive

malaria PCR and low-grade parasitemia their febrile illness may have had a different etiology.

Etiology of bacterial and fungal bloodstream infection

The results of the pilot study performed in 2012/2013, are the first published data on the etiology of bacterial and fungal bloodstream infections in Unguja, Zanzibar (paper I). In this period, gram-negative bacteria were predominating with *K. pneumoniae*, *E. coli*, and *Acinetobacter* species on the first three ranks, followed by the gram-positive *S. aureus.*

In contrast, in the 2015/2016 study period *S.* Typhi was the by far most frequent microbe accounting for 38% of the patients with BSIs (paper II) compared to 11% in 2012/2013.

The fact that different pathogens were predominating in the two study periods emphasizes the importance of continuous surveillance of the etiology of BSIs in the local setting.

Similar to our findings in the 2015/2016 study period, the study from Pemba found that *S.* Typhi was the prevailing microbe representing 58% (46/79) of the pathogens (120). *S.* Typhi is a predominant microbe in BSIs in low- and middle-income countries (LMIC) (115) including sub-Saharan Africa (7, 116), especially in children (116). In two studies from north-eastern Tanzania it was the most frequent cause of BSI in younger children (332, 333), in another study from northern Tanzania it was the most frequent pathogen in HIV-negative children with invasive bacterial infection (334). Lack of access to safe drinking water and of functioning sanitation contribute significantly to the risk of acquiring typhoid fever (23, 60).

The Centers for Disease Control and Prevention (CDC), Atlanta, USA, definition of outbreak is “the occurrence of more cases of disease than expected in a given area or among a specific group of people over a particular period of time” (https://www.cdc.gov/). Our study found a very high prevalence of typhoid fever in 2015/16 compared to 2012/2013. We considered this to represent an outbreak of

typhoid fever, warned the health authorities, and informed the local population in a TV interview.

By WGS analysis including phylogenetic mapping, we could show that all 62 study isolates which were accessible to WGS analysis (58 of the main study and three of the pilot study) belong to the 4.3.1 genotype, with all but one part of the lineage I subtype (4.3.1.1), and the remaining one a lineage II subtype (4.3.1.2) (paper II).

Recently, epidemiological analyses based on WGS have added important knowledge on the clonal spread of *S.* Typhi, including a large surveillance in sub-Saharan Africa on strains isolated in 2008-2015. Here, most of the strains (90%) belonged to just three genotypes, with the 4.3.1. genotype representing 40% and the 3.1.1 genotype 39%. Geographically, the 4.3.1 clade (including the subtypes 4.3.1.1 and 4.3.1.2) is limited to eastern and southern Africa, the 3.1.1 clade to West Africa (335). Both lineage I and lineage II of the 4.3.1 genotype have propagated from South Asia to eastern and southern Africa and represent a continuous burden in the region (102, 103, 335-337). In line with our findings, outbreaks caused by the 4.3.1 genotype have been reported from several countries including Malawi, Zambia and Zimbabwe (103, 336, 337). With our results, we have contributed important knowledge that may lead to a better understanding of the spread of *S.* Typhi both locally in Zanzibar and internationally. The findings also show the importance of routine blood culturing not only for the optimal treatment of the individual patient, but also for the surveillance of typhoid fever. Such information may lead to the detection of outbreaks and it may aid to identify and eradicate the source, and to plan countermeasures including vaccination.

Non-typhoid *Salmonella* commonly account for at least half of *Salmonella enterica* isolates causing BSIs in Africa (7, 47). In the pilot study no non-typhoid *Salmonella* were detected. As BSI caused by non-typhoid *Salmonella* is associated with HIV infection (7, 43), the relatively low HIV prevalence in Zanzibar compared to other regions in Africa including mainland Tanzania may be an explanation for the absence of non-typhoid *S. enterica* in our study.

In contrast to the predominance of *S.* Typhi in the main study, the most frequent pathogens in the pilot study were *K. pneumoniae* (14%), *E. coli* (13%), both belonging

to the order *Enterobacterales*, the gram-negative non-fermenting rod *Acinetobacter* species (13%), and *S. aureus* (11%). Gram-negative bacteria accounted for the majority (75%) of the detected pathogens.

The BSI study from Pemba found 6% (5 isolates) *E. coli* and did not report any *Klebsiella* species or *Acinetobacter* (120). One reason for the lack of *Klebsiella* spp. may be that the Pemba study did not include neonates. Seventy-three percent of the *Klebsiella* spp. in the pilot study were isolated from neonates, and they were the most frequent pathogen in this age group. In a study on neonatal sepsis in mainland Tanzania, *K. pneumoniae* was the leading bacteria (34%) (285), and in a meta-analysis on neonatal BSIs in sub-Saharan Africa, *Klebsiella* spp. was the second most common pathogen (21%) (46).

In three reviews on BSIs in Africa (two of them including all age groups, and one study including children aged 1 month to 18 years), *E. coli* was ranked the third or fourth most frequent pathogen accounting for 7% to 11%, whereas *Klebsiella* and *Acinetobacter* species accounting for less than three and two percent of the isolates, respectively (7, 40, 41). For *E. coli*, both the results of the pilot study and of the study from Pemba are close to this range, whereas our *Klebsiella* spp. and *Acinetobacter* spp. results differ.

In accordance with our findings, Tanzanian studies on BSI including younger children (47, 324) and all age groups (323) found that *Klebsiella* spp. was the most frequent pathogen. However, the frequency of *Acinetobacter* spp. (up to 5%) was lower in these studies compared to our results (47, 323, 324). *Acinetobacter* spp. has been shown to cause serious illness (338), especially in tropical climates (338), and has been reported in neonatal bacteremia (339). In a comprehensive South African study on the etiology of BSIs in hospitalized neonates, it was the second most frequent pathogen representing 13% of the pathogens (340), a similar proportion as in the neonates of the pilot study (14%, data not shown in paper).

In a large review and meta-analysis on neonatal sepsis including studies from low- and lower-middle-income countries in Africa and Asia, gram-negative bacteria accounted

for 60% of the pathogens, with *Klebsiella* spp. (38%), *E. coli* (15%), *Pseudomonas* spp. (7%) and *Acinetobacter* spp. (6%) most frequently isolated (341).

S. aureus was the most frequent gram positive (50%, 9/18) and accounted for 11% of all pathogens detected in blood culture in the pilot study. In contrast, in the Pemba study on BSIs, *S. aureus* was on rank two of the most frequent gram positives after *S. pneumoniae* and accounted for 6% of all pathogens (120). Similar to our findings, in three studies on BSI performed in mainland Tanzania, *S. aureus* was the most frequent gram-positive bacteria constituting 10% and 17% (both studies in younger children) and 22% (all age groups) of the pathogens, respectively (47, 323, 324). In a Tanzanian study on neonatal BSI, *S. aureus* was the second most common pathogen (21%) (285).

In reviews on the etiology of BSIs in Africa, the findings are more diverse. One study reports *S. aureus* on rank three of the pathogens, representing 10% of the pathogens (7), whereas in a meta-analysis on BSIs in children it is the most frequent one (18%) (41). In another review and meta-analysis on community-acquired BSI, the frequency of *S. aureus* was not reported, yet it was not among the three most frequent pathogens (40). These variations may be explained by differences in the study populations.

In our study, *S. pneumoniae* was detected only once, accounting for less than two percent of patients with pathogens in blood culture. In contrast, in the study from Pemba, *S. pneumoniae* was the second most frequent pathogen (15%) in BSI (120), in line with the three earlier mentioned large reviews on the etiology of BSIs in Africa which found 13% to 17% prevalence of pneumococci (7, 40, 41). However, our study results align with findings from nearby Dar es Salaam where pneumococci represented 4% of isolates in one study (324), and was not detected at all in two other studies (47, 323). The very low prevalence of these fastidious pathogens in our study could be due to high rates of antimicrobial treatment prior to blood culture sampling, and possibly delay between collection and processing of blood cultures in the laboratory. In contrast to our study, the Pemba study on BSI used resin-containing blood culture bottles which have been shown to perform better in patients under antibiotic therapy compared to bottles without resin, presumably due to the ability of resin to neutralize antibiotics (342). Unfortunately, in most patients we lack information on antibiotic

therapy before blood culture sampling. One may suspect that a considerable number of patients had already taken antimicrobials. This is quite common as antibiotics are easily accessible over-the-counter without prescription in Tanzania. In one of the earlier mentioned studies performed in Dar es Salaam, fifty eight percent of the patients had taken antibiotics before the blood culture was taken (323).
In neighboring Malawi, the proportion of *S. pneumoniae* among pathogens causing BSIs dropped significantly from 20% (1072 isolates) in 2006 to 2009 to 3% (123 isolates) in the period 2014 to 2016 (343). This reduction may be explained by the introduction of the pneumococcal vaccine in 2011 (343). In Zanzibar, vaccination against pneumococci in the first year of life had just been introduced in 2012, the first year of the pilot study (344), and might therefore only have impact on youngest study participants.

6.1.2 Antimicrobial resistance in bacterial bloodstream infection in Zanzibar

Antimicrobial resistance and phylogenetic relatedness in Salmonella Typhi

Multidrug resistance in *S.* Typhi

In both of our study periods, the proportion of multidrug resistance in *S.* Typhi was very high (86% in 2012/2013, and 98% in 2015/2016), limiting the treatment choices of typhoid fever considerably.

In the Pemba study, a lower, but still considerable number of MDR *S.* Typhi was detected (42%, 19/45 isolates) (120). These findings are in accordance with the results of a large global study which detected increasing numbers of MDR *S.* Typhi in Africa since the year 2000, whereas classic multidrug resistance has been decreasing in South Asia (108).

The 4.3.1 *S.* Typhi genotype is associated with a high percentage of multidrug resistance (102). In a large world-wide study, ninety-eight percent of the isolates with acquired multidrug resistance belonged to the 4.3.1 genotype (108). A comprehensive study on African *S.* Typhi isolates found 62% of the 4.3.1 genotype to be MDR (335). Furthermore, the 4.3.1 genotype carrying multidrug resistance has been connected to outbreaks in eastern and southern Africa, including Malawi, Zambia and Zimbabwe

(103, 335, 336). In Tanzania, in isolates from 2011/2012, multidrug resistant *S.* Typhi of the 4.3.1.1 and the 4.3.1.2 sub-lineage were detected, both presumably historically related to isolates from Kenya. In the Tanzanian 4.3.1.1 subgroup, signs for ongoing local expansion were detected (335). Consistent with these findings, all but one of the study isolates are both MDR and belong to the 4.3.1.1 subclade (paper II).

According to a large global study, in the 1990s AMR determinants coding for multidrug resistance have been associated with a certain plasmid (IncHI1), enabling horizontal transfer of AMR (108). Newer studies from Zambia and Bangladesh did not detect this plasmid, but showed that the genes coding for multidrug resistance were located on a transposon integrated in the chromosome (102, 103, 335, 345). In our study, analysis of long and short read sequences demonstrated that the multidrug resistance determinants are located on the chromosome of the bacteria, in line with the findings from Zambia and Bangladesh. A study on a large number of global 4.3.1 genotype isolates of *S.* Typhi isolates found that the amount of plasmid-related multidrug resistance has been decreasing since the year 2000 and that in 2016 the majority (75%) of the genes coding for multidrug resistance were located in the chromosomes of the bacteria (108). This positioning may consume less energy compared to the location on a plasmid and guarantees vertical transmission of this resistance traits, and hence represent an advantage for the bacterium in the Darwinian fight for survival (97, 107, 108, 335).

Ciprofloxaxin-resistance in *S.* Typhi

The majority of the *S.* Typhi study isolates displayed low-level resistance to ciprofloxacin. While 69% of isolates from the main study were ciprofloxacin-resistant, only one of seven isolates from the pilot study had this resistance trait.

In the Pemba study, only one of 45 *S.* Typhi was resistant to ciprofloxacin, with further four isolates showing resistance to nalidixin (120).

Mutations in the quinolone-resistance-determining regions (QRDR) of *S.* Typhi have appeared in all *S.* Typhi genotypes (108). Most predominant genotypes carrying QRDR mutations have originated from southern Asia and have since disseminated

worldwide (108). Yet, AMR determinants linked to fluoroquinolone resistance are more prevalent in the 4.3.1 genotype, compared to other *S.* Typhi, with *gyrA* mutations being most common, and, within the *gyrA* mutations, alterations of the 83 codon (as *gyrA*-S83F) or the 87 codon most frequent (97, 102).

A large international study on genomic epidemiology of *S.* Typhi, found high rates (71%) of fluoroquinolone non-susceptibility in *S.* Typhi in South-East Asia in 2011 (108). Since 2010, multiple QRDR mutations have become more common (108).

In contrast to Asia, fluoroquinolone resistance has previously been rare in Africa (96, 97), although it has been speculated that this could partly be due to the fact that little data were collected (346). Since 2010, the frequency has risen (97, 104, 108). In a large study based on WGS analyses of *S.* Typhi isolates from sub-Saharan Africa from 2010 to 2014, sixteen percent (39/247) were not fully susceptible to ciprofloxacin and carried antimicrobial resistance determinants *gyrA* at codon 83, with the nine isolates from Kenya harboring the same S83F mutation as the one non-MDR *S.* Typhi isolate of our study (335). In a genetic surveillance of *S.* Typhi isolates from a large informal urban settlement in Kenya, in the years 2007 to 2012 16% of the isolates had resistance markers for fluoroquinolone, in 2013 to 2019 46% (347). A study from Rwanda including historical and recent *S.* Typhi isolates found recent emergence of the 4.3.1 genotype, which has become the predominant clade and is highly associated with decreased ciprofloxacin susceptibility, all isolates harboring the gyrA S83Y mutation, and all but one MDR (348), underlining the association of the 4.3.1 genotype with AMR.

Likewise, in our study, low-grade resistance to ciprofloxacin was related to a *gyrA* mutation, with all but one of the resistant isolates carrying a *gyrA*-D87G mutation and belonging to the local MDR/low-level ciprofloxacin resistant (cipR) sub-lineage of the 4.3.1.1 genotype (MDR/cipR 4.3.1.1), and one single non-MDR isolate with a *gyrA*-S83F mutation, belonging to the 4.3.1.2 clade. In East Africa, the *gyrA*-D87G mutation is rare in *S.* Typhi. In Tanzania, it has to our knowledge only been described in two 4.3.1.1 genotype *S.* Typhi strains isolated in the year 2015 from returning travelers (107). In our phylogenetic analysis these two isolates had no single nucleotide

polymorphism (SNP) difference to the MDR/cipR 4.3.1.1 isolates of our study, indicating a very close relatedness. Evaluating the AMR pattern including the genotypic AMR results together with the epidemiological analysis, the study isolates belonging to the local MDR/cipR 4.3.1.1 may represent a new subtype and an outbreak strain, however, this is speculation.

The only study isolate with a different mutation coding for fluoroquinolone resistance, a *gyrA*-S83F point mutation, was non-MDR. It belonged to the 4.3.1.2 subtype and was most closely related to an isolate from Tanzania from the year 2009, which had the same point mutation, but was MDR carrying an IncHI1 plasmid. The second closely related isolates were from Tanzania. They had the identical point mutation, but no multidrug resistance and no plasmid. From Asia, a decline of classical multidrug resistance is related to reduced presence of these plasmids (108). We speculate that the study isolate may have lost the IncHI1 plasmid. The study isolate was more closely related to Indian isolates than to isolates from Kenya and Zambia which may indicate a dissemination of a common ancestor from Asia to Tanzania.

Worryingly, in our study the majority of the isolated *S.* Typhi strains were carrying both multidrug resistance and, in addition, low-grade fluoroquinolone resistance, underlining the high ability of the 4.3.1 genotype to gain AMR. Resistance to fluoroquinolones in *S.* Typhi evolves stepwise with the acquisition of additional mutations coding for fluoroquinolone resistance leading to gradual increase of the MIC. With three mutations, the strain is expected to become high-level fluoroquinolone resistant (96, 107).

Implications of our AMR findings in *S.* Typhi

Our study has important take-home messages regarding diagnosis and treatment of typhoid fever. First, clinicians should maintain high awareness of typhoid fever as a differential diagnosis in acute febrile illness, as it is the predominating pathogen in blood cultures in Zanzibar, and outbreaks may reappear at any time. Second, the findings can guide treatment. In *S.* Typhi with low-level resistance to ciprofloxacin, the drug is presumably still effective if the dose is increased or the treatment prolonged. Use of ciprofloxacin will put additional pressure on *S.* Typhi with the risk

that high-level resistant strains may be selected, leaving azithromycin the only peroral alternative. Ceftriaxone is still effective but has to be given parenteral. Oral third-generation cephalosporins, such as cefixime, are not commonly used in Tanzania, and are associated with poorer outcomes than fluoroquinolones (349). Introduction of easy-to-use oral broad-spectrum cephalosporins might be ecologically hazardous, as it could promote further emergence of AMR.

Our findings underline the need to survey the prevalence and AMR patterns of typhoid fever and to establish effective control measures including vaccination. Currently, important public health challenges of typhoid fever are high-level resistance to ciprofloxacin, and ESBL-production (107) illustrated by the outbreak of XDR ESBL-producing *S.* Typhi in Pakistan where the strains belong to the 4.3.1 genotype (93). Also, azithromycin resistance may emerge, further limiting therapeutic options. Antimicrobial stewardship may contribute to prudent use of antimicrobials and thereby minimize the selection pressure on the local bacteria.

For successful surveillance, a functioning well supplied microbiological laboratory including blood culturing, antimicrobial susceptibility testing with external quality control is important. Whole genome sequencing analyses are valuable tools in the surveillance of *S.* Typhi.

Extended-spectrum beta-lactamase (ESBL) producing Enterobacterales

In the pilot study (paper I), with six isolates in five patients with proven or probable ESBL-producing bacteria, accounting for 39% of all *Klebsiella pneumoniae* and *Escherichia coli* isolates, we documented for the first time bloodstream infections (BSIs) in Zanzibar caused by this difficult-to-treat microbes. Only about half (6/11) of all *K. pneumoniae* were sensitive to third generation cephalosporins, nine of ten *E coli*.

In the Pemba study on BSIs, no ESBL-producing bacteria were found (120). In comparison to Unguja, Pemba is more rural, and antibiotics may be harder to get. Also, it has less connections and exchange with continental Tanzania where a high level of ESBL-positive microbes is recorded, both as colonizers and as cause of infections. In 2010/2011, a study on children under two years of age in Dar es Salaam found an ESBL-carrier rate of 34%, significant higher in hospitalized children (50%)

compared to children in the community (12%) (350), and a study on street children in Mwanza performed in 2015 reported a 32% carrier rate (351). Two studies on hospitalized patients in Dar es Salaam found 56% and 60% ESBL-positive (352, 353).

Similarly, high ESBL rates are detected in infections, including BSIs. In a study performed in 2018 at a tertiary hospital in the neighboring town of Dar es Salaam, sixty-eight percent of the *Enterobacterales* isolates causing BSIs were ESBL-producing (323), and in another study on BSIs in children from four hospitals in Dar es Salaam performed in 2017/2018, over 50% of the gram-negative pathogens were ESBL-positive (324). A review on AMR to third-generation cephalosporins in BSIs in Africa, including studies from 1990 to 2019, found a very high median prevalence of 54% (IQR 24-81) in *Klebsiella* spp. and of 18% (IQR 11-35) in *E. coli* (354). An even higher pooled resistance rate of *Klebsiella* spp. to third-generation cephalosporins of 88% (95%, confidence interval (CI) 72% to 96%) was reported in a large review and meta-analysis on neonatal sepsis in LMIC (341). As an archipelago, Zanzibar has possibly less communication compared to many regions in mainland Africa which may explain the slightly lower ESBL prevalence in BSIs, however, it is still worryingly high.

In the pilot study, ESBL-producing bacteria were detected in three community-acquired infections. This means that these difficult-to-treat microbes are not only disseminated in the hospital, but that they have already spread in the local population. To our knowledge, no other studies documenting infections caused by ESBL-positive microbes in Zanzibar exist. A very high prevalence of gut colonization with extended-spectrum cephalosporin resistant bacteria (91.5%) has been documented in a study on local hotel employees in the year 2018 (355). Although the study group may represent a subgroup which is more exposed to resistant microbes due to their contact to travelers from many parts of the world, this finding may indicate that colonization with ESBL-positive bacteria is not uncommon in the population of Unguja. Over the counter availability of antimicrobial drugs, and unregulated use of antimicrobials in livestock farming may be contributing factors (356, 357).

Of the four ESBL-positive study isolates accessible to PCR-analyses, three were CTX-M PCR positive, the remaining was CMY-PCR positive, consistent with an AmpC betalactamase. The predominance of CTX-M type ESBLs is in line with other studies from Africa (47, 324, 358).

MDR in ESBL

Besides resistance to third generation cephalosporins and ampicillin, all of the six ESBL-positive bacteria isolated in the pilot study were resistant to trimethoprim-sulfamethoxazole. In addition, half of these microbes were also resistant to gentamicin which means that they were MDR, defined as resistance to at least three classes of antimicrobials. This is in line with previous reports that the plasmids of ESBL-producing microbes often carry AMR determinants for other classes of antimicrobials such as aminoglycosides and trimethoprim-sulfamethoxazole (207), limiting the treatment choices considerably. Two of the six isolates (33%) were resistant to ciprofloxacin, one (17%) to piperacillin-tazobactam. No resistance to meropenem was found.

Implications of our findings on ESBL-producing strains in *Enterobacterales*

At the time of the study, the standard first-line treatment of patients with BSIs at MMH was either the third generation cephalosporin ceftriaxone, or the combination of the aminoglycoside gentamicin with penicillin or ampicillin. Considering the 50% gentamicin co-resistance of the study's ESBL-producing bacteria, maximally half of these patients would have been covered by the standard treatment (the gentamicin plus penicillin/ampicillin regimen provided), leaving carbapenems with their broad antimicrobial spectrum as the "drug-of-choice" (207, 359).

In the study setting, carbapenems were not routinely available, which means that patients with BSIs caused by ESBL-producing microbes were potentially untreatable. Consequently, treatment recommendations should presumably include the more costly carbapenems. Yet, the challenging question is, in which situation they should be applied. With no routine blood culture diagnostic accessible it is impossible to detect infections caused by these resistant microbes. Hence, there is a great danger of misuse

and overuse of these broad-spectrum antibiotics, potentially leading to further development of AMR including the selection of carbapenemase-producing strains. In Zanzibar, carbapenemase-producing strains have not yet been detected, in contrast to mainland Tanzania (360-362).

The implementation of blood culture diagnostics, the surveillance of AMR, education on the correct use of antimicrobials and an antimicrobial stewardship program are tools to counteract this development. A review on antimicrobial stewardship in LMIC emphasizes accessibility of diagnostic testing, education of health personal on antimicrobial resistance and the performance of easy to perform studies for measuring the effect of interventions, as important points (363). Such an antimicrobial stewardship program should probably include restrictive usage of certain antimicrobials (364), such as carbapenems, following the WHO recommendations (https://www.who.int/publications/i/item/WHO-MHP-HPS-EML-2023.04).

Further findings of antimicrobial resistance

AMR in *Staphylococcus aureus*

Among the nine *S. aureus* isolates of the pilot study, no methicillin-resistant *S. aureus* (MRSA) was detected in line to the study on BSIs from Pemba (120). In contrast, in mainland Tanzania two studies on BSIs from Dar es Salaam found a considerable amount of MRSA (26% and 40% of the S. aureus, respectively) (323, 324). Likewise, two reviews on AMR in BSIs in Africa found high numbers of MRSA (30%, including pediatric studies from 1990 to 2019, and 18%, including studies from 2008 to 2019) (41, 49).

AMR in *S. pneumoniae*

In the pilot study, the only *S. pneumoniae* strain isolated was resistant to oxacillin, hence indicating resistance mechanisms to beta-lactam antibiotics. Unfortunately, the strain was not accessible to further testing. Yet, our finding is not contradicting the results of the Pemba study, where a considerable number of *S. pneumoniae* (25%, 3/12) were resistant to penicillin (120). In contrast, a review and meta-analysis on

BSIs in children found little beta-lactam resistance in pneumococci from Africa, with 93% susceptible to ampicillin, and 100% to third-generation cephalosporins (41).

7.1.3 Malaria routine test evaluation

To distinguish malaria from BSI or febrile diseases of other etiology, quick, reliable, and, especially in low-resource areas, affordable and easy-to-use tests are needed both for diagnosing and for ruling out malaria. The WHO in vitro evaluations of malaria are useful (175). Yet, the malaria epidemiology in different populations varies significantly, and these differences may have an impact on the clinical performance of a test (145).

Our study contributes by assessing the in vivo performance of RDT as well as microscopy, both routine malaria diagnostic tests, in a low-transmission area for malaria (154) with the study population consisting of hospitalized patients with fever, by using PCR as gold standard (paper III).

Microscopy's role as gold standard for evaluation of RDTs (145), has recently been challenged by PCR (177, 328). Due to its superior sensitivity, we chose PCR as gold standard for this study.

We selected RDTs based on local availability, detection of both HRP2 and LDH antigen and satisfying score in the WHO evaluation. The score for finding 200 *P. falciparum* per µl was 85% and 90% for RDT First Response Malaria Ag. LDH/HRP2 Combo Card Test and for CareStartTM Malaria HRP2/pLDH (Pf/PAN) Combo Test, respectively (365).
Whereas we found a high specificity of both RDT and microscopy (98% and 99%, respectively), the sensitivity was a relatively low (64% for the RDT and 50% for microscopy) compared to PCR, meaning a considerably higher detection rate of malaria by PCR.
An earlier PCR based RDT evaluation from Zanzibar found a higher sensitivity of 77% (328) using a test with a higher score in the WHO evaluation (96% sensitivity, Paracheck Pf Test, Orchid Biomedical Systems, Goa, India) (365). Yet, the PCR tests used as reference in the different studies may have different lower detection limits which may have an impact on the comparability of the study results. The PCR assay

applied in our study has been shown to be very sensitive (178) which may, to some extent, be an explanation for the relatively low sensitivity of the RDT.
We found that only half of the PCR positive cases were detected by microscopy. This is in accordance with the results of a review comparing the performance of microscopy with PCR in malaria diagnosis in endemic regions (180). There is correlation between the severity of the disease and the degree of parasitemia (136, 139, 176). In our study, false negative results in RDT and microscopy were associated with low-grade parasitemia in PCR. These findings may, at least in some patients, represent asymptomatic parasitemia, and not be related to the actual illness (184). Conversely, in the clinical diagnostic of symptomatic patients, the use of very sensitive methods as PCR may lead to overtreatment of patients (184). Less sensitive methods as RDT and microscopy may be more adequate in the diagnostic of clinical malaria.
Yet, it is concerning that false negative results in both RDTs and microscopy also occurred in some study patients with high-level parasitemia, similar to two other studies from Zanzibar (177, 366).
It has been speculated that the lack of sensitivity of RDTs could be due to the presence of *P. falciparum* variants with HRP2-deletions (177, 366). However, the RDTs used in our study should, in addition to HRP2, also detect parasite LDH. Hence, the lack of sensitivity of the RDTs in our study is not explained by *P. falciparum* variants with HRP2-deletions. In some cases, mistakes of the staff when performing the RDTs could explain the false negative result of the RDT (328, 366). Another explanation could be the prozone effect meaning a false negative test result caused by an overabundance of antigen. Yet, in malaria, this phenomenon has mainly been reported in patients with extremely high parasitemia (328).
In malaria microscopy, sensitivity depends on training and experience of the person performing it, and the quality of microscope and slides (145, 167). In our study, these points may have contributed to false negative microscopy results in patients with high-level parasitemia by PCR.

In summary, we showed that both RDTs and microscopy are relatively reliable in the assessment of febrile patients in the local setting. Yet, our data also highlight that the

clinician must evaluate the test results with caution and must be particularly aware of the limited negative predictive value of these tests.

For routine use in LMIC, PCR needs too much resources and is too slow. Although faster, less costly and easier to-use, loop-mediated isothermal amplification (LAMP) (153, 177) still requires considerable resources (5). For research and surveillance, highly sensitive molecular methods are excellent (5, 178) and superior to both RDT and microscopy. This includes the detection of asymptomatic low-grade parasitemia as source for transmission (154, 177), which was possibly one reason for the increasing malaria numbers in Zanzibar in 2015-2020 (330).

7.1.4 Import of malaria and autochthonous transmission

Knowledge of the epidemiology of malaria, including the relative importance of imported cases and autochthonous transmission, is key in the fight against malaria (367).

In malaria PCR positive patients, we found a significantly higher probability of travel outside of Zanzibar within the past six months (47%) compared to those with negative PCR (6%) (paper III). This is in line with a study on malaria dynamics in Zanzibar in the years 2015 to 2020 which found a positive travel history (defined as journey outside the archipelago in the past month) in more than 40% of the malaria cases (330). In the local fight against malaria it has been emphasized to concentrate on imported cases (367). However, about 50% (10/19) of the malaria positive patients of our study had not left Zanzibar implying that autochthonous transmission contributes significantly to malaria infections in Zanzibar. Similarly, a recently published study estimates that the majority (56%) of new cases in Unguja is indigenous (367). This finding is supported by genetic investigations on *P. falciparum* parasites from Zanzibar which found evidence of import as well as of indigenous transmission (368, 369) (the latter a not yet peer reviewed preprint). Hence, local control measures should target both ways of transmission (369, 370), as Zanzibar remains an area with a population susceptive to infection and capable vectors (369).

7.1.5 Comparison of clinical outcome in bloodstream infections with ESBL+ and ESBL- strains

In the pilot study, sixty percent (three of 5) of the patients with ESBL-producing *E. coli* and *K. pneumoniae* in blood culture died, in contrast to 36% (four of 11) of patients with ESBL-negative *E. coli* and *K. pneumoniae* (paper I). However, the difference in mortality was not statistically significant (p=0.6). In contrast, two Tanzanian blood culture studies, on children and on neonates, respectively, found a significant association between BSIs caused by ESBL-producing pathogens and a fatal outcome (280, 285).

The higher mortality in patients with ESBL-positive pathogens is possibly explained by a higher risk of inappropriate treatment. The lack of significance in our findings could be due to the small numbers of patients. Hence, more data including a larger number of patients may lead to a statistically significant result.

7.2 Discussion of methods

7.2.1 Study design

Choice of inclusion criteria

We aimed at investigating patients with BSIs caused by bacteria, fungi, or malaria. The definition of a BSI is based on a laboratory finding combined with clinical symptoms of overt infection. For an appropriate selection of patients, we chose typical clinical symptoms for a systemic infection, including fever, hypothermia, or be "otherwise suspected to have systemic bacterial infection as judged by the clinician". Hence, the inclusion criteria consisted of both measurable criteria (high or low body temperature) and a subjective one.

Fever is an unspecific symptom. It can be caused by a large spectrum of illnesses from infections caused by bacteria, including microbes requiring special culture methods as mycobacteria, by fungal, viral and parasitical infections, encompassing innocent transient viral infections and potentially life-threatening diseases as bloodstream infections (44, 170), and also by non-infectious illnesses as auto-immune diseases and inflammatory conditions (371). Yet, acute undifferentiated fever is frequently used in studies investigating the etiology of severe systemic infections as BSIs caused by

bacteria, fungi and malaria (44, 170), therefore we considered it an appropriate criterion.

In bloodstream infections, high and low body temperature can occur (3, 372), hence we included also low body temperature as inclusion criterion.

Some patients, such as neonates, patients with impaired immune system and old people, may have normal body temperature, although they are suffering from a serious systemic infection (325, 372). Hence, we included the subjective criterion "or otherwise suspected of having a systemic infection by the clinician".

As all clinical doctors could include patients, the experience of the responsible clinicians varied. We tried to minimize the risk of false patient selection by repeated review of the interpretation of inclusion criteria with the study clinicians. However, other diseases than systemic infections may give similar clinical symptoms, and it can be impossible to distinguish, even for an experienced clinician. In summary, the subjective criterion may have biased the selection of study patients.

Presumably, some selection bias occurred. However, the prevalence of pathogens in blood culture in the pilot study (14%) and in the main study (16%) is comparable with the prevalence reported in other studies on BSIs. This may be regarded as an indicator that the study population is sufficiently representative for the target population (373).

We did not take malaria tests from neonates (under 15 days of age) for two reasons: we assumed that they had a low risk for malaria due to maternal protection, and we wanted to avoid taking unnecessary blood volume from these vulnerable babies.

