The carriage of antimicrobial resistance in community children – a TB-CHAMP sub-study.

by

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Abstract

Introduction: The world-wide rise in antimicrobial resistance (AMR) is threatening the effectivity of antibiotics and the control of infectious diseases. The challenge of AMR is considerably exacerbated by the presence of mobile genetic elements harbouring various antibiotic resistance genes, which can easily spread to other species by horizontal gene transfer. This poses serious risks for clinical infections, however, reports on the carriage of these plasmid-mediated resistance genes are still rare in South Africa. This study aimed to describe the rates of carriage and mechanisms of antimicrobial resistance at baseline, as well as the effect of levofloxacin exposure on these rates in children in communities in Cape Town.

Methods: Stool samples were collected from 100 children enrolled in the Tuberculosis Child Multidrug-resistant Preventive Therapy Trial (TB-CHAMP) at baseline and at 16- and 24-week follow up visits between November 2017 and November 2019. The stool samples were cultured onto MacConkey agar plates with cefpodoxime and ertapenem disks and in some cases, nalidixic acid and ciprofloxacin disks, in order to select for cephalosporin, carbapenem and quinolone resistant and susceptible *E. coli* and *Klebsiella* isolates. Kirby Bauer disk diffusion was used to determine the susceptibility profiles of the organisms and PCR and Sanger sequencing were used for subsequent detection of cephalosporin and quinolone resistance mechanisms. DNA was extracted directly from the stools and targeted molecular detection and quantification of plasmid-mediated quinolone resistance (PMQR) genes using real-time PCR.

Results: High levels of antibiotic resistance were detected at baseline, with 81% of participants carrying an organism resistant to at least one antibiotic and 49% and 33% carrying quinolone and cephalosporin resistant organisms respectively. These rates increased over time, with significant increases in quinolone resistance after 16 weeks (69.8%). The presence of the extended spectrum β-lactamase gene, *bla*$_{\text{CTX-M}}$, in cephalosporin resistant *E. coli* and *Klebsiella* spp. remained relatively constant over time, ranging between 19.7 – 26.8% and 33.3 – 37.5% respectively over 24 weeks. However, this gene was observed in higher proportions in *Klebsiella* spp. compared to *E. coli.* We saw high rates of carriage of *qnrB* (53.3%) and ***aac(6')-Ib-cr*** (66.7%) in *Klebsiella* spp. at baseline and significant increases in *qnrS* and ***aac(6')-Ib-cr,*** as well as mutations in *gyrA* and *parC* after 16 and, in some cases, 24 weeks. The presence

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of *aac(6')-Ib-cr* also increased significantly in *E. coli* from baseline (3.8%) to 16 weeks (21.3%). *qnrS* was detected in 86% of stools in the targeted molecular analysis, while *qnrB* was only detected in 14%, although it was more abundant than *qnrS* in the stool samples.

Conclusions: We report high rates of resistance to various antibiotics, as well as the presence of β-lactamase and PMQR genes, in commensal gut bacteria in children in Cape Town communities before and over 24 weeks of levofloxacin treatment. The high rate of quinolone resistance at baseline is especially worrying as roughly half of these children started levofloxacin treatment after the baseline stool samples were collected. The increase in rates of resistance and presence of PMQR genes is interesting, however, the TB-CHAMP trial is still ongoing and we do not yet know which participants are receiving levofloxacin or placebo. Once the TB-CHAMP study has been completed and the blind has been broken, the results can be stratified according to treatment group to determine the impact of levofloxacin on resistance carriage.

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Opsomming

Inleiding: Die wêreldwye toename in antibiotiese weerstand bedreig die effektiwiteit van antibiotika, asook die beheer van aansteeklike siektes. Die uitdaging van antibiotiese weerstand word aansienlik vererger deur die teenwoordigheid van mobiele genetiese elemente wat verskeie antibiotiese weerstandsgene dra en maklik kan versprei na ander spesies deur horisontale geenoordrag. Dit hou ernstige risikos in vir kliniese infeksies, maar inligting oor die dra van hierdie plasmied-bemiddelde weerstandsgene is egter nog baie skaars in Suid-Afrika. Hierdie studie beskryf die vlakke en meganismes van antibiotiese weerstand by baslislyn, asook die effek van levofloksasien blootstelling hierop in kinders in gemeenskappe in Kaapstad.

Metodes: Stoelgang monsters is ingesamel vanaf 100 kinders wat deelneem aan die “Tuberculosis Child Multidrug-resistant Preventive Therapy Trial (TB-CHAMP)” by basislyn en 16-en 24-week opvolg besoeke tussen November 2017 en November 2019. Die stoelgang monsters is gekweek op MacConkey agar met kefpodoksiem en ertapenem skyfies en, in sommige gevalle, nalidiksiese suur and siprofloksasien skyfies, om kefalosporien-, karbapenem- en kinoloon-weerstandige en -vatbare *Escherichia coli* and *Klebsiella* spp. isolate te selekteer. Die Kirby Bauer skyfie diffusiemetode is gebruik om die antibiotiese weerstandsprofiele van die organismes te bepaal en polimerasekettingreaksie (PKR) en Sanger DNS-volgordebepaling is gebruik vir die opsporing van kefalosporien- en kinoloon-weerstandsmeganismes. DNS is ook direk vanaf die stoelgang monsters onttrek om geteikende molekulêre opsporing en kwantifisering van plasmied-bemiddelde kinoloon weerstands- (PBKW) gene uit te voer deur middel van ware-tyd PKR.

Resultate: Hoë vlakke van antibiotiese weerstand is opgespoor by basislyn, waar 81% van deelnemers ‘n organisme gedra het wat weerstandig is teen ten minste een antibiotika en 49% en 33% kinoloon- en kefalosporien-weerstandige organismes onderskeidelik gedra het. Hierdie vlakke het toegeneem oor tyd, met betekenisvolle toenames in kinoloon-weerstand na 16 weke (69.8%). Die teenwoordigheid van die verlengde-spektrum β-laktamase (VSBL) geen, *bla*$_{\text{CTX-M}}$, in kefalosporien-weerstandige *E. coli* en *Klebsiella* spp. het redelik konstant gebly met tyd, tussen 19.7 – 26.8% en 33.3 – 37.5% onderskeidelik oor 24 weke. Hierdie geen is egter in hoër vlakke waargeneem in *Klebsiella* spp. in vergelyking met *E. coli.* Ons het hoë vlakke

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van die PBKW-gene *qnrB* (53.3%) en *aac(6')-Ib-cr* (66.7%) in *Klebsiella* spp. waargeneem by basislyn en betekenisvolle toenames in *qnrS* en *aac(6')-Ib-cr,* asook mutasies in *gyrA* en *parC* na 16 en, in sommige gevalle, 24 weke. Die teenwoordigheid van *aac(6')-Ib-cr* het ook betekenisvol toegeneem in *E. coli* vanaf basislyn (3.8%) na 16 weke (21.3%). *qnrS* is opgespoor in 86% van die stoelgange in die geteikende molekulêre analise, terwyl *qnrB* slegs in 14% opgespoor is, alhoewel dit meer oorvloedig was in die stoelgange as *qnrS*.

Gevolgtrekkings: Ons het hoë vlakke van antibiotiese weerstand teenoor verskeie antibiotika aangemeld, asook die teenwoordigheid van VSBL en PBKW-gene, in kommensale bakterieë in kinders in Kaapstadse gemeenskappe voor en oor 24 weke van levofloksasien behandeling. Die hoë vlakke van kinoloon-weerstand by basislyn is veral 'n bekommernis, aangesien omtrent die helfte van hierdie kinders met levofloksasien behandeling begin het nadat die basislyn monsters ingesamel is. Die toenames in die vlakke van weerstand en teenwoordigheid van PBKW-gene is interessant, maar die TB-CHAMP studie is egter nog aan die gang en ons weet nog nie watter deelnemers ontvang levofloksasien of plasebo nie. Sodra die TB-CHAMP studie voltooi is en ontblinding plaasgevind het, kan die resultate geskei word volgens behandelingsgroep om die invloed van levofloksasien op die dra van antibiotiese weerstand te bepaal.

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List of abbreviations

A	Amoxicillin
AK	Amikacin
AMR	Antimicrobial resistance
AUG / Co-amoxiclav	Amoxicillin + clavulanic acid
BLI(s)	β-lactamase inhibitor(s)
BRICS	Brazil, Russia, India, China, and South Africa.
CAP	Community-acquired pneumonia
CAR	Central African Republic
CAZ	Ceftazidime
CDC	US Centers for Disease Control and Prevention
CDDEP	Centre for Disease Dynamics, Economics & Policy
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CMY	Cephamycin
CPM	Cefepime
CRE	Carbapenem resistant Enterobacteriaceae
C_T	Cycle threshold
CTX	Cefotaxime
CXM	Cefuroxime
ΔC_T	Cycle threshold relative to reference gene (16S)
DDD	Defined daily doses
DHA	AmpC β-lactamase named after the site of discovery (Dharhan hospital, Saudi-Arabia)
DNA	Deoxyribonucleic acid
DTTC	Desmond Tutu TB Centre
E. coli	*Escherichia coli*
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended spectrum beta-lactamase
ESKAPE	*Enterococcus* spp., *Staphylococcus aureus, Klebsiella* spp., *Acinetobacter* spp., *Pseudomonas aeruginosa, Enterobacter* spp.
ETP	Ertapenem
GES	Guiana extended-spectrum

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GIT	Gastro-intestinal tract
GM	Gentamicin
HGT	Horizontal gene transfer
HIC(s)	High-income country(ies)
HIV	Human immunodeficiency virus
IDSA	Infectious Diseases Society of America
IMI	Imipenem
IMP	β-lactamase active on imipenem
IRT	Inhibitor-resistant TEM
K. oxytoca	*Klebsiella oxytoca*
K. pneumoniae	*Klebsiella pneumoniae*
Klebsiella spp.	*Klebsiella species*
KPC	*Klebsiella pneumoniae* carbapenemase
KZN	KwaZulu-Natal
LMIC(s)	Low and middle-income country(ies)
MBL	Metallo-β-lactamases
MDR	Multidrug-resistant(ce)
MDR-TB	Multidrug-resistant Tuberculosis
MH	Mueller Hinton
MIC(s)	Minimal inhibitory concentration(s)
MRC-CTU	Medical Research Council Clinical Trials Unit
NA	Nalidixic acid
NDM	New Delhi Metallo-β-lactamase
NHLS	National Health Laboratory Services
OXA	Oxacillinase
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PMQR	Plasmid-mediated quinolone resistance
QRDR	Quinolone resistance-determining regions
rDNA	Ribosomal deoxyribonucleic acid
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TB-CHAMP	Tuberculosis Child Multidrug-resistant Preventive Therapy Trial
TBA	Tryptose blood agar
UK	United Kingdom

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US	United States
USA	United States of America
VIM	Verona Integron-encoded Metallo-β-lactamase
WHO	World Health Organisation
XDR	Extensively drug-resistant
ZOI(s)	Zone(s) of inhibition

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Chapter 1: Literature review of antimicrobial resistance in Enterobacterales

1.1. The global problem of antimicrobial resistance

The emergence and rapid world wide spread of antimicrobial resistance (AMR) in bacteria that cause hospital- as well as community-acquired infections is threatening the effectiveness of antibiotics (WHO, 2017a). This has raised concerns that we are on the verge of a 'post antibiotic era' where common bacterial infections will become completely untreatable (Laxminarayan *et al.*, 2013; Auer and Rahman, 2015). In addition, this growing resistance poses broader economic and societal threats that ultimately jeopardise the achievement of the Sustainable Development Goals (Jasovsky *et al.*, 2016; WHO, 2017a). For example, AMR is associated with increased morbidity and mortality, extended duration of illness and infectivity (Laxminarayan and Heymann, 2012) and, therefore, extended hospitalisation periods and increased healthcare costs (Bryce *et al.*, 2016; Hamilton and Wenlock, 2016). It will, however, require the collaborative action of various stakeholders, at regional, national and global levels, to address this issue at its core (Roca *et al.*, 2015; WHO, 2017a).

1.1.1. Overuse of antimicrobials

During his Nobel lecture in 1945, Alexander Fleming already warned of the potential of microorganisms to develop resistance to antibiotics when exposed to concentrations insufficient to kill them (Fleming, 1945). This allows surviving strains to grow and spread, as there is less competition from other strains (O'Neill, 2016). AMR emerges naturally over time, as organisms undergo genetic changes in order to adapt to their environment and survive (Alanis, 2005; WHO, 2016). These genetic changes are selected for if they are beneficial to the organism but can also be lost if they impede survival (Miko and LeJeune, 2009).

The overuse and misuse of antimicrobials is speeding up the process (WHO, 2016) and providing a competitive advantage for mutated strains of microorganisms (Laxminarayan *et al.*, 2013). The continued extensive use of antibiotics in high-income countries (HICs) contributes to this selection pressure that sustains resistant organisms. This forces a shift towards newer broad-spectrum antibiotics in order to overcome the reduced effectiveness of first line antibiotics (Laxminarayan and

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Heymann, 2012). In most low and middle-income countries (LMICs), the use of newer broad spectrum antibiotics is limited by their high cost and reduced accessibility (Bryce *et al.*, 2016). This fuels resistance to cheaper first-line antibiotics, which are increasingly in use because of their unregulated availability without prescription in most shops and pharmacies in these countries (Ayukekbong *et al.*, 2017).

Van Boeckel *et al*. studied trends of antibiotic consumption between 2000 and 2010 in 71 countries and reported that even though more antibiotics are consumed per person in HICs, the rates of consumption in emerging economies are rapidly rising (Van Boeckel *et al.*, 2014). They found that BRICS countries[1] made up 75% of the 35% global increase in human antibiotic consumption observed during their study, despite only representing a combined 40% of the global population (Van Boeckel *et al.*, 2014). Van Boeckel *et al*. found that China and India, where access to previously unaffordable antibiotics is improving due to rapid economic expansion, accounted for a great deal of the increase in antibiotic consumption (Van Boeckel *et al.*, 2014).

More recently, Klein *et al.* reported a 65% increase in global antibiotic use and, as with Van Boeckel's study four years earlier, they observed that this increase was largely driven by LMICs, with India and China as the leading LMIC antibiotic consumers (Klein *et al.*, 2018). Like Van Boeckel *et al.*, they noted that these severe changes in consumption patterns could indicate increased access to antibiotics due to economic growth. Another suggestion is the expiration of patents, which make these medications cheaper and easier to access (Klein *et al.*, 2018).

Van Boeckel *et al.* also found that acute diarrhoeal disease and acute undifferentiated febrile diseases, like malaria, dengue fever and rickettsiosis, are partly accountable for the increase in cephalosporin and fluoroquinolone use in LMICs, although most of these diseases are not caused by bacteria (Van Boeckel *et al.*, 2014). They therefore suggest inappropriate use as a highly likely cause of increased antibiotic consumption. A study on antibiotic prescribing patterns in the USA from 2007 to 2009, also revealed that more than 25% of prescriptions were for respiratory infections like bronchitis, for which antibiotics are not usually indicated (Shapiro *et al.*, 2014).

1 An association of five major developing national economies, consisting of Brazil, Russia, India, China, and South Africa.

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Marked increases in the use of cephalosporins and fluoroquinolones in India between 2000 and 2015 can also be seen on ResistanceMap, a web-based collection of tools summarizing data and trends on AMR and antibiotic use in various countries across the globe, developed by the Centre for Disease Dynamics, Economics & Policy (CDDEP) in 2010 (CDDEP, 2018a). Constant high levels (70-90%) of *E. coli* and *K. pneumoniae* resistant to these antibiotics were reported between 2008 and 2015 CDDEP, 2018b). Although the site does not provide data on resistance levels prior to 2008, one would assume that the resistance is likely to have risen with the increase in consumption, as seen in other cases provided by this source.

Van Boeckel *et al.* also reported significant increases in the rates of consumption of carbapenems (45%) and polymixins (13%), two last-resort antibiotic classes (Van Boeckel *et al.*, 2014). They suggest that the increased use of carbapenems could, to a certain extent, be explained by the worldwide rise in extended spectrum beta-lactamase (ESBL)-producing Gram-negative bacteria and that the global rise in carbapenem resistant Enterobacteriaceae (CRE) could explain the increase in polymixin use. While one driver of the rise in CRE is likely to be increased carbapenem use, poor infection control and hygiene likely contributes as well. For example, inadequate sanitation is believed to be a reason behind the extensive challenge of CRE in India (Walsh *et al.*, 2011). In HICs, antibiotics were introduced after a decrease in infectious disease mortality rates following the effect of water treatment, improved sanitation, and vaccination (Armstrong *et al.*, 1999).

However, in many LMICs, antibiotics are used as an alternative to public health measures (Van Boeckel *et al.*, 2014). Considering the extent of the increase in antibiotic consumption, as well as the growing population in LMICs, urgent measures are needed to put a stop to the regular use of antimicrobials as an alternative to infection control (Van Boeckel *et al.*, 2014). Reducing, or even better, eliminating antibiotic misuse is considered the best way to fight AMR, however, we cannot discredit the importance of developing new antibiotics (Nelson, 2003).

1.1.2. The deteriorating antibiotic pipeline

The continuous introduction of novel antimicrobial agents had minimised the problem of resistance for a few decades (Martinez, 2014). However, after the “golden age” of antibiotic discovery in the 1950s and 1960s, hardly any new antibiotic classes, with

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novel chemical structures, modes of action or targets, have been successfully introduced to market (Theuretzbacher, 2017; WHO, 2017a). In 2011, during a study on the status of the antibacterial drug development pipeline, Freire-Moran *et al*. reported that 73% of the 90 antibacterial agents included in the study were new substances, but only 17% were found to act via a possible new mode of action and on a possibly new target (Freire-Moran *et al.*, 2011).

During their review of the antibacterial agents currently in the clinical development pipeline, the World Health Organisation (WHO) indicated a lack of potential treatments for the priority resistant bacteria, especially for multidrug-resistant[2] (MDR) and extensively drug-resistant[3] (XDR) Gram-negative pathogens. The study by Freire-Moran *et al*. also demonstrates the dire need to develop novel antimicrobials against particularly challenging Gram-negative bacteria, as only 4.4% of the antibacterial agents with new modes of action or targets demonstrated *in vitro* activity against Gram-negative infections (Freire-Moran *et al.*, 2011). The WHO also suggests that more investment is required in the discovery and clinical development of antimicrobial agents against the critical priority pathogens, such as carbapenem-resistant Enterobacteriaceae (WHO, 2017a).

The supply of novel antibiotics is insufficient to keep up with the constant increase in drug resistance (O'Neill, 2016; WHO, 2017a). Both Van Boeckel *et al.* and the WHO advise that the development of novel antimicrobials should therefore coincide with improvement of hygiene, infection prevention and control practices, and promotion of appropriate present and future antibiotic use through stewardship measures (Van Boeckel *et al.*, 2014; WHO, 2017a).

1.2. Clinical importance of Enterobacterales

The Enterobacteriaceae are a family of rod-shaped Gram-negative bacteria that colonise the gastrointestinal tract of humans and animals, and are also found in plants, soil and water (Georgopapadakou, 2008). This family contains some of the deadliest pathogens and roughly a third of the genera comprise human pathogens which result in various hospital- and community-aquired infections. Urinary tract infections (UTIs) remain the most frequent bacterial infections in humans (Harwalkar *et al.*, 2013) and

2 Nonsusceptibility to more than one antibiotic
3 Nonsusceptibility to at least one agent in all but two or fewer antimicrobial categories

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are also the primary source of bacteraemia, a serious complication of Enterobacteriaceae infection which can lead to severe sepsis, organ failure and septic shock (Paterson, 2016). Enterobacteriaceae are important opportunistic pathogens (Livermore, 2012), with the two most noteworthy members being *Escherichia coli* and *Klebsiella pneumoniae* (Surgers *et al.*, 2018).

Antibiotics are becoming less effective in treating these infections as a result of the world-wide rise in multidrug-resistant *E. coli* and *Klebsiella* species (Diancourt *et al.*, 2005; Mavroidi *et al.*, 2012). Several plasmid-mediated fluoroquinolone resistance genes and ESBLs and have emerged in Enterobacteriaceae from community and clinical settings all over the world in the last few years (Fortini *et al.*, 2015). Due to the severity of infections caused by these organisms, their resistance to antibiotics has significant clinical implications and ranks among the most significant threats to public health globally (Georgopapadakou, 2008; WHO, 2017b).

Enterobacteriaceae are considered a major public health issue by various health organisations such as the European Centre for Disease Prevention and Control (ECDC) and the US Centers for Disease Control and Prevention (CDC), in addition to the WHO (Roca *et al.*, 2015). Moreover, carbapenem- and 3rd-generation cephalosporin resistant strains of Enterobacteriaceae, including *E. coli* and *Klebsiella* spp., are listed by the WHO (WHO, 2017c) and the Infectious Diseases Society of America (IDSA) (Isendahl *et al.*, 2012) as priority pathogens for which new antibiotics need to be developed in order to fight AMR.

1.2.1. *Escherichia coli*

E. coli is a component of the natural gut microbiota in humans, with the majority of *E. coli* being harmless commensal bacteria (Steiner, 2016). However, certain strains have acquired pathogenicity genes which allow them to cause severe infections (Steiner, 2016). The differentiation of these pathogenic strains of *E. coli* from harmless commensal strains poses a unique challenge to clinicians and microbiology laboratories in order to provide more appropriate diagnosis and treatment (Paterson, 2016). *E. coli* is the most common source of bacteraemia and hospital- and community-acquired UTIs, accounting for more than 80% of urine isolates in nearly all clinical conditions (Paterson, 2016). It is also associated with skin and soft tissue infections, neonatal meningitis, spontaneous and post-surgical

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peritonitis, and is among the leading causes of food-borne infections globally (ECDC, 2017).

E. coli can also cause pyogenic liver abscess and intra-abdominal abscess (Paterson, 2016). Enteric *E. coli* infections can affect the colon, small intestine, or both, depending on the genetically encoded virulence traits of the organism (Steiner, 2016). These include various adherence factors, toxins and secreted mediators that join forces to disturb the intestinal physiology of the host. Six major pathotypes of diarrheogenic *E. coli* are produced by certain combinations of these virulence factors (Steiner, 2016).

1.2.2. *Klebsiella* spp.

Like *E. coli*, bacteria of the *Klebsiella* genus are also common colonisers of the human gut, particularly in recently hospitalised individuals (WHO, 2017b). The bulk of human infections caused by this genus are health-care associated, with *K. pneumoniae* being the most clinically important species (Podschun and Ullmann, 1998). *Klebsiella oxytoca* has also been isolated from clinical samples, but to a much lesser degree; as have the new species *Klebsiella planticola* and *Klebsiella terrigena,* which were formerly considered “environmental” species of *Klebsiella.* However, a clonal outbreak of carbapenemase producing *K. oxytoca* has recently been reported in a haematology ward in Austria (Leitner *et al.*, 2015)

K. pneumoniae mostly colonise the skin and the gastrointestinal and respiratory tracts of hospitalised individuals, where they can cause intra-abdominal and lower respiratory tract infections, as well as UTIs (ECDC, 2017). They can also be sources of pneumonia, more commonly in hospital-acquired pneumonia than community-aquired pneumonia (Paterson, 2016). *K. pneumoniae* is the second most common cause of Gram-negative bacteraemia, after *E. coli,* and is associated with opportunistic infections in immunocompromised individuals like new-borns, cancer patients, alcoholics, diabetics and hospitalised patients with indwelling devices (ECDC, 2013). These infections spread rapidly via the hands of hospital staff and between patients, resulting in nosocomial outbreaks (ECDC, 2017), such as that of ESBL-producing *K. pneumoniae* which became common during the 1980s and 1990s (Paterson, 2016).

