Developing an Antimicrobial Strategy for Sepsis in Malawi

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Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor in Philosophy by Joseph Michael Lewis

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Introduction

1.1 Chapter Overview

The syndrome of sepsis is an ancient one; from Hippocrates to Galen and Semmelweis, the potentially serious consequences of infection have long been recognized. Modern definitions of sepsis conceptualise it as a syndrome of life threatening organ dysfunction due to a deleterious and dysregulated host response to infection, but despite increased understanding of its pathogenesis, mortality from sepsis remains high. Progress has been made in improving sepsis mortality in high income settings through timely application of basic care: early appropriate antimicrobials, aggressive fluid resuscitation and organ support largely in a critical care environment. Limited data from low resource settings including sub-Saharan Africa (sSA) suggest that mortality remains high, and increasing evidence suggests that exporting high-income setting sepsis protocols to sSA has the potential to do harm. Data to guide sepsis management protocols for sSA are urgently needed.

Data on sepsis aetiology from sSA to guide antimicrobial strategies are lacking; currently, in Blantyre Malawi, for example, empirical management of sepsis is the norm and patients often receive prolonged empiric courses of broad spectrum antimicrobials – largely ceftriaxone, a third-generation cephalosporin antibiotic. The effects of this at an individual level are unknown, but on a population level invasive Escherichia coli and Klebsiella pneumoniae bacteria are showing an alarming increase in ceftriaxone resistance since the drug was introduced in Malawi in 2005. The majority of these resistant bacteria are so-called extended-spectrum beta lactamase producers (ESBL-producers) and are often untreatable with locally available antimicrobials. Novel antimicrobial strategies are needed to safely preserve ceftriaxone - a first and last line antibiotic - for those who need it.

It is the hypothesis of this thesis, then, that sepsis is Malawi is caused by a wide variety of infections that are currently unrecognised and untreated, and that this is contributing to high sepsis mortality. Conversely, prolonged ceftriaxone exposure in sepsis survivors is causing acquisition and carriage of resistant bacteria (principally ESBL Enterobacteriaceae, henceforth ESBL-E) and their transportation into the community. I will argue that sustainable antimicrobial strategies for sepsis in sSA can not only consider mortality; the unintended consequences in terms of antimicrobial resistance (AMR) acquisition in a setting where empiric management of infection is the norm must also be considered, and mitigated against where possible. In this chapter, I will review, firstly, the definitions, epidemiology, aetiology and management of sepsis, with a focus on aetiology and antimicrobial treatment; and secondly, the epidemiology and drivers of ESBL-E carriage, both with a focus on sSA.

1.2 Sepsis in sub-Saharan Africa

1.2.1 Search strategy

A review of the literature was undertaken to identify prospective cohort, case control studies or randomised controlled trials (RCTs) of sepsis in sub-Saharan Africa with the search terms sepsis *and* ((Angola *or* Benin *or* Botswana *or* Burkina Faso *or* Burundi *or* Cameroon *or* Cape Verde *or* Central African Republic *or* Chad *or* Comoros *or* Republic of the Congo *or* Congo Brazzaville *or* Democratic republic of the Congo *or* Cote d'Ivoire *or* Djibouti *or* Equatorial Guinea *or* Eritrea *or* Ethiopia *or* Gabon *or* The Gambia *or* Ghana *or* Guinea *or* Guinea-Bissau *or* Kenya *or* Lesotho *or* Liberia *or* Madagascar *or* Malawi *or* Mali *or* Mauritania *or* Mauritius *or* Mozambique *or* Namibia *or* Niger *or* Nigeria *or* Reunion *or* Rwanda *or* Sao Tome and Principe *or* Senegal *or* Seychelles *or* Sierra Leone *or* Somalia *or* South Africa *or* Sudan *or* Swaziland *or* Eswatini *or* Tanzania *or* Togo *or* Uganda *or* Western Sahara *or* Zambia *or* Zimbabwe) *or* Africa). Pubmed and scopus were searched, yielding 5460 unique studies on 17 July 2018. Inclusion criteria were any prospective cohort, RCT or case-control studies of sepsis in sSA (defined as taking place in the countries listed in search terms panel) recruiting patients using sepsis 1,2 or 3 definitions. Abstract review was undertaken resulting in inclusion of 91 studies for full text review. Eleven publications providing data on eight prospective cohorts[1–8] and three intervention studies (two RCTs[9,10] and one before-after intervention[11]) were identified. These data inform the following review, alongside non-systematically searched studies examining sepsis in high-resource settings.

In order to put sepsis aetiology data in context, systematic searches of the Pubmed and Scopus databases for leptospirosis, brucellosis, Q fever, Rickettsioses, arboviruses (dengue, or chikungunya) and histoplasmosis prevalence in unselected sepsis or fever cohorts in sSA were undertaken. Because a recent systematic review has examined these pathogens up to 2013 (see "sepsis aetiology" below), the date of these searches were restricted the 2014 to the present. Any studies examining disease prevalence in cohorts of febrile adults or children were included; outbreaks were excluded. Studies where the inclusion criteria were not clear (including those with, for example, "suspected leptospirosis" with no further details) were excluded. Finally, systematic searches of Pneumocystis Jiroveci pneumonia (PCP) were made using the search terms below; because a recent systematic review has examined the role of PCP up to 2015, the date on this search was restricted to 2015 or later. Table 1.1 shows the search terms, number of of hits and number of included studies after full text review: nine studies provided data on Leptospirosis[12–20], seven on Brucellosis[21–27], seven on Q-fever[19,23,28–31] , six on Rickettsioses[19,28,32–35], eighteen on Dengue[13,15,19,20,28,34,36–47], thirteen on Chikingunya[15,20,34,37,40,42,44–50], three on Zika [43–45], two on Histoplasmosis[51,52] and none on PCP. Details of the included studies are provided below.

Table 1.1: Search terms for fever studies

Organism	Search	n_abstracts	n_included
Leprospirosis	Leptospir AND	187	9
Brucellosis	Brucell AND	123	7
Q-fever	((Q fever) OR (coxiella)) AND	315	7
Rickettsioses	(Ricketts OR typhus OR (spotted fever)) AND	375	6
Arboviruses	(dengue OR chikungunya OR arbovir) AND	1422	Dengue 18, Chikungunya 13, Zika 3
Histoplasmosis	Histoplasm AND	72	2
PCP	(((((PCP) OR pneumocystis) OR "pneumocystis carini*") OR "pneumocystis jiroveci")) AND	87	0

Note:
All searches inlcuded the sSA country list in addition to the disease-specific terms above.

1.2.2 Defining sepsis

Sepsis is a heterogenous syndrome, with no diagnostic gold standard. In 1991 the first modern sepsis diagnostic criteria were defined in a consensus conference of key opinion makers[53] (Table 1.2). Sepsis was defined as the presence of the systemic inflammatory response syndrome (SIRS) plus infection, with a gradient of severity increasing through severe sepsis (sepsis plus organ dysfunction) to septic shock. These definitions were widely adopted as entry points into clinical trials, but ongoing concerns that SIRS was both insensitive and nonspecific for the diagnosis of sepsis led to an expansion of the diagnostic criteria in 2001[54] again by expert consensus. Despite these revised guidelines the SIRS criteria largely continued to be preferred both as the entry point to clinical trials of sepsis and in clinical practice until the development of the current sepsis-3 definitions in 2016[55].

The sepsis-3 definitions redefined sepsis as "life threatening organ dysfunction triggered by infection", a definition that rendered the sepsis-2 severe sepsis category obsolete. In contrast to the previous diagnostic criteria that had relied largely on expert opinion, the sepsis-3 criteria attempted to use a probabilistic approach to defining sepsis, by mandating that sepsis should be associated with excess mortality. The sequential organ dysfunction score (SOFA, Table 1.9, Appendix), an organ-dysfunction score already in use in high income settings, and shown to be associated with mortality[56] was selected to operationalise the definition of sepsis. An acute change in SOFA of 2 or more points defines sepsis under sepsis-3.

Mindful that the SOFA score requires a large number of variables and is difficult to apply at the bedside, the consensus guideline group suggest the use of a simpler score, quick SOFA to identify patients who may have sepsis. Any two of: altered mental status, SBP < 100mmHg or respiratory rate > 22 defines a positive qSOFA score. qSOFA does not define sepsis; rather, under sepsis-3 patients with a qSOFA score of 2 or more are at increased risk of poor outcomes and should be screened for sepsis using a full SOFA score. The qSOFA was derived by identifying factors associated with mortality in large datasets of patients with infection from the United States and validated in further US and German datasets; in these datasets it showed good discriminant ability to predict mortality, equivalent to full SOFA score outside the intensive therapy unit (ITU)[57].

Finally, sepsis-3 defines septic shock as persistent hypotension requiring vasopressors to maintain mean arterial blood pressure (MAP) above 65mmHg and serum lactate greater than 2mmol /L. This definition was arrived at by a combination of consensus and systematic review to identify potential defining variables and validation in large datasets from the United States, where it was found to be strongly associated with mortality[58].

1.2.3 Applicability of sepsis-3 definitions in sub-Saharan Africa

Application of the sepsis-3 definitions, both in terms of clinical use and as inclusion criteria for research studies in sub-Saharan African low resource settings, is problematic. Several of the domains of SOFA require the results of blood tests, which may not be available. In Blantyre, and elsewhere in sSA, intensive organ support with inotropes or mechanical ventilation (invasive or non-invasive) may not be available[59] or be difficult to access[60], yet use of these treatment modalities form components of the SOFA score. Both lactate measurement and inotropic support may be unavailable in some settings and yet these define septic shock. Five studies have validated the qSOFA score in sub-Saharan African settings[6,61–64] and found variable discriminant ability for mortality but it is not clear how this score should be deployed in this setting; no studies have been undertaken to link qSOFA score to clinical action, and it is not intended to define sepsis under sepsis-3. The optimal sepsis definitions (both clinical and for research) for sSA are therefore not clear.

Table 1.2: Sepsis diagnostic criteria

Definition	Diagnosis	Criteria
Sepsis-1 (1991)	SIRS	Two or more of: Temperature > 38°C or < 36°C, Heart rate > 90 /min, Respiratory rate > 20 /min or PaCO2 < 32mmHg (4.3 kPa), White blood cell count > 12 x 10-9 /Lor < 4 x 10-9 /L or > 10% immature forms
	Sepsis	SIRS plus proven or suspected infection
	Severe Sepsis	Sepsis plus acute organ dysfunction
	Septic shock	Sepsis with persistent hypotension after fluid resuscitation
Sepsis-2 (2001)	Sepsis	Infection documented or suspected and some of the following General variables: temperature > 38°C or < 36°C, heart rate > 90 min-1 or > SD above normal for age, tachypnoea, altered mental status, significant oedema or positive fluid balance (> 20ml/kg over 24hrs), hyperglycaemia > 7.7mmol /L Inflammatory variables: white blood cell count > 12 x 10-9 /L or < 4 x 10-9 /L or > 10% immature forms, plasma C-reactive protein > SD above normal, plasma procalcitonin > 2 SD above normal Haemodynamic variables: arterial hypotension (SBP < 90 mmHg or MAP < 70 mmHg or SBP decrease > 40mmHg in adults or 2SD below normal range, SvO2 > 70%, Cardiac index > 3.5
	Severe sepsis	Sepsis plus organ dysfunction Organ dysfunction variables: arterial hypoxaemia (PaO2 / FiO2) < 300, acute oliguria (urine output < 0.5 ml kg-1 hr -1 for at least 2 hours), creatinine increase > 0.5mg/ dL, coagulation abnormalities (INR > 1.5 or aPTT > 60s), ileus, thrombocytopenia (platelet count < 100,000 /mL, hyperbilirubinaemia (plasma bilirubin > 4mg /dL or 70 mmol /L
	Septic shock	Sepsis plus hypotension SBP < 90mmHg or MAP < 60mmHg or reduction in SBP of 40mmHg from baseline despite adequate volume resuscitation
Sepsis-3 (2016)	Sepsis	Infection plus life threatening organ dysfunction defined by an acute change in SOFA score of 2 or more
	Septic shock	Persisting hypotension requiring vasopressors to maintain MAP 65mmHg AND serum lactate below 2mmol /L

Note:
SIRS = Systemic Inflammatory Response Syndrome, SD = Standard deviation, SBP = Systolic blood pressure, MAP = Mean arterial blood pressure

1.2.4 Sepsis epidemiology in sub-Sahara Africa

1.2.4.1 Incidence

The changing case definition of sepsis over time hampers estimation of incidence even in high-income settings, furthermore sepsis is not included in global burden of disease estimates. Different methods of

defining sepsis from disease registries can result in very different estimates[65], but a recent systematic review and meta-analysis of 27 studies from 9 high income countries found a recent population incidence rate of 437/100,000 person-years (95% CI 334-571) for sepsis and 270 (95% CI 176 – 412) for severe sepsis with an increasing incidence over time from 1979 to 2015[66]. Crudely extrapolating these estimates to the worldwide population would result in 20.7 million sepsis and 10.7 million severe sepsis cases a year, largely in low resource settings. However, no data are available from low or middle income settings and these estimates must be treated with caution.

1.2.4.2 Risk factors: the sepsis population in sub-Saharan Africa

In high-income settings, risk factors for sepsis have been identified, though once again changing definitions as well as a lack of large scale community based studies make it difficult to draw definitive conclusions. However, chronic diseases (including HIV) and immunosuppressive agents have been associated with increased sepsis incidence, as well as older age[67,68]. In the United States, male sex and black ethnicity (vs white) and poverty are associated with increased sepsis incidence and severity[69].

Though equivalent studies aiming to identify risk factors for sepsis in adults in sSA are lacking, it is clear from the available data that HIV-infection is the dominant risk factor there. Summary patient demographics from the 10 identified sepsis studies are shown in Table 1.3; of 2788 included patients with available HIV status, 69% (1809/2788) were HIV infected, and often with advanced disease; of 1278 HIV-infected patients from 5 studies the study median CD4 count ranges from 52-168 cells/μL. In keeping with the epidemiology of the HIV epidemic in Africa, these patients are young, with average ages (variably reported as mean or median) ranging from 30-39 across the studies. These studies recruited an equal proportion of males and females (1444/2812 males, 51%), suggesting that sex is not a risk factor.

These data contrast sharply with the sepsis population in high income settings, from whom the majority of sepsis data have been generated, and who are older and mostly HIV uninfected[67,70,71]. The need for data from sSA to guide sepsis treatment protocols, rather than extrapolating from the high-income setting sepsis population, is clear.

1.2.4.3 Outcomes

Summary outcomes for sepsis and severe sepsis in sSA from the identified studies are presented in Figure 1.1 below. Summary statistics of 28 or 30-day mortality were extracted from identified studies or, if 28- or 30-day data were not available, in-hospital mortality was used. For interventional studies, in order to reflect the "usual-care" mortality, only the usual care arms were included. Pooled mortality estimates were then generated using a random effect meta-analysis of proportions with a generalised linear mixed model (GLMM, the so called binomial-normal model) using the meta package in R. Exact binomial 95% mortality confidence intervals were used throughout.

It is clear that there is significant heterogeneity in outcomes of sepsis and severe sepsis in sSA, likely reflecting diverse patient and pathogen populations and variation in availability of available resources. This heterogeneity means that summary estimates should be interpreted with extreme caution but severe sepsis (49% [95% CI 39-58]), as expected, seems to carry a higher mortality hazard than sepsis (23% [95% CI 12-38]). Data of outcomes beyond 30 days are absent.

How does this compare to high income settings? A recent meta-analysis of population level estimates in high income settings found that a pooled sepsis 30-day mortality estimate of 17% (95% CI 11-26%)[66], though even older cohort studies as well as the more recent large sepsis-3 derivation cohorts have found considerably lower mortalities for sepsis (as defined by sepsis-2) ranging from 4-7%[57,72,73]. Most recent (largely post-2005) estimates of 30-day mortality from severe sepsis range from from 18-29%[65,66,71,74,75]. It seems likely therefore, that both sepsis and severe sepsis 30-day mortality is considerably higher in sSA than in high-income settings. The reasons for this are not clear, but are likely to be multifactorial; resource limitation is likely to play a part but the HIV epidemic in

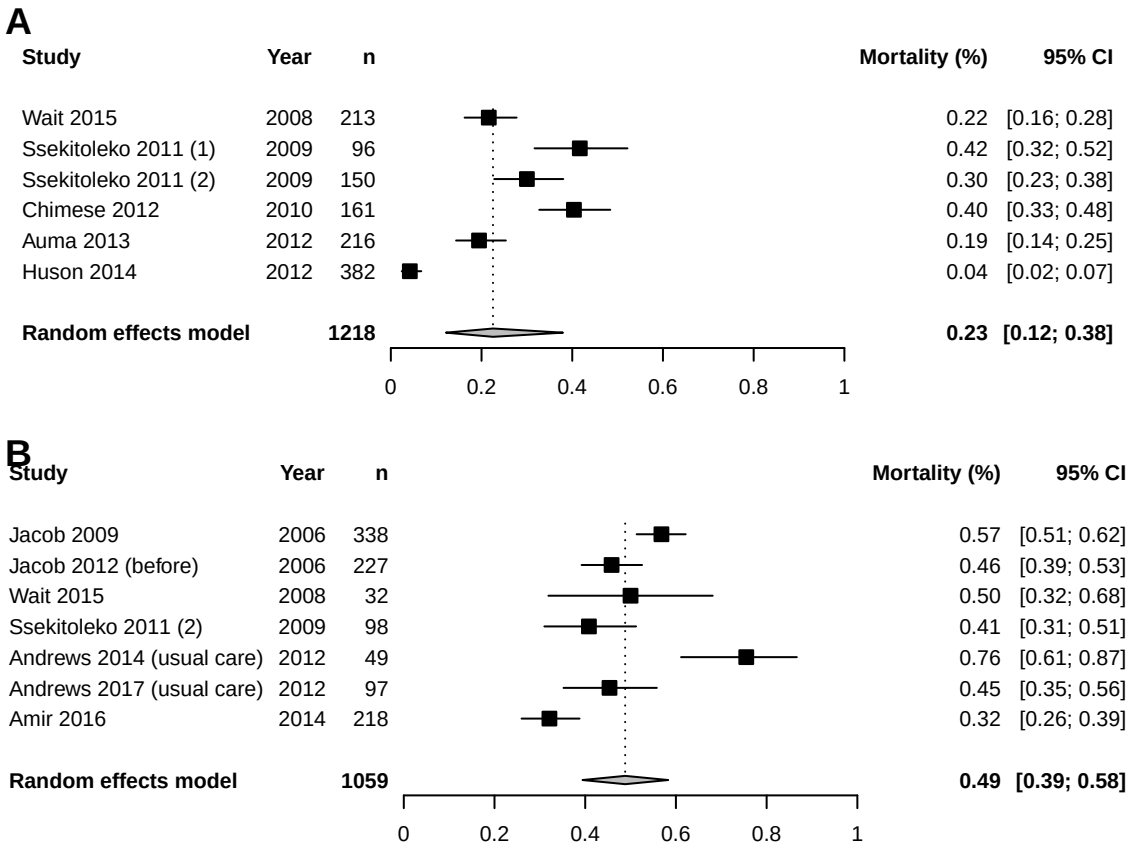

Figure 1.1: Pooled sepsis (A, top) and severe sepsis (B, bottom) inpatient mortality in sSA

Table 1.3: Characteristics of patients recruited to sSA sepsis studies

Study	Type	Year	Country	Inc. criteria	n	Male	Age	HIV infected	Median CD4
Jacob 2009	Cohort	2006	Uganda	Severe sepsis	382	156/382 (41%)	34.8 (11.2)	320/382 (85%)	52 (16-131)
Jacob 2012	Before-after	2006	Uganda	Severe sepsisc	245	95/245 (39%)	34 (28-41)	207/245 (86%)	43 (11-178)
		2008-09			426	207/426 (49%)	34 (27-40)	362/426 (85%)	63 (15-178)
Waitt 2015	Cohort	2008-09	Malawi	Sepsis	213	87/213 (41%)	30 (25-39)	161/213 (76%)	NR
Ssekitoleko 2011 (1)	Cohort	2009	Uganda	Sepsis	96	193/418 (46%)	35.1 (12.0)	331/418b (83%)	NR
Ssekitoleko 2011 (2)	Cohort	2009	Uganda	Sepsis	150	94/150 (63%)	35 (13)	96/150 (64%)	NR
Chimese 2012	Cohort	2010	Zambia	Sepsis	161	79/161 (49%)	39 (15.6)	110/138 (80%)	NR
Andrews 2014	RCT	2012	Zambia	Severe sepsis	112	58/109 (53%)	35 (1.4)	88/109 (81%)	NR
Auma 2013	Cohort	2012	Uganda	Sepsis	216	106/216 (49%)	32 (27-43)	122/216 (56%)	NR
Andrews 2017	RCT	2012-13	Zambia	Severe sepsis	209	117/209 (56%)	36.7 (12.4)	187/209 (89.5%)	66 (21-143)
Huson 2014	Cohort	2012-13	Gabon	Sepsis	384	142/382 (37%)	34 (25-46)	77/384 (20%)	168 (61-438)
Amir 2016	Cohort	2014-15	Uganda	Severe sepsis	218	110/218 (50%)	35 (26-50)	125/218 (57%)	78 (20-202)

Note:
RCT = randomised controlled trial. All studies use a modified sepsis-2 definition of sepsis or severe sepsis. Age is given as median (IQR) or mean (SD). Units of CD4 count are cells/microlitre. Jacob 2012 includes two cohorts of patients – results shown for both separately - and includes data from patients included in Jacob 2009. The n here includes those not included in this publication but the summary estimates include all patients as they cannot be disaggregated

sSA, differing pathogen burden and lack of data and evidence based guidelines to inform optimal management in sSA may also play a role.

In the longer term, sepsis mortality continues to rise after the usual sepsis-study primary end point of 28 or 30 days, though data from sSA are absent. A systematic review in 2010 of long term sepsis mortality identified 26 studies (with none from low-resource settings) that reported long term sepsis mortality; 1 year mortality ranged from 22-72%, increasing to 45-75% at greater than 3 years[76]. Both short and long term morbidity is formidable also, though, once again, data from low income settings including sSA are absent. Cohort studies with no comparator group may not identify morbidity that is sepsis-specific (rather morbidity that is related to critical illness) but new, long-lasting reduction in physical and cognitive function with associated functional impairment have been identified in matched cohort studies in sepsis survivors[77,78]. Health-related quality of life in sepsis survivors in high-income settings have been found to be persistently below population norms[76]. Increased incidence of cardiovascular disease, renal failure and further episodes of infection are seen following a hospital discharge for sepsis[79–81]. Long term sepsis outcomes in sSA are unknown.

Table 1.4: Aetiology of sepsis in sSA

Study	BSI	MTB BSI	Malaria
Jacob 2009	48/382 (13%)	156/382 (22%)	34.8 (15%)
Jacob 2012	83/671 (12%)	104/576 (18%)	83/671 (12%)
Waitt 2015	33/213 (15%)	ND	26/213 (12%)
Ssekitoleko 2011 (1)	ND	ND	ND
Ssekitoleko 2011 (2)	39/150 (26%)	ND	7/150 (5%)
Chimese 2012	27/161 (17%)	ND	ND
Andrews 2014	26/109 (24%)	32/81 (40%)	2/109 (2%)
Auma 2013	41/216 (19%)	ND	9/216 (4%)
Andrews 2017	29/209 (14%)	43/187 (23%)	3/47 (6%)
Huson 2014	39/384 (10%)	NR	130/384 (33%)
Amir 2016	ND	ND	ND
TOTAL	**365/2493 (15%)**	**234/1093 (21%)**	**311/2139 (15%)**

Table 1.5: BSI isolates in sepsis in sSA

Organism	N
S. aureus	109
Non-Typhoidal Salmonellae	84
S. pneumoniae	67
Non-salmonellae Enterobacteriaceae	46
Cryptococcus spp.	20
S. Typhi	6
Other	33
TOTAL	**365**

Note:
Excluded are coagulase-negative Staphylococci, alpha-haemolytic Streptococci other than Pneumococcus, Bacillus spp. and Micrococci as likely contaminants.

1.2.5 Sepsis aetiology in sub-Saharan Africa

The 11 identified prospective sepsis studies in sSA carried out various combinations of diagnostic testing for malaria (either microscopy or rapid diagnostic test) and aerobic and mycobacterial blood culture; a summary is shown in Table 1.4 and 1.5 below. The commonest bloodstream infection (BSI) in all studies where mycobacterial blood cultures were carried out was tuberculosis – present in a higher proportion than of all BSI isolates from aerobic culture combined - though it is important to note that mycobacterial blood cultures in most studies were carried out in HIV infected people and bacteraemic tuberculosis is almost exclusively HIV-associated. The importance of bacteraemic tuberculosis as a cause of sepsis is further examined in an individual patient data meta analysis in chapter 3. With the exception of one study, malaria was less common than BSI, highlighting the importance of non-malarial fever in sSA as malaria control efforts reduce the burden of malaria.

