

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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5.4.1 Study population

In total, 425 participants were recruited to the study between 19th February 2017 and 2nd October 2018; 225 participants with sepsis (arm 1), 100 inpatients without antimicrobial exposure at baseline (arm 2) and 100 community members (arm 3). Flow of participants through the study is shown in Figure 5.2. It was often challenging to collect stool samples from participants but 87% (1416/1631) eligible patient-visits resulted in the collection of a stool sample. Drop out from the study and failure to collect stool samples were similar in arm 1 and 2 and with no apparent systematic bias, but both drop out and missing samples were less frequent in arm 3 (Figure 5.1A). There was significant variation in the timing of stool sample collection, with a distribution around the ostensible collation day (Figure 5.1B).

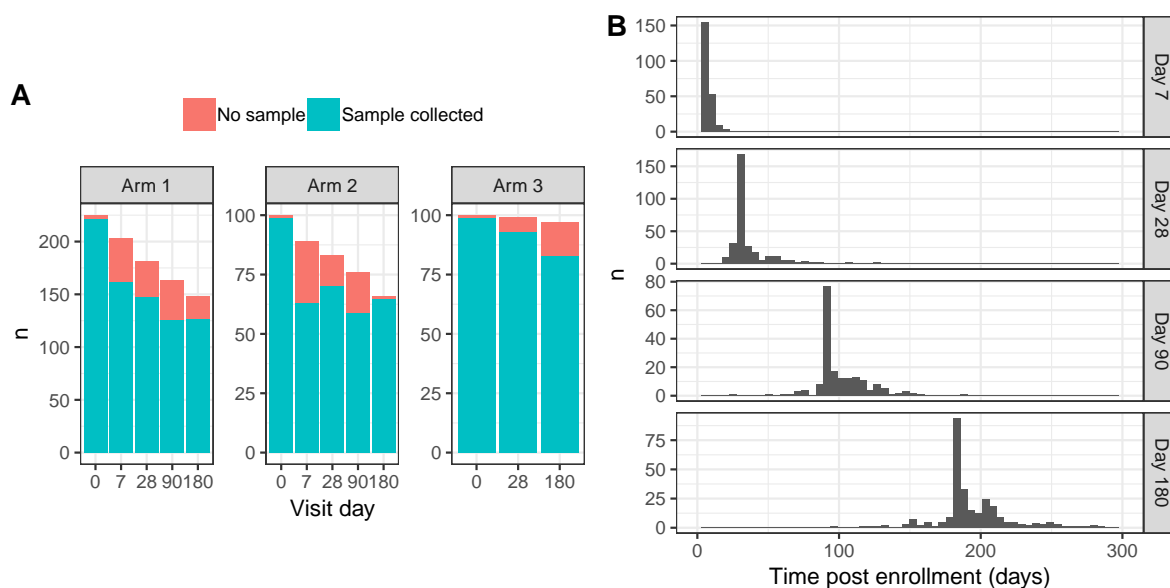


Figure 5.1: A: Missing stool samples stratified by arm and visit. Bar height at a given visit represents the number of eligible participants, coloured by successful sample collection (blue) or failure to collect a sample (red). B: Distribution of actual day of sample collection for ostensible day 7, 28, 90 and 180 samples showing considerable variation.

The baseline characteristics of the enrolled participants are shown in Table 5.1. There were some important differences between the arms of the study: despite matching on age and sex, antimicrobial-unexposed participants were older. They were also less likely to be HIV-infected than participants with sepsis (13% [12/89] of those with known HIV status were HIV-infected versus 67% [143/213] with sepsis), and less likely to have been treated for TB. Sepsis participants were more likely to have received antimicrobials or been hospitalised in the previous 4 weeks. In the community arm of the study, there were a high proportion of participants (60% [60/100]) with an unknown HIV status, and there were some differences in toilet facilities, water sources, cooking fuel and presence of animals at home across the three groups.