K. pneumoniae was also one of the first microorganisms to become resistant to aminoglycosides (Christensen and Korner, 1972), with outbreaks due to gentamicin-resistant strains in the 1970s. *K. pneumoniae* has played a key role in the spread of

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antibiotic resistance (Surgers *et al.*, 2018) and has, therefore, been classified as an ESKAPE[4] organism, together with other important MDR pathogens (Boucher *et al.*, 2009). These pathogens are all are intrinsically resistant to one or more antibiotics. For example, *K. pneumoniae* harbours a chromosomal gene that is the precursor of the plasmid-mediated SHV-type ESBLs, causing it to be intrinsically resistant to aminopenicillins. This makes it especially concerning as the aminopenicillins are commonly used as first line antibiotics for community-acquired pneumonia (CAP), caused by *K. pneumoniae,* in many LMICs (Rammaert *et al.*, 2012; ECDC, 2017). Moreover, several new ESBL variants were originally discovered in *K. pneumoniae* and only subsequently identified in *E. coli* (ECDC, 2017).

1.3. Antimicrobial resistance in Enterobacterales

The three major classes of antimicrobial agents used to treat Gram-negative infections are the β-lactams (especially combinations of β-lactams and β-lactamase inhibitors, as well as later-generation cephalosporins and carbapenems), fluoroquinolones and aminoglycosides (Doi *et al.*, 2016). However, various Enterobacteriaceae are now multidrug resistant, making it extremely challenging to manage severe infections (Paterson, 2016).

Bacteria can become resistant to antibiotics by a variety of biochemical mechanisms, including antibiotic-inactivating enzymes, antibiotic target modification and reduced uptake of an antibiotic (Rossolini *et al.*, 2017). When susceptible species obtain these resistance mechanisms by mutations in chromosomal genes or horizontal gene transfer (HGT), it is called acquired resistance (MacGowan and Macnaughton, 2017; Rossolini *et al.*, 2017). Intrinsic resistance, on the other hand, is when a certain mechanism of resistance occurs naturally in all or most strains of a bacterial species.

Gram-negative bacteria are intrinsically more resistant to antibiotics compared to Gram-positive species, due to their virtually impermeable double membrane and effective clearance of molecules that succeed in crossing it. The additional outer membrane prohibits large or hydrophobic molecules, like glycopeptides, from entering the cell and slows the entry of drugs that are able to pass through it (Livermore, 2012).

[4] A group of priority pathogens, recognised by the Infectious Diseases Society of America (IDSA), consisting of two Gram-positive (*Enterococcus faecium* and *Staphylococcus aureus*) and four Gram-negative organisms (*Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa*, and *Enterobacter* species).

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This enhances the efficiency of the next line of defence, such as periplasmic-β-lactamases and efflux pumps in the cytoplasmic membrane, which hunt for amphipathic molecules (like antibiotics) and pump them back out of the cell (Li and Nikaido, 2009). This explains why new antibiotics that are active against Gram-negatives are harder to find.

1.3.1. β-lactams

β-lactam antibiotics comprise a variety of compounds, all sharing a common feature: a β-lactam ring (Mascaretti, 2003). The active β-lactam ring of penicillin, the oldest β-lactam antibiotic, has been exploited to produce a wide range of β-lactam antibiotics, consisting of four classes – penicillins, cephalosporins, carbapenems, and monobactams – which have been extensively prescribed in clinical practice due to their highly effective broad spectrum activity, safety and versatility (Rossolini *et al.*, 2017).

Penicillins are directly or indirectly derived from strains of fungi of the Penicillium genus, as well as other soil-dwelling fungi (Whitehouse *et al.*, 2018). Natural penicillins are not stable against penicillinase (enzymes that break down penicillins) and have a narrow spectrum of activity – mainly against Gram-positive bacteria. Semi-synthetic penicillins, on the other hand, have a greater tolerance against penicillinases and an extended spectrum of activity. They are mainly used to treat infections caused by penicillinase-producing staphylococci and some are effective against various Gram-negative bacilli (Whitehouse *et al.*, 2018).

These drugs can be used alone or in combination with beta-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam, to treat penicillin-resistant organisms (Wanger *et al.*, 2017). The *β*-lactamase inhibitors bind to *β*-lactamases and inactivate them, thereby allowing the co-administered β-lactam to regain activity and perform its task of inactivating the penicillin binding protein (PBP) target and ultimately kill the bacteria (Papp-Wallace and Bonomo, 2016). These combinations, for example amoxillin-clavulanic acid and piperacillin-tazobactam, were introduced into clinics during the 1980s and 1990s and were highly effective because they imitated the β-lactam core (Papp-Wallace and Bonomo, 2016). Their frequent use in hospitals and in general practice has, however, led to the selection of further mutant enzymes that resist inhibition (Mascaretti, 2003).

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Cephalosporins have a broader antibacterial spectrum against both Gram-positive and Gram-negative organisms, as well as increased stability against many types of *β*-lactamases. They are often classified into four, and more recently even five, generational classes by their antimicrobial spectrum properties, with first-generation cephalosporins being mostly active against Gram-positive bacteria, and later generations having extended activity against Gram-negative bacteria and being generally more resilient against *β*-lactamases (Wanger *et al.*, 2017; Whitehouse *et al.*, 2018).

Carbapenems have an even broader spectrum of antimicrobial activity and are referred to as “last resort” antibiotics. They are highly tolerant to a variety of *β*-lactamases and many MDR hospital-acquired bacteria are often susceptible to carbapenems. However, the increasing use of carbapenems has resulted in carbapenem resistance in Gram-negative organisms such as certain Enterobacteriaceae (Whitehouse *et al.*, 2018). Monobactam agents, on the other hand, have a narrow and distinctive spectrum of activity, working only against Gram-negative bacilli. They are relatively stable against *β*-lactamases, however, the emergence of resistant organisms is increasing with their common use (Whitehouse *et al.*, 2018).

β-lactam resistance can arise through various mechanisms, such as 1) reduced access of β-lactams to their PBP targets in Gram-negative bacteria; 2) modified PBPs with lower affinity for β-lactams; and 3) the production of enzymes, called β-lactamases, that break the β-lactam ring (Rossolini *et al.*, 2017). Although the combination and interaction of all three mechanisms is an important factor in the susceptibility of Enterobacteriaceae to β-lactams (Georgopapadakou, 2008), the latter is the most crucial β-lactam resistance mechanism in Gram-negative pathogens (Rossolini *et al.*, 2017). For the purpose of this study we will, therefore, only be focusing on this mechanism of β-lactam resistance.

1.3.1.1. β-lactamases

Gram-negative β-lactamases are characterised into different classes via various schemes, with the most common being the Ambler molecular classification scheme (Ambler *et al.*, 1991), which groups them into four classes, A to D, according to their amino acid sequence similarity and functional properties (Rossolini *et al.*, 2017). Class

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A, C and D enzymes contain a serine residue at their active site, forming an acyl enzyme which enables them to hydrolyse their substrates (Bush and Jacoby, 2010). The class B enzymes are metalloenzymes that require a zinc co-factor to be active (Rossolini *et al.*, 2017).

The production of β-lactamases is a widespread mechanism of intrinsic as well as acquired β-lactam resistance (Rossolini *et al.*, 2017). Class A β-lactamases, for example, are inherently encoded on the chromosomes of some species, like *K. pneumoniae,* and are also some of the most common plasmid-encoded β-lactamases found in healthcare settings. These include the broad-spectrum TEM-1, TEM-2 and SHV-1 enzymes that have widely spread in Enterobacteriaceae since emerging in the 1970s (Rossolini *et al.*, 2017). TEM and SHV β-lactamases are not active against the extended-spectrum cephalosporins (Rossolini *et al.*, 2017) which were designed to withstand the hydrolytic action of β-lactamases and are commonly used to treat severe Gram-negative infections (Monstein *et al.*, 2007).

The increased use of these compounds, however, generated a selective pressure which allowed the TEM and SHV enzymes to evolve an expanded spectrum of activity (Rossolini *et al.*, 2017). These mutant genes are similar to SHV or TEM but possess point mutations that result in an altered amino acid sequence. The consequent structural change around the enzyme's active site brings about the ability to hydrolyse and thereby inactivate third- and fourth-generation cephalosporins and monobactams before they can bind to the PBPs at the cytoplasmic membrane (Falagas *et al.*, 2009; ECDC, 2013; Paterson *et al.*, 2016). Given the extended spectrum of antibiotic-hydrolysing ability in comparison with the parent SHV and TEM enzymes, these β-lactamases were called extended-spectrum β-lactamases (ESBLs) (Paterson, 2016).

1.3.1.2. Extended Spectrum β-lactamases

The first reports of ESBLs came from a *Klebsiella ozaenae* isolate in Germany in 1983, which produced a SHV-type ESBL, designated SHV-2 (Knothe *et al.*, 1983). This was followed by the emergence of the TEM-type ESBLs, first reported in France in 1987, detected in *K. pneumoniae* isolated as early as 1984. The enzyme was initially coined CTX-1, owing to its extended activity against cefotaxime, but has since been renamed TEM-3. It was later discovered that a third-generation cephalosporin resistant *K. oxytoca* strain isolated in 1982 from a neonatal unit in a hospital in Liverpool, England

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(Hart and Percival, 1982), harboured a TEM-type ESBL, which was later named TEM-12 (Du Bois *et al.*, 1995).

A new group of ESBLs, the CTX-M family, unrelated to TEM or SHV enzymes, was first described in Germany in 1989, detected in *E. coli* and called CTX-M-1 due to its ability to hydrolyse cefotaxime (Bauernfeind *et al.*, 1990). Soon after, another CTX-M enzyme, later designated CTX-M-2, was found to be widespread among Salmonella strains in Argentina (Bauernfeind *et al.*, 1992). However, the first-published CTX-M β-lactamase was actually characterised in an *E. coli* isolate from Japan in 1986, but it was initially named FEC-1 (Matsumoto *et al.*, 1988). Only after nucleotide sequencing in 1996, was it found to be almost identical to CTX-M-3, which was described in Poland that year (Gniadkowski *et al.*, 1998).

The *bla*$_{\text{CTX-M}}$ genes stem from clinical strains, but have now spread to community-acquired strains, making this family of ESBLs extremely worrisome (Cha *et al.*, 2008). The production of ESBLs by Gram-negative bacilli is increasingly recognized as a major public health issue in LMICs as well as HICs (Rammaert *et al.*, 2012). Today, ESBLs are the most common cause of bacterial resistance to β-lactam antibiotics and are frequently produced by several members of Enterobacteriaceae, particularly *E. coli* and *K. pneumoniae*, with CTX-M being the most prominent (Paterson, 2016; Rossolini *et al.*, 2017).

ESBLs were initially unable to hydrolyse cephamycins and carbapenems (Bradford, 2001) as their activity could usually be inhibited by β-lactamase inhibitors (BLIs) such as clavulanic acid (Rossolini *et al.*, 2017). However, as mentioned earlier, mutant enzymes that are resistant to their inhibition have emerged (Mascaretti, 2003). This inhibitor-resistant phenotype was originally limited to the TEM subfamily, hence the term “inhibitor-resistant TEM” (IRT) was coined (Cha *et al.*, 2008). Inhibitor-resistant β-lactamases have now also been found in the SHV family (Cha *et al.*, 2008) and have become extremely widespread in clinical settings (Papp-Wallace and Bonomo, 2016). This means that previously suitable treatments for common bacterial strains, such as extended-spectrum β-lactam antibiotics or the combination of a penicillin and a BLI, have become more and more ineffective (Mascaretti, 2003).

Another group of β-lactamases that are not inhibited by tazobactam, sulbactam or clavulanic acid, is the class C β-lactamases (Rossolini *et al.*, 2017). These β-

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lactamases do not stem from narrower-spectrum parent β-lactamases, so they cannot be called extended-spectrum β-lactamases, but are known as AmpC β-lactamases (Rossolini *et al.*, 2017).

1.3.1.3. AmpC β-lactamases

Together with class A β-lactamases, the class C enzymes, also known as AmpC enzymes, are the most frequently occurring enzymes (Bradford, 2001). AmpC β-lactamases are chromosomally encoded by various genera of Enterobacteriaceae and confer resistance to all penicillins and cephalosporins such as cephamycins and some expanded-spectrum cephalosporins, but rarely cefepime (Paterson, 2016; Rossolini *et al.*, 2017). Interestingly, an AmpC cephalosporinase identified from *E. coli* in 1940 was the first bacterial enzyme to destroy penicillin, many years before penicillin was introduced into clinical practice (Abraham and Chain, 1940).

Many common Enterobacteriaceae, including the Enterobacter, Serratia, Citrobacter, Providencia and Morganella genera, have chromosomal *bla*$_{AmpC}$ genes with constitutive low-level expression. However, AmpC enzymes may be overproduced during exposure to antibiotics such as amoxicillin, and imipenem, as the expression of *bla*$_{AmpC}$ genes is inducible by derepression upon exposure to these antibiotics (Tang *et al.*, 2014; Paterson, 2016). Mutations in AmpR, a regulator of AmpC transcription, can result in constitutive hyperexpression of AmpC, with a resistance phenotype similar to that of an ESBL producing organism (Jacoby, 2009).

The *bla*$_{AmpC}$ genes have now also been found on plasmids in various Gram-negative bacteria (Paterson, 2016) which allows them to disseminate by HGT (Rossolini *et al.*, 2017). The plasmid-encoded AmpC β-lactamases, like DHA, named after the site of discovery (Dharhan hospital, Saudi-Arabia) and CMY, named for activity against cephamycins, etc., are becoming increasingly prevalent among Enterobacteriaceae (Rossolini *et al.*, 2017). Some of the genes encoding these enzymes are inducible, whereas others are not (Jacoby, 2009). Like the chromosomally encoded enzymes, the plasmid-mediated AmpC β-lactamases confer resistance to a broad range of β-lactam antibiotics. These plasmids can also carry various genes conferring resistance to other antibiotics such as quinolones, aminoglycosides, etc. as well as genes for other β-lactamases (Jacoby, 2009). Moreover, the first carbapenem resistant Enterobacteriaceae (CRE) was documented in the USA to be a result of an

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overexpressed plasmid-mediated AmpC enzyme together with the loss of a key outer membrane protein (Bradford *et al.*, 1997).

1.3.1.4. Carbapenemases

The extensive use of carbapenems, fuelled by the increasing rate of ESBL-producing Enterobacteriaceae (ECDC, 2013), is thought to be a contributing factor to the emergence of CRE (Lowe *et al.*, 2013; Williams *et al.*, 2018). Carbapenems are usually considered a last resort for effective treatment of infections with resistant organisms, but the emergence and spread of CRE over the past decade has left us with limited treatment options and another severe public health threat (Limbago *et al.*, 2011).

Efflux pumps or mutations that change the expression and/or function of PBPs and porins can confer resistance to carbapenems (Papp-Wallace *et al.*, 2011). More recently, the production of carbapenemase enzymes was described in Enterobacteriaceae (Hamilton and Wenlock, 2016). These enzymes efficiently hydrolyse carbapenems and virtually all current β-lactam antibiotics (Limbago *et al.*, 2011; ECDC. All of these mechanisms are associated with carbapenem resistance in Gram-negative bacilli, and combinations thereof result in high levels of carbapenem resistance in species like *K. pneumoniae, A. baumannii* and *P. aeruginosa*. The production of carbapenemases is, however, the mechanism that has been studied in the most detail (Papp-Wallace *et al.*, 2011).

Class A β-lactamases include a few carbapenemases, with *K. pneumoniae* carbapenemase (KPC) being one of the most important. This enzyme was first isolated and characterised in *K. pneumoniae* in the USA in 1996, but it has since spread globally as a major carbapenem resistance mechanism in CRE (Rossolini *et al.*, 2017). A few variants of the Guiana extended-spectrum (GES) β-lactamases have also become able to hydrolyse carbapenems, in addition to broad-spectrum cephalosporins, due to specific amino acid changes within the active site (Naas *et al.*, 2008). GES enzymes are quite rare but have been identified worldwide.

Class B metallo-β-lactamases (MBLs) are also known for their effective carbapenemase activity (Rossolini *et al.*, 2017). Unlike the serine-β-lactamases, MBLs are inhibited by EDTA, but are resistant to β-lactamase inhibitors such as avibactam (Rossolini *et al.*, 2017). MBL enzymes are encoded on the chromosomes of certain

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environmental species with low pathogenic potential, however, various plasmid-mediated MBLs have emerged in non-fermentative Gram-negative isolates and other Enterobacteriaceae since the mid-1990s (Rossolini *et al.*, 2017). The Verona integron-encoded metallo β-lactamase (VIM)-, IMP- (named for activity against imipenem) and New Delhi metallo β-lactamase (NDM)-type β-lactamases are the most common acquired MBLs found among clinical isolates (Rossolini *et al.*, 2017).

The *bla*$_{\text{NDM-1}}$ gene has also been reported to coexist with the class D carbapenemase gene *bla*$_{\text{OXA-23}}$ in clinical *Acinetobacter baumannii* isolates in India (Karthikeyan *et al.*, 2010). These class D β-lactamases, also known as OXA-type enzymes after their ability to effectively hydrolyse oxacillin, are encoded on the chromosomes of various bacterial species, but can also occur on plasmids (Rossolini *et al.*, 2017). Plasmid-encoded class D carbapenemases have also recently emerged and are disseminating and conferring acquired carbapenem resistance among members of the Enterobacteriaceae (e.g. OXA-48) (Rossolini *et al.*, 2017).

A systematic review of carbapenemase-producing bacteria in Africa revealed that outbreaks due to OXA-48-producing Enterobacterales have been reported in numerous African countries, including South Africa (Manenzhe *et al.*, 2015). The earliest report of OXA-48 in South Africa was in Johannesburg in 2011, in a *K. pneumoniae* strain isolated from a patient previously hospitalised in Egypt (Brink *et al.*, 2013). Recently, OXA-48–like enzymes have been shown to be the second most common carbapenemase, after NDM, in various healthcare centres across the country (Perovic *et al.*, 2016).

Carbapenemase genes can also occur on the same plasmids as other resistance genes, such as plasmid-mediated AmpC and ESBLs, as well as plasmid-mediated quinolone resistance (PMQR) genes (Hu *et al.*, 2014). The close correlation between the production of ESBLs and ciprofloxacin resistance is of particular concern, as it limits the range of alternative therapy for isolates harbouring both of these mechanisms simultaneously (Colodner *et al.*, 2004).

1.3.2. Quinolones

Quinolones are a class of synthetic antibiotics that are used to treat various Gram-positive and Gram-negative bacterial infections, and have become one of the most frequently prescribed antibiotic classes worldwide (Aldred *et al.*, 2014). The first

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member of this class, nalidixic acid, was isolated in 1962 as a by-product of chloroquine synthesis (Lesher *et al.*, 1962) and was introduced into clinical use in 1983 as a treatment for UTIs caused by enteric bacteria (Bagel *et al.*, 1999; Emmerson and Jones, 2003). This first quinolone was used to synthesize the even more active fluoroquinolones (Mitscher, 2005), containing a fluorine at position C6, as well as a ring substituent (piperazine or methyl-piperazine) at C7 (Fu *et al.*, 2013).

With the declining efficacy of earlier antibiotics such as the aminopenicillins and β-lactam/β-lactamase combinations due to rapidly rising rates of AMR, fluoroquinolones quickly became among the most widely used antimicrobials worldwide as effective alternative treatments for common and life-threatening infections (Green and Tillotson, 1997). Ciprofloxacin and levofloxacin are the two most common fluoroquinolones, with ciprofloxacin being very effective against Gram-negative bacteria, particularly the Enterobacteriaceae, and levofloxacin being especially active against Gram-positive bacteria (Fu *et al.*, 2013). Quinolones target the bacterial type II topoisomerases, DNA gyrase and topoisomerase IV enzymes, which regulate conformational changes in the chromosome of bacteria during replication and transcription (Hooper, 1999). This interaction results in an irreversible inhibition of the enzyme activity, followed by fragmentation of DNA and ultimately cell death (ECDC, 2013).

The extensive use of quinolones for the treatment of various bacterial infections has recently resulted in increased bacterial resistance to quinolones worldwide (Mavroidi *et al.*, 2012). Fluoroquinolone resistance in Enterobacteriaceae is an especially concerning and growing problem (Paterson, 2016) and the WHO recently highlighted fluoroquinolone resistance in *E. coli* and related organisms as a major threat to public health (WHO, 2014). Quinolone resistance mechanisms are categorised into three groups which will be discussed below (figure 1.1). The changes in the bacterial cell caused by each mechanism are not mutually exclusive and can accumulate to result in highly quinolone-resistant strains.

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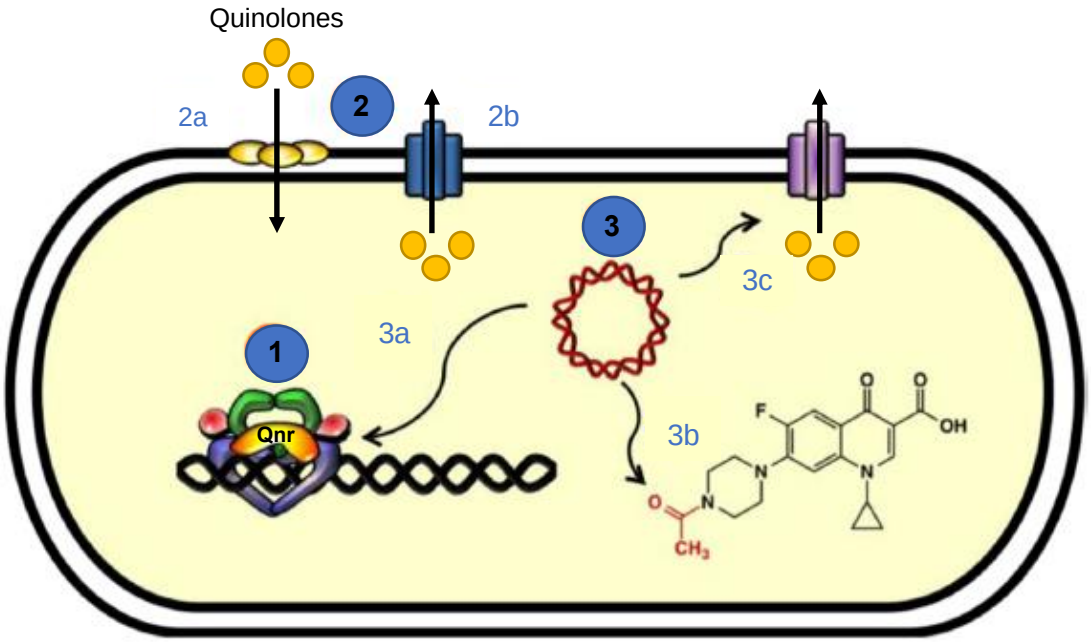

Figure 1.1: Mechanisms of quinolone resistance. (1) Target modification by mutations in gyrase and topoisomerase IV which weaken interactions between these enzymes and quinolones. (2) Chromosome-mediated resistance: (2a) In Gram-negative species, decreased drug uptake due to underexpression of porins or (2b) decreased drug retention by overexpression of chromosome-mediated efflux pumps. (3) Plasmid mediated resistance: (3a) Qnr proteins reduce the binding of topoisomerase to DNA and protect DNA-enzyme complexes from the action of quinolones. (3b) An aminoglycoside acetyltransferase, encoded by *aac(6')-Ib-cr*, acetylates the free nitrogen on the C7 ring of ciprofloxacin and norfloxacin and decreases their effectiveness, (2c) Plasmid-mediated efflux pumps reduce the concentration of quinolones in the cell. (Adapted from Aldred et al., 2014).