1.2.5.1 Bacterial zoonoses, Rickettsioses and arboviruses

There are several reasons to suspect that aetiologic agents other than bacterial BSI and tuberculosis may be significant in sSA, though data in sepsis are sparse. Studies of febrile illness in sSA have

implicated Rickettsioses, arboviruses and bacterial zoonoses as causes of fever, accounting for a third of fever in hospitalised adults in one study in Tanzania[82]. Historically, however, data on these pathogens have been lacking. A 2015 systematic review of fever aetiology in LMIC (considering studies from 1980-2013) found that small numbers of patients had been systematically screened for these pathogens: in sSA 40/453 (8.8%) of adults with fever fulfilled diagnostic criteria for Leptospirosis, 16/453 (3.5%) for Brucellosis, 36/450 (8.0%) for spotted fever group Rickettsiosis, 24/482 (5%) for Q-fever and 55/700 (7.9%) for Chikingunya[83].

Increasing interest in non-malarial fever, however, has meant that data are accumulating from different settings in sSA, post-2013, as identified by the systematic review of the literature performed for this thesis. Details of the studies identified from this review are shown below in Table 1.10 in the chapter Appendix. These data highlight, firstly, the heterogeneity in diagnostics which are used for these pathogens – a combination of serology, PCR and antigen testing (often not using gold-standard case definitions), and secondly, the spatial and temporal heterogeneity across the continent.

These studies also demonstrate an increase, post-2013, in the use of molecular tests, particularly multiplex PCR assays (TaqMan array cards or PCR macroarrays) to detect multiple pathogens in fever aetiology studies. Despite the attractiveness of these assays – the ability to detect tens of pathogens in one assay on one body fluid sample – many infections will have only transiently detectable pathogen genetic material in blood and as such may have limited sensitivity. The post-2013 fever aetiology data strongly suggest paired sera will maximise the diagnostic yield of bacterial zoonoses and Rickettsioses: for example, in studies of leptospirosis using PCR only 23/2533 (0.9%) of samples were positive versus 75/1464 (5.1%) in studies using paired sera; for Q-fever 9/3811 (0.2%) of samples were positive in PCR only studies versus 25/370 (6.8%) for paired sera studies; for Brucellosis PCR only studies 15/1005 (1.5%) of samples were positive versus 39/562 (6.9%) for paired sera studies; and for Rickettsioses 55/1932 (2.8%) of samples were positive for PCR studies vs paired sera 63/364 (17%). Some care must be taken with this conclusion: there are no studies that aim to directly compare paired sera and PCR assays for diagnosis of febrile illness, so the possibility of confounding remains.

Available data therefore suggest that bacterial zoonoses, Rickettsioses and arboviruses are significant causes of febrile illness in sSA. Their role is sepsis however is unknown. Only two studies have directly addressed the question of sepsis aetiology beyond BSI, malaria and TB: the first[84] performed PCR for 43 pathogens (using a TaqMan array card) including viruses (including dengue, chikungunya, and causes of viral haemorrhagic fever), bacteria (including *S. pneumoniae*, *E. coli*, *Salmonella spp.*, *S. aureus* as well as *Coxiella burnetti*, *Rickettsia spp.*, *Brucella spp.* and *Leptrospira spp.*), Mycobacterial (including *M. tuberculosis* (MTB) and *M. avium* complex), fungal (*Cryptococcus* and *Histoplasma spp.*) and parasitic (including malaria) on a convenience sample of 336 stored plasma samples from a Ugandan sepsis study. In keeping with the original study, MTB was frequently identified as was pneumococcus and malaria. Cytomegalovirus (CMV) was detected in 139/336 (41%) of patients, and was found to be independently associated with death, a finding which has been seen in sepsis studies in high-income settings[85] and may be related to the immune paresis of sepsis and CMV viraemia rather than disease. This study had no pathologic specimens and could not address this question. Dengue was detected in 17/336 (5%) of patients; Rickettsia spp. in 6/336 (2%), Leptospira spp. in 2/366 (0.6%) and Coxiella burnetti and Brucella spp. in 1/336 (0.3%) each. The true burden of disease of these pathogens may be higher, given the potential for increased diagnostic yield fom serological assays.

The second study[86] is a retrospective analysis of a fever aetiology cohort from Tanzania, in which paired serology for bacterial zoonoses and Rickettsioses was carried out, as well as arboviral PCR. Of 423 enrolled adults, 25 were retrospectively classified as having septic shock, 37 severe respiratory distress without shock and 109 severe pneumonia by WHO Integrated Management of Adolescent and Adult Illness (IMAI) District Clinician Manual criteria[87]. These patients would likely fulfil sepsis criteria under sepsis-2 or 3 guidelines, and were found to have a variety of diagnoses, though not all patients had all diagnostic tests: Chikungunya (6/154 [3%]),Leptospirosis (5/82 [6%]), Coxiella burnetti (7/83 [8%]) and spotted fever group Rickettsioses (6/83 [7%]).

1.2.5.2 HIV opportunistic infections: PCP, histoplasmosis and cryptococcal disease

The burden of HIV opportunistic infections in sepsis in sSA (including PCP, cryptococcal disease and including here Histoplasmosis as an opportunistic infection) is unclear. Beyond blood culture identification of *Cryptococcus neoformans* (present in 20/365 of positive blood cultures in the sepsis studies identified in this review) none of these pathogens have been systematically sought in sepsis cohorts in sSA, and their role as causative agents of sepsis is far from clear. Cryptococcal disease most commonly manifests as cryptococcal meningitis, is common in HIV infection and is thought to account globally for 15% of AIDS-related deaths[88]. It is likely therefore to contribute significantly to aetiology of sepsis; of the 11 identified sSA sepsis cohorts, three[4,5,9] provide data on suspected site of infection, and CNS infection accounts for 14-31% of the total, of which cryptococcal disease is likely to be responsible for a large proportion. One study2 performed CSF examination on 41/213 patients for suspected meninigoencephalitis. Of these, 3/41 cultured *C. neoformans*.

No study has attempted to define the burden of PCP in sepsis in sSA, though a 2016 systematic review[89] addressed the prevalence and attributable mortality of PCP. Searches were limited to post-1995; 48 studies were identified comprising 6884 individuals from 18 countries, with a varying patient population including inpatients and outpatients with respiratory presentation or clinical or radiological community acquired pneumonia, often sputum smear negative for TB, and some autopsy studies. A number of diagnostic tests including bronchoscopy and bronchoalveolar lavage were carried out. Many of the inpatient cohorts would include patients with sepsis; the pooled prevalence of PCP in inpatients (n = 2593, 23 studies) was 22% (90% CI 17 – 27%) in random effect meta-analysis. Clearly there are significant difficulties with obtaining lower respiratory tract specimens in unwell hypoxic, shocked or obtruded patients; newer serologic tests (1,3, beta-d glucan) which have reasonable diagnostic characteristics for PCP in high-income settings[90] and may have a role to play, but no study in sSA has attempted to use or validate this assay in any condition.

Data examining the role of Histoplasmosis as a cause of fever or sepsis in sSA are sparse. A 2015 systematic review[83] identified only one study up to 2013 which Histoplasma urine antigen testing in 628 febrile adults and children in Tanzania and acute serum testing on a subset of 200, finding 9/628 (1%) probable cases, 6/9 of whom were HIV infected. Since then, two studies have addressed histoplasma prevalence in varying conditions: the first, in Uganda, enrolled HIV-infected patients with suspected meningitis[51] and found 0/151 patients had detectable IgM to *Histoplasma capsulatum* and no Histoplasma antigen was detected in serum (n = 57), urine (n = 37) or CSF (n=63). The second study in Cameroon[52] recruited HIV infected patients with CD4 $<$ 200 cells/μL, chronic cough and Histoplasmosis like skin manifestations. Histopathologic examination and culture found Histoplasmosis in 7/56 (13%) of patients over 3 years.

1.2.6 Sepsis management

The cornerstone of sepsis management is rapid administration of appropriate antimicrobial therapy, source control of any infectious focus and normalisation of tissue perfusion using intravenous fluids and, if necessary, inotropes, with other organ support as necessary (e.g. intubation and mechanical ventilation and renal replacement therapy). Several international guidelines for sepsis care are available; this section will examine these and specific guidance for sepsis in adults in sSA followed by a review of the evidence to inform these guidelines.

The surviving sepsis campaign has published four editions of comprehensive guidance on the management of sepsis in adults, which are endorsed by all the major critical care organisation in high income settings and form the basis of most sepsis care in high income settings; selected major recommendations of the latest guidance[91] are shown in Table 1.6 below.

Mindful that guidelines aimed at high-income settings may be impossible to implement in low-resource settings (including large areas of sSA) the Global Intensive Care Working Group of the European Society

Table 1.6: Surviving sepsis campaign guidelines

Recommendation	Strength of recommendation	Quality of evidence
Resusitation		
Administer 30ml/kg of intravenous crystalloid solution, within 3hr of diagnosis of sepsis	Strong	Low
Use frequent reassessment to guide further fluid	BPS	BPS
Use dynamic variables to assess fluid responsiveness (e.g. cardiac output)	Weak	Low
Use vasopressors in patients who remain hypotensive despite adequate fluid resuscitation; target a MAP of 65mmHg	Strong	Moderate
Use noradrenaline as first-line vasopressor	Strong	Moderate
Measure lactate and use lactate normalisation to guide resuscitation in patients with elevated lactate	Weak	Low
Antimicrobials		
Administer broad spectrum antibiotics within 1hr of diagnosis of sepsis	Strong	Moderate
Adjunctive therapies		
Use hydrocortisone 200mg IV per day if adequate fluid resuscitation and vasopressor therapy are unable to restore haemodynamic stability	Weak	Low

Note:
BPS = best practice statement

of Intensive Care Medicine (ESICM) published recommendations for sepsis management in resource-limited settings in 2012[92], endorsed by a number of national and international sepsis organisations, and supplements in 2016-17 covering general supportive care[93], infection management[94], management of severe malaria and severe dengue[92] and haemodynamic assessment and support[95] in sepsis in low-resource settings. The major recommendations of this guidance are consolidated in Table 1.7 below.

The World Health Organisation (WHO) in 2011 published the integrated management of adolescent and adult illness (IMAI) guidance[87], which includes guidance on the management of septic shock and is aimed at district-level clinicians in resource limited settings rather than critical care clinicians. This suggests defining shock as SBP < 90mmHg or pulse > 110/minute and suggest that, once shock is identified, oxygen should be given, a 1 litre bolus of fluid should be given immediately and pulse, SBP and signs of perfusion (urine output, mental status) should be rechecked. If shock persists, another litre should be given; if shock persists after the second litre then help should be sought. Antimicrobials should be administered: ceftriaxone IV or IM, and antimalarials if indicated. No evidence base is referenced for these recommendations.

1.2.6.1 Early goal directed therapy

In 2001 a pivotal single centre study in the United States of 263 patients with severe sepsis or septic shock[96] found that protocolised aggressive early resuscitation (called Early Goal Directed Therapy, EGDT) significantly reduced mortality from 46.5% to 30.5%. EGDT called for early central venous catheterisation and protocolised resuscitation to central venous pressure (CVP), MAP and central venous oxygen saturation targets (ScvO2), and was widely adopted. However three large multicentre randomised controlled trials of EGDT – ProCESS in the United States[97], ARISE in Australasia[98] and ProMISe[99] in the United Kingdom, reporting in 2014 and 2015 failed to show any difference in outcomes between the EGDT and usual-care arms. A pre-planned individual level meta-analysis of

Table 1.7: ESICM low resource setting sepsis recommendations

Recommendation	Strength of recommendation	Quality of evidence
Resusitation		
Use capillary refill time, skin mottling scores or skin temperature gradients to assess adequacy of tissue perfusion.	Weak	Ungraded
Use passive leg raise (PLR) to guide fluid resuscitation in sepsis or septic shock	Weak	High
Use crystalloid for fluid resuscitation	Strong	Moderate
Give 30ml/kg of fluid over the first 3hr following sepsis diagnosis, to start within 30mins of recognition	Strong	High
Larger volumes of fluid may be needed if the patient remains fluid responsive and still shows signs of tissue hypoperfusion	Strong	Low
Be extremely cautious in settings with no or limited access to vasopressors and mechanical ventilation and consider stopping fluid if respiratory distress or lung crepitations develop	Strong	High
Use noradrenaline as first line vasopressor	Strong	Moderate
Target a MAP of $>$ 65mmHg	Strong	Moderate
Antimicrobials		
Appropriate antibiotics should be given within the first our following septic shock	Strong	Low
Source control should occur within 12hr of admission to hospital	Ungraded	Ungraded
Adjunctive therapies		
Use hydrocortisone 200mg IV per day if adequate fluid resuscitation and vasopressor therapy are unable to restore haemodynamic stability	Weak	Low

Note:
MAP = Mean arterial blood pressure

the 3723 patients included in these trials confirmed similar 90 day mortality in both arms (24.9% for EGDT vs 25.4% for usual care, aOR 0.97 [95% CI 0.82-1.14]) with no benefit found in pre-planned subgroup analysis for patients with worse shock or in hospitals with lower propensity for vasopressors or fluid administration[71]. It is likely therefore that the tenets of EGDT that improve outcomes (early antimicrobials and aggressive fluid resuscitation) have been absorbed into usual care in the fifteen years since the original EGDT study, as evidenced by the reduction is sepsis mortality over this time period, and so the specific package of protocolised care and EGDT targets does not in itself improve outcomes. Unanswered questions now remain regarding the most effective use of the individual components of EGDT (fluids, vasopressors etc). A number of attempts have been made to develop protocolised sepsis care packages in the style of EGDT for sSA; these are described below in relation to the individual components of sepsis care.

1.2.6.2 Evidence to guide antimicrobial therapy in sSA

There is evidence from high income settings that delay in appropriate antimicrobial administration is associated with worse outcomes in sepsis. The first study to investigate this relationship, published in 2006, found a very strong relationship between time to appropriate antimicrobial administration from onset of hypotension and mortality with an absolute increase in mortality of 7.6% for each hour

delay over the first six hours[100]. Subsequent data have been more nuanced: a 2015 meta-analysis addressing this question identified 11 studies of 16,178 patients and found no relationship between antimicrobial delay and mortality[101], though many of the included studies are open to confounding by indication (sicker patients are given antimicrobials more quickly), timed antimicrobial administration to non-physiological events (e.g. arrival to hospital or time of blood culture draw rather than onset of hypotension) and did not assess the appropriateness of antimicrobial therapy, all of which could mask a relationship. Appropriate antimicrobial therapy has certainly been shown to be associated with improved survival: a 2010 meta-analysis quantified the pooled adjusted odds ratio to be 1.6 (95% CI 1.4-1.9) from 26 studies for appropriate versus inappropriate antimicrobial therapy[Paul2010]. A recent large retrospective study of 49,331 patients in New York hospitals[70] confirmed the relationship between antimicrobial delay and mortality with an adjusted odds ratio of in-hospital death of 1.04 per hour delay (95% CI, 1.03 – 1.06), and rapid antimicrobial administration forms a key recommendation of current sepsis guidelines.

Data from sSA are lacking, however; neither of the meta analyses above (including between them 37 studies) included any data from sSA, but three of the sepsis studies identified in this systematic review attempt to address the question. The first[1], in an observational study of 382 adults with severe sepsis in Uganda found no association between administration of antibiotics within 1 hour and mortality (OR 0.9 [95% CI 0.6-1.6]) but a total of 42 antibiotic regimens were used and there was a high proportion (22%) of bacteraemic tuberculosis; no assessment of appropriateness of antimicrobials was undertaken and it is possible that inappropriate antimicrobials could mask any association between time of administration and mortality, if one existed.

The second[11], interventional, study in the same centres in Uganda used a before-after design with 661 patients to implement a clinical-officer delivered fluid resuscitation protocol (see below) and administration of antimicrobials. 426 patients were included in the intervention with 245 in the usual care group. The protocol resulted in more rapid administration of antibiotics (67% administered within 1hr versus 30%, $p < 0.001$) and less (though still very prevalent) inappropriate antimicrobial administration (81% versus 95%, $p < 0.001$). Antimicrobial administration was associated with a reduced hazard of death in a multivariable Cox proportional hazards model, but the comparator group used was patients who received no antimicrobials and the hazard ratio for rapid administration ($<$ 1hr HR 0.44 [95% CI 0.21 – 0.89]) was not significantly different from delayed administration ($>$ 6hr HR 0.39 [95% CI 0.19 – 0.81]). This type of study design is very prone to bias due to confounding as sepsis management changes over time, especially as the "before" arm was recruited two years before the "after" arm, so results from this study should be interpreted with caution.

A third observational study in a Ugandan teaching hospital[102] provides data on the effect of rapidity of administration of antimicrobials; this study enrolled 218 patients; 89% of them received any antibiotics within 6 hours, with a median time to antibiotic administration of 30mins. Antibiotic administration within 6hr (versus not) was not significantly associated with in hospital mortality in univariate analysis (OR 1.5 95% CI 0.6 – 3.8) though the confidence intervals are wide and could incorporate a clinically significant effect. Again, no assessment of appropriateness of antimicrobials was made.

Only one study provides limited evidence that appropriate antimicrobial therapy improves outcomes in infection in sSA[103]: a combined retrospective-prospective analysis of 104 patients with typhoid perforation (defined by clinical and operative findings rather than culture) from a single Tanzanian teaching hospital found that adequate antimicrobial exposure (defined as at least 3 days of antimicrobial active against S. Typhi prior to hospital admission) was associated with improved in-hospital survival in multivariable analysis (aOR 2.9 [95% CI 2.1-4.5]), however it is doubtful that this very specific complication of typhoid fever is generalizable.

1.2.6.3 Evidence to guide intravenous fluid therapy in sub-Saharan Africa

The evidence base for rapid fluid administration – and the surviving sepsis recommendation of 30ml/kg within 3hrs following diagnosis - is less secure than for rapid antimicrobial administration. As with

antimicrobial administration, adoption of guidelines in response to the EGDT study has meant that disentangling the independent effect of fluid administration is difficult. The data are contradictory. Several large retrospective observational analyses have found no impact on rapidity of fluid bolus administration following sepsis diagnosis: one multicentre study of 2796 adults with severe sepsis[104] found no propensity adjusted difference in in-hospital mortality for patients with shock or elevated lactate whether they received fluid bolus within the first 6hr following diagnosis (aOR 1.01 [95% CI 0.73 – 1.39]); the New York study of 49,331 septic adults described above[70] found no association between time to completion of fluid bolus and mortality (aOR 1.01 per hour [95% CI 0.99 – 1.02]). Indeed, fluid clearly has the potential for harm; positive fluid balance for patients with sepsis in the ITU has been persistently linked with worse outcomes[@[[70]; Boyd2011; Vincent2006].

In contrast, several studies contradict these findings; a retrospective single centre of 594 adults with severe sepsis or septic shock[105] found improved mortality in patients who had a higher proportion of 6-hour fluid administered in the first 3hr, when adjusted for total volume of fluid administered over 6hr (aOR 0.34 [95% CI 0.15 – 0.75]); a larger retrospective multicentre study of 11,182 patients with sepsis and hypotension[106] found an independent mortality benefit for early intravenous crystalloid administration, with fluid administration within 30mins having the largest effect (aOR 0.74 [95% CI 0.62 – 0.87] versus > 120mins). A prospective study of 1866 patients from the same authors[107] had similar findings (aOR 0.63 [95% CI 0.46-0.86]).

It may be that heterogeneity in response to fluids plays a role in these conflicting findings; a retrospective multicentre cohort analysis of 3686 patients[108] found that 64% were "fluid responders" – that is, they had a sustained blood pressure response to initial fluid resuscitation without need for vasopressors. Heart failure, hypothermia, altered gas exchange, initial lactate > 4.0mmol/L, coagulopathy and immune compromise (including HIV/AIDS) were associated with fluid nonresponse, as was fluid initiation greater than 2 hours after sepsis diagnosis. Mortality was 15% greater (95% CI 10-18%) in fluid nonresponders.

In sSA, there is increasing evidence that liberal intravenous fluid administration to septic patients causes harm. The landmark FEAST trial[109] randomised 3141 children with severe febrile illness in Kenya, Uganda and Tanzania to receive either albumin bolus or 0.9% saline bolus or usual care and found an increased risk of death by 48 hours in both bolus groups (RR 1.45 [95% CI 0.78-1.29] for any bolus compared to no bolus). In a secondary analysis[110] this was thought to be due to cardiovascular collapse rather than pulmonary oedema; the mechanism of this is unclear.

Only three controlled studies have addressed the question of optimal intravenous fluid resuscitation for septic adults in sSA; the first is the before-after intervention study in septic shock patients carried out in Uganda and described above[11]. 426 patients were included in the intervention with 245 in the usual care group; the intervention consisted of clinical-officer delivered protocolised care over the first 6 hours of hospital admission. The intervention increased fluid administration over 6 hours (3.0L vs 0.5L, $p < 0.001$) and 24 hours (3.5L vs 1.0L, $p < 0.001$), and more patients received fluid within 1 hour (97% vs 55%, $p < 0.001$). The study found a mortality benefit of > 1L fluid over the first 6hr compared to < 1L in multivariable Cox proportional hazard model (HR 0.54 [95% CI 0.35-0.82] for 1.0 – 2.5 L vs < 1.0L) though with the absence of any further dose-response effect above 1L. As stated above, the before-after study design means that this result should be interpreted with caution.

Two randomised controlled trials of protocolised early sepsis care in adults have been carried out at a single centre in Zambia, with a focus on fluid. The first[9] recruited patients with severe sepsis with organ dysfunction criteria including respiratory rate > 40/min. Patients were randomised to usual care or an intervention protocol consisting of a 2L bolus of crystalloid (lactated Ringer's or 0.9% saline) over 1 hour and then, if the jugular venous pressure (JVP) was below 3cm, a further 2L over 4 hours. Fluids were stopped if worsening respiratory signs or symptoms developed. If MAP was below 65mmHg after 2L of fluid, a dopamine infusion was started. Blood was transfused if Hb was < 7g/dL. This trial was stopped early (after recruitment of 109 patients) as it was felt that participants with baseline respiratory compromise (RR > 40 or oxygen saturation < 90%) might be at risk of harm; 7/10 (80%) of this subgroup died in the usual care group, compared to 8/8 (100%) in the intervention group ($p = 0.09$).

The same intervention was then used at the same centre in a similar trial[Andrews2017], this time recruiting patients with two SIRS criteria and hypotension (SBP < 90mmHg or MAP < 65mmHg), but excluding patients with baseline respiratory compromise(RR > 40/min or oxygen saturation < 90%) and randomising them 1:1 to the intervention protocol. 209 patients were recruited and patients in the intervention group (n = 106) at 6 hours received more fluid (median 3.5L vs 2.0L, p <0.001) with more vasopressor use (12% vs 2%, p = 0.01), but similar proportions of blood transfusion (16% vs 12%, p = 0.48). Lactate change by 6 hours was greater in the intervention group (median -1.2 vs -0.5 mmol/L, p = 0.02), but so too was in hospital mortality (48% vs 33%, p = 0.03). The reasons for this are not clear. More respiratory compromise (defined as increase in respiratory rate by 5 breaths/min or reduction in oxygen saturation of 3% or more) occurred in the intervention group (35% vs 22%, p =0.03) but persisted beyond 6 hours in similar numbers in both groups (17% vs 15%, p = 0.63).

1.3 ESBL-E in sub-Saharan Africa

1.3.1 Search strategy

A systematic review of the literature was undertaken to answer the following questions: firstly, what is the prevalence of ESBL-E amongst invasive isolates of *Klebsiella pneumoniae* and *Escherichia coli* infecting humans in sub-Saharan Africa? Secondly, what is the prevalence of gut mucosal carriage of ESBL-E amongst humans in sSA, and what risk factors for carriage have been identified? To this end a search of PubMed and Scopus was carried out using the search terms (((ESBL) OR Extended-spectrum beta-lactamase)) AND (((Angola OR Benin OR Botswana OR Burkina Faso OR Burundi OR Cameroon OR Cape Verde OR Central African Republic OR Chad OR Comoros OR Republic of the Congo OR Congo Brazzaville OR Democratic republic of the Congo OR Cote d'Ivoire OR Djibouti OR Equatorial Guinea OR Eritrea OR Ethiopia OR Gabon OR The Gambia OR Ghana OR Guinea OR Guinea-Bissau OR Kenya OR Lesotho OR Liberia OR Madagascar OR Malawi OR Mali OR Mauritania OR Mauritius OR Mozambique OR Namibia OR Niger OR Nigeria OR Reunion OR Rwanda OR Sao Tome and Principe OR Senegal OR Seychelles OR Sierra Leone OR Somalia OR South Africa OR Sudan OR Swaziland OR Eswatini OR Tanzania OR Togo OR Uganda OR Western Sahara OR Zambia OR Zimbabwe) OR Africa)).