7.2.2 Microbiological methods

Blood culture

We used blood culture for detection of the etiology of bacterial bloodstream infections, as it is the standard and reference method for detection of bacteremia (10, 287, 372). Whereas it takes longer time than the newer molecular biological approaches (374, 375), it has the advantage that broad antimicrobial susceptibility testing can be performed on the detected pathogen.

For blood culturing, automated blood systems are the standard in high-resource areas, while manual blood culture systems often are used in many LMIC. Manual systems are labor intensive due to need for daily evaluation for microbial growth, but widely used due to lower cost (287). The sensitivity of these manual blood cultures may vary, especially regarding more fastidious microbes as *Haemophilus influenzae* and *S. pneumoniae* (7). Because of limited resources we used bottles produced for both automated systems and for manual reading, placed them in an incubator and checked them manually for microbial growth (303). This blood culturing system was also chosen because it supports growth of fungi and mycobacteria, in addition to common bacterial pathogens. An evaluation of this method concluded that it is sensitive for the detection of bacteria, including fastidious pathogens as *S. pneumoniae*, and fungi (303). Yet, another study suggests that it is not excellent for the detection of *S. aureus* and *S. pneumoniae* (376).

Phenotypic identification and antimicrobial resistance tests

For the identification of bacteria and fungi, we used routine laboratory methods. Antimicrobial susceptibility testing was performed according to the standardized rules of EUCAST (https://www.eucast.org). To improve the quality of the investigations performed at MMH in Zanzibar, we reanalyzed the accessible study isolates at the microbiological laboratory of VVHT, Norway, which is accredited by the Norwegian authorities.

Usually, AMR testing by disc diffusion is valid and sufficient in frequently detected bacterial pathogens. However, in some cases additional tests as MIC-strips are required, as no breakpoints for disc diffusion exist, as for azithromycin in *Salmonella* (https://www.eucast.org). Also, *Salmonella* ciprofloxacin low-level resistance cannot be reliably detected by ciprofloxacin disc diffusion, but needs either MIC tests or pefloxacin disc diffusion test (https://www.eucast.org) (377).

We chose to use the EUCAST guidelines for AMR testing and interpretation, as they are accessible online free of charge (https://www.eucast.org) in contrast to the Clinical and Laboratory Standards Institute (CLSI) guidelines (https://clsi.org/standards/products/microbiology/documents/).

7.2.3 Whole genome sequencing

Whole genome sequencing (WGS) has in the later years become an increasingly useful method as one analysis basically provides a broad spectrum of information including identification, investigation of genes coding for antimicrobial resistance and virulence and epidemiological mapping (314, 378-383). Challenges of WGS are high expenses including advanced equipment, the need of expertise within bioinformatics and long turnaround time (381, 384), yet newer developments including advanced tools have reduced both cost and time for completion (379). Initially used for research purpose, in the later years it has become more applicable within Public Health including outbreak investigations (314, 379, 382).

The large number of *S.* Typhi isolated during the main study, consistent with an outbreak, and their worrying AMR patterns combined with awareness on the global spread of certain genotypes associated with AMR, led to our decision to add WGS analysis to the investigations of the study strains. We aimed at further characterization including mapping the AMR determinants and at epidemiological analyses.

Short-read sequencing is precise, but it utilizes small fragments minor in size as compared to several repetitive elements of the genome of bacteria. Therefore, it is not possible to define the exact position of genes, for example to detect whether they are localized on a plasmid or are part of the chromosome. In contrast, long-read sequencing is less accurate, but it enables to assemble the whole bacterial genome as it uses long fragments (101, 310, 384). An open access online platform enables a so-called hybrid assembly where the long-read sequences are used to build a scaffold into which the accurate short-read sequences can be added (310). Hence, we added long read sequencing on selected *S.* Typhi isolates, performed a hybrid assembly and could show that the multidrug resistance determinants are located on the chromosome, as previously demonstrated for example on strains from Zambia (103).

The quality of WGS results depends on several factors including the quality of sequencing data, the platforms applied and the evaluation of the findings. Discordance between genotypic AMR analysis by WGS and phenotypic findings occurs, and WGS may lead to false AMR results (385, 386). The significance of unknown mutations

cannot be predicted (383), and genes may be expressed differently, which may have an impact on the phenotypic AMR pattern of a microbe (387).

In order to get reliable quality, we used large, well established platforms, including different tools offered by the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/), for testing of antimicrobial resistance determinants, for plasmid analysis, identification, sequence typing, and epidemiologic analyses. For confirmation, we performed the analyses on AMR determinants and sequence typing also on the Pathogenwatch platform (https://pathogenwatch.com) and got the same results. Pathogenwatch is a free accessible online platform which has been designed for the surveillance of *S.* Typhi. It represents a valuable and well-documented tool both for identifying AMR determinants and for comparing local data with global public data on genomes, creating a context between local data and world-wide patterns, and possibly detecting high-risk clones (314).

The detected genotypic AMR predictors in *S.* Typhi were in line with the results of phenotypic susceptibility testing. This is consistent with previous findings on *S.* Typhi (314).

8 Strengths and limitations

8.1 Strengths

1. This is the first study on the etiology and prevalence of BSIs caused by bacteria, fungi and of malaria, as well as the AMR patterns of the bacterial pathogens detected in hospitalized patients from Unguja, Zanzibar. Hence it is adding valuable data in an area where knowledge is both limited and needed.

2. By using advanced methods such as WGS, this study adds important information on the local dissemination of *S.* Typhi including prevailing antimicrobial resistance patterns and contributes to the global surveillance of typhoid fever especially of the 4.3.1 clone and to the spread of antimicrobial resistance. The *S.* Typhi sequence data of the study are uploaded to open access databases (paper II), enabling researchers to use them for further studies (388).

3. Our study adds important data on the in vivo performance of standard diagnostic malaria tests, RDT and microscopy, in symptomatic patients in the local setting by using PCR, a very sensitive method, as gold standard.

4. The study data revealed autochthonous malaria transmission in an area with presumed good malaria control.

5. The study findings identified an outbreak of typhoid fever in Zanzibar. We alerted the health authorities, and we informed the local population via a TV interview.

8.2 Limitations

1. Although originally planned, we decided to leave out investigations for BSIs caused by *Mycobacteria* and dengue virus, mainly due to limited resources. Furthermore, we did not have resources to perform testing for other febrile illness, including zoonoses caused by the bacteria *Rickettsia, Leptospira*, *Borrelia* spp. and *Coxiella burnetii* (44, 170, 389, 390), infections caused by various viruses (170, 391), and by blood parasites other than malaria (170), as well as non-infectious etiology of fever (371) was not investigated. Innocent transient viral infections have presumably caused fever in some of our study patients, especially in children (171), yet we lack investigations.

2. In many study patients, important clinical information is lacking including antimicrobial treatment, both before and after the blood culture sample was taken, and, in most of the patients of the pilot study, the outcome. Reasons are weaknesses in the design of the case report forms, and incompletely filled forms. Presumably, by a better design of the case report forms and by having dedicated persons following up the forms continuously, we could have gained better information.

3. As we used a single blood culture bottle per patient, growth of low pathogenic microbes belonging to normal skin flora or the environment was defined as contamination. In some cases, these microbes might indeed represent relevant pathogens causing BSI. This could have been uncovered, if they had been detected in more than one blood culture taken by separate venipunctures (287).

4. Common reasons for contamination are probably insufficient skin antisepsis and inappropriate technique when taking the BC (287). We found 11% (pilot study, paper I) and 13% (main study, data not yet published) of the blood culture samples to be contaminated. From high-income countries lower rates of up to 10% are reported (287). Yet, our numbers are similar or even lower compared to publications from different African countries (287, 392, 393). Commonly a contamination rate of under three percent is recommended (287), as these samples are lost for diagnostics and add unnecessary costs. Several measures have been shown to reduce the number of contaminated cultures (287). Presumably, by better training of the staff we could have reached a lower contamination rate.

5. Our quality control at VVHT in Norway uncovered that some of the identifications and susceptibility tests performed at MMH were incorrect, but the majority was correct. Most noteworthy is the weak performance of the meropenem disc during the main study, including eight of the 61 *S.* Typhi isolates, which were falsely categorized as resistant at MMH. These problems could be significantly reduced, possibly even avoided, if quality control of AMR testing would be performed frequently.

9 Conclusions

In these first data on bacterial and fungal bloodstream infections in Unguja, Zanzibar, the high number of multi-drug resistant and low-grade ciprofloxacin resistant *S.* Typhi imply few therapeutic choices in typhoid fever. The discovery of community-acquired ESBL-producing bacteria causing bloodstream infections is highly concerning, as it demonstrates that these difficult-to treat microbes have spread in the general population. In effect, infections by these bacteria can be incurable in the local situation.

The study results demonstrate that malaria still represents a relevant differential diagnosis in patients with fever in Zanzibar. In the routine diagnostic of malaria, RDTs and microscopy are helpful tools, yet, negative results must be assessed with caution.

The whole genome sequencing findings on the *S.* Typhi isolates add to the understanding of the local and global spread of the 4.3.1 genotype, which is associated with multidrug resistance.

The results can aid in developing local treatment guidelines and suggest that prevention measures against typhoid fever are needed, including vaccination.

10 Further recommendations

The study results point out the need for evidence based antimicrobial treatment, based on local etiology and susceptibility data. To avoid further escalation of AMR prudent and restrictive antibiotic use is needed. Ciprofloxacin may be losing ground in the treatment of typhoid, in refractory cases (or proven resistance) azithromycin or cephalosporins may be the only options. The introduction of "reserve" broad-spectrum antibiotics such as carbapenems has to be considered including guidance on criteria for when they can be applied.

The study results underline the need of further surveillance of the etiology of BSIs and AMR patterns of the bacteria and of setting up plans of action to combat further dissemination of AMR.

A sufficiently supplied microbiological laboratory including blood culture diagnostic and AMR testing is needed both for surveillance, for antibiotic stewardship, and for the optimal treatment of the individual patient (27, 287).

Reliable, affordable, easy to perform rapid diagnostic tests including typhoid fever and malaria should be developed.

Further research is necessary looking for various etiology of severe febrile illness requiring different management such as more fastidious and difficult to detect microbes including *Mycobacterium* spp., *Leptospira, Rickettsia* spp. (44, 170), various viruses including dengue (170), and various parasites (44, 170), as well as co-infections (44, 389, 391), and noninfectious etiology (371).

11 References

1. Timsit JF, Ruppé E, Barbier F, Tabah A, Bassetti M. Bloodstream infections in critically ill patients: an expert statement. Intensive Care Med. 2020;46(2):266-84.
2. Kontula KS, Skogberg K, Ollgren J, Järvinen A, Lyytikäinen O. Early deaths associated with community-acquired and healthcare-associated bloodstream infections: a population-based study, Finland, 2004 to 2018. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2022;27(36).
3. Network CsNHS. Bloodstream Infection Event (Central Line-Associated Bloodstream Infection and Non-central Line Associated Bloodstream Infection): Centers for Disease Control and Prevention; 2023 [30.01.2023]. Available from: https://www.cdc.gov/nhsn/pdfs/pscmanual/4psc_clabscurrent.pdf.
4. Murray P RK, Pfaller M. Laboratory Diagnosis of Bacterial Diseases. Medical Microbiology. 9th ed: Elsevier; 2020. p. 161-8.e1.
5. Oyegoke OO, Maharaj L, Akoniyon OP, Kwoji I, Roux AT, Adewumi TS, et al. Malaria diagnostic methods with the elimination goal in view. Parasitol Res. 2022;121(7):1867-85.
6. Kern WV, Rieg S. Burden of bacterial bloodstream infection-a brief update on epidemiology and significance of multidrug-resistant pathogens. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2020;26(2):151-7.
7. Reddy EA, Shaw AV, Crump JA. Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. Lancet Infect Dis. 2010;10(6):417-32.
8. Pays E, Vanhollebeke B, Uzureau P, Lecordier L, Pérez-Morga D. The molecular arms race between African trypanosomes and humans. Nat Rev Microbiol. 2014;12(8):575-84.
9. Michel CS, Teschner D, Wagner EM, Theobald M, Radsak MP. Diagnostic value of sTREM-1, IL-8, PCT, and CRP in febrile neutropenia after autologous stem cell transplantation. Ann Hematol. 2017;96(12):2095-101.
10. Reimer LG, Wilson ML, Weinstein MP. Update on detection of bacteremia and fungemia. Clinical microbiology reviews. 1997;10(3):444-65.
11. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest. 1992;101(6):1644-55.
12. van der Poll T WW. Sepsis and Septic Shock. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 9th ed: Elsevier; 2020. p. 990-1008.e3.
13. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). Jama. 2016;315(8):801-10.
14. McNamara JF, Righi E, Wright H, Hartel GF, Harris PNA, Paterson DL. Long-term morbidity and mortality following bloodstream infection: A systematic literature review. J Infect. 2018;77(1):1-8.
15. Laupland KB, Church DL. Population-based epidemiology and microbiology of community-onset bloodstream infections. Clinical microbiology reviews. 2014;27(4):647-64.
16. Spellberg B. Principles of antiinfective therapy. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 9th edition ed: Elsevier Inc.; 2020. p. 211-21.e2.
17. Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010;74(3):417-33.

18. Selbie FR. Microbial resistance to chemotherapeutic drugs. Br Med Bull. 1946;4(4):267-71.
19. Kowalska-Krochmal B, Dudek-Wicher R. The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. Pathogens. 2021;10(2).
20. Matuschek E, Brown DF, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2014;20(4):O255-66.
21. EUCAST. New definitions of S, I and R from 2019: European Comittee on Antimicrobial Susceptibility Testing (EUCAST); 2019 [Available from: https://www.eucast.org/newsiandr/.
22. Nabal Díaz SG, Algara Robles O, García-Lechuz Moya JM. New definitions of susceptibility categories EUCAST 2019: clinic application. Rev Esp Quimioter. 2022;35 Suppl 3(Suppl 3):84-8.
23. Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ. Typhoid fever. The New England journal of medicine. 2002;347(22):1770-82.
24. Dyson ZA, Klemm EJ, Palmer S, Dougan G. Antibiotic Resistance and Typhoid. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2019;68(Supplement_2):S165-s70.
25. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2012;18(3):268-81.
26. Peirano G, Chen L, Kreiswirth BN, Pitout JDD. Emerging Antimicrobial-Resistant High-Risk Klebsiella pneumoniae Clones ST307 and ST147. Antimicrob Agents Chemother. 2020;64(10).
27. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 2022;400(10369):2221-48.
28. Global Health Estimates 2019: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2019. Geneva, World Health Organization; 2020.: World Health Organization, Geneva, Switzerland; 2020 [Available from: https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-leading-causes-of-death.
29. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 2020;396(10258):1204-22.
30. Levels and Trends in Child Mortality Report 2020: United Nations Children's Fund; 2020 [Available from: https://www.unicef.org/media/79371/file/UN-IGME-child-mortality-report-2020.pdf.pdf.
31. Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, et al. Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet. 2015;385(9966):430-40.
32. Feikin DR, Olack B, Bigogo GM, Audi A, Cosmas L, Aura B, et al. The burden of common infectious disease syndromes at the clinic and household level from population-based surveillance in rural and urban Kenya. PloS one. 2011;6(1):e16085.
33. WHO Sustainable Development Goals Target 3.3 Communicable Diseases: WHO, Geneva, Switzerland; 2023 [cited 2023. Available from: https://www.who.int/data/gho/data/themes/topics/sdg-target-3_3-communicable-diseases.
34. Goto M, Al-Hasan MN. Overall burden of bloodstream infection and nosocomial bloodstream infection in North America and Europe. Clinical microbiology and infection : the

official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2013;19(6):501-9.
35. Fleischmann-Struzek C, Goldfarb DM, Schlattmann P, Schlapbach LJ, Reinhart K, Kissoon N. The global burden of paediatric and neonatal sepsis: a systematic review. Lancet Respir Med. 2018;6(3):223-30.
36. Wolf BH, Ikeogu MO, Vos ET. Effect of nutritional and HIV status on bacteraemia in Zimbabwean children who died at home. Eur J Pediatr. 1995;154(4):299-303.
37. Becker JU, Theodosis C, Jacob ST, Wira CR, Groce NE. Surviving sepsis in low-income and middle-income countries: new directions for care and research. The Lancet Infectious Diseases. 2009;9(9):577-82.
38. Evans JA, Adusei A, Timmann C, May J, Mack D, Agbenyega T, et al. High mortality of infant bacteraemia clinically indistinguishable from severe malaria. Qjm. 2004;97(9):591-7.
39. Diekema DJ, Hsueh PR, Mendes RE, Pfaller MA, Rolston KV, Sader HS, Jones RN. The Microbiology of Bloodstream Infection: 20-Year Trends from the SENTRY Antimicrobial Surveillance Program. Antimicrob Agents Chemother. 2019;63(7).
40. Marchello CS, Dale AP, Pisharody S, Rubach MP, Crump JA. A Systematic Review and Meta-analysis of the Prevalence of Community-Onset Bloodstream Infections among Hospitalized Patients in Africa and Asia. Antimicrob Agents Chemother. 2019;64(1).
41. Droz N, Hsia Y, Ellis S, Dramowski A, Sharland M, Basmaci R. Bacterial pathogens and resistance causing community acquired paediatric bloodstream infections in low- and middle-income countries: a systematic review and meta-analysis. Antimicrob Resist Infect Control. 2019;8:207.
42. Uche IV, MacLennan CA, Saul A. A Systematic Review of the Incidence, Risk Factors and Case Fatality Rates of Invasive Nontyphoidal Salmonella (iNTS) Disease in Africa (1966 to 2014). PLoS neglected tropical diseases. 2017;11(1):e0005118.
43. Archibald LK, den Dulk MO, Pallangyo KJ, Reller LB. Fatal Mycobacterium tuberculosis bloodstream infections in febrile hospitalized adults in Dar es Salaam, Tanzania. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1998;26(2):290-6.
44. Maze MJ, Bassat Q, Feasey NA, Mandomando I, Musicha P, Crump JA. The epidemiology of febrile illness in sub-Saharan Africa: implications for diagnosis and management. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2018;24(8):808-14.
45. Pavlinac PB, Lokken EM, Walson JL, Richardson BA, Crump JA, John-Stewart GC. Mycobacterium tuberculosis bacteremia in adults and children: a systematic review and meta-analysis. Int J Tuberc Lung Dis. 2016;20(7):895-902.
46. Okomo U, Akpalu ENK, Le Doare K, Roca A, Cousens S, Jarde A, et al. Aetiology of invasive bacterial infection and antimicrobial resistance in neonates in sub-Saharan Africa: a systematic review and meta-analysis in line with the STROBE-NI reporting guidelines. Lancet Infect Dis. 2019;19(11):1219-34.
47. Blomberg B, Manji KP, Urassa WK, Tamim BS, Mwakagile DS, Jureen R, et al. Antimicrobial resistance predicts death in Tanzanian children with bloodstream infections: a prospective cohort study. BMC infectious diseases. 2007;7:43.
48. McKay R, Bamford C. Community- versus healthcare-acquired bloodstream infections at Groote Schuur Hospital, Cape Town, South Africa. S Afr Med J. 2015;105(5):363-9.
49. Haindongo EH, Ndakolo D, Hedimbi M, Vainio O, Hakanen A, Vuopio J. Antimicrobial resistance prevalence of Escherichia coli and Staphylococcus aureus amongst bacteremic patients in Africa: a systematic review. J Glob Antimicrob Resist. 2023;32:35-43.

50. Williams PCM, Isaacs D, Berkley JA. Antimicrobial resistance among children in sub-Saharan Africa. Lancet Infect Dis. 2018;18(2):e33-e44.
51. Pocket book of hospital care for children: World Health Organization, Geneva, Switzerland; 2013 [2nd:[Available from: https://iris.who.int/bitstream/handle/10665/81170/9789241548373_eng.pdf?sequence=1.
52. Recommendations for management of common childhood conditions: World Health Organization, Geneva, Switzerland; 2012 [Available from: https://iris.who.int/bitstream/handle/10665/44774/9789241502825_eng.pdf?sequence=1.
53. Louis PCA. Recherches anatomiques, pathologiques et thérapeutiques sur la maladie connue sous les noms de fièvre typhoïde, putride, adynamique, ataxique... comparée avec les maladies aigues les plus ordinaires. 1841 [Available from: https://gallica.bnf.fr/ark:/12148/bpt6k5445514c/f41.double.
54. Etymologia: Typhus. Emerging infectious diseases. 2009;15(6):977.
55. Budd W. TYPHOID FEVER ITS NATURE, MODE OF SPREADING, AND PREVENTION. Am J Public Health (N Y). 1918;8(8):610-2.
56. Barnett R. Typhoid fever. Lancet. 2016;388(10059):2467.
57. Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. Salmonella nomenclature. Journal of clinical microbiology. 2000;38(7):2465-7.
58. Xie L, Ming L, Ding M, Deng L, Liu M, Cong Y. Paratyphoid Fever A: Infection and Prevention. Frontiers in microbiology. 2022;13:945235.
59. Parry CM, Wijedoru L, Arjyal A, Baker S. The utility of diagnostic tests for enteric fever in endemic locations. Expert review of anti-infective therapy. 2011;9(6):711-25.
60. Crump JA, Sjolund-Karlsson M, Gordon MA, Parry CM. Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive Salmonella Infections. Clinical microbiology reviews. 2015;28(4):901-37.
61. Pakkanen SH, Kantele JM, Kantele A. Cross-reactive immune response induced by the Vi capsular polysaccharide typhoid vaccine against Salmonella Paratyphi strains. Scand J Immunol. 2014;79(3):222-9.
62. de Jong HK, Parry CM, van der Poll T, Wiersinga WJ. Host-pathogen interaction in invasive Salmonellosis. PLoS Pathog. 2012;8(10):e1002933.
63. Parkhill J, Dougan G, James KD, Thomson NR, Pickard D, Wain J, et al. Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18. Nature. 2001;413(6858):848-52.
64. Kidgell C, Reichard U, Wain J, Linz B, Torpdahl M, Dougan G, Achtman M. Salmonella typhi, the causative agent of typhoid fever, is approximately 50,000 years old. Infect Genet Evol. 2002;2(1):39-45.
65. Roumagnac P, Weill FX, Dolecek C, Baker S, Brisse S, Chinh NT, et al. Evolutionary history of Salmonella typhi. Science (New York, NY). 2006;314(5803):1301-4.
66. Blaser MJ, Newman LS. A review of human salmonellosis: I. Infective dose. Rev Infect Dis. 1982;4(6):1096-106.
67. Awofisayo-Okuyelu A, McCarthy N, Mgbakor I, Hall I. Incubation period of typhoidal salmonellosis: a systematic review and meta-analysis of outbreaks and experimental studies occurring over the last century. BMC infectious diseases. 2018;18(1):483.
68. Johnson R, Mylona E, Frankel G. Typhoidal Salmonella: Distinctive virulence factors and pathogenesis. Cell Microbiol. 2018;20(9):e12939.
69. Cruz Espinoza LM, McCreedy E, Holm M, Im J, Mogeni OD, Parajulee P, et al. Occurrence of Typhoid Fever Complications and Their Relation to Duration of Illness Preceding Hospitalization: A Systematic Literature Review and Meta-analysis. Clinical

infectious diseases : an official publication of the Infectious Diseases Society of America. 2019;69(Suppl 6):S435-s48.
70. Gaines LM. Mortality and Prevalence of Typhoid Fever. Atlanta J Rec Med. 1913;60(2):54-60.
71. Pieters Z, Saad NJ, Antillón M, Pitzer VE, Bilcke J. Case Fatality Rate of Enteric Fever in Endemic Countries: A Systematic Review and Meta-analysis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2018;67(4):628-38.
72. Marchello CS, Birkhold M, Crump JA. Complications and mortality of typhoid fever: A global systematic review and meta-analysis. J Infect. 2020;81(6):902-10.
73. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis. 2019;19(4):369-81.
74. Gonzalez-Escobedo G, Marshall JM, Gunn JS. Chronic and acute infection of the gall bladder by Salmonella Typhi: understanding the carrier state. Nat Rev Microbiol. 2011;9(1):9-14.
75. Vogelsang TM, BØE J. Temporary and chronic carriers of Salmonella typhi and Salmonella paratyphi B. J Hyg (Lond). 1948;46(3):252-61.
76. Crump JA, Heyderman RS. A Perspective on Invasive Salmonella Disease in Africa. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2015;61 Suppl 4(Suppl 4):S235-40.
77. Baker S, Blohmke CJ, Maes M, Johnston PI, Darton TC. The Current Status of Enteric Fever Diagnostics and Implications for Disease Control. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2020;71(Suppl 2):S64-s70.
78. Antillon M, Saad NJ, Baker S, Pollard AJ, Pitzer VE. The Relationship Between Blood Sample Volume and Diagnostic Sensitivity of Blood Culture for Typhoid and Paratyphoid Fever: A Systematic Review and Meta-Analysis. The Journal of infectious diseases. 2018;218(suppl_4):S255-s67.
79. Mogasale V, Ramani E, Mogasale VV, Park J. What proportion of Salmonella Typhi cases are detected by blood culture? A systematic literature review. Annals of clinical microbiology and antimicrobials. 2016;15(1):32.
80. Thriemer K, Ley B, Ame SS, Deen JL, Pak GD, Chang NY, et al. Clinical and epidemiological features of typhoid fever in Pemba, Zanzibar: assessment of the performance of the WHO case definitions. PloS one. 2012;7(12):e51823.
81. Parry CM, Hoa NT, Diep TS, Wain J, Chinh NT, Vinh H, et al. Value of a single-tube widal test in diagnosis of typhoid fever in Vietnam. Journal of clinical microbiology. 1999;37(9):2882-6.
82. Wain J, Hendriksen RS, Mikoleit ML, Keddy KH, Ochiai RL. Typhoid fever. Lancet. 2015;385(9973):1136-45.
83. Wijedoru L, Mallett S, Parry CM. Rapid diagnostic tests for typhoid and paratyphoid (enteric) fever. The Cochrane database of systematic reviews. 2017;5(5):Cd008892.
84. Sapkota J, Roberts T, Basnyat B, Baker S, Hampton LM, Dittrich S. Diagnostics for Typhoid Fever: Current Perspectives and Future Outlooks for Product Development and Access. Open Forum Infect Dis. 2023;10(Suppl 1):S17-s20.
85. Najib MA, Mustaffa KMF, Ong EBB, Selvam K, Khalid MF, Awang MS, et al. Performance of Immunodiagnostic Tests for Typhoid Fever: A Systematic Review and Meta-Analysis. Pathogens. 2021;10(9).
86. Baker S, Favorov M, Dougan G. Searching for the elusive typhoid diagnostic. BMC infectious diseases. 2010;10:45.
87. Camprubí-Ferrer D, Cobuccio L, Van Den Broucke S, Balerdi-Sarasola L, Genton B, Bottieau E, et al. Clinical evaluation of BioFire® multiplex-PCR panel for acute

undifferentiated febrile illnesses in travellers: a prospective multicentre study. J Travel Med. 2023;30(3).
88. Mather RG, Hopkins H, Parry CM, Dittrich S. Redefining typhoid diagnosis: what would an improved test need to look like? BMJ Glob Health. 2019;4(5):e001831.
89. Bhutta ZA. Impact of age and drug resistance on mortality in typhoid fever. Arch Dis Child. 1996;75(3):214-7.
90. Murdoch DA, Banatvaia N, Bone A, Shoismatulloev BI, Ward LR, Threlfall EJ. Epidemic ciprofloxacin-resistant Salmonella typhi in Tajikistan. Lancet. 1998;351(9099):339.
91. Le TA, Fabre L, Roumagnac P, Grimont PA, Scavizzi MR, Weill FX. Clonal expansion and microevolution of quinolone-resistant Salmonella enterica serotype typhi in Vietnam from 1996 to 2004. Journal of clinical microbiology. 2007;45(11):3485-92.
92. Parry CM, Qamar FN, Rijal S, McCann N, Baker S, Basnyat B. What Should We Be Recommending for the Treatment of Enteric Fever? Open Forum Infect Dis. 2023;10(Suppl 1):S26-s31.
93. Klemm EJ, Shakoor S, Page AJ, Qamar FN, Judge K, Saeed DK, et al. Emergence of an Extensively Drug-Resistant Salmonella enterica Serovar Typhi Clone Harboring a Promiscuous Plasmid Encoding Resistance to Fluoroquinolones and Third-Generation Cephalosporins. mBio. 2018;9(1).
94. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18(3):318-27.
95. Bhutta ZA. Current concepts in the diagnosis and treatment of typhoid fever. Bmj. 2006;333(7558):78-82.
96. Britto CD, Wong VK, Dougan G, Pollard AJ. A systematic review of antimicrobial resistance in Salmonella enterica serovar Typhi, the etiological agent of typhoid. PLoS neglected tropical diseases. 2018;12(10):e0006779.
97. Carey ME, Dyson ZA, Ingle DJ, Amir A, Aworh MK, Chattaway MA, et al. Global diversity and antimicrobial resistance of typhoid fever pathogens: Insights from a meta-analysis of 13,000 Salmonella Typhi genomes. eLife. 2023;12.
98. Phan MD, Kidgell C, Nair S, Holt KE, Turner AK, Hinds J, et al. Variation in Salmonella enterica serovar typhi IncHI1 plasmids during the global spread of resistant typhoid fever. Antimicrob Agents Chemother. 2009;53(2):716-27.
99. Kariuki S, Revathi G, Kiiru J, Mengo DM, Mwituria J, Muyodi J, et al. Typhoid in Kenya is associated with a dominant multidrug-resistant Salmonella enterica serovar Typhi haplotype that is also widespread in Southeast Asia. Journal of clinical microbiology. 2010;48(6):2171-6.
100. Holt KE, Phan MD, Baker S, Duy PT, Nga TV, Nair S, et al. Emergence of a globally dominant IncHI1 plasmid type associated with multiple drug resistant typhoid. PLoS neglected tropical diseases. 2011;5(7):e1245.
101. Ashton PM, Nair S, Dallman T, Rubino S, Rabsch W, Mwaigwisya S, et al. MinION nanopore sequencing identifies the position and structure of a bacterial antibiotic resistance island. Nature biotechnology. 2015;33(3):296-300.
102. Wong VK, Baker S, Pickard DJ, Parkhill J, Page AJ, Feasey NA, et al. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of Salmonella Typhi identifies inter- and intracontinental transmission events. Nature genetics. 2015;47(6):632-9.
103. Hendriksen RS, Leekitcharoenphon P, Lukjancenko O, Lukwesa-Musyani C, Tambatamba B, Mwaba J, et al. Genomic signature of multidrug-resistant Salmonella enterica serovar typhi isolates related to a massive outbreak in Zambia between 2010 and 2012. Journal of clinical microbiology. 2015;53(1):262-72.