1.3.2.1. Target modification

The most common mechanism of quinolone resistance is specific mutations resulting in amino acid alterations in the quinolone resistance-determining regions (QRDRs) in the DNA gyrase subunits (GyrA and GyrB) and DNA topoisomerase IV subunits (ParC and ParE) (Mavroidi *et al.*, 2012; Fu *et al.*, 2013). The QRDR of GyrA was designated by Yoshida *et al.* in 1990 as a small region in the amino terminus, between amino acids 67 and 106 (Yoshida *et al.*, 1990). ParC contains an analogous QRDR region (Mascaretti, 2003). Amino acid substitutions within this region mostly involve a hydroxyl group being replaced with a bulky hydrophobic residue. Therefore, mutations in these regions cause changes in the conformation and/or charge of the respective enzymes, causing them to prevent quinolones from binding to the DNA-substrate complex, yet still preserving their enzymatic function (Namboodiri *et al.*, 2011).

DNA gyrase is the primary target for quinolones in Gram-negative organisms as it is the only enzyme that can alter the supercoiling of DNA; therefore its inhibition by quinolones is accompanied by rapid killing of the bacterial cell (Schmitz and Fluit,

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2010). Quinolone resistance mutations in the *gyrA* gene were first characterized and reported by Cullen *et al*. in a uropathogenic strain of *E. coli* in 1989, however only one out of the three mutations detected was found to cause an amino acid change (Ser83Trp) (Cullen *et al.*, 1989). This mutation is located in a highly conserved, relatively hydrophilic part of the polypeptide chain, close to a tyrosine residue at position 122 at the active site which binds covalently to the DNA (Schmitz and Fluit, 2010).

Although quinolones are believed to interact mostly with GyrA, certain mutations in the *gyrB* gene also confer quinolone resistance in certain species, like *E. coli*. The frequency of *gyrB* mutations has, however, been shown to be rather low compared to that of *gyrA* mutations in clinical isolates of various Gram-negative organisms (Schmitz and Fluit, 2010). Studies have found that resistance-conferring mutations usually occur first in *gyrA*, and then *parC* (Namboodiri *et al.*, 2011). While topoisomerase IV seems to be the primary quinolone target in Gram-positive organisms such as *S. aureus*, it is a secondary target for quinolones in *E. coli* in the absence of a sensitive DNA gyrase.

Topoisomerase IV regulates the topological separation of daughter chromosomes during cell division (Schmitz and Fluit, 2010) and is also able to unwind negatively supercoiled DNA (Chen *et al.*, 2000). Like DNA gyrase, topoisomerase IV consists of two subunits, ParC and ParE, which are homologous to GyrA and GyrB. Mutations in ParC have been reported to occur at Ser80 and Glu84, which are analogous to codons Ser83 and Asp87 of GyrA in *E. coli*, where mutations also commonly occur. These *parC* mutations are common in quinolone-resistant clinical isolates of *E. coli*, whereas *parE* mutations which result in decreased quinolone susceptibility are not as common, as with *gyrB* (Mascaretti, 2003; Schmitz and Fluit, 2010).

It is supposed that the serine and acidic amino acid residues play an essential role in regulating quinolone−enzyme interactions (Aldred *et al.*, 2014), with a water−metal ion (Mg^{2+}) bridge between these residues aligning it to the enzyme. Studies have shown that this water−metal ion bridge is the main interaction between bacterial type II enzymes and quinolones. They also indicate that mutation of either of these residues limits the range of metal ions that can be used to sustain quinolone activity and reduces the affinity of the quinolone−enzyme complex for the noncatalytic Mg^{2+} ion (Aldred *et al.*, 2014).

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The accumulation of mutations in these genes increases the minimum inhibitory concentration (MIC) of quinolones in a stepwise manner (ECDC, 2013). A mutation in either residue significantly decreases the binding of quinolones to gyrase or topoisomerase IV, typically conferring ≤10-fold drug resistance, while mutations in both abolish the activity of quinolones to stabilize cleavage complexes, resulting in higher levels of resistance (∼10−100-fold) (Aldred *et al.*, 2014). The latter also results in a broadening of the resistance spectrum to include later generation quinolones, in other words, the fluoroquinolones (Namboodiri *et al.*, 2011).

1.3.2.2. Chromosome-mediated resistance

In order to reach DNA gyrase and topoisomerase IV, located in the cytoplasm of the bacterial cell, quinolones need to cross the cell envelope. In contrast to Gram-positive bacteria, the Gram-negative outer membrane poses yet another barrier for the quinolone to cross (Schmitz and Fluit, 2010; Aldred *et al.*, 2014). Drug influx in Gram-negative species is, therefore, mediated by protein channels called porins, such as OmpA, OmpC, OmpD and OmpF, with the latter being the major channel for ciprofloxacin entry in *E. coli* (Vinué *et al.*, 2016). Underexpression of these chromosomal porins results in decreased outer membrane permeability, which decreases drug uptake (Aldred *et al.*, 2014) and has been associated with increased antibiotic resistance to quinolones and other drugs.

Furthermore, the overexpression of OmpX, a downregulator of porin expression, causes reduced expression of the OmpC, OmpD, OmpF, LamB and Tsx porins, which also results in increased resistance to various antibiotics, including quinolones (Correia *et al.*, 2017). In addition, the overexpression of chromosome-encoded efflux pumps, commonly caused by mutations in regulatory proteins, reduces drug retention in the cell and can lead to quinolone resistance (Aldred *et al.*, 2014). For example, mutations in *acrR*, the gene encoding a repressor of the *acrAB* pump genes, have been associated with quinolone resistance (Namboodiri *et al.*, 2011).

Modifications in quinolone uptake and retention generally result in low-level resistance and do not seem to do major damage in the absence of additional resistance mechanisms (Aldred *et al.*, 2014). However, decreasing the concentration of quinolones in the cell creates a favourable environment for the development of other

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resistance mechanisms, such as plasmid-mediated quinolone resistance (Aldred *et al.*, 2014).

1.3.2.3. Plasmid-mediated quinolone resistance

Quinolone efflux pumps can also be plasmid-mediated, encoded by the *qepA* and *oqxAB* genes, conferring resistance to hydrophilic fluoroquinolones such as ciprofloxacin, with 32- to 64-fold increases in MICs (Hansen *et al.*, 2007; Yamane *et al.*, 2007). Moreover, QepA is also able to export a narrow range of other agents such as ethidium bromide, erythromycin, and acriflavine, while OqxAB is able to extrude a wider range of agents such as tetracyclines, trimethoprim, chloramphenicol, etc. (Hansen *et al.*, 2007).

The first PMQR determinant to be reported, was named *qnr* but later changed to *qnrA* as more *qnr* alleles were identified (Martínez-Martínez *et al.*, 1998). The *qnr* genes encode pentapeptide repeat proteins that bind to gyrase and topoisomerase IV, thereby inhibiting quinolones from entering cleavage complexes formed by these enzymes (Aldred *et al.*, 2014). Numerous *qnrA* variants have been described, as well as more distant relatives called *qnrB* and *qnrS*, of which several variants are also known. Isolates harbouring these *qnr* genes have been reported globally and this mechanism of quinolone resistance appears to be increasingly important (Schmitz and Fluit, 2010).

The Qnr-proteins can also protect cells from quinolones by limiting the binding of gyrase and topoisomerase IV to DNA, which lowers the amount of available enzyme targets for quinolones on the chromosome (Aldred *et al.*, 2014). These proteins result in a 10- to 100-fold increase in the MIC of quinolones. However, as these values are mostly very low to start with, the expression of *qnr* might not be sufficient to reach the resistant or even intermediately resistant breakpoint (Schmitz and Fluit, 2010). It is, however thought that this could offer the bacterium a larger time window to acquire mutations in gyrase or topoisomerase IV which will increase the MIC tolerated and, therefore, the resistance level (Robicsek *et al.*, 2006).

In 2006, a study on a *qnrA*-harbouring plasmid from Shanghai that resulted in a higher than expected level of ciprofloxacin resistance led to the discovery of another PMQR mechanism: modification of certain quinolones by a variant of an aminoglycoside acetyltransferase, AAC(6′)-Ib-cr (Robicsek *et al.*, 2006). It contains two specific point

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mutations which allow it to acetylate the unsubstituted nitrogen of the C7 piperazine ring in fluoroquinolones like ciprofloxacin and norfloxacin, thereby decreasing drug activity. While the wild-type and mutant enzymes are both able to acetylate aminoglycosides, only the mutant is active against quinolones (Aldred *et al.*, 2014). PMQR mechanisms are especially worrying due to their frequent association with resistance to other antimicrobials as well as the easily transferable nature of these resistance genes.

1.4. Epidemiology of resistant *E. coli* and *K. pneumoniae*

Antimicrobial resistance rates and mechanisms vary depending not only on the bacterial species and the group of antimicrobials, but also on the geographical region (ECDC, 2018). These variations most likely correlate with differences in the use of antimicrobials, healthcare utilisation patterns, and infection prevention and control practices; factors which are likely to influence resistance rates world-wide.

1.4.1. Global spread of resistant *E. coli* and *Klebsiella spp.*

The thriving worldwide spread of ESBL producing *E. coli* and *K. pneumoniae* remains a serious concern for the treatment of MDR bacteria (Chong *et al.*, 2018). Rates of between 7.4 - 18% have been reported for cephalosporin resistant and ESBL-producing *E. coli* in clinical settings in Europe and North America (Johnson *et al.*, 2010; Gibreel *et al.*, 2012; Giufré *et al.*, 2012; Wickramasinghe *et al.*, 2012; Nüesch-Inderbinen *et al.*, 2017). Considerably higher rates of ESBL-producing *E. coli* and *K. pneumoniae*, between 38.4 – 56.3%, have been reported in hospitals in LMICs like China, Mexico and Uruguay (Zhao *et al.*, 2015; Bado *et al.*, 2016; Xiao *et al.*, 2017; Garza-Ramos *et al.*, 2018).

In Africa, rates within the first range have been reported in abattoir workers in Cameroon (11.3%), hospitals in Nigeria (12.8%) and HIV-infected children in Zimbabwe (13.7%) (Aibinu *et al.*, 2012; Wilmore *et al.*, 2017; Founou *et al.*, 2018). However, other studies involving hospital patients and staff in Cameroon and healthy pregnant women in Nigeria have reported higher rates, at 54.1% and 31.7%, respectively (Magoué *et al.*, 2013; Fortini *et al.*, 2015).

Conjugative horizontal transfer of plasmids and clonal spread have contributed to an increased prevalence of *bla*$_{\text{CTX-M}}$-type ESBL genes in Enterobacteriaceae, especially in *E. coli* and *K. pneumoniae,* which has been reported globally (Bonnet, 2004; Cantón

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and Coque, 2006). Dominant *bla*$_{CTX-M}$ variants differ geographically, however, *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-14}$ are the most common worldwide, followed by *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-3}$ and *bla*$_{CTX-M-1}$ (Zhao and Hu, 2013). The worldwide spread of *bla*$_{CTX-M}$ is evident in table 1.1, where it is seen to be reported in 36 (92.7%) of 41 studies describing the distribution of plasmid-mediated ESBL, carbapenemase and quinolone resistance genes in *E. coli* and *K. pneumoniae*, whereas *bla*$_{SHV}$ and *bla*$_{TEM}$ (whether ESBL or not) were reported in 20 (48.8%) and 18 (43.9%) studies, respectively. The global spread of *bla*$_{CTX-M-15}$ has mostly been attributed to the pandemic *E. coli* ST131 clone, which has been associated with competitive advantages such as multidrug-resistance and improved virulence factors (Peirano and Pitout, 2010).

Varying rates of carriage of quinolone resistant *E. coli* have also been reported in Nigeria, at 12.8% amongst the abattoir workers in Lagos (Aibinu *et al.*, 2012) and 25.7% in hospital patients and staff in Ibadan (Fortini *et al.*, 2015). In clinical settings in the USA, Mexico and Switzerland, the carriage of quinolone resistant *E. coli* and *Klebsiella* spp. has also been reported to range between 20.5% and 24.5% (Johnson *et al.*, 2010; Nüesch-Inderbinen *et al.*, 2017; Garza-Ramos *et al.*, 2018), while higher rates have been reported in hospitals in Italy and China, ranging from 41.3% to 51.2% (Giufré *et al.*, 2012; Zhao *et al.*, 2015; Xiao *et al.*, 2017). The *qnr* and *aac(6')-Ib-cr* PMQR genes are common worldwide, both reported in 21 studies (77.8%) in table 1.1, while *oqx* and *qep* are only reported in 4 (14.8%) and 5 (18.5%) of the studies in table 1.1.

The world-wide spread and high rates of third-generation cephalosporin and fluoroquinolone resistant *E. coli* and *K. pneumoniae* can also be seen on ResistanceMap, where they are reported at high rates in various countries across the world (CDDEP, 2018b). Carbapenem resistant *E. coli* is, however, still present at lower rates across the globe, with the highest rates in Nigeria (15%) and India (18%), as reported in 2017 (CDDEP, 2018b).

Carbapenem resistant *K. pneumoniae*, on the other hand, is more widespread, with the highest rates of 31-68% in Eastern Europe and the Middle East, 59% in India and 72% in Egypt (CDDEP, 2018b). *Klebsiella* spp. has commonly been reported to carry carbapenemase genes, with *bla*$_{KPC}$ being reported in five studies in table 1.1, *bla*$_{OXA}$-carbapenemases in four, *bla*$_{NDM}$ in three and *bla*$_{IMP}$ and *bla*$_{VIM}$ in only one study each.

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Table 1.1: Global epidemiology of plasmid-mediated ESBL, carbapenemase and quinolone resistance genes in *E. coli* and *Klebsiella spp.*

Continent	Country	Organism	Beta-lactamase genes (incl. ESBL-, non-ESBL- & carbapenemase genes)	PMQR genes	Collection period	Reference
Africa	Cameroon	*K. pneumoniae*	*bla*CTX-M-15, *bla*SHV-11, *bla*SHV-28, *bla*SHV-134, *bla*TEM-1, *bla*TEM-116, *bla*OXA-9	*aac(6')-Ib-cr, qnrB, oqxA, oqxB*	2016	Founou et al., 2018
			*bla*CTX-M-15, *bla*SHV-1, *bla*SHV-12, *bla*TEM-1, *bla*OXA-1	–	2010	Magoue et al., 2013
		E. coli	*bla*CTX-M-15, *bla*TEM-1, *bla*OXA-1	*aac(6')-Ib-cr*		
	Central African Republic (CAR)	*E. coli*	*bla*CTX-M15	*aac(6')-Ib-cr, qnrB, qnrS*	2011 - 2012	Rafai et al., 2015
	Ghana	*E. coli*	ND	*qnrS, qnrB, qepA*	2006 - 2008	Namboodiri et al., 2011
	Madagascar	*E. coli*	*bla*CTX-M15, *bla*TEM-1, *bla*SHV-12, *bla*OXA-1	*aac(6')-Ib-cr, qnrB*	2006 - 2007	Rakotonira et al., 2013
		K. pneumoniae				
		K. oxytoca	*bla*CTX-M-15, *bla*SHV-12	–		
	Mozambique	*E. coli*	*bla*CTX-M-15, *bla*SHV-12, *bla*TEM-1, *bla*OXA-1	*qnrB*	2004 - 2009	Guiral et al., 2018
		K. pneumoniae	*bla*CTX-M-15, *bla*SHV-1, *bla*SHV-2, *bla*SHV-5, *bla*SHV-11, *bla*SHV-32, *bla*SHV-33, *bla*SHV-121,*bla*TEM-1, *bla*OXA-30, *bla*CMY-2	ND	2004 - 2009	Pons et al., 2015
			*bla*CTX-M15	*aac(6')-Ib-cr, qnrB, oqxAB*	2012	Zhou et al., 2015
	Nigeria	*E. coli*	*bla*CTX-M15, *bla*TEM-1, *bla*CMY-2	*aac(6')-Ib-cr, qnrS, qnrB, qepA*	2011	Fortini et al., 2015
		E. coli	*bla*CTX-M15, *bla*TEM-1	*aac(6')-Ib-cr, qnrB*	2010 - 2011	Inwezerua et al., 2014
		E. coli	*bla*CTX-M15	*aac(6')-Ib-cr, qnrA, qnrB*	2008 - 2009	Aibinu et al., 2012
	Tunisia	*K. pneumoniae*	*bla*CTX-M-15, *bla*SHV-28, *bla*TEM-1	*qnrA, qnrB, qnrS*	2005 - 2007	Dahmen et al., 2010
		E. coli	*bla*CTX-M-15, *bla*TEM-1	*qnrB, qnrS*		
		E. coli	*bla*CTX-M-1, *bla*CTX-M-15, *bla*CMY-2	*aac(6')-Ib-cr*	2012 - 2013	Ferjani et al., 2017

– : Not detected

ND: Not determined

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Table 1.1 continued

Continent	Country	Organism	Beta-lactamase genes (incl. ESBL-, non-ESBL- & carbapenemase genes)	PMQR genes	Collection period	Reference
Africa	Zimbabwe	*E. coli*	*bla*CTX-M (group 1 & 9), *bla*SHV-2a, *bla*TEM-1, *bla*OXA-1, *bla*CMY-2, *bla*DHA-1, *bla*LEN-4	ND	2014 - 2015	Wilmore et al., 2017
		K. pneumoniae	*bla*CTX-M (group 1), *bla*OXA-1, *bla*TEM-1, *bla*SHV-11, *bla*LEN-11			
Asia	China	*K. pneumoniae*	*bla*CTX-M-14, *bla*CTX-M-15, *bla*SHV-1, *bla*SHV-11, *bla*SHV27, *bla*OXA-1, *bla*KPC-2, *bla*NDM-1, *bla*IMP-4, *bla*IMP-8	*aac(6')-Ib-cr, oqxAB, qnrB, qnrS*	2014 - 2015	Zhang et al., 2018
		E. coli	*bla*CTX-M14, *bla*CTX-M-15, *bla*CTX-M-23	*aac(6')-Ib-cr, qnrB, qnrS*	2014 - 2015	Li et al., 2017
	Japan	*E. coli*	*bla*CTX-M-15, *bla*CTX-M-14, *bla*CTX-M-27, *bla*SHV-12, *bla*TEM-1, *bla*OXA-1	*aac(6')-Ib-cr, qnrB*	2008 - 2011	Yano et al., 2013
	Korea	*K. pneumoniae*	*bla*CTX-M-15, *bla*SHV-12, *bla*TEM-1, *bla*OXA-9	*qnrB, oqxA, oqxB*	NS	Souza et al., 2017
	Singapore	*K. pneumoniae*	*bla*CTX-M-15, *bla*SHV-1, *bla*SHV-11, *bla*TEM-1, *bla*TEM-116, *bla*OXA-1, *bla*OXA-9, *bla*OXA-181	ND	2010 - 2012	Balm et al., 2013
Europe	France	*E. coli*	*bla*CTX-M-1, *bla*CTX-M-15, *bla*CTX-M-24, *bla*CTX-M-27, *bla*SHV-12, *bla*TEM-52	*aac(6')-Ib-cr, qnrA, qnrB*	2008 - 2009	Guillard et al., 2014
	Hungary	*K. pneumoniae*	*bla*CTX-M-15, *bla*SHV-11, *bla*SHV-12, *bla*TEM-1, *bla*KPC2	ND	2008 - 2009	Toth et al., 201
	Italy	*E. coli*	*bla*CTX-M-1, *bla*CTX-M-15, *bla*CTX-M-27, *bla*SHV-12	ND	2009	Giufre et al., 2012
	Netherlands	*K. pneumoniae*	*bla*CTX-M-3, *bla*CTX-M-14, *bla*CTX-M-9, *bla*CTX-M-15, *bla*SHV-71, *bla*SHV-12, *bla*SHV-28, *bla*SHV-36, *bla*DHA-1	*aac(6')-Ib-cr, qnrA, qnrB, qnrS*	2008	Paltansing et al. 2013
		E. coli	*bla*CTX-M-1, *bla*CTX-M-9, *bla*CTX-M-14, *bla*CTX-M-15, *bla*CTX-M-27, *bla*CTX-M-55, *bla*SHV-12, *bla*TEM-28, *bla*TEM-52	*aac(6')-Ib-cr, qnrA*		
	Norway	*E. coli*	*bla*SHV-2, *bla*TEM-1, *bla*CMY-7	–	1999-2008	Knudsen et al., 2017
	Spain	*K. pneumoniae*	*bla*CTX-M-9, *bla*VIM-1	*qnrA*	2009 - 2011	Coelho et al., 2012
		E. coli	*bla*CTX-M15	ND	2006	Diaz et al., 2010

NS: Not specified

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Table 1.1 continued

Continent	Country	Organism	Beta-lactamase genes (incl. ESBL-, non-ESBL- & carbapenemase genes)	PMQR genes	Collection period	Reference
Europe	Switzerland	*E. coli*	*bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$	*aac(6')-Ib-cr*	2016	Nüesch-Inderbinen et al. 2017
	United Kingdom	*E. coli*	*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-15}$	ND	2004 - 2006	Dhanji et al., 2011
			bla$_{CTX-M-14}$, *bla*$_{CTX-M-15}$	ND	2010	Wickramasinghe et al. 2012
			bla$_{CTX-M15}$	*aac(6')-Ib-cr*	2007 & 2009	Gibreel et al., 2012
North America	Canada	*K. pneumoniae*	*bla*$_{CTX-M14}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$, *bla*$_{SHV-14}$	*aac(6')-Ib-cr, qnrB, qnrS*	2000 - 2009	Peirano et al., 2012 (1)
		E. coli	*bla*$_{CTX-M14}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV}$, *bla*$_{TEM-52}$	*aac(6')-Ib-cr, qepA*	2000 - 2010	Peirano et al., 2012 (2)
	Mexico	*K. pneumoniae*	*bla*$_{CTX-M15}$	–	2013 - 2014	Garza-Ramos et al., 2018
			bla$_{SHV-1}$, *bla*$_{SHV-12}$, *bla*$_{NDM-1}$, *bla*$_{KPC-2}$	ND	2016	Aquino-Andrade et al., 2018
		K. oxytoca	*bla*$_{OXA-48}$			
		E. coli	*bla*$_{CTX-M15}$, *bla*$_{OXA-232}$, *bla*$_{NDM-1}$, *bla*$_{KPC-2}$			
			bla$_{CTX-M-14}$, *bla*$_{CTX-M15}$, *bla*$_{TEM-1}$, *bla*$_{OXA-1}$	*aac(6')-Ib-cr, qnrB*	2012	Cortes-Cortes et al., 2017
	USA	*E. coli*	*bla*$_{CTX-M-1}$, *bla*$_{CTX-M-15}$	ND	2007	Johnson et al., 2010
		K. pneumoniae	*bla*$_{KPC-2}$, *bla*$_{KPC-3}$	*oqxAB*	NS	Perez et al., 2013
South America	Brazil	*E. coli*	*bla*$_{CTX-M-15}$, *bla*$_{CTX-M\ group\ 8\ \&\ 9}$	ND	2013 - 2014	Goncalves et al., 2016
			bla$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-59}$	ND	2000 - 2001	Quieroz et al., 2012
	Bolivia	*E. coli*	*bla*$_{CTX-M-2}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-8}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-65}$	*aac(6')-Ib-cr, qnrB, qepA*	2011	Bartoloni et al., 2012
	Uruguay	*K. pneumoniae*	*bla*$_{CTX-M14}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-5}$	*aac(6')-Ib-cr, qnrB*	2010 - 2011	Bado et al., 2016
		E. coli	*bla*$_{CTX-M15}$	–		

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1.4.2. Dissemination of resistant *E. coli* and *Klebsiella spp.* in South Africa

Reports describing the epidemiology of antimicrobial resistant Enterobacteriaceae in South Africa, especially in community settings, are still very rare, as depicted by the limited number of studies described in table 1.2. One of these studies reported on the carriage of ESBL-producing MDR ESKAPE bacteria in hospitals in KwaZulu-Natal, which was 37.21% (16/43) at admission, 42.31% (11/26) after 48 h and 57.14% (4/7) at discharge, with 61.3% of the 31 isolates (n=19) resistant to ciprofloxacin (R. C. Founou *et al.*, 2018). The prevalence of these bacteria in faecal carriage (46%) was also higher than in clinical samples (28%). In the Cape Town study, 72.7% (16/22) of ESBL-producing *E. coli* isolated from hospitalised patients were quinolone resistant (Peirano *et al.*, 2011). The study by Perovic et al. (2014) reported ESBL production with resistance to cefotaxime, ceftazidime and cefepime in 68% and ciprofloxacin resistance in 46% of *K. pneumoniae* isolated from patients with bacteraemia from sentinel laboratories across various provinces. (The authors did not specify whether only one organism was isolated per patient, so these rates may or may not reflect the carriage rate).