Inclusion criteria were any study that took place in sSA and allowed the calculation of a prevalence of ESBL-E in *K. pneumoniae* or *E. coli* amongst invasive human isolates, or prevalence of human gut mucosal carriage of ESBL-E. Studies were excluded if no ESBL-E confirmatory testing was performed using phenotypic (double disc or combination disc or E-test) or molecular (PCR) methods. Invasive isolates were defined to be any blood or CSF sample other usually sterile fluid, or urine or wound swabs with clinical suspicion of infection. On 8th December 2018 this search identified 2975 unique studies; after abstract review 192 underwent full-text review, resulting in the inclusion of 86 studies, 54[111–163] providing data on invasive infection and 32 [164–196] 167–199 on carriage. Details of these studies are given below. A broad non-systematic review of the literature was also undertaken to place these studies in context and provide a background understanding of the classification and global epidemiology of ESBL-E, using the same literature databases.

1.3.2 Introduction: definition and classification of ESBL-E

Beta-lactamases are enzymes that hydrolyse the active beta lactam ring in beta lactam antimicrobials. Though no standardised definition of ESBL exists, they are usually defined as enzymes which confer resistance via hydrolysis to penicillins, cephalosporins of the first, second or third generation (excluding cephamycins), aztreonam, but not carbapenems, and are inhibited by beta-lactamase inhibitors such as clavulanic acid[197].

Two classification schemes are usually used for ESBL: the molecular (or structural) classification of Ambler[198], or the Bush-Jacoby-Medeiros functional classification[199] (Table 1.8). Molecular

classification is straightforward and depends on protein homology; class A, C and D enzymes are serine beta-lactamases and class B are metallo-beta lactamases, named for the composition of their active site. The functional classification is complex and clusters enzymes into four groups, with a number of subgroups, based on substrates and the effect of beta-lactamase inhibitors and EDTA: class 1 (corresponding to Ambler class C) are cephalosporinases that are not inhibited by clavulanic acid, and includes the AmpC enzymes of the Enterobacteriaceae; class 2 enzymes are beta lactamases that are largely inhibited by clavulanic acid and belong to Ambler class A or C; and class 3 are the metallo-beta-lactamases corresponding to Ambler class B. Class 4 enzymes are penicillinases which are not inhibited by clavulanic acid, though are of limited significance and not included in Table 14. The vast majority of clinically relevant ESBLs (and all of those defined as above) belong to Ambler class A, functional class 2be.

Table 1.8: ESBL classification. Adapted from Bush (2010)

Bush Jacoby group	Molecular class	Distinctive substrates	Inhibited by BLI	Inhibited by EDTA	Defining hydrolysis spectrum characteristics	Representative enzymes
1	C	Cephalosporins	No	No	cephalosporins > benpen, hydrolyzes cephamycins	E. coli AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	No	No	ceftazidime and often other oxyimino-beta-lactams	GC1, CMY-37
2a	A	Penicillins	Yes	No	benzylpen > cephalosporins	PC1
2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin, cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	oxyimino-beta lactams	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	Penicillins	No	No	Resistance to BLI	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	oxyimino-beta lactams plus resistance to BLI	TEM-50
2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	No	cloxacillin or oxacillin and oxyimino-beta-lactams	OXA-11, OXA-15
2df	D	Carbapenems	Variable	No	cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	No	Inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Variable	No	carbapenems, oxyimino-beta-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	Yes	includes carbapenems but not monobactams	IMP-1, VIM-1,CcrA, IND-1
	B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1

Note:
BLI = Beta-lactamase inhibitor

1.3.3 Global molecular epidemiology of ESBL-E: an overview

The history of the global spread of ESBL-E is complex and an enormous number of unique ESBL amino acid sequences have been described; at the time of writing the NCBI beta-lactamase directory contains 1557 named beta-lactamase genes, many of them ESBL. However, there are 3 families which cause the majority of infections in humans: TEM, SHV, and CTX-M. They will be briefly described here in turn in the context of their putative origins and global dissemination in the latter half of the 20th century. A diverse range of other ESBL enzymes have been described, but are largely of less clinical significance than those described above, and are beyond the scope of this review: most notably the OXA type, which in contrast to TEM, SHV and CTX-M, are of the molecular class D and functional class 2d, and are characterised by a high rate of hydrolysis of cloxacillin[200]; like TEM and SHV, OXA beta-lactamases are not always extended-spectrum.

1.3.3.1 1980s-1990s: First identificatiom of ESBL in nosocomial pathogens

Beta-lactamases form an integral part of the natural armamentarium of many genera of bacteria – particularly gram negatives, including Enterobacteriaceae - and predate the antibiotic era; penicillinases were identified in E. coli, for example, prior to the widespread introduction of penicillin for treatment of human disease[201]. These beta-lactamases are often chromosomally located; the first plasmid-mediated narrow-spectrum beta-lactamase, TEM-1 -named for the patient, Temoneira, from whose blood it was first isolated – was found in Athens in the 1960s[202]. It rapidly disseminated globally and is thought to be responsible for a high proportion of ampicillin resistance in E. coli, for example[200]. This worldwide spread spurred the development and use of beta-lactamase resistant extended-spectrum cephalosporin antimicrobials, which found wide use in the 1980s. Perhaps inevitably, an enzyme conferring resistance to extended-spectrum oxyimino-cephalosporins was subsequently identified in a German clinical *Klebsiella ozaenae* isolate in 1983, carried on a pBP60 plasmid and enzymes of this sort were named ESBLs[203,204].

This first ESBL enzyme was found to be similar to an existing plasmid-borne narrow spectrum beta lactamase, SHV-1, which had been described in the 1970s in E. coli, and was thought to itself be descended from a chromosomal *K. pnemoniae* narrow spectrum beta lactamase which was liberated onto a plasmid[205]. The point mutations in SHV-1 conferred the ESBL phenotype, and this enzyme was named SHV-2. This pattern - mutation of a narrow spectrum beta-lactamase to produce an ESBL phenotype - also occurred in TEM, and the first ESBL TEM was described in France in 1989[206] and named TEM-3. Many TEM and SHV variants were subsequently described[207]. However, in this early stage of the epidemic, ESBL enzymes were largely nosocomial, and often associated with *Klebsiella spp.*[208].

1.3.3.2 1990s-2010s: Emergence and globalisation of CTX-M

From the late 1990s onwards, there were profound changes in the global epidemiology of ESBL-E, on three fronts, all intricately interrelated, and occurring simultaneously: first, the rapid emergence and globalisation of the successful CTX-M ESBL enzyme family[209], aided by mobile genetic elements; second, *E. coli* joining *Klebsiella spp.* as a major ESBL host[210], and the emergence of so-called high risk bacterial clones; and third, the spread of ESBL-E into the community[211]. CTX-M-1 was first identified and named in Germany in 1989[212] and many variants were subsequently identified, largely in *E. coli* and *K. pneumoniae*, from isolates all over the world[213]. CTX-M genes are clustered by homology into 5 groups (CTX-M groups 1,2,8,9 and 25) and each group is thought to have descended from a chromosomal beta lactamase from *Kluyvera spp.*[209]

A year-on-year rise in incidence of invasive ESBL-E infection was seen in most high-income settings (Figure 1.2) throughout the 2000s and 2010s, the majority of which were CTX-M producers, though with varying proportions of different CTX-M enzymes in different locations[[210]; Bevan2017]. Risk factors for ESBL-E infection in high income settings have persistently been shown to be hospital or

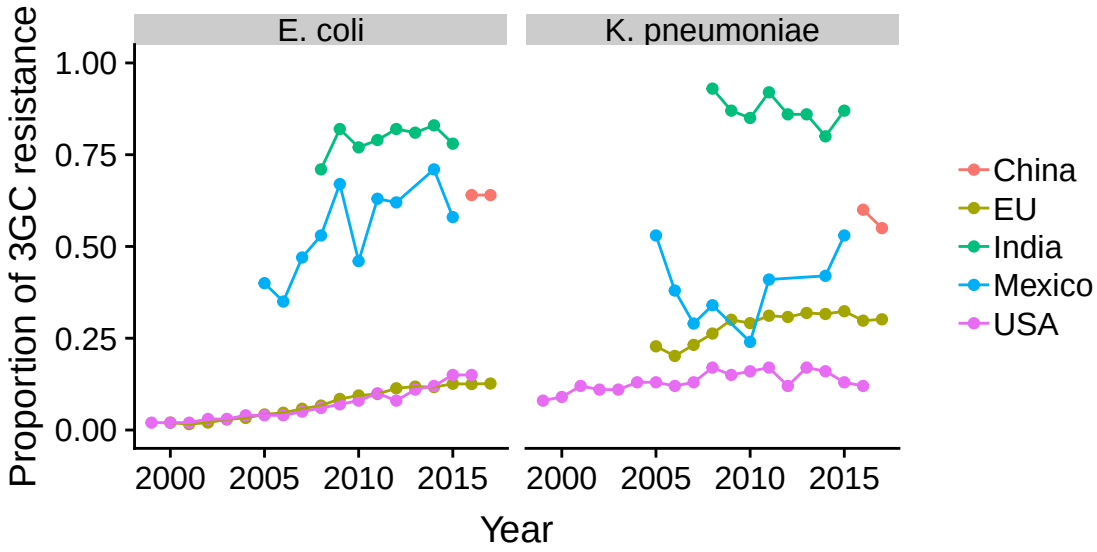

Figure 1.2: Prevalence of third generation cephalosporin resistance in representative high income (EU, USA) and middle income (China, Mexico, India) areas in invasive E. coli (left panel) and K. pneumoniae (right panel) isolates. Data for EU are from ECDC surveillance atlas (https://ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc) and for other countries are from CCDEP resistance map (https://resistancemap.cddep.org/AntibioticResistance.php) both accessed 28 December 2018. 3GC = third generation cephalosporin. Note that these data are 3GC-resistant isolates rather than confirmed ESBL-producers, but would be expected to be ESBL-producers in the vast majority of cases.

long-term care facility exposure, antimicrobial exposure and chronic health conditions though it was recognised in the 2000s that a large proportion of patients with invasive ESBL-E lack any of these risk factors[211], suggesting acquisition in the community. Colonisation prior to infection is thought to be the norm; prior colonisation is a significant risk factor for infection and indeed when sought ESBL-E are found in the stool of healthy community members worldwide (see carriage, below).

Though less comprehensive, data from middle income countries suggests that prevalence of ESBL producers amongst invasive *E. coli* and *K. pneumoniae* are very high (Figure 1.2) and in countries such as India invasive *E. coli* and *K. pneumoniae* that are sensitive to third-generation cephalosporins are in the minority. The reasons for this are not clear but country and regional level associations (which are open to ecologic bias) have been shown with antimicrobial consumption[[214]; Lai2011] and economic status; GDP per capita has been found to correlate inversely at a country level with third-generation cephalosporin resistance rates[215]. Data from sSA have historically been lacking and are systematically reviewed below.

1.3.3.3 Epidemiology of gut mucosal carriage of ESBL-E: the first step towards invasive infection

Invasive infections with Enterobacteriaceae are thought to usually result from infection from an individual's own gut microbiota, irrespective of resistance pattern[216], and whole genome sequencing has confirmed that invasive isolates are often closely related to prior gut carriage isolates[217]. Strategies to minimise carriage are therefore potentially attractive as interventions to reduce invasive infection and a number of studies have attempted to understand the dynamics of gut mucosal ESBL-E carriage in health and disease. A brief overview of ESBL-E community carriage is presented here, and ESBL-E carriage in sSA is systematically reviewed below.

ESBL-E community carriage in Spanish outpatients[218] and healthy children in Poland[219] was first

described in 2001, and subsequently has been identified worldwide when sought[220], though there are heterogeneities between and within countries which mirror the prevalence of invasive ESBL-E prevalence amongst *E. coli* and *K. pneumoniae*. In Europe, for example, community prevalence of ESBL-E carriage was estimated be 7.3% in the UK in 2014 in a large community study[221], 4.5% in the Netherlands in 2012[222] and 4.7% in Sweden in 2012/13[223] and 3.7% in Spain in 2003[224], significantly lower than community carriage prevalence of 50.9% seen in China in 2009[225] or 33.8% in India in 2011-2013[226].

Risk factors for colonisation have been identified in many studies and antimicrobial exposure[227,228] and healthcare facility exposure[[226]; Luvsansharav2012] (including long term care facilities[229]) are consistently identified as such. Colonisation of a household member has also been identified as a risk factor[[230]; Rodriguez-Bano2008], suggesting significant within-household spread. Antacid use has been associated with ESBL-E colonisation[227] as has exposure to farming[@[222]. In low prevalence areas, travel to high prevalence areas is a risk factor[221,223,227,228,231].

The majority of studies of ESBL-E carriage are cross sectional and only a handful have attempted to characterise longitudinal carriage of ESBL-E with a longitudinal sampling approach. Estimates of carriage duration vary, partly because of the difficulty in inferring them from interval-censored rectal swab or stool data, but it is clear that some patients remain colonised for many months. Following a Swedish ESBL-E outbreak, 12% of patients still carried ESBL-E at the final sampling visit, a median 58 months after the outbreak[232]. French and German studies found a median duration of carriage of 4.3[233] and 12.5[234] months respectively following hospitalisation or outbreak. More transient carriage following international travel seems to be the norm with a median of 30 days in a large Dutch study[235]; the reasons for this are not clear.

The largest longitudinal community study of ESBL-E carriage took place in the Netherlands which recruited 76 ESBL-E colonised and 249 uncolonised community members and carried out longitudinal stool sampling at 5 time points over 8 months. 25/76 (32.9%) of initially-colonised participants remained persistently colonised after a median 242 days. Antimicrobial exposure in the past 6 months, proton-pump inhibitor use, colonisation with *E. coli* phylogroup B2 or D and presence of CTX-M-27 or CTX-M-14 was associated with persistent carriage, suggesting both host and bacterial factors may be important determinants of carriage duration. *K. pneumoniae* colonisation seemed to be less common in the persistent carriage group[231,236]. This study also found significant heterogeneity of *E.coli* sequence type in longitudinal samples of persistent carriers but that ESBL genes and often detectable plasmid replicons remained unchanged, suggesting a significant role for mobile genetic elements.

1.3.3.4 Molecular mechanisms underlying success of CTX-M: mobile genetic elements and high-risk clones

The remarkable success of CTX-M has led to efforts to understand the molecular mechanisms by which this enzyme spread so rapidly. The system is complex, and poorly understood, but should be considered at multiple levels including that of the organism; the plasmid; the transposon, which may contain integrons or insertion sequences and, at the lowest level the ESBL gene. These will briefly be reviewed here.

The initial mobilisation event of CTX-M from *Kluyvera spp.* is thought to have been mediated by capture of transposable insertion sequences; the insertion sequence ISEcp1 has been experimentally demonstrated to mobilise the CTX-M precursor from *Kluyvera ascorbata*[237] and ISEcp1 is most consistently associated with CTX-M genes but IS26, ISCR1 and IS10 have also persistently been described upstream from CTX-M genes, suggesting multiple mobilisation events[238]. There is also an association between particular pairs of CTX-M gene clusters and insertion sequences, consistent with a hypothesis of multiple mobilisation events[239]. These insertion sequences provide two roles: they encode a transposase enabling gene mobilisation but act as a strong promotor of CTX-M, without which phenotypic cephalosporin resistance is absent or reduced[240].

After mobilisation from the *Kluyvera* genome, the CTX-M genes were integrated onto a plasmid

backbone, a process which is likely ongoing as a substantial number of diverse CTX-M carrying plasmids have been described: there is, however, an association between CTX-M genotype and plasmid incompatibility group. The successful CTX-M 15 gene is very strongly associated with the narrow host-range IncF plasmid group, for example, which are restricted to Enterobacteriaceae[[240]; Carattoli2009]. Identical CTX-M containing plasmids have been found across diverse geographical regions and have been termed "epidemic plasmids"[238] though the mechanism of persistence of these plasmids within a bacterial population remains unclear.

In addition to frequently co-occurring CTX-M genes, transposable elements and plasmids, some clonal groups of *E. coli* and *K. pneumoniae* are both globally successful and associated with particular CTX-M genes and plasmids. These successful sequence types (STs) are known as "high risk clones." The archetypal example is *E. Coli* ST131 which is often associated with an IncFII plasmid containing CTX-M-15[241]. First described in 2008, *E. coli* ST131 is thought to be responsible for around 80% of extra-intestinal ESBL E. coli infection[242]. Population genomics studies have demonstrated that a particular clade, ST131 clade C, is globally dominant and have shown a sequential acquisition of virulence determinants followed by mobile genetic elements conferring fluoroquinolone and ESBL resistance[243,244]. These events may have contributed to the global success of ST131, but the precise mechanism of its apparent fitness advantage remains unknown.

1.3.4 Epidemiology of ESBL-E in sub-Saharan Africa

Of the 86 studies identified by the systematic literature review, 54 studies provided data on invasive ESBL-E and 32 provided data on human carriage is sSA. These are considered in turn below.

1.3.4.1 Invasive ESBL-E infection

Table xx in appendix xx shows the 54 included studies in this analysis, which provide data on 6067 *E. coli* and 2974 *K. pneumoniae* isolates. All studies were cross sectional in design. Of the 54, 18/54 were laboratory based (i.e. a survey of all samples received in the laboratory); 17/54 were truly invasive in that they included predominantly blood culture; a combination of urine, CSF, and wound swabs were included in the remaining studies. 36/54 studies provided data on adults and children; 6/54 on adults only; and 12/54 on children only. The majority of studies (42/54) include both community and nosocomial acquired infection. Of the remainder, 3/54 provided data on nosocomial infection only. Figure **??**A shows a map of available data by country; data are available from across the continent though Nigeria (8 studies) and Tanzania (7 studies) are over represented and many countries provide no data.

The proportion of ESBL producers amongst invasive E. coli and K. pneumoniae in sSA is heterogeneous but many studies show extremely an extremely high prevalence (Figures **??**B and **??**C), comparable to that seen in the Indian subcontinent and other high-prevalence areas and highlighting the scale of the public health problem posed by ESBL-E in sSA. Meta regression shows clear temporal trends of an increase over time: addition of time as a fixed-effect covariate to the random effects model gives improved fit on likelihood ratio testing of nested models ($p < 0.001$ for both *E. coli* and *K. pneumoniae*). Though data are sparse pre-2000, those data that are available suggest that ESBL producing *E. coli* and *K. pneumoniae* were identified in West Africa even in the 1990s: a retrospective laboratory based study in Yaounde, Cameroon on isolates from a variety of clinical samples from 1995-1998 found that 13/91 *E coli.* and 12/64 *K. pneumoniae* were ESBL producers, with the SHV-12 enzyme predominant[155]; in Dakar, Senegal, 6/97 *K. pneumoniae* isolates from community acquired urinary tract infections in 1999-2000 were found to be ESBL producers[158].

Some of the heterogeneity in prevalence does however seem to be explained by sample type; a clearer picture appears when the analysis is restricted to the 16 studies including predominantly blood culture (Figure 1.4)[115,117–122,124,127,128,130,132,137,140,154,163]. In this analysis it seems clear that the worldwide epidemiology of ESBL-E was mirrored in sSA; ESBL initially spread amongst invasive K.

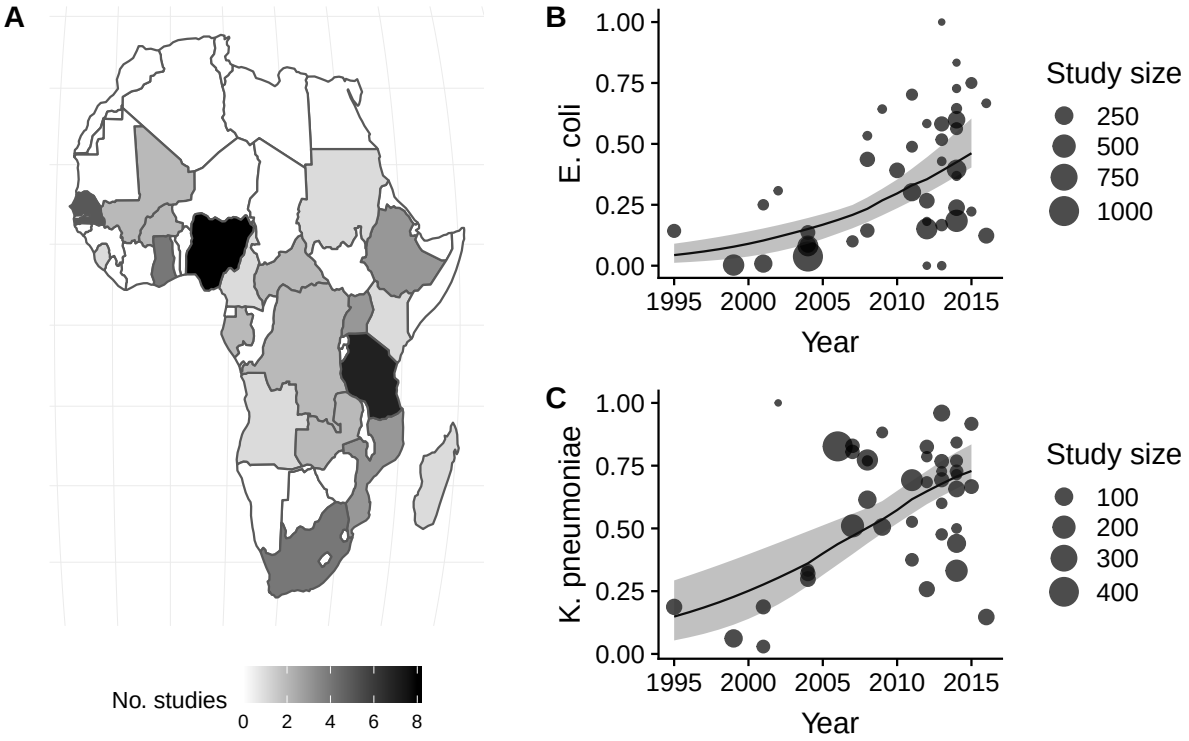

Figure 1.3: Invasive ESBL-E in sSA. A: Available studies by country. B and C: meta regression of proportion of invasive *E. coli* and *K. pneumoniae* respectively as a function of time. In both cases time is statistically significantly associated with proportion of ESBL ($p < 0.001$ on likelihood ratio testing of nested models). 95% CI generated from 1000 bootstrap replicates.

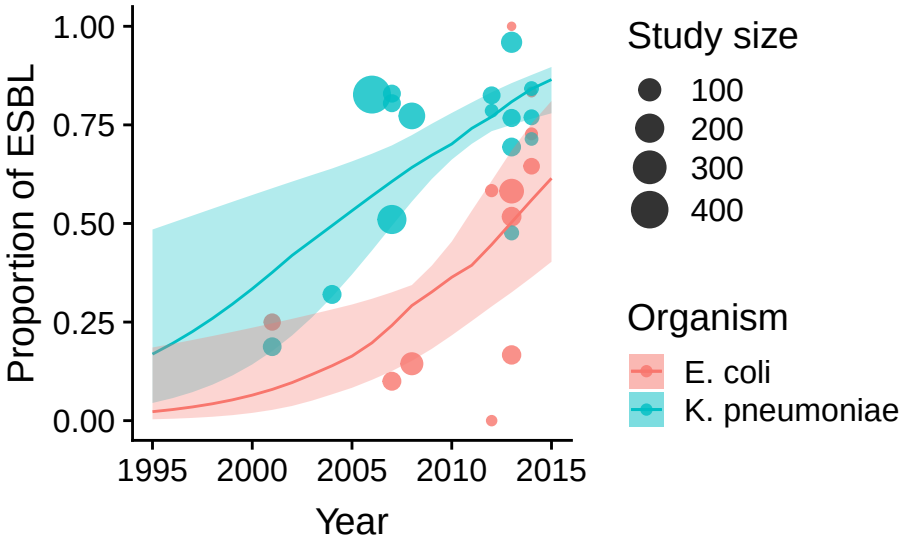

Figure 1.4: Meta regression of proportion of ESBL producing *E. coli* and *K. pneumoniae* amongst invasive isolate in sSA from studies carrying out blood culture, as a function of time. Includes 1242 *K. pneumoniae* and 489 *E. coli* isolates. 95% CI generated from 1000 bootstrap replicates from fitted models. In both cases time is statistically significantly associated with proportion of ESBL ($p < 0.001$ on likelihood ratio testing of nested models).

pneumoniae post 2000 (particularly post 2005) before becoming established in E. coli after 2010. In 2014, the latest available data, the pooled population prevalence of ESBL from binomal-normal random effects meta analysis was 61% [95% CI 40-80%] amongst *E. coli* and 86% [95% CI 73-92%] amongst *K. pneumoniae* bloodstream infection isolates, suggesting endemicity of ESBL amongst these pathogens in sSA, and comparable to the highest prevalence areas in the world.