104. Al-Emran HM, Eibach D, Krumkamp R, Ali M, Baker S, Biggs HM, et al. A Multicountry Molecular Analysis of Salmonella enterica Serovar Typhi With Reduced Susceptibility to Ciprofloxacin in Sub-Saharan Africa. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2016;62 Suppl 1:S42-6.
105. Hopkins KL, Davies RH, Threlfall EJ. Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. International journal of antimicrobial agents. 2005;25(5):358-73.
106. Tadesse G, Tessema TS, Beyene G, Aseffa A. Molecular epidemiology of fluoroquinolone resistant Salmonella in Africa: A systematic review and meta-analysis. PloS one. 2018;13(2):e0192575.
107. Ingle DJ, Nair S, Hartman H, Ashton PM, Dyson ZA, Day M, et al. Informal genomic surveillance of regional distribution of Salmonella Typhi genotypes and antimicrobial resistance via returning travellers. PLoS neglected tropical diseases. 2019;13(9):e0007620.
108. da Silva KE, Tanmoy AM, Pragasam AK, Iqbal J, Sajib MSI, Mutreja A, et al. The international and intercontinental spread and expansion of antimicrobial-resistant Salmonella Typhi: a genomic epidemiology study. Lancet Microbe. 2022;3(8):e567-e77.
109. Polonsky JA, Martínez-Pino I, Nackers F, Chonzi P, Manangazira P, Van Herp M, et al. Descriptive epidemiology of typhoid fever during an epidemic in Harare, Zimbabwe, 2012. PloS one. 2014;9(12):e114702.
110. Appiah GD, Chung A, Bentsi-Enchill AD, Kim S, Crump JA, Mogasale V, et al. Typhoid Outbreaks, 1989-2018: Implications for Prevention and Control. Am J Trop Med Hyg. 2020;102(6):1296-305.
111. Kariuki S, Revathi G, Muyodi J, Mwituria J, Munyalo A, Mirza S, Hart CA. Characterization of multidrug-resistant typhoid outbreaks in Kenya. Journal of clinical microbiology. 2004;42(4):1477-82.
112. Breiman RF, Cosmas L, Njuguna H, Audi A, Olack B, Ochieng JB, et al. Population-based incidence of typhoid fever in an urban informal settlement and a rural area in Kenya: implications for typhoid vaccine use in Africa. PloS one. 2012;7(1):e29119.
113. Crump JA. Progress in Typhoid Fever Epidemiology. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2019;68(Suppl 1):S4-s9.
114. Global Burden of Disease Collaborative Network. GBD 2020 Cause and Risk Summaries: Typhoid fever — level 4 cause. Seattle, United States: Institute for Health Metrics and Evaluation (IHME). 2020 [cited 2022 07.02.2022]. Available from: https://www.healthdata.org/results/gbd_summaries/2019/typhoid-fever-level-4-cause.
115. Antillón M, Warren JL, Crawford FW, Weinberger DM, Kürüm E, Pak GD, et al. The burden of typhoid fever in low- and middle-income countries: A meta-regression approach. PLoS neglected tropical diseases. 2017;11(2):e0005376.
116. Marks F, von Kalckreuth V, Aaby P, Adu-Sarkodie Y, El Tayeb MA, Ali M, et al. Incidence of invasive salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study. Lancet Glob Health. 2017;5(3):e310-e23.
117. Wong VK, Baker S, Connor TR, Pickard D, Page AJ, Dave J, et al. An extended genotyping framework for Salmonella enterica serovar Typhi, the cause of human typhoid. Nature communications. 2016;7:12827.
118. Dyson ZA, Holt KE. Five years of GenoTyphi: updates to the global Salmonella Typhi genotyping framework. The Journal of infectious diseases. 2021.
119. Ombelet S, Ronat JB, Walsh T, Yansouni CP, Cox J, Vlieghe E, et al. Clinical bacteriology in low-resource settings: today's solutions. Lancet Infect Dis. 2018;18(8):e248-e58.
120. Thriemer K, Ley B, Ame S, von Seidlein L, Pak GD, Chang NY, et al. The burden of invasive bacterial infections in Pemba, Zanzibar. PloS one. 2012;7(2):e30350.

121. World Health O. Typhoid vaccines: WHO position paper, March 2018 - Recommendations. Vaccine. 2019;37(2):214-6.
122. Frost I, Sati H, Garcia-Vello P, Hasso-Agopsowicz M, Lienhardt C, Gigante V, Beyer P. The role of bacterial vaccines in the fight against antimicrobial resistance: an analysis of the preclinical and clinical development pipeline. Lancet Microbe. 2023;4(2):e113-e25.
123. Laurens MB. RTS,S/AS01 vaccine (Mosquirix™): an overview. Hum Vaccin Immunother. 2020;16(3):480-9.
124. White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM. Malaria. Lancet. 2014;383(9918):723-35.
125. World malaria report 2022: World Health Organization, Geneva, Switzerland; 2022 [Available from: https://www.who.int/publications/i/item/9789240064898.
126. Walker IS, Rogerson SJ. Pathogenicity and virulence of malaria: Sticky problems and tricky solutions. Virulence. 2023;14(1):2150456.
127. Amir A, Cheong FW, De Silva JR, Lau YL. Diagnostic tools in childhood malaria. Parasit Vectors. 2018;11(1):53.
128. Cox FE. History of the discovery of the malaria parasites and their vectors. Parasit Vectors. 2010;3(1):5.
129. Bruce-Chwatt LJ. Alphonse Laveran's discovery 100 years ago and today's global fight against malaria. J R Soc Med. 1981;74(7):531-6.
130. A. L. Fievres palustres avec la description des microbes du paludisme 1884 [Available from: https://ia600500.us.archive.org/25/items/traitdesfivr00lave/traitdesfivr00lave.pdf.
131. Ross R. On some Peculiar Pigmented Cells Found in Two Mosquitos Fed on Malarial Blood. Br Med J. 1897;2(1929):1786-8.
132. Ross R. The role of the mosquito in the evolution of the malarial parasite: the recent researches of Surgeon-Major Ronald Ross, I.M.S. 1898. Yale J Biol Med. 2002;75(2):103-5.
133. Grassi B. The Transmission of Human Malaria. Nature. 1924;113(2835):304-7.
134. Feachem RG, Phillips AA, Hwang J. Shrinking the malaria map: progress and prospects. Lancet. 2010;376.
135. Bykowski A, Hashmi MF, Logan TD. Cinchonism. StatPearls. Treasure Island (FL): StatPearls Publishing

136. White NJ. Severe malaria. Malaria journal. 2022;21(1):284.
137. Doolan DL, Dobaño C, Baird JK. Acquired immunity to malaria. Clinical microbiology reviews. 2009;22(1):13-36, Table of Contents.
138. Cowman AF, Healer J, Marapana D, Marsh K. Malaria: Biology and Disease. Cell. 2016;167(3):610-24.
139. Plewes K, Leopold SJ, Kingston HWF, Dondorp AM. Malaria: What's New in the Management of Malaria? Infect Dis Clin North Am. 2019;33(1):39-60.
140. Bhattarai A, Ali AS, Kachur SP. Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar. PLoS medicine. 2007;4.
141. World malaria report 2023: World Health Organization, Geneva, Switzerland; 2023 [Available from: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2023.
142. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature. 2015;526(7572):207-11.
143. D'Acremont V, Lengeler C, Genton B. Reduction in the proportion of fevers associated with Plasmodium falciparum parasitaemia in Africa: a systematic review. Malaria journal. 2010;9:240.

144. Pryce J, Medley N, Choi L. Indoor residual spraying for preventing malaria in communities using insecticide-treated nets. The Cochrane database of systematic reviews. 2022;1(1):Cd012688.
145. Wilson ML. Laboratory diagnosis of malaria: conventional and rapid diagnostic methods. Arch Pathol Lab Med. 2013;137(6):805-11.
146. WHO. WHO Guidelines for malaria: WHO, Geneva, Switzerland; 2023 [Available from: https://www.who.int/teams/global-malaria-programme/guidelines-for-malaria.
147. Cockburn IA, Seder RA. Malaria prevention: from immunological concepts to effective vaccines and protective antibodies. Nat Immunol. 2018;19(11):1199-211.
148. WHO Malaria: World Health Organization, Geneva, Switzerland; 2023 [Available from: https://www.who.int/news-room/fact-sheets/detail/malaria.
149. Datoo MS, Natama MH, Somé A, Traoré O, Rouamba T, Bellamy D, et al. Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial. Lancet. 2021;397(10287):1809-18.
150. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. The New England journal of medicine. 2011;365(20):1863-75.
151. Wilson ML. Malaria rapid diagnostic tests. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2012;54(11):1637-41.
152. Sachs J, Malaney P. The economic and social burden of malaria. Nature. 2002;415(6872):680-5.
153. Cook J, Xu W, Msellem M, Vonk M. Mass screening and treatment on the basis of results of a Plasmodium falciparum-specific rapid diagnostic test did not reduce malaria incidence in Zanzibar. The Journal of infectious diseases. 2015;211.
154. Björkman A, Shakely D, Ali AS, Morris U, Mkali H, Abbas AK, et al. From high to low malaria transmission in Zanzibar—challenges and opportunities to achieve elimination. BMC medicine. 2019;17(1):14.
155. Ashton RA, Bennett A, Al-Mafazy AW, Abass AK, Msellem MI, McElroy P, et al. Use of Routine Health Information System Data to Evaluate Impact of Malaria Control Interventions in Zanzibar, Tanzania from 2000 to 2015. EClinicalMedicine. 2019;12:11-9.
156. Ali MH, Kitau J, Ali AS, Al-Mafazy AW, Tegegne SG, Ussi O, et al. Malaria elimination in Zanzibar: where next? Pan Afr Med J. 2023;45(Suppl 1):7.
157. Hawadak J, Dongang Nana RR, Singh V. Epidemiological, Physiological and Diagnostic Comparison of Plasmodium ovale curtisi and Plasmodium ovale wallikeri. Diagnostics (Basel). 2021;11(10).
158. Stevenson MM, Riley EM. Innate immunity to malaria. Nat Rev Immunol. 2004;4(3):169-80.
159. Croft AM. Malaria: prevention in travellers. BMJ Clin Evid. 2007;2007.
160. Sleiman E, Upadhyaya A, Glaser A, Krzyzak M. A Case of Plasmodium falciparum Malaria with a Prolonged Incubation Period of Four Years. Cureus. 2020;12(3):e7176.
161. Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. Nat Rev Microbiol. 2014;12(12):833-40.
162. Berkley JA, Bejon P, Mwangi T, Gwer S, Maitland K, Williams TN, et al. HIV infection, malnutrition, and invasive bacterial infection among children with severe malaria. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2009;49(3):336-43.

163. Gonçalves BP, Huang CY, Morrison R, Holte S, Kabyemela E, Prevots DR, et al. Parasite burden and severity of malaria in Tanzanian children. The New England journal of medicine. 2014;370(19):1799-808.
164. Donnelly E, de Water JV, Luckhart S. Malaria-induced bacteremia as a consequence of multiple parasite survival strategies. Curr Res Microb Sci. 2021;2:100036.
165. Park SE, Pak GD, Aaby P, Adu-Sarkodie Y, Ali M, Aseffa A, et al. The Relationship Between Invasive Nontyphoidal Salmonella Disease, Other Bacterial Bloodstream Infections, and Malaria in Sub-Saharan Africa. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2016;62 Suppl 1(Suppl 1):S23-31.
166. Esoh K, Wonkam A. Evolutionary history of sickle-cell mutation: implications for global genetic medicine. Hum Mol Genet. 2021;30(R1):R119-r28.
167. Fitri LE, Widaningrum T, Endharti AT, Prabowo MH, Winaris N, Nugraha RYB. Malaria diagnostic update: From conventional to advanced method. J Clin Lab Anal. 2022;36(4):e24314.
168. Elven J, Dahal P, Ashley EA, Thomas NV, Shrestha P, Stepniewska K, et al. Non-malarial febrile illness: a systematic review of published aetiological studies and case reports from Africa, 1980-2015. BMC medicine. 2020;18(1):279.
169. Crump JA, Kirk MD. Estimating the Burden of Febrile Illnesses. PLoS neglected tropical diseases. 2015;9(12):e0004040.
170. Prasad N, Murdoch DR, Reyburn H, Crump JA. Etiology of Severe Febrile Illness in Low- and Middle-Income Countries: A Systematic Review. PloS one. 2015;10(6):e0127962.
171. Rhee C, Kharod GA, Schaad N, Furukawa NW, Vora NM, Blaney DD, et al. Global knowledge gaps in acute febrile illness etiologic investigations: A scoping review. PLoS neglected tropical diseases. 2019;13(11):e0007792.
172. Wainaina M, Vey da Silva DA, Dohoo I, Mayer-Scholl A, Roesel K, Hofreuter D, et al. A systematic review and meta-analysis of the aetiological agents of non-malarial febrile illnesses in Africa. PLoS neglected tropical diseases. 2022;16(1):e0010144.
173. Moody A. Rapid diagnostic tests for malaria parasites. Clinical microbiology reviews. 2002;15(1):66-78.
174. Wu L, van den Hoogen LL, Slater H, Walker PG, Ghani AC, Drakeley CJ, Okell LC. Comparison of diagnostics for the detection of asymptomatic Plasmodium falciparum infections to inform control and elimination strategies. Nature. 2015;528(7580):S86-93.
175. Cunningham J, Jones S, Gatton ML, Barnwell JW, Cheng Q, Chiodini PL, et al. A review of the WHO malaria rapid diagnostic test product testing programme (2008-2018): performance, procurement and policy. Malaria journal. 2019;18(1):387.
176. Björkman A, Morris U. Why Asymptomatic Plasmodium falciparum Infections Are Common in Low-Transmission Settings. Trends Parasitol. 2020;36(11):898-905.
177. Stuck L, Fakih BS, Al-Mafazy AH, Hofmann NE, Holzschuh A, Grossenbacher B, et al. Malaria infection prevalence and sensitivity of reactive case detection in Zanzibar. Int J Infect Dis. 2020;97:337-46.
178. Haanshuus CG, Mørch K, Blomberg B, Strøm GEA, Langeland N, Hanevik K, Mohn SC. Assessment of malaria real-time PCR methods and application with focus on low-level parasitaemia. PloS one. 2019;14(7):e0218982.
179. Feleke DG, Alemu Y, Yemanebirhane N. Performance of rapid diagnostic tests, microscopy, loop-mediated isothermal amplification (LAMP) and PCR for malaria diagnosis in Ethiopia: a systematic review and meta-analysis. Malaria journal. 2021;20(1):384.
180. Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in Plasmodium falciparum-endemic populations: a systematic review and meta-analysis. The Journal of infectious diseases. 2009;200(10):1509-17.

181. Hopkins H, González IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C, et al. Highly sensitive detection of malaria parasitemia in a malaria-endemic setting: performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda. The Journal of infectious diseases. 2013;208(4):645-52.
182. Ramírez AM, Tang THT, Suárez ML, Fernández A, García CM, Hisam S, Rubio JM. Assessment of Commercial Real-Time PCR Assays for Detection of Malaria Infection in a Non-Endemic Setting. Am J Trop Med Hyg. 2021;105(6):1732-7.
183. Haanshuus CG, Mørch K. Detection of remaining Plasmodium DNA and gametocytes during follow up after curative malaria treatment among returned travellers in Norway. Malaria journal. 2020;19(1):296.
184. Hartley MA, Hofmann N, Keitel K, Kagoro F, Antunes Moniz C, Mlaganile T, et al. Clinical relevance of low-density Plasmodium falciparum parasitemia in untreated febrile children: A cohort study. PLoS medicine. 2020;17(9):e1003318.
185. Polley SD, González IJ, Mohamed D, Daly R, Bowers K, Watson J, et al. Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. The Journal of infectious diseases. 2013;208(4):637-44.
186. van Dijk NJ, Menting S, Wentink-Bonnema EMS, Broekhuizen-van Haaften PE, Withycombe E, Schallig H, Mens PF. Laboratory evaluation of the miniature direct-on-blood PCR nucleic acid lateral flow immunoassay (mini-dbPCR-NALFIA), a simplified molecular diagnostic test for Plasmodium. Malaria journal. 2023;22(1):98.
187. Holmes AH, Moore LS, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, et al. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 2016;387(10014):176-87.
188. Bush K. Past and Present Perspectives on β-Lactamases. Antimicrob Agents Chemother. 2018;62(10).
189. Browne K, Chakraborty S, Chen R, Willcox MD, Black DS, Walsh WR, Kumar N. A New Era of Antibiotics: The Clinical Potential of Antimicrobial Peptides. Int J Mol Sci. 2020;21(19).
190. Ehrlich P. Address in Pathology, ON CHEMIOTHERAPY: Delivered before the Seventeenth International Congress of Medicine. Br Med J. 1913;2(2746):353-9.
191. Fleming A. On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ. The British Journal of Experimental Pathology. 1929;10(3):226–36.
192. Otten H. Domagk and the development of the sulphonamides. The Journal of antimicrobial chemotherapy. 1986;17(6):689-96.
193. Quinn R. Rethinking antibiotic research and development: World War II and the penicillin collaborative. Am J Public Health. 2013;103(3):426-34.
194. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. 1940. Rev Infect Dis. 1988;10(4):677-8.
195. Livermore DM. Antibiotic resistance in staphylococci. International journal of antimicrobial agents. 2000;16 Suppl 1:S3-10.
196. Davies J. Vicious circles: looking back on resistance plasmids. Genetics. 1995;139(4):1465-8.
197. Livermore DM. Discovery research: the scientific challenge of finding new antibiotics. The Journal of antimicrobial chemotherapy. 2011;66(9):1941-4.
198. Powers JH. Antimicrobial drug development--the past, the present, and the future. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2004;10 Suppl 4:23-31.
199. Hutchings MI, Truman AW, Wilkinson B. Antibiotics: past, present and future. Curr Opin Microbiol. 2019;51:72-80.

200. Bush K, Bradford PA. Epidemiology of β-Lactamase-Producing Pathogens. Clinical microbiology reviews. 2020;33(2).
201. Durand GA, Raoult D, Dubourg G. Antibiotic discovery: history, methods and perspectives. International journal of antimicrobial agents. 2019;53(4):371-82.
202. Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, et al. Antibiotic resistance-the need for global solutions. Lancet Infect Dis. 2013;13(12):1057-98.
203. Bradford PA. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clinical microbiology reviews. 2001;14(4):933-51, table of contents.
204. Knothe H, Shah P, Krcmery V, Antal M, Mitsuhashi S. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of Klebsiella pneumoniae and Serratia marcescens. Infection. 1983;11(6):315-7.
205. Kliebe C, Nies BA, Meyer JF, Tolxdorff-Neutzling RM, Wiedemann B. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob Agents Chemother. 1985;28(2):302-7.
206. Giske CG, Sundsfjord AS, Kahlmeter G, Woodford N, Nordmann P, Paterson DL, et al. Redefining extended-spectrum beta-lactamases: balancing science and clinical need. The Journal of antimicrobial chemotherapy. 2009;63(1):1-4.
207. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. Clinical microbiology reviews. 2005;18(4):657-86.
208. Castanheira M, Simner PJ, Bradford PA. Extended-spectrum β-lactamases: an update on their characteristics, epidemiology and detection. JAC Antimicrob Resist. 2021;3(3):dlab092.
209. Alvarez M, Tran JH, Chow N, Jacoby GA. Epidemiology of conjugative plasmid-mediated AmpC beta-lactamases in the United States. Antimicrob Agents Chemother. 2004;48(2):533-7.
210. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. Clinical microbiology reviews. 2007;20(3):440-58, table of contents.
211. Temkin E, Adler A, Lerner A, Carmeli Y. Carbapenem-resistant Enterobacteriaceae: biology, epidemiology, and management. Ann N Y Acad Sci. 2014;1323:22-42.
212. Llor C, Bjerrum L. Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. Ther Adv Drug Saf. 2014;5(6):229-41.
213. Dadgostar P. Antimicrobial Resistance: Implications and Costs. Infect Drug Resist. 2019;12:3903-10.
214. Khawbung JL, Nath D, Chakraborty S. Drug resistant Tuberculosis: A review. Comp Immunol Microbiol Infect Dis. 2021;74:101574.
215. Lange C, Dheda K, Chesov D, Mandalakas AM, Udwadia Z, Horsburgh CR, Jr. Management of drug-resistant tuberculosis. Lancet. 2019;394(10202):953-66.
216. Friedrich MJ. Drug-Resistant Tuberculosis Predicted to Increase in High-Burden Countries. Jama. 2017;318(3):231.
217. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022;399(10325):629-55.
218. Mathers AJ, Peirano G, Pitout JD. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. Clinical microbiology reviews. 2015;28(3):565-91.
219. Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2005;40(9):1333-41.

220. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 2016;16(2):161-8.
221. Venne DM, Hartley DM, Malchione MD, Koch M, Britto AY, Goodman JL. Review and analysis of the overlapping threats of carbapenem and polymyxin resistant E. coli and Klebsiella in Africa. Antimicrob Resist Infect Control. 2023;12(1):29.
222. Årdal C, Balasegaram M, Laxminarayan R, McAdams D, Outterson K, Rex JH, Sumpradit N. Antibiotic development - economic, regulatory and societal challenges. Nat Rev Microbiol. 2020;18(5):267-74.
223. WHO. 2021 Antibacterial agents in clinical and preclinical development: an overview and analysis: World Health Organization, Switzerland, Geneva; 2022 [Available from: https://www.who.int/publications/i/item/9789240047655.
224. WHO. WHO publishes list of bacteria for which new antibiotics are urgently needed: World Health Organization, Geneva, Switzerland; 2017 [Available from: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed.
225. De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, et al. Antimicrobial Resistance in ESKAPE Pathogens. Clinical microbiology reviews. 2020;33(3).
226. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. Clinical microbiology reviews. 2018;31(4).
227. CDC. Antibiotic resistance threats in the United States, 2019: U.S. Centers for Disease Contral and Prevention, Atlanta, Georgia, USA; 2019 [Available from: https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf.
228. Cohen ML. Epidemiology of drug resistance: implications for a post-antimicrobial era. Science (New York, NY). 1992;257(5073):1050-5.
229. Global action plan on antimicrobial resistance: WHO, Geneva, Switzerland; 2015 [Available from: https://www.who.int/publications/i/item/9789241509763.
230. Antimicrobial resistance key facts: WHO, Geneva, Switzerland; 2021 [Available from: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.
231. UN. Antimicrobiol resistance: a global threat: United Nations; [Available from: https://www.unep.org/explore-topics/chemicals-waste/what-we-do/emerging-issues/antimicrobial-resistance-global-threat.
232. Tackling drug-resistance globally: final report and recommendations. The review on antimicrobial resistance, chaired by Jim O'Neill 2016 [Available from: https://apo.org.au/sites/default/files/resource-files/2016-05/apo-nid63983.pdf.
233. Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. 2014. Chaired by Jim O'Neill. 2014 [Available from: https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf.
234. Global antimicrobial resistance and use surveillance system (GLASS) report 2022: WHO, Geneva, Switzerland; 2022 [Available from: https://www.who.int/publications/i/item/9789240062702.
235. Gandra S, Tseng KK, Arora A, Bhowmik B, Robinson ML, Panigrahi B, et al. The Mortality Burden of Multidrug-resistant Pathogens in India: A Retrospective, Observational Study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2019;69(4):563-70.
236. Lester R, Musicha P, Kawaza K, Langton J, Mango J, Mangochi H, et al. Effect of resistance to third-generation cephalosporins on morbidity and mortality from bloodstream

infections in Blantyre, Malawi: a prospective cohort study. Lancet Microbe. 2022;3(12):e922-e30.
237. WHO GLASS report 2022 release: Report signals increasing resistance to antibiotics in bacterial infections in humans and need for better data: World Health Organization, Geneva, Switzerland; 2022 [Available from: https://www.who.int/news/item/09-12-2022-report-signals-increasing-resistance-to-antibiotics-in-bacterial-infections-in-humans-and-need-for-better-data.
238. Founou RC, Founou LL, Essack SY. Clinical and economic impact of antibiotic resistance in developing countries: A systematic review and meta-analysis. PloS one. 2017;12(12):e0189621.
239. Christaki E, Marcou M, Tofarides A. Antimicrobial Resistance in Bacteria: Mechanisms, Evolution, and Persistence. J Mol Evol. 2020;88(1):26-40.
240. Chen Y, Nie F, Xie SQ, Zheng YF, Dai Q, Bray T, et al. Efficient assembly of nanopore reads via highly accurate and intact error correction. Nature communications. 2021;12(1):60.
241. Wright GD. Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biol. 2010;8:123.
242. Opal SM, Pop-Vicas A. Molecular mechanisms of antibiotic resistance in bacteria. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 9th edition ed: Elsevier Inc., Amsterdam, Netherlands; 2019. p. 222-39.e3.
243. Liu G, Thomsen LE, Olsen JE. Antimicrobial-induced horizontal transfer of antimicrobial resistance genes in bacteria: a mini-review. The Journal of antimicrobial chemotherapy. 2022;77(3):556-67.
244. McEwen SA, Collignon PJ. Antimicrobial Resistance: a One Health Perspective. Microbiol Spectr. 2018;6(2).
245. Toleman MA, Walsh TR. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. FEMS Microbiol Rev. 2011;35(5):912-35.
246. Goossens H, Ferech M, Vander Stichele R, Elseviers M. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. Lancet. 2005;365(9459):579-87.
247. Klein EY, Van Boeckel TP, Martinez EM, Pant S, Gandra S, Levin SA, et al. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci U S A. 2018;115(15):E3463-e70.
248. Oldenkamp R, Schultsz C, Mancini E, Cappuccio A. Filling the gaps in the global prevalence map of clinical antimicrobial resistance. Proc Natl Acad Sci U S A. 2021;118(1).
249. Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol. 2014;12(7):465-78.
250. Ayukekbong JA, Ntemgwa M, Atabe AN. The threat of antimicrobial resistance in developing countries: causes and control strategies. Antimicrob Resist Infect Control. 2017;6:47.
251. Laxminarayan R, Heymann DL. Challenges of drug resistance in the developing world. Bmj. 2012;344:e1567.
252. Fick J, Söderström H, Lindberg RH, Phan C, Tysklind M, Larsson DGJ. Contamination of surface, ground, and drinking water from pharmaceutical production. Environmental Toxicology and Chemistry. 2009;28(12):2522-7.
253. Lääveri T, Vlot JA, van Dam AP, Häkkinen HK, Sonder GJB, Visser LG, Kantele A. Extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-PE) among travellers to Africa: destination-specific data pooled from three European prospective studies. BMC infectious diseases. 2018;18(1):341.

254. Woerther PL, Andremont A, Kantele A. Travel-acquired ESBL-producing Enterobacteriaceae: impact of colonization at individual and community level. J Travel Med. 2017;24(suppl_1):S29-s34.
255. Evans L, Rhodes A, Alhazzani W, Antonelli M, Coopersmith CM, French C, et al. Surviving sepsis campaign: international guidelines for management of sepsis and septic shock 2021. Intensive Care Med. 2021;47(11):1181-247.
256. Ferrer R, Martin-Loeches I, Phillips G, Osborn TM, Townsend S, Dellinger RP, et al. Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: results from a guideline-based performance improvement program. Crit Care Med. 2014;42(8):1749-55.
257. Liu VX, Fielding-Singh V, Greene JD, Baker JM, Iwashyna TJ, Bhattacharya J, Escobar GJ. The Timing of Early Antibiotics and Hospital Mortality in Sepsis. Am J Respir Crit Care Med. 2017;196(7):856-63.
258. Seymour CW, Gesten F, Prescott HC, Friedrich ME, Iwashyna TJ, Phillips GS, et al. Time to Treatment and Mortality during Mandated Emergency Care for Sepsis. The New England journal of medicine. 2017;376(23):2235-44.
259. Rottier WC, Ammerlaan HS, Bonten MJ. Effects of confounders and intermediates on the association of bacteraemia caused by extended-spectrum β-lactamase-producing Enterobacteriaceae and patient outcome: a meta-analysis. The Journal of antimicrobial chemotherapy. 2012;67(6):1311-20.
260. Raman G, Avendano E, Berger S, Menon V. Appropriate initial antibiotic therapy in hospitalized patients with gram-negative infections: systematic review and meta-analysis. BMC infectious diseases. 2015;15:395.
261. Cosgrove SE, Carmeli Y. The impact of antimicrobial resistance on health and economic outcomes. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2003;36(11):1433-7.
262. Marquet K, Liesenborgs A, Bergs J, Vleugels A, Claes N. Incidence and outcome of inappropriate in-hospital empiric antibiotics for severe infection: a systematic review and meta-analysis. Crit Care. 2015;19(1):63.
263. Ohnuma T, Chihara S, Costin B, Treggiari MM, Bartz RR, Raghunathan K, Krishnamoorthy V. Association of Appropriate Empirical Antimicrobial Therapy With In-Hospital Mortality in Patients With Bloodstream Infections in the US. JAMA Netw Open. 2023;6(1):e2249353.
264. Kadri SS, Lai YL, Warner S, Strich JR, Babiker A, Ricotta EE, et al. Inappropriate empirical antibiotic therapy for bloodstream infections based on discordant in-vitro susceptibilities: a retrospective cohort analysis of prevalence, predictors, and mortality risk in US hospitals. Lancet Infect Dis. 2021;21(2):241-51.
265. Hung YP, Lee CC, Ko WC. Effects of Inappropriate Administration of Empirical Antibiotics on Mortality in Adults With Bacteraemia: Systematic Review and Meta-Analysis. Front Med (Lausanne). 2022;9:869822.
266. Cosgrove SE. The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. Clin Infect Dis. 2006;42 Suppl 2:S82-9.
267. Kaye KS, Engemann JJ, Mozaffari E, Carmeli Y. Reference group choice and antibiotic resistance outcomes. Emerging infectious diseases. 2004;10(6):1125-8.
268. Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y. Comparison of mortality associated with methicillin-resistant and methicillin-susceptible Staphylococcus aureus bacteremia: a meta-analysis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2003;36(1):53-9.

269. Naylor NR, Atun R, Zhu N, Kulasabanathan K, Silva S, Chatterjee A, et al. Estimating the burden of antimicrobial resistance: a systematic literature review. Antimicrob Resist Infect Control. 2018;7:58.
270. Stewardson AJ, Allignol A, Beyersmann J, Graves N, Schumacher M, Meyer R, et al. The health and economic burden of bloodstream infections caused by antimicrobial-susceptible and non-susceptible Enterobacteriaceae and Staphylococcus aureus in European hospitals, 2010 and 2011: a multicentre retrospective cohort study. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2016;21(33).
271. de Kraker ME, Davey PG, Grundmann H. Mortality and hospital stay associated with resistant Staphylococcus aureus and Escherichia coli bacteremia: estimating the burden of antibiotic resistance in Europe. PLoS medicine. 2011;8(10):e1001104.
272. Ling W, Furuya-Kanamori L, Ezure Y, Harris PNA, Paterson DL. Adverse clinical outcomes associated with infections by Enterobacterales producing ESBL (ESBL-E): a systematic review and meta-analysis. JAC Antimicrob Resist. 2021;3(2):dlab068.
273. Sostarich AM, Zolldann D, Haefner H, Luetticken R, Schulze-Roebecke R, Lemmen SW. Impact of multiresistance of gram-negative bacteria in bloodstream infection on mortality rates and length of stay. Infection. 2008;36(1):31-5.
274. Neidell MJ, Cohen B, Furuya Y, Hill J, Jeon CY, Glied S, Larson EL. Costs of healthcare- and community-associated infections with antimicrobial-resistant versus antimicrobial-susceptible organisms. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2012;55(6):807-15.
275. Falagas ME, Tansarli GS, Karageorgopoulos DE, Vardakas KZ. Deaths attributable to carbapenem-resistant Enterobacteriaceae infections. Emerging infectious diseases. 2014;20(7):1170-5.
276. Budhram DR, Mac S, Bielecki JM, Patel SN, Sander B. Health outcomes attributable to carbapenemase-producing Enterobacteriaceae infections: A systematic review and meta-analysis. Infect Control Hosp Epidemiol. 2020;41(1):37-43.
277. Avendano EE, Raman G, Chan J, McCann E. Burden of carbapenem non-susceptible infections in high-risk patients: systematic literature review and meta-analysis. Antimicrob Resist Infect Control. 2020;9(1):193.
278. Kohler PP, Volling C, Green K, Uleryk EM, Shah PS, McGeer A. Carbapenem Resistance, Initial Antibiotic Therapy, and Mortality in Klebsiella pneumoniae Bacteremia: A Systematic Review and Meta-Analysis. Infect Control Hosp Epidemiol. 2017;38(11):1319-28.
279. Schwaber MJ, Carmeli Y. Mortality and delay in effective therapy associated with extended-spectrum beta-lactamase production in Enterobacteriaceae bacteraemia: a systematic review and meta-analysis. The Journal of antimicrobial chemotherapy. 2007;60(5):913-20.
280. Blomberg B, Jureen R, Manji KP, Tamim BS, Mwakagile DS, Urassa WK, et al. High rate of fatal cases of pediatric septicemia caused by gram-negative bacteria with extended-spectrum beta-lactamases in Dar es Salaam, Tanzania. Journal of clinical microbiology. 2005;43(2):745-9.
281. de Kraker ME, Wolkewitz M, Davey PG, Koller W, Berger J, Nagler J, et al. Clinical impact of antimicrobial resistance in European hospitals: excess mortality and length of hospital stay related to methicillin-resistant Staphylococcus aureus bloodstream infections. Antimicrob Agents Chemother. 2011;55(4):1598-605.
282. Shrestha P, Cooper BS, Coast J, Oppong R, Do Thi Thuy N, Phodha T, et al. Enumerating the economic cost of antimicrobial resistance per antibiotic consumed to inform the evaluation of interventions affecting their use. Antimicrob Resist Infect Control. 2018;7:98.