Across the 10 studies in table 1.2, *bla*$_{\text{CTX-M-15}}$ was the most common β-lactamase (60%), followed by *bla*$_{\text{SHV}}$ (50%) and *bla*$_{\text{TEM}}$ (40%). Only three studies reported quinolone resistance mechanisms, where *aac(6')-Ib-cr* was detected in all three, *qnrS* and *qnrB* in two, and *oqxA* and *oqxB* only in the Eastern Cape. The limited data on quinolone resistance mechanisms is worrying, as these antibiotics are increasingly used in South Africa, as seen on ResistanceMap, where the use of fluoroquinolones specifically has increased from 168 to 607 defined daily doses (DDD) per 1000 individuals between 2000 and 2010, after which it fluctuated in the range of 545 to 589 DDD per 1000 individuals until 2015, which is the last available data point (The Center for Disease Dynamics Economics & Policy, 2018b). ResistanceMap also reports third-generation cephalosporin and fluoroquinolone resistance rates in South Africa in 2016 at 23% and 28% for *E. coli* and 65% and 36% for *K. pneumoniae*, as well as a 7% carbapenem resistance rate for the latter.

Another nationwide study focussed on CRE voluntarily submitted by 46 laboratories to the national Antimicrobial Resistance Laboratory (AMRL), where 68% of the isolates harboured one or more carbapenemase gene (Singh-Moodley and Perovic, 2016). *bla*$_{\text{NDM}}$ was the carbapenemase gene harboured by most of the organisms, the majority

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of which were *Klebsiella* spp., while *bla*$_{OXA-48}$ (including variants) was the predominating carbapenemase gene in *E. coli*. Although the rates of carbapenem resistance in South Africa are still low, CRE is becoming a big problem in South Africa and it is clear from the studies in table 1.2 that carbapenemase genes are common in clinical settings across the country.

Table 1.2: Epidemiology of plasmid-mediated ESBL, carbapenemase and quinolone resistance genes in *E. coli* and *Klebsiella spp.* in South Africa.

Province	Organism	Beta-lactamase genes	PMQR genes	Isolate collection period	Reference
Eastern Cape	*E. coli*	*bla*$_{CTX-M-9}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *bla*$_{OXA-1}$	*aac(6')-Ib-cr, qnrB, qnrS*	2012 - 2013	Gqunta & Govender 2015
		bla$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *bla*$_{TEM-1}$, *bla*$_{TEM-2}$	ND	NS	DeFrancesco et al., 2017
Gauteng	*K. pneumoniae*	*bla*$_{SHV-1}$, bla$_{VIM-1}$	–	NS	Peirano et al., 2010
		bla$_{CTX-M-15}$, *bla*$_{SHV-1}$, *bla*$_{SHV-2}$, *bla*$_{TEM-1}$, *bla*$_{OXA-48}$, *bla*$_{OXA-181}$	ND	2011	Brink et al., 2013
KZN	*E. coli*	ND	–	2012 - 2013	Sekyere et al., 2017
	K. michiganensis		*aac(6')-Ib-cr, oqxA, oqxB, qnrS1*		
	K. pneumoniae		*aac(6')-Ib-cr, oqxA, oqxB, qnrS, qnrB*		
		bla$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{OXA-1}$-like, *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$	ND	2017	Founou et al., 2018
Western Cape	*E. coli*	*bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *bla*$_{OXA-1}$	*aac(6')-Ib-cr*	2008 - 2009	Peirano et al., 2011
	K. pneumoniae	*bla*$_{CTX-M-15}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{OXA-181}$	ND	2012	Jacobson et al., 2015
	K. oxytoca	*bla*$_{CTX-M-15}$, *bla*$_{TEM}$, *bla*$_{OXA-181}$			
Various provinces	*Klebsiella* spp.	*bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, *bla*$_{SHV}$, *bla*$_{TEM}$	ND	2010 - 2012	Perovic et al (2014)
		bla$_{OXA-48}$ (& variants), *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$	ND	2012 – 2015	Singh-Moodley & Perovic, 2016
	E. coli	*bla*$_{OXA-48}$ (& variants), *bla*$_{NDM}$, *bla*$_{VIM}$			

– : Not detected

ND: Not determined

NS: Not specified

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1.5. Gastro-intestinal carriage of antimicrobial resistant *E. coli* in community children in LMICs

While very few South African studies have characterised resistance rates in clinical settings, even less have described this in the gut microbial flora of children in communities, which are important reservoirs for AMR genes. A recent study on *E. coli* isolated from the stools of children in rural areas in Limpopo, South Africa, reported a penicillin (ampicillin or amoxicillin) resistance rate of 47.1% (DeFrancesco *et al.*, 2017). This study also reported a considerably lower rate of resistance to amoxicillin-clavulanic acid (co-amoxiclav), a β-lactam+β-lactamase inhibitor combination, at 1.2%, as well as low rates of resistance to cefotaxime (2.3%) and nalidixic acid (5.7%), while none of the isolates were resistant to ciprofloxacin, gentamicin, amikacin or imipenem.

Looking at other LMICs, 72% of community children in India were reported to carry *E. coli* resistant to at least one antibiotic (Shakya *et al.*, 2013). The ampicillin resistance rate was a slightly lower than that of the South African study, at 37%, while the rate of resistance to co-amoxiclav (29%) was substantially higher. The Indian study also reported an MDR *E. coli* carriage rate of 33% in children in the community. The carriage of resistance has been reported to be even higher in Taiwan, where 74.5% of *E. coli* from community children were resistant to at least one antibiotic, 37% were MDR, 70.1% resistant to ampicillin, and 65.6% to co-amoxiclav (Huang *et al.*, 2018).

The Taiwanese study also reported 22.9% gentamicin resistance and 18.5% ciprofloxacin resistance. Moreover, in Vietnam, 96% of rural children (<5 years) carried *E. coli* resistant to at least one antibiotic, of which 60% of the were MDR (Dyar *et al.*, 2012). The ampicillin resistance rate in this study was 65%, while 27% of the *E. coli* were nalidixic acid resistant and <1% (only 2 out of 818) were ciprofloxacin resistant. Higher levels of quinolone resistance were reported in community children in Bolivia, where 76% and 44% of *E. coli* isolates were resistant to nalidixic acid and ciprofloxacin, respectively (Bartoloni *et al.*, 2013). However, only 12.4% of the *E. coli* isolates were resistant to one or more cephalosporins.

The limited available data on the baseline rates of AMR carriage in children in communities in South Africa is worrying, especially since these rates are very high in other LMICs, as seen from these few studies. In order to improve antibiotic treatment

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strategies and to develop new antimicrobials to manage this problem, it is important to know which mechanisms of resistance are circulating in communities.

1.6. Problem statement

The global problem of antimicrobial resistance is especially concerning in LMICs, where the burden of infectious diseases is extremely high (Okeke *et al.*, 2005). Community-acquired infections, especially respiratory and gastrointestinal infections, such as those caused by Enterobacterales, remain the leading cause of death in these countries (Okeke *et al.*, 2005). Moreover, the complex structure of the Gram-negative cell wall, especially the rather impervious outer membrane, makes it extremely difficult to find novel antibiotics that are able to permeate this barrier and not be transported out of the cell by efflux pumps (Silver, 2016). This has left the development of novel antimicrobials against MDR Gram-negative bacteria virtually at a standstill, with the focus over the past few decades being entirely on Gram-positive organisms.

It is therefore alarming that limited research has been published on the carriage of resistant clinical as well as commensal Enterobacterales isolates in communities in South Africa, as seen in the two previous sections. Moreover, studies have shown that younger children, who are generally more exposed to antibiotics than older population groups in the community (Bryce *et al.*, 2016), have the highest risk of carrying resistant commensal bacteria (Duerink *et al.*, 2007). Commensals in the gut are important reservoirs of antibiotic resistance genes (Marshall *et al.*, 2009) and are able to spread these genes to pathogenic microorganisms by HGT (Blake *et al.*, 2003).

Furthermore, the simultaneous transfer of various resistance genes has left us with greater concerns, such as the frequent association between fluoroquinolone resistance and the production of ESBLs in Enterobacteriaceae (Dalhoff, 2012; Fortini *et al.*, 2015). The co-selection of β-lactam and even aminoglycoside resistance by fluoroquinolones and vice versa, severely complicates the treatment of community- and healthcare acquired infections, such as urinary tract-and intra-abdominal infections and travellers' diarrhoea (Dalhoff, 2012).

There is a lack of information on the carriage of ESBL-producing and fluoroquinolone resistant Enterobacteriaceae in children in communities in South Africa and this gap needs to be filled in order to even begin addressing the serious health issues in this country.

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1.7. TB-Champ study

This study forms part of the Tuberculosis Child Multidrug-resistant Preventive Therapy Trial (TB-CHAMP), conducted by the Desmond Tutu TB Centre (DTTC), Stellenbosch University. This is an ongoing randomized double-blind placebo-controlled trial to evaluate the efficacy of levofloxacin prophylaxis in multidrug-resistant Tuberculosis (MDR-TB) exposed children in South Africa. Children under the age of 5 with a known household MDR-TB adult contact are randomized and subjected to 24 weeks of daily dosing of either levofloxacin or placebo in a 50:50 ratio. Stool samples are collected from children enrolled at the Cape Town study sites at Khayelitsha and Philippi before starting treatment (baseline), during treatment (8, 16 and 24 weeks) and after 6 months after completion of treatment (48 weeks).

Children under the age of five were included in the study if: 1) they were household contacts of recently diagnosed (within 6 months) adult MDR-TB cases, 2) their primary residence was in such a household and 3) written informed consent was obtained from their parent(s) or legal guardian(s) to be enrolled and 4) tested for human immunodeficiency virus (HIV). Consent was obtained from parents/ legal guardians of children after the aims, methods, benefits and potential hazards of the trial had been explained and before any trial-specific procedures were performed. Children were excluded from the study if they had: 1) already been diagnosed with TB disease 2) treated for TB disease, 3) receiving isoniazid (INH) or a fluoroquinolone for ≥14 days at enrolment, 4) exposed to an INH-susceptible index case or 5) diagnosed with myasthenia gravis or Guillain-Barré syndrome. Participants were randomised at the household level and all eligible children within the household (2 on average) were treated with the same intervention.

The TB-CHAMP study provides an opportunity to describe the rates and mechanisms of resistant bacterial carriage in healthy children in community settings in South Africa. However, it also poses further dangers, as the use of levofloxacin in these healthy children could be selecting for ESBL-producers or organisms resistant to various antibiotics, leaving these children vulnerable to other serious infections.

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1.8. Aims & Objectives

The aim of this study is to describe the carriage of antimicrobial resistance in children in communities in Cape Town. This will be done by:

- Describing the baseline community carriage of antimicrobial resistant *E. coli* and *Klebsiella* spp. in children.
- Describing the mechanisms of resistance present in antimicrobial resistant *E. coli* and *Klebsiella* spp. isolated from stool samples of children at baseline.
- Describing the changes in the carriage and mechanisms of AMR in children enrolled in the TB-CHAMP trial.
- Describing the carriage of plasmid mediated fluoroquinolone resistance genes in the gut bacterial population of children at baseline.

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Chapter 2: Gastro-intestinal carriage of antimicrobial resistant *E. coli* and *Klebsiella* spp.

2.1. Introduction

While most studies on the carriage of antimicrobial resistance have focused on clinical isolates causing disease, very few have looked at resistance in commensal bacteria circulating in communities, which are considered important reservoirs for antimicrobial resistance genes, which can be transferred to pathogens (Marshall *et al.*, 2009). As the largest population of commensals is found in the gut, it is important to study the fecal carriage of antimicrobial resistance in common commensals like *Escherichia coli* and *Klebsiella* species especially in children, who are at the highest risk of carrying resistant commensal bacteria (Duerink *et al.*, 2007).

Moreover, multidrug-resistance (MDR) is increasingly being detected in several Gram-negative bacteria, like *E. coli* and *Klebsiella* spp., due to the extensive use of antibiotics in hospitals and communities (R. C. Founou *et al.*, 2018). The significant problem of antimicrobial resistance (AMR) and MDR, in particular, has not been described sufficiently in Africa due to financial constraints (R. C. Founou *et al.*, 2018). More information on this serious issue in South Africa, especially in children in communities, is needed to raise awareness about the burden of AMR in a community setting. This can both motivate for better stewardship of antimicrobials as well as possibly inform treatment guidelines and clinical practice (R. C. Founou *et al.*, 2018).

Therefore, this chapter aims to provide more data on the prevalence of AMR *E. coli* and *Klebsiella* spp. in the gut flora of children in communities in Cape Town, South Africa, while subsequent chapters will go into more detail on the molecular mechanisms contributing to the phenotypic resistance.

2.2. Materials and methods

2.2.1. Sample collection and bacterial culture

Stool culturing and processing were carried out in the NHLS microbiology laboratory at Tygerberg Hospital, and unless stated otherwise, all media used were supplied by the National Health Laboratory Services (NHLS) Media Laboratory, Greenpoint, South Africa. Stool samples were collected from children enrolled in TB-CHAMP at baseline

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and at 8-, 16-, 24- and 48-week follow up visits between November 2017 and November 2019. For the purpose of this study, only the 16- and 24-week follow up samples were included for the first 100 participants for which baseline samples were received. The stool samples were either immediately cultured onto MacConkey agar plates with the addition of cefpodoxime (10 μg) and ertapenem (10 μg) disks (Mast, Laboratories, UK) at 37°C overnight or, where not possible, stored on a sterile swab in Cary Blair Medium (Diagnostic Media Products, NHLS, South Africa) at 4°C and cultured within 7 days.

Based on typical colony morphology (including features such as colour, size, mucoid nature), suspected *E. coli* and *Klebsiella* spp. isolates from within and outside of the zones of inhibition (ZOI) around either of the disks were selected as cephalosporin and carbapenem resistant and potentially susceptible isolates, respectively. Nalidixic acid (30 μg) and ciprofloxacin (5 μg) disks were included from the 65th baseline sample received (and subsequent follow up samples) to potentially improve the detection of *E. coli* and *Klebsiella* spp. that are resistant to quinolones. Isolates were then sub-cultured onto tryptose blood agar (TBA) plates in order to obtain pure colonies for identification and susceptibility testing.

2.2.2. Identification and antibiotic susceptibility testing of bacterial isolates

Colonies of bacterial isolates from the TBA plates were inoculated in purified water and turbidities were determined using a DensiCHEK (bioMérieux, France) and adjusted to a McFarland standard of 0.45 - 0.55 (equivalent to 1 - 2 x 10^8 CFU/mL). For species identification, the suspensions were inoculated onto Analytical Profile Index (API) 10S or 20E strips (bioMérieux, France) and incubated for 18-24h at 37°C before interpretation according to the manufacturer's instructions and using the apiweb™ software.

The bacterial suspensions were also inoculated onto Mueller Hinton (MH)-Sens agar plates with sterile swabs in order to perform standard Kirby Bauer disk diffusion (Bauer *et al.*, 1966) to determine susceptibility to amoxicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), cefuroxime (30μg), cefotaxime (30 μg), ceftazidime (30 μg), cefepime (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), amikacin (30 μg), ertapenem (10 μg) and imipenem (10 μg). After incubation at 37°C for ~18h, the ZOIs were measured and the isolates were classified as either susceptible,

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intermediate or resistant (the latter two will further be referred to together as “non-susceptible”) to each antibiotic according to the Clinical and Laboratory Standards Institute (CLSI) 2019 guidelines (CLSI, 2019). The isolates were classified as multidrug-resistant (MDR) if they were non-susceptible to at least one agent in three or more antimicrobial categories, with the exception of the intrinsic amoxicillin non-susceptibility of *Klebsiella* spp. (Magiorakos *et al.*, 2012). This intrinsic non-susceptibility of *Klebsiella* spp. was also excluded when classifying the isolates as non-susceptible to at least one antibiotic.

Any isolates that were not identified as *E. coli* or *Klebsiella* spp., as well as duplicate isolates from the same sample (same species with the same susceptibility pattern) were discarded. Loopfuls of the remaining isolates were picked from the TBA plates and stored on Microbank™ beads (Pro Lab Diagnostics, Canada) at -80°C for future analysis. The bacterial culture, identification and susceptibility testing of isolates from the first 50 baseline samples were performed by fellow MSc student, Mr. Remous Ocloo, and his findings were combined with our findings on the subsequent 50 baseline samples.

2.2.3. Statistical analysis

Chi-squared tests were performed using the “CHITEST” function on Microsoft® Excel to determine the significance of differences between levels of non-susceptibility to antibiotics at various time points, as well as the difference between rates of non-susceptibility to amoxicillin and amoxicillin/clavulanic acid, to determine whether there was a significant reduction in non-susceptibility to amoxicillin with the addition of a β-lactamase inhibitor (BLI). Fisher’s exact tests were performed in cases where sample sizes were smaller than 20 and observed values less than five during the chi-squared tests. Differences with a *p*-value of ≤ 0.05 were considered statistically significant.

2.3. Results

2.3.1. Baseline carriage of non-susceptible *E. coli* and *Klebsiella* spp.

A total of 157 isolates were cultured from the 100 baseline stool samples, consisting of 116 *E. coli* and 41 *Klebsiella* spp., of which 52 and 17, respectively, were isolated from the first 50 baseline samples and described by Mr. Ocloo (R. Ocloo, MSc, 2019). The *Klebsiella* spp. isolates consisted of 34/41 (82.9%) *Klebsiella pneumoniae* and 7/41 (17.1%) *Klebsiella oxytoca*. Of the 100 participants, 81% carried an organism

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which was non-susceptible to at least one antibiotic, and 27% carried multidrug-resistant (MDR) organisms. The carriage of MDR *E. coli* and MDR *Klebsiella* spp., respectively, was 19% and 10%. Seventy percent of participants carried an organism non-susceptible to the penicillins, followed by quinolones (49%), cephalosporins (33%), and aminoglycosides (18%), while a carbapenem resistant organism was only carried by one participant. This trend can be seen in figure 2.1 which indicates the proportions of *E. coli* and *Klebsiella* spp. that were non-susceptible to the 12 antibiotics tested.

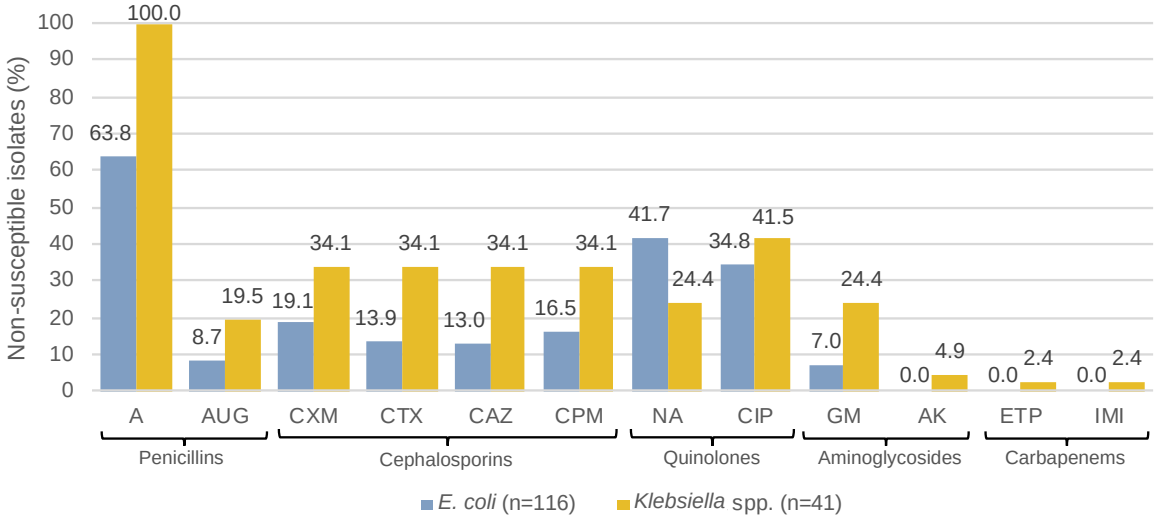

Figure 2.1: Baseline rates of non-susceptibility of *E. coli* and *Klebsiella spp*. to 12 antibiotics based on disk diffusion. Key: A = amoxicillin, AUG = amoxicillin + clavulanic acid, CXM = cefuroxime, CTX = cefotaxime, CAZ = ceftazidime, CPM = cefepime, NA = nalidixic acid, CIP = ciprofloxacin, GM = gentamicin, AK = amikacin, ETP = ertapenem, IMI = imipenem.

Non-susceptibility to at least one antibiotic was seen in 70.7% (n=82/116) of the *E. coli* and 46.3% (n=19/41) of the *Klebsiella* spp. isolates. This was driven primarily by resistance to amoxicillin; we did not include *Klebsiella* species resistant to amoxicillin in this comparison since they are intrinsically resistant. However, the *Klebsiella* spp. isolates displayed higher rates of non-susceptibility than *E. coli* against all antibiotics, except for nalidixic acid (figure 2.1), and multidrug resistance was more common in the *Klebsiella* spp. at 24.4% (n=10/41), compared to 18.1% (n=21/116) in *E. coli.* The high rates of non-susceptibility to amoxicillin were significantly reduced with the addition of a BLI, as seen with the lower rates of co-amoxiclav non-susceptibility compared to that of amoxicillin alone in figure 2.1 ($p<0.001$ for both *E. coli* and *Klebsiella* spp.).

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The isolates also displayed high rates of non-susceptibility to the quinolones, with nalidixic acid non-susceptibility being more common in *E. coli* and ciprofloxacin non-susceptibility more common in *Klebsiella* spp. (figure 2.1). The proportion of participants from which quinolone non-susceptible isolates were cultured was 51.6% before and 52.8% after the change in culturing method to include the nalidixic acid and ciprofloxacin disks (p=0.907). The rates of quinolone resistance per isolate was slightly higher before (46.7%) than after (41.8%), however not significantly (p=0.543). Therefore, inclusion of the quinolone disks did not affect the yield of quinolone resistant organisms.

A third of the *Klebsiella* spp. isolates were non-susceptible to all four of the cephalosporins tested (34.1%), while 20.7% (n=24) *E. coli* were non-susceptible to at least one of the cephalosporins. Reduced susceptibility to aminoglycosides was driven by non-susceptibility to gentamicin, as amikacin non-susceptibility was only detected in two *Klebsiella* spp. isolates (4.9%) and not in *E. coli.* Only one carbapenem-non-susceptible *Klebsiella pneumoniae* was found (R. Ocloo, MSc, 2019).

2.3.2. Carriage of non-susceptible *E. coli* and *Klebsiella* spp. after 16- and 24-weeks

At the 16- and 24-week follow up visits, stool samples were collected from 53 and 48 of the 100 participants, respectively. At 16 weeks, 71 *E. coli* and 16 *Klebsiella* spp. were isolated from the stool samples, where the latter consisted of 12 (75%) *K. pneumoniae* and 4 (25%) *K. oxytoca*. At 24 weeks, 71 *E. coli* and 12 *Klebsiella* spp., consisting of 8 (66.7%) *K. pneumoniae* and 4 (33.3%) *K. oxytoca*, were isolated.