ESBL genes were characterised in 10 studies by whole genome sequencing[163] (n=1) or by PCR[116,119,120,122,129,137,138,154,157] (n=9) for 821 *E. coli* and 791 *K. pneumoniae* isolates (Figure 1.5). CTX-M enzymes were the most commonly occurring ESBL genes, and the majority of these were CTX-M-15 in both organisms. OXA, TEM and SHV genes were also commonly found but were often not further characterised, presenting some problems of interpretation, as these enzymes can be narrow or broad-spectrum beta-lactamases. Certainly, SHV-1 and TEM-1 are narrow spectrum beta lactamase enzymes, which were commonly identified in these studies, though only a handful of isolates had characterisation of SHV enzymes beyond identification of the SHV group. All the identified OXA genes were narrow spectrum beta lactamases (OXA-1). These data suggest that the genomic landscape of invasive ESBL-E in sSA is dominated by CTX-M, and CTX-M-15 in particular, mirroring that seen worldwide.

Though no data were identified from Malawi that fulfilled the inclusion criteria of the systematic review, there are three studies that suggest the epidemiology of invasive ESBL-E in Malawi is similar to that described above. A study from Blantyre in 2004-2005 found that ESBL-E were unusual in blood stream infection (BSI) isolates: of 1191 Enterobacteriaeciae BSI, only 8 unique isolates showed an ESBL phenotype (K. pneumoniae 4/8, K. oxytoca, 1/8, Enterobacter cloacae 2/8 and E. coli 1/8) though no denominators are provided to allow calculation of prevalence. CTX-M-15 (n = 1) was described, though in the minority: SHV-11 (n = 1), SHV-12 (n = 3), SHV-27 (n = 1) and TEM-63 (n = 2) were the other enzymes identified[245]. Longitudinal blood culture surveillance in Blantyre suggests that after 2005 – which coincided with the introduction of ceftriaxone in government hospitals – the prevalence of ceftriaxone resistance rapidly increased, to 90.5% in K. pneumoniae and 30.3% in E. coli BSI isolates by 2016[246], though this study did not carry out confirmatory ESBL testing. Finally, a retrospective whole-genome sequencing study which selected 94 diverse (largely invasive) clinical E. coli isolates from Blantyre from 1996-2014 found that 21/94 isolates carried an ESBL gene, with CTX-M predominating

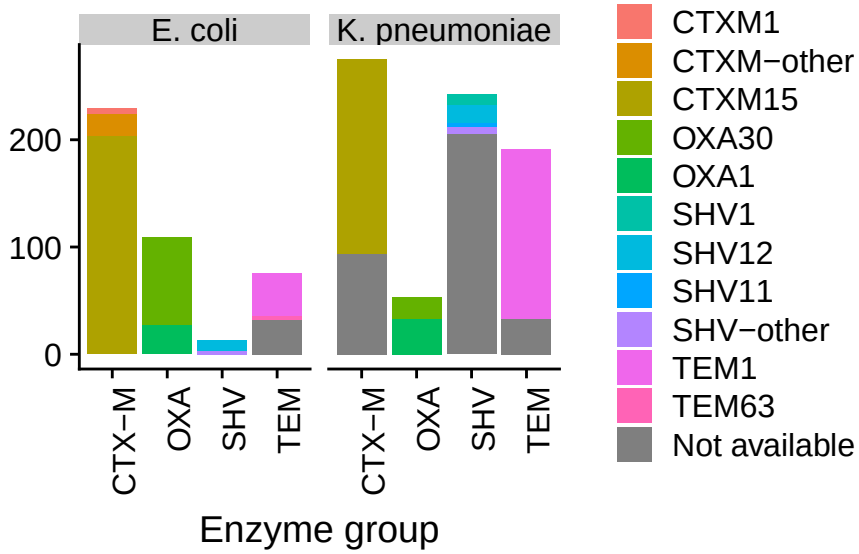

Figure 1.5: Disribution of beta-lactamase genes in invasive ESBL producing *E. coli* (n= 821) and *K. pneumoniae* (n = 791) from 10 studies

(20/21)[247].

1.3.4.2 Gut mucosal carriage of ESBL-E in sub-Saharan Africa

Table xx in appendix xx shows the 32 identified studies that provide data on gut mucosal carriage in different populations in sSA. The populations recruited to the studies are heterogeneous but include community members, hospitalised patients, outpatients, orphanage residents, hospital workers and food handlers in schools. Adults and children are included. Data on 10,232 individuals from 19 countries are available in total (Figure A). The earliest samples were collected from staff and children in a Malian orphanage in 2003, when 49/68 participants were found to be colonised with ESBL-E[190]. There is significant heterogeneity in prevalence, some of which is explained by the study population (Figure **??**); inpatients tend to have a higher ESBL-E carriage prevalence than community members. Outpatients have similar carriage prevalence to community members but inpatients even on hospital admission seem to have a higher carriage prevalence than community members.

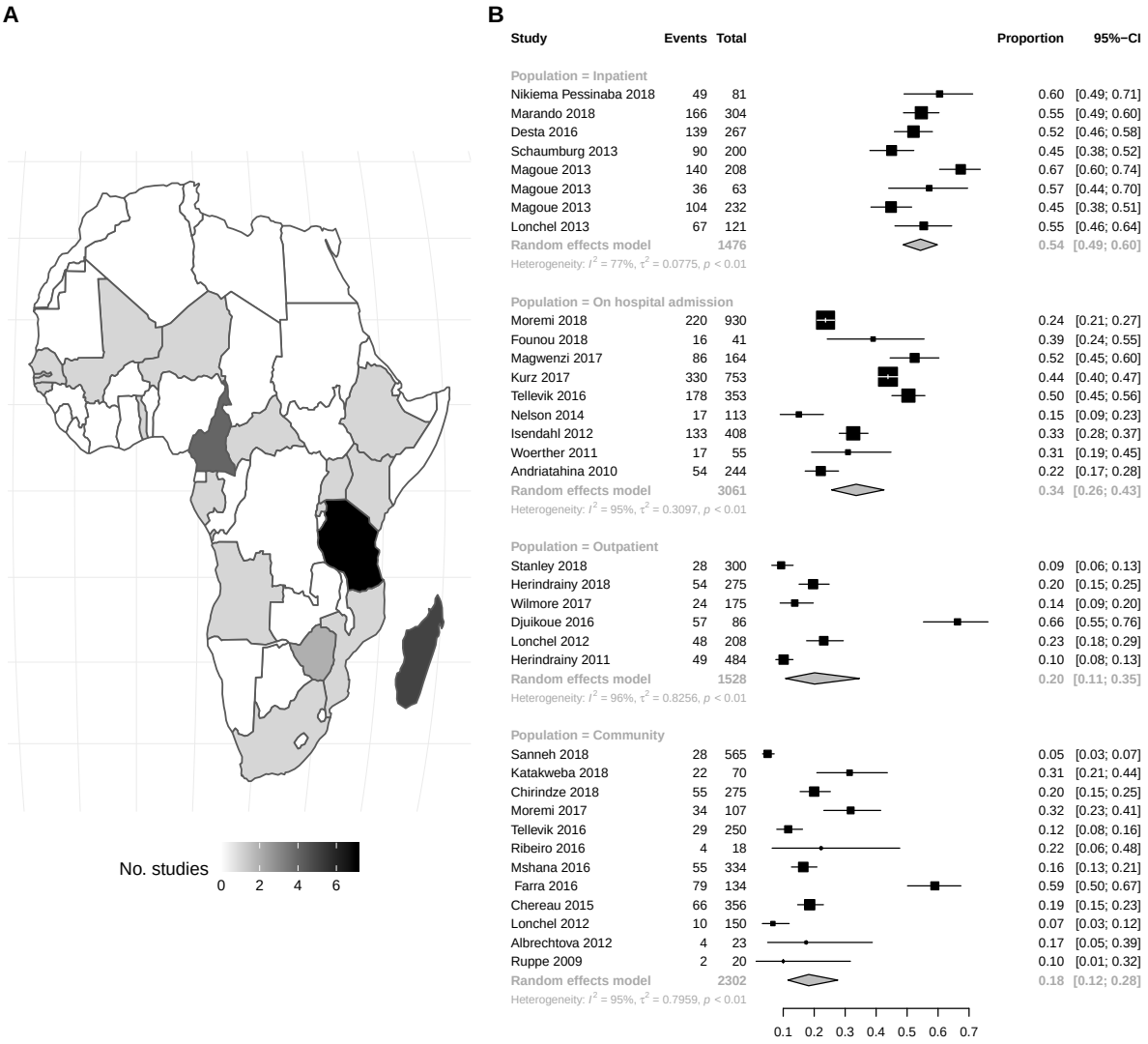

Figure 1.6: ESBL-E gut mucosal carriage in sSA. A: included studies by country; B: forest plot of ESBL-E carriage prevalence stratified by population. Pooled random effect summary estimates shown.

Significant heterogeneity in prevalence persists across all groups meaning that summary estimates should be interpreted with caution; community carriage if ESBL-E ranges from 5% in adults in The Gambia in 2015[194] to 59% in children in the Central African Republic in 2013[169], but a summary estimate from a random effect meta analysis is that 18% (95% CI 12-28%) of community members in sSA are colonised with ESBL-E, significantly higher than the prevalence in high-income settings.

Hospitalisation is clearly a driver of ESBL-E colonisation in the included studies - hospitalised cohorts have persistently higher prevalence of ESBL-E carriage – and prior antimicrobial exposure is consistently identified as a risk factor for carriage[171,178,194]. Consistent with a putative faecal-oral transmission route, boiling water and using a borehole as a source of water were identified as protective factors in studies in Rwanda[193] and Togo[196] respectively. Data to elucidate the role of within-household transmission are sparse, though one study in Rwanda found that a colonised family member was independently associated with ESBL-E carriage on admission to hospital[196]. Lower socioeconomic status was found to both be protective against ESBL-E colonisation in the Central African Rebulblic[169] and be associated with ESBL-E colonisation in Madagascar[185]; this relationship is likely to be complex and mediated by, for example, local availability and cost of antimicrobials. The role of HIV is not

clear: in children in Dar-es-salaam, Tanzania, ESBL-E carriage was much more common amongst HIV infected children[174], and in Harare Zimbabwe, receipt of ART for less than a year was associated with carriage[168]. This relationship is very open to confounding and many studies have not found an association between ESBL-E carriage and HIV infection[171,178,184,192,194,196].

Data on beta lactamase genes present in carriage isolates are available for 996 E. coli and 607 K. pneumoniae from 8 studies (Figure **??**), showing a similar picture to invasive isolates; the landscape is dominated by CTX-M-15. One study used whole-genome sequencing [191], the remainder used a variety of PCR techniques[167,179–181,184,186,192].

Only 4 studies are longitudinal cohorts which could provide insight into temporal trends and determinants of carriage[186,188,192,196]; all of these studies were health facility based and ascertained ESBL status on admission and discharge. Significant increases in ESBL-E carriage were seen in all studies: from 50 to 65% in Rwanda; from 30 to 95% in Niger; from 21.2 to 57% in Madagascar; and from 23% to 36% in Tanzania. No studies followed patients into the community, thus carriage duration of ESBL-E in sSA remains unknown and no interventional studies identified aiming to reduce ESBL-E carriage were identified.

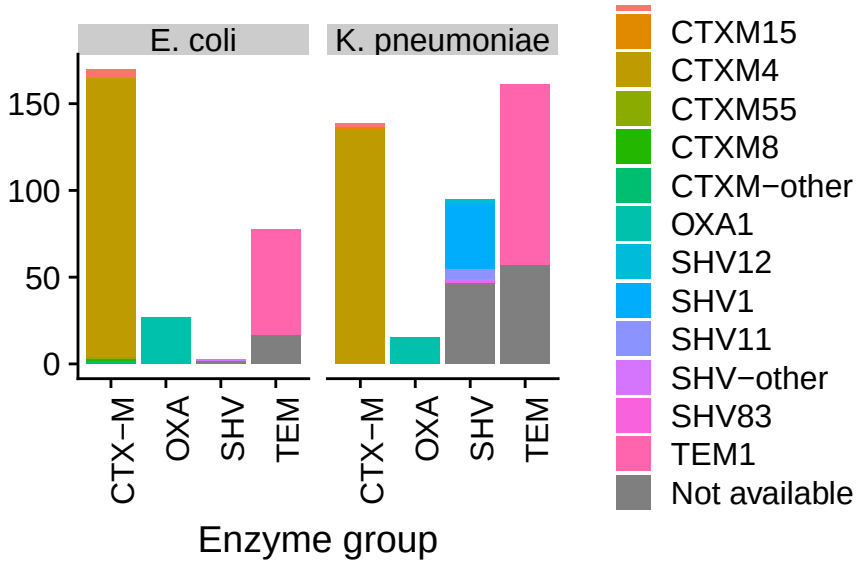

Figure 1.7: Distribution of beta-lactamase genes in carriage ESBL producing *E. coli* (n= 996) and *K. pneumoniae* (n = 607) from 9 studies.

1.4 Conclusions

The aetiology of sepsis in sSA is poorly defined, hence optimal antimicrobial strategies are unknown; disseminated TB is likely to play a significant role, but data to guide tuberculosis therapy strategies in the critically unwell are lacking. The role of bacterial zoonoses, arboviruses and HIV opportunistic infections are not well defined, but may be significant. Diagnostic uncertainty and paucity of microbiologic support across sSA may be creating a permissive environment for the widespread broad spectrum antimicrobial use, often third-generation cephalosporins. It is likely that dose, duration and indication are frequently inappropriate, and thus could contribute both to increased mortality and to spread of ESBL-E.

Certainly, ESBL-E are endemic in sSA and are a problem of serious public health concern; sSA has rates of ESBL-E in invasive disease that are comparable to the highest in the world and ESBL-E gut mucosal carriage in healthy populations across the continent is common. Whilst it is clear that

health care facilities are strongly associated with ESBL-E acquisition, a deeper understanding of the determinants and sources of acquisition, and carriage duration is lacking. In order to understand the role of health facilities in driving the ESBL-E pandemic, a high quality longitudinal ESBL-E carriage data from both healthy and sick (health facility exposed) populations are required.

It may be that optimising the treatment of severe febrile illness in hospitals is the best place to start to reduce over prescription of broad-spectrum antimicrobials and reduce selection pressure for ESBL-E whilst ensuring timely and appropriate access to the right treatments for those who need them. This is the central hypothesis of this thesis, and the following chapters present the data that can be used to define such a strategy.

1.4.1 Specific aims

The specific aims of this thesis are: 1. To describe the presentation, aetiology, outcome, and determinants of mortality from sepsis in adults presenting to Queen Elizabeth Central Hospital, Blantyre Malawi; 2. To describe the acquisition and carriage of ESBL-E in sepsis survivors, with an analysis of determinants of carriage.

1.5 Thesis overview

Chapter 2 (methods) presents the clinical study that forms the basis of the rest of this thesis; given the likely importance of disseminated TB in sepsis in sSA, chapter 3 presents a systematic review and individual patient data meta anlaysis of prevalence, diagnosis, and mortality hazard or TB bloodstream infection. Chapter 4 presents data on the clinical presentation, aetiology and outcomes of sepsis in Blantyre, Malawi; chapter 5 describes the determinants of long-term carriage of ESBL-E amongst sepsis survivors. Chapter 6 presents an overview of the genomic landscape of ESBL-E in Blantyre, Malawi whilst chapter 7 combines the genomic and epidemiologic data from chapters 5 and 6 to understand mechanisms and drivers of ESBL-E carriage in health and disease in Malawian adults. [microbiome stuff if available]. Finally, chapter 8 provides suggestions of further work.

1.6 Appendix

Table 1.9: Sequential organ failure assessment (SOFA) score

	Score				
System	0	1	2	3	4
Respiratory					
Pao2 / FiO2 mmHg (kPa)	400 (53.3)	< 400 (53.3)	< 300 (40)	< 200 (26.7) with respiratory support	< 100 (13.3) with respiratory support
Coagulation					
Platelets x100,000/ mL	150	< 150	< 100	< 50	< 20
Liver					
Bilirubin mg /dL (mmol/ L)	<1.2 (20)	1.2-1.9 (20 – 32)	2.0 – 5.9 (33-101)	6.0 – 11.9 (102 – 204)	> 12.0 (204)
Cardiovascular					
Cardiovascular	MAP > 70mmHg	MAP < 70mmHg	Dopamine < 5 or dobutamine any dose	Dopamine 5.1 – 15 or epinephrine < 0.1 or norepinephrine < 0.1	Dopamine > 15 or epinephrine > 0.1 or norepinephrine > 0.1
CNS					
Glasgow coma scale	15	13-14	10-12	7-9	< 6
Renal					
Creatinine mg/dL (mmol /L)	< 1.2 (110)	1.2 – 1.9 (110 -170)	2.0 – 3.4 (171 – 299)	3.5 – 4.9 (300 – 440)	> 5.9 (440)
Urine output (ml /day)				< 500	< 200

Note:
PaO2 = Arterial partial pressure of oxygen, FiO2 = Inspired fraction of oxygen, MAP = mean arterial blood pressure, CNS = Central nervous system. All doses of inotropes are micrograms/kg/min

Table 1.10: Selected causes of fever in sSA since 2013

Study	Year	Country	Setting	Patient Population	Test used	Case definition	Confirmed acute disease
Leptospirosis							
Zida 2018	2014-15	Burkina Faso	Central reference lab	Febrile Jaundice adults and children	In house IgM followed by MAT and PCR (acute only, > 1:400)	MAT > 1:400	27/781 (3.5%)

Table 1.10: Selected causes of fever in sSA since 2013 *(continued)*

Study	Year	Country	Setting	Patient Population	Test used	Case definition	Confirmed acute disease
Guillebaud 2018	2014-2015	Madagascar	21 health-care centres	Febrile adults and children	PCR array	Positive PCR	1/682 (0.2%)
Maze 2018	2012-2014	Tanzania	2 Referral Hospitals	Febrile adults and children	MAT (acute + conv)	MAT > 1:800 or fourfold rise	24/1239 (1.9%)
Gadia 2017		Central African Republic	Central reference lab	Febrile Jaundice adults and children	IgM ELISA (acute only)	Any IgM positive	0/198 (0%)
Hagen 2017	2011-2013	Madagascar	District Hospital	Adults and children FUO	PCR	Positive PCR	0/1009 (0%)
Biscornet 2017	2014-2015	Seychelles	Reference leptospirosis clinic	13 or above FUO, referred to central leptospirosis clinic	In house IgM followed by MAT and PCR (acute + conv)	MAT > 1:400 or fourfold rise	51/225 (23%)
Dreyfus 2017	2014	Uganda	2 Health centres	Any adult heath centre attendee	MAT (acute only)	MAT > 1:800	7/359 (1.9%)
Hercik 2017	2014-2015	Tanzania	District hospital	Febrile adults and children	Taqman PCR array	Positive PCR	22/842 (2.6%)
Chipwaza 2015	2014	Tanzania	District hospital	Outpatient febrile children	IgM IgG ELISA then MAT (acute only)	MAT > 1:160	26/200 (13%)
Q-fever							
Amoako 2018	2016-17	Ghana	2 distrct hospitals	Febrile children	Taqman PCR array	Positive PCR	1/166 (0.6%)
Hercik 2017	2014-2015	Tanzania	District hospital	Febrile adults and children	Taqman PCR array	Positive PCR	2/842 (0.2%)
Boone 2017	2011-13	Madagascar	Two public health care facilities	Febrile adults and children	PCR	Positive PCR	0/1005

Table 1.10: Selected causes of fever in sSA since 2013 *(continued)*

Study	Year	Country	Setting	Patient Population	Test used	Case definition	Confirmed acute disease
Njeru 2016	2014-15	Kenya	Two district hospitals	Febrile adults and children	Phase I/II IgG ELISA and IFA; PCR on subset (acute only)	Phase II IgG IFA titre > 1:128	163/1067 (15%), 10/448 (2.2%) PCR positive
Mourembou 2016	2013-14	Gabon	Four health centres	Febrile children	PCR	Positive PCR	0/410 (0%)
Maina 2016	2011-12	Kenya	District Hospital	Febrile children	IgM/IgG ELISA phase I and II (acute and conv)	Phase II IgG seroconversion	25/370 (8.9%)
Angelaksis 2014	2010-12	Senegal, Mali, Gabon	Six health centres	Febrile adults and children	PCR	Positive PCR	6/1388 (0.4%)
Brucellosis							
Cash-Goldwasser 2018	2012-14	Tanzania	Two referral hospitals	Febrile adults and children	MAT and blood culture (acute + conv)	Fourfold rise in MAT	39/562 (6.9%)
Gafiritia 2017	2014	Rwanda	District hospital	Adults, fever	Rose Bengal test	Positive test	10/198 (6.1%)
Boone 2017	2011-13	Madagascar	Two public health care facilities	Febrile adults and children	PCR	Positive PCR	15/1005 (1.5%)
De Glanville 2017	2012	Kenya	Referral hospital and private clinic	Febrile adults and children	Rose Bengal test	Positive test	8/825 (9.7%)

Table 1.10: Selected causes of fever in sSA since 2013 *(continued)*

Study	Year	Country	Setting	Patient Population	Test used	Case definition	Confirmed acute disease
Njeru 2016	2014-15	Kenya	Two district hospitals	Febrile adults and children	Rose bengal test, IgG/IgM ELISA, PCR (acute only)	Positive ELISA or PCR	146/1067 (13.7%)
Chipwaza 2015	2014	Tanzania	District hospital	Outpatient febrile children	IgM and IgG and tube agglutination (acute only)	Positive IgM	26/370 (7.0%)
Feleke 2015	2011	Ethiopia	Health centre	Febrile adults and children	Brucella antigen test	Positive test	3/280 (1%)
Rickettsioses							
Amoako 2018	2016-17	Ghana	2 distrct hospitals	Febrile children	Taqman PCR array	Positive PCR	5/166 (3.0%) RS
Hercik 2017	2014-2015	Tanzania	District hospital	Febrile adults and children	Taqman PCR array	Positive PCR	2/842 (0.2%) RF
Sothmann 2017	2012	Ghana	Referral hospital	Febrile Children	PCR	Positive PCR	6/431 (1.4%) RF
Maina 2016	2011-12	Kenya	District Hospital	Febrile children	IgG ELISA (acute and conv)	Fourfold rise in IgG titre	63/364 (22.4%) SFG 3/364 (1.1%) TG, 10/364 (3.6%) STG
Elfving 2016	2011	Zanzibar	District hospital	Febrile children with no diagnosis	PCR	Positive PCR	0/83 RS
Mourembou 2015	2013-14	Gabon	4 heath centres	Febrile children	PCR	Positive PCR	42/410 (10.2%) RF
Dengue							
Amoako 2018	2016-17	Ghana	2 district hospitals	Febrile children	Taqman PCR array	Positive PCR	2/166 (1.2%)

Table 1.10: Selected causes of fever in sSA since 2013 *(continued)*

Study	Year	Country	Setting	Patient Population	Test used	Case definition	Confirmed acute disease
Guillebaud 2018	2014-2015	Madagascar	21 health-care centres	Febrile adults and children	PCR macroarray	Positive PCR	0/682 (0%)
Kayiwa 2018	2014-2017	Uganda	District hospital	Febrile adults and children	PCR	Positive PCR	1/384 (0.26%)
Makiala-Mandanda 2018	2003-2012	Democratic Republic of Congo	Central lab	Febrile Jaundice, yellow fever IgM negative	PCR	Positive PCR	16/453 (3.5%)
Muianga 2018	2014	Mozambique	Not clear	Febrile adults and children	IgG, IgM and PCR (acute only)	Positive PCR	37/99 by PCR (37.4%)
Mugabe 2018	2016	Mozambique	Five health centres	Febrile adults and children	IgM, IgG, PCR (acute only)	Positive PCR	PCR 0/163
Hercik 2018	2014-2015	Tanzania	District hospital	Febrile adults and children	Taqman PCR array	Positive PCR	1/191 (0.5%)
Gadia 2017		Central African Republic	Central reference lab	Febrile Jaundice adults and children	IgM (Acute only)	Positive IgM	0/198 (0%)
Vu 2017	2014-2015	Kenya	Two health centres	Febrile children	PCR	Positive PCR	82/1104 (7.4%)
Waggoner 2017	2014-2015	Kenya	Two health centres and two district hospitals	Children with fever	PCR	Positive PCR	0/385 (0%)
Kolawole 2017	2016	Nigeria	Two heath centres	Adults and children with fever	IgM, IgG, PCR (Acute only)	Positive PCR	11/176 (6.2%)
Nasir 2017	2016	Nigeria	Teaching hospital	Adults and children with fever	NS1 antigen	Positive antigen	15/171 (8.8%)

Table 1.10: Selected causes of fever in sSA since 2013 *(continued)*

Study	Year	Country	Setting	Patient Population	Test used	Case definition	Confirmed acute disease
Ngoi 2016	2014-2015	Kenya	Five health clinics, one district hospital	Adults with fever, negative for acute HIV and malaria	PCR	Positive PCR	43/489 (8.8%)
Onoja 2016	2014	Nigeria	One district hospital	Adults and children with fever	IgM (Acute only)	Positive IgM	64/274 (23.3%)
Kajeguka 2016	2013-2014	Tanzania	Three district hospitals	Probable Dengue (on clinical and IgM)	PCR	Positive PCR	0/381 (0%)
Elfving 2016	2011	Zanzibar	District hospital	Febrile children with no diagnosis	PCR	Positive PCR	0/83
Sow 2016	2009-2013	Senegal	Seven health-care facilities	Adults and children with fever	IgM, PCR (acute only)	Positive PCR	3/13,845 (0.02%)
Chipwaza 2014	2013	Tanzania	One district hospital	Children with fever	IgM, PCR (acute only)	Positive PCR	29/364 (8.0%)
Chikingunya							
Kayiwa 2018	2014-2017	Uganda	District hospital	Febrile adults and children	PCR	Positive PCR	19/384 (4.9%)
Makiala-Mandanda 2018	2003-2012	Democratic Republic of Congo	Central lab	Febrile Jaundice, yellow fever IgM negative	PCR	Positive PCR	2/453 (0.4%)
Muianga 2018	2014	Mozambique	Not clear	Febrile adults and children	IgG, IgM (acute only)	Positive IgM	8/114 by IgM (7%)
Antonio 2018	2015-16	Mozambique	Eight health centres	Undifferentiated fever	IgM, IgG (Acute only)	Positive IgM	6/392 (1.5%)
Mugabe 2018	2016	Mozambique	Five health centres	Febrile adults and children	IgM, IgG, PCR (Acute only)	Positive PCR	PCR 0/163, IgM 17/163 (10.4%)
Sow 2017	2009-2010	Senegal	Fiver health centres and four schools	Febrile adults and children	IgM, IgG, PCR (Acute only)	Positive PCR	20/1049 (1.4%)

Table 1.10: Selected causes of fever in sSA since 2013 *(continued)*

Study	Year	Country	Setting	Patient Population	Test used	Case definition	Confirmed acute disease
Gadia 2017		Central African Republic	Central reference lab	Febrile Jaundice adults and children	IgM (Acute only)	Positive IgM	0/198 (0%)
Olajiga 2017	2015-2016	Nigeria	Seven hospitals	Fever or joint pain or rash, over 10 years	IgM, IgG (acute only)	Positive IgM	66/172 (38.4) by IgM
Waggoner 2017	2014-2015	Kenya	Two health centres and two district hospitals	Children with fever	PCR	Positive PCR	32/385 (8.3%)
Ngoi 2016	2014-2015	Kenya	Five health clinics, one district hospital	Adults with fever, negative for acute HIV and malaria	PCR	Positive PCR	0/489 (0%)
Kajeguka 2016	2013-2014	Tanzania	Three district hospitals	Probable Chikungunya (on clinical and IgM)	PCR	Positive PCR	11/263 (4.2%)
Elfving 2016	2011	Zanzibar	District hospital	Febrile children with no diagnosis	PCR	Positive PCR	0/83
Sow 2016	2009-2013	Senegal	Seven health-care faciities	Adults and children with fever	IgM, PCR (acute only)	Positive PCR	13/13,845 (0.1%)
Chipwaza 2014	2013	Tanzania	One district hospital	Children with fever	IgM (acute only)	Positive IgM	17/364 (4.7%)
Zika							
Kayiwa 2018	2014-2017	Uganda	District hospital	Febrile adults and children	PCR	Positive PCR	5/384 (1.3%)
Makiala-Mandanda 2018	2003-2012	Democratic Republic of Congo	Central lab	Febrile Jaundice, yellow fever IgM negative	PCR	Positive PCR	0/453 (0%)
Sow 2016	2009-2013	Senegal	Seven health-care faciities	Adults and children with fever	IgM, PCR (Acute only)	Positive PCR	9/13,845 (0.1%)

Note:
RS = Rickettsia spp., RF = R. felis, SFG/TG/STG = spotted fever/ typhus/scrub typhus group

Table 1.11: included studies providing an estimate of proportion of ESBL producers in invasive *E. coli* and *K. pneumoniae* isolates in sSA.