283. McGowan JE, Jr. Economic impact of antimicrobial resistance. Emerging infectious diseases. 2001;7(2):286-92.
284. Becker JU, Theodosis C, Jacob ST, Wira CR, Groce NE. Surviving sepsis in low-income and middle-income countries: new directions for care and research. Lancet Infect Dis. 2009;9(9):577-82.
285. Kayange N, Kamugisha E, Mwizamholya DL, Jeremiah S, Mshana SE. Predictors of positive blood culture and deaths among neonates with suspected neonatal sepsis in a tertiary hospital, Mwanza-Tanzania. BMC pediatrics. 2010;10:39.
286. O'Brien TF. The global epidemic nature of antimicrobial resistance and the need to monitor and manage it locally. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1997;24 Suppl 1:S2-8.
287. Ombelet S, Barbé B, Affolabi D, Ronat JB, Lompo P, Lunguya O, et al. Best Practices of Blood Cultures in Low- and Middle-Income Countries. Front Med (Lausanne). 2019;6:131.
288. Zanzibar at a glance: Zanzibar Investment Promotion Authority (ZIPA); 2023 [Available from: https://www.zipa.go.tz/zanzibar-at-a-glance/.
289. The World Bank Country economy classification income: The World Bank, Washington DC, USA; 2023 [Available from: https://datahelpdesk.worldbank.org/knowledgebase/articles/906519-world-bank-country-and-lending-groups.
290. United Nations Least Developed Country Category: United Republic of Tanzania Profile: United Nations, New York, USA; 2021 [Available from: https://www.un.org/development/desa/dpad/least-developed-country-category-united-republic-of-tanzania.html.
291. Tanzania National Bureau of Statistics: Tanzania National Bureaau of Statistics, Dodoma, Tanzania; 2023 [Available from: https://www.nbs.go.tz/index.php/en/.
292. Tanzania Economic Update 2022: The World Bank, Washington DC, USA; 2022 [Available from: https://documents.worldbank.org/en/publication/documents-reports/documentdetail/099141002082366224/p17961001428f80cf0a6700fadf5ecf43ef.
293. World Bank Zanzibar Poverty Assessment 2022: World Bank, Washington DC, USA; 2022 [Available from: file:///P:/_Zanzibar%20fever%20study/Litteratur%20Zanzibar/World%20Bank%20Zanzibar%20poverty%20assessment%202022.pdf.
294. Revolutionary Government of Zanzibar, Ministry of Health, RCH, 2018. Zanzibar Reproductive, Maternal, Newborn, Child and Adolescent Reproductive Health Strategic Plan (2019-2023): Ministry of Health, Zanzibar; 2019 [Available from: file:///P:/_Zanzibar%20fever%20study/Litteratur%20Zanzibar/Zanzibar_Reproductive_Maternal_Newborn_Health%20plan%202019%20to%202023.pdf.
295. Kessy F OM. Status and progress of human development and implications for achieving Zanzibar development vision 2020: The Economic and Social Research Foundation, Dar es Salaam, Tanzania; 2014 [Available from: https://esrf.or.tz/wp-content/uploads/2021/01/THDR-BP-10.pdf.
296. Integrated Reproductive and Child Health Unit MoH, Social Welfare, Elderly, Gender and Children Zanzibar. Zanzibar Reproductive, Maternal, Newborn, Child and Adolescent Health Strategic Plan 2019-2023 [Available from: https://mohz.go.tz/eng/irch/.
297. Diguisto C, Saucedo M, Kallianidis A, Bloemenkamp K, Bødker B, Buoncristiano M, et al. Maternal mortality in eight European countries with enhanced surveillance systems: descriptive population based study. Bmj. 2022;379:e070621.
298. Global tuberculosis report: World Health Organization, Geneva, Switzerland; 2022 [Available from: https://reliefweb.int/report/world/global-tuberculosis-report-

2022?psafe_param=1&gclid=EAIaIQobChMI6YSkiIq2_gIV7UeRBR1G1gfOEAAYASAAEgKYZPD_BwE.
299. Senkoro M, Mfinanga S, Egwaga S, Mtandu R, Kamara DV, Basra D, et al. Prevalence of pulmonary tuberculosis in adult population of Tanzania: a national survey, 2012. Int J Tuberc Lung Dis. 2016;20(8):1014-21.
300. Bi Q, Abdalla FM, Masauni S, Reyburn R, Msambazi M, Deglise C, et al. The Epidemiology of Cholera in Zanzibar: Implications for the Zanzibar Comprehensive Cholera Elimination Plan. The Journal of infectious diseases. 2018;218(suppl_3):S173-s80.
301. Revolutionary Government of Zanzibar, Mnazi Mmoja Hospital, Zanzibar: Mnazi Mmoja Hospital, Zanzibar; 2020 [Available from: https://mmh.go.tz/.
302. Ministry of Health and Social Welfare Zanzibar: Ministry of Health Zanzibar, Tanzania; 2023 [Available from: https://mohz.go.tz/eng/.
303. Archibald LK, McDonald LC, Addison RM, McKnight C, Byrne T, Dobbie H, et al. Comparison of BACTEC MYCO/F LYTIC and WAMPOLE ISOLATOR 10 (lysis-centrifugation) systems for detection of bacteremia, mycobacteremia, and fungemia in a developing country. Journal of clinical microbiology. 2000;38(8):2994-7.
304. Collee JG MB, Irvine R, Fraser AG, Simmons A, editor. Mackie & McCartney Practical Medical Microbiology. 14th edition ed. New York: Churchill Livingstone; 1996.
305. Birkett CI, Ludlam HA, Woodford N, Brown DF, Brown NM, Roberts MT, et al. Real-time TaqMan PCR for rapid detection and typing of genes encoding CTX-M extended-spectrum beta-lactamases. Journal of medical microbiology. 2007;56(Pt 1):52-5.
306. Sturød K. DUR, Berg E.S., Steinbakk M., Wester A.L. Evaluation of the ability of four ESBL-screening media to detect ESBL-producing *Salmonella* and *Shigella*. BMC microbiology. 2014;14.
307. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-9.
308. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics (Oxford, England). 2009;25(16):2078-9.
309. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics (Oxford, England). 2011;27(21):2987-93.
310. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol. 2017;13(6):e1005595.
311. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PloS one. 2014;9(11):e112963.
312. Vaser R, Sović I, Nagarajan N, Šikić M. Fast and accurate de novo genome assembly from long uncorrected reads. Genome Res. 2017;27(5):737-46.
313. Bosi E, Donati B, Galardini M, Brunetti S, Sagot MF, Lió P, et al. MeDuSa: a multi-draft based scaffolder. Bioinformatics (Oxford, England). 2015;31(15):2443-51.
314. Argimon S, Yeats CA, Goater RJ, Abudahab K, Taylor B, Underwood A, et al. A global resource for genomic predictions of antimicrobial resistance and surveillance of Salmonella Typhi at pathogenwatch. Nature communications. 2021;12(1):2879.
315. Sikorski MJ, Hazen TH, Desai SN, Nimarota-Brown S, Tupua S, Sialeipata M, et al. Persistence of Rare Salmonella Typhi Genotypes Susceptible to First-Line Antibiotics in the Remote Islands of Samoa. mBio. 2022;13(5):e0192022.
316. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 2021;49(W1):W293-w6.

317. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server: rapid annotations using subsystems technology. BMC genomics. 2008;9:75.
318. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10.
319. ResFinder, Danish Technical University, Denmark [20.10.2022]. Available from: https://cge.food.dtu.dk/services/ResFinder/.
320. Guy L, Kultima JR, Andersson SG. genoPlotR: comparative gene and genome visualization in R. Bioinformatics (Oxford, England). 2010;26(18):2334-5.
321. Haanshuus CG, Mohn SC, Morch K, Langeland N, Blomberg B, Hanevik K. A novel, single-amplification PCR targeting mitochondrial genome highly sensitive and specific in diagnosing malaria among returned travellers in Bergen, Norway. Malaria journal. 2013;12:26.
322. Padley D, Moody AH, Chiodini PL, Saldanha J. Use of a rapid, single-round, multiplex PCR to detect malarial parasites and identify the species present. Ann Trop Med Parasitol. 2003;97(2):131-7.
323. Manyahi J, Kibwana U, Mgimba E, Majigo M. Multi-drug resistant bacteria predict mortality in bloodstream infection in a tertiary setting in Tanzania. PloS one. 2020;15(3):e0220424.
324. Moyo SJ, Manyahi J, Blomberg B, Tellevik MG, Masoud NS, Aboud S, et al. Bacteraemia, Malaria, and Case Fatality Among Children Hospitalized With Fever in Dar es Salaam, Tanzania. Frontiers in microbiology. 2020;11:2118.
325. Zea-Vera A, Ochoa TJ. Challenges in the diagnosis and management of neonatal sepsis. J Trop Pediatr. 2015;61(1):1-13.
326. Seale AC, Blencowe H, Manu AA, Nair H, Bahl R, Qazi SA, et al. Estimates of possible severe bacterial infection in neonates in sub-Saharan Africa, south Asia, and Latin America for 2012: a systematic review and meta-analysis. Lancet Infect Dis. 2014;14(8):731-41.
327. Russell NJ, Stöhr W, Plakkal N, Cook A, Berkley JA, Adhisivam B, et al. Patterns of antibiotic use, pathogens, and prediction of mortality in hospitalized neonates and young infants with sepsis: A global neonatal sepsis observational cohort study (NeoOBS). PLoS medicine. 2023;20(6):e1004179.
328. Shakely D, Elfving K, Aydin-Schmidt B, Msellem MI, Morris U, Omar R, et al. The usefulness of rapid diagnostic tests in the new context of low malaria transmission in Zanzibar. PloS one. 2013;8(9):e72912.
329. Elfving K, Shakely D, Andersson M, Baltzell K, Ali AS, Bachelard M, et al. Acute Uncomplicated Febrile Illness in Children Aged 2-59 months in Zanzibar - Aetiologies, Antibiotic Treatment and Outcome. PloS one. 2016;11(1):e0146054.
330. Bisanzio D, Lalji S, Abbas FB, Ali MH, Hassan W, Mkali HR, et al. Spatiotemporal dynamics of malaria in Zanzibar, 2015-2020. BMJ Glob Health. 2023;8(1).
331. : World Health Organization, Geneva, Switzerland; 2022 [Available from: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022.
332. Mahende C, Ngasala B, Lusingu J, Butichi A, Lushino P, Lemnge M, et al. Bloodstream bacterial infection among outpatient children with acute febrile illness in north-eastern Tanzania. BMC research notes. 2015;8:289.
333. Msemo OA, Mbwana J, Mahende C, Malabeja A, Gesase S, Crump JA, et al. Epidemiology and Antimicrobial Susceptibility of Salmonella enterica Bloodstream Isolates Among Febrile Children in a Rural District in Northeastern Tanzania: A Cross-sectional Study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2019;68(Supplement_2):S177-s82.

334. Crump JA, Ramadhani HO, Morrissey AB, Msuya LJ, Yang LY, Chow SC, et al. Invasive bacterial and fungal infections among hospitalized HIV-infected and HIV-uninfected children and infants in northern Tanzania. Trop Med Int Health. 2011;16(7):830-7.
335. Park SE, Pham DT, Boinett C, Wong VK, Pak GD, Panzner U, et al. The phylogeography and incidence of multi-drug resistant typhoid fever in sub-Saharan Africa. Nature communications. 2018;9(1):5094.
336. Mashe T, Leekitcharoenphon P, Mtapuri-Zinyowera S, Kingsley RA, Robertson V, Tarupiwa A, et al. Salmonella enterica serovar Typhi H58 clone has been endemic in Zimbabwe from 2012 to 2019. The Journal of antimicrobial chemotherapy. 2021;76(5):1160-7.
337. Feasey NA, Gaskell K, Wong V, Msefula C, Selemani G, Kumwenda S, et al. Rapid emergence of multidrug resistant, H58-lineage Salmonella typhi in Blantyre, Malawi. PLoS neglected tropical diseases. 2015;9(4):e0003748.
338. Munoz-Price LS, Weinstein RA. Acinetobacter infection. The New England journal of medicine. 2008;358(12):1271-81.
339. Hsu JF, Chu SM, Lien R, Chiu CH, Chiang MC, Fu RH, et al. Case-control analysis of endemic Acinetobacter baumannii bacteremia in the neonatal intensive care unit. American journal of infection control. 2014;42(1):23-7.
340. Mashau RC, Meiring ST, Dramowski A, Magobo RE, Quan VC, Perovic O, et al. Culture-confirmed neonatal bloodstream infections and meningitis in South Africa, 2014-19: a cross-sectional study. Lancet Glob Health. 2022;10(8):e1170-e8.
341. Wen SCH, Ezure Y, Rolley L, Spurling G, Lau CL, Riaz S, et al. Gram-negative neonatal sepsis in low- and lower-middle-income countries and WHO empirical antibiotic recommendations: A systematic review and meta-analysis. PLoS medicine. 2021;18(9):e1003787.
342. Rohner P, Pepey B, Auckenthaler R. Advantage of combining resin with lytic BACTEC blood culture media. Journal of clinical microbiology. 1997;35(10):2634-8.
343. Musicha P, Cornick JE, Bar-Zeev N, French N, Masesa C, Denis B, et al. Trends in antimicrobial resistance in bloodstream infection isolates at a large urban hospital in Malawi (1998-2016): a surveillance study. Lancet Infect Dis. 2017;17(10):1042-52.
344. Elfving K, Strömberg LG, Geravandi S, Andersson M, Bachelard M, Msellem M, et al. Pneumococcal concentration and serotype distribution in preschool children with radiologically confirmed pneumonia compared to healthy controls prior to introduction of pneumococcal vaccination in Zanzibar: an observational study. BMC infectious diseases. 2022;22(1):925.
345. Chiou CS, Alam M, Kuo JC, Liu YY, Wang PJ. Chromosome-mediated multidrug resistance in Salmonella enterica serovar Typhi. Antimicrob Agents Chemother. 2015;59(1):721-3.
346. Cuypers WL, Jacobs J, Wong V, Klemm EJ, Deborggraeve S, Van Puyvelde S. Fluoroquinolone resistance in Salmonella: insights by whole-genome sequencing. Microb Genom. 2018;4(7).
347. Ochieng C, Chen JC, Osita MP, Katz LS, Griswold T, Omballa V, et al. Molecular characterization of circulating Salmonella Typhi strains in an urban informal settlement in Kenya. PLoS neglected tropical diseases. 2022;16(8):e0010704.
348. Rutanga JP, de Block T, Cuypers WL, Cafmeyer J, Peeters M, Umumararungu E, et al. Salmonella Typhi whole genome sequencing in Rwanda shows a diverse historical population with recent introduction of haplotype H58. PLoS neglected tropical diseases. 2023;17(6):e0011285.

349. Kuehn R, Stoesser N, Eyre D, Darton TC, Basnyat B, Parry CM. Treatment of enteric fever (typhoid and paratyphoid fever) with cephalosporins. The Cochrane database of systematic reviews. 2022;11(11):Cd010452.
350. Tellevik MG, Blomberg B, Kommedal Ø, Maselle SY, Langeland N, Moyo SJ. High Prevalence of Faecal Carriage of ESBL-Producing Enterobacteriaceae among Children in Dar es Salaam, Tanzania. PloS one. 2016;11(12):e0168024.
351. Moremi N, Claus H, Vogel U, Mshana SE. Faecal carriage of CTX-M extended-spectrum beta-lactamase-producing Enterobacteriaceae among street children dwelling in Mwanza city, Tanzania. PloS one. 2017;12(9):e0184592.
352. Kibwana UO, Majigo M, Kamori D, Manyahi J. High fecal carriage of extended Beta Lactamase producing Enterobacteriaceae among adult patients admitted in referral hospitals in Dar es Salaam, Tanzania. BMC infectious diseases. 2020;20(1):557.
353. Kibwana UO, Manyahi J, Sandnes HH, Blomberg B, Mshana SE, Langeland N, Moyo SJ. Gastrointestinal colonization of extended-spectrum beta-lactamase-producing bacteria among children below five years of age hospitalized with fever in Dar es Salaam, Tanzania. J Glob Antimicrob Resist. 2022;30:107-14.
354. Lester R, Musicha P, van Ginneken N, Dramowski A, Hamer DH, Garner P, Feasey NA. Prevalence and outcome of bloodstream infections due to third-generation cephalosporin-resistant Enterobacteriaceae in sub-Saharan Africa: a systematic review. The Journal of antimicrobial chemotherapy. 2020;75(3):492-507.
355. Büdel T, Kuenzli E, Clément M, Bernasconi OJ, Fehr J, Mohammed AH, et al. Polyclonal gut colonization with extended-spectrum cephalosporin- and/or colistin-resistant Enterobacteriaceae: a normal status for hotel employees on the island of Zanzibar, Tanzania. The Journal of antimicrobial chemotherapy. 2019;74(10):2880-90.
356. Group TGARPG-TW. Antibiotic Use and Resistance in Tanzania: The Center for Disease Dynamics, Ecomomics and Policy, Washington DC, USA, and New Delhi, India; 2015 [Available from: https://onehealthtrust.org/wp-content/uploads/2017/06/garp-tz_situation_analysis-1.pdf.
357. Tompkins K, Juliano JJ, van Duin D. Antimicrobial Resistance in Enterobacterales and Its Contribution to Sepsis in Sub-saharan Africa. Front Med (Lausanne). 2021;8:615649.
358. Onduru OG, Mkakosya RS, Aboud S, Rumisha SF. Genetic Determinants of Resistance among ESBL-Producing Enterobacteriaceae in Community and Hospital Settings in East, Central, and Southern Africa: A Systematic Review and Meta-Analysis of Prevalence. Can J Infect Dis Med Microbiol. 2021;2021:5153237.
359. Paterson DL. Resistance in gram-negative bacteria: Enterobacteriaceae. American journal of infection control. 2006;34(5 Suppl 1):S20-8; discussion S64-73.
360. Mushi MF, Mshana SE, Imirzalioglu C, Bwanga F. Carbapenemase genes among multidrug resistant gram negative clinical isolates from a tertiary hospital in Mwanza, Tanzania. BioMed research international. 2014;2014:303104.
361. Moyo S HB, Aboud S, et al. . Identification of VIM-2-producing Pseudomonas aeruginosa from Tanzania is associated with sequence types 244 and 640 and the location of blaVIM-2 in a TniC integron. Antimicrobial agents and chemotherapy 2015;59:682-5.
362. Moyo SJ, Manyahi J, Hubbard ATM, Byrne RL, Masoud NS, Aboud S, et al. Molecular characterisation of the first New Delhi metallo-β-lactamase 1-producing Acinetobacter baumannii from Tanzania. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2021;115(9):1080-5.
363. Cox JA, Vlieghe E, Mendelson M, Wertheim H, Ndegwa L, Villegas MV, et al. Antibiotic stewardship in low- and middle-income countries: the same but different? Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2017;23(11):812-8.

364. Amponsah OKO, Courtenay A, Ayisi-Boateng NK, Abuelhana A, Opoku DA, Blay LK, et al. Assessing the impact of antimicrobial stewardship implementation at a district hospital in Ghana using a health partnership model. JAC Antimicrob Resist. 2023;5(4):dlad084.
365. WHO malaria rapid diagnostic test performance: round 6 (2014-2015) [17.02.2021]. Available from: https://apps.who.int/iris/bitstream/handle/10665/204118/9789241510035_eng.pdf;jsessionid=0D38F7525A81AF24C9346C1ADF302A61?sequence=1.
366. Baltzell KA, Shakely D, Hsiang M, Kemere J, Ali AS, Björkman A, et al. Prevalence of PCR detectable malaria infection among febrile patients with a negative Plasmodium falciparum specific rapid diagnostic test in Zanzibar. Am J Trop Med Hyg. 2013;88(2):289-91.
367. Das AM, Hetzel MW, Yukich JO, Stuck L, Fakih BS, Al-Mafazy AH, et al. Modelling the impact of interventions on imported, introduced and indigenous malaria infections in Zanzibar, Tanzania. Nature communications. 2023;14(1):2750.
368. Holzschuh A, Lerch A, Gerlovina I, Fakih BS, Al-Mafazy AH, Reaves EJ, et al. Multiplexed ddPCR-amplicon sequencing reveals isolated Plasmodium falciparum populations amenable to local elimination in Zanzibar, Tanzania. Nature communications. 2023;14(1):3699.
369. Connelly SV, Brazeau NF, Msellem M, Ngasala BE, Aydemir O, Goel V, et al. Strong isolation by distance and evidence of population microstructure reflect ongoing Plasmodium falciparum transmission in Zanzibar. medRxiv. 2023.
370. Fakih BS, Holzschuh A, Ross A, Stuck L, Abdul R, Al-Mafazy AH, et al. Risk of imported malaria infections in Zanzibar: a cross-sectional study. Infect Dis Poverty. 2023;12(1):80.
371. Long SS. Diagnosis and management of undifferentiated fever in children. J Infect. 2016;72 Suppl:S68-76.
372. Martinez RM, Wolk DM. Bloodstream Infections. Microbiol Spectr. 2016;4(4).
373. Delgado-Rodríguez M, Llorca J. Bias. J Epidemiol Community Health. 2004;58(8):635-41.
374. Opota O, Jaton K, Greub G. Microbial diagnosis of bloodstream infection: towards molecular diagnosis directly from blood. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2015;21(4):323-31.
375. Huerta LE, Rice TW. Pathologic Difference between Sepsis and Bloodstream Infections. J Appl Lab Med. 2019;3(4):654-63.
376. Vetter E, Torgerson C, Feuker A, Hughes J, Harmsen S, Schleck C, et al. Comparison of the BACTEC MYCO/F Lytic bottle to the isolator tube, BACTEC Plus Aerobic F/bottle, and BACTEC Anaerobic Lytic/10 bottle and comparison of the BACTEC Plus Aerobic F/bottle to the Isolator tube for recovery of bacteria, mycobacteria, and fungi from blood. Journal of clinical microbiology. 2001;39(12):4380-6.
377. Inayath SB, Broor S, Gupta R, Agarwal P, Majumder S, Anveshi AK, Gaind R. Validation of Pefloxacin for detection of fluoroquinolone (FQ) resistance among Salmonella Typhi with special reference to GyrB mutations. Journal of medical microbiology. 2021;70(8).
378. Deurenberg RH, Bathoorn E, Chlebowicz MA, Couto N, Ferdous M, García-Cobos S, et al. Application of next generation sequencing in clinical microbiology and infection prevention. J Biotechnol. 2017;243:16-24.

379. Quainoo S, Coolen JPM, van Hijum S, Huynen MA, Melchers WJG, van Schaik W, Wertheim HFL. Whole-Genome Sequencing of Bacterial Pathogens: the Future of Nosocomial Outbreak Analysis. Clinical microbiology reviews. 2017;30(4):1015-63.
380. Tagini F, Greub G. Bacterial genome sequencing in clinical microbiology: a pathogen-oriented review. Eur J Clin Microbiol Infect Dis. 2017;36(11):2007-20.
381. Rossen JWA, Friedrich AW, Moran-Gilad J. Practical issues in implementing whole-genome-sequencing in routine diagnostic microbiology. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2018;24(4):355-60.
382. Gilchrist CA, Turner SD, Riley MF, Petri WA, Jr., Hewlett EL. Whole-genome sequencing in outbreak analysis. Clinical microbiology reviews. 2015;28(3):541-63.
383. Ellington MJ, Ekelund O, Aarestrup FM, Canton R, Doumith M, Giske C, et al. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2017;23(1):2-22.
384. Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. Nat Rev Genet. 2016;17(6):333-51.
385. Doyle RM, O'Sullivan DM, Aller SD, Bruchmann S, Clark T, Coello Pelegrin A, et al. Discordant bioinformatic predictions of antimicrobial resistance from whole-genome sequencing data of bacterial isolates: an inter-laboratory study. Microb Genom. 2020;6(2).
386. Su M, Satola SW, Read TD. Genome-Based Prediction of Bacterial Antibiotic Resistance. Journal of clinical microbiology. 2019;57(3).
387. Ruppé E, Cherkaoui A, Charretier Y, Girard M, Schicklin S, Lazarevic V, Schrenzel J. From genotype to antibiotic susceptibility phenotype in the order Enterobacterales: a clinical perspective. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2020;26(5):643.e1-.e7.
388. Waddington C, Carey ME, Boinett CJ, Higginson E, Veeraraghavan B, Baker S. Exploiting genomics to mitigate the public health impact of antimicrobial resistance. Genome Med. 2022;14(1):15.
389. Shrestha P, Roberts T, Homsana A, Myat TO, Crump JA, Lubell Y, Newton PN. Febrile illness in Asia: gaps in epidemiology, diagnosis and management for informing health policy. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2018;24(8):815-26.
390. Kigozi BK, Kharod GA, Bukenya H, Shadomy SV, Haberling DL, Stoddard RA, et al. Investigating the etiology of acute febrile illness: a prospective clinic-based study in Uganda. BMC infectious diseases. 2023;23(1):411.
391. Moreira J, Bressan CS, Brasil P, Siqueira AM. Epidemiology of acute febrile illness in Latin America. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2018;24(8):827-35.
392. Obeng-Nkrumah N, Labi AK, Addison NO, Labi JE, Awuah-Mensah G. Trends in paediatric and adult bloodstream infections at a Ghanaian referral hospital: a retrospective study. Annals of clinical microbiology and antimicrobials. 2016;15(1):49.
393. Mtunthama N, Gordon SB, Kusimbwe T, Zijlstra EE, Molyneux ME, French N. Blood culture collection technique and pneumococcal surveillance in Malawi during the four year period 2003-2006: an observational study. BMC infectious diseases. 2008;8:137.

12 Papers

Paper I

PLOS ONE

RESEARCH ARTICLE

Prevalence and Antimicrobial Resistance of Microbes Causing Bloodstream Infections in Unguja, Zanzibar

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Abstract

Background

Bloodstream infections (BSI) are frequent and cause high case-fatality rates. Urgent antibiotic treatment can save patients' lives, but antibiotic resistance can render antibiotic therapy futile. This study is the first to collect epidemiological data on BSI from Unguja, Zanzibar.

Methods

Clinical data and blood for culturing and susceptibility testing of isolated microbes were obtained from 469 consecutively enrolled neonates, children and adults presenting with signs of systemic infections at Mnazi Mmoja Hospital (MMH), Zanzibar.

Results

Pathogenic bacteria were recovered from the blood of 14% of the patients (66/469). The most frequently isolated microbes were *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter* spp. and *Staphylococcus aureus*. Infections were community-acquired in 56 patients (85%) and hospital-acquired in 8 (12%) (data missing for 2 patients). BSI caused by extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* (*E. coli*, *K. pneumoniae*) was found in 5 cases, of which 3 were community-acquired and 2 hospital-acquired. Three of these patients died. Six of 7 *Salmonella* Typhi isolates were multidrug resistant. *Streptococcus pneumoniae* was found in one patient only.

Conclusions

This is the first report of ESBL-producing bacteria causing BSI from the Zanzibar archipelago. Our finding of community-acquired BSI caused by ESBL-producing bacteria is alarming, as it implies that these difficult-to-treat bacteria have already spread in the society. In the local setting these infections are virtually impossible to cure. The findings call for

to Norway as well as payment to the local collaborators in Zanzibar, both at the laboratory and at the wards, and part of the travel expenses and accommodation expenses of AO.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: BSI, Bloodstream-infection; MMH, Mnazi Mmoja Hospital; ESBL, extended-spectrum beta-lactamase; HIV, human immunodeficiency virus; PCR, polymerase chain reaction.

increased awareness of rational antibiotic use, infection control and surveillance to counteract the problem of emerging antimicrobial resistance.

Introduction

Sepsis is a major health problem associated with high mortality rates [1,2]. Data on both mortality and incidence of sepsis in Africa are limited. A mean mortality rate of 18.1% is reported in a meta-analysis on community acquired bloodstream-infection (BSI) in Africa [1]. In a study on a pediatric population in Tanzania a mortality rate of 34.9% was found [3]. High prevalence of immunosuppression due to malnutrition and other infectious diseases including human immunodeficiency virus (HIV) infection and measles may contribute to an increased burden of severe bacterial infections in African countries [3,4]. BSI caused by multidrug-resistant, extended-spectrum beta-lactamase (ESBL) producing Gram-negative bacilli is associated with very high case-fatality rates approaching those of the pre-antibiotic era [3]. Epidemiological data from specific geographic regions are needed to optimize guidelines for empirical treatment. In Zanzibar, data on the etiology of BSI have only been published from Pemba, the less populated of the two main islands comprising Zanzibar [5]. We performed a prospective cohort study in patients suspected of having BSI at Mnazi Mmoja Hospital (MMH) on Unguja, the most populated island of the Zanzibar archipelago, Zanzibar, Tanzania. The aim was to identify the most common bacterial pathogens causing BSI and to determine their antimicrobial susceptibility.

Material and Methods

Study site

Mnazi Mmoja hospital (MMH), Zanzibar, Tanzania, is the main referral and teaching hospital of the Zanzibar Archipelago with a population estimated to about 1.3 million in 2012 (http://www.nbs.go.tz). The hospital also offers primary and secondary health care for the residents of Zanzibar city with a population of about 600,000. The hospital has 544 beds.

Study design

Patients in the medical, pediatric and neonatal departments were enrolled in the study if they, either on admission or during their hospital stay, had fever ($\geq$ 38.3°C in adults, $\geq$ 38.5°C in children) or hypothermia (<36.0°C), tachypnoea >20/min, tachycardia >90/min or were otherwise suspected to have systemic bacterial infection as judged by the clinician. Demographic and clinical information was obtained. Infections were defined as community-acquired and hospital-acquired, if pathogens were detected in samples taken within 2 days after admission and > 2 days after admission, respectively.

Methods

Patients were included over a period of 7 months (26th March to 22nd June 2012, 26th October to 21st December 2012, and 4th February to 22nd April 2013). Blood specimens were inoculated in BACTEC Myco/F lytic blood culturing vials (Becton Dickinson, Franklin Lakes, N.J.), one bottle per episode of febrile illness. The bottles were incubated at 37°C for 7 days and checked daily on Monday to Friday and once on either Saturdays or Sundays for microbial growth by inspecting the bottom indicator of the bottle for fluorescence [6].

Positive samples were examined by microscopy of Gram-stained preparations and subcultivated for two days on chocolate agar and on human blood agar in 5% CO_2, and on MacConkey agar in aerobe atmosphere. The isolates were identified according to established conventional procedures [7]. Samples with polymicrobial growth were included, if at least one of the microbes was considered a pathogen. As most patients had only one blood culture drawn, it was not possible to ascertain the role of bacteria of uncertain clinical relevance, such as coagulase-negative staphylococci, diphtheroids and *Bacillus* species. Thus, these species were considered contaminants.

Gram-negative rods were identified using standard biochemical tests and the API 20E or the API 20NE system (bioMérieux, Marcy-l'Etoile, France). Susceptibility testing was performed by disc diffusion technique as described in the EUCAST guidelines [8]. Reports on the results were sent to the wards.

Isolates of pathogenic microbes were sent to Bærum Hospital, Vestre Viken Health Trust, Norway, for quality control and further identification by VITEK 2 and, if necessary, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and/or 16S rDNA polymerase chain reaction (PCR) sequencing (performed at Oslo University Hospital and/or the Norwegian Institute of Public Health, Oslo, Norway). In Norway, susceptibility testing was performed by disc diffusion technique and/or Etest gradient system (bioMérieux) according to the EUCAST guidelines and/or by VITEK 2 and interpreted by the S-I-R system [9]. *Enterobacteriaceae* isolates resistant to cefotaxime or ceftazidime, were further assessed for ESBL type resistance by ESBL Etest gradient system (bioMérieux, France), ESBL CTX-M in-house PCR [10] and AmpC in-house PCR [11].

Statistical analysis and ethical approval

Differences between proportions were compared using Fisher's exact test with cutoff for statistical significance at $p = 0.05$. The research protocol was approved by the Zanzibar Medical Research and Ethical Committee (ZAMREC), record no ZAMREC /0004/JAN/012, and by the Regional Committee for Medical Research Ethics Health Region West (REK III), Norway, record no 201124397/2011/2439/REK vest. Written informed consent was obtained from the patient or, in the case of children, from a parent or a responsible family member.