Forty-nine (92%) of 53 participants carried an organism non-susceptible to at least one antibiotic at 16 weeks, while 21 (38.9%) carried an MDR organism. Non-susceptible and MDR isolates were carried by 46 (95.8%) and 16 (34.8%) of the 48 participants respectively at 24 weeks. The carriage of organisms non-susceptible to at least one antibiotic therefore increased from baseline (81%) to 16 weeks (p=0.059) and significantly to 24 weeks (p=0.015). The carriage of MDR organisms also increased from baseline (27%) to 16 weeks, although not significantly (p=0.109), before decreasing slightly. This was driven by a significant increase in the carriage of MDR *E. coli* from 19% of participants at baseline to 35.8% at 16 weeks (p=0.022), before decreasing again to 25% at 24-weeks, although not significantly (p=0.401

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between baseline and 24 weeks). The carriage of MDR *Klebsiella* spp., on the other hand, decreased from 10% at baseline to 7.4% at 16 weeks (p=0.617) and 4.3% at 24 weeks (p=0.224 between baseline and 24 weeks), however the samples sizes for *Klebsiella* spp. at 16 and 24 weeks were very small.

As seen in table 2.1, the rates of carriage of penicillin non-susceptible organisms increased significantly from baseline to 24 weeks, as did the carriage of quinolone non-susceptible organisms between baseline and 16 weeks, which returned to the baseline level after 24 weeks. No significant changes in the rates of non-susceptibility to cephalosporins and aminoglycosides were observed over the three time points.

Table 2.1: Proportion of participants carrying organisms non-susceptible to various antibiotic classes

	Baseline (%)	**16 weeks (%, p-value)**	**24 weeks (%, p-value)**
Penicillins	69.0	81.1 (0.107)	87.5 (0.015*)
Cephalosporins	33.0	35.8 (0.723)	31.3 (0.831)
Quinolones	49.0	69.8 (0.014*)	50.0 (0.909)
Aminoglycosides	18.0	20.8 (0.679)	25.0 (0.321)

** Significantly different from baseline.*

An additional analysis was performed on the 53 baseline samples for which 16-week samples were received, as well as the 48 baseline samples for which 24-week samples were received to determine whether the rates of resistance were influenced by the loss to follow up and, for most of the antibiotics, it remained the same as for the 100 baselines in table 2.1. Only the penicillin resistance rates were much lower after this analysis, at 56.6% for baseline samples with 16-week samples and 50% for baseline samples with 24-week samples. This resulted in smaller p-values for the difference between these new baseline values and those at 16- (p=0.006) and 24-weeks (p<0.001) respectively, making the increase after 16 weeks significant as well.

The rates of non-susceptibility of the *E. coli* and *Klebsiella* spp. isolates to each of the 12 antibiotics over the three time points are shown in figure 2.2. In *E. coli* there was a significant increase in non-susceptibility to both co-amoxiclav (p=0.028) and cefotaxime (p=0.009) at 16 weeks. Co-amoxiclav non-susceptibility remained higher than at baseline (p=0.050), while cefotaxime non-susceptibility decreased to the

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baseline level at 24 weeks. The overall cephalosporin resistance rate in *E. coli* at 16 weeks (28.2%) was also slightly higher than baseline (20.7%) and returned to baseline level at 24 weeks (19.7%). The rate of amoxicillin resistance remained at 100% across all three time points and it is interesting to note that co-amoxiclav non-susceptibility decreased (although not significantly) over 24 weeks in *Klebsiella* spp. (p=0.259). As with *E. coli,* the rate of cephalosporin non-susceptible *Klebsiella* spp. remained relatively constant over time, increasing only slightly from 34.1% at baseline to 37.5% at 16 weeks and returning to baseline level at 24 weeks (33.3%).

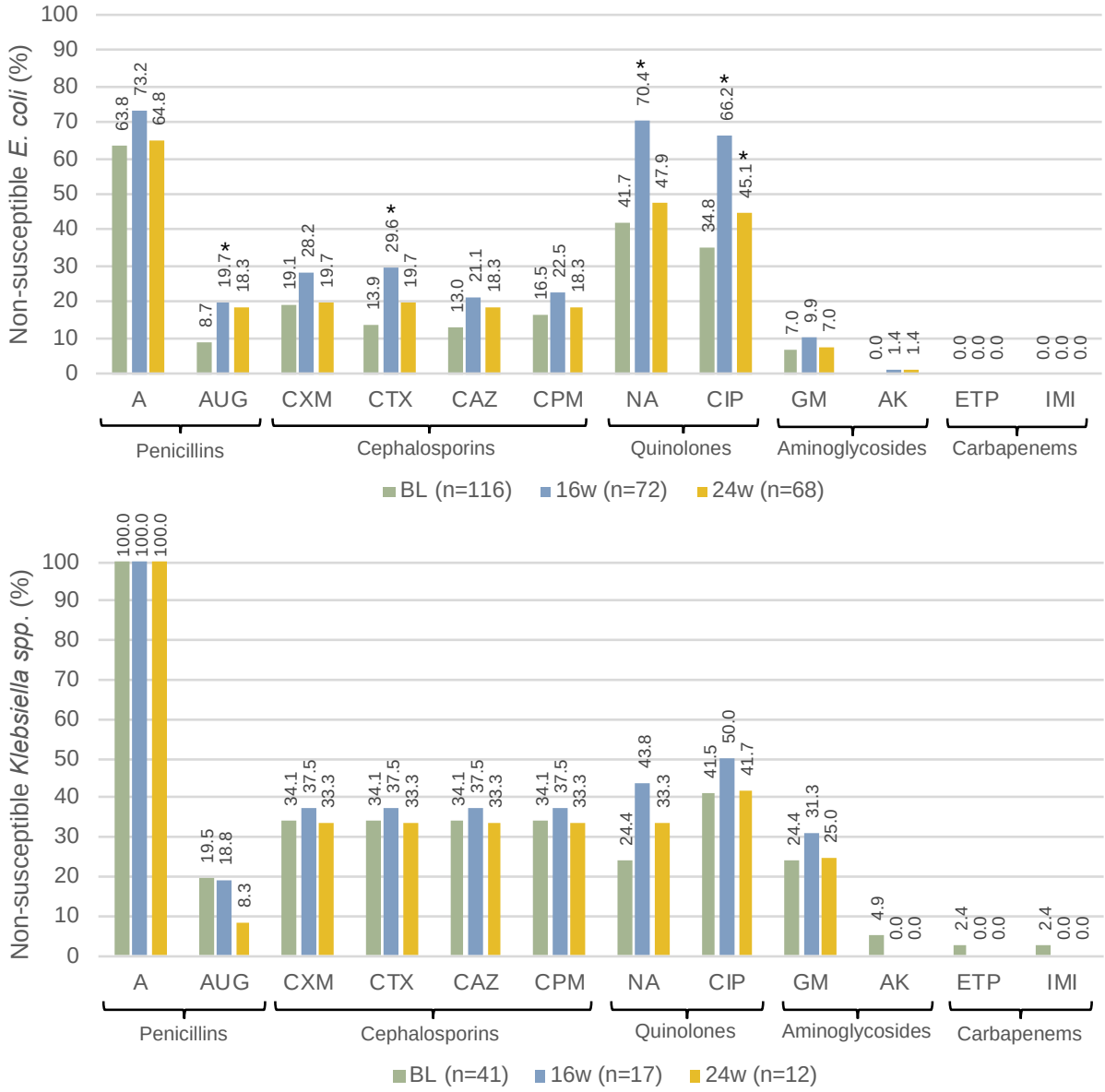

Figure 2.2: Rates of non-susceptibility of *E. coli* (a) and *Klebsiella* spp. (b) to 12 different antibiotics at baseline, 16 weeks and 24 weeks. (Refer to key in fig. 2.1 for antibiotic abbreviations). **Significantly different from baseline.*

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The rates of quinolone non-susceptibility increased after 16 weeks in *E. coli* ($p<0.001$ for both nalidixic acid and ciprofloxacin), but not significantly in *Klebsiella* spp. ($p=0.110$ for nalidixic acid and $p=0.211$ for ciprofloxacin) before decreasing again at 24 weeks. The rates of non-susceptibility to nalidixic acid at 24 weeks were still slightly higher than the baseline rates in *E. coli* ($p=0.384$) and *Klebsiella* spp. ($p=0.231$), however not significantly. The rate of ciprofloxacin non-susceptibility in *E. coli* at 24 weeks was significantly higher than that at baseline ($p=0.011$), however the rate of ciprofloxacin non-susceptibility in *Klebsiella* spp. at 24 weeks was the same as at baseline.

There was no significant increase in gentamicin non-susceptibility in *E. coli* ($p=0.469$) or *Klebsiella* spp. ($p=0.233$) at 16 weeks. Amikacin non-susceptibility remained very rare in both organisms over time and two *E. coli* isolates (one at 16 weeks and one at 24 weeks) were non-susceptible to amikacin without being non-susceptible to gentamicin. No carbapenem non-susceptible isolates were detected in the 16- and 24-week samples. As with the baseline samples, *Klebsiella* spp. displayed higher rates of non-susceptibility than *E. coli* to most antibiotics in the follow-up samples.

2.4. Discussion

In this chapter, we reported on the proportions of healthy children in the community carrying antimicrobial resistant *E. coli* and *Klebsiella* spp. before antibiotic treatment, as well as over 24 weeks of treatment with levofloxacin or placebo. As the rates of carriage of resistant organisms per individual have not commonly been reported, we have also reported the resistance rates in the *E. coli* and *Klebsiella* spp. isolates, in order to better compare our results with other studies. We have, however, not yet received the extensive participant information which was collected during the clinical trial, nor have we been unblinded to which participants are receiving levofloxacin or placebo. We can therefore not yet comment on the effect of levofloxacin treatment on the carriage of resistant organisms or correlate our results with previous antibiotic use, hospitalisation, etc. The 16- and 24-week sample sizes were also much smaller than that of baseline, as there were high rates of loss to follow up.

We reported a high prevalence of participants carrying an organism that was non-susceptible to at least one antibiotic at baseline (81%), which increased significantly after 24 weeks (92.5%). These values are higher than that of the Indian study on

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community children mentioned in chapter 1 (72%) (Huang *et al.*, 2018). The rates of participants carrying MDR organisms at baseline (27%) also compare to the 33% reported in India (Shakya *et al.*, 2013). Only one South African study on the GIT carriage of antibiotic resistant *E. coli* in the stools of children in communities was found to which we can compare our results (DeFrancesco *et al.*, 2017). However they do not report on the proportion of children carrying resistant organisms, but on the proportion of resistant isolates, with more than one *E. coli* isolated per child. The proportion of *E. coli* isolates in our study at baseline which were non-susceptible to at least one antibiotic (70.7%) is similar to what was reported in the South African study (66.6%), however, the proportion of MDR *E. coli* (18.1%) is lower than the 27% reported by DeFrancesco *et al*. (2017).

The significant increase in carriage of MDR *E. coli* in our participants after 16 weeks is worrying for healthy children in the community and presents a serious public health concern, as the chances of acquiring infections with resistant organisms, either in the community or hospital, increases with the increasing number of individuals carrying these resistant organisms as part of their normal flora (Huang *et al.*, 2018). The transmissibility of plasmid-mediated resistance determinants is a key contributing factor in the emergence of multidrug resistant pathogens (Moyo *et al.*, 2010). Therefore, the increase in carriage of MDR organisms, or MDR *E. coli* specifically, could reflect an increased presence of these plasmid-mediated resistance genes circulating in the community, as genes conferring resistance to various antibiotics can be carried on the same plasmid. The decrease in the carriage of MDR organisms at 24 weeks, as well as the lack of significance thereof, could be a reflection of the smaller sample size at 24 weeks.

The high rate of carriage of penicillin non-susceptibility in our participants (69%, 81.1% and 87.5% at baseline, 16 and 24 weeks, respectively) is not surprising, as these antibiotics have been on the market the longest and resistance has been documented since 1942 (Rammelkamp and Maxon, 1942). Various strains of Enterobacteriaceae are now intrinsically resistant to aminopenicillins such as amoxicillin (Bouza and Cercenado, 2002). For example, 100% of the *Klebsiella* spp. isolates were non-susceptible to amoxicillin at all three time points in our study (fig. 2.2), possibly due to a chromosomally encoded SHV β-lactamase which makes these organisms

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intrinsically resistant to aminopenicillins (European Centre for Disease Prevention and Control, 2017).

The baseline rate of non-susceptibility to amoxicillin (and penicillins overall) in our *E. coli* isolates (63.8%, fig. 2.1) is high compared to the 47.1% penicillin resistance reported in the South African study (DeFrancesco *et al.*, 2017). The high rates of amoxicillin non-susceptibility could be due to the overuse and misuse of this antibiotic in our setting. A recent study on antibiotic prescribing practices in community health care centres in the Cape Town Metro district reported that amoxicillin was the most commonly prescribed antibiotic and that it was one of the antibiotics prescribed for the largest proportion of unspecified diagnoses, after ciprofloxacin (Gasson *et al.*, 2018). The previous South African study, and various others, reported the presence of TEM β-lactamases in 95.1% of ampicillin-resistant *E. coli* (DeFrancesco *et al.*, 2017). As with the increase in MDR *E. coli* after 16 weeks, the increase in amoxicillin non-susceptibility could be a reflection of an increase in the prevalence of these plasmid-mediated genes in the community.

The observed levels of co-amoxiclav non-susceptibility in *E. coli* (8.7%, 19.7% and 18.3% at baseline, 16 and 24 weeks, respectively) were much lower than what was reported in the studies from Taiwan (65.6%) (Huang *et al.*, 2018) and India (29%) (Shakya *et al.*, 2013). The South African study also reported a considerably lower rates of co-amoxiclav resistance, compared to that of ampicillin or amoxicillin alone (DeFrancesco *et al.*, 2017). This suggests that co-amoxiclav could still be an effective treatment option in our setting, and our participants in particular, should these children become infected with an amoxicillin resistant organism.

The higher level of co-amoxiclav non-susceptibility in *Klebsiella* spp. compared to *E. coli* at baseline (fig. 2.1), as well as the increase in co-amoxiclav non-susceptibility in *E. coli* after 16 weeks (fig. 2.2a), could be due to the presence or hyperproduction of inhibitor-resistant β-lactamases such as OXA-1, which is poorly inhibited by clavulanic acid (Pérez-Moreno *et al.*, 2011), or inhibitor-resistant TEM-1 in *E. coli* and SHV-1 in *Klebsiella* spp. (Lemozy *et al.*, 1995). These genes could have proliferated in the community due to widespread use of co-amoxiclav in the community, following high levels of amoxicillin resistance.

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We reported a disturbingly high proportion of children (49%) carrying quinolone non-susceptible organisms at baseline prior to treatment in the TB-CHAMP study. Although no other South African studies reporting on proportions of children carrying quinolone resistant organisms could be found, the rate is higher than what was reported for community children in Vietnam (27%) (Dyar *et al.*, 2012). The baseline rate of nalidixic acid non-susceptible *E. coli,* at 41.7% (fig. 2.1), is much higher than the 10.3% reported in the South African study (DeFrancesco *et al.*, 2017).

It is alarming that our quinolone non-susceptibility carriage rates overall are higher than or comparable to rates of resistance in clinical isolates from hospitalised children in South Africa, as well as Taiwan, China, Kenya and various other African countries (Mshana *et al.*, 2013; Taitt *et al.*, 2017; Xue *et al.*, 2017; Huang *et al.*, 2018). Again, the high levels of non-susceptibility to quinolones could be a reflection of prescribing practice in Cape Town, as Gasson *et al.* reported ciprofloxacin as the antibiotic with the smallest proportion of prescriptions adhering to guidelines (17.4%) and the largest proportion of prescriptions for unspecified diagnoses (17%) (Gasson *et al.*, 2018).

The high rate of quinolone non-susceptibility at baseline is especially worrying as approximately half of these children would have started treatment with levofloxacin, based on the TB-CHAMP trial protocol, after the baseline stool samples were taken. It is very tempting to speculate that the increase in quinolone non-susceptibility after 16 weeks is driven by the use of levofloxacin in these participants. It will be interesting to evaluate the rates of resistance in the cases vs. controls once the TB-CHAMP study has been completed and the blind has been broken.

Even without the unblinding, the significant increase in quinolone resistant *E. coli* after 16 weeks, as well as 24 weeks for ciprofloxacin specifically, is interesting and worrying. The neonatal study from India also reported an increase in ciprofloxacin non-susceptibility in Enterobacterales from 15% to 38% after the first 60 days of life, without antibiotic use or selective culture (Saksena *et al.*, 2018). Our rates of ciprofloxacin non-susceptible *E. coli* (66.2%) and *Klebsiella* spp. (50.0%) at 16 weeks are much higher than this, however as 60 days is closer to 8 weeks, these rates may not be directly comparable. Our children are also older and may have had more exposure to resistant organisms and to antibiotics. The increase in carriage of resistant organisms after 16 weeks might be associated with a general increase in resistant organisms over time and not necessarily with antimicrobial exposure. Similarly to the Indian study,

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a study from America reported the isolation of ciprofloxacin non-susceptible *E. coli* from the stools of 19% of children at least once in the first two years of life, even in the absence of antibiotic pressure (Gurnee *et al.*, 2015).

Studies from India and Bolivia also reported higher levels of resistance to nalidixic acid compared to ciprofloxacin in *E. coli* (Bartoloni *et al.*, 2013; Shakya *et al.*, 2013), as in our study (fig. 2.2a). This could be due to the widespread use of this older antibiotic, which came onto the market two decades before ciprofloxacin, as well as the ubiquitous nature of *E. coli*. The higher rates of non-susceptibility to ciprofloxacin compared to nalidixic acid in the *Klebsiella* spp. isolates in our study (fig. 2.1 & 2.2b) could be due to the presence of the plasmid-mediated *aac(6')-Ib-cr* gene, which confers non-susceptibility to ciprofloxacin but not nalidixic acid, which *Klebsiella* spp. with its plastic genome can easily acquire. This will be discussed further in the next chapter which investigates the molecular mechanisms contributing to resistance.

The carriage of cephalosporin non-susceptible organisms in our study at all three time points is much higher than the 12.4% reported in Bolivia (Bartoloni *et al.*, 2013) and the 13.3% in India (Shakya *et al.*, 2013). Looking at non-susceptibility to cephalosporins amongst the isolates (as opposed to carriage in participants), the rates in our *E. coli* isolates (ranging between 14-24% across the three time points) are much higher than the 2.3% of cefotaxime non-susceptible *E. coli* in the South African study (DeFrancesco *et al.*, 2017). About a third of the *Klebsiella* spp. isolates in our study were non-susceptible to all of the cephalosporins over all three time points, which is also much higher than the 10% of commensal *K. pneumoniae* which were resistant to at least one third generation cephalosporin, carried by outpatients presenting with acute diarrhoea at medical facilities in Kenya (Taitt *et al.*, 2017).

Non-susceptibility to aminoglycosides was found in an average of 21% of the participants in our study over the three time points, which is similar to that of hospitalised children in Taiwan (22.9%) (Huang *et al.*, 2018). The rates of gentamicin non-susceptible *E. coli* isolated from the stools of our participants, at an average of 8% over the three time points, is comparable to the 4% in India where only one *E. coli* was isolated per child (Shakya *et al.*, 2013), while no aminoglycoside non-susceptible *E. coli* were found in the South African study by DeFrancesco *et al*. (2017). The carriage of 24.4% and 4.9% gentamicin and amikacin resistant *Klebsiella* spp. respectively, is much higher than the 8% and 2% reported for the

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K. pneumoniae isolates in the Kenyan study (Taitt *et al.*, 2017). Therefore, as with the quinolones, our rates of non-susceptibility to aminoglycosides in community isolates are similar to or higher than what has been reported for clinical isolates elsewhere.

The Kenyan study, as well as two studies from Iran, also reported higher levels of gentamicin resistance, compared to amikacin (Raei *et al.*, 2014; Hasani *et al.*, 2017), as in our study. Gentamicin is an older antibiotic which came into commercial use twelve years before amikacin in 1976 (Alapi and Fischer, 2006), so bacteria have had more time to develop resistance to this antibiotic. Once we have received the participant data collected during the TB-CHAMP clinical trial, we can determine whether the carriage of aminoglycoside resistant organisms in the children correlates with previous amikacin or kanamycin treatment for MDR-TB in their parents, which could then indicate transfer of aminoglycoside resistant organisms from the parents to the children.

Although only one carbapenem resistant isolate (*K. pneumoniae*) was found, it is worrying that it was isolated from the gut of a healthy child in the community prior to levofloxacin treatment. However, until we have the clinical data on previous antibiotic use or hospitalisation, we can only speculate on how the child could have acquired this organism.

The plasticity of the *K. pneumoniae* genome related to the acquisition of plasmid-mediated resistance determinants, as well as the association of *K. pneumoniae* with the earliest reports of numerous resistance genes before their distribution amongst other clinically important Gram negative bacteria (Holt *et al.*, 2015) could explain the generally higher rates of non-susceptibility in *Klebsiella* spp. compared to *E. coli* (figures 2.1 and 2.2). This finding is also in line with another study conducted across various African countries, where *K. pneumoniae* showed higher levels of resistance to most antibiotics tested, despite being isolated less frequently than *E. coli* (Mshana *et al.*, 2013). It is also to be expected that *E. coli* was isolated almost three times more than *Klebsiella* spp. in our participants, as it is well known that *E. coli* is the predominant aerobic species in the gut (Tenaillon *et al.*, 2010; Delmas *et al.*, 2015).

Although there were no significant increases in non-susceptibility of the *Klebsiella* spp. to any of the antibiotics over time (fig. 2.2b), this should not detract from the concerns regarding the high resistance rates in this organism, as the numbers of *Klebsiella* spp.

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isolated in our study were very small, compared to *E. coli*. The decrease in MDR and co-amoxiclav non-susceptible *Klebsiella* spp. over the 24 weeks could also reflect the considerably lower numbers of *Klebsiella* spp. isolated from follow up stools compared to that at baseline.

In summary, we reported high rates of non-susceptibility to various antibiotics in commensal gut bacteria in children in Cape Town communities before and over 24 weeks of levofloxacin treatment; which is extremely worrying. These rates are higher than those reported by other community studies from South Africa and other LMICs, and even clinical studies. This could possibly be attributed to the misuse of antibiotics in our setting. The study by Gasson *et al*., reported low adherence to antibiotic prescribing guidelines in community health centres in the Cape Town Metro district (only 32.1% of cases where antibiotics were prescribed), especially in paediatric patients, mostly because there was no diagnosis specified in the medical records (Gasson *et al.*, 2018). The authors also found that dosing error was significantly more likely in paediatric patients than adult patients (Gasson *et al.*, 2018). The high level of unnecessary antibiotic prescriptions in Cape Town could have contributed to increased amounts of resistant organisms circulating in the community and, therefore, possibly explain the high rates of resistance carriage in our participants. Without data on prior antibiotic use or the unblinding of treatment groups, however, it is difficult to rationalise the baseline and follow up findings, respectively.

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Chapter 3: Molecular characterisation of antimicrobial resistant *E. coli* and *Klebsiella* spp.