Year	First author	Country	Population	Sample	E coli	K pneumoniae
2018	Guiral	Mozambique	A C IP	Blood urine	13/151 (9%)	ND
2018	Karppinen	Angola	C IP OP	Wound swab	8/15 (53%)	10/13 (77%)
2018	Kpoda	Burkina Faso	A C IP OP	Various	117/296 (40%)	48/109 (44%)
2018	Onanuga	Nigeria	A OP	Urine	4/18 (22%)	30/45 (67%)
2018	Seni	Nigeria	A C IP OP	Various	41/60 (68%)	ND
2018	Zeynudin	Ethiopia	NA IP OP	Various	13/13 (100%)	30/31 (97%)
2017	Ampaire	Uganda	A C IP OP	Various	18/146 (12%)	10/68 (15%)
2017	Andrew	Uganda	A C IP OP	Various	33/44 (75%)	33/36 (92%)
2017	Archary	South Africa	C IP	Various	2/11 (18%)	13/19 (68%)
2017	Henson	Kenya	A C IP OP	Blood	ND	101/198 (51%)
2017	Ibrahim	Nigeria	A C IP OP	Urine wound swab	68/140 (49%)	76/108 (70%)
2017	Kassam	Tanzania	A C IP OP	Wound swab	6/14 (43%)	8/11 (73%)
2017	Legese	Ethiopia	C IP	Blood urine	5/6 (83%)	16/19 (84%)
2017	Manyahi	Tanzania	A C IP OP	Urine	15/110 (14%)	9/27 (33%)
2017	Sangare	Mali	A C IP	Blood	20/31 (65%)	20/26 (77%)
2017	Vasaikar	South Africa	A C IP OP	Various	ND	117/169 (69%)
2016	Abera	Ethiopia	A C IP OP	Blood urine	71/122 (58%)	34/49 (69%)
2016	Agyekum	Ghana	A C IP OP	Blood urine	30/58 (52%)	33/43 (77%)
2016	Breurec	Senegal	C IP	Blood CSF	ND	33/41 (80%)
2016	Buys	South Africa	C IP	Blood	ND	339/410 (83%)
2016	Eibach	Ghana	A C IP	Blood	5/50 (10%)	34/41 (83%)
2016	Kabwe	Zambia	C IP	Blood	5/5 (100%)	71/74 (96%)
2016	Leski	Sierra Leone	A C OP	Urine	0/13 (0%)	9/15 (60%)
2016	Mohammed	Nigeria	A C IP OP	Various	41/172 (24%)	59/178 (33%)
2016	Naas	Madagascar	C IP OP	Blood	0/7 (0%)	11/14 (79%)
2016	Ndir	Senegal	C IP	Blood	7/12 (58%)	33/40 (82%)
2016	Ouedraogo	Burkina Faso	A C IP OP	Various	121/202 (60%)	46/70 (66%)
2016	Sangare	Mali	A C IP	Blood	8/11 (73%)	10/14 (71%)
2016	Seni	Tanzania	A IP	Pertitoneal fluid	7/19 (37%)	5/10 (50%)
2015	Dramowski	South Africa	C IP OP	Blood	14/97 (14%)	119/154 (77%)
2015	Irenge	Democratic Republic of Congo	A C IP OP	Blood	9/54 (17%)	10/21 (48%)
2015	Kateregga	Uganda	A C IP OP	Various	36/64 (56%)	24/33 (73%)
2015	Opintan	Ghana	A C IP OP	Various	81/440 (18%)	ND
2015	Pons	Mozambique	A C IP OP	Blood urine	ND	16/50 (32%)

Table 1.11: included studies providing an estimate of proportion of ESBL producers in invasive *E. coli* and *K. pneumoniae* isolates in sSA. *(continued)*

Year	First author	Country	Population	Sample	E coli	K pneumoniae
2015	Rafa	Central african republic	A C IP	Wound swab	33/47 (70%)	10/19 (53%)
2014	Adeyankinnu	Nigeria	A C IP OP	Various	36/135 (27%)	16/62 (26%)
2014	Irenge	Democratic Republic of Congo	A C IP OP	Urine	57/376 (15%)	ND
2014	Scherbaum	Gabon	A IP	Various	5/14 (36%)	3/6 (50%)
2014	Yusuf	Nigeria	A IP OP	Various	47/278 (17%)	19/128 (15%)
2013	Alabi	Gabon	A C IP OP	Various	ND	43/85 (51%)
2013	Ibrahim	Sudan	A C IP OP	Various	70/232 (30%)	ND
2013	Obeng-Nkrumah	Ghana	A C IP OP	Various	55/126 (44%)	59/96 (61%)
2013	Raji	Nigeria	A C IP OP	Various	21/43 (49%)	12/32 (38%)
2013	van der Meeren	Mozambique	C IP	Urine	9/14 (64%)	15/17 (88%)
2011	Idowu	Nigeria	A IP	Wound swab	6/15 (40%)	ND
2010	Moyo	Tanzania	A C IP OP	Urine	54/138 (39%)	ND
2009	Bercion	Central African Republic	A C OP	Urine	29/357 (8%)	17/57 (30%)
2009	Mshana	Tanzania	A C IP OP	Various	31/127 (24%)	58/91 (64%)
2007	Sire	Senegal	A C IP OP	Urine	38/1010 (4%)	ND
2005	Blomberg	Tanzania	C IP OP	Blood	9/36 (25%)	9/48 (19%)
2005	Gangoue Pieboji	Cameroon	A C IP	Various	13/91 (14%)	12/64 (19%)
2005	Ndugulile	Tanzania	A IP	Various	4/13 (31%)	2/2 (100%)
2004	Dromigny	Senegal	A C OP	Urine	2/233 (1%)	1/34 (3%)
2002	Dromigny	Senegal	A C OP	Urine	1/386 (0%)	6/97 (6%)

Note:
A = Adults, C = children, IP = inpatients OP = outpatients, ND = not done.

1.7 References

Chapter 2

Methods

2.1 Chapter Overview

This chapter gives an overview of the clinical study which underpins this thesis, and the laboratory and computational procedures used in analysis. Further details are given in the individual chapters, where necessary.

2.2 Study site

2.2.1 Malawi

Malawi is a country of 17.5 million people in South-Eastern Africa[248]. It is one of the poorest countries in the world: it is a low income country under the World Bank classifcation, with a 2017 Gross National Income (GNI) per capita of $320 in US dollars[249], and was ranked 171st of 189 countries in 2017 by the human development index (HDI), a composite statistic of life expectancy, education and income per capita indicators[(UNDP human development reports)][250]. In 2010, 71% of the population was estimated to survive on less than $1.90 per day. Life expectancy at birth in 2017 was 63 years, and though significant progress is being made, neonatal and under-5 mortality remains high at 23 and 55 per 1000 live births, respectively. The population is largely rural (83% in 2017), with a young population (44% under the age of 15, 2017) and high fertility rate[249]. Malaria is endemic, and there is an ongoing generalised HIV epidemic: adult HIV prevalance (age 15-49) was estimated to be 9.6% in 2017 (UNAIDS), though falling from a peak of 16.6% in 1999[251]. HIV antiretroviral therapy (ART) national scale up began in 2004 and in 2017 71% of elibigible adults and children were estimated to be recieveing ART[251]. It is classed by the WHO as a high-TB/high-HIV burden country, with an estimated TB incidence rate of 131 (95% CI 70-210) cases per 100000 population per year[WorldHealthOrganisation2018].

It has a subtropcial climate, with three main seasons: a warm wet season from November to April, a cooler dry winter period from May to August and a hot dry period from September to October. Blantyre city, the location of the study in this thesis, is the second city of Malawi with a population of 585000. It is located in Blantyre district, population 995000 in 2018[248], in the Shire highlands at an altitude of 1000m (Figure 2.1).

Figure 2.1: Malawi, showing administrative boundaries (North, Central, and South regions), Lilongwe, the capital city and Blantyre, the study location. Source: openstreetmap.org, used under Creative Commons Attribution ShareAlike 2.0 licence CC-BY-SA

2.2.2 Queen Elizabeth Central Hospital

Queen Elizabeth Central Hospital (QECH), located in Blantyre city, is the tertiary referral hospital for the Southern Region of Malawi. It has 1300 beds but often operates above capacity, and is the only site providing free inpatient healthcare to the adult population of Blantyre district. Since 2011 it has had a dedicated emergency department for adults, the Adult Emergency and Trauma Centre, staffed 24 hours a day. Since 2015 (and for the whole of the study period), attendees to the AETC must be referred from a primary health clinic. Adults attending the AETC are triaged by a nurse and then reviewed by a doctor or clinical officer; if admission under a specialty team (including medicine) is deemed appropriate then a patient will be reviewed by an intern or registrar from the admitting specialty and usually by a consultant within 24 hours. There is a 6-bed AETC resusitation area in which oxygen concentrators, cardiac monitors and a defibrillator are available; none of these items are available in the rest of the AETC.

There are two dedicated single-sex medical wards, each of approximately 60 beds, and one mixed-sex TB ward. Male and female high-dependency units (HDUs), each with a capacity of six beds, have oxygen concentrators (or, if available, oxygen cylinders) to deliver supplemental oxygen. The medical wards are staffed by two or three trained nurses and a variable number of nursing students. Basic nursing care is usually provided by a patients relative or friend, called a 'guardian.' Patients on the medical wards are reviewed twice-weekly by a consultant physician and then variably at other times by junior doctors, clinical officers or medical students depending on the availability of staff. Malawi national treatment guidelines suggest ceftriaxone for the treatment of sepsis requiring hospitalisation.

2.2.3 Participating Laboratories

2.2.3.1 Malawi-Liverpool-Wellcome Clinical Research Programme

The Malawi-Liverpool Wellcome Trust Clinical Research Programme was established in 1995 and since them has been active in researching priority health issues in Malawi. It is an affiliate of the Malawi College of Medicine, and is based in the grounds of QECH in Blantyre. It runs an on-site microbiology laboratory which has provided an aerobic blood culture service to QECH since 1998, and also provides CSF microscopy and culture. Bacterial culture is carried out as per British Society of Antimicrobial Chemotherapy (BSAC) guidelines[252] and the laboratory adheres to UK NEQAS external quality control. It is core funded by the Wellcome Trust.

2.2.3.2 Malawi College of Medicine Tuberculosis Laboratory

2.2.3.3 Wellcome Trust Sanger Institute

The Wellcome Sanger Institute is a research institute based in Hinxton, UK, which was established in 1993, and undertakes research in all aspects of genomics including bacterial genomics as part of the parasite and microbes programme. It has one of the largest DNA sequencing facilities in the world as well as high performace computing clusters. It is funded by the Wellcome Trust.

2.3 Clinical Study

The DASSIM (Developing an Antimicrobial Strategy for Sepsis in Malawi) study was an observational cohort study, recruiting from the AETC at QECH with two broad aims: firstly, to describe the presentation, aetiology and determinents of outcome in sepsis in Malawi; and secondly to determinents of carriage of ESBL-E in sepsis survivors.

2.3.1 Entry Criteria

The study was open for recruitment between 19 February 2017 and 2 October 2018; there were three arms. Firstly, in order to define the aetiology of sepsis, adults with sepsis attending AETC were recruited as early as possible in their attendance to QECH following triage. Secondly, in order to clearly define the relative effects of antimicrobial exposure versus hospital admission in ESBL-E acquisition and carriage, antibiotic unexposed adults attending AETC with no plan for antimicrobial administration were recruited. Finally, to define baseline flux of ESBL-E antibiotic-unexposed community members were recruited. Detailed inclusion and exclusion criteria for each arm are given shown in Table 2.1. For logistic reasons, recruitment occured 7am - 5pm Monday to Friday.

Exclusion criteria were the same for all arms of the study: Particiapnts were not eligible for enrollment if they were unable to give informed consent and no guardian available to provide proxy consent; they spoke neither English nor Chichewa; or they lived > 30km from Blantyre city. The antibiotic-unexposed inpatients and community members were matched on age (+/- 5yr) and sex to sepsis survivors (defined as patients surviving to 28 days). In addition community members were matched on location to sepsis survivors; putative households for recruitment were identified by random walk from the houses of surviving sepsis participants, with initial direction established by spinning a bottle on the floor.

Table 2.1: Study inclusion criteria

Study Arm	Inclusion Criteria
Arm 1 - Sepsis	Adults (16 years or over) attending AETC AND Axillary temperature > 37.5C or history of fever within 72 hours AND Life threatening organ dysfunction defined by any one of: Oxygen saturations < 90 percent on air, respiratory rate > 30 breaths/minute, systolic blood pressure < 90mmHg, glasgow coma scale < 15
Arm 2 - Inpatient	Adults (16 years or over) attending AETC AND No suspicion of infection or plan for antimicrobial administraton AND No antimicrobial therapy within last 4 weeks
Arm 3 - Community	Adults (16 years or over) AND No antimicrobial therapy within last 4 weeks

Note:
AETC = Adult Emergency and Trauma Centre

2.3.2 Study Visits and Patient Sampling

2.3.2.1 Enrollment assessment and first six hours

An overview of the study visit schedule is shown in Figure 2.2. At enrollment, following consent a baseline questionnaire was completed (by the guardian if the participant was obtunded) to capture background demographic and clinical data, a Chichewa-language EQ-5D heath related quality of life questionairre completed, and sample collection undertaken (see below). For hospitalised patients, enrollment aimed to be as soon after triage as possible, and arm 1 (sepsis) patients underwent a

more intensive assessment over the first six hours of enrollment. Data on therapies administered over this time period were captured by hourly review by a member of the study team, including the time of administration of antimicrobial therapy and volumes of intravenous fluid administered, the latter visually comfirmed by the study team member. Hourly vital signs and standardised bedside assessment of intravascular status were performed: central capillary refill, limb temperature and passive leg raise. For this latter test, blood pressure and pulse rate were measured with the participant sitting at 45 degrees, and one minute after lying the participant supine and lifting the legs to 45 degrees. All blood pressure mesurements were made noninvasively with an automated cuff (Omron M2, Omron, Japan), oxygen saturations measured with a dedicated study pulse oximeter (Contec CM50, Contec Medical Systems, China), and temperature measured in the axilla with a digital thermometer (Omron FWH000, Omron, Japan).

All treatment decisions were at the discretion of the parrticipant's attending healthcare worker.

2.3.2.2 Subsequent visits

Hospitalised participants were briefly reviewed daily Monday to Friday following admission whilst on the wards to capture details of therapies administered. For the 72 hours following admission, vital signs were repeated and an assessment made of intravenous fluid administered; if records of fluid balance were incomplete (as wa anticipated to be the case in the majority of cases) then fluid administered was estimated from participant- or guardian-reported number of bags of fluid administered.

The visit schedule for the study is shown in Figure 2.2. Arm 1 (sepsis) and 2 (hospital inpatients) were reviewed by a study team member 7 and 28 days following enrollment, and at 3 and 6 months; comunity members were reviewed at 28 days and 6 months. At each visit, details of any antimicrobials or other therapies recieved and any health care facility contact were collected, and the Chichewa language EQ-5D was repeated. Samples were collected as below.

These study visits occured preferentially at QECH, but at the patient's home if it was difficult for them to attend the hospital. If patients missed a scheduled visit, then attempts were made to contact them by telephone. If these attempts were unsucessful then attempts to visit them at home were undertaken by a member of the study team.

2.3.2.3 Blood, urine, and stool, sputum and CSF collection

Blood was collected from arm 1 (sepsis) participants only on enrollment and at the 4-week visit. At baseline, blood was collected aseptically directly into collection tubes with a vacutainer device with the following order of draw and volumes: one BacT/Alert (BioMerieux, France) aerobic blood culture bottle (7-10ml), one BD BACTEC Myco/F Lytic mycobacterial culture bottle (3-5ml) (Beckton Dickinson, United States), one serum (10ml) and two EDTA (4ml, both Grenier Bio-One, Austria) samples. Finger prick for capillary blood was used for point of care diagnostics as described below. At the 4-week visit, 10mls of convalescent serum was collected. At baseline, urine was collected from all arm 1 (sepsis) participants into a sterile polypropylene universal container (Alpha Laboratories, UK) either by the participant themselves (if this was possible) or with the aid of a disposable bedpan.

At the enrollment visit and all other visits for all arms of the study stool was collected into a sterile polypropylene universal container (Alpha Laboratories, UK) with aseptic technique. If it was not possible for a patient to provide stool then a rectal swab was taken using a sterile rayon-tipped swab (Technical Service Consultants Ltd, UK) inserted into 2-3cm into the rectum, rotated for approximately 10 seconds and placed into Amies gel media for transport to the laboratory.

The decision to collect sputum for Xpert testing for tuberculosis or to perform lumbar puncture (LP) rested with the participant's attending healthcare worker. Lumbar puncture, where done, was carried out by QECH AETC or medicine department doctors or clinical officers and 7-10ml of cerebrospinal fluid (CSF) was aseptically collected. Sputum samples were collected in sterile polypropylene universal

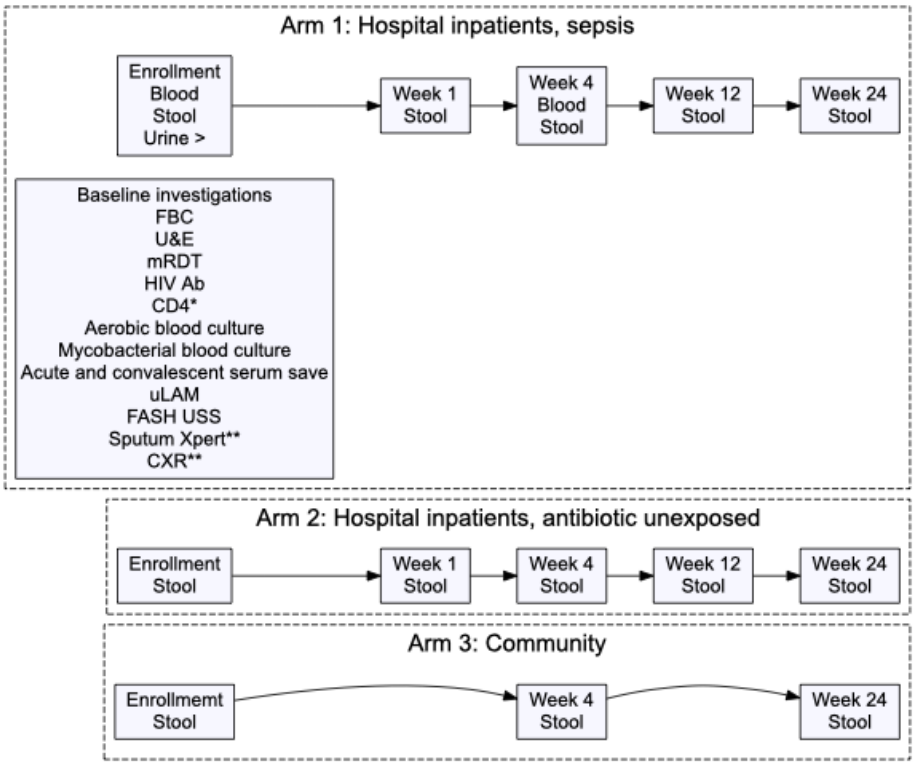

Figure 2.2: Overview of patient sampling schedule.FBC = Full blood count, U&E = Urea and elctrolytes, mRDT = Malaria rapid diagnostic test, uLAM = Urinary LAM, FASH USS = Focussed assessment for HIV-associated tubercusosis ultrasound scan

container and trasported to the Malawi College of Medicine (CoM) tuberculosis laboratory. Myco/F lytic bottles for mycobacterial blood culture were also transported to the CoM tuberculosis laboratory; all other samples were transported to the MLW laboratories in the first instance.

2.3.2.4 Imaging: chest x-ray and ultrasound scanning

Ultrasound examination following the FASH (Focussed ASsessment for HIV-associated tuberculosis) protocol[253] was undertaken for all sepsis participants by me, following training with a UK-registered consultant radiologist. The protocol aims to ascertain presence or absence of six features likely to be associated with extrapulmonary TB: pericardial effusion, abdominal lymph nodes, pleural effusion, ascites, hypoechoic focal liver lesions or hypoechoic focal splenic lesions. Scanning was undertaken with a xxxxxx device with a curvilinear probe; images were saved and 10% of scans were read by a UK-registered consultant radiologist for quality control.

Decision to undertake chest x-ray was at the discretion of the participant's attending healthcare worker. If chest x-ray was done then digital pictures were taken and stored in the study database.

2.3.3 Outcomes and sample size calculations

The two co-primary outcomes for the study are:

1) 28 day mortality.

2) Aquisition of ESBL-E detected in stool by aerobic culture by 28 days.

Two power calculations were undertaken: to inform the size of the sepsis cohort, and one for the non-sepsis participants. The sepsis component was powered to detect clinically relevant risk factors for death; we made the a priori assumption that a risk ratio of 2 or more is clinically relevant in this setting and patient population. The initial calculation suggested 250 patients with severe sepsis with 50% mortality (as seen in previous studies in Malawi) would have 80% power to detect risk factors for death with prevalence of 20-50% that confer a risk ratio (RR) of 1.5-3; therefore, a RR of 2 is likely to be detected. Logistic considerations resulted in a reduction of a feasible sample size to 225; post-hoc power calculation suggested 80% power to detect risk factors coferring a RR of 2 with 25-50% prevalence, assuming 50% mortality.