Results

Microbes

A total of 470 blood culture samples from 469 patients (242 male, 226 female, 1 with gender not reported) were consecutively included in the study, including 148 (31%) from children (aged >1 month to 5 years of age), and 113 (24%) from neonates (age $\leq$ 1 month) (Table 1). One blood culture bottle per patient was taken except for one patient who had two blood cultures taken 3 weeks apart, because of a new episode of febrile illness. Pathogenic microbes were isolated from 14.0% (66/470) of the blood cultures (66 patients), including 15 cultures with growth of multiple isolates, out of which at least one pathogen. The most frequent isolated pathogens were *K. pneumoniae* (n = 11), *E. coli* (n = 10), *Acinetobacter* spp. (n = 10) and *S. aureus* (n = 9) (Tables 2 and 3). A further 53 (11%) blood cultures yielded growth of bacteria considered to be probable contaminants: coagulase-negative staphylococci (n = 46, 10%), diphtheroids (n = 2), *Bacillus* sp. (n = 1), Gram-positive rods (n = 3), and mixture of coagulase negative staphylococcus and diphtheroids (n = 1).

Among the 66 patients with pathogenic microbes in the blood culture, 56 patients (85%) had community-acquired infection, and 8 patients (12%) had hospital-acquired infection. Mode of acquisition could not be assessed for 2 patients due to missing information.

Table 1. Distribution of 469 patients by gender and age group.

	Neonate* (≤ 1 month)	<5 years (>1 month to <5 years)	5–15 years	Adults
Male	57	84	25	76
Female	55	64	15	92
Total	113	148	40	168

*1 patient no info on gender.

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Table 2. Frequency of bacterial and fungal pathogens causing community- and hospital-acquired bloodstream-infections in patients admitted at Mnazi Mmoja Hospital, Zanzibar.

Pathogens	Total	Commu-nity acquired**	Hospital acquired***	Missing data
Total	**79 (100%)**	**67 (100%)**	**10 (100%)**	**2**
Total Gram-negative bacteria**	**59 (75%)**	**51 (76%)**	**7 (70%)**	**1**
Total *Enterobacteriaceae*	**36**	**31**	**4**	**1**
-*E.coli**	**10**	**9**	**1**	
-*K. pneumoniae**	**11**	**8**	**3**	
-*Salmonella serovar* Typhi*	**7**	**6**		**1**
-*Enterobacter cloacae**	**4**	**4**		
-*Enterobacter aerogenes*	**1**	**1**		
-*Proteus mirabilis*	**2**	**2**		
-*Citrobacter freundii*	**1**	**1**		
Total non-*Enterobacteriaceae*	**19**	**17**	**2**	
-*Pseudomonas putida*	**1**	**1**		
-*Acinetobacter baumannii**	**6**	**6**		
-*Acinetobacter* spp. non- *baumannii*	**4**	**3**	**1**	
-*Ochrobactrum anthropi*	**3**	**2**	**1**	
-*Achromobacter* spp.	**5**	**5**		
Unidentified Gram-negative rods	**4**	**3**	**1**	
Total Gram-positive bacteria	**18 (23%)**	**14 (21%)**	**3 (30%)**	**1**
Staphylococcus aureus	**9**	**7**	**1**	**1**
Enterococcus faecalis	**4**	**3**	**1**	
Enterococcus faecium	**1**		**1**	
*Streptococcus pneumoniae**	**1**	**1**		
Group B *streptococcus*	**2**	**2**		
Rhodococcus equi	**1**	**1**		
Total yeast	**2 (2%)**	**2 (3%)**		
Candida guillermondii	**1**	**1**		
Other yeast	**1**	**1**		

*Isolates not stored in Zanzibar: *E. coli* 3, *K. pneumoniae* 2, *S.* Typhi 1, *A. baumannii* 2, unidentified gram-negative rods 2, *S. aureus* 2.

Isolates that succumbed during transport to Norway: *E. coli* 2, *K. pneumoniae* 1, unidentified gram-negative rod 1, *S. pneumoniae* 1.

6 strains of *S.* Typhi and 1 unidentified gram-negative rod (possible Brucella) sent to Norway were inactivated because of transport regulations.

** Community-acquired infection, i.e. blood-culture obtained ≤ 48 hours from of admission.

***Hospital-acquired infection: Blood-culture obtained > 48 hours from admission.

****The percentage refers to the proportion of all pathogenic bacterial and fungal isolates.

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Table 3. Frequency of bacterial and fungal pathogens causing bloodstream infection in among patients admitted to Mnazi Mmoja Hospital, Zanzibar, by age groups.

Pathogen	Total	Neonate (≤1 month)	<5 years (>1 month to <5 years)	5–15 years	Adult
Total	**79**	**43 (54%)**	**10 (13%)**	**8 (10%)**	**18 (23%)**
Total Gram-negatives	**59**	**33**	**9**	**4**	**13**
Total *Enterobacteriaceae*	**36**	**19**	**5**	**3**	**9**
-E.coli	**10**	**6**	**1**	**1**	**2**
-K. pneumoniae	**11**	**8**	**1**		**2**
-Salmonella serovar Typhi	**7**		**1**	**2**	**4**
-Other *Enterobacteriaceae*	**8**	**5**	**2**		**1**
Total non-*Enterobacteriaceae*	**19**	**11**	**4**	**1**	**3**
Unidentified Gram-negative rods	**4**	**3**			**1**
Total Gram-positives	**18**	**10**		**4**	**4**
S. aureus	**9**	**3**		**4**	**2**
Enterococcus faecalis	**4**	**4**			
Enterococcus faecium	**1**	**1**			
S. pneumoniae	**1**				**1**
Group B *streptococcus*	**2**	**1**			**1**
Rhodococcus equi	**1**	**1**			
Total yeast	**2**		**1**		**1**

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Eighteen isolates of pathogens could not be retested in Norway as they either were not stored in the freezer in Zanzibar (n = 12) or did not survive the transport (n = 6).

Antimicrobial resistance (Table 4)

Six isolates (from 5 patients) of *Enterobacteriaceae* (*Klebsiella pneumoniae* (5) and *E. coli* (1)) were suspect of ESBL-production as they displayed resistance to cefotaxime or/and ceftazidime on disc-diffusion testing. ESBL-Etest was positive for 4 isolates (from 3 patients), *K. pneumoniae* (3) and *E. coli* (1). For 2 of the *K. pneumoniae* isolates including 1 isolate testing intermediate for meropenem, the results on further testing for ESBL production are lacking.

Table 4. Antimicrobial susceptibility (number tested) of *Enterobacteriaceae* isolates causing bloodstream infection in patients admitted to Mnazi Mmoja Hospital, Zanzibar.

	K. pneumoniae **(n = 11)**	***E. coli*** **(n = 10)**	***S.* Typhi (n = 7)**	**other *Entero-bacteriaceae* (n = 8)**	**Total *Entero-bacteriaceae* (n = 36)***	**Total susceptibility percentage**
Ampicillin	0**	6	0	1	7	19%
Cefotaxime	6	9	7	7	29	81%
Piperacillin/tazobactam	7	10		8	25	86%
Meropenem	10***	10	7	8	35	97%
Gentamicin	8	10		8	26	90%
Ciprofloxacin	10	9	6	8	33	92%
Trimethoprim/ sulfamethoxazole	6	3	0	5	14	39%

*for Piperacillin/tazobactam only 29 isolates were analysed. For *S.* Typhi, the susceptibility to gentamicin was not analyzed: aminoglycosids are not recommended for treatment of *S.* Typhi infections because they lack activity against intracellular Salmonella[12].

***K. pneumoniae* are naturally resistant to ampicillin.

***one intermediate to meropenem, only tested in Zanzibar, cefotaxime, ampicillin, trimethoprim-sulfamethoxazole R, probable ESBL positive.

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The 4 ESBL E-test positive isolates were tested with PCR for CTX-M genotype. Three of the isolates were CTX-M PCR positive. The fourth isolate (*K. pneumoniae*) was CTX-M PCR negative, but CMY-PCR positive (belonging to the AmpC β-lactamases).

Among the 5 patients with BSI caused by bacteria with confirmed or probable ESBL-production, the infection was classified as hospital-acquired in 2 patients and community-acquired in 3 patients. In 13 patients, ESBL-negative *E. coli* or *K. pneumoniae* or both (two patients had mixed infection with both *E.coli* and *K. pneumoniae*) were isolated, of which the majority were community-acquired (10/13). Three of the 5 (60%) patients with infection caused by confirmed or probable ESBL-positive bacteria died. Four of the 11 (36%) of the patients with infection caused by bacteria without ESBL-production died (data were missing for 2 patients). This difference in case-fatality rates was not statistically significant ($p = 0.6$).

All confirmed and probable ESBL-positive isolates were resistant to trimethoprim-sulfamethoxazole, while rates of co-resistance to other antibiotics were 3/5 to gentamicin, 2/5 to ciprofloxacin, 1/5 to piperacillin-tazobactam. No isolate displayed phenotypic carbapenem resistance. The majority of the *S.* Typhi isolates 6/7 (86%) were multidrug-resistant (i.e. resistant to ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol), but susceptible to cefotaxime.

Only one isolate of *S. pneumoniae* was recovered. Resistance to oxacillin indicated a reduced susceptibility to penicillin G. Further testing was not possible as the isolate died. The only *Enterococcus faecium* that was isolated was high level gentamicin resistant, but susceptible to vancomycin. All nine *S. aureus* strains were susceptible to cefoxitin, clindamycin and erythromycin. No methicillin-resistant *S. aureus* (MRSA) was found. All 7 *S. aureus* isolates tested for pencillinase-production were positive, 2/9 were resistant to trimethoprim-sulfamethoxazole, 1/9 to tetracycline.

Discussion

While ESBL producing microbes in clinical samples including blood cultures have been reported from other parts of the African continent [13], including the mainland of Tanzania [14–16], this is the first report of ESBL-producing bacteria causing bloodstream infections from the Zanzibar archipelago. In a recent study on bacteremia from the neighbor island Pemba [5] no ESBL-positive bacteria were found. The finding of ESBL-positive microbes in blood culture is associated with increased mortality [3]. We did not find significantly higher case-fatality rate in patients with bloodstream-infections caused by ESBL producing *Enterobacteriaceae* (3/5) compared to those caused by ESBL negative microbes (4/11), but the numbers of the patients were small.

Differences between the findings of the studies from MMH/ Unguja and Pemba in both etiology of bloodstream infections and the susceptibility patterns of the isolated microbes may be explained by the fact that Unguja has a more urban infrastructure and people have easier access to antimicrobials. Furthermore, Unguja has more extensive contact with mainland Tanzania, where a high prevalence of resistant microbes has been documented [15,16], and also more exposure to tourists and international travelers. These differences may imply a higher rate of preadmission antimicrobial treatment, although we have no evidence to support this.

While the study from Pemba assessed community-acquired infections at three district hospitals, our study was performed at an urban referral hospital and included nosocomial infections and patients in a neonatal intensive care unit. This may have led to selection of more severely ill patients, infections with more resistant microbes, and more frequent use of broad-spectrum antimicrobials, which in turn may also have contributed to the higher rate of resistant bacteria in our study.

Previously, ESBL-producing bacteria were largely associated with nosocomial infections, but according to more recent studies, infections caused by community-acquired ESBL-producing bacteria are increasing [17]. ESBL-positive bacteria at MMH were found not only in hospital-acquired, but also in community-acquired infections. Our finding of community-acquired bloodstream infections caused by ESBL producing bacteria is alarming, as it implies that these difficult-to-treat bacteria have already spread in the society. Treatment of infections caused by ESBL-producing bacteria is much more costly, if at all available, and leads to prolonged hospital stays for those who survive [18,19].

ESBL-positive bacteria are resistant to third generation cephalosporins. These are often used as first line medication in sepsis at MMH. ESBL resistance is plasmid-mediated. These plasmids also often carry resistance genes to other groups of antibiotics [20]. Therefore carbapenems are the cornerstone of treatment of infections caused by ESBL-producing bacteria. However, these antibiotics are expensive and generally not available in resource-constrained settings such as Zanzibar, rendering such infections virtually untreatable in the local setting. Even if carbapenems were available, their use in the absence of accessible microbiological diagnostic services is problematic. Low treatment success due to high prevalence of infections caused by resistant bacteria likely results in increasing empiric use of broad-spectrum antibiotics, which exerts a strong selection pressure favoring further emergence of multidrug-resistant bacteria in the hospital and the society. Infections caused by carbapenem-resistant bacteria have already been documented in nearby Dar es Salaam, in mainland Tanzania [21]. Globally, antimicrobial resistance to Gram-negative microbes is rising faster than in Gram-positive bacteria and there are no new antibiotics effective against Gram-negative bacteria in the immediate pipeline [22]. In countries with limited resources, the rapid emergence of antibiotic-resistant bacteria is furthermore promoted by patient overcrowding, overwhelmed health-care workers, limited hospital infrastructure, poor compliance with hand hygiene, and, lack of infection control programs [23]. Improved microbiological diagnosis, antibiotic susceptibility testing and epidemiological studies, may help guide sustainable, rational antibiotic use.

Comparison of the etiology of sepsis among different studies in Africa [1] is challenging, as different populations are included (adults, children, neonates, all age groups, community-acquired or/and nosocomial infections). The varying prevalence of other diseases, such as HIV-infections and malaria probably also have an impact on the findings [24], as well as the geographical region and the socio-economic structure. Our study population consists of all age groups, with both community-acquired and nosocomial infection, from an area with low prevalence of malaria and HIV-infection [25–27].

The prevalence of bacteremia in our study (14%) is in line with findings of a meta-analysis on the cause of community-acquired bloodstream infections in Africa, which found a prevalence of 13.4% among patients with fever [1]. *Salmonella enterica*, of which 41% were *Salmonella* Typhi, followed by *Streptococcus pneumoniae* were the most frequent isolated microbes, with *S. enterica* being the most common isolate in adults, and *S. pneumoniae* the most frequent in children. Other common bacteria were *S. aureus* and *E. coli*. [1]. We found only one isolate of *S. pneumoniae*, and we suspect, as in the study from Dar es Salaam [3], that frequent prehospital antibiotic use may have precluded the recovery of pneumococci from blood cultures, resulting in underestimation of the proportion of pneumococcal infections.

Multi-resistance in *S.* Typhi (resistant to ampicillin, cotrimaxozole and chloramphenicol) was more frequent in our study (86%, n = 6/7) than reported from Pemba (42%, n = 19/45, p = 0.046, Fisher's exact test) [5]. Low and similar rates of resistance to ciprofloxacin were found at both sites.

The only *S. pneumoniae* in our study was oxacillin resistant implicating a decreased sensitivity to penicillin G. In the study from Pemba, *S. pneumoniae* was the second most common microbe (15%), after S. Typhi (58%), and 25% (3/12) of the pneumococcal isolates were penicillin resistant [5]. We found low rates of resistance among Gram-positive bacteria. No methicillin-resistant *S. aureus* (MRSA) was isolated. This is in line with the study from Pemba, but contrary to findings from other African countries and mainland Tanzania [3,28,29].

Non-fermentative gram-negative rods were frequently isolated from neonatal patients and must be regarded as real pathogens [30] as the immune system in neonates is still immature. *Acinetobacter* has been shown to cause severe disease, particularly in tropical countries [30]. The study from Dar es Salaam also found a high proportion of BSI attributable to non-fermenters (11.6%, 34/294) [3]. Non-fermentative gram-negative bacteria are generally isolated more frequently in sepsis especially in patients with underlying diseases [31]. However, contamination must also be considered in these cases.

Polymicrobial infection, i.e. growth of 2 or 3 different microbes from the same blood culture, occurred frequently (19% of BSIs) in our study, as in the study from Dar es Salaam (12%) [3]. Polymicrobial BSI may have been caused by translocation from gastrointestinal focus of infection, possibly in very sick or immunocompromised patients. Contamination of the samples may be another possible explanation. Better staff training in the technique of taking samples can reduce the risk of contamination.

The main limitation of the study is the low number of patients included. Another limitation is that only one sample per patient was taken, due to limited resources. Coagulase-negative staphylococci were therefore counted as contaminants, although they may have had clinical significance in some cases of immune-compromised patients or patients with indwelling devices and among neonates. No anaerobe culture was performed. Data were lacking on pre-treatment with antibiotics and outcome. As the study did not cover all seasons, possible seasonal variations may have been missed. Limitations in laboratory facilities and transport caused loss of some data [32,33].

Conclusions

This is the first study of bloodstream-infections from Unguja, Zanzibar, and the first to document the presence of ESBL-producing multidrug-resistant *Enterobacteriaceae* as a cause of bloodstream infections in the Zanzibar archipelago. These infections are difficult to treat in the local setting and were associated with a high case-fatality rate. The finding of community-acquired infections caused by ESBL-resistant bacteria in Zanzibar is particularly worrying, as it indicates a general spread of these resistant bacteria in the society. The study findings call for prudent antibiotic use and focus on infection control in health-care settings.

More data are needed on the etiology and antimicrobial susceptibility in bloodstream infections in Zanzibar including the prevalence of multidrug-resistant ESBL-producing bacteria, and this knowledge can be used to guide the development of new treatment guidelines for MMH and Zanzibar. The education of health workers in rational use of antimicrobials as well as in infection control should be intensified.

Supporting Information

S1 Table. Data on study participants.
(XLS)

S2 Table. Data on bacterial isolates.
(XLS)

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Author Contributions

Conceived and designed the experiments: AO BB AKS PJ. Analyzed the data: AO BB AKS MJ PJ. Wrote the paper: AO BB PJ AKS MJ.

References

1. Reddy EA, Shaw AV, Crump JA (2010) Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. Lancet Infect Dis 10: 417–432. doi: 10.1016/S1473-3099(10)70072-4 PMID: 20510282
2. Gomes Silva BN, Andriolo RB, Atallah AN, Salomao R (2010) De-escalation of antimicrobial treatment for adults with sepsis, severe sepsis or septic shock. Cochrane Database Syst Rev: Cd007934. doi: 10.1002/14651858.CD007934.pub2 PMID: 21154391
3. Blomberg B, Manji KP, Urassa WK, Tamim BS, Mwakagile DS, Jureen R, et al. (2007) Antimicrobial resistance predicts death in Tanzanian children with bloodstream infections: a prospective cohort study. BMC Infect Dis 7: 43. PMID: 17519011
4. Becker JU, Theodosis C, Jacob ST, Wira CR, Groce NE (2009) Surviving sepsis in low-income and middle-income countries: new directions for care and research. The Lancet Infectious Diseases 9: 577–582. doi: 10.1016/S1473-3099(09)70135-5 PMID: 19695494
5. Thriemer K, Ley B, Ame S, von Seidlein L, Pak GD, Chang NY, et al. (2012) The burden of invasive bacterial infections in Pemba, Zanzibar. PLoS One 7: e30350. doi: 10.1371/journal.pone.0030350 PMID: 22363426
6. Archibald LK, McDonald LC, Addison RM, McKnight C, Byrne T, Dobbie H, et al. (2000) Comparison of BACTEC MYCO/F LYTIC and WAMPOLE ISOLATOR 10 (lysis-centrifugation) systems for detection of bacteremia, mycobacteremia, and fungemia in a developing country. J Clin Microbiol 38: 2994–2997. PMID: 10921966
7. Collee JG M B, Irvine R, Fraser AG, Simmons A, editor (1996) Mackie & McCartney Practical Medical Microbiology. 14th edition ed. New York: Churchill Livingstone.
8. http://eucast.org/.
9. (1981) A revised system for antibiotic sensitivity testing.The Swedish Reference Group for Antibiotics. Scandinavian Journal of Infectious Diseases 13: 148–152. PMID: 7313568
10. Birkett CI, Ludlam HA, Woodford N, Brown DF, Brown NM, Roberts MT, et al. (2007) Real-time TaqMan PCR for rapid detection and typing of genes encoding CTX-M extended-spectrum beta-lactamases. J Med Microbiol 56: 52–55. PMID: 17172517
11. Sturød K. D UR, Berg E.S., Steinbakk M., Wester A.L. (2014) Evaluation of the ability of four ESBL-screening media to detect ESBL-producing *Salmonella* and *Shigella*. BMC Microbiol 14.
12. Mandell G.L. B JE, Dolin R., editor (2005) Principles and Practice of Infectious Diseases. 6 ed: Elsevier Churchill Livingstone.
13. Tansarli GS, Poulikakos P, Kapaskelis A, Falagas ME (2014) Proportion of extended-spectrum beta-lactamase (ESBL)-producing isolates among Enterobacteriaceae in Africa: evaluation of the evidence

—systematic review. J Antimicrob Chemother 69: 1177–1184. doi: 10.1093/jac/dkt500 PMID: 24398340

14. Mshana SE, Hain T, Domann E, Lyamuya EF, Chakraborty T, Imirzalioglu C (2013) Predominance of Klebsiella pneumoniae ST14 carrying CTX-M-15 causing neonatal sepsis in Tanzania. BMC Infect Dis 13: 466. doi: 10.1186/1471-2334-13-466 PMID: 24099282
15. Mshana SE, Kamugisha E, Mirambo M, Chakraborty T, Lyamuya EF (2009) Prevalence of multiresistant gram-negative organisms in a tertiary hospital in Mwanza, Tanzania. BMC Res Notes 2: 49. doi: 10.1186/1756-0500-2-49 PMID: 19323805
16. Blomberg B, Jureen R, Manji KP, Tamim BS, Mwakagile DS, Urassa WK, et al. (2005) High rate of fatal cases of pediatric septicemia caused by gram-negative bacteria with extended-spectrum beta-lactamases in Dar es Salaam, Tanzania. J Clin Microbiol 43: 745–749. PMID: 15695674
17. Kothari C, Gaind R, Singh LC, Sinha A, Kumari V, Arya S, et al. (2013) Community acquisition of beta-lactamase producing Enterobacteriaceae in neonatal gut. BMC Microbiol 13: 136. doi: 10.1186/1471-2180-13-136 PMID: 23773627
18. Cosgrove SE (2006) The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. Clin Infect Dis 42 Suppl 2: S82–89. PMID: 16355321
19. Tansarli GS, Karageorgopoulos DE, Kapaskelis A, Falagas ME (2013) Impact of antimicrobial multidrug resistance on inpatient care cost: an evaluation of the evidence. Expert Rev Anti Infect Ther 11: 321–331. doi: 10.1586/eri.13.4 PMID: 23458771
20. Paterson DL (2006) Resistance in gram-negative bacteria: Enterobacteriaceae. Am J Infect Control 34: S20–28; discussion S64–73. PMID: 16813978
21. Moyo S, Haldorsen B, Aboud S, Blomberg B, Maselle SY, Sundsfjord A, et al. (2015) Identification of VIM-2-producing Pseudomonas aeruginosa from Tanzania is associated with sequence types 244 and 640 and the location of blaVIM-2 in a TniC integron. Antimicrobial agents and chemotherapy 59: 682–685. doi: 10.1128/AAC.01436-13 PMID: 25331700
22. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, et al. (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis 10: 597–602. doi: 10.1016/S1473-3099(10)70143-2 PMID: 20705517
23. Alp E, Leblebicioglu H, Doganay M, Voss A (2011) Infection control practice in countries with limited resources. Ann Clin Microbiol Antimicrob 10: 36. doi: 10.1186/1476-0711-10-36 PMID: 22018286
24. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA (2012) Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. Lancet 379: 2489–2499. doi: 10.1016/S0140-6736(11)61752-2 PMID: 22587967
25. Aregawi MW, Ali AS, Al-mafazy AW, Molteni F, Katikiti S, Warsame M, et al. (2011) Reductions in malaria and anaemia case and death burden at hospitals following scale-up of malaria control in Zanzibar, 1999–2008. Malar J 10: 46. doi: 10.1186/1475-2875-10-46 PMID: 21332989
26. Mnyika KS, Makwaya CK, Lyamuya EF, Nyamuryekung'e K, Ndyetabura FE, Dahoma MU, et al. (2012) Prevalence of HIV-1 infection in Zanzibar: results from a national HIV-1 serosurvey 2002. East Afr J Public Health 9: 123–127. PMID: 23136709
27. WHO facts on Tanzania updated august 2009.
28. Vlieghe E, Phoba MF, Tamfun JJ, Jacobs J (2009) Antibiotic resistance among bacterial pathogens in Central Africa: a review of the published literature between 1955 and 2008. Int J Antimicrob Agents 34: 295–303. doi: 10.1016/j.ijantimicag.2009.04.015 PMID: 19540725
29. Saied T, Elkholy A, Hafez SF, Basim H, Wasfy MO, El-Shoubary W, et al. (2011) Antimicrobial resistance in pathogens causing nosocomial bloodstream infections in university hospitals in Egypt. Am J Infect Control 39: e61–65. doi: 10.1016/j.ajic.2011.04.009 PMID: 21835504
30. Munoz-Price LS, Weinstein RA (2008) Acinetobacter infection. N Engl J Med 358: 1271–1281. doi: 10.1056/NEJMra070741 PMID: 18354105
31. Aisenberg G, Rolston KV, Safdar A (2004) Bacteremia caused by Achromobacter and Alcaligenes species in 46 patients with cancer (1989–2003). Cancer 101: 2134–2140. PMID: 15389476
32. Archibald LK, Reller LB (2001) Clinical microbiology in developing countries. Emerg Infect Dis 7: 302–305. PMID: 11294729
33. Petti CA, Polage CR, Quinn TC, Ronald AR, Sande MA (2006) Laboratory medicine in Africa: a barrier to effective health care. Clin Infect Dis 42: 377–382. PMID: 16392084

Paper II

Title: Predominance of multidrug-resistant *Salmonella* Typhi genotype 4.3.1 with low-level ciprofloxacin resistance in Zanzibar

Short title: Typhoid in Zanzibar

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Abstract

Background: Typhoid fever is a common cause of febrile illness in low- and middle-income countries. While multidrug-resistant (MDR) *Salmonella* Typhi (*S.* Typhi) has spread globally, fluoroquinolone resistance has mainly affected Asia.

Methods: Consecutively, 1038 blood cultures were obtained from patients of all age groups with fever and/or suspicion of serious systemic infection admitted at Mnazi Mmoja Hospital, Zanzibar in 2015-2016. *S.* Typhi were analyzed with antimicrobial susceptibility testing and with short read (61 strains) and long read (9 strains) whole genome sequencing, including three *S*. Typhi strains isolated in a pilot study 2012-2013.

Results: Sixty-three *S*. Typhi isolates (98%) were MDR carrying $bla_{TEM\text{-}1B}$, *sul1* and *sul2*, *dfrA7* and *catA1* genes. Low-level ciprofloxacin resistance was detected in 69 % (43/62), with a single gyrase mutation *gyrA*-D87*G* in 41 strains, and a single *gyrA*-S83F mutation in the non-MDR strain. All isolates were susceptible to ceftriaxone and azithromycin. All MDR isolates belonged to genotype 4.3.1 lineage I (4.3.1.1), with the antimicrobial resistance determinants located on a composite transposon integrated into the chromosome. Phylogenetically, the MDR subgroup with ciprofloxacin resistance clusters together with two external isolates.

Conclusions: We report a high rate of MDR and low-level ciprofloxacin resistant *S.* Typhi circulating in Zanzibar, belonging to genotype 4.3.1.1, which is widespread in Southeast Asia and African countries and associated with low-level ciprofloxacin resistance. Few therapeutic options are available for treatment of typhoid fever in the study setting. Surveillance of the prevalence, spread and antimicrobial susceptibility of *S.* Typhi can guide treatment and control efforts.

Author summary

Salmonella Typhi causes typhoid fever. Multi-drug resistant (MDR) *S.* Typhi is spreading globally. Local and regional surveillance of MDR *S*. Typhi populations using both blood culture and whole genome sequencing can uncover outbreaks and help mapping the spread of *S*. Typhi and resistance mechanisms, which, in turn, can guide both control and prevention efforts and clinical management. Data regarding the distribution of MDR *S.* Typhi genotypes and resistance mechanisms is scarce in Zanzibar, Tanzania, as in many other African countries. In this study we characterize *S.* Typhi phenotypically and genotypically. This study shows high rate of MDR *S.* Typhi, hence few therapeutic options are available for treatment of typhoid fever in the study setting. Our findings contribute to the knowledge base on typhoid fever in the region and to guide correct treatment of individual patients and control of the disease.

Introduction

Salmonella enterica subspecies *enterica* serovar Typhi (*S.* Typhi) causes typhoid fever, an important global health problem with an estimated worldwide annual incidence of over 9 million cases, and about 100,000 to 200,000 deaths (1-4). The estimated burden is uncertain due to diagnostic shortcomings and poor access to health care (5), but there are indications that the burden has declined lately (2). In different geographic regions the incidence varies substantially, with incidence in excess of 800 cases per 100 000 persons per year in some settings in sub-Saharan Africa (6, 7), and higher numbers in certain urban areas compared to rural settings (7). Typhoid fever prevails in regions with poor sanitation facilities and limited access to clean drinking water (8). Infections caused by multidrug-resistant (MDR) *S.* Typhi strains, defined by resistance to the prior first-line treatments ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole, are complicated to treat and are associated with increased mortality (9, 10). The appearance of MDR *S*. Typhi in the 1970s led to widespread use of fluoroquinolones, and subsequent emergence of fluoroquinolone resistant *S*. Typhi in the early 1990s (11-13). A combination of MDR and fluoroquinolone resistant *S.* Typhi leaves clinicians with few therapeutic options especially in developing countries. Since 2017, WHO ranked fluoroquinolone resistant *S.* Typhi as a high priority pathogen for research and development of new antibiotics (14).

Genetically *S*. Typhi is monomorphic (13). A recent introduction of a phylogenetic genotyping scheme, GenoTyphi, has facilitated the interpretation of whole genome sequencing data (WGS) for *S*. Typhi (15-17). Using this scheme, a global collection of *S*. Typhi isolates showed that the *S*. Typhi population is comprised of dozens of

subclades which are specific for different geographical locations (15, 17, 18). These global surveillance studies have shown that the majority of MDR *S.* Typhi infections worldwide belong to genotype 4.3.1 (18), which originated from South Asia and spread up to East Africa (15, 17-19).

MDR in *S.* Typhi is linked to the presence of a composite transposon. In the 4.3.1. genotype, it was first introduced via the IncHI1-PST6 plasmid (18-21). Later, reports have shown that the transposon carrying the genes associated with MDR was integrated into the *S.* Typhi chromosome (18, 22, 23). This composite transposon carries antimicrobial resistance determinants which confer resistance towards penicillins (bla_{TEM-1}), trimethoprim (*dfrA7*) and sulfonamides (*sul1, sul2*), chloramphenicol (*catA1)* and to streptomycin (*strA, strB)* (18, 21, 23).

Resistance against fluoroquinolones can result from mutations in the quinolone-resistance-determining regions (QRDRs) of the chromosomal *gyr* and *par* genes (10, 24, 25) and/or plasmid-mediated quinolone resistance (PMQR) which involves acquisition of e.g. *qnr* genes (24, 26-28). Using WGS, a large study (18) reported that the global population of MDR *S.* Typhi with reduced susceptibility to fluoroquinolones was associated with QRDR mutations. The MDR *S.* Typhi 4.3.1 subclade, which commonly also has reduced susceptibility to fluoroquinolones, is responsible for inter- and intra-continental spread, regional circulation, as well as local outbreaks in different parts of the world (18).

As the MDR *S.* Typhi population is increasing and spreading in different parts of the world, local/regional surveillance of MDR *S.* Typhi populations using both blood culture and WGS approach is important to report data on mechanisms responsible for

resistance for control and prevention of its spread, and to guide clinicians with available treatment options. However, in the African setting where typhoid fever is endemic, blood cultures for diagnostic confirmation and molecular characterization of *S.* Typhi are not performed routinely due to cost and infrastructure constraints (29). Thus, there is paucity of data regarding the distribution of MDR *S.* Typhi genotypes and resistance mechanisms in Zanzibar, Tanzania, as in many other African countries. As part of a prospective study collecting blood-cultures from patients admitted with acute undifferentiated fever in Zanzibar, we here characterize *S.* Typhi phenotypically and genotypically to contribute to the knowledge base on typhoid fever in the region and to guide treatment and control.

Methods

Study site

Mnazi Mmoja hospital (MMH), Zanzibar, Tanzania, is the main referral hospital of the Zanzibar Archipelago with a population estimated to about 1.4 million in 2015 (30). The 544-bed hospital also offers primary and secondary health care for the residents of Zanzibar city with a population of about 600,000 and is a teaching hospital for the State University of Zanzibar.