3.1. Introduction

The gut represents a potential reservoir for antimicrobial resistance (AMR), as well as a site where resistance genes can be transferred from commensal microorganisms to pathogens (Marshall *et al.*, 2009). Common gut commensals, *Escherichia coli* and *Klebsiella pneumoniae,* gather and transfer countless AMR determinants and, therefore, can act as key transmitters of resistance genes within the human gut (Blake *et al.*, 2003; Salyers *et al.*, 2004; Wyres and Holt, 2018) and could increase the risk of resistant infections in hospital settings (Taitt *et al.*, 2017). *E. coli* and *K. pneumoniae* are regarded as the predominant species of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (Yang *et al.*, 2010) and are the main ESBL-producers isolated worldwide (Pitout and Laupland, 2008). Fecal *E. coli* is also considered a useful indicator of the spread of acquired AMR genes in the community (Nys *et al.*, 2004) and, as the main transmitters of infections within communities, children could be important vessels for the spread of antimicrobial-resistant microbes and infections (Bryce *et al.*, 2018).

Plasmid-mediated ESBL transmission gives rise to increasing resistance to non-β-lactams, as these plasmids can carry genes conferring resistance to various antimicrobial agents (Barguigua *et al.*, 2015; Arhoune *et al.*, 2017), such as carbapenemase genes, plasmid-mediated quinolone resistance (PMQR) genes, as well as aminoglycoside resistance determinants. These plasmids can easily spread to other species by horizontal gene transfer, which exacerbates the challenge of AMR (Andam *et al.*, 2011). The presence of mobile elements conferring resistance to multiple antibiotics in various members of the Enterobacteriaceae family, especially *E. coli* and *K. pneumoniae*, is of great clinical and microbiological importance (Falagas and Karageorgopoulos, 2009) and a huge global health concern (Pilmis *et al.*, 2018). It is therefore important to study not only the rates of bacterial resistance but, maybe more importantly, the prevalence of the underlying mechanisms causing this resistance.

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3.2. Materials and methods

3.2.1. DNA extraction

Crude DNA was extracted from pure cultures of all isolates from trypose blood agar (TBA) plates, either concurrent with storage on microbank beads, as described in chapter 2, or after storage and re-culture onto TBA. A loopful of colonies was suspended in 400 µl nuclease-free water in a microcentrifuge tube, vortexed to homogenize the solution and incubated in a heating block for 30 minutes at 95°C. This was followed by freezing for 30 minutes at -80°C, after which the suspensions were centrifuged for 10 minutes at 14000 x g. The supernatant containing the DNA was transferred to a clean microcentrifuge tube and stored at -20°C for use in further molecular analyses.

3.2.2. Molecular detection of cephalosporin resistance mechanisms

Based on the phenotypic susceptibility results (chapter 2), cephalosporin non-susceptible isolates were screened for the β-lactamase genes *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$, with a multiplex polymerase chain reaction (PCR) using 12.5 µL of KAPA 2G Mulitplex mix (KAPA Biosystems, South Africa) and 0.2 µM of each primer (table 3.1), except for SHV (0.24 µM), in a final reaction volume of 25 µL. Primers for *rpoB* (table 3.1) were included as an internal amplification control and an isolate harbouring the *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$, genes obtained from a previous study in our department (L. Paterson, MSc 2018) was used as a positive control. The PCR conditions consisted of an initial cycle at 95°C for 3 min, followed by 35 cycles at 95°C for 15s, 67°C for 30s and 72°C for 1 min, and a final extension cycle of 72°C for 3 min.

The amplification products were separated on 2% Seakem LE agarose (Lonza, South Africa) gels, with the addition of 1 µl of Novel Juice (GeneDireX, United States) to 5 µl of each product, in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) for 50 minutes at 120 volts. A 100bp Ladder (New England Biolabs Inc.) was included in each gel to determine the band size of each product. The gels were visualised under UV light with the UVItec Cambridge Alliance 2.7 gel documentation system (UVItec, UK).

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Table 3.1: Primer sequences for detection of cephalosporin and quinolone resistance mechanisms.

Target gene	Primers	Sequences	Product size (bp)	Reference
bla$_{CTX-M}$	CTX-M-F	ATGTGCAGYACCAGTAARGTK ATGGC	593	Monstein *et al*., 2007
	CTX-M-R	TGGGTRAARTARGTSACCAGA AYCAAYCAGCGG		
bla$_{SHV}$	SHV-F	ATGCGTTATATTCGCCTGTG	747	
	SHV-R	TGCTTTGTTATTCGGGCCAA		
bla$_{TEM}$	TEM-F	TCGCCGCATACACTATTCTCA GAATGA	445	
	TEM-R	ACGCTCACCGGCTCCAGATTT AT		
rpoB	rpoB-F	AACCAGTTCCGCGTTGGCCTG G	1088	Hoffmann *et al*., 2003
	rpoB-R	CCTGAACAACACGCTCGGA		
gyrA	gyrA-F	ACGTACTAGGCAATGACTGG	191	Liu *et al*., 2012
	gyrA-R	AGAAGTCGCCGTCGATAGAA		
parC (E. coli)	parC-F	TGTATGCGATGTCTGAACTG	264	
	parC-R	CTCAATAGCAGCTCGGAATA		
parC (K. pneumoniae)	KPN parC-F	TCCAGCGTCGCATCGTCTAT	420	R. Ocloo, MSc 2019
	KPN parC-R	GAATATCGGTCGCCATGCC		
qnrB	qnrBmF	GGMATHGAAATTCGCCACTG	263	Marti *et al*., 2013
	qnrBmR	TTYGCBGYYCGCCAGTCGAA		
qnrS	qnrSrtF11	GACGTGCTAACTTGCGTGAT	118	
	qnrSrtR11	TGGCATTGTTGGAAACTTG		
aac(6')-Ib-cr	aac(6')-Ib-cr-F	TTGGAAGCGGGGACGGAM	265	(Arhin *et al*., 2010)
	aac(6')-Ib-cr-R	ACACGGCTGGACCATA		

3.2.3. Molecular detection of quinolone resistance mechanisms

For quinolone non-susceptible isolates, based on the phenotypic susceptibility results (chapter 2), the quinolone resistance determining regions (QRDR) of the *gyrA* and *parC* genes were amplified using singleplex PCR reactions with 12.5 μL of KAPA Taq ReadyMix and 2 μM of each primer (table 3.1) in a final reaction volume of 25 μL. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as positive controls. PCR conditions were the same as for the ESBL PCR (3.2.2), using an annealing temperature of 60°C. PCR products were separated by agarose gel electrophoresis and visualised as described for the ESBL PCR. Amplified products were sent for Sanger sequencing at Inqaba Biotech™ (South Africa) and a wild-type Enterobacteriaciae sequence (accession number: NC_000913) was aligned to the

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obtained *gyrA* and *parC* sequences using BioEdit (Hall, 1999) or Unipro Ugene (Okonechnikov *et al.*, 2012) software to detect resistance conferring mutations in these genes.

The quinolone non-susceptible isolates were also screened for the *aac(6')-Ib-cr* gene, conferring resistance to amikacin as well as ciprofloxacin, using singleplex PCR with 12.5 μL of KAPA Taq ReadyMix and 2 μM of each primer (table 3.1) in a final reaction volume of 25 μL. A previously sequenced isolate carrying this gene, which was obtained from another study in our department (L. Stein, BScHons 2017), was used as a positive control. The PCR conditions were the same as for that of the *gyrA* and *parC* PCRs, but with a final extension step at 72°C for 5 min. PCR products were separated by agarose gel electrophoresis and visualised as described for the ESBL PCR (3.2.2).

All isolates, regardless of susceptibility profile, were screened for the presence of *qnrB* and *qnrS* genes by singleplex real time PCR on a RotorgeneQ real-time PCR cycler (Qiagen). KAPA SYBR FAST (10 μL) and either 0.2 μM each of *qnrB* primers or 0.125 μM each of *qnrS* primers (table 3.1) were used in a final reaction volume of 20 μL. A *qnrB*- and *qnrS*-positive isolate obtained from this study was used as positive control after confirmation of the presence of these genes by Sanger sequencing. The cycling conditions consisted of an initial cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 15s and 62°C for 20s for *qnrB*. The last step was performed at 65°C for *qnrS.* Melting curves were constructed in the range of 60 to 95°C to verify the specificity of the amplified products. The melting temperatures were 85.5 - 86.8°C for *qnrB* and 82.0 - 82.8°C for *qnrS*. Detection limits were defined as change in fluorescence (df/dt) values of 0.2 for *qnrB* and 0.5 for *qnrS* and any peaks below these values were regarded as "not detected".

DNA extractions and molecular detection of ESBL genes in cephalosporin non-susceptible isolates from the first 50 baseline samples as well as molecular detection of *qnr* genes and mutations in *gyrA* and *parC* of quinolone non-susceptible isolates from the first 50 baseline samples were performed by Mr. Ocloo (R. Ocloo, MSc 2019). DNA extractions and molecular detection of *qnr* genes in quinolone susceptible isolates and *aac(6')-Ib-cr* in quinolone non-susceptible isolates from the first 50

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baseline samples were performed during this study, in addition to that of isolates from subsequent baseline samples and the 16- and 24-week samples.

3.2.4. MIC testing

Gradient diffusion tests were performed on a selection of *E. coli* and *Klebsiella* spp. isolates in order to describe the effect of different resistance mechanisms on the MICs of nalidixic acid and ciprofloxacin. Isolates were selected based on the results of the phenotypic quinolone disk diffusion tests (chapter 2) and molecular detection of quinolone resistance mechanisms. Bacterial suspensions were prepared as described in chapter 2 (2.2.2), using turbidities of 1 McFarland for the *Klebsiella* spp. isolates, as instructed by the manufacturer of the gradient diffusion strips (Liofilchem®). The suspensions were inoculated on Mueller-Hinton (MH)-Sens agar plates (NHLS Media Laboratory, Greenpoint, South Africa) before adding the gradient diffusion strip, and incubated at 37°C for ~18h. Clinical and Laboratory Standards Institute (CLSI) 2019 breakpoints were used to interpret the results (CLSI, 2019).

3.2.5. Statistical analysis

Chi-squared tests were performed using Microsoft® Excel to determine the significance of differences in the rates of carriage of various resistance genes across the three time points. Where sample sizes were too small, as in the case of the participants carrying quinolone non-susceptible *Klebsiella* spp. isolates, as well as *E. coli* with *qnrB*, Fisher's exact tests were performed using Microsoft® Excel. *p*-values ≤ 0.05 were considered statistically significant.

3.3. Results

3.3.1. Molecular detection of β-lactamase genes in cephalosporin non-susceptible *E. coli* and *Klebsiella* spp.

As reported in in chapter 2, 20.7% (n=24/116), 28.2% (n=20/72) and 19.7% (n=14/68) of the *E. coli* isolates and 34.1% (n=14/41), 37.5% (n=6/17) and 33.3% (n=4/12) of the *Klebsiella* spp. isolates were resistant to cephalosporins at baseline, 16 weeks and 24 weeks, respectively. The *bla*$_{\text{CTX-M}}$ ESBL gene was detected in all of the cephalosporin non-susceptible *Klebsiella* spp. (table 3.2), and in 23 (95.8%), 19 (95%) and 14 (100%) of the cephalosporin non-susceptible *E. coli* at baseline, 16 weeks and 24 weeks respectively (table 3.2).The *bla*$_{\text{TEM}}$ gene was the next most common β-lactamase gene

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detected, present in all of the *Klebsiella* spp. isolates and 62.5%, 50% and 42.9% of the *E. coli* isolates at baseline, 16 and 24 weeks, respectively (table 3.2). The *bla*SHV gene was detected in all but one of the *Klebsiella* spp. isolates (isolated at 16 weeks) and in only one *E. coli* isolate (isolated at 24 weeks). These rates all refer to the presence of these genes alone or in combination with other β-lactamase genes. Looking at common combinations of these genes, *bla*CTX-M and *bla*TEM commonly occurred together in *E. coli*, while most of the *Klebsiella* spp. harboured all three genes together (table 3.2). The single *E. coli* isolate at 24 weeks with *bla*SHV also contained *bla*CTX-M and *bla*TEM.

Both of the *E. coli* isolates without *bla*CTX-M, one isolated at baseline and one at 16 weeks, carried only a *bla*TEM gene. However, the PCR does not discriminate between ESBL and non-ESBL variants of the *bla*TEM and *bla*SHV genes and sequencing would be needed to further characterise the spectrum of activity. The carbapenem-resistant isolate found by Mr. Ocloo (*K. pneumoniae*) was reported to harbour the *bla*NDM carbapenemase gene (R. Ocloo, MSc, 2019).

Table 3.2: β-lactamase genes detected in cephalosporin non-susceptible *E. coli* and *Klebsiella* spp. isolates at baseline, 16 weeks and 24 weeks.

β-lactamase genes detected	**Cephalosporin non-susceptible isolates; n (%)**		
E. coli	**BL (n=24)**	**16w (n=20)**	**24w (n=14)**
*bla*CTX-M	23 (95.8)	19 (95.0)	14 (100.0)
*bla*TEM	17 (70.8)	10 (50.0)	6 (42.9)
*bla*SHV	0 (0.0)	0 (0.0)	1 (7.1)
*bla*CTX-M & *bla*TEM together	16 (66.7)	9 (45.0)	6 (42.9)
Klebsiella spp.	**BL (n=14)**	**16w (n=6)**	**24w (n=4)**
*bla*CTX-M	14 (100.0)	6 (100.0)	4 (100.0)
*bla*TEM	14 (100.0)	6 (100.0)	4 (100.0)
*bla*SHV	14 (100.0)	5 (83.3)	4 (100.0)
*bla*CTX-M, *bla*TEM & *bla*SHV together	14 (100.0)	5 (83.3)	4 (100.0)

3.3.2. Molecular detection of quinolone resistance mechanisms in quinolone non-susceptible *E. coli* and *Klebsiella* spp.

In the quinolone non-susceptible *E. coli* at baseline, at least one mutation in *gyrA*, was the most common quinolone resistance mechanism (84.6%), followed by at least one mutation in *parC* (52.4%). The presence of PMQR genes was less common, with *qnrS*

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detected most frequently, at 26.9%, while *qnrB* and ***aac(6')-Ib-cr*** were each detected in only 3.8% of the *E. coli* isolates. In the quinolone non-susceptible *Klebsiella* spp., however, ***aac(6')-Ib-cr*** (66.7%) was the most common mechanism of quinolone resistance at baseline, followed by *qnrB* (53.3%), mutations in *gyrA* and *parC* (both 26.7%), while *qnrS* (20%) was the least common. The proportion of quinolone non-susceptible *E. coli* and *Klebsiella* spp. with various resistance mechanisms at baseline, 16 weeks and 24 weeks is demonstrated in figure 3.1.

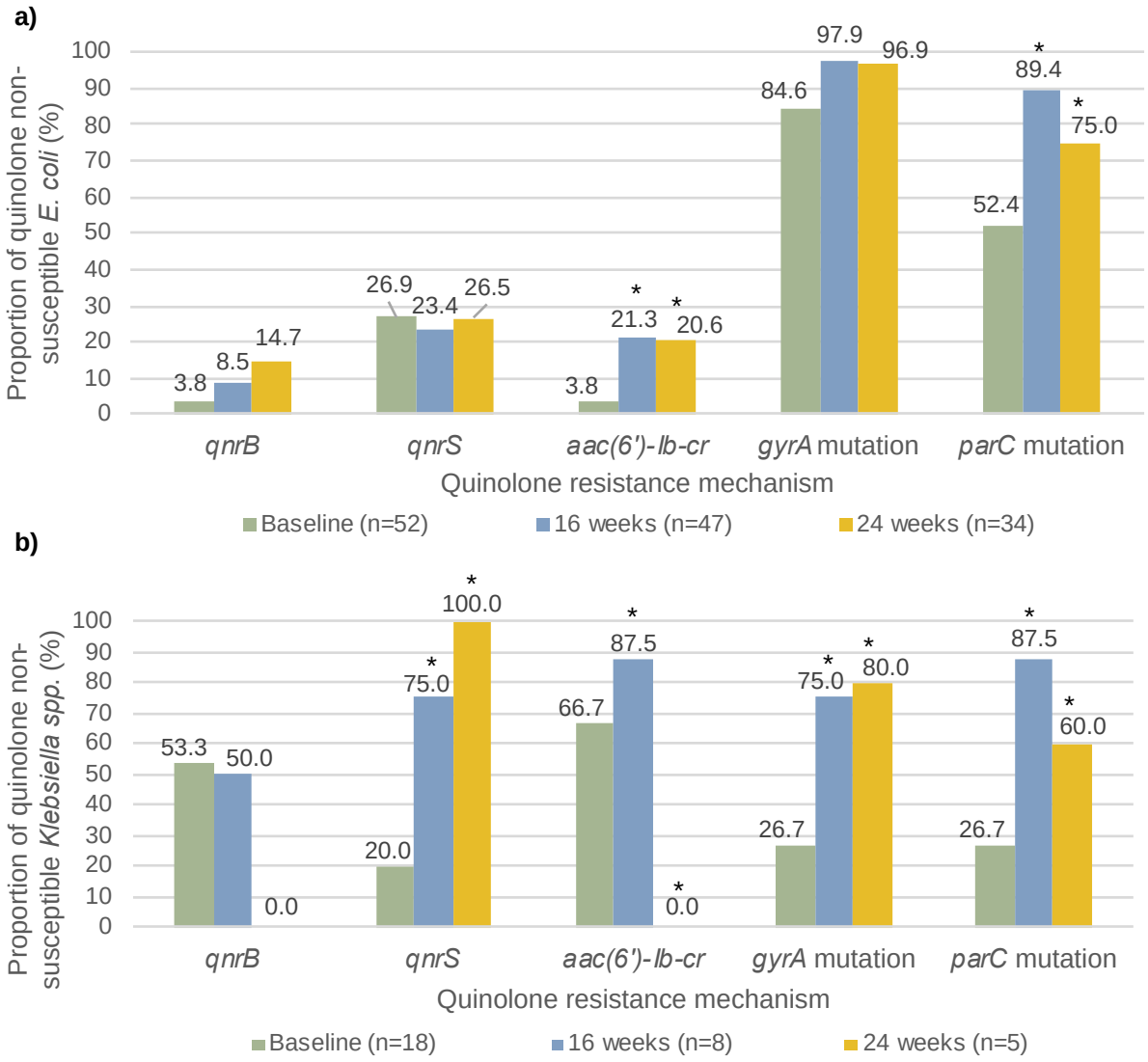

Figure 3.1: Percentage of quinolone resistant *E. coli* (a) and *Klebsiella spp*. (b) with various quinolone resistance mechanisms at baseline, 16 and 24 weeks. **Significantly different to baseline.*

Mutations in *gyrA* remained the most common mechanism of quinolone resistance in the quinolone non-susceptible *E. coli*, with no significant differences in detection rates after 16 and 24 weeks. Mutations in *parC* increased significantly after 16 weeks (p=0.002) and remained significantly higher than that of baseline at 24 weeks

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(p=0.003) (Figure 3.1a). The presence of *qnrS* remained stable in the *E. coli* isolates over the 24 weeks, while *qnrB* increased slightly, although not significantly. The *aac(6')-Ib-cr* gene, though rare at baseline, showed a significant increase at both 16 weeks (p=0.008) and 24 weeks (p<0.001).

In the quinolone non-susceptible *Klebsiella* spp., the presence of *aac(6')-Ib-cr* increased significantly at 16 weeks (p<0.001), as did mutations in *parC* (p<0.001), making these the most common mechanisms of quinolone resistance at 16 weeks (Figure 3.1b). The presence of *qnrS* (p=0.027) and mutations in *gyrA* (p=0.009) also increased significantly after 16 weeks, while the presence of *qnrB* remained stable. At 24 weeks we see a shift towards *qnrS* as the most common mechanism of quinolone resistance after a significant increase (p<0.001), followed by mutations in *gyrA* which increased similarly (p<0.001). Although mutations in *parC* were less common at 24 weeks than at 16 weeks, they were still present at significantly higher rates than at baseline (p<0.001). *qnrB* and *aac(6')-Ib-cr* were not detected in any of the *Klebsiella* spp. isolates at 24 weeks, however the small number of *Klebsiella* spp. isolated at this time point limits the interpretation of these results.

Only two quinolone non-susceptible *Klebsiella* spp. isolates (from baseline samples) did not harbour any of quinolone resistance mechanisms detected in this study, and all of the quinolone non-susceptible *E. coli* isolates had at least one of the investigated quinolone resistance mechanisms. The *qnrS* and *qnrB* genes were very rarely detected in the same isolate (only in two *Klebsiella* spp. and one *E. coli,* isolated at 16 weeks). *qnr* genes were not detected in any quinolone susceptible isolates and the *aac(6')-Ib-cr* gene was detected in all amikacin non-susceptible *E. coli* and *Klebsiella* spp. across the three time points (in addition to ciprofloxacin non-susceptibility in these isolates).

The most commonly observed mutations within the QRDRs of GyrA and ParC are listed in table 3.2. In the *E. coli* isolates, mutations of serine to leucine at amino acid position 83 (S83L) and aspartic acid to asparagine at position 87 (D87N) in GyrA and serine to isoleucine at amino acid position 80 (S80I) in ParC were the most common. S83L mutations in GyrA did occur in *Klebsiella* spp. isolates, but mutations from serine to phenylalanine (S83F) or isoleucine (S83I) were more common at this position, while aspartic acid to alanine at position 87 (D87A) occurred more commonly than D87N. The S80I ParC mutation was detected amongst the *Klebsiella* spp. isolates, in addition

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to two sequence variants which were detected in almost all of the *Klebsiella* spp. isolates: serine to alanine mutation at position 129 (S129A) and alanine to valine at position 141 (A141V). These two variants were not included in table 3.3 or figure 3.1 as they were also found in quinolone susceptible *Klebsiella* spp..

Table 3.3: Commonly observed mutations in GyrA and ParC in *E. coli* and *Klebsiella* spp..

Mutations per organism		**Proportion of quinolone non-susceptible isolates; n (%)**		
E. coli		**Baseline (n=52)**	**16 weeks (n=47)**	**24 weeks (n=34)**
GyrA	S83L	37 (71.2)	47 (100.0)	33 (97.1)
	D87N	21 (40.4)	66.0 (31)	26 (76.5)
ParC	S80I	20 (38.5)	43 (91.5)	27 (79.4)
	E84V	2 (3.8)	8 (17.0)	7 (20.6)
Klebsiella spp.		**Baseline (n=15)**	**16 weeks (n=8)**	**24 weeks (n=5)**
GyrA	S83F	2 (13.3)	1 (12.5)	2 (40.0)
	S83I	0 (0.0)	3 (37.5)	2 (40.0)
	D87A	3 (20.0)	1 (12.5)	2 (40.0)
ParC	S80I	3 (20.0)	4 (50.0)	3 (60.0)

3.3.3. Co-carriage of β-lactamase and PMQR genes

Quinolone resistance was more common amongst isolates that carried *bla*$_{CTX-M}$, *bla*$_{TEM}$ and/or *bla*$_{SHV}$ than those that did not, at 91.2% vs 41.3% in *E. coli* and 91.7% vs 11.1% in *Klebsiella* spp, respectively (p<0.001 for both organisms). In the case of *Klebsiella* spp., only two β-lactamase carriers (isolated at baseline) were susceptible to quinolones. The β-lactamase carriers were also more likely to carry PMQRs than the non-β-lactamase carriers, at 45.6% vs 13.4% in *E. coli* and 91.7% vs 8.9% in *Klebsiella* spp. (p<0.001 for both organisms).

3.3.4. The effect of molecular resistance mechanisms on MIC

In *E. coli,* isolates with *qnrS* alone displayed slightly higher MICs for nalidixic acid and ciprofloxacin, compared to those without any quinolone resistance mechanisms, although not high enough to cross the non-susceptible breakpoint for either agent (table 3.4). However, based on the disk diffusion results (not included in table 3.4), the *qnr* genes were detected in all isolates that displayed non-susceptibility to nalidixic acid or ciprofloxacin in the absence of mutations in *gyrA* and *parC*. An isolate with both *aac(6')-Ib-cr* gene and *qnrS* displayed higher MICs for nalidixic acid and ciprofloxacin

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than those with *qnrS* alone. This isolate showed high level resistance to ciprofloxacin, despite the absence of mutations in *gyrA* or *parC*. Unfortunately there was only one isolate with this genotype, so we could not replicate this finding.