For the second co-primary outcome, assuming 30% of participants with sepsis (and hence antimicrobial exposure) aquire ESBL-E by 28 days, and that 50% of them die by day 28, 125 antibiotic unexposed hospitalised participants would give 80% power to detect a 50% relative difference in aquisition. Logistic considerations resulted in the target sample size being reduced to 100 participants; revised post-hoc power calculation suggested that the 80% power to detet a 50% relative difference was maintained.

In order to define the baseline flux of ESBL-E organisms and ESBL genes, one community member for each antibiotic unexposed hospitalised participant, giving a target sample size of 100.

2.4 Diagnostic Laboratory Procedures

Results of all laboratory diagnostic procedures were fed back to participant's attending healthcare worker in real time. If any investigation result became available after the participant was discharged (e.g. Mycobacteraemia was identified) then they were contacted and called back to the hospital to be reviewed by me, and referred for care to the relevant QECH department.

2.4.1 Point of care diagnostics

Point of care tests were carried out on capillary blood for capillary lactate (Lactate Pro 2, Arkray, Japan), for *P. falciparum* HRP-2 antigen (Paracheck Pf, Orchid Biomedical, India) and, if HIV status was unknown, to test for HIV antibodies using Determine HIV 1/2 kit (Abbott Diagnostic Division) and Unigold HIV 1/2 kit (Trinity Biotech Inc.) following Malwian national guidelines[254].

2.4.2 Laboratory diagnostics

2.4.2.1 Haematology and biochemistry

Automated full blood counts (Beckman Coulter HmX Haematology Analyser, Beckman Coulter, USA) were undertaken in the MLW laboratories on EDTA whole-blood samples, as were CD4 cell counts (Becton Dickinson FACSCount, Becton Dickinson, USA) for HIV-infected participants. Serum samples were allowed to settle upright for 30-60 minutes and then centrifuged (at 1300 g for 10 min). Biochemistry testing for urea and electrolytes (Beckman Coulter AU480 Chemistry Analyser, Beckman Coulter, USA) was undretaken immediately and a maximum of three 1.8ml aliquots of serum stored at -80C for later analysis.

2.4.2.2 Aerobic blood and CSF culture

Blood in aerobic culture bottles was incubated in an automated system (BacT/Alert BioMerieux) and identified to species level using the API system (Biomerieux, France) and standard techniques (ref). Anaerobic culture was not available. Coagulase-negative *Staphylococci*, *bacillus spp.*, *diptheroids* and

alpha-haemolytic *Streptococci* other than *S. pneumoniae* were considered as contaminants. Antimicrobial sensitivity testing was undertaken using the disc diffusion method following BSAC guidelines to amoxicillin, chloramphenicol, co-trimoxazole, gentamicin, ciprofloxacin and ceftriaxone.

CSF, where available, was cultured onto blood, chocolate and Sabouraud agar and then identification of any growth undertaken as above.

2.4.2.3 Mycobacterial blood culture

Blood in Myco/F Lytic bottles was cultured at 37C in and inspected daily for the first 14 days with a handheld UK Wood's lamp, and once every two days thereafter. Contents of the bottles were centrifuged (3000xg for 20 mninutes) within 48hr of detection of flourescence, examined by ZN and gram stain to exclude contamination, then inoculated into mycobacterial growth indicator tubes (MGIT, Becton Dickinson Diagnostic Systems, United States) for up to 6 weeks. Isolates were classified as *M. tuberculosis* or nontuberculous Mycobacteria by microscopic cording and MPT-64 lateral flow assays (TAUNS Laboratories, Japan).

After 8 weeks of culture of Myco/F Lytic bottles if no flourescence was seen, then centrifugation and microscopy was carried out. If no organisms wereseen, the culture was reported as "no growth."

2.4.2.4 Sputum Xpert

Sputum Xpert testing (Cepheid, United States) was carried out as per the manufacturers instructions: sputum specimens were mixed with sample reagent and incubated at room temperature for 15 minutes. The liquefied specimen was then loaded into the Xpert MTB/RIF test cartridge for processing.

2.4.2.5 Urinary LAM

Urinary LAM testing was carried out on all available urine samples from HIV-infected participants, using the Alere Deterine TB LAM Ag lateral flow assay (Alere, United States) following the manufacturer's instructions. Frozen urine samples were used: they were allowed to come to room temperature and then briefly centrifuged to remove debris. 60 microliters of urine was applied to the sample pad and the result read after 25 minutes by comparing to the provided reference scale card. The results were read independently by two readers, with a tie-break read by a third reader inthe even of disagreement.

2.4.2.6 Selective stool culture for ESBL-E

Stool and rectal swabs recieved in the laboratory were stored at 4C pending processing, before being plated onto commercially available ESBL selective media (CHROMagar ESBL media, CHROMagar, France) and cultured aerobically at 37C overnight. Rectal swabs were streaked directly onto the plate, or a cotton tipped applicator used for solid stool. CHROMagar is also chromagenic: *E. Coli* colonies are pink, *Klebsiella spp.*, *Enterobacter spp.* and *Citrobacter spp.* blue and other species white. Blue or white colonies were speciated using the API 20E system. Morphpologically distinct colonies were confirmed to be ESBL producers using combination disc methods: the putative ESBL producer was cultured overnight on ISO-sensitest agar with discs of cefotaxime and ceftazidime (30 micrograms) with and without clavulanic acid (10 micrograms), and ESBL production confirmed if ther was a difference of at least 5mm between discs with and without clavulanic acid . For organisms likely to carry a chromasomal AmpC and hence be capable of hydrolysing cefotaxime and ceftazadime, (*Enterobacter spp.*, *Citrobacter freundii*, *Morganella morganii*, *Providencia stuartii*, *Serratia spp.*, *Hafnia alvei*) cefipime (30 micrograms), an AmpC-stable cephalosporin was used with and without clavulanic acid (10 micrograms). Antimicrobial sensitivity testing to meropenam, amikacin, gentamicin, co-trimoxazole, chloramphenicol and ciprofloxacin following BSAC guidelines[252] was undertaken for a subset of confirmed ESBL-producers.

2.4.2.7 Acute and convalescent serologies

PHE stuff

2.5 Molecular methods

One of each morphologically distinct colony from ESBL-E selective culture was taken forward for DNA extraction and whole genmone sequencing. DNA was extracted from overnight nutrient broth culture using the Qiagen DNA mini kit (Qiagen, Germany) following the manufacturers instructions. Extracted DNA was shipped to the Wellcome Sanger Institute where it underwent library preperation according to the Illumina protocol and paired-end sequencing on Illumina HiSeqX (*check this*) by the WSI DNA pipelines team.

2.6 Bioinformatics

All analyses were undertaken on the WSI computing cluster running Ubuntu v12.04.2, Precise Pangolin. Quality control of reads for each sample was carried out using metrics provided the WSI QC pipleine including total number of reads, and statistics obtained by mapping a random 100 Mbases fron each sample to a reference and calculating depth of coverage, number of heterogeneous SNPs, GC content and insert size. Kraken[255] v0.10.6, a kmer-based taxonomic assignemnt tool, was used to assign reads to a species and to identify suspected sample contamination or species misclassification. Reads were *de novo* assembled with SPAdes[256] v3.11.0, with default settings. Assembly metrics of quality (e.g. number of contigs, largest contig, N50 [the contig length on which at least half of the bases in an assembly have been assembled]) were calculated using QUAST[257] v4.6.0 and completeness and contamination of the assemblies assessed by checkM[258] v1.0.7, a tool that uses co-located sets of lineage associated genes to assess genome completeness and contamination. Contaminated samples (with $>$ 25% contamination as defined by CheckM) or poor assemblies (with N50 $<$ xxxxx) were discarded.

Prokka[259] v1.5 with a genus specific database from RefSeq was used to annotate the retained assemblies. The Roary v1.007 pan-genome pipeline[260] was used to construct a core genome with a blastp percentage identity of 95%; genes were considered core if they were present in 99% or more of isolates. A core gene multiple sequence alignment was generated using maaft[261] v7.205, SNP-sites identified using SNP-sites[262] v2.4.1 and the resultant SNP alignment used to build a maximum likelihood phylogenetic tree using IQ-TREE[263] v1.6.3. Trees were visualised in the ggtree v1.14.4 package[264] in R.

Ariba[265] v2.12.1 was used to identify AMR-associated genes using the SRST2[266] database, to identify plasmid replicons using the PlasmidFinder database[267] and to perform *in silico* muli-locus sequence typing (MLST) using the database from http://mlst.warwick.ac.uk/mlst/dbs/Ecoli accessed via www.pubmlst.org. *E. coli* phylogrouping was performed with a quadruplex *in silico* PCR using the Clermont scheme[268]. To track carriage of similar bacteria within and between individuals, clustering of the core gene alignment was performed using hierarchical BAPS (bayesian analysis of population structure) algorithm[269], implemented in the rhierbaps package v1.1.0 in R, with 2 levels. To track putative mobile genetic elements (MGE), a difficult task with short-read sequencing, isolates ESBL-gene containing contigs were identified using BLASTn[270] v2.7.0 with and then contigs clustered using cd-hit[271] v4.6 with sequence identity threshold 0.9, word size 5 and accurate mode (command line flag -g) to produce mutually exclusive ESBL-gene contig groupings.

2.7 Statistical Analysis

Details of anaysis methods are given in the relevant chapters. All analyses were undertaken using R v3.5.1 and any Bayesian modelling using Markov Chain Monte Carlo (MCMC) methods using Stan v2.18 via the Rstan interface with R. This thesis was written using the bookdown package in R and the code to generate it as well as all analysis scripts are avaiable at https://github.com/joelewis101/thesis.

2.8 Study Team

I am the principal investigator of the study, and led a study team which consisted of: Emma Smith, a medical doctor who managed the running of the study from March 2017 - May 2017; study nurses Lucy Keyala, Grace Mwaminawa and Tusekile Phiri who recruited patients, collected samples and completed follow up visits; field workers Witness Mtambo and Gladys Namacha who recruited patients, collected samples and completed follow up visits; and laboratory technicians Madlitso Mphasa and Rachel Banda who processed laboratory specimens, did stool culture and DNA extractions, and the uLAM testing. The MLW core laboratory staff undertook haematology and biochemistry testing and blood and CSF culture, and the Malawi CoM tuberculosis laboratory staff carried out tuberculosis culture and Xpert testing.

2.9 Data Collection and Storage

Data collection used both the Open Data Kit software (ODK) and structured TeleForm paper forms. Electronic ODK forms were loaded onto Asos ZenTouch tablets running Android using the ODK Collect Android app. Completed forms were pushed daily to the dedicated secure study SQL (structired query language) database built and administered by the MLW data team. Teleform forms were checked by me, batched and scanned by the MLW data team, and variable values automatically extracted by the TeleForm system; validation was undertaken by the MLW data teame and data queries generated for missing or invalid values. Queries were resolved either by manually reconciling with the forms or by discussion with the clinical team. Once the data passed validation it was pushed to the SQL database. Completed paper TeleForm records were stored securely in the MLW data department. All data on the study database is stored securely with access restricted to the study PI and the database adminstrators in the MLW data department. Other paper records including consent forms were stored in a locked cabinet with access restricted to members of the study team. Results of laboratory investigations in the MLW laboratory were stored in the MLW PreLink laboratory information management system (LIMS), anonymised and linked only to the participant unique study ID number. For analysis, anonymised data were extracted from the study SQL and LIMS databases as comma delimited files.

2.10 Ethical Approval, Consent and Participant Remuneration

The study protocol was granted ethical approval by the Malawi College of Medicine Research Ethics Committee (COMREC), protocol number P.11/16/2063 and the Liverpool School of Tropical Medicine (LSTM) Research Ethics Committee, protocol number 16-062. LSTM acted as the study sponsor. All study team member were trained in NIHR Good Clinical Practive (GCP).

All participants in the study provided infomred, written consent, as follows: if a patient lacked capacity to provide informed consent themselves then informed consent was sought from the patient's representative, usually their guardian. Patients with no representative and lacking capacity to provide informed comsent were not recruited. Initially, eligible patients (or their representative) were approached

by a member of the study team and the study was explained to them including study procedures, risks and benefits, financial and confidentiality considerations and how to obtain more information. Written patient information leaflets were provided in English and Chichewa. If the patient/representative was willing to enter the study then they were asked to sign and date two copies of the consent form, and provided with a copy of the form to keep. If either the patient or representative was unable to read then the consent form wasread to them by the study team, and witnessed by an additional staff member who is not a member of the study team. If the patient (or their representative) agreed to enter the study, then the witness signed and dated the form. Any patient who was enrolled to the study after consent from a representative and who then regained the capacity for informed consent was then approached independently for informed consent. A patient could withdraw at any time without giving a reason.

Hospitalised patients were not financially compensated for their time and/or transport costs, but all other participants were, follwing standard MLW guidelines: 500MWK (approximately USD$0.7 at February 2019 exchange rates) was provided to participants if they were visited at home and 2000MWK (approximately USD$2.8 at February 2019 exchange rates) or their reported transport costs (whichever was higher) for participants who attended QECH for study purposes.

Chapter 3

Mycobacterium tuberculosis BSI: an IPD meta analysis

Chapter 4

Sepsis in Blantyre, Malawi

4.1 Chapter overview

4.2 Methods

blah blah

4.3 Study population

Figure - Consort diagram

Table - demographics

Table - presentation

Table - health seeking behaviour

4.4 Aetiology

Table

Figure to show crossover

4.5 Treatment

Table: Time to antimicrobials Time to fluid Amount of fluid

4.6 Outcome

Table - 28 and 90 day mortality

Figure - KM survival curve

Logistic regression - determinants of 28 day mortality

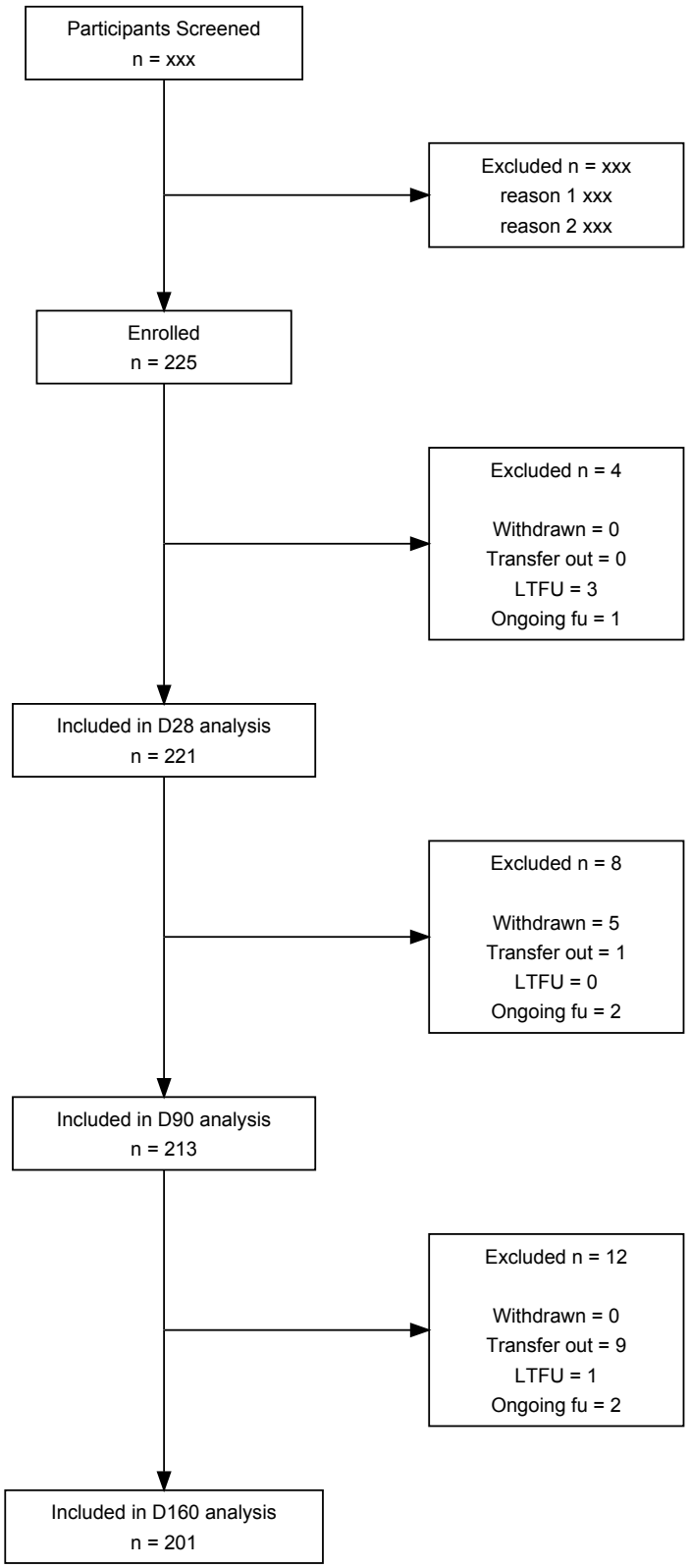

Figure 4.1: Study recruitment and follow up.

Chapter 5

Early response to resusitation in sepsis

Chapter 6

Gut mucosal carriage of ESBL-E in Blantyre, Malawi

Chapter 7

Whole genome sequencing of ESBL *E. coli* carriage isolates

7.1 Chapter overview

This chapter describes the use of whole-genome sequencing (WGS) of ESBL producing *E. coli* to understand the drivers of gut mucosal ESBL-E carriage. I will begin with a description of the genomic landscape of the isolates form this study: starting with simple descriptions of *E. coli* phylogroup and multilocus sequence type (MLST) I will place the isolates from this study in the context of the *E. coli* population, followed by higher-resolution contextulaistaion using phylogenetics to place isolates from this study in the context of a global *E. coli* collection. I will describe the genetic basis of antimicrobial resistance in these isolates and explore the extent to which AMR genes tend to cluster together beyond what would be expected by chance. Finally, I will attempt to use the resolution offered by WGS to attempt to answer two specific questions: firstly, what is the mechanism of rapid increase in ESBL-E carriage prevalence following hospital admission and antimicrobial exposure we see in this study? Secondly, what is the likely unit of ESBL-E transmission in this study? Are bacteria, or mobile genetic elements (MGE) implicated? And if, MGE, which: plasmids, transposons, integrons - or a combination?

These questions, phrased in this way, seem difficult or impossible to answer given the available WGS data, but by slightly reframing them they become tractable: first, what is the diversity of apparent hospital-acquired ESBL *E. coli* in comparison to apparent community-acquired isolates? Apparent hospital acquisitions could represent true acquisitions of, for example, a hospital-associated clone - but equally they could be an "unmasking" of minority variant *E. coli* in the microbiota, acquired in the community but not detected by culture because of low abundance, until enriched for by antimicrobial exposure. If the diversity of apparently hospital aquired isolates is contained within the diversity of community isolates, this would lend support to this latter hyothesis. The second question - what is the unit of transmission in this system - can be reframed by asking: what is the unit that is most conserved within patients, as compared to between patients? The questions then reduce to a dimensionality redcution problem: in order to address them both, it is necessary to classify either bacteria or MGE into mutually exclusive categories, in order to compare hospital to community isolates, and between-patient to within-patient. I describe the approach I have taken to this below.

7.2 Methods

7.2.1 Bioinformatic pipeline

The basic bioinformatic pipeline used is described in detail in Chapter 2, methods. Briefly, one *E. coli* colony from each patient sample was taken forward for DNA extraction and paired-end short-read whole genome sequencing using Illumina HiSeq X at the Wellcome Sanger Institute. Read quality control was undertaken with Kraken[255] v0.10.6 to assign reads to species and WSI QC pipeline which maps a random 100 Mbases fron each sample to a reference and calculates depth of coverage, number of heterogeneous SNPs, GC content and insert size. Samples that contained > 80% non *E coli.* reads were discarded and *de novo* assembly was undertaken with SPAdes[256] v3.11.0. Assembly statistics were calculated with QUAST[257] v4.6.0 and completeness and contamination of the assemblies assessed by checkM[258] v1.0.7. Contaminated assemblies (with checkM-defined contamination of > 25%) or poor assemblies (with less than 1Mb assembled length) were discarded. Annotation was carried out with Prokka[259] v1.5 with a genus specific database from RefSeq and the Roary v1.007 pan-genome pipeline[260] was used to identify a core genome. A core gene multiple sequence alignment was generated using maaft[261] v7.205, SNP-sites identified using SNP-sites[262] v2.4.1 and the resultant SNP alignment used to build a maximum likelihood phylogenetic tree using IQ-TREE[263] v1.6.3, using ascertainment bias correction to correct for the fact that the input pseudosequence contained only variable sites, and using the ModelFinder module used to find the best fitting nucleotide substition model. This calculates the likeliood of a number of different models and chooses the model with the lowest (best fitting) Bayesian Information Criterion, a statistic which penalises model parameters. Reliability of inferred branch partitions was assessed with 1000 bootstrap replicates. Trees were visualised in the ggtree v1.14.4 package[264] in R.

Ariba[265] v2.12.1 was used to identify AMR-associated genes using the SRST2[266] database, to identify plasmid replicons using the PlasmidFinder database[267] and to perform *in silico* muli-locus sequence typing (MLST) using the database from http://mlst.warwick.ac.uk/mlst/dbs/Ecoli accessed via www.pubmlst.org. The β-lactamase genes *ampC*1, *ampC*2 and *ampH* were excluded from the analysis of AMR determinents as they do not usually cause a resistant phenotype in *E. coli*. Because quinolone resistance often results from SNPs in the chromasome in the quinolone resistance determining regions (QRDRs) of the *gyrA*, *gyrB*, *parE* and *parC* genes - rather than acquisition of whole AMR-determining genes, as is the case with the other genes sought by Ariba - these genes were downloaded from the comprehensive antimicrobial resistance database (CARD, https://card.mcmaster.ca/) and Ariba used to call SNPs in them, with default settings. *E. coli* phylogrouping was performed with a quadruplex *in silico* PCR using the Clermont scheme[268] and isPcr v33x2 (https://github.com/bowhan/kent/tree/master/src/isPcr)

Rhierbaps package v1.1.0 in R[269] was used to cluster the core genome pseudosequence into sequence clusters (SCs). Two levels were used and these level 2 clusters used to test associations (see statistical analysis, below). To track putative mobile genetic elements ESBL-gene containing contigs were identified using BLASTn[270] v2.7.0 of all contigs against the SRST2 database and then contigs containing any given ESBL gene were grouped by the ESBL gene they contained (for example, all bla_{ctxm15} gene-containing clusters were grouped together), and each group clustered using cd-hit[271] v4.6 to produce mutually exclusive ESBL-gene-containing contig clusters for each identified ESBL gene. Henceforth, these clusters will be referred to as ESBL-clusters, for brevity. In order to attempt to determine the biological significance of the identified ESBL-clusters (i.e. what kind of MGE element they are likely to represent), basic statistics were plotted (number of samples contained withing each cluster, length of longest contig in cluster in kbases, length distribution of all contigs is cluster relative to longest contig and distribution of sequence identity compared to the longest contig in the cluster). Presence of compound trasposons, AMR determinents and plasmid replicons were identified by using BLAST with default settings of each ESBL-cluster representative sequence (as determined by cd-hit i.e one, the longest, for each ESBL-cluster) against the insertion sequence finder (ISfinder) database and the SRST2 database, taking the top hit (as determined by bitscore) for any given location, and visualising the results in gggenes v0.3.2. To assess lineage association, the ESBL-clusters were mapped back to the

core genome SNP tree.

7.2.2 Global *E. coli* collection

In order to place the isolates from this study in a global context, published *E. coli* assemblies were downloaded from the WSI servers. These included 149 ESBL-producing *E. coli* from a single centre study in Chachoengsao province, eastern Thailand[272]. In this study, human clinical isolates from standard care in Bhuddhasothorn hospital were selected on the basis of the ESBL phenotype, and environmental samples were collected as part of a cross sectional study and selectively cultured for ESBL-E in 2014-2015. I also downloaded assemblies of 362 enterotoxogenic *E. coli* (ETEC), selected for an ETEC genomic study from the Gothenburg University ETEC collection to represent a broad collection of ETEC isolated worldwide from 1980-2011[273]; 185 atypical enteropathogenic *E. coli* (aEPEC) sequenced for a study of aEPEC and selected from samples frrm the Global Enteric Multicentre Study (GEMS) in seven centres in Africa and Asia between 2007-2011[274]; and 94 *E. coli* from QECH in Blantyre, Malawi, a combination of invasive (bloodstream and CSF) and carriage isolates, selected for diversity in AMR phenotype from 1996-2014[275]. Details of the year, sample andcountry of isolation for these samples are given in the appendix to this chapter.

Phylogroup and MLST were determined for these context genomes as described above. AMR genes were identified with Ariba and the SRST2 database, as above, and context genomes were classified as ESBL if they contained any Bush-Jacoby group 2be ESBL gene .