Study population

Patients in the medical, pediatric and neonatal departments were enrolled in the study if they, either on admission or during their hospital stay, had fever (≥38.3°C in adults, ≥38.5°C in children) or hypothermia (<36.0°C), or were otherwise suspected to have

144 systemic bacterial infection as judged by the clinician. Demographic and clinical
145 information was obtained. A total of 1037 of 1043 eligible patients with fever and/or
146 suspicion of serious systemic infection admitted to Mnazi Mmoja Hospital, Zanzibar,
147 were consecutively included from March 17, 2015, to October 4, 2016 (in one patient
148 two blood cultures were taken. Six patients were excluded because the blood culture
149 sample was lacking). In addition, the three accessible (of in total seven) *S.* Typhi strains
150 isolated during the pilot study in the years 2012 to 2013 (31) were included in the
151 analyses. The details of the methodology of pilot study have been previously described
152 (31). Briefly, the pilot study was performed at the same departments of Mnazi Mmoja
153 Hospital as the main study, whereas 469 participants (neonates, children and adults)
154 presenting with signs of systemic infections were included. As for the present study, the
155 pilot study included clinical data, and blood was collected for culture and susceptibility
156 testing of isolated microbes (31).

157 ## Bacterial isolation and identification

158 Blood specimens were inoculated in BACTEC Myco/F lytic blood culturing vials (Becton
159 Dickinson, Franklin Lakes, N.J.), one bottle per episode of febrile illness, incubated and
160 analyzed as described earlier (31). *Salmonella* Typhi isolates were identified by
161 standard biochemical tests, the API 20E, VITEK 2 analysis using the identification cards
162 for gram-negatives (both bioMérieux, Marcy-l'Etoile, France), and serogrouping by
163 omnivalent A-67 and Vi-antigen (sifin diagnostics gmbh, Berlin, Germany). In the 61
164 isolates accessible for further analyses, the phenotypic identification was confirmed by
165 whole genome sequencing.

166 ## Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for ampicillin, cefotaxime, ceftazidime, ciprofloxacin, trimethoprim-sulfamethoxazole, and chloramphenicol was performed by disc diffusion technique (Oxoid™, Basingstoke, United Kingdom), and, for azithromycin, by minimal inhibitory concentration (MIC) gradient test (Liofilchem®, Roseto degli Abruzzi, Italy) as described in the EUCAST guidelines (32). Fifty-nine of the 61 isolates (the remaining two isolates were not frozen) were sent to Bærum Hospital, Vestre Viken Hospital Trust, Norway, for quality control of identification and susceptibility testing as well as for further characterization. For susceptibility testing, the same antimicrobials and the same disc diffusion technique as in Zanzibar were used. In addition, ceftriaxone, ciprofloxacin and azithromycin were tested by the MIC gradient test (Liofilchem®). The three isolates of the pilot study were analyzed at the Department of Clinical Science, University of Bergen, Norway, using the same techniques. The results of the susceptibility testing were interpreted by the S-I-R system according to the EUCAST guidelines v 12.0 (33). For the testing for ciprofloxacin, the EUCAST criteria for *Salmonella* species have been applied, classifying the strains with MIC >0.06 mg/L as resistant.

Whole Genome Sequencing

Short read whole genome sequencing (WGS) was performed on 58 of the 61 *Salmonella* Typhi isolates (3 isolates not accessible) and on three *S*. Typhi strains of the pilot study (31). For the 58 study strains, genomic DNA for the sequencing was extracted from single colonies using the Wizard® genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. QIAxpert® (QIAGEN, Valencia, CA, USA) was used for purity control. For ample library preparation TruSeq™ Nano reagents (Illumina, San Diego, CA, USA) were used. For the three

strains of the pilot study, DNA isolation was performed using Invitrogen™ PureLink™ genomic DNA kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's recommendations. DNA library preparation was carried out using the standard protocol of the Nextera XT DNA Library Preparation Kit v. 3 (Illumina) up to the library amplification. Sequencing was performed on two runs of a MiSeq instrument (Illumina) with 300 bp paired end reads, according to the manufacturer´s instructions. All 61 short-read sequences were *de novo* assembled using the Pathogenwatch online platform (https://pathogen.watch). Fifty-eight of these 61 short-read sequences, all from the main study, were, in addition, assembled against a reference genome using Bowtie v. 2.3.4.2 (34), Samtools v.1.7 (35) and BCFTools v. 1.9 (35, 36), using the largest closed genome representative for one of the two lineages as reference.

Long read sequencing was performed on six selected *S.* Typhi isolates of the main study (representing isolates with different antimicrobial resistance patterns and from both years 2015 and 2016), as well as three *S*. Typhi strains from the pilot study (31). DNA isolation was performed using PureLink genomic DNA kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. Nanopore sequencing was performed using the rapid barcoding kit (SQK-RBK004) and MinION sequencer (Oxford Nanopore Technologies Ltd., Oxford, United Kingdom), and Guppy v. 3.2.10 for basecalling (37). Hybrid-assembly was carried out using Unicycler v.0.4.8 (38) running with Pilon v. 1.23 (39) and Racon v. 1.4.3 (40) for error correction and sequence polishing on 'normal' settings. Five of the six isolates were completely assembled resulting in one contig, while isolate 50-M123 was returned with 14 contigs. Medusa v. 1.6 (41) together with one closed assembly from the different lineages was used to sort

213 the 14 contigs so that they matched the other *Salmonella* isolates as closely as
214 possible. Manual editing of contigs were subsequently carried out on the 50-M123
215 isolate to align sequence start with the other isolates in the study.

216 Identification of resistance genes genotypes and sequence types

217 We used ResFinder (v4.1) with the default setting (90% threshold and 60% coverage)
218 and MLST (v. 2.) with the default setting for the species, both from the Centre for
219 Genomic Epidemiology CGE server (http://www.genomicepidemiology.org/), to identify
220 acquired and chromosomal antimicrobial resistance determinants, and to assign
221 sequence types, respectively. An online platform, Pathogenwatch
222 (https://pathogen.watch) (42), was used to screen and assign *S*. Typhi genotypes. The
223 Pathogenwatch tool uses GenoTyphi (code available
224 at https://github.com/katholt/genotyphi) (42). The GenoTyphi genotyping scheme
225 divides the *Salmonella* Typhi population into four major lineages, and >75 different
226 clades and subclades with the globally disseminated 4.3.1 (H58) subclade further
227 delineated into lineages I and II (4.3.1.1 and 4.3.1.2) (15).

228 Plasmid analysis

229 The presence of plasmids was investigated using PlasmidFinder v. 2.1 with the default
230 setting (Danish Technical University, Denmark:
231 http://cge.cbs.dtu.dk/services/PlasmidFinder/).

232 Phylogenetic analysis

233 Conserved signature inserts phylogeny server (v. 1.4)
234 (https://cge.food.dtu.dk/services/CSIPhylogeny/) was used to create a single nucleotide
235 polymorphism (SNP)-based phylogenetic tree. All default values of the software were

applied including minimum depth at SNP positions 10x, minimum relative depth at SNP positions 10%, minimum SNP quality 30. The first phylogenetic tree compared the relatedness of our *S*. Typhi isolates, in the tree we included all 61 *S.* Typhi from this study which underwent WGS. The *S.* Typhi strain with accession number ERL12960 of genotype 4.3.1.1, isolated in India in 2012 (43) was used as a reference genome for the phylogenetic tree. The second phylogenetic tree was created to compare the 61 *S*. Typhi isolates from this study which belong to 4.3.1 genotype and map their relationship with other strains to get more insights into the spread of the 4.3.1 genotype. We included 38 published WGS sequences of *S*. Typhi of the same genotype from different parts of the world (18, 22, 28, 44), including 16 isolates from Tanzania, fourteen from Kenya, two from Zambia, five from India and one from Pakistan, from the years 2007 to 2017. Both phylogenetic trees were annotated using the Interactive Tree of Life (v. 5.6.3) (45).

Genetic environment for the MDR *S.* Typhi

The two representative *S*. Typhi from this study (ZNZ13L78 and ZNZ57M188) were annotated manually using a combination of RAST (46), Basic Local Alignment Search Tool (BLAST) (v. 2.11.0) (47), ResFinder (v. 4.1) (48) and MobileElementFinder (v. 1.0.3) from the CGE server and in SnapGene (v. 3.3.4) from GSL Biotech (available at snapgene.com). A comparative analysis using genoPlotr (49) was done for the chromosomal MDR gene segment of about 25kb of the two strains in this study and strain ERL12960.

Ethical statement

The research protocol was approved by the Zanzibar Medical Research and Ethical Committee (record no ZAMREC/0002/November/2014, renewal no ZAHREC/02/June/2019/41), and by the Regional Committee for Medical Research Ethics Health Region South East Norway (record no 2014/1940/REK South-East). Written informed consent was obtained from the patients or, in the case of children, from a parent or a custodian.

Data availability

The data for this study (fastq files of all short read whole genome sequencing analyzes and five of the long read assemblies of the nine selected strains) have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB59168 (https://www.ebi.ac.uk/ena/browser/view/PRJEB59168), the accession numbers of the genomes are listed in the Supplementary information (Supplementary Table 1). The remaining four long read assemblies have been deposited in GenBank, National Institutes of Health (NIH), BioProject number PRJNA982791 (GenBank Overview (nih.gov)), accession numbers SAMN35714885 (ZNZ13L78), SAMN35713968 (ZNZ17F60), SAMN35714917 (ZNZ55M142), and SAMN35714939 (ZNZ57M158).

Results

During the study period of about 19 months in 2015-2016, 1038 blood cultures of 1037 patients were obtained, and 161 (16%) of these had growth of pathogens. *S.* Typhi was the most common pathogen, found in 61 of the 161 (38%) positive blood cultures (including nine patients with two and two patients with three isolates), corresponding to

35% (61/174) of the pathogens recovered. Additionally, among seven *S.* Typhi detected during the eight months pilot study in 2012-2013 (31), three isolates were available for further analyses. During the main study, the average isolation rate was more than three isolates per month compared to one isolate per month during the pilot study.

Antimicrobial resistance pattern of *S.* Typhi

As shown in Table 1, MDR (defined as resistance towards ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol) was observed in 98% of the isolates (63/64, including 60/61 from the main study and all three from the pilot study).

Table 1: Antimicrobial resistance pattern for *S.* Typhi isolates

Antimicrobial agent	**Number (%) of resistant isolates**						
	Total	**Genotype**		**Year**			
		4.3.1.1	**4.3.1.2**	**2012**	**2013**	**2015**	**2016**
Disc diffusion							
Ampicillin (n=64)	63* (98%)	60 (100%)	0 (0%)	2 (100%)	1 (100%)	34 (100%)	24 (96%)
Chloramphenicol (n=64)	63* (98%)	60 (100%)	0 (0%)	2 (100%)	1 (100%)	34 (100%)	24 (96%)
Trimethoprim-sulfamethoxazole (n= 64)	63* (98%)	60 (100%)	0 (0%)	2 (100%)	1 (100%)	34 (100%)	24 (96%)
Cefotaxime (n=64)	0* (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Ceftazidime (n=64)	0* (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
MIC gradient test							
Ceftriaxone (n= 62)	0* (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Ciprofloxacin (n=62)	43* (69%)	41 (68%)	1 (100%)	0 (0%)	0 (0%)	22 (65%)	21 (84%)
Azithromycin (n=63)	0* (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

289

290 * including 3 isolates of the pilot study 2012-2013

291 A total of 62 *S*. Typhi isolates (59 from the main study and three from the pilot study)
292 were tested for ciprofloxacin resistance and 69% (43/62) showed low-level resistance
293 with MIC values ranging from 0.125 – 0.25 mg/L. Of note, one of the *S.* Typhi isolates
294 with low-level ciprofloxacin resistance was not MDR.
295 Results from disc diffusion showed all 64 *S.* Typhi isolates were susceptible to
296 cefotaxime and ceftazidime. All tested isolates (fifty nine from the main study and three
297 from the pilot study) were also susceptible to ceftriaxone by MIC gradient test with MIC
298 values of 0.047-0.125 mg/L.
299 All 63 *S.* Typhi isolates were susceptible to azithromycin with MIC values 8-16 mg/L.

300 ## Whole genome sequencing results

301 Short read sequencing was performed on 61 *S.* Typhi isolates (fifty-eight from the main
302 study and three from the pilot study). A total of 60 isolates (98.3%) which phenotypically
303 were MDR carried antimicrobial resistance determinants associated with resistance to

ampicillin (*bla*$_{TEM-1B}$), trimethoprim-sulfamethoxazole (*sul1* and *sul2* plus a *dfrA7* gene) and chloramphenicol (*catA1*). In addition to MDR determinants, these isolates also carried AMR determinants conferring resistance towards aminoglycosides (*aph(6)-Id* and *aph(3")-Ib*). One *S.* Typhi which was not MDR phenotypically, did not carry any AMR determinants. Table 2 shows the distribution of AMR determinants including QRDR mutations of the three different AMR genotype profiles. Detailed information on the 61 genomes is listed in Supplementary Table 1.

Table 2: Antimicrobial resistance genes and QRDR mutations of the three AMR genotype profiles

AMR genotype profile	**Aminoglycoside**	**Beta lactam**	**Phenicol**	**Sulfamethoxazole, trimethoprim**	**QRDR mutations**
MDR/cipR sub-lineage of 4.3.1.1	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G
MDR/cipS sub-lineage of 4.3.1.1	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-
nonMDR/cipR 4.3.1.2 isolate	-	-	-	-	*gyrA*: p.S83F

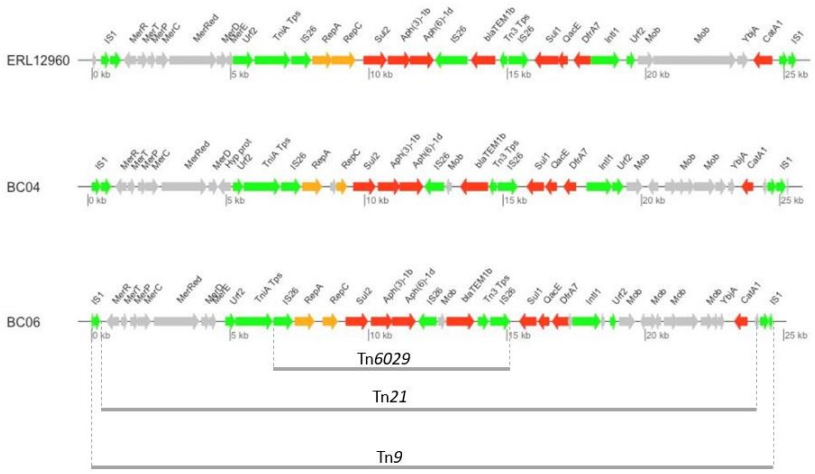
Resistance toward fluoroquinolones was associated with a single QRDR mutation in 42/43 *S.* Typhi which showed phenotypic low-level resistance to ciprofloxacin (the remaining strain was not accessible to WGS). Forty-one strains had single *gyrA*-D87G QRDR mutation, while one strain which was not MDR had a different single *gyrA*-S83F mutation.

As shown in table 2, all MDR *S.* Typhi isolates were assigned to the 4.3.1.1 genotype using the GenoTyphi scheme (15, 42). On the contrary, one *S.* Typhi isolate (ZNZ50M123) which was not MDR was assigned to the 4.3.1.2 genotype. Furthermore, all *S.* Typhi isolates belong to MLST sequence type ST1.

The genetic environment of resistance genes

Long-read sequencing was performed on six selected strains of the main study to investigate whether the AMR island is integrated in the chromosome or located on a plasmid. The gene contents of the five MDR *S.* Typhi isolates were highly similar, and the antimicrobial resistance determinants coding for MDR were located on a composite transposon integrated in the chromosome within a segment encompassed by insertion sequences and transposases. Figure 1 shows the structure of composite transposon of two *S.* Typhi isolates from this study ZNZ13L78 and ZNZ55M142 (with ENA accession numbers ERR11413875 and ERR11413524 respectively, for the short reads, and GenBank accession numbers SAMN35714885 and SAMN35714917 respectively, for the long reads) and ERL12960 as a comparison *S.* Typhi. All MDR *S*. Typhi isolates carried a composite transposon Tn*6029* (encoding bla_{TEM-1B}, *sul2*, *aph(6)-Id and aph(3")-Ib*) which was inserted in Tn*21* (carrying a class I integron encoding *dfrA* alleles in the gene cassette and *sul1*), which was in turn inserted within Tn*9* (encoding *catA1*) with IS*1* on both ends. No plasmid was found, but the MDR *S*. Typhi strains contained the IncQ1 plasmid replicon sequence (*repA* and *repC*) as shown in Figure 1. Furthermore, we detected chromosomal integration of the IS*1* at the known site downstream of *cyaY* as previously described (22).

Figure 1. The structure of MDR composite transposon



342

343 Figure 1 legend: The red arrows show antimicrobial resistance genes, MDR genes

344 are *sul1/sul2*, *dfrA7*, bla_{TEM-1} and *catA1*. Green arrows show insertion sequences (IS) or

345 transposases and orange arrow show the replicons. BC04 is study isolate ZNZ13L78,

346 BC06 is study isolate ZNZ55M142.

347 **Phylogenetic analysis**

348 Figure 2A is a whole genome SNP-based phylogenetic tree containing the 61 *S*. Typhi

349 isolates from this study and one reference strain ERL12960. As shown in Figure 2A,

350 one isolate ZNZ50M123 (ENA accession numbers ERR11414360 and ERZ18316203,

351 project PRJEB59168), highlighted in green belongs to genotype 4.3.1.2. This isolate

352 has reduced susceptibility to ciprofloxacin (due to *gyrA*-S83F), and no acquired

353 resistance genes. All other isolates were genotype 4.3.1.1, these were all MDR (due to

354 bla_{TEM-1}, *catA1*, *sul1*, *sul2*, *DfrA7*). The majority of these belonged to a monophyletic

355 clade (highlighted purple in tree) and had reduced susceptibility to ciprofloxacin (due to

gyrA-D87G), herein referred as MDR/cipR. In contrast the others (highlighted yellow in tree) including the older isolates from the pilot study, are fully sensitive to ciprofloxacin, and lack QRDR mutations referred as MDR/cipS. Within the study isolates, the SNP difference ranged between 0 and 41 (median 7.5), with the biggest SNP between the only isolate belonging to the 4.3.1.2 genotype (ZNZ50M123) and an isolate which is MDR/cipR. For the genomes belonging to the 4.3.1.1 genotype, the overall difference of all study isolates ranged between 0 and 27 SNPs (median 7), within MDR/cipR isolates between 0 and 16 SNPs (median 6), within MDR/cipS isolates between 3 and 17 SNPs (median 9.5). The MDR/cipS isolates had a minimum difference of 11 SNPs to the closest neighbor of MDR/cipR isolates, with two of the pilot study isolates being closest related to MDR/cipR isolates. The only isolate of the genotype 4.3.1.2 (ZNZ50M123), had a difference between 25 and 41 SNPs (median 34) to the other study isolates (all belonging to 4.3.1.1 genotype).

Figure 2A: Phylogenetic tree of *S.* Typhi isolates from this study using the reference genome ERL 12960 as midpoint root

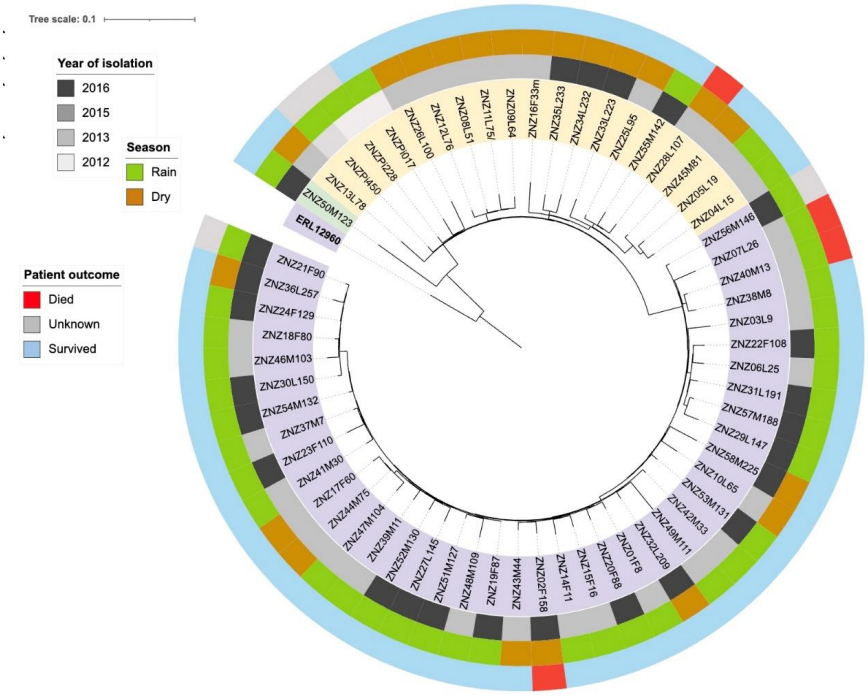


Figure 2A Legend: The inner circle shows three clusters of our *S*. Typhi: purple highlighted is a monophyletic clade (MDR/cipR), these are MDR and have reduced susceptibility to ciprofloxacin due to gyrA-D87G; yellow highlighted are strains which are MDR but sensitive to ciprofloxacin (MDR/cipS); one strain in green highlight is not MDR and has low-level ciprofloxacin resistance due to single *gyrA*-S83F QRDR mutation. The second circle shows the year of isolation of *S*. Typhi; black represents *S.* Typhi isolated in 2016, grey in 2015, light grey and very light grey were isolated in 2013 and 2012 respectively. The next circle shows the season when *S.* Typhi were isolated, green represents isolates collected during the rainy season while orange highlighted *S*. Typhi were collected during the dry season. The outer circle shows the outcome of the

patients; red represents *S.* Typhi from patients who died, blue represents *S.* Typhi from patients who survived while grey represents *S.* Typhi from patients with unknown outcome.

In figure 2B, to provide context, the relationship of the 61 study strains to 38 strains from other publications including the reference strain ERL12960 is shown in a whole genome SNP-based phylogenetic tree. The minimum difference of the study isolates belonging to MDR/cipR group (genotype 4.3.1.1, MDR, and *gyrA*-D87G QRDR mutation) to isolates from other studies was 0 SNP in two isolates obtained in 2015 from travelers returning from Tanzania, both of which also were MDR and had identical *gyrA* point mutation, but no IncHI1 plasmid (28). The 2nd most closely related isolate was also from Tanzania, and had 5 SNPs difference (year 2009, MDR, no IncHI1 plasmid, no FQ point mutation, ERR108659) (18). The closest isolate from Kenya had a difference of 17 SNPs. The isolates from Zambia had a difference of at least 15 SNPs, the minimum difference to isolates from Asia was 29 SNPs (India).

Figure 2B: Phylogenetic tree of *S.* Typhi isolates from this study and of isolates from other publications using the reference genome ERL 12960 as midpoint root

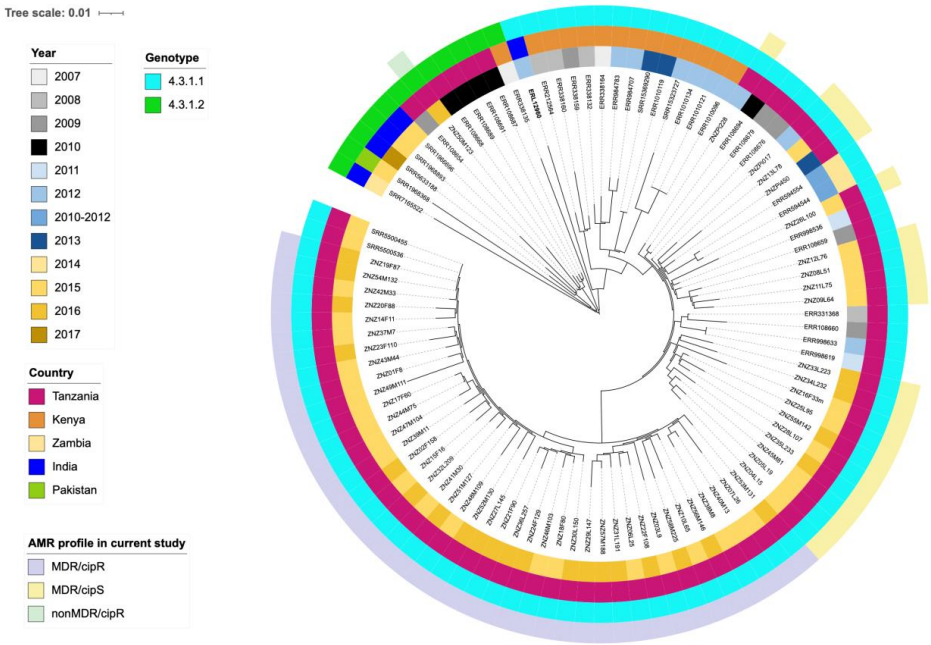


Figure 2B Legend: From inside out, the inner circle shows the year of isolation of *S.* Typhi. The second circle shows the country where the strains were isolated. The next circle shows the genotype of the *S.* Typhi strains. The outer circle shows the AMR profile in the current study. The monophyletic MDR/cipR is highlighted in purple, the MDR/cipS cluster is yellow, the only nonMDR/cipR strain is highlighted in green.

Isolates which are MDR/cipS had a minimum difference of 2 SNPs to isolates from East Africa (Tanzania, year 2009, with MDR, without the IncHI1 plasmid and without FQ point mutation) (18). The closest isolate from Kenya had 11 SNPs difference. The difference

to the Zambian isolates was at least 8 SNPs, and to the Asian isolates at least 22 SNPs (an isolate from India).

The non MDR genotype 4.3.1.2 isolate with the point mutation *gyrA*-S83F ZNZ50M123 had a minimum difference of 8 SNPs from another previously published Tanzanian isolate (year 2009, MDR, with IncHI1 plasmid and same *gyrA* point mutation, ERR108654) (18). The second most closely related are four isolates from Tanzania with 10 SNPs distance (all from 2010, no MDR, no IncHI1 plasmid, all with the point mutation *gyrA*-S83F) (18). The minimum distance to Kenyan isolates was 27 SNPs, to isolates from Zambia 29 SNPs, and to isolates from Asia (India) 21 SNPs.

All mentioned related external isolates belong to the ST1, as do the isolates from the current study.

Discussion

The study shows a high rate of multidrug resistance in *S.* Typhi from Zanzibar throughout the study period, concurring with findings from previous studies from Tanzania (25), neighboring Malawi, Kenya and Zambia and other African countries (19, 22, 44, 50). The prevalence of typhoid fever increased from 1% (7/469) of patients in the pilot study in 2012/2013 to 6% (61/1037) in the current study from 2015/2016. Correspondingly, *S.* Typhi accounted for 9% (7/79) and 35% (61/174) of pathogenic bacteria in blood cultures in the pilot and the current study, respectively, suggesting an outbreak in 2015/2016. Our findings support the report of an ongoing epidemic of MDR *S*. Typhi in Africa (18). The high prevalence of MDR and reduced susceptibility to fluoroquinolones limit the treatment options of typhoid fever in Zanzibar considerably.

In this study all MDR *S.* Typhi belong to genotype 4.3.1.1. This genotype has been linked with inter- and intra-continental spread of MDR *S.* Typhi (18). Therefore, it is likely that genotype 4.3.1 has been imported from South Asia in the last two decades to East African countries including Tanzania, Kenya and Uganda (44) resulting in further spread of the genotype and local outbreaks. This genotype is associated with resistance to all three former first line drugs; ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole. The availability over the counter and widespread use of these antibiotics in the study setting may exert a selective pressure that contributes to maintaining the persistence of MDR *S*. Typhi in the region. The only *S.* Typhi isolate which is not MDR belongs to genotype 4.3.1.2. This genotype has been reported in Southeast Asian countries such as India, Nepal, and Bangladesh (18), neighboring countries Kenya and Uganda (16, 44, 51), as well as in Tanzania (44).

Ciprofloxacin and other fluoroquinolones are widely used for the treatment of typhoid fever as a consequence of the high prevalence of MDR (52). This study found that a high proportion of *S.* Typhi genotype 4.3.1.1 had low-level ciprofloxacin resistance. This is alarming as treatment with fluoroquinolones may lead to treatment failure (53) and relapse. Increased doses and prolonged therapy may be effective but may fuel emergence of high-level resistance (8). In a multi-country typhoid fever surveillance study in Africa, isolates from Tanzania showed a high percentage of MDR (89%, 8/9 isolates), but no fluoroquinolone resistance was found (25). However, the fluoroquinolone resistance rates currently increase on the African continent (24), and overuse of fluoroquinolones is likely partly to blame (54). Fortunately, high-level

fluoroquinolone resistance was not detected in the study strains, but continued widespread use of fluoroquinolone may bring it on.

In the present study, fluoroquinolone resistance was conferred by chromosomal single point mutations leading to structural alterations in topoisomerases as DNA gyrase. Increasing numbers of point mutations are correlated with a cumulative increase in MIC values, with the simultaneous acquisition of at least three-point mutations resulting in high-level fluoroquinolone resistance (24). Changes in positions 83 and 87 of the *gyrA* gene are commonly reported point mutations (10, 24). All *S.* Typhi study isolates belonging to a monophyletic clade (MDR/cipR) had a single *gyrA*-D87G QRDR mutation and were phenotypically expressing low-level fluoroquinolone resistance. To our knowledge, in East Africa the *gyrA*-D87G point mutation has only been described in two *S*. Typhi strains isolated from returning travelers from Tanzania (28).

In contrast, the only isolate without MDR (isolate ZNZ50M123, belonging to the 4.3.1.2 genotype) harbors a different single mutation (*gyrA*-S83F), another common mutation in fluoroquinolone resistant *S*. Typhi, including East African isolates (25). Furthermore, all *S.* Typhi isolates in this study were sensitive to azithromycin which would remain the only effective oral treatment for typhoid fever. Globally, azithromycin resistant *S.* Typhi is rare, but increasingly reported in South-East Asia, notably in Bangladesh (55). Increased use of azithromycin will subsequently pose a risk of introducing and spreading azithromycin-resistant *S*. Typhi in the African region (55). Third generation cephalosporins such as ceftriaxone are still effective in Zanzibar, unlike in Pakistan, that currently experiences a long-lasting outbreak with an extensive drug-resistant *S.* Typhi

478 strain, resistant to ceftriaxone, ciprofloxacin, ampicillin, trimethoprim-sulfamethoxazole,
479 and chloramphenicol (52, 55, 56).
480 Earlier studies in Asia and some African countries have shown that MDR in *S*. Typhi is
481 associated with the presence of an IncHI1 plasmid (18, 44). This means the main
482 spread of antimicrobial resistance determinants has been horizontal transfer using a
483 plasmid. Later studies have shown that MDR *S*. Typhi isolates from Zambia (22),
484 Tanzania (44) and Asia (18, 57) did not harbor plasmids associated with MDR
485 determinants, suggesting that the genes conferring MDR have been incorporated in the
486 chromosome of the bacteria. Concurring with previous findings (18, 22, 44), all MDR
487 isolates in the present study carried a composite transposon integrated into the
488 chromosome. Both the data from our study and from recent studies are compatible with
489 spread of AMR through clonal expansion (18). Previous studies suggest that the
490 chromosomal location of the MDR determinants may confer a competitive advantage for
491 the bacteria as it is less energy consuming compared to harboring a plasmid (28, 44).
492 The integration of AMR determinants into the *S*. Typhi chromosome is worrying, as the
493 chromosomal location reduces the likelihood of bacteria losing the antimicrobial
494 resistance determinants (10). The presumably low fitness cost associated with carriage
495 of the MDR transposon (28) can provide a mechanism for sustained vertical
496 transmission of MDR *S.* Typhi, even in the absence of selection pressure for the specific
497 resistances (28).
498 In the phylogenetic comparison of the study isolates, monophyletic clade (MDR/cipR)
499 isolates showed no SNP difference to the genotypes of *S.* Typhi isolated from two
500 travelers returning to the United Kingdom from Tanzania during the same period (28) .