Table 3.4: MICs of nalidixic acid (NA) and ciprofloxacin (CIP) in *E. coli* isolates with various combinations of quinolone resistance mechanisms. MICs crossing the non-susceptible breakpoints are indicated in bold.

PMQRs	Chromosomal mutations				NA MICs [a]			CIP MICs [b]		
	GyrA		ParC		Isolate 1	Isolate 2	Isolate 3	Isolate 1	Isolate 2	Isolate 3
–	none				2	2	4	0.008	0.012	0.016
qnrS	none				4	8	8	0.125	0.125	0.25
qnrS & aac(6')-Ib-cr	none				16			**>32**		
–	S83L				**64**	**64**	**>256**	0.25	0.125	**0.5**
qnrS	S83L				**64**	**>256**	**>256**	**0.5**	**0.5**	**1**
–	S83L		S80I		8	**128**	**>256**	0.25	0.25	**0.5**
–	S83L	D87N	S80I		**>256**	**>256**	**>256**	**4**	**8**	**16**
qnrS and/or *qnrB* and/or *aac(6')-Ib-cr*	S83L/F	D87N/A	S80I		**>256**	**>256**	**>256**	**>32**	**>32**	**>32**
	S83L	D87N	S80I	E84V	**>256**	**>256**	**>256**	**>32**	**>32**	**>32**

[a] *Nalidixic acid breakpoints: 16 = susceptible, 32 = resistant*
[b] *Ciprofloxacin breakpoints: 0.25 = susceptible, 0.5 = intermediate, 1 = resistant* (CLSI, 2019).

Isolates with a single mutation in *gyrA* displayed increased MICs for nalidixic acid and ciprofloxacin, although only one of these crossed the non-susceptible breakpoint for ciprofloxacin. This isolate also had a higher nalidixic acid MIC, compared to the others with a single mutation in *gyrA*. This may indicate additional mechanisms contributing to quinolone resistance, such as efflux pumps. Some of the isolates with *qnrS* in addition to a single *gyrA* mutation displayed higher MICs (up to two doubling dilutions) than isolates with only a single *gyrA* mutation, while those with both a single mutation in gyrA and a single mutation in *parC* displayed similar ciprofloxacin MICs to those with only a single mutation in *gyrA*.

One of the isolates with single mutations in both *gyrA* and *parC*, however, had a lower MIC for nalidixic acid which did not cross the non-susceptible breakpoint, even after repeating the nalidixic acid e-test. However, this isolate was non-susceptible to

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nalidixic acid by disk diffusion. This isolate's MIC for ciprofloxacin also did not cross the non-susceptible breakpoint. *E. coli* isolates with double mutations in *gyrA* and single mutations in *parC* displayed maximum recordable MICs for nalidixic acid and increased MICs for ciprofloxacin, in the resistant range. With the addition of PMQRs, the MICs for both nalidixic acid and ciprofloxacin were beyond the upper end of the range of the gradient strip and we were unable to assess the effect of a second mutation in *parC*.

In the *Klebsiella* spp. isolates (table 3.5), an S83T mutation in GyrA, did not result in non-susceptibility to nalidixic acid or ciprofloxacin, with the isolates displaying similar MICs to those without any resistance mechanisms. A single isolate carrying *qnrS* alone displayed slightly higher MICs for nalidixic acid and ciprofloxacin, resulting in ciprofloxacin resistance while remaining susceptible to nalidixic acid. Isolates with a single mutation at position 84 in ParC together with *qnrS* displayed similar MICs to the isolate with *qnrS* alone.

Table 3.5: MICs of nalidixic acid (NA) and ciprofloxacin (CIP) in *Klebsiella* spp. isolates with various combinations of quinolone resistance mechanisms. MICs crossing the non-susceptible breakpoints are indicated bold.

PMQRs	**Chromosomal mutations**				**NA MICs** [a]			**CIP MICs** [b]		
	GyrA		**ParC**		Isolate 1	Isolate 2	Isolate 3	Isolate 1	Isolate 2	Isolate 3
–		none			4	4	4	0.032	0.064	0.064
–	S83T				2	2	4	0.032	0.032	0.032
qnrS		none			8			**2**		
qnrS				E84	8	8		**1**	**1**	
qnrS / qnrB & aac(6')-Ib-cr		none			16	16	16	**1**	**2**	**2**
qnrS and/or qnrB and/or aac(6')-Ib-cr	S83L/I		S80I		**>256**	**>256**	**>256**	**>32**	**>32**	**>32**
	S83L/I/F	D87N/A	S80I		**>256**	**>256**	**>256**	**>32**	**>32**	**>32**

[a] *Nalidixic acid breakpoints: 16 = susceptible, 32 = resistant*
[b] *Ciprofloxacin breakpoints: 0.25 = susceptible, 0.5 = intermediate, 1 = resistant* (CLSI, 2019)

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Klebsiella spp. isolates with ***aac(6')-Ib-cr*** together with either *qnrS* or *qnrB,* on the other hand, displayed slightly increased nalidixic acid MICs (at most one doubling dilution, although still in the susceptible range), compared to those with *qnrS* alone. These isolates displayed similar ciprofloxacin MICs to those with *qnrS* alone and, interestingly, these values were much lower than that of the *E. coli* isolate with ***aac(6')-Ib-cr*** together with *qnrS*. No *Klebsiella* spp. isolates with only mutations in *gyrA* and *parC* were found, and the presence of a single or double mutation in *gyrA* plus the S80I mutation in *parC* together with one or more PMQR resulted in MICs above the range of the gradient diffusion strip.

3.4. Discussion

3.4.1. Molecular detection of β-lactamase genes in cephalosporin non-susceptible *E. coli* and *Klebsiella* spp.

In this chapter, we described the rates of carriage of β-lactamase and PMQR-genes and quinolone resistance determining mutations in *E. coli* and *Klebsiella* spp. carried by children in Cape Town communities. The most frequently detected β-lactamase gene was *bla*$_{CTX-M}$, carried by all of the cephalosporin non-susceptible *Klebsiella* spp. isolates and the majority (95-100%) of the cephalosporin non-susceptible *E. coli* isolates over the 24 weeks. These rates compare to other South African studies, at 100% of *E. coli* in Port Elizabeth (Gqunta and Govender, 2015) and 90% in *Klebsiella* spp. in Johannesburg (Brink *et al.*, 2013). Our *E. coli* rate also compares to the 97% in Bolivia (Bartoloni *et al.*, 2013) and is slightly higher than what has been reported in cephalosporin resistant *E. coli* in children from communities in Tunisia, at 85.7% (Ferjani *et al.*, 2017). The rate of *bla*$_{CTX-M}$ carriage in cephalosporin non-susceptible *Klebsiella* spp. is also higher that of clinical *Klebsiella* spp. isolates in Kenya, at 55.5% (Taitt *et al.*, 2017). There were no significant increases in the presence of this gene over time in either *E. coli* or *Klebsiella* spp.

The *bla*$_{SHV}$ gene is usually located on the chromosome of *K. pneumoniae*, however, in one of our ampicillin and cephalosporin non-susceptible *K. pneumoniae* isolates, it was not detected. Other studies have reported a lack of *bla*$_{SHV}$ in *K. pneumoniae* (García *et al.*, 2016; Horna *et al.*, 2017), with some suggested explanations. These include possible genetic deletion or insertion of a mobile element within the gene, resulting in its inactivation, which may influence the detection of the gene by PCR

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(Horna *et al.*, 2017). The presence of *bla*$_{SHV}$ in only one *E. coli* isolate in our study is also in keeping with other South African studies which have either rarely detected this gene in *E. coli* (Peirano *et al.*, 2011) or not at all (Young *et al.*, 2019).

The *bla*$_{TEM}$ gene detected in the 16-week *E. coli* isolate in the absence of *bla*$_{CTX-M}$ is probably a non-ESBL variant, since the isolate was susceptible to the third generation cephalosporins cefotaxime and ceftazidime, and the fourth-generation cephalosporin, cefepime. However, the *bla*$_{TEM}$ gene detected in a baseline *E. coli* isolate in the absence of *bla*$_{CTX-M}$ could be an ESBL- and possibly inhibitor resistant variant of *bla*$_{TEM}$, as this isolate was non-susceptible to amoxicillin, amoxicillin/clavulanic acid, cefuroxime, cefotaxime and ceftazidime.

3.4.2. Molecular detection of quinolone resistance mechanisms in quinolone non-susceptible *E. coli* and *Klebsiella* spp.

The distribution of quinolone resistance mechanisms in the isolates in our study is comparable to others around the world. Compared to some of the South African studies discussed in chapter 1, we reported higher baseline rates of *qnrS* (26.9%), similar rates of *qnrB* (3.8%) and lower rates of *aac(6')-Ib-cr* (3.8%) in quinolone non-susceptible *E. coli,* compared to the 14.3%, 4.7% and 61.5% of these genes, respectively, from clinical samples in Port Elizabeth (Gqunta and Govender, 2015).

Numerous studies have reported mutations in *gyrA* and *parC* to be the most prominent mechanisms of quinolone resistance in Enterobacterales, with a lower prevalence of *qnr* genes (Sekyere and Amoako, 2017; Saksena *et al.*, 2018), as found in our *E. coli* isolates. This could explain the higher levels of nalidixic acid non-susceptibility compared to that of ciprofloxacin in *E. coli* as reported in chapter 2, as target site mutations are known to more readily confer resistance to nalidixic acid than ciprofloxacin (Deguchi *et al.*, 1998). The presence of *aac(6')-Ib-cr* was the most common mechanism of quinolone non-susceptibility in *Klebsiella* spp. at baseline, with target site mutations being far less common than observed in *E. coli*. This helps to explain the higher level of non-susceptibility to ciprofloxacin than nalidixic acid in *Klebsiella* spp. as reported in chapter 2, as *aac(6')-Ib-cr* confers resistance to ciprofloxacin and not nalidixic acid. However, it still raises the question of why *aac(6')-Ib-cr* is more common in *Klebsiella* spp., and target site mutations are more common in *E. coli.*

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We reported higher baseline rates of detection of *qnrS* (20%), *qnrB* (53.3%) and *aac(6')-Ib-cr* (66.7%) in *Klebsiella* spp. compared to the 4.7%, 9.5% and 47.6% of these genes reported in *Klebsiella* spp. in Durban, respectively (Sekyere and Amoako, 2017). Studies on children in Niger (Moumouni *et al.*, 2017) and Korea (Kim *et al.*, 2013) also reported *qnrB* to be more common in *Klebsiella* spp. than *E. coli*, as with our study, and, in fact, this gene was first detected in *K. pneumoniae* (Jacoby *et al.*, 2006).

We observed slight increases in *qnrB* in *E. coli* and significant increases in *qnrS* in *Klebsiella* spp., as well as significant increases in *aac(6')-Ib-cr* in both organisms over 24 weeks. The lack of detection of *qnr* or *aac(6')-Ib-cr* genes in *Klebsiella* spp. at 24 weeks is difficult to interpret, due to the small sample size – only 4 participants carried quinolone non-susceptible *Klebsiella* spp. at this time point. The two *Klebsiella* spp. which were quinolone non-susceptible without any *qnr* or *aac(6')-Ib-cr* genes or mutations in the QRDR may have carried other mechanisms such as the *oqxAB* or *qepA* efflux pump genes which were not screened for in this study.

3.4.3. Co-carriage of β-lactamase and PMQR genes

We reported higher rates of resistance to quinolones in *E. coli* (91.2%) and *Klebsiella* spp. (91.7%) that harboured β-lactamase genes compared to those that did not (43.9% and 11.1% respectively). With the plastic genome of *Klebsiella* spp., it is not surprising that almost all of the β-lactamase-carrying *Klebsiella* spp. isolates were quinolone non-susceptible and harboured PMQR genes (91.7%). A recent review has highlighted that *K. pneumoniae* genomes contain double the amount of acquired AMR genes present in *E. coli*, and 50% more than in a few other ESKAPE species (Wyres and Holt, 2018). The authors suggest that *K. pneumoniae* is able to receive a more extensive range of AMR genes from environmental microbes.

Our rate of co-carriage in *E. coli* is slightly higher than the 82% reported in *E. coli* causing bloodstream infections in China (Wang *et al.*, 2016) and compares to the 91% reported in community children in Bolivia (Bartoloni *et al.*, 2013). Additionally, the 45.6% of β-lactamase-carrying *E. coli* which harboured PMQR genes in our study compares to the 55% in the Bolivian study. As mentioned earlier, plasmids carrying β-lactamase genes often harbour genes conferring resistance to other antimicrobials. Therefore, as expected, the *E. coli* harbouring β-lactamase genes were more likely

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than the non-β-lactamase-carriers to be quinolone non-susceptible and harbour PMQR genes (13.4%). Plasmid characterisation in *E. coli* and *Klebsiella* spp. isolates with co-carriage of ESBL- and PMQR-genes is being investigated in more detail as part of a separate study in our department.

The proportion of non-β-lactamase-carrying *E. coli* isolates with reduced susceptibility to quinolones in our study (41.1%), however, is higher than that reported in the Chinese study (27.6% and 24.1% for ciprofloxacin and levofloxacin, respectively) (Wang *et al.*, 2016). This, as well as the high rates of quinolone non-susceptibility in our β-lactamase-carrying isolates could be a reflection of our culture-based selection for quinolone non-susceptible and ESBL-producing isolates. While we did endeavour to pick isolates that were outside the zone of inhibition of either the quinolone or cefpodoxime disc, this was a random process. Selection of multiple colonies on each plate may have provided a more robust selection of isolates; however, this was outside the scope of this particular project.

3.4.4. The effect of molecular resistance mechanisms on MIC

It is well known that the *qnr* genes only slightly increase the MICs of the quinolones resulting in reduced susceptibility, which rarely cross the non-susceptible breakpoint. This explains the slightly higher MICs for nalidixic acid and ciprofloxacin in *E. coli* isolates with *qnrS* alone, compared to those without any resistance mechanisms. As *aac(6')-Ib-cr* confers non-susceptibility to ciprofloxacin, it is to be expected that the ciprofloxacin MIC of the *E. coli* isolate with *aac(6')-Ib-cr* and *qnrS* would be higher than that of the isolates with *qnrS* alone (table 3.4). Moreover, it is interesting to note that this isolate's ciprofloxacin MIC (>32µg/mL) was higher than the MICs in the *Klebsiella* spp. isolates with *qnrS* or *qnrB* and *aac(6')-Ib-cr* (1-2µg/mL) (table 3.5). This could be due to the presence of additional quinolone resistance mechanisms we had not screened for.

Of the *E. coli* isolates with single mutations in *gyrA*, all were non-susceptible to nalidixic acid, but only one displayed non-susceptibility to ciprofloxacin. This isolate also had a higher nalidixic acid MIC compared to the others, which could again allude to the presence of other quinolone resistance mechanisms. As DNA gyrase and topoisomerase IV are the targets of the quinolones, it is to be expected that mutations in the genes encoding these enzymes would result in higher levels of quinolone non-

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susceptibility, compared to the PMQR enzymes which only confer low-level resistance.

Studies have shown that DNA gyrase is the primary target of quinolones and that mutations in *parC* are complementary to the development of higher-levels of fluoroquinolone resistance (Deguchi *et al.*, 1998). This explains why the isolates with a single mutation in both *gyrA* and *parC* displayed similar MICs for nalidixic acid and ciprofloxacin to those with only a single mutation in *gyrA*. One of these isolates, however, displayed a very low MIC for nalidixic acid compared to the other isolates, even after the nalidixic acid gradient diffusion test was repeated, although this isolate was non-susceptible to nalidixic acid with disk diffusion, with no zone around the nalidixic acid disk. As the e-tests were performed after the isolates had been stored, a susceptible subpopulation of the organism might have been stored and used for MIC testing. Re-amplification and -sequencing of the *gyrA* and *parC* genes would be required to further investigate this unusual result.

Resistance to nalidixic acid was achieved with fewer quinolone resistance mechanisms than resistance to ciprofloxacin (table 3.4), which required double mutations in gyrA (except for the isolate with *qnrS* and *aac(6')-Ib-cr*). This is in line with what was found by Vila et al. (1994), where a single mutation at amino acid position 83 in *gyrA* was sufficient to generate high levels of nalidixic acid non-susceptibility, while a second mutation at position 87 was required to result in high levels of ciprofloxacin resistance in *E. coli* (Vila *et al.*, 1994). It has also been documented that mutations in *parC* do not affect susceptibility to fluoroquinolones unless *gyrA* is also altered (Bagel *et al.*, 1999). This explains why the *Klebsiella* spp. isolates with a single *parC* mutation together with *qnrS* did not display higher MICs for naladixic acid and ciprofloxacin than isolates with *qnrS* alone (table 3.5).

Klebsiella spp. isolates carrying *aac(6')-Ib-cr* together with either *qnrS* or *qnrB* displayed higher nalidixic acid MICs, compared to those with *qnrS* alone and *qnrS* with a single mutation in *parC*. This could not be due to the presence of *aac(6')-Ib-cr* and it is likely that these isolates harboured additional quinolone resistance mechanisms. Amino acid position 83 in GyrA has been commonly associated with quinolone resistance, and common mutations described include S83L/I/F/Y. However, in three of our isolates, the S83T mutation did not result in increased MICs and non-susceptibility to nalidixic acid or ciprofloxacin. This mutation has been previously

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documented in quinolone susceptible isolates (Fu *et al.*, 2008, 2013) and, interestingly, these studies also reported S83L/I/F/Y in quinolone susceptible isolates.

In summary, in this chapter we described high rates of carriage of *bla*$_{\text{CTX-M}}$ in cephalosporin non-susceptible Enterobacterales in community children, compared to community as well as clinical studies in other LMICs, however similar to other studies in South Africa. We also reported high rates of co-carriage of β-lactamase and PMQR genes and increases in the presence of quinolone resistance mechanisms over time in most cases. Our findings regarding the effect of quinolone resistance mechanisms on MICs were in line with previous studies. We detected higher proportions of *qnrS* in *E. coli* and *qnrB*, *qnrS* and *aac(6')-Ib-cr* in *Klebsiella* spp. at baseline than other South African studies. This is concerning, as we expect that approximately half of our participants would have been exposed to levofloxacin after the baseline samples were taken. Until the TB-CHAMP study is completed and we have been unblinded, we can only speculate that exposure to levofloxacin has contributed to the increases in the presence of these genes observed at 16 and 24 weeks.

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Chapter 4: Targeted molecular screening for plasmid-mediated quinolone resistance genes in the stools of children prior to levofloxacin exposure

4.1. Introduction

The human gut microbiota, with its extremely high density of microorganisms and constant exposure to a wide range of external influences, including environmental bacteria from water, food, other humans, animals and soil, requires particular attention as a reservoir of antimicrobial resistance (AMR). This constant influx of bacteria into an already densely populated environment not only facilitates the transfer of AMR genes to the commensal microbial communities, enriching the pool of available AMR determinants in the gut microbiota, but also to potentially pathogenic bacteria (Penders *et al.*, 2013; von Wintersdorff *et al.*, 2014). High relative abundances of antimicrobial resistant Enterobacteriaceae have been reported to increase the risk of bacteraemia and UTIs caused by resistant bacteria originating from the gut (Taur *et al.*, 2012; Ruppé *et al.*, 2013).

Over the last few decades, most studies on AMR within the gut microbiome have focused on selective culture and antibiotic susceptibility profiling of specific indicator bacteria like *E. coli.* These studies have produced valuable insights on the frequency and geographical spread of AMR (Nys *et al.*, 2004), as well as the associations between AMR in humans and food animals (van den Bogaard and Stobberingh, 2000) and between AMR in intestinal microorganisms and antibiotic use (van der Veen *et al.*, 2009). They have also revealed the emergence of resistance in populations with minimal antibiotic exposure (Bartoloni *et al.*, 2013). However, culture-based techniques are only capable of capturing a small fraction of the vast number of species in the GIT (Ashbolt *et al.*, 2018), since more than 80% of the gut microbiota remains unculturable (Eckburg *et al.*, 2005; von Wintersdorff *et al.*, 2014).

The potential for transfer of AMR genes in the human gut microbiota calls for research that focuses on AMR in the gut microbiome as a whole, by using metagenomic approaches which avoid the bias of selective culture. Various types of metagenomic approaches, which analyse all the genetic material of all microorganisms recovered directly from environmental samples, have been applied to study the resistome,

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consisting of all of the resistance genes within the entire microbiota (Penders *et al.*, 2013). Recent studies using functional metagenomics have drawn attention to the underestimated diversity of AMR genes in the gut microbiome and identified genes that had not been previously described, while more targeted approaches for the detection and quantification of AMR genes within a population, such as PCR, have great potential for extensive epidemiological investigations (Penders *et al.*, 2013).

Moreover, the use of real-time PCR allows for the generation of quantitative results to determine the relative abundance of resistance genes. Using a targeted real-time PCR, this chapter aims to provide information on the prevalence and relative abundance of plasmid mediated quinolone resistance (PMQR) genes in community children before commencement of levofloxacin treatment.

4.2. Materials and methods

4.2.1. Stool DNA extraction

DNA was extracted directly from 200 mg of each of the 100 baseline stool samples that had been frozen at -80°C (Chapter 2) using the PSP® Spin Stool DNA Kit (Stratec Molecular, Germany) according to the manufacturer's instructions. DNA was eluted in 100 µL of elution buffer before storage at -20°C until further analysis. The DNA concentrations were determined using a BioDrop spectrophotometer, before diluting to concentrations of 50±10 µg/mL.

4.2.2. Detection and quantification of *qnr* genes in gut bacterial population

Real-time PCR assays were used to detect and quantify the plasmid-mediated quinolone resistance (PMQR) genes *qnrB* and *qnrS* in stool DNA extractions of the first 100 baseline stool samples. The same reagents, instrument and reaction conditions were used as described in chapter 3 (section 3.2.3). However, in this assay, the same primer concentrations (0.2 µM) and annealing temperature (62°C) were used for the detection of both *qnrS* and *qnrB*. Universal bacterial 16S rDNA was also amplified as a reference gene in order to normalise the abundance of *qnr* genes relative to the amount of bacterial DNA in the stool samples (primer sequences given in table 4.1).

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Table 4.1: Primer sequences for amplification of universal bacterial 16S rDNA.

Target gene	Primers	Sequences	Size (bp)	Reference
Universal bacterial 16S rDNA	HDA-1-F	ACTCCTACGGGAGGCAGCAGT	174 - 199	Walter *et al.*, 2000
	HDA-1-R	GTATTACCGCGGCTGCTGGCAC		

Melt curves were constructed in the range of 60°C - 95°C to verify the specificity of the amplified products for detection of *qnrB* and *qnrS*. The quantitative real-time PCR assays were validated using ten-fold serial dilutions of previously amplified and purified *qnrB* and *qnrS* products and subsequent construction of standard curves to determine the cycle threshold ranges for quantification using R_2>0.99 and efficiency values between 0.90 and 1.1. Any samples which amplified beyond the upper limit of the quantification ranges were regarded as “not quantifiable”. DNA from the 10^{-5} dilution for *qnrB* and 10^{-8} dilution for *qnrS* were included in each reaction as controls for inter-assay reproducibility, which was defined as C_T values within one cycle of the C_T in the standard curve. C_T values of the 16S rDNA were subtracted from that of each *qnr* gene to obtain ΔC_T values indicative of the relative abundance of each *qnr* gene relative to the total bacterial abundance in the stool (Pfaffl, 2006).