7.2.3 Statistical analysis

In order to explore clustering of AMR genes, the Jaccard index was calculated for a given AMR-gene pair using he Jaccard v0.1.0 package in R. The Jaccard index, a measure of the similarity of two sets of data, is defined as *intersection over union*; in this context, for a given pair of AMR genes x and y, the Jaccard index $J(x, y)$ is the number of isolates that contain both gene x and y divided by the total number that contain either x or y:

$$J(x, y) = \frac{|x \cap y|}{|x \cup y|}$$

By definition it lies between 0 (x and y never co-occur) and 1 (x and y always co-occur). Co-occurance matrices using the Jaccard index were plotted using the pheatmap v 1.0.12 package in R. The statistical significance of co-occurance of genes was assessed by generating 2x2 contingency tables for a given gene pair and p values generated using a Fisher's test with Bonferroni correction; a p value of less that 0.05 was considered statistically significant. Co-occurance networks of genes occuring commonly together (defined as Jaccard index > 0.5) at a rate greater than expected by chance ($p < 0.05$ following Bonferroni correction) and uncommonly occuring together (defined as Jaccard index < 0.1 and $p < 0.05$ following Bonferroni correction) were plotted using igraph v1.2.2 and ggraph v1.0.2 in R.

To explore hospital or community associations of any given *E. coli* clade, the location of isolation was first plotted against the phylogenetic tree; location of isolation was classified as hospital, community, or recent hospital discharge (defined as a date of isolation within 2 weeks of hospital discharge). This latter category was used because it is possible that a patient could acquire an ESBL-E clone in hospital but only be sampled once leaving hospital; using only hospital isolated and community isolted categories could therefore introduce bias. Hospital or community association of each sequence cluster was assessed using a Fisher's test of proportion of hospital associated samples (defined as sum of hospital isolated and recent hospital discharge) for the given sequence cluster as compared to proportion of hospital associated samples in the remainder of the samples, with a Bonferroni correction for multiple comparisons. $p < 0.05$ was again considered statistically significant.

To compare within-patient to between-patient conservation of bacteria (as represented by core genome alignment and sequence cluster) and ESBL-containing MGE (as represented by the ESBL-clusters) several approaches were taken. Firstly, I assessed whether either sequence cluster or ESBL-cluster were conserved within an individual at all. I hypothesised that any within-patient correlation is likely to be a function of time: samples closer together in time may be more likely to be similar. To assess if this was the case for bacteria, pairwise core genome pseudosequence SNP distance was was calculated using snp-dists v0.4 (https://github.com/tseemann/snp-dists) for all samples and plotted against the time difference (in days) between samples, within and between patients, and with a smoothed curve fitted using a general additive model with cubic splines. Because of significant overplotting, this was also plotted as a 2D density plot. Based on these plots, the within and between patient SNP distances were compared in two post-hoc defined groups binned by time distance between the samples (50 days or less vs. more than 50 days), and distributions compared with Kruskal-Wallace tests.

I then compared the within patient temporal clustering of ESBL-clusters and sequence clusters, by estimating the proportion of within-patient samples that contain the same ESBL-cluster or sequence cluster, as a function of time; essentially a temporal autocorrelation function. To estimate this, I considered pairwise comparsion of all within-patient samples. For any given time between samples, t I defined a window of +/-5 days and estimated the probabilities as the number of all within-patient sample pairs in the window $[t-5, t+5]$ that contained the same sequence cluster or ESBL-cluster divided by the total number of all within-patient sample pairs within that time window. Exact binomial confidence intervals for these proportions were generated and probabilities plotted as a function of time. In order to estimate the probability of two samples containing the same sequence cluster of contig-cluster purely by chance, 1000 sample pairs were randomly drawn from all samples with replacement and the proportion of these samples that contained the same sequence cluster or ESBL-cluster calculated.

Finally, to inform the question as to what the likely unit of transmission in this system is, I assessed what was most conserved within patients, in pairwise sample comparison: bacteria (as represented by core gene sequence cluster), ESBL-containing MGE (as represented by ESBL-cluster), or both. Simple proportions in all-against-all pairwise comparison - stratified by whether between-patient or within-patient - were calculated: the proportion of samples that contain the same core gene sequence cluster only, the proportion of samples contain the same ESBL-cluster only, and the proportion that contain both sequence cluster and ESBL-cluster. Proportions were compared between within and between-patient strat in these three groups using Fisher's exact test, with $p < 0.05$ considered statistically significant.

7.3 Results

7.3.1 Samples and quality control

In total, 520 *E. coli* underwent DNA extraction and were shipped from Malawi to WSI; these represented all sequential isolates at the time of final DNA extraction, which occured in two batches in February 2018 and October 2018. Kracken/Bracken read assignment of these samples is shown in Figure 7.1. The majority of samples have $> 90\%$ or reads assigned to *E. coli*; a minority have $< 90\%$ of reads assigned to *E. coli* but a very closely related species such as *Shigella*, amd as such are likely to be pure *E. coli* culture with read misclassification. However, 12 samples have $> 80\%$ reads assigned to a non-*E. coli* species such as *Klebsiella pneumoniae*. These samples were assumed to represent upstream species misidentification or, perhaps more likely, selection of the wrong sample from the freezer archive for culture and DNA extraction, given that for any sample ID there are often several bacterial species identified and cryopreserved. These samples were excluded from further analysis.

Of the remaining 508 samples, there were a median (IQR) of 2339594 (2112842.5-2533930.5) reads, with a median (IQR) depth of coverage (obtained by mapping a random 100Mbases to a refernce *E. coli* genome, Escherichia coli strain K-12 substrain MG1655, NCBI reference NC_000913.3) of 58 (51-66).

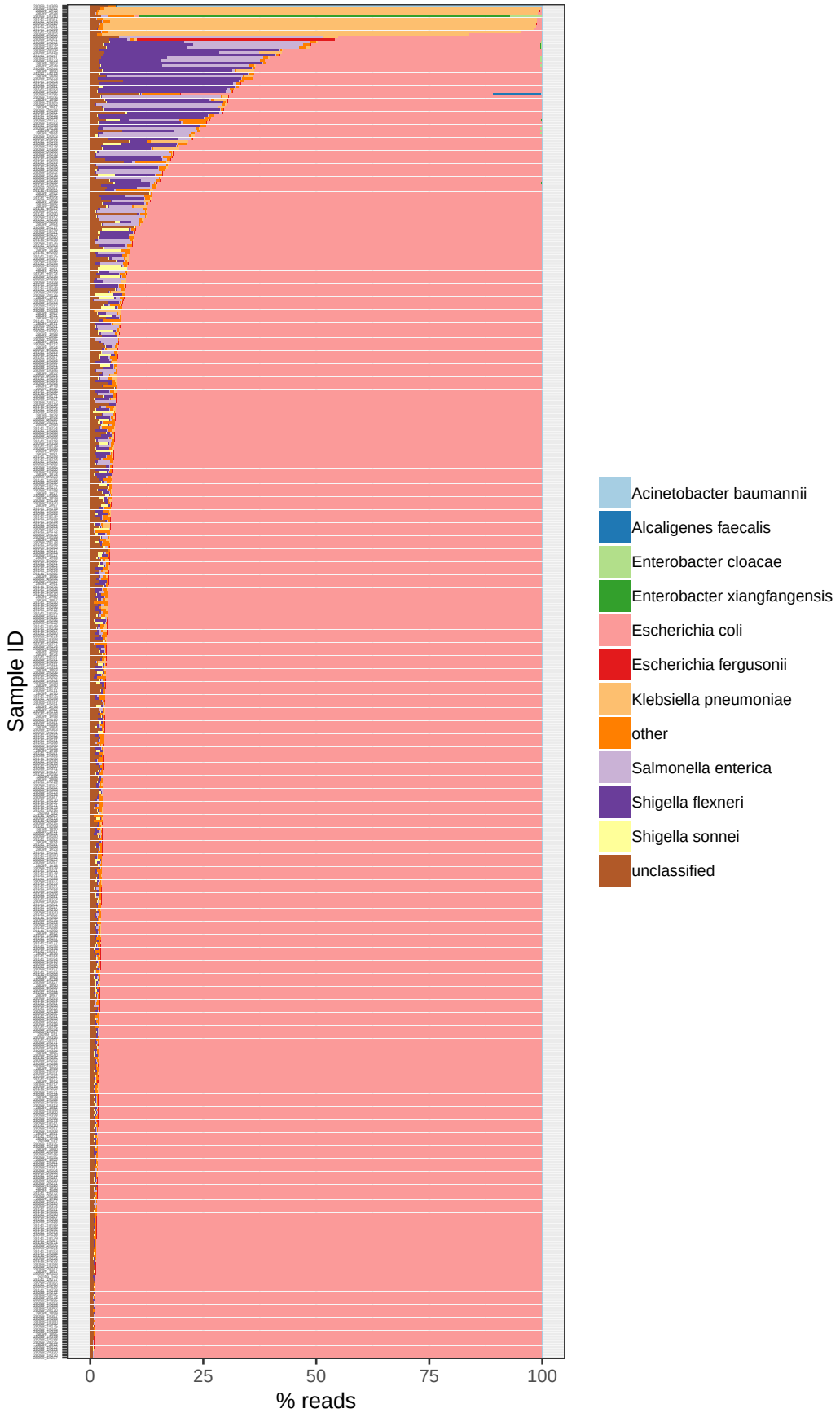

Figure 7.1: Species read assignment of all samples

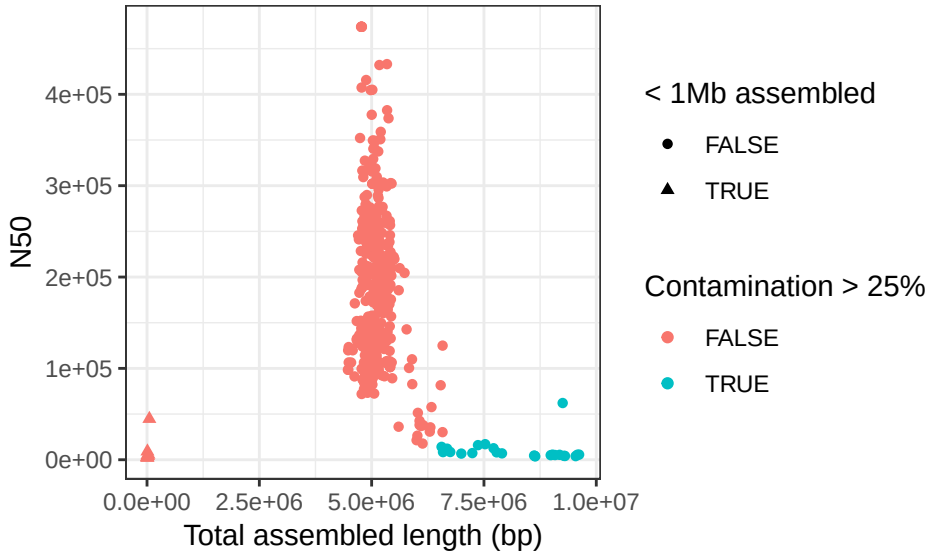

Figure 7.2: N50 as a function of total assembled length. Failed assemblies with less than 1Mb assembled shown as triangles. Contaminated assemblies with checkM-defined contamination above 25% shown in blue.

One sample had an order of magnitude lower number of reads (291556) with depth of coverage 0; this was assumed to represent sequencing failure and it was excluded from further analysis.

The output from quast and checkM are shown in Figure 7.2, where N50 (the minimum contig length upon which at least half assembled bases are contained) is plotted as a function of total assembled length. The expected *E. coli* genome length is around 4.6Mb and most samples cluster close to this at a total assembled length of ~ 5Mb. Howvever it is clear that some assemblies have failed, with low N50 and low assembled length. It is also apparent that some samples seem to be contaminated, as indicated by low N50 and much longer than expected total assembled length. Defining assembly failure as < 1Mb assembled length (triangles in the plot, n = 9) and contamination as checkM-defined contamination of $> 25\%$ (blue points in the plot, n = 24) and excluding both groups results in 33 further samples being excluded from further analysis.

In total, therefore, 46/520 (11%) of samples which were submitted for sequencing were excluded from downstream analysis. The remaining 474 samples represent 69% (474/686) of the cutured *E. coli* in this study; 354 are from patients with sepsis, 86 are from hospitalised inpatients and 33 are from community members, with a median of 2 (range 1-5) samples per participant. N50, total assembled length and number of assembled contigs are shown in the appendix to this chapter.

7.3.2 Phylogroup, MLST and core genome phylogeny of study isolates

The commonset *E. coli* phylogroup was phylogroup A: 204/473 (43%) samples belonged to phylogroup A, followed by phylogroup B2 (96/473 [20%]), F (53/473 [11%]), B1 (43/473 [9%]) and C (43/473 [9%]) and D (26/473 [5%]). Two samples were Clade I or II (so called cryptic clades) and 6/473 (1%) were unknown phylogroup using the Clermont PCR scheme. In the MLST analysis, 56 recognised sequence types (STs) were identified, and 12 samples were novel STs; however over half (249/473 [53%]) of samples were represented by the top seven most frequent STs (Figure 7.3). ST131 was the most commonly isolated sequence type (64/473 [14%] of isolates) followed by ST410 (45/473 [10%] of isolates) and ST167 (38/473 [8%] of isolates).

The Roary pan-genome pipeline identified a core genome in the study isolates of 2966 genes, with a pan-genome of 26840 genes. The resultant core gene pseudosequence of length 1388742 bases contained

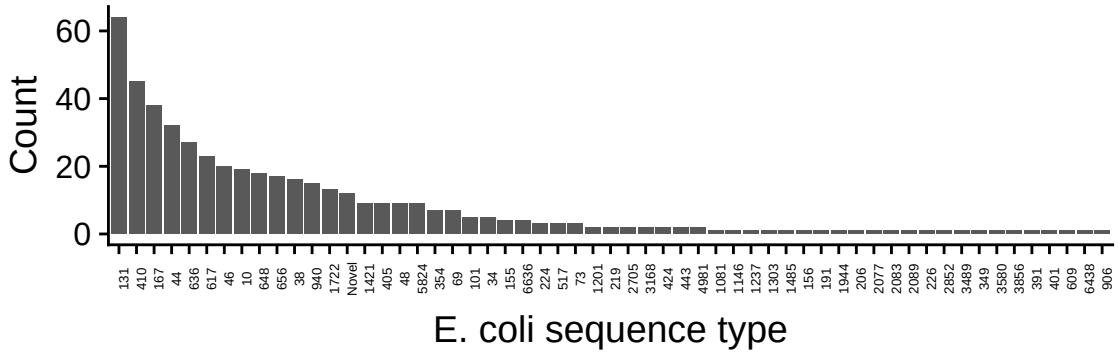

Figure 7.3: *E. coli* sequence type distribution

99693 variable sites, which were used to infer the maximum likelihood phylogenetic tree. The IQTREE ModelFinder module determined that a general time reversible (GTR) model with FreeRate site heterogeneity with 5 parameters provided the best fit to the data. The inferred tree is shown in Figure 7.3 along with isolate phylogroup and sequence types; in general, as expected, sequence types were largely monophyletic and phylogroups tended to cluster together.

7.3.3 Study isolates in a global context

The global collection of *E. coli* comprised 1273 samples, including the 473 from this study. 753/1253 (60%) were from Africa, 335/1253 (27%) from Asia and 167 (13%) from South America. The majority of samples, 1026/1253 (82%), were from stool, with 106/1253 (8%) truly invasive samples from blood or CSF and 63/1253 (5%) possibly invasive samples from urine, pus, or sputum. 65/1253 (5%) of samples were environmental, all from Thailand. 670/1253 (53%) of samples contained at least one ESBL-encoding gene. The majority of isolates with ESBL gene (622/670 [92%]) came from this study or the Thai ESBL study. Phylogroup A was the commonest phylogroup in the global collection (482/1273 [38%]), followed by B1 (333/1273 [26%]) and B2 (191/1273 [15%]); phylogroup C was uncommon in the global collection (74/1273 [6%]) but the majority of the phylogroup C samples came from this study (43/74 [58%]). All of these 43 phyogroup C isolates belonged to a single ST, ST410; this ST was not seen at all in the previous Malawian study of largely invasive isolates, despite beingthe second-commonest ST in this study. ST131 was again the commonest ST in the global collection.

The Roary pan-genome pipeline identified 2872 core genes in a pan genome of 44840 genes; this large pan-genome is consistent with the open *E. coli* pan genome that will continue to increase in size as isolates are added. The core gene alignment contained 604817 bases with 77194 variable sites, which were used to infer the maximum likelihood phylogenetic tree, using same nucleotide substitution model as previously.

The inferred tree is shown in Figure 7.5). Isolates from this study are distributed throughout the tree, and there is widespread mixing of isolates from diverse geographic regions. Though invasive isolates are spread throughout the tree, there is a tendance for them to cluster together, particularly in phylogroup B2, a phylogroup with has a recognised association with ExPEC (needs ref). The Malawian ST410 isolates clusterd tightly together, though are most closely related to clinical ESBL-procucing ST410 isolates from Thailand. By comparison, ST131 isolates from this study were distributed amongst ST131 isolates from other studies, both in Malawi and elsewhere.

7.3.4 Antimicrobial resistance determinents

All identified AMR genes are shown in Figure 7.6A, alongside a summary of number of isolates with resistnce mutations to given antimicrobial classes (Figure 7.6B) and the phenotypic resistance of the

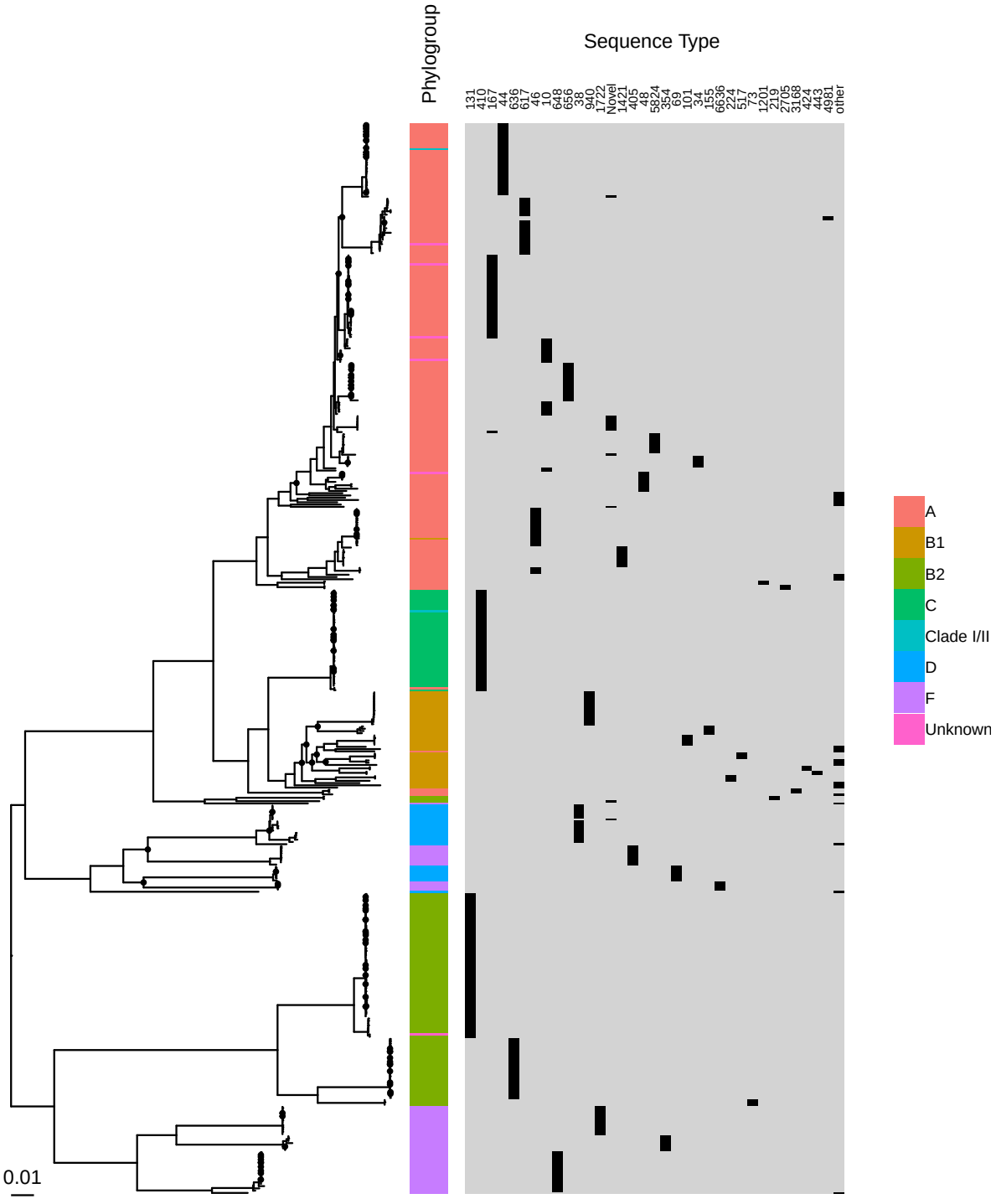

Figure 7.4: Maximum likelihood phylogenetic tree of included study *E. coli* isolates showing phylogroups and sequence types. Bootstrap support of less than 90% is indicated by a black circle at a given node. Scale bar indicates 0.01 SNPs/site.

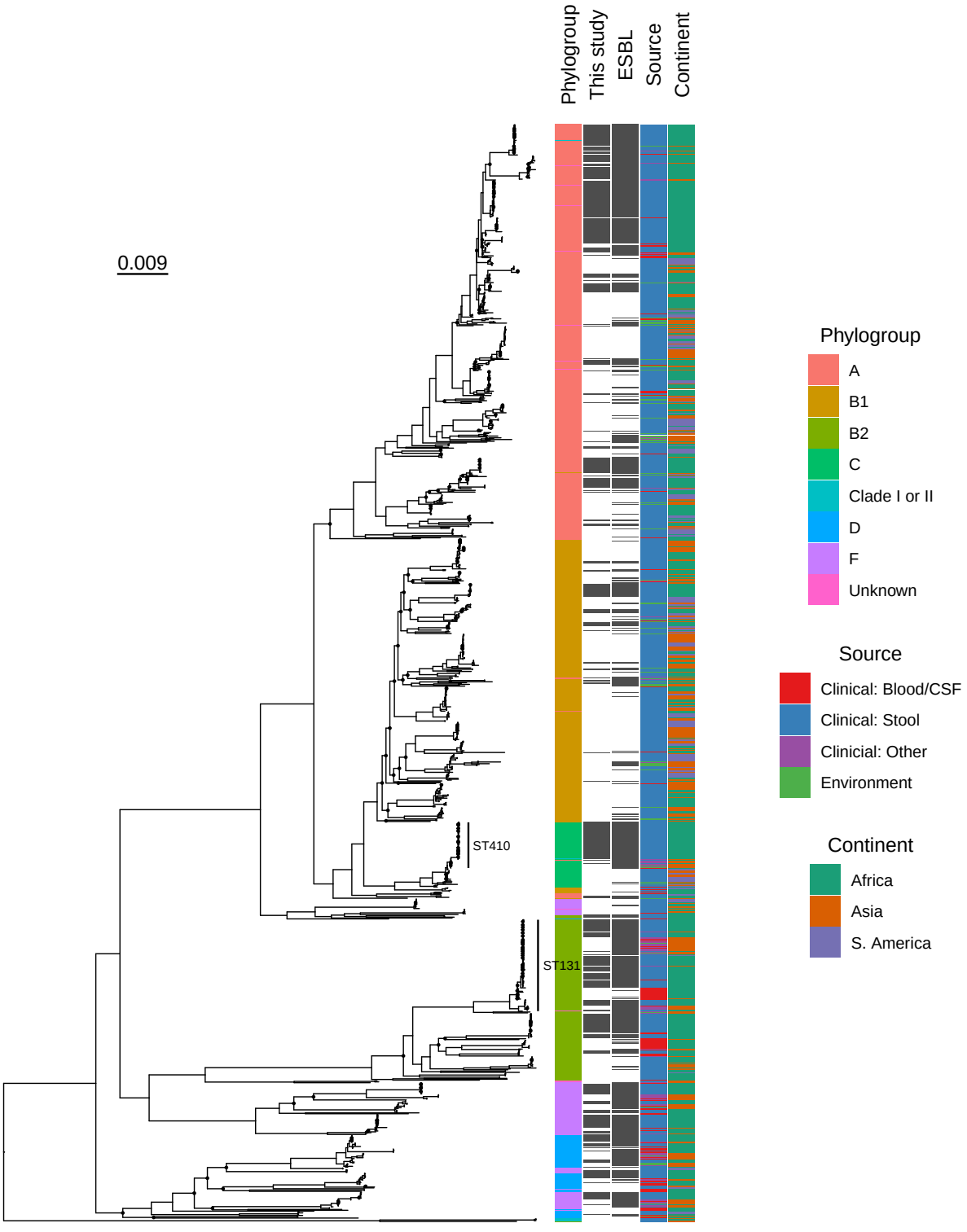

Figure 7.5: Midpoint rooted maximum likelihood phylogenetic tree of included study *E. coli* isolates along with global context isolates, showing phylogroups, source sample type and continent of isolation (coloured bars). Dark grey bars indicate isolates from this study or isolates with ESBL gene presence, as labelled (this study or ESBL, respectively). Two most frequently isolated STs (131 and 410) labelled. Bootstrap support of less than 90% is indicated by a black circle at a given node. Scale bar indicates 0.009 SNPs/site.

isolates for which phenotypic antimicrobial resistance testing was carried out (449/473 [95%]). A description of resistance gene by class, along with a consdideration of concordance (or otherwise) of phenotypic resistance and predicted resistance from genotype, are given in turn below.