They additionally share the same determinants coding for AMR including the identical *gyrA* point mutation, which, to our knowledge, has not been described in other *S.* Typhi isolates from East Africa. Isolates which are MDR/cipS, are closely related to other strains from Tanzania. Compared to MDR/cipR isolates, MDR/cipS has a smaller SNP difference to isolates from both Kenya and, especially, to those from Zambia. Assessing the results of the epidemiological and the antimicrobial resistance analyses, we speculate that the monophyletic clade (MDR/cipR) isolates may represent a new subtype and an outbreak strain, whereas MDR/cipS may be endemic.

The only non-MDR study strain is belonging to the genotype 4.3.1.2 and harboring a *gyrA*-S83F point mutation. It is closest related to a Tanzanian isolate from 2009 which had the same point mutation but differed by also harboring MDR and an IncHI1 plasmid. The second most closely related are four Tanzanian isolates from 2010 without MDR and without the IncHI1 plasmid, also with the same point mutation. In Asia, a decrease of MDR is associated with a corresponding decrease in carriage of IncHI1 plasmids (58), and we may speculate that the isolate from our study may also have lost the IncHI1 plasmid. This study isolate showed a smaller SNP difference to isolates from India than to isolates from neighboring Kenya or Zambia. This may support the earlier introduction and spread of a common ancestor from India to Tanzania.

In line with previous reports (42), the genetic findings in our study match the phenotypical results, emphasizing the potential utility of WGS for the prediction of AMR. The results are underlining that WGS is an important tool for surveillance of typhoid fever for uncovering outbreaks, and for understanding epidemiological relationships and the spread of antimicrobial resistance locally and globally. The high rate of MDR *S.*

Typhi demonstrate the need of both antimicrobial stewardship for the treatment of suspected typhoid fever as well as surveillance. The study hospital is located on Unguja, the largest island of the Zanzibar Archipelago, which has a multicultural population with historical links to mainland Tanzania, India and the Arabian Peninsula, in addition many international tourists visit the island. Extensive international travel may render the island vulnerable to the spread of resistant microbes (59), underlining the importance of continuous surveillance both locally and internationally.

Conclusions

We report high rate of MDR and low-level ciprofloxacin resistant *S.* Typhi genotype 4.3.1.1 circulating in Zanzibar. The findings support that this clade now prevails in East Africa (16, 44, 50), leaving few therapeutic options available for treatment of typhoid fever in the setting. Surveillance of the prevalence, spread and antimicrobial susceptibility of *S.* Typhi can guide treatment and control efforts.

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References

1. Crump JA. Progress in Typhoid Fever Epidemiology. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2019;68(Suppl 1):S4-s9.
2. Global Burden of Disease Collaborative Network. GBD 2020 Cause and Risk Summaries: Typhoid fever — level 4 cause. Seattle, United States: Institute for Health Metrics and Evaluation (IHME). 2020 [cited 2022 07.02.2022]. Available from: https://www.healthdata.org/results/gbd_summaries/2019/typhoid-fever-level-4-cause.
3. Antillón M, Warren JL, Crawford FW, Weinberger DM, Kürüm E, Pak GD, et al. The burden of typhoid fever in low- and middle-income countries: A meta-regression approach. PLoS neglected tropical diseases. 2017;11(2):e0005376.
4. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis. 2019;19(4):369-81.
5. Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ. Typhoid fever. The New England journal of medicine. 2002;347(22):1770-82.
6. Marks F, von Kalckreuth V, Aaby P, Adu-Sarkodie Y, El Tayeb MA, Ali M, et al. Incidence of invasive salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study. Lancet Glob Health. 2017;5(3):e310-e23.
7. Breiman RF, Cosmas L, Njuguna H, Audi A, Olack B, Ochieng JB, et al. Population-based incidence of typhoid fever in an urban informal settlement and a rural area in Kenya: implications for typhoid vaccine use in Africa. PloS one. 2012;7(1):e29119.
8. Crump JA, Sjolund-Karlsson M, Gordon MA, Parry CM. Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive Salmonella Infections. Clinical microbiology reviews. 2015;28(4):901-37.
9. Bhutta ZA. Impact of age and drug resistance on mortality in typhoid fever. Arch Dis Child. 1996;75(3):214-7.
10. Dyson ZA, Klemm EJ, Palmer S, Dougan G. Antibiotic Resistance and Typhoid. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2019;68(Supplement_2):S165-s70.
11. Le TA, Fabre L, Roumagnac P, Grimont PA, Scavizzi MR, Weill FX. Clonal expansion and microevolution of quinolone-resistant Salmonella enterica serotype typhi in Vietnam from 1996 to 2004. Journal of clinical microbiology. 2007;45(11):3485-92.
12. Murdoch DA, Banatvaia N, Bone A, Shoismatulloev BI, Ward LR, Threlfall EJ. Epidemic ciprofloxacin-resistant Salmonella typhi in Tajikistan. Lancet. 1998;351(9099):339.
13. Roumagnac P, Weill FX, Dolecek C, Baker S, Brisse S, Chinh NT, et al. Evolutionary history of Salmonella typhi. Science (New York, NY). 2006;314(5803):1301-4.
14. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18(3):318-27.
15. Wong VK, Baker S, Connor TR, Pickard D, Page AJ, Dave J, et al. An extended genotyping framework for Salmonella enterica serovar Typhi, the cause of human typhoid. Nature communications. 2016;7:12827.
16. Dyson ZA, Holt KE. Five years of GenoTyphi: updates to the global Salmonella Typhi genotyping framework. The Journal of infectious diseases. 2021.
17. Carey ME, Dyson ZA, Ingle DJ, Amir A, Aworh MK, Chattaway MA, et al. Global diversity and antimicrobial resistance of typhoid fever pathogens: Insights from a meta-analysis of 13,000 Salmonella Typhi genomes. eLife. 2023;12.

18. Wong VK, Baker S, Pickard DJ, Parkhill J, Page AJ, Feasey NA, et al. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of Salmonella Typhi identifies inter- and intracontinental transmission events. Nature genetics. 2015;47(6):632-9.
19. Kariuki S, Revathi G, Kiiru J, Mengo DM, Mwituria J, Muyodi J, et al. Typhoid in Kenya is associated with a dominant multidrug-resistant Salmonella enterica serovar Typhi haplotype that is also widespread in Southeast Asia. Journal of clinical microbiology. 2010;48(6):2171-6.
20. Phan MD, Kidgell C, Nair S, Holt KE, Turner AK, Hinds J, et al. Variation in Salmonella enterica serovar typhi IncHI1 plasmids during the global spread of resistant typhoid fever. Antimicrob Agents Chemother. 2009;53(2):716-27.
21. Holt KE, Phan MD, Baker S, Duy PT, Nga TV, Nair S, et al. Emergence of a globally dominant IncHI1 plasmid type associated with multiple drug resistant typhoid. PLoS neglected tropical diseases. 2011;5(7):e1245.
22. Hendriksen RS, Leekitcharoenphon P, Lukjancenko O, Lukwesa-Musyani C, Tambatamba B, Mwaba J, et al. Genomic signature of multidrug-resistant Salmonella enterica serovar typhi isolates related to a massive outbreak in Zambia between 2010 and 2012. Journal of clinical microbiology. 2015;53(1):262-72.
23. Ashton PM, Nair S, Dallman T, Rubino S, Rabsch W, Mwaigwisya S, et al. MinION nanopore sequencing identifies the position and structure of a bacterial antibiotic resistance island. Nature biotechnology. 2015;33(3):296-300.
24. Britto CD, Wong VK, Dougan G, Pollard AJ. A systematic review of antimicrobial resistance in Salmonella enterica serovar Typhi, the etiological agent of typhoid. PLoS neglected tropical diseases. 2018;12(10):e0006779.
25. Al-Emran HM, Eibach D, Krumkamp R, Ali M, Baker S, Biggs HM, et al. A Multicountry Molecular Analysis of Salmonella enterica Serovar Typhi With Reduced Susceptibility to Ciprofloxacin in Sub-Saharan Africa. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2016;62 Suppl 1:S42-6.
26. Hopkins KL, Davies RH, Threlfall EJ. Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. International journal of antimicrobial agents. 2005;25(5):358-73.
27. Tadesse G, Tessema TS, Beyene G, Aseffa A. Molecular epidemiology of fluoroquinolone resistant Salmonella in Africa: A systematic review and meta-analysis. PloS one. 2018;13(2):e0192575.
28. Ingle DJ, Nair S, Hartman H, Ashton PM, Dyson ZA, Day M, et al. Informal genomic surveillance of regional distribution of Salmonella Typhi genotypes and antimicrobial resistance via returning travellers. PLoS neglected tropical diseases. 2019;13(9):e0007620.
29. Ombelet S, Ronat JB, Walsh T, Yansouni CP, Cox J, Vlieghe E, et al. Clinical bacteriology in low-resource settings: today's solutions. Lancet Infect Dis. 2018;18(8):e248-e58.
30. Tanzania NBoS. Tanzania in figures 2015 2016 [29.08.2022]. Available from: https://www.nbs.go.tz/index.php/en/tanzania-in-figures.
31. Onken A, Said AK, Jorstad M, Jenum PA, Blomberg B. Prevalence and Antimicrobial Resistance of Microbes Causing Bloodstream Infections in Unguja, Zanzibar. PloS one. 2015;10(12):e0145632.
32. EUCAST. [29.08.2022]. Available from: https://www.eucast.org/ast_of_bacteria/previous_versions_of_documents/.
33. EUCAST clinical breakpoints (bacteria) v 12.0: EUCAST; 2022 [updated 01.01.2022. Available from: https://www.eucast.org/clinical_breakpoints.
34. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-9.
35. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics (Oxford, England). 2009;25(16):2078-9.

36. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics (Oxford, England). 2011;27(21):2987-93.
37. Wick RR, Judd LM, Holt KE. Performance of neural network basecalling tools for Oxford Nanopore sequencing. Genome Biol. 2019;20(1):129.
38. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol. 2017;13(6):e1005595.
39. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PloS one. 2014;9(11):e112963.
40. Vaser R, Sović I, Nagarajan N, Šikić M. Fast and accurate de novo genome assembly from long uncorrected reads. Genome Res. 2017;27(5):737-46.
41. Bosi E, Donati B, Galardini M, Brunetti S, Sagot MF, Lió P, et al. MeDuSa: a multi-draft based scaffolder. Bioinformatics (Oxford, England). 2015;31(15):2443-51.
42. Argimón S, Yeats CA, Goater RJ, Abudahab K, Taylor B, Underwood A, et al. A global resource for genomic predictions of antimicrobial resistance and surveillance of Salmonella Typhi at pathogenwatch. Nature communications. 2021;12(1):2879.
43. Sikorski MJ, Hazen TH, Desai SN, Nimarota-Brown S, Tupua S, Sialeipata M, et al. Persistence of Rare Salmonella Typhi Genotypes Susceptible to First-Line Antibiotics in the Remote Islands of Samoa. mBio. 2022;13(5):e0192022.
44. Park SE, Pham DT, Boinett C, Wong VK, Pak GD, Panzner U, et al. The phylogeography and incidence of multi-drug resistant typhoid fever in sub-Saharan Africa. Nature communications. 2018;9(1):5094.
45. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 2021;49(W1):W293-w6.
46. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server: rapid annotations using subsystems technology. BMC genomics. 2008;9:75.
47. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10.
48. ResFinder, Danish Technical University, Denmark [20.10.2022]. Available from: https://cge.food.dtu.dk/services/ResFinder/.
49. Guy L, Kultima JR, Andersson SG. genoPlotR: comparative gene and genome visualization in R. Bioinformatics (Oxford, England). 2010;26(18):2334-5.
50. Feasey NA, Gaskell K, Wong V, Msefula C, Selemani G, Kumwenda S, et al. Rapid emergence of multidrug resistant, H58-lineage Salmonella typhi in Blantyre, Malawi. PLoS neglected tropical diseases. 2015;9(4):e0003748.
51. Kariuki S, Dyson ZA, Mbae C, Ngetich R, Kavai SM, Wairimu C, et al. Multiple introductions of multidrug-resistant typhoid associated with acute infection and asymptomatic carriage, Kenya. eLife. 2021;10.
52. Marchello CS, Carr SD, Crump JA. A Systematic Review on Antimicrobial Resistance among Salmonella Typhi Worldwide. Am J Trop Med Hyg. 2020;103(6):2518-27.
53. Threlfall EJ, Ward LR, Skinner JA, Smith HR, Lacey S. Ciprofloxacin-resistant Salmonella typhi and treatment failure. Lancet. 1999;353(9164):1590-1.
54. Mashe T, Leekitcharoenphon P, Mtapuri-Zinyowera S, Kingsley RA, Robertson V, Tarupiwa A, et al. Salmonella enterica serovar Typhi H58 clone has been endemic in Zimbabwe from 2012 to 2019. The Journal of antimicrobial chemotherapy. 2021;76(5):1160-7.
55. Saha S, Sajib MSI, Garrett D, Qamar FN. Antimicrobial Resistance in Typhoidal Salmonella: Around the World in 3 Days. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2020;71(Suppl 2):S91-s5.
56. Klemm EJ, Shakoor S, Page AJ, Qamar FN, Judge K, Saeed DK, et al. Emergence of an Extensively Drug-Resistant Salmonella enterica Serovar Typhi Clone Harboring a

717 Promiscuous Plasmid Encoding Resistance to Fluoroquinolones and Third-Generation
718 Cephalosporins. mBio. 2018;9(1).
719 57. Chiou CS, Alam M, Kuo JC, Liu YY, Wang PJ. Chromosome-mediated multidrug
720 resistance in Salmonella enterica serovar Typhi. Antimicrob Agents Chemother.
721 2015;59(1):721-3.
722 58. da Silva KE, Tanmoy AM, Pragasam AK, Iqbal J, Sajib MSI, Mutreja A, et al. The
723 international and intercontinental spread and expansion of antimicrobial-resistant Salmonella
724 Typhi: a genomic epidemiology study. Lancet Microbe. 2022;3(8):e567-e77.
725 59. Woerther PL, Andremont A, Kantele A. Travel-acquired ESBL-producing
726 Enterobacteriaceae: impact of colonization at individual and community level. J Travel Med.
727 2017;24(suppl_1):S29-s34.
728

Supplementary information

Supplementary Table 1: Per-isolate information on AMR genotype profile, year, AMR determinants and accession numbers of genomes (submitted to ENA project PRJEB59168 and to GenBank BioProject PRJNA982791)

ID	AMR genotype profile	Year	Amino-glycoside	Beta lactam	Phenicol	Sulfa-methoxazole, trimethoprim	QRDR mutations	Accession number short read	Accession number long read
ZNZ01F8	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11412256	
ZNZ02F158	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11412266	
ZNZ03L9	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11270576	
ZNZ04L15	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11412010	ERZ18315935
ZNZ05L19	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11412012	
ZNZ06L25	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11412277	
ZNZ07L26	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11412278	
ZNZ08L51	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11412269	
ZNZ09L64	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11412270	
ZNZ10L65	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11412271	
ZNZ11L75	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11414157	
ZNZ12L76	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11414158	
ZNZ13L78	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11413875	SAMN35714885
ZNZ14F11	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11414161	
ZNZ15F16	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11413762	
ZNZ16F33m	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11414162	
ZNZ17F60	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11414163	SAMN35713968
ZNZ18F80	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11414164	
ZNZ19F87	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11412666	
ZNZ20F88	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11414165	
ZNZ21F90	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	gyrA: p.D87G	ERR11412281	
ZNZ22F108	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	gyrA: p.D87G	ERR11414242	
ZNZ23F110	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11413529	
ZNZ24F129	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11414243	
ZNZ25L95	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11414244	
ZNZ26L100	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3'')-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11412282	

ZNZ27L145	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: p.D87G	ERR11414246	
ZNZ28L107	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11414247	
ZNZ29L147	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*:p.D87G	ERR11412670	
ZNZ30L150	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414248	
ZNZ31L191	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA* : D87G	ERR11414250	
ZNZ32L209	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414251	
ZNZ33L223	MDR/cipS 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11414252	
ZNZ34L232	MDR/cipS 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11414253	
ZNZ35L233	MDR/cipS 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11413528	
ZNZ36L257	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414254	
ZNZ37M7	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414255	
ZNZ38M8	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414257	
ZNZ39M11	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414258	
ZNZ40M13	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11412669	
ZNZ41M30	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414353	
ZNZ42M33	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414354	
ZNZ43M44	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11412654	
ZNZ44M75	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11413527	
ZNZ45M81	MDR/cipS 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11414355	
ZNZ46M103	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414356	
ZNZ47M104	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414358	
ZNZ48M109	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11414359	
ZNZ49M111	MDR/cipR 4.3.1.1	2015	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11412667	
ZNZ50M123	Non MDR/cipR 4.3.1.2	2016	-	-	-	-	***gyrA*: S83F**	ERR11414360	ERZ18316203
ZNZ51M127	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA* : D87G	ERR11414919	
ZNZ52M130	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11415478	
ZNZ53M131	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11416037	
ZNZ54M132	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11416596	
ZNZ55M142	MDR/cipS 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11413524	SAMN35714917
ZNZ56M146	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11413526	
ZNZ57M188	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11411994	SAMN35714939
ZNZ58M225	MDR/cipR 4.3.1.1	2016	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	*gyrA*: D87G	ERR11416599	
ZNZPi017	MDR/cipS 4.3.1.1	2012	*aph(6)-Id, aph(3")-Ib*	*bla*$_{TEM-1B}$	*catA1*	*sul1, sul2, dfrA7*	-	ERR11411976	ERZ18315661

ZNZPi228	MDR/cipS 4.3.1.1	2012	*aph(6)-Id, aph(3")-Ib*	bla_{TEM-1B}	*catA1*	*sul1, sul2, dfrA7*	-	ERR11412001	ERZ18315814
ZNZPi450	MDR/cipS 4.3.1.1	2013	*aph(6)-Id, aph(3")-Ib*	bla_{TEM-1B}	*catA1*	*sul1, sul2, dfrA7*	-	ERR11411981	ERZ18315820

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736 MDR/cipR sub-lineage of 4.3.1.1 = MDR/cipR 4.3.1.1; MDR/cipS sub-lineage of
737 4.3.1.1=MDR/cipS 4.3.1.1

Paper III

Malaria Journal

RESEARCH

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Malaria prevalence and performance of diagnostic tests among patients hospitalized with acute undifferentiated fever in Zanzibar

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Abstract

Background: Control efforts in Zanzibar reduced the burden of malaria substantially from 2000 to 2015, but re-emergence of falciparum malaria has been observed lately. This study evaluated the prevalence of malaria and performance of routine diagnostic tests among hospitalized fever patients in a 1.5 years period in 2015 and 2016.

Methods: From March 2015 to October 2016, paediatric and adult patients hospitalized with acute undifferentiated fever at Mnazi Mmoja Hospital, Zanzibar were included. The malaria prevalence, and performance of rapid diagnostic test (RDT) and microscopy, were assessed using polymerase chain reaction (PCR) as gold standard.

Results: The malaria prevalence was 9% (63/731). Children under 5 years old had lower malaria prevalence (5%, 14/260) than older children (15%, 20/131, $p=0.001$) and persons aged 16 to 30 years (13%, 15/119, $p=0.02$), but not different from persons over 30 years old (6%, 14/217, $p=0.7$). All cases had *Plasmodium falciparum* infection, except for one case of *Plasmodium ovale*. Ten malaria patients had no history of visiting mainland Tanzania. The RDT had a sensitivity of 64% (36/56) and a specificity of 98% (561/575), and microscopy had a sensitivity of 50% (18/36) and a specificity of 99% (251/254), compared to PCR. The malaria parasitaemia was lower in patients with false negative results on RDT (median 7×10^3 copies/µL, interquartile range [IQR] $2 \times 10^3 - 8 \times 10^4$, $p=0.002$) and microscopy (median 9×10^3 copies/µL, IQR $8 \times 10^2 - 7 \times 10^4$, $p=0.006$) compared to those with true positive RDT (median 2×10^5 copies/µL, IQR $3 \times 10^4 - 5 \times 10^5$) and microscopy (median 2×10^5 copies/µL, IQR $6 \times 10^4 - 5 \times 10^5$).

Conclusions: The study emphasizes that malaria was a frequent cause of febrile illness in hospitalized patients in Zanzibar in the years 2015-2016, particularly among school age children and young adults. We found evidence of autochthonous malaria transmission in Zanzibar. Compared to PCR, both RDT and microscopy had low sensitivity, and false negative results were associated with low parasitaemia. While low parasitaemia identified only by PCR in a semi-immune individual could be coincidental and without clinical relevance, clinicians should be aware of the risk of false negative results on routine tests.

Keywords: Malaria, Prevalence, Surveillance, Fever, Microscopy, Point-of-care diagnostic tests, Polymerase chain reaction, Zanzibar, Tanzania, Eastern Africa

Background

Successful control efforts reduced the burden of malaria in Zanzibar substantially from 2000 to 2015 [1]. However, this progress has halted in recent years. According

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to the World Health Organization (WHO), sub-Saharan Africa suffered 384,000 estimated malaria deaths in 2020, equalling 94% of the global malaria death toll [2, 3]. In the Zanzibar archipelago, a comprehensive control and elimination programme was implemented in 2001, introducing artemisinin-based combination therapy, intermittent treatment in pregnancy, nationwide distribution of long-lasting insecticide-treated bed nets, indoor residual spraying, active case detection among contacts and larvicidal treatment of mosquito breeding sites [4]. The interventions reduced malaria-prevalence by 96% from 2002 to 2015 [5], malaria in-patient cases by 78% from 1999 to 2008 [6], and cut reported deaths to negligible. However, since 2016 the number of reported cases in Zanzibar has increased [2], and in 2020, the Zanzibar Ministry of Health intensified control measures after a surge in malaria cases during a prolonged rainy season.

Commercially available malaria rapid diagnostic tests (RDTs) differ widely in sensitivity and specificity [7], and accurate microscopy depends on high quality technical equipment and experience [8]. While the sensitivity of polymerase chain reaction (PCR) also varies between assays, PCR has generally high sensitivity and detect parasitaemia lower than 1 parasite/μL, while the detection limits for microscopy and sensitive RDTs are around 50–200 p/μL and 100 p/μL, respectively [9].

The main objective of this study was to evaluate the prevalence of malaria identified by PCR, and the performance of the routine tests RDT and microscopy, in febrile patients admitted to Mnazi Mmoja Hospital (MMH), Zanzibar.

Methods

Patient material

From 17th March 2015 to 4th October 2016, we consecutively enrolled patients with acute undifferentiated febrile illness admitted to the Department of Internal Medicine and the Department of Paediatrics at MMH. With 544 beds, this hospital in Zanzibar city is the referral hospital for the 1.3 million population of the Zanzibar Archipelago [10]. Inclusion criteria were fever ($\geq$ 38.3 °C in adults, $\geq$ 38.5 °C in children) or hypothermia (< 36.0 °C), tachypnoea > 20/min, tachycardia > 90/min on admission, or attending clinicians' diagnosis of severe acute infection. Neonates under 15 days of age were excluded. Demographic and clinical information was obtained using a standardized case-report form.

Blood for on-site RDT and microscopy, and blood in EDTA tubes was obtained, the latter stored at − 20 °C and shipped on dry ice to Norway for malaria-PCR to be done later. Malaria microscopy was performed if requested by attending clinician, while PCR and RDT was performed on all patients for the sake of the study.

PCR was defined as gold standard for assessment of prevalence and for evaluation of performance of routine diagnostic tests.

Microscopy and rapid diagnostic test

For microscopy, a 10% Giemsa solution was used to stain both thick and thin blood films, in accordance with hospital procedures. The RDT First Response Malaria Ag pLDH/HRP2 Combo Card Test (Premier Medical Corporation Ltd., India) was used until 20.08.2016. At this time, for the remaining 6 weeks, it was replaced by CareStartTM Malaria HRP2/pLDH (Pf/PAN) Combo (Access Bio, Inc., Somerset, NJ, USA) due to stock-out. 96% (685/714) of the patients were tested with the first RDT.

PCR methods

DNA was extracted from 500 μL whole blood using MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche Diagnostics GmbH, Mannheim, Germany). Presence of *Plasmodium* DNA was assessed applying a genus-specific PCR, targeting *cytochrome b (cytb)* on the mitochondrial genome, and quantitative analysis (q-PCR) was performed using a customized plasmid, as previously described [11]. Parasitaemia by PCR was given in copies/μL blood as unit of measurement. The mitochondrial *cytb* target exists in about 20–160 copies depending on the different development stages. One mitochondrion harbours about 20 copies of the *Plasmodium* genome. It is reported that early ring stage parasites have one mitochondrion, while mature gametocytes have up to eight fold higher quantity of the mitochondrial genome (about 80–160 copies) [12, 13]. Due to unknown variation of development stages in a sample, the unit copies/μL blood cannot be converted into the unit parasites/μL. For quality assurance, results with cycle threshold values ≥30 (low amplification) were re-analysed in triplicates, and in case of discordant results between PCR, RDT or routine microscopy, samples were retested from DNA extraction, using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genus-specific PCR positive samples were further analysed by species-specific real-time PCR assays targeting the 18 S gene of *Plasmodium falciparum*, *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae*. Previously published primers [14, 15] were applied in separate master mixes with the following concentrations: 200 nM for *P. falciparum*, 100 nM for *P. vivax*, 300 nM for *P. ovale*, and 200 nM for *P. malariae*. The species-specific amplifications were performed using the following cycling parameters: Step 1, 50 °C for 2 min; step 2, 95 °C for 10 min; step 3, denaturation at 95 °C for 15 s and step 4, annealing at 60 °C for 1 min; steps 3–4 repeated 40 times. All reaction mixtures, both genus-/

and species-specific, contained 2 µL template (DNA), and 12.5 µL SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA, USA), at a total volume of 25 µL. To identify species in samples not detected by species-specific PCR, relevant genus-specific positive PCR-products were sequenced in one direction applying primer PgMt19 F3, sequences run by BLAST, and specific polymorphisms confirmed, as previously described [14].

Statistics

Dichotomous variables were assessed by Chi-square test, and by binomial logistic regression for factors with multiple levels (i.e. age groups). Continuous variables such as age and level of parasitaemia, were assessed by pairwise Wilcoxon rank-sum test for two groups and by Kruskal Wallis test for multiple groups. Analyses were performed in R version 4.1.2, Rstudio version 2021.09.1 (R Core Team, Vienna, Austria) [16].

Results

Among 1044 patients fulfilling the inclusion criteria, we excluded 207 neonates < 15 days, 17 patients from whom we could not obtain blood for testing and 89 patients lacking a result for PCR, resulting in a study population of 731 (Fig. 1). 58% (421/731) were males. Median age was 13 years, range 16 days–95 years. 50% were admitted to the Department of Paediatrics (n = 362, age range 16 days–14 years) and 50% to the Department of Internal Medicine (n = 369, age range 13–95 years).

Malaria prevalence defined by positive PCR was 9% (63/731) (Fig. 2). The median parasitaemia was 5×10^4 copies/µL blood (interquartile range [IQR] 4×10^3 to 4×10^5). All positive cases had *P. falciparum* infection, except for one, who had *P. ovale* identified by sequencing (parasitaemia 1.1×10^5 copies/µL blood, negative RDT, positive microscopy). Malaria RDT was performed in 631 patients, of whom 8% (50/631) had positive test results. Compared to PCR, the RDT had a sensitivity of 64% (36/56), a specificity of 98% (561/575), a positive predictive value (PPV) of 72% (36/50) and a negative predictive value (NPV) of 97% (561/581) (Table 1). Malaria microscopy was performed for 40% (290/731) of whom 7% (21/290) had positive results. Compared to PCR, microscopy had a sensitivity of 50% (18/36), a specificity of 99% (251/254), a PPV of 86% (18/21) and a NPV of 93% (251/269).

Malaria patients with false negative microscopy results had significantly lower parasitaemia (median 9×10^3 copies/µL, IQR $8 \times 10^2 - 7 \times 10^4$) than the true positive ones (microscopy and PCR positive, median 2×10^5 copies/µL, IQR $6 \times 10^4 - 5 \times 10^5$, p = 0.006). Similarly, patients with false negative RDT had

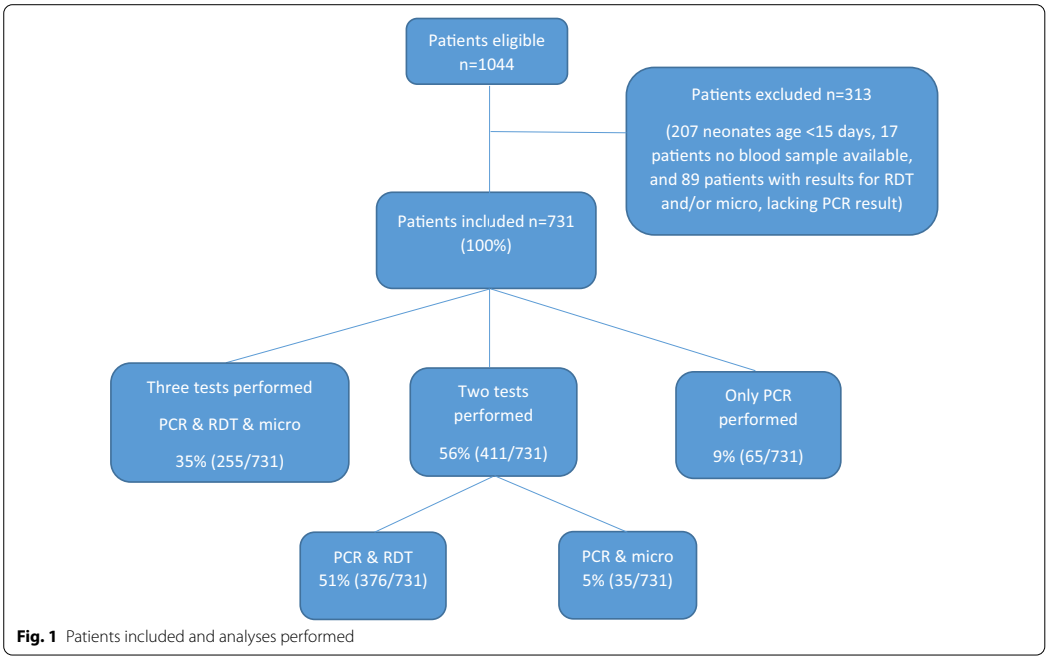

Fig. 1 Patients included and analyses performed

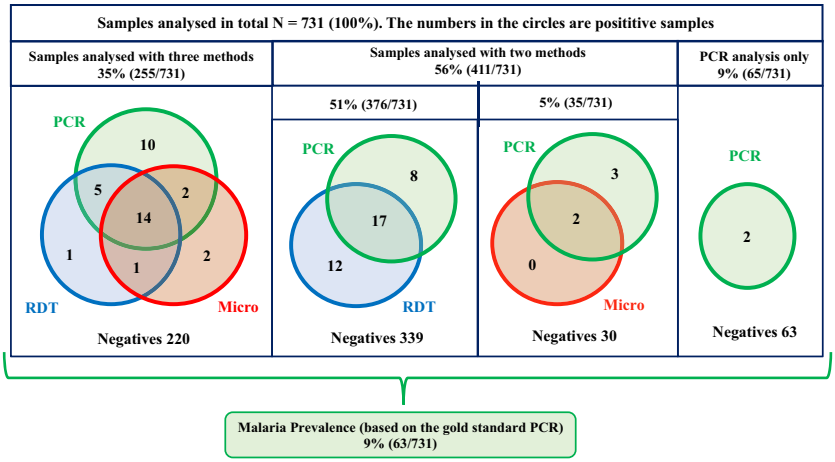

Fig. 2 An overview and results from the analyses performed by PCR, RDT and routine microscopy. The numbers in the circles refer to malaria positive results performed by each method. The numbers of malaria negatives are given in the bottom of the squares. Except for the malaria prevalence, all numbers are given for the performance of each method independently of false positives/negatives by the gold standard method PCR

Table 1 Performance of RDT and microscopy compared to PCR among patients hospitalized with fever in Zanzibar (total n = 820)

	RDT (n = 631) Percentage (n/total)	Microscopy (n = 290) Percentage (n/total)
Sensitivity	64% (36/56)	50% (18/36)
Specificity	98% (561/575)	99% (251/254)
Positive predictive value	72% (36/50)	86% (18/21)
Negative predictive value	97% (561/581)	93% (251/269)

Numbers given for patients investigated with PCR and each test. Discrepancies are due to missing values

RDT, rapid diagnostic test; PCR, polymerase chain reaction

significantly lower parasitaemia (median 7×10^3 copies/μL, IQR 2×10^3 – 8×10^4) than the true positive ones (median 2×10^5 copies/μL, IQR 3×10^4 – 5×10^5, p = 0.002 (Fig. 3).