4.2.3. Detection of *aac(6')-Ib-cr* in gut bacterial population

The stool DNA extractions were subjected to singleplex conventional PCR reactions screening for the presence of the *aac(6')-Ib-cr* gene as described for the isolates in chapter 3 (section 3.2.3) but using 40 cycles. This was followed by visualisation using agarose gel electrophoresis as described in chapter 3.

4.3. Results

4.3.1. Detection PMQR genes in total gut bacterial population

The melting temperature for *qnrS* was 82.3 – 83.3°C and 85.7 – 86.5°C for *qnrB* (Figure 4.1). Detection limits were defined as change in fluorescence (df/dt) values of 0.4 for *qnrB* (figure 4.1a) and 0.5 for *qnrS* (figure 4.1b); any peaks below these values were regarded as “not detected”.

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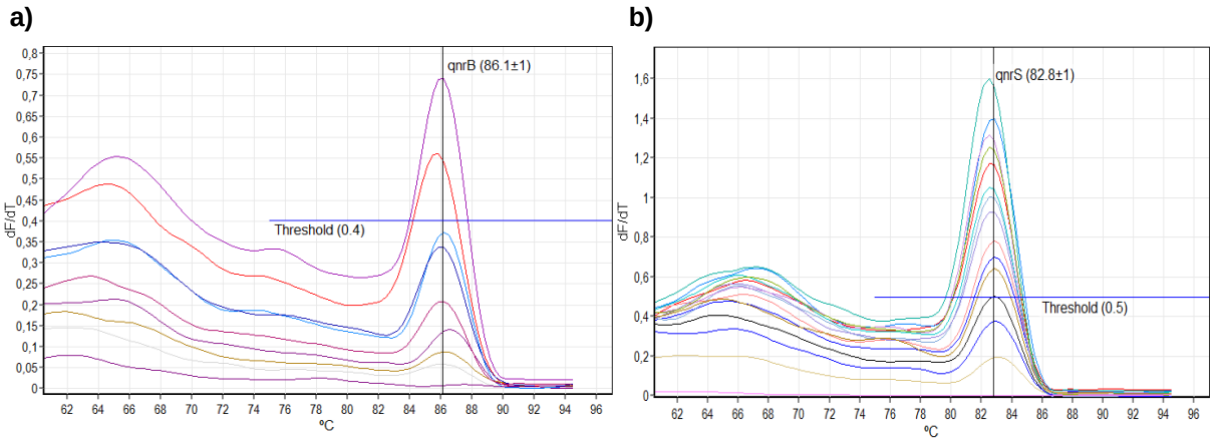

Figure 4.1: Visual representation of the melting temperatures and detection limits for *qnrB* (a) and *qnrS* (b**)**. Temperature thresholds were set at 75°C as melt peaks below this temperature were not visible with gel electrophoresis and were ignored. Peak bins were set at the mean values of the melting temperature ranges, with a bin width of 1°C.

The *qnrS* gene was detected in 86 of the 100 baseline stool samples while *qnrB* was only detected in in 14 (table 4.2). *qnrS* and *qnrB* were only detected in isolates from 15 and 9 stools, respectively (table 4.2), with one isolate positive for both genes. Interestingly, a *qnrS*-positive isolate was cultured from the stool of a participant in which the gene was not detected in the stool DNA and isolates positive *for qnrB* were cultured from five stool samples in which the *qnrB* gene was not detected directly (table 4.2).

The *aac(6')-Ib-cr* gene was detected in the stool of nine participants (9%; table 4.2). The gene was detected in isolates cultured from the stools of 11 participants (Chapter 3 and table 4.2); although the gene was only detected directly in the stool DNA of five of these participants (table 4.2). A more detailed description of the presence of different combinations of the *qnrB*, *qnrS* and *aac(6')-Ib-cr* genes in stool DNA vs. cultured isolates, is given in appendix A.

Table 4.2: Comparison of detection of *qnrS*, *qnrB* and *aac(6')-Ib-cr* in stools directly or in isolates cultured from the stools of 100 participants at baseline.

	Participants with PMQR-positive stool DNA	Participants with PMQR-positive isolates cultured	Stool + / culture +	Stool + / culture -	Stool - / culture +
qnrS	86	15	14	72	1
qnrB	14	9	4	10	5
aac(6')-Ib-cr	9	11	5	4	6

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4.3.2. Quantification of *qnr* genes in total gut bacterial population

The quantification ranges were defined as cycle threshold (C_T) values of 3 - 25 for *qnrB* (figure 4.2a), 10 - 31 for *qnrS* (figure 4.2b) and 4 - 18 for 16S (not included in figure 4.2), based on the construction of standard curves (figure 4.2c and 4.2d for *qnrB* and *qnrS*, respectively.

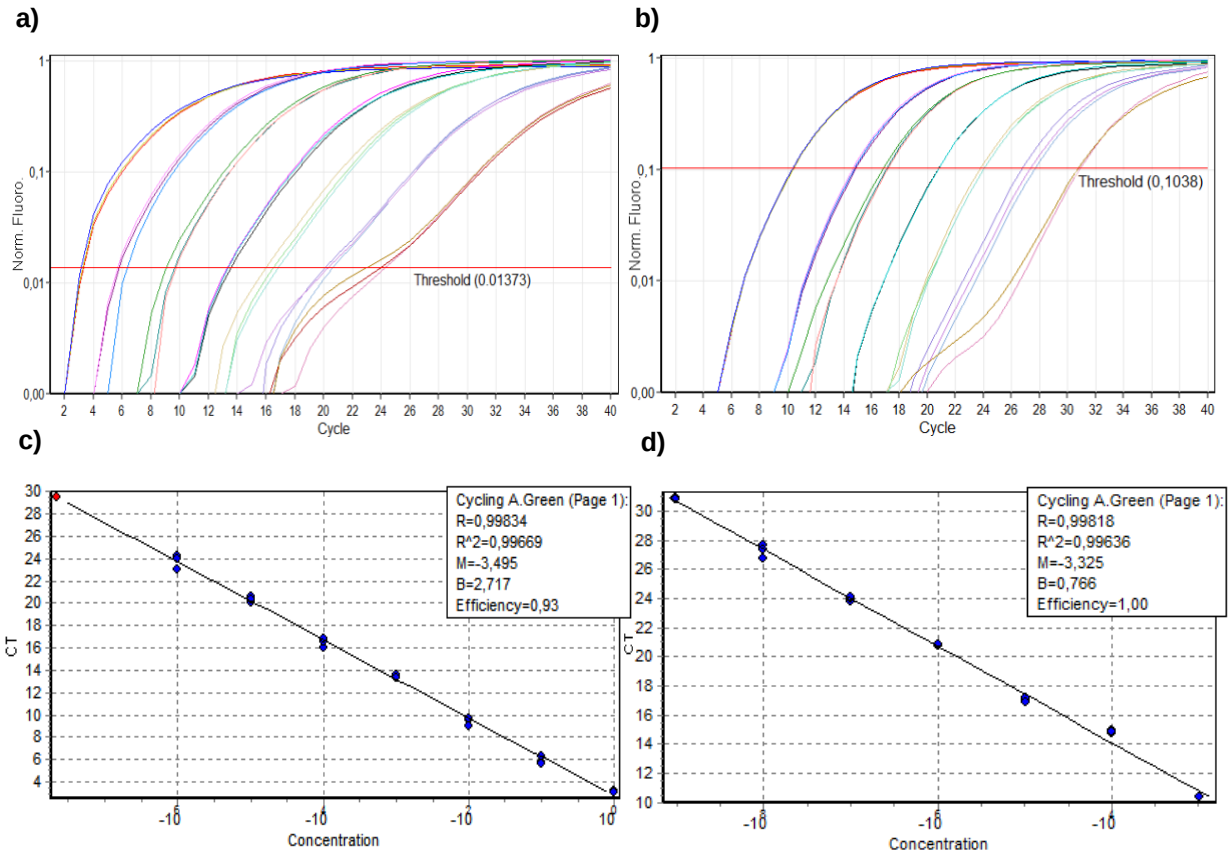

Figure 4.2: Visual representation of the quantification ranges and standard curves of the *qnrB* and *qnrS* assays. Figures a and b represent the quantification curves of the standard dilutions which were used for the validation of the quantification ranges of the *qnrB* (5-25) and *qnrS* (10-31) assays, respectively. Cycle threshold values were determined using the standard curves for *qnrB* (c) and *qnrS* (d).

The *qnrS* and *qnrB* genes could only be quantified in 53 (61.6%) and 11 (78.6%) of the stool samples in which they were detected, respectively, based on the defined ranges of quantification. The median ΔC_T relative to universal bacterial DNA in the stool was 20.1 for *qnrS* and 16.7 for *qnrB* for the stools in which these genes were quantifiable. This indicates a higher abundance of *qnrB* in the stool samples, although it was detected in a much smaller proportion of stool samples than *qnrS*.

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4.4. Discussion

In this chapter we used a targeted molecular approach to describe the prevalence of the plasmid mediated quinolone resistance genes *qnrB*, *qnrS* and *aac(6')-Ib-cr* in the gut microbiota of community children before exposure to a fluoroquinolone antibiotic. The detection of *qnrS* in the stools of 86% of participants is comparable to the 74.5% of Vietnamese children with acute respiratory tract infections carrying this gene in their stools (Vien *et al.*, 2012), as well as the 85% reported in the fecal samples of community individuals (aged 4-75) in India (Rutgersson *et al.*, 2014). The 14% detection rate of *qnrB* is, however, much lower than the 44.1% in the Vietnamese study, and the 83% in the Indian individuals, but is comparable to the 8% that Rutgersson *et al.* reported in Swedish individuals (aged 22-34). The presence of *qnrS* was also much lower in Swedish individuals, at 11% (Rutgersson *et al.*, 2014), contrasting the levels of both of these genes present in low and middle income countries (LMICs) vs. high income countries (HICs). The finding of a high prevalence of *qnrS* and a low prevalence of *qnrB* in our population is therefore somewhat at odds with other studies which have shown higher levels of both (in LMICs) or lower levels of both (in HIC).

The Vietnamese study also reported a higher prevalence of *qnrS* compared to *qnrB* in the stool DNA of children, as with our study. Other than the studies by Vien *et al*. (2012) and Rutgersson *et al.* (2014), no other studies screening for *qnr* genes in total fecal DNA could be found. In bacterial isolates, however, *qnrB* is usually reported to be more prevalent around the world (Yang *et al.*, 2008; Poirel *et al.*, 2012; Alheib *et al.*, 2015; Chmielarczyk *et al.*, 2015; Mokhtari-Farsani *et al.*, 2016; Albornoz *et al.*, 2017; Salah *et al.*, 2019), although a few studies in the Middle East have reported *qnrS* to be more common (Al-Agamy *et al.*, 2018; Ranjbar *et al.*, 2018; Farajzadehsheikh *et al.*, 2019). The South African studies on *K. pneumoniae* in Durban (Sekyere and Amoako, 2017) and *E. coli* in Port Elizabeth (PE) (Gqunta and Govender, 2015) also report a higher prevalence of *qnrS* compared to *qnrB*, which could mean that unlike most of the world, *qnrS* is more prevalent than *qnrB* in South Africa.

However, *qnrB* could still be present in the stool samples, just at levels below our detection limits (df/dt<0.4). In more than half of the samples in which *qnrB*-positive isolates were cultured, *qnrB* was not detected by direct PCR, suggesting that the PCR

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assay may not be sensitive enough to detect relatively low abundances of the gene in the stool. The higher prevalence of *qnrS* in the stools (86%) compared to the isolates cultured from stools (15%) could be due to our culturing method which may not have been sensitive enough for selecting quinolone non-susceptible organisms – especially given the relatively low-level quinolone resistance associated with *qnrS* alone. However, the gene could also be present in the multitude of organisms other than *E. coli* and *Klebsiella* spp. that colonise the gut. For example *qnrB* and *qnrS* have been reported in *Salmonella* spp. isolated from stool samples in Iraq (Ghaima *et al.*, 2016). For the single stool sample from which a *qnrS*-positive isolate was cultured but the gene was not detected in the stool by direct PCR, the gene could, again, just be present at levels too low for the PCR to detect (df/dt<0.5).

The *aac(6')-Ib-cr* gene was rarely detected in the stool DNA (9%), which correlates with the 11% detected in the isolates themselves. A study from Korea detected this variant in all six tested human faecal samples, with this specific variant of *aac(6')-Ib* being the most abundant in four of these (Kim *et al.*, 2018). This is the only study we found which has screened for the presence of *aac(6')-Ib-cr* in total stool DNA. It will be interesting to see how the presence of this variant changes over time with levofloxacin treatment in the 16- and 24-week samples.

We were able to quantify *qnrS* in 61.6% and *qnrB* in 78.6% of the stools in which they were detected. In the remaining stools where these genes were detected, the abundances of these genes were too low. The quantification results in isolation are difficult to comment on, since there is little data that correlate relative abundance with, for example, risk of HGT or infection with a resistant isolate. However, it would be interesting to see how the relative abundance of these genes are impacted by levofloxacin treatment in the 16- and 24- week samples, once we have been unblinded. In future, it would also be interesting to investigate whether the relative abundance of specific resistance genes is associated with other risks and clinically relevant outcomes.

The Vietnamese study demonstrated an increase in the prevalence and copy number of *qnr*S and *qnrB* over 7 days in the absence of fluoroquinolone treatment, but associated with amoxicillin/clavulanic acid treatment alone or in combination with a cephalosporin (Vien *et al.*, 2012). This suggests that the presence and quantity of PMQR genes may even increase in our placebo-group participants, if these children

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received any other antibiotics, as PMQR plasmids commonly harbour other resistance genes, especially those encoding resistance to β-lactams. Even if the children weren't receiving any other antibiotics, they may acquire bacteria with these genes from others in the community who are on antibiotic treatment, as well as from the environment and livestock.

Studies have reported *qnrS* as the most common *qnr* gene identified in the environment, usually detected in waterborne species (Poirel *et al.*, 2012; Marti and Balcázar, 2013), but also in *E. coli* from pigs (Szmolka *et al.*, 2011), horses (Dolejska *et al.*, 2011) and poultry (Yue *et al.*, 2011). The authors of the Korean study reporting on *aac(6')-Ib-cr* have even suggested that this variant originated from the environment (Kim *et al.*, 2018). Although we do not yet have access to the baseline socio-economic data of our participants, it would be important to look for correlations between environmental exposures and carriage of these genes.

Nevertheless, the high rates of *qnrS* in the stools is worrying. Theoretically, half of these children will have been exposed to long term levofloxacin treatment as part of the TB-CHAMP trial, which may select for these resistance genes and facilitate their dissemination amongst potentially pathogenic organisms. More broadly, if these findings are generalisable, then wider use of levofloxacin for prophylaxis in childhood MDR-TB contacts may create societal risks of dissemination of resistance. This of course needs to be balanced with the potential benefit of levofloxacin in preventing MDR-TB. The high prevalence of *qnrS* in the gut microbiota of our participants also suggests that organisms carrying this gene are circulating in individuals in the community. This emphasises the notion that children may be important vessels for the dissemination of antimicrobial-resistant organisms in the community (Bryce *et al.*, 2018).

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Chapter 5: Concluding remarks

This study aimed to describe the gastro-intestinal carriage of antimicrobial resistant *E. coli* and *Klebsiella* spp. in multidrug-resistant Tuberculosis (MDR-TB) exposed children in Cape Town communities. Previous studies have mostly focussed on the rates of resistant organisms in hospitalised patients or clinical isolates; although communities, and especially children in communities, may be a major source of antibiotic resistant organisms. This type of information is especially limited in South Africa, where the burden of infectious diseases is high and awareness needs to be raised urgently in order to fight resistant bacteria and preserve the effectivity of antibiotics.

We reported disturbingly high baseline rates of carriage of organisms resistant to various antibiotics in children under 5 years of age, prior to levofloxacin treatment, especially the 49% of children who already carried quinolone non-susceptible organisms. Additionally, the finding that 27% of children carried multidrug-resistant (MDR) organisms in their gut microbiota is concerning. Cephalosporin non-susceptible organisms were carried by 33% of children at baseline, the majority of which harboured the *bla*$_{\text{CTX-M}}$ gene, possibly pointing towards a high prevalence of this extended-spectrum β-lactamase (ESBL) gene in the community. We could, however, not correlate these levels of resistance with any participant data, such as previous antibiotic use, hospitalisation, pets or day-care exposure, as we are still awaiting this data from the Medical Research Council Clinical Trials Unit (MRC-CTU) who manages all the data collected as a part of the TB-CHAMP clinical trial. Once we have received this information we will be able to better describe whether any of these demographic factors are associated with the carriage of non-susceptible Enterobacterales.

We detected higher proportions of *Klebsiella* spp. carrying *qnrB* (53.3%) and *aac(6')-Ib-cr* (66.7%) at baseline, compared to *E. coli* (3.8% for both genes). This, as well as the significant increases in *qnrS* and *aac(6')-Ib-cr* observed over time in *Klebsiella* spp., highlights the danger of this organism with its ability to easily acquire plasmid-mediated resistance determinants. In the targeted molecular screening for these genes in baseline stool samples, we saw extremely high proportions of participants carrying *qnrS* in their total gut microbiota (86%) and lower proportions carrying *qnrB* (14%) and *aac(6')-Ib-cr* (9%). Further screening could be performed on the 16- and

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24-week samples to investigate how the detection and quantification of these genes is affected by antibiotic treatment over time.

We reported increasing rates of carriage of antimicrobial resistant organisms after 16 and 24 weeks. The most striking was the rate of non-susceptibility to quinolones which increased to 69.8% at 16 weeks. However, as the TB-CHAMP trial is still in progress, we do not know which of the children were treated with levofloxacin and which received placebo treatment. Once unblinded, we will be able to stratify our results according to treatment groups and determine whether levofloxacin treatment influenced the carriage of non-susceptible organisms. Nevertheless, fluoroquinolone resistance can develop even in the absence of exposure to antibiotics of this class, as various mechanisms of co-selection, such as plasmids carrying genes conferring resistance to multiple antibiotics, aid in the emergence of quinolone resistance (Dalhoff, 2012).

Another limitation of our study is that our sample set might not be truly representative of the community. The children were recruited from two low income communities and may therefore not represent the greater Cape Town community, as there may be varying socio-economic and demographic factors which could have influenced our results. Moreover, although we have included 100 participants in the baseline analysis, the communities where these children reside are very densely populated and this number could be a very small representation of the greater population in these communities.

Furthermore, our participants are exposed to very ill parents/caregivers who are susceptible to infectious diseases other than MDR-TB and commonly exposed to healthcare settings and, therefore, antimicrobial resistant organisms. This could have introduced bias, as our participants could thus be commonly exposed to resistant nosocomial bacteria through their parents/caregivers. Future studies should aim to include more individuals from the community, in the absence of antibiotic treatment and from TB-unexposed homes or communities to describe resistance rates in other population groups.

Given the poor overall socio-economic status of the communities in this study, there have been concerns that these children may be anaemic, malnourished or have worms. These may be contributing factors to the carriage of resistant organisms.

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Again, access to the participant data will help to assess this and highlight possible risk factors.

We also struggled with high rates of loss to follow up, almost 50% at 16- and 24-weeks, which means that we might not have a 50:50 ratio of levofloxacin exposed vs. unexposed children in the follow up samples. One of the common reasons we received for stools not being collected from participants during visits at the Philippi clinic was due to the child visiting or moving to the Eastern Cape. The majority of the residents of this township migrated from the former Ciskei and Transkei homelands during the apartheid era (Anderson *et al.*, 2009), therefore many still have ties to the Eastern Cape and commonly visit or move there. The other common reason was that the parent/caregiver could simply not collect stool from the child or that the child did not produce stool at the time of the visit. Two children were reported to start TB treatment and were removed from the study after baseline and/or 8-week samples were received and one child passed away after a baseline sample had been collected.

Additionally, our methodology could have introduced bias due to selection of cephalosporin, carbapenem and quinolone resistant isolates using antibiotic disks during initial culturing of the stool samples. We did attempt to mitigate this by sub-culturing a colony remote from the antibiotic disc; however, the selection of this colony was random, and in future, selection of multiple colonies from the agar plate may create a more representative selection. This was unfortunately impractical for the purposes of this research study.

Additionally, the small portion of sample cultured might not be truly representative of the entire stool sample, if the sample was not properly homogenised. This may have contributed to the detection of plasmid-mediated quinolone resistance (PMQR) genes in stools from which no PMQR containing *E. coli* or *Klebsiella* spp. isolates were cultured; however, these genes may also have been present in bacteria other than *E. coli* and *Klebsiella*.

The molecular screening of *qnr* genes using real time PCR also had limits of detection and quantification and we could have underreported the presence of these genes in the stools of our participants. Another drawback to this method is that it cannot tell us which organisms are carrying these resistance genes, nor if they are being expressed (von Wintersdorff *et al.*, 2014). Metagenomic sequencing, especially long-read

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technology such as PacBio or Oxford Nanopore, could provide information on which genes were present in which organisms in the stools. Moreover, sequence-based metagenomics can also provide information on community composition and metabolic profile (Penders et al., 2013). However, this method does not provide information on gene expression. Future studies on this sample set could use reverse-transcription real-time PCR (RT-qPCR) to investigate how the levels of expression changes over time with antibiotic treatment in the 16- and 24-week samples.

Epidemiological methods can also be performed on the cultured isolates to see which strains are circulating and dominating in the community and whether this changes over the course of 24 weeks of levofloxacin treatment. Whole genome sequencing of the isolates could provide information on strain types, as well as a wealth of possible resistance mechanisms that we had not screened for. It would be interesting to see whether our participants carried organisms that harbour the *qep* and *oqx* plasmid mediated quinolone resistance (PMQR) genes. The latter has been reported to be the most common PMQR in Durban (Sekyere and Amoako, 2017) and the *qepA* gene has been reported to be a part of the same transposable element as an aminoglycoside ribosome methyltransferase (Yamane *et al.*, 2007). Therefore, as with *aac(6')-Ib-cr*, there is a potential of selection of *qepA* genes by aminoglycosides and aminoglycoside resistance by quinolones. Additionally, these genes might be increasingly present in our isolates over time, as we have seen with the *qnr* and *aac(6')-Ib-cr* genes.

This co-selection of resistance to other antibiotics, could make our participants extremely vulnerable with limited treatment options should they acquire a bacterial infection. Our findings, with high rates of resistant organisms with various resistance genes carried by young children prior to antibiotic treatment and increasingly over time, should increase awareness of the major problem of antibiotic resistance right here in our city. Moreover, this should also be a call for more studies focussing on antibiotic resistance in South Africa, as this type of information is extremely scarce in our country and could aid in the choice of treatment regimens in health care facilities in our communities.

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Appendix A

Combinations of PMQR genes detected in isolates cultured from stools vs. stool DNA directly.

		Targeted PCR directly on stool DNA (n=100)								
		qnrS	*qnrB*	*qnrS & qnrB*	*aac(6')-Ib-cr*	*aac(6')-Ib-cr & qnrB*	*aac(6')-Ib-cr & qnrS*	*aac(6')-Ib-cr & qnrS & qnrB*	None	Total
PCR on isolates cultured from stools (n=100 participants)	*qnrS*	8	0	2	0	0	2	0	1	13
	qnrB	1	0	1	0	0	1	0	0	3
	qnrS & qnrB	1	0	0	0	0	0	0	0	1
	aac(6')-Ib-cr	0	0	2	0	0	1	0	0	3
	aac(6')-Ib-cr & qnrB	1	0	1	0	0	1	2	0	5
	aac(6')-Ib-cr & qnrS	0	0	0	0	0	1	0	0	1
	aac(6')-Ib-cr & qnrS & qnrB	0	0	0	0	0	0	0	0	0
	None	54	1	4	0	0	2	1	12	74
	Total	65	1	10	0	0	8	3	13	100

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