7.3.4.1 β-lactam resistance

All isolates contained at least one gene that conferred resistance to third-generation cephalosporins, either an ESBL gene (n= 472) or a carbapenamase (n=1). The majority of ESBL-gene containing isolates contained only one ESBL gene (459/472 [97%]); fewer contained 2 (13/472 [3%]) and none contained more than 2. bla_{CTX-M} was the commonest ESBL gene, and over two thirds (319/473 [67%]) of isolates contained $bla_{CTX-M-15}$. ESBL bla_{SHV} (26/473 [5%] of isolates) genes were also seen. ESBL bla_{TEM} (1/473 isolates) and bla_{OXA} (1/473 isolates) were very unusual; however, narrow spectrum bla_{TEM} and bla_{OXA} β-lactamases were common: bla_{OXA-1} and bla_{TEM-95} were present in 186/473 [39%] and 289/473 [61%] of isolates respectively. Plasmid-mediated bla_{ampC} genes were identified in 45/473 (9%) of isolates, almost all (44/45) bla_{CMY-42}; this was unexpected as all of these isolates were comfirmed to be ESBL-producers by combination disc testing. This testing uses cephalosporin-containing discs both with and without clavulanic acid, and confirms EBSL production by a difference in zone size between these discs, as ESBL enzymes are inactivated by clavulanic acid. However, the cephalosporins used in this test are likely to be hydrolysed by *ampC* enzymes, and if these isolates were producing such enzymes it could confer cephalosporin resistance regardless of the presence or absence of clavulanic acid. This was not the case for any of these isolates; none of them hydorolised the cephalosporins used in the presence of clavulanic acid. It may be that the bla_{CMY} genes were not expressed.

The carbapenamase gene identified was s bla_{NDM-5}; the isolate harbouring this gene was recovered from the stool of a 67-year old man with no history of foreign travel nor hospitalistion. He had been admitted to the hospital with fever seven days previously and treated with seven days of intravenous ceftriaxone for sepsis, the source of which was not clear. He made an uneventful recovery, and no carpapenamase-containing isolate was recovered from his stool at any other time. The bla_{NDM-5} gene was carried on a partially assembled IncX3 plasmid. BLAST of this assembly against the NCBI database showed that this contig had 99% sequence identity with a previously sequenced pNDM-MGR194 46.2 kbp blaNDM-5 containing Inc-X3 plasmid found in India between 2011-13[276]. We fully assembled the plasmid by mapping reads back to pNDM-MGR194 with Burrows-Wheeler alignment and found it to be extremely similar, with only 13 SNPs compared to pNDM-MGR194.

7.3.4.2 Quinolone resistance

108/473 (23%) of isolates contained plasmid-mediated quinolone resistance PMQR) genes, either *qnr* or *qep*. Nonsynonymous mutations were identified in at least one of the quinolone resistance-determining regions (QRDR) - *gyrA*, *gyrB*, *parC*, or *parE* - in 349/449 (78%) of isolates. The majority of mutations were well-described QRDR mutations (codon 83 and 87 in *gyrA*, codon 80 and 84 in *parC* and codon 458 in *parE*, Figure 7.7A). QRDR mutations tended to cluster togather (Figure 7.7B) but alone they correlated poorly with phenotypic resistance. Of the 449 samples with available phenotypic sensitivity data, 294/449 (65%) were intermediate or resistant to ciprofloxacin, but 349/449 (78%) had a mutation in any codon in one of the four QRDR; presence of any QRDR mutation together with presence of PMQR had sensitivity of 95% (95% CI 93-98%) but specificity of 27% (95% CI 20-34%) for phenotypic quinolone resistance. Presence of mutations at all of codon 83 and 87 of *gyrA* and at codon 80 of *parC* has previously been shown to have the best predictive ability of phenotypic resistance[277], and this showed improved, but still poor, discrimination for phenotypic resistance with sensitivity 89% (95% 85-93%) and specificity 54% (95% CI 46-62%) in this dataset.

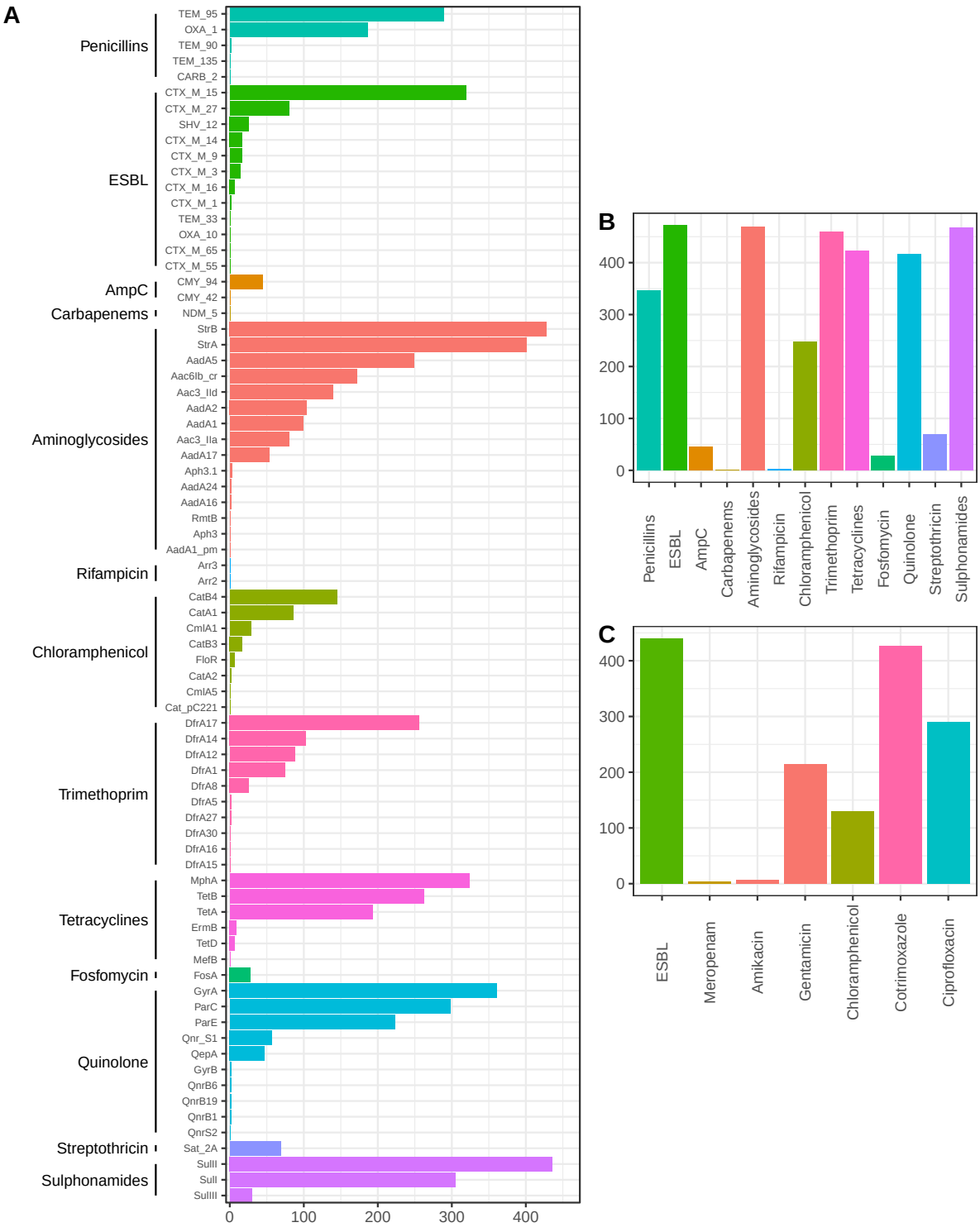

Figure 7.6: A: Frequency distribution of AMR genes identified in isolates. Class of antimicrobial to which gene confers resistance is shown. B: Number of isolates with any mutation to a given class. Any mutation that could possibly confer resistance to a given class is included, including any mutation in the QRDR for quinolones. C: Phenotypic resistance patterns for subset of samples in this analysis that also underwent phenotypic testing (n = 449)

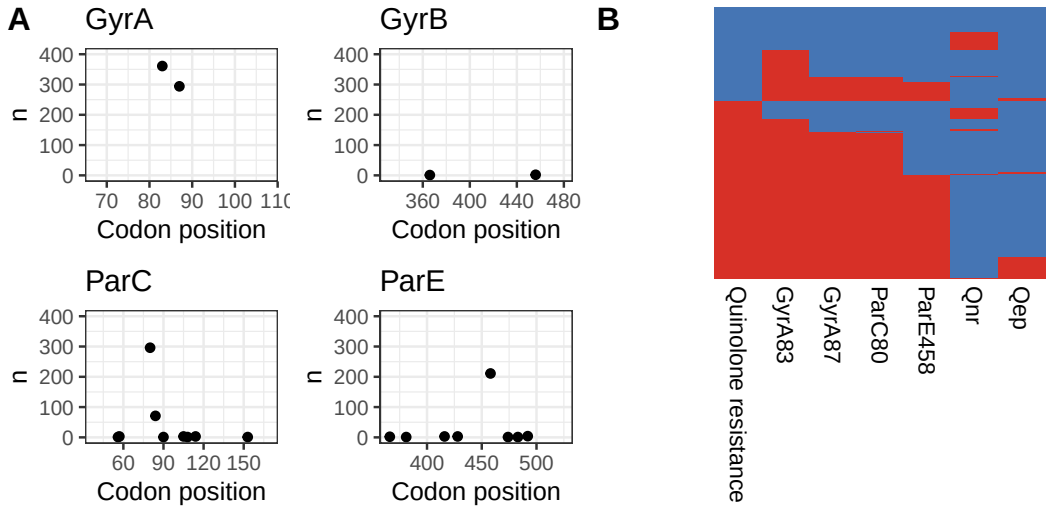

Figure 7.7: A: Mutation positions in quinolone resistance-determining regions, showing that most mutations are well-recognised (see text for details) B: Co-occurance heatmap of QRDR mutations (*gyrA*, *parC*, or *parE*) plamid-mediated quinolone resistance mutations (*qnr* or *qep*) and phenotypic resistance. Each row is one sample, red = presence, blue = absence.

7.3.4.3 Aminoglycoside resistance

Aminoglycoside resistance genes were very common in the sequenced isolates, with 469/473 (99%) of isolates containing at least one aminoglycoside gene, and most containing multiple different genes: median number of aminoglycoside resistance genes per isolate was 4 (IQR 3-5). Despite streptomycin being absent from all Malawian treatment guidelines save for retreatment of tuberculosis, the streptomycin resistance genes *strA*, *strB* and *aadA* family of genes (also called *aad(3")*) were very commonly seen (Figure 7.8A). Genes that would be expected to confer gentamicin resistance - *aac(3)-IIa*, *aac(3)-IId* and *aac(6')-Ib-cr* were common, but genes that would be expected to confer amikacin resistance (*rmtB*) and kanamycin resistance (*aph(3')*) were unusual (Figure 7.8B)[278,279]

The predictive value of presence of *aac(3)-IIa*, *aac(3)-IId* or *aac(6')-Ib-cr* for phenotypic gentamicin resistance was moderate at best with sensitivity 77% (95% CI 71-83%) and specificity 73% (95% CI 67-79%). Of 6 phenotypically amikacin resistant or intermediate isolates, all had recognised streptomycin resistance determinents (*strA*, *strB* or *aadA*) but 4/6 had no other aminoglycoside determinent identified. Of the remaining two, one isolate contained *aac(6')-Ib-cr* and one both *aac(3)-IIa*, *aac(3)-IId*.

7.3.4.4 Chloramphenicol, co-trimoxazole and tetracycline resistance

248/473 (52%) of isolates contained at least one chloramphenicol resistance gene (Figure 7.6), usually 1 (210/248 [85%]), less commonly 2 (37/248 [15%]) or 3 (1/248 [<1%]). *catB4* was the most commonly identified gene but once again phenotypic chloramphenicol resistance correlated poorly with presence of chloramphenicol resistnce genes (7.9A)) with presence of any chloramphenicol resistance gene predicting phenotypic resistance with a sensitivity of 70% (95% 62-78%) and specificity of 55% (95% CI 49-60%).

Almost all isolates contained either a trimethoprim resistance (459/473 [97%]) or a sulphonamide resistance gene (468/473 [99%]); only 3/473 isolates did not contain either. Trimethoprim resistance genes were all of the *dfrA* family; *sulII* was the commonest sulphonamide resistance determinent (Figures 7.6 and 7.9B). Summary sensitivity of presence of any *dfrA* or *sul* gene as a predictor of phenotypic resistance was 100% [95% CI 99- 100%] but (partially due to the rarity of cotrimoxazole

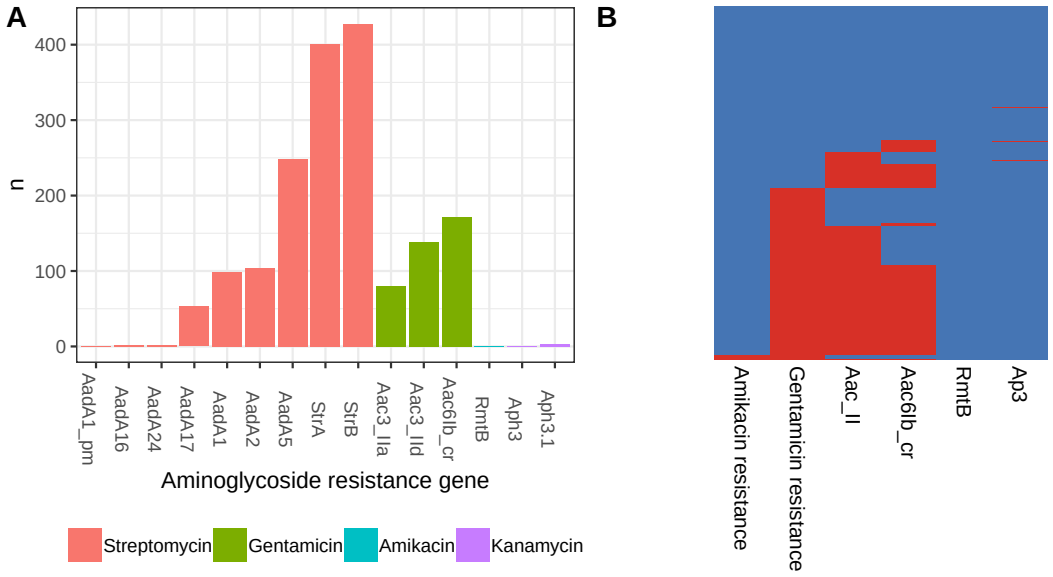

Figure 7.8: A: Aminoglycoside mutations and expected resistance to gentamicin, amikacin and kanamycin B: Heatmap showning phenotypic amikacin and gentamicin resistance and identified resistance genes that could be expected to confer resistance to these agents (see text for details). Aac_II in heatmap indicates presence of either *aac(3)-IIa* or*aac(3)-IIa* gene. Each row is one sample, red = presence, blue = absence.

sensitivity in this datset) specificity was 13% [95% CI 2-40%].

Tetracycline resistance genes were also very common, identified in 422/473 (89%) of isolates, most commonly *mphA* (324/473 [68%] of isolates), followed by *tetB* (262/473 [55%] of isolates) and *tetA* (193/473 [41%] of isolates). No antimicrobial sensitivity testing was carried out for any agent of the tetracycline class.

7.3.5 Lineage association and clustering of AMR determinents

Next, I explored associations of AMR determinents, both with bacterial lineages, and with other AMR determinents. First, I mapped the identified AMR determinents back to the maximum likelihood tree (Figure 7.10), demonstrating a clear lineage association, in that certain AMR genes tend to cluster within lineages. To explore this further, the matrix of pairwise jaccard indices was constructed

7.3.6 Plasmid replicons

7.3.7 Testing metadata associations: Sequence clusters and ESBL-clusters

Finally,

7.4 Appendix

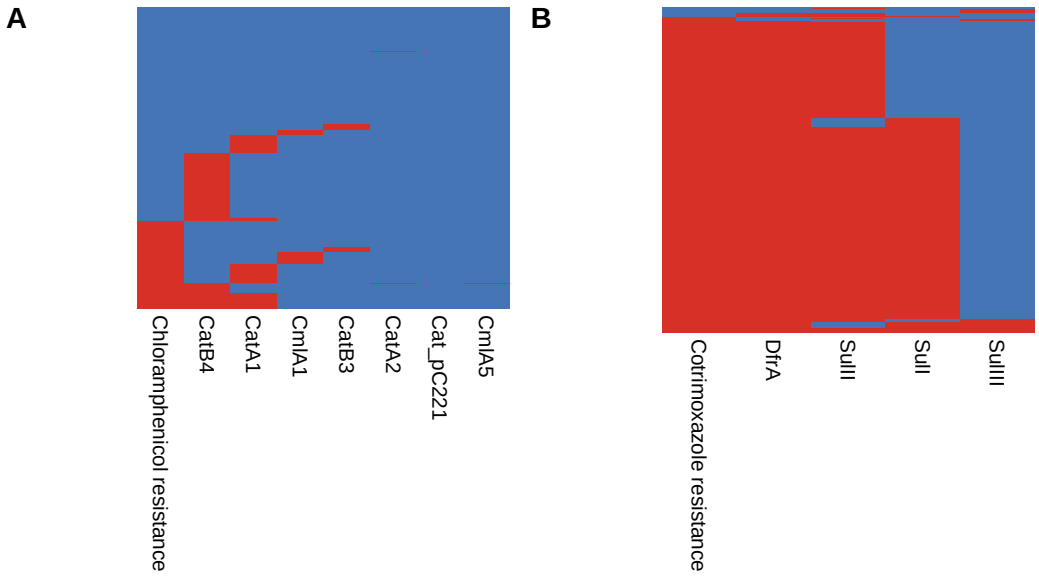

Figure 7.9: Heatmap showning phenotypic chlramphenicol (A) and cotrimoxazole (B) resistance and identified resistance genes that could be expected to confer resistance to these agents. Each row is one sample, red = presence, blue = absence

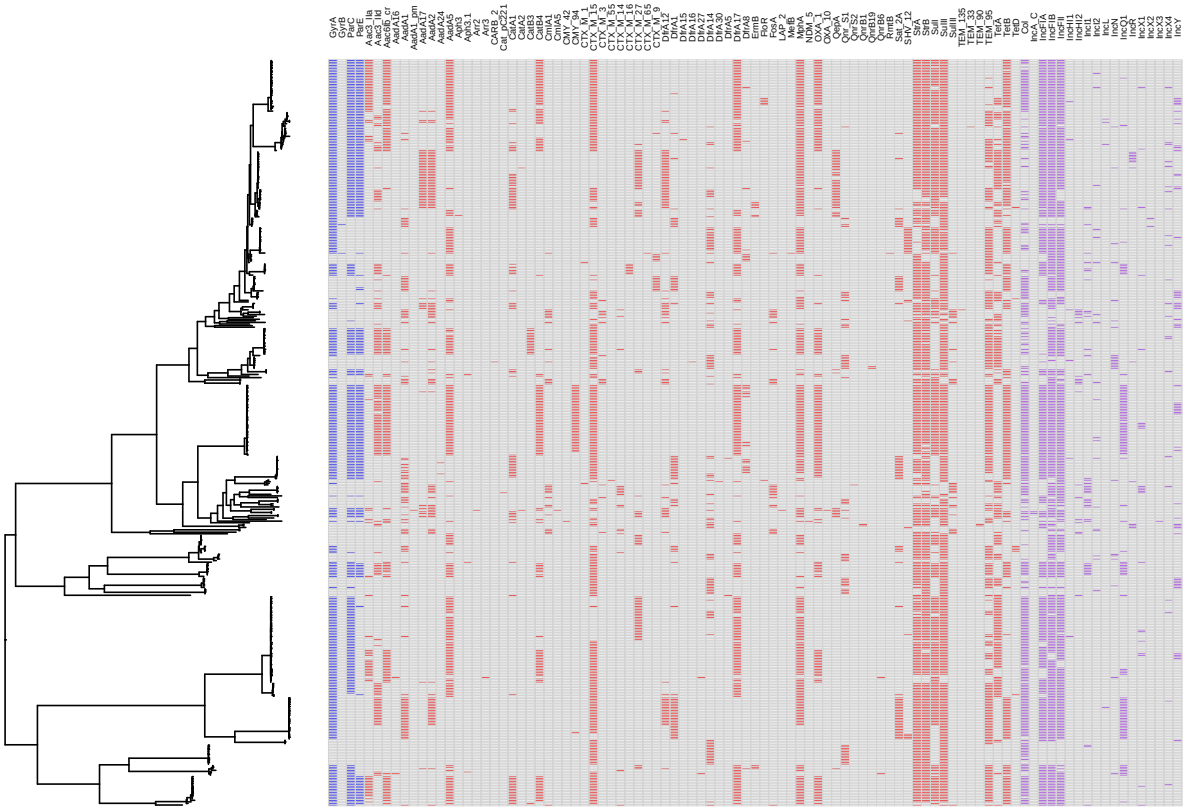

Figure 7.10: AMR determinents mapped back to tree, showing lineage associations of AMR genes and plasmid replicons. Presence of QRDR mutations is indicated by blue (any nonsynonymous mutation in any QRDR shown), plasmid replicons purple and other AMR genes red.

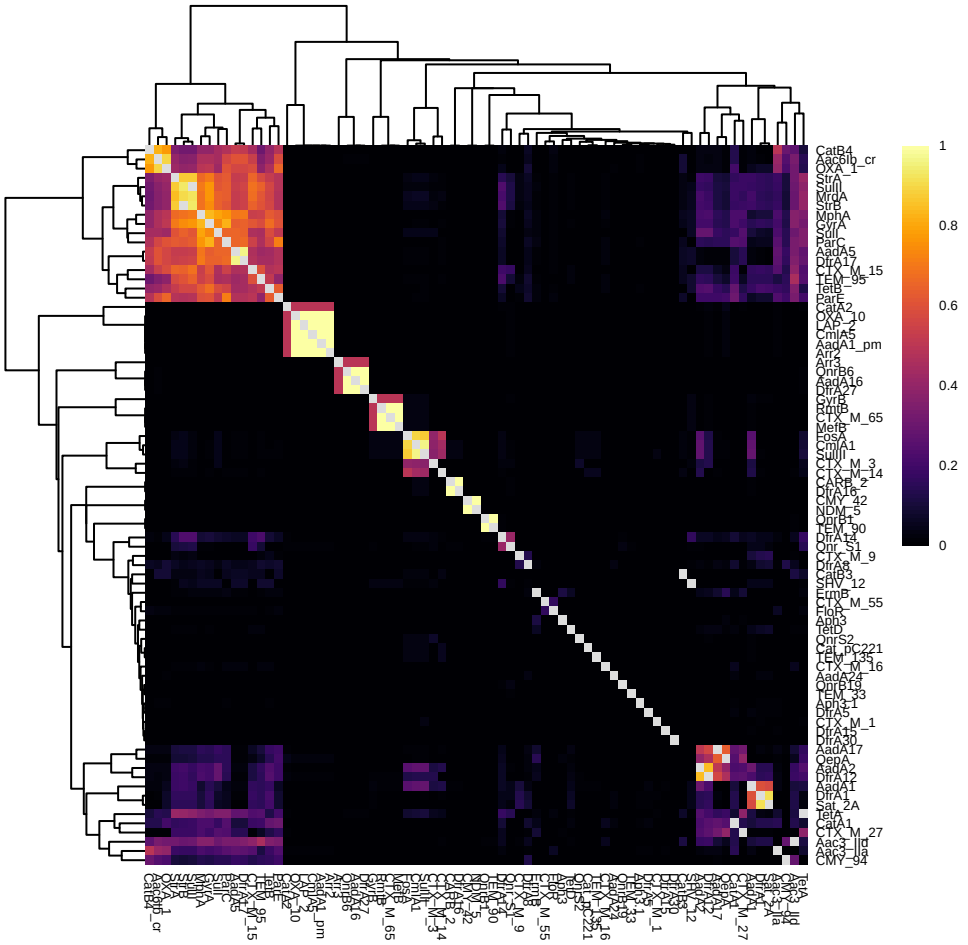

Figure 7.11: Heatmap of row and column clustered matrix of pairwise Jaccard indices to compare co-occurance of AMR genes

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