Malaria prevalence was 10% (41/421) in males and 7% (22/310) in females, however, this difference was not statistically significant (OR 1.4, CI 0.8–2.4, p = 0.3). The prevalence of malaria among children under 5 years old (5%, 14/260) was significantly lower compared to children aged 5 to 15 years (15%, 20/131, p = 0.001) and young adults aged 16 to 30 years (13%, 15/119, p = 0.02), but not different from that in people over 30 years old (6%, 14/217, p = 0.7, Fig. 4). However, the level of parasitaemia was not significantly different across the age groups (Table 2).

Travel history was recorded for 33% (243/731). Malaria patients were more likely to have travelled to mainland Tanzania within the past six months (47%, 9/19), than those testing negative (6%, 13/224, OR 32, CI 5–42, p < 0.0001). Ten of the 19 malaria patients with known travel history had not visited the mainland, four of these had negative RDT. However, all ten cases of presumed autochthonous malaria were positive on PCR with a median parasitaemia of 5.0×10^4 copies/μL blood (IQR 1×10^2 – 2×10^5). Monthly variations in malaria prevalence are shown in Fig. 5. An increase of malaria cases was observed at the end and shortly after the rainy season.

Discussion

A malaria prevalence of 9% was identified by PCR in this study on patients hospitalized for acute febrile illness in Zanzibar in 2015–2016. This is higher than in a survey in 2009 to 2010 at three hospitals in Pemba, the second

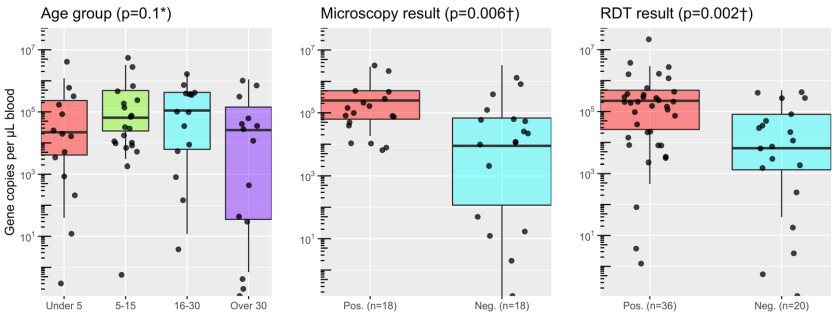

Fig. 3 Malaria parasitaemia by age group (years) and result of microscopy and malaria rapid diagnostic test (RDT). Quantitation of parasitaemia by real-time PCR by diagnostic modalities expressed as log-transformed values of copies per µL blood. Unit of measurement for parasitaemia by PCR is described in the section PCR methods. Dots represent individual observations. Number tested in brackets. * Kruskal Wallis test † Wilcoxon rank sum test

largest island of the Zanzibar archipelago, where only 1% of febrile patients were positive for malaria with RDT and/or microscopy [17].

There are no previous PCR-based malaria prevalence studies in hospitalized patients in Zanzibar. Previous community-based studies in Zanzibar reported a prevalence below 3% up to 2015, including PCR-based studies [18–20]. In 2015, a PCR-based study documented a 2% malaria prevalence in out-patients from rural areas of the two main islands of Zanzibar [5].

In line with that publication [5], the present study found a lower malaria prevalence in children under 5 years compared to school aged children and young adults (Fig. 4; Table 2). The relatively lower malaria prevalence in children < 5 years may be related to a higher probability of using insecticide-treated nets [5]. The higher prevalence in school age children and younger adults may reflect higher exposure to mosquitos during hours of transmission, but could also be related to a relatively lower immunity in this group, compared to the potentially semi-immune older population who were exposed to malaria prior to implementation of the comprehensive malaria control programme.

Malaria was strongly associated with travel to mainland Tanzania within the past six months. This is also shown in recent molecular studies substantiating malaria import from the mainland [21, 22]. However, ten patients had no travel history, confirming autochthonous malaria transmission inside the Zanzibar archipelago in the years 2015 and 2016. Four of these had negative RDT and would have been missed by routine diagnostics. In a survey of out-patients from rural areas of the two main islands of Zanzibar in the period from 2003 to 2015, findings imply ongoing autochthonous transmission [5]. Considering the presence of the effective malaria vector *Anopheles gambiae*, and increasing resistance of vectors to pyrethroid [23], re-introduced malaria can spread quickly in the population.

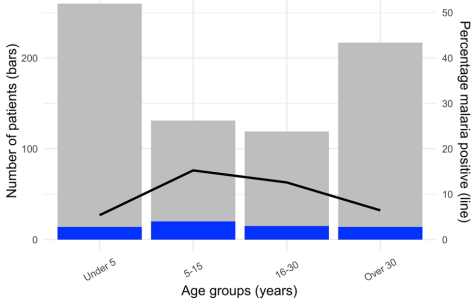

Fig. 4 Malaria cases by age groups. Number of malaria patients (blue bars) among febrile patients (grey bars) and percentage positive (line) in different age groups

Table 2 Comparison of malaria prevalence and parasitaemia by age groups

Age group	Positive	OR (CI)p*	Parasitaemia in copies/µL median (IQR)	p†
Under 5	5% (14/260)	ref.	22 (4-236)	ref.
5-15 years	15% (20/131)	1.10 (1.04-1.17) **0.001**	65 (25-488)	0.30
16-30 years	13% (15/119)	1.07 (1.01-1.14) **0.020**	111 (6-427)	0.41
Over 30	6% (14/217)	1.01 (0.96-1.06) 0.678	28 (0.05-170)	0.65

* Logistic regression (glm in R)

† Kruskal–Wallis test and pairwise Wilcoxon rank sum test for multiple comparisons

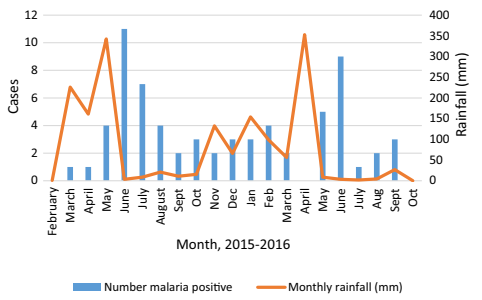

Fig. 5 Monthly number of malaria cases and monthly rainfall from February 2015 to October 2016. Rainfall data for Dar es Salaam from the Tanzanian Meteorological Agency (TMA) [35, 36]. Study start March 17, 2015, study end October 4, 2016

The finding of rising malaria prevalence shortly after rainy periods is in line with the study from Zanzibar [5] and numerous other studies.

The present study indicates that malaria had resurged as an important cause of febrile illness in Zanzibar by 2015. While fifteen years of comprehensive malaria control greatly reduced malaria incidence in the archipelago, it may also have rendered school age children and young adults with less immunity and increased the susceptibility to malaria. The finding of higher malaria prevalence in these age groups underlines the risk of severe malaria in a non-immune population.

The RDT showed a slightly poorer performance compared to PCR than reported previously from Zanzibar [18]. The lower sensitivity of RDTs in the present study (64%) compared to 77% in a study of Shakely et al. [18] may be explained by an inferior performance of the RDTs. In a WHO evaluation, the sensitivity for detecting 200 *P. falciparum* parasites per µL was scored 85% and 90% for the tests used in the present study (RDT First Response Malaria Ag. pLDH/HRP2 Combo Card Test and CareStartTM Malaria HRP2/pLDH (Pf/PAN) Combo Test) [24]. In comparison, the Paracheck Pf Test (Orchid Biomedical Systems, Goa, India) used in the study by Shakely et al. [18] had a sensitivity of 96% [24]. However, a limitation of studies comparing the performance of RDTs, is that the different PCR assays used as gold standards may have varying limits of detection. Thus, the very low detection limit of the PCR assay used in the current study [11] could, at least partly, explain the apparent lower sensitivity of the RDT in this study. The false-positivity rate in the WHO-evaluation of the three tests was 0.0%, 0.4% and 1.3%, respectively [24]. In the present study 36% of malaria cases were missed by RDT and 50% by microscopy compared to PCR. Since PCR has higher sensitivity in low level parasitaemia, it is possible that some of the discrepancy between PCR and RDT/microscopy could be due to coincidental non-significant low-level parasitaemia in semi-immune individuals suffering from febrile illness of other causes. Indeed, patients positive only by PCR had significantly lower parasitaemia than those who also had positive RDT and/or microscopy (Fig. 1). The limitation of RDT in low level parasitaemia is in line with a study reporting 34% sensitivity of RDT compared to PCR in reactive case detection programs in Zanzibar [22].

The superior sensitivity of PCR compared to microscopy is well known [9], and may, apart from inherent methodological issues, be due to suboptimal staining of blood slides, malfunctioning microscopes and deficient training of the laboratory technician [25]. In the present study, sensitivity of microscopy is still substantially higher than in several other surveys [26–29]. Our findings are in line with a review comparing PCR and microscopy for malaria diagnosis in endemic areas, which found that PCR identified on average twice the number of malaria infections compared to microscopy [30]. While PCR is highly sensitive, the level of parasitaemia detected by RDT and microscopy corresponds well with clinically relevant malaria [31].

With its high sensitivity, PCR may be useful in malaria surveillance, including reactive case detection in elimination programs as shown in recent publications from Zanzibar [22, 32]. In a study from 2015, PCR was positive in 2% of asymptomatic individuals in Zanzibar [33]. For clinical diagnosis of acute undifferentiated febrile illness, limitations of PCR are a longer turn-around time, higher cost and higher technical requirements than RDTs, as well as the potential for detecting non-significant low level malaria parasitaemia, or DNA remains of non-viable parasites weeks after parasite clearance [34].

Conclusions

The study emphasizes the importance of malaria as a cause of febrile illness in patients admitted to hospital in Zanzibar, and confirms autochthonous malaria-transmission in Zanzibar in the years 2015 and 2016. The higher malaria prevalence in school age children and young adults could partly be related to waning immunity during the last decades of strict malaria control, in addition to factors such as behaviour and mosquito exposure. The study shows that currently used routine diagnostics may miss up to one-third of malaria positive patients in Zanzibar. Low sensitivities of routine diagnostic tests were related to poor test performance in patients with low parasitaemia.

Abbreviations
IQR: Interquartile range; MMH: Mnazi Mmoja Hospital; NPV: Negative predictive value; PCR: Polymerase chain reaction; PPV: Positive predictive value; RDT: Rapid diagnostic test.

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Authors' contributions
AO, BB, KM and CGH conceived the study. AO, MKM, KAA, KOK, MR and KM recruited patients and collected data for the study. CGH and KOK performed laboratory analyses. AO, BB and CGH analysed the data. BB and AO wrote the first draft of the manuscript. All authors contributed to discussion of the data and revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The research protocol was approved by the Zanzibar Medical Research and Ethical Committee (record no ZAMREC/0002/November/2014, renewal no ZAHREC/02/June/2019/41), and by the Regional Committee for Medical Research Ethics Health Region South East Norway (record no 2014/1940/REK South-East). Inclusion was subject to informed consent from patient or custodian.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References

1. WHO. World Malaria Report 2018: Country profile United Republic of Tanzania. Geneva, World Health Organization. 2018. https://www.who.int/malaria/publications/country-profiles/profile_tz1_en.pdf.
2. WHO. World Malaria Report 2020. Geneva WH, Organization. 2020. https://www.mmv.org/sites/default/files/uploads/docs/publications/World_Malaria_Report_2020.pdf.
3. Ghebreyesus TA, Admasu K. Countries must steer new response to turn the malaria tide. Lancet. 2018;392:2246–7.
4. Bhattarai A, Ali AS, Kachur SP. Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar. PLoS Med. 2007;4:e309.
5. Bjorkman A, Shakely D, Ali AS, Morris U, Mkali H, Abbas AK, et al. From high to low malaria transmission in Zanzibar-challenges and opportunities to achieve elimination. BMC Med. 2019;17:14.
6. Aregawi MW, Ali AS, Al-mafazy AW, Molteni F, Katikiti S, Warsame M, et al. Reductions in malaria and anaemia case and death burden at hospitals following scale-up of malaria control in Zanzibar, 1999-2008. Malar J. 2011;10:46.
7. WHO. Malaria rapid diagnostic test performance: round 8 (2016-2018). Geneva: World Health Organization. https://www.who.int/malaria/publications/atoz/9789241514965/en/.
8. Long EG. Requirements for diagnosis of malaria at different levels of the laboratory network in Africa. Am J Clin Pathol. 2009;131:858–60.
9. Pham NM, Karlen W, Beck HP, Delamarche E. Malaria and the 'last' parasite: how can technology help? Malar J. 2018;17:260.
10. National Bureau of Statistics Tanzania. 2012 Population and Housing Census - DGP Tanzania. http://www.tzdpg.or.tz/fileadmin/documents/dpg_internal/dpg_working_groups_clusters/cluster_2/water/WSDP/Background_information/2012_Census_General_Report.pdf.
11. Haanshuus CG, Mørch K, Blomberg B, Strøm GEA, Langeland N, Hanevik K, et al. Assessment of malaria real-time PCR methods and application with focus on low-level parasitaemia. PLoS One. 2019;14:e0218982.
12. Krungkrai J. The multiple roles of the mitochondrion of the malarial parasite. Parasitology. 2004;129:511–24.
13. Petmitr S, Krungkrai J. Mitochondrial cytochrome b gene in two developmental stages of human malarial parasite *Plasmodium falciparum*, Southeast Asian. J Trop Med Public Health. 1995;26:600–5.
14. Haanshuus CG, Mohn SC, Morch K, Langeland N, Blomberg B, Hanevik K. A novel, single-amplification PCR targeting mitochondrial genome highly sensitive and specific in diagnosing malaria among returned travellers in Bergen, Norway. Malar J. 2013;12:26.
15. Padley D, Moody AH, Chiodini PL, Saldanha J. Use of a rapid, single-round, multiplex PCR to detect malarial parasites and identify the species present. Ann Trop Med Parasitol. 2003;97:131–7.
16. Core Team R. Vienna, Austria. https://www.r-project.org/.
17. Thriemer K, Ley B, Ame S, von Seidlein L, Pak GD, Chang NY, et al. The burden of invasive bacterial infections in Pemba, Zanzibar. PLoS One. 2012;7:e30350.
18. Shakely D, Elfving K, Aydin-Schmidt B, Msellem MI, Morris U, Omar R, et al. The usefulness of rapid diagnostic tests in the new context of low malaria transmission in Zanzibar. PLoS One. 2013;8:e72912.
19. Baltzell KA, Shakely D, Hsiang M, Kemere J, Ali AS, Björkman A, et al. Prevalence of PCR detectable malaria infection among febrile patients with a negative *Plasmodium falciparum* specific rapid diagnostic test in Zanzibar. Am J Trop Med Hyg. 2013;88:289–91.
20. Elfving K, Shakely D, Andersson M, Baltzell K, Ali AS, Bachelard M, et al. Acute uncomplicated febrile illness in children aged 2-59 months in Zanzibar - aetiologies, antibiotic treatment and outcome. PLoS One. 2016;11:e0146054.
21. Morgan AP, Brazeau NF, Ngasala B, Mhamilawa LE, Denton M, Msellem M, et al. Falciparum malaria from coastal Tanzania and Zanzibar remains highly connected despite effective control efforts on the archipelago. Malar J. 2020;19:47.
22. Stuck L, Fakih BS, Al-Mafazy AH, Hofmann NE, Holzschuh A, Grossenbacher B, et al. Malaria infection prevalence and sensitivity of reactive case detection in Zanzibar. Int J Infect Dis. 2020;97:337–46.
23. Haji KA, Khatib BO, Smith S. Challenges for malaria elimination in Zanzibar: pyrethroid resistance in malaria vectors and poor performance of long-lasting insecticide nets. Parasit Vectors. 2013;6:82.

24. WHO. Malaria rapid diagnostic test performance: round 6 (2014-2015). Geneva, World Health Organization. https://apps.who.int/iris/bitstream/handle/10665/204118/9789241510035_eng.pdf;jsessionid=0D38F7525A81AF24C9346C1ADF302A61?sequence=1.
25. Murphy SC, Shott JP, Parikh S, Etter P, Prescott WR, Stewart VA. Malaria diagnostics in clinical trials. Am J Trop Med Hyg. 2013;89:824–39.
26. Haanshuus CG, Chandy S, Manoharan A, Vivek R, Mathai D, Xena D, et al. A high malaria prevalence identified by PCR among patients with acute undifferentiated fever in India. PLoS One. 2016;11:e0158816.
27. Wang B, Han SS, Cho C, Han JH, Cheng Y, Lee SK, et al. Comparison of microscopy, nested-PCR, and Real-Time-PCR assays using high-throughput screening of pooled samples for diagnosis of malaria in asymptomatic carriers from areas of endemicity in Myanmar. J Clin Microbiol. 2014;52:1838–45.
28. Rantala AM, Taylor SM, Trottman PA, Luntamo M, Mbewe B, Maleta K, et al. Comparison of real-time PCR and microscopy for malaria parasite detection in Malawian pregnant women. Malar J. 2010;9:269.
29. Tajebe A, Magoma G, Aemero M, Kimani F. Detection of mixed infection level of *Plasmodium falciparum* and *Plasmodium vivax* by SYBR Green I-based real-time PCR in North Gondar, north-west Ethiopia. Malar J. 2014;13:411.
30. Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in *Plasmodium falciparum*-endemic populations: a systematic review and meta-analysis. J Infect Dis. 2009;200:1509–17.
31. Hartley MA, Hofmann N, Keitel K, Kagoro F, Antunes Moniz C, Mlaganile T, et al. Clinical relevance of low-density *Plasmodium falciparum* parasitemia in untreated febrile children: a cohort study. PLoS Med. 2020;17:e1003318.
32. Grossenbacher B, Holzschuh A, Hofmann NE, Omar KA, Stuck L, Fakih BS, et al. Molecular methods for tracking residual *Plasmodium falciparum* transmission in a close-to-elimination setting in Zanzibar. Malar J. 2020;19:50.
33. Aydin-Schmidt B, Morris U, Ding XC. Field evaluation of a high throughput loop mediated isothermal amplification test for the detection of asymptomatic *Plasmodium* infections in Zanzibar. PLoS One. 2017;12:e0169037.
34. Haanshuus CG, Mørch K. Detection of remaining *Plasmodium* DNA and gametocytes during follow up after curative malaria treatment among returned travellers in Norway. Malar J. 2020;19:296.
35. Tanzania National Bureau of Statistics. Tanzania in Fig. 2015. https://www.nbs.go.tz/index.php/en/tanzania-in-figures.
36. Tanzania National Bureau of Statistics. Tanzania in Fig. 2016. https://www.nbs.go.tz/index.php/en/tanzania-in-figures.

Publisher's Note

13 Appendices

Appendix A: Study questionnaire pilot study 2012/2013

CASE REPORT FORM – BLOOD CULTURE STUDY MNAZIMMOJA HOSPITAL

Case number

Name of the patient

Age/ date of birth of the patient

Gender (M/F)

Date of admission

Date of blood culture

Duration of fever

Temperature on admission

Pulse

Respiratory rate

Other clinical signs and symptoms

HIV status (if known)

Outcome of hospital stay (survival/ death)

Appendix B: Study questionnaires main study 2015/2016

Case report form ward

CASE REPORT FORM – FEVER STUDY MMH. *TO BE KEPT IN WARD*

Study number:_____ Date of admission (DD/MM/YY):_____

Admission number:_____ Name of the patient:_____

Age or date of birth of the patient:_____
Gender: Male ☐ Female ☐

Duration of fever (days):_____

HIV status (if known): HIV Positive ☐ HIV Negative ☐ HIV status not known ☐

Examination:
Temperature:_____
Pulse rate:_____ Respiratory rate:_____
Oxygen saturation (if available):_____ Blood pressure:_____
Conscious ☐ Not conscious ☐
Convulsions ☐ No convulsions ☐
Palmar pallor ☐ No palmar pallor ☐
Malnourished ☐ Not malnourished ☐
Abnormal bleeding ☐ No abnormal bleeding ☐
Unable to sit (in babies: unable to suck) ☐ Able to sit (able to suck) ☐

Malaria microscopy:
Positive ☐ If positive, write species and parasitaemia (%):_____
Negative☐ Not done ☐

Malaria rapid test: Positive ☐ If positive, write species: _____
Negative☐ Not done☐

Dengue rapid test: Positive ☐ Negative ☐ Not done ☐

Antibiotics during hospitalization: Yes ☐ No ☐
If given during hospitalization, name of antibiotics:_____
_____
Antimalaria treatment during hospitalisation: Yes ☐ No ☐
If given, name of antimalaria medication:_____

Outcome of hospital stay: Survived ☐ Died ☐

Case report form laboratory

CASE REPORT FORM – FEVER STUDY MMH.

TO BE SENT TO LABORATORY

Study number:_____Date of admission (DD/MM/YY):_____

Admission number:_____ Name of the patient:_____

Date of blood samples for the study:_____

Malaria microscopy:

Done ☐ Not done ☐

Appendix C: informed consent form in English and Kiswahili - adults

Informed consent form in English – adults

Information letter for the patient / patient's relatives on participating in the study of bloodstream infections (adult)

My name is _____. I am a research assistant/nurse/doctor working in a research project studying bloodstream infections in patients with fever admitted to Mnazi Mmoja Hospital.

You have been admitted to this hospital with fever or other signs of infection. Therefore, we would like to ask whether you agree to participate in this study. But first I will explain you about the study and answer any questions you may have.

The purpose of the study is to get better knowledge of the diseases causing bloodstream infections and which drugs work best against these diseases.

A blood test will be taken from each patient who is admitted with fever if he or she, in children his or her parents/relatives agree. Participating in the study is completely voluntary. If you chose not to take part in the study, you will continue to receive the services one normally gets from the hospital. The blood will be examined for microbial infections, and we will test which drugs work best for any bacteria we find.

All the information we collect will be handled confidentially.

The result of the malaria rapid test will be ready at once so that you can get antimalarial treatment, if necessary.

We expect that the results of the tests can help you to get a better treatment for bacterial infections, because we may find out exactly which bacteria cause the infection and which drugs work best for those particular bacteria. In addition, we expect that the results of the whole study may help doctors give better treatment to other patients with infections of the bloodstream in the future.

The blood test is a very common test for patients who are admitted to the hospital with fever. The test is not dangerous for the patient in any way and we don't expect patients to experience any problems as a result of the study.

Specimens will also be taken abroad (to Norway) for further tests to find out which microbe caused the fever. The tests will be done to learn more on diagnosis and treatment of febrile diseases. The result of these tests from abroad will not be given to you as these tests will be done a long time, possibly years, after you have been discharged from the hospital. The results will be given to the Hospital Director and to the Ministry of Health and can help improve patient treatment in the future.

.

Do you agree to take part in this study?

I do agree I do not agree

Signature of the patient

Mobile phone number of the patient

Contact persons: Dr. Marianne Arnestad, mobile phone 772 477 406
Dr. Muhammed Khamis Miraji, mobile phone 777 678 088

Informed consent form in Kiswahili - adults

MAELEZO KWA MGONJWA/JAMAA WA MGONJWA KATIKA UTAFITI WA MARADHI YANAYOSABABISHWA NA VIJIDUDU

Jina langu ……., ni mfanyakazi wa afya. Pia nimo katika kufanya utafiti wa maradhi yanayosababishwa na vijidudu katika damu ya mgonjwa mwenye homa.

Umelazwa hospitali kwa sababu ya homa au dalili ya maradhi mengine yanayosababishwa na vijidudu. Kwa hiyo tunataka kuchunguza damu yako tujue chanzo cha homa yako kama imesababishwa na vijidudu aina ya bakteria au fangas; tunaomba ruhusa yako ili tuweze kukutoa damu kwa ajili ya utafiti huu. Kabla ya yote nitakupa maelezo ya namna ya utafiti wetu na kama utakuwa na swali lolote nipo tayari kukujibu.

Madhumuni ya utafiti wetu ni kutaka kujua vijidudu aina gani kati ya bakteria au fangas wanaosababisha homa hapa kwetu na ni dawa gani hasa ambazo zinatibu kwa kiwango gani.

Kipimo cha damu kitachukuliwa ikiwa utaridhia kufanya hivyo. Kushiriki katika utafiti huu ni hiari, na kukataa kwako hakutasababisha kukosa huduma katika hospitali hii. Damu tutaichunguza kama ina vijidudu na pia ni kwa dawa ipi ambayo inaua vijidudu hivyo kwa kasi zaidi.

Habari za ugonjwa wako hatopewa mtu mwengine asiyehusika. Matokeo ya kipimo cha malaria yatakuwa tayari moja kwa moja ili uweza kupata dawa ya malaria itapohitajika.

Tunatarajia majibu ya vipimo hivi yatasaidia kupata matibabu mazuri zaidi baada ya kugundua ni vijidudu gani vimeathiri damu yako na dawa gani hasa inayotibu vizuri zaidi. Mbali ya hayo, utafiti huu utasaidia madaktari wetu kuweza kujua vijidudu vinavyotuathiri hapa kwetu, na hivyo kutoa dawa nzuri zaidi.

Vipimo vya damu ni vya kawaida kufanywa wakati mgonjwa anapolazwa kwa homa. Kipimo hiki hakina dhara kwa mgonjwa, na hatutarajii mgonjwa kupata tatizo lolote katika utafiti huu. Vipimo vya damu vinaweza kupelekwa nje ya nchi (mpaka Norway) kwa uchunguzi zaidi. Kazi hii itaweza kutuengezea ujuzi wa kuwachunguza na kuwatibu wagonjwa. Matokeo ya vipimo vinavyopelekwa nje ya nchi yatachukua muda mrefu, baada ya wewe kutoka hospitali, kwa hivyo hamtapata majibu ya motokea hayo. Lakini matokeo hayo yatapelekwa kwa mkurugenzi wa hospitali na waziri wa afya, ili yasaidie kutibu wagonjwa vizuri zaidi siku za mbeleni.

Jee unakubali kushirikishwa katika utafiti huu na hivyo kutolewa damu?

Nakubali Sikubali

Sahihi ya mgonjwa au ndugu ya karibu

Namba ya simu ya mgonjwa au ndugu

Wahusika unaoweza kuwasiliana nao:
Daktari Marianne Arnestad, namba ya simu ya mkononi: 0772 477 406
Daktari Mohammed Khamis Miraji, namba ya simu ya mkononi: 0777 678 088

Appendix D: informed consent form in English and Kiswahili - children

Informed consent form in English - children

Information letter for the patient / patient's relatives on participating in the study of bloodstream infections (child)

My name is _____. I am a research assistant/nurse/doctor working in a research project studying bloodstream infections in patients with fever admitted to Mnazi Mmoja Hospital.

Your child has been admitted to this hospital with fever or other signs of infection. Therefore, we would like to ask whether you agree to let your child participate in this study. But first I will explain you about the study and answer any questions you may have.

The purpose of the study is to get better knowledge of the diseases causing bloodstream infections and which drugs work best against these diseases.

A blood test will be taken from each patient who is admitted with fever if he or she, in children his or her parents/relatives agree. Participating in the study is completely voluntary. If you chose not to let your child take part in the study, the child will continue to receive the services one normally gets from the hospital. The blood will be examined for microbial infections, and we will test which drugs work best for any bacteria we find.

All the information we collect will be handled confidentially.

The result of the malaria rapid test will be ready at once so that your child can get antimalarial treatment, if necessary.

We expect that the results of the tests can help your child get a better treatment for bacterial infections, because we may find out exactly which bacteria cause the infection and which drugs work best for those particular bacteria.

In addition, we expect that the results of the whole study may help doctors give better treatment to other patients with infections of the bloodstream in the future.

The blood test is a very common test for patients who are admitted to the hospital with fever. The test is not dangerous for the patient in any way and we don't expect patients to experience any problems as a result of the study.

Specimens will also be taken abroad (to Norway) for further tests to find out which microbe caused the fever. The tests will be done to learn more on diagnosis and treatment of febrile diseases. The result of these tests from abroad will not be given to you as these tests will be done a long time, possibly years, after your child has been discharged from the hospital. The results will be given to the Hospital Director and to the Ministry of Health and can help improve patient treatment in the future.

Do you agree that your child will take part in this study?

I do agree I do not agree

Signature of parent or close family member of the patient

Mobile phone number of parent or close family member of the patient

Contact persons: Dr. Marianne Arnestad, mobile phone 772 477 406
Dr. Muhammed Khamis Miraji, mobile phone 777 678 088

Informed consent form in Kiswahili - children

MAELEZO KWA MGONJWA/JAMAA WA MGONJWA KATIKA UTAFITI WA MARADHI YANAYOSABABISHWA NA VIJIDUDU

Jina langu ……., ni mfanyakazi wa afya. Pia nimo katika kufanya utafiti wa maradhi yanayosababishwa na vijidudu katika damu ya mgonjwa mwenye homa.
Mtoto wako amelazwa hospitali kwa sababu ya homa au dalili ya maradhi mengine yanayosababishwa na vijidudu. Kwa hiyo tunataka kumchunguza mtoto wako sababu hasa ya chanzo cha homa yake; tunaomba ruhusa yako ili tumchunguze mtoto wako katika utafiti wetu huu. Kabla ya yote nitakupa maelezo ya namna ya utafiti wetu na kama utakuwa na swali lolote nipo tayari kukujibu.
Madhumuni ya utafiti wetu ni kutaka kujua ni aina gani ya vijidudu vinavyosababisha homa hapa kwetu na ni dawa gani hasa ambazo zinatibu kwa kiwango gani.
Kipimo cha damu kitachukuliwa kutoka kwa mgonjwa wako aliyelazwa na homa ikiwa utaridhia kufanya hivyo. Kushiriki katika utafiti huu ni hiari, na kukataa kwako hakutasababisha mtoto wako asiendelee kupata huduma katika hospitali hii. Damu tutaichunguza kama ina vijidudu na pia ni kwa dawa ipi ambayo inaua vijidudu hivi kwa kasi zaidi.
Habari za mgonjwa hatopewa mtu mwengine asiyehusika. Matokeo ya kipimo cha malaria yatakuwa tayari moja kwa moja ili mtoto wako aweza kupata dawa ya malaria itapohitajika. Tunategemea kama matokeo ya vipimo vya utafiti huu yanaweza kumsaidia mtoto wako apate matibabu bora kwa ugonjwa wake, kwa sababu tunaweza kujua kikamilifu ni vijidudu gani vinamsumbua na dawa gani zinaweza kufanya kazi vizuri zaidi.
Tunatarajia majibu ya vipimo hivi yatasaidia mtoto wako kupata matibabu mazuri zaidi baada ya kugundua ni vijidudu gani vimemuathiri na dawa gani hasa inayotibu vizuri zaidi. Mbali ya hayo, utafiti huu utasaidia madaktari wetu waweze kujua vijidudu vinavyotuathiri hapa kwetu na hivyo kutoa dawa nzuri zaidi.
Vipimo vya damu ni vya kawaida kufanywa wakati mgonjwa anapolazwa na homa. Kipimo hiki hakina dhara kwa mgonjwa, na hatutarajii mgonjwa kupata tatizo lolote katika utafiti huu. Vipimo vya damu vinaweza kupelekwa nje ya nchi (mpaka Norway) kwa uchunguzi zaidi. Kazi hii itaweza kutuengezea ujuzi wa kuwachunguza na kuwatibu wagonjwa. Matokeo ya vipimo vitavyopelekwa nje ya nchi yatachukua muda mrefu, baada ya mtoto wako ameshatoka hospitali, kwa hivyo hamtapata majibu ya motokea hayo. Lakini matokeo hayo yatapelekwa kwa waziri wa afya na mkurugenzi wa hospitali ili yasaidie kutibu wagonjwa vizuri zaidi siku za mbeleni.
Jee unakubali mtoto wako kushirikishwa katika utafiti huu na hivyo kutolewa damu?

Nakubali Sikubali

Sahihi ya mzazi au ndugu wa karibu

Namba ya simu ya mzazi au ndugu wa karibu
Wahusika unaoweza kuwasiliana nao:
Daktari Marianne Arnestad, namba ya simu ya mkononi: 0772 477 406
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