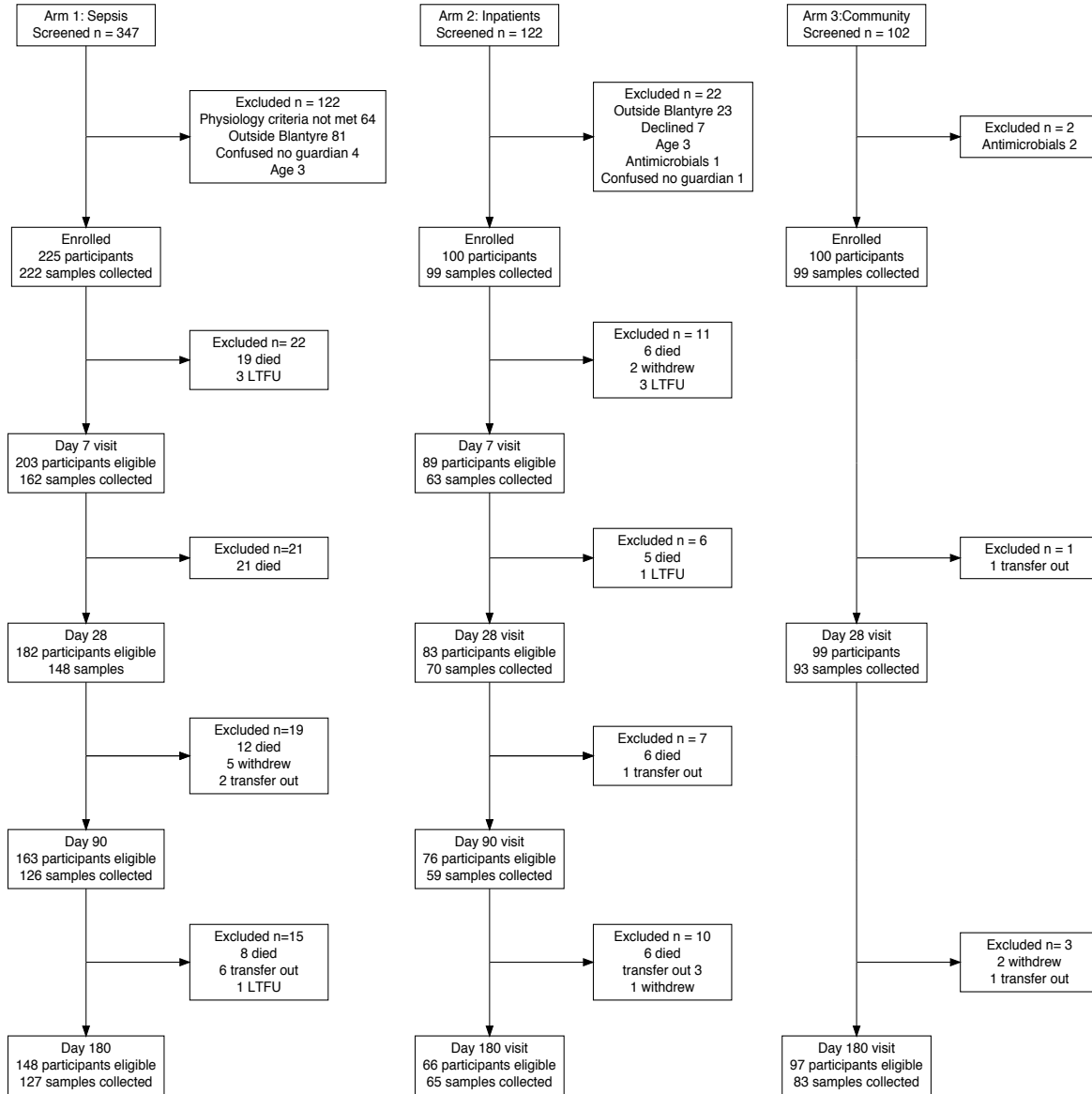


Figure 5.2: Study recruitment and follow up. At each time point *eligible participants* refers to participants who are known to be alive and have not withdrawn from the study by that time point, and *samples collected* refers to patients from whom a stool sample was successfully collected for that visit.

Table 5.1: Participant Characteristics

Variable	Sepsis	Inpatient	Community	p	Total
Demographics					
Age (yr)	35.9 (27.8-43.5)	40.4 (29.1-48.3)	32.5 (24.0-38.4)	<0.001	35.6 (26.9-43.9)
Male sex	114/225 (51%)	51/100 (51%)	40/100 (40%)	0.533	205/425 (48%)
HIV/TB status					
HIV Reactive	143/225 (64%)	12/100 (12%)	18/100 (18%)	<0.001	173/425 (41%)
HIV Non Reactive	70/225 (31%)	77/100 (77%)	22/100 (22%)	<0.001	169/425 (40%)
HIV Unknown	12/225 (5%)	11/100 (11%)	60/100 (60%)	<0.001	83/425 (20%)
Ever treated for TB	37/225 (16%)	5/100 (5%)	4/100 (4%)	0.002	46/425 (11%)
Of those, current TB treatment	10/37 (27%)	0/5 (0%)	4/4 (100%)	0.098	14/46 (30%)
ART status*					
Current ART*	117/143 (82%)	9/12 (75%)	18/18 (100%)	0.859	144/173 (83%)
Months on ART	28.7 (3.7-72.6)	35.1 (2.9-79.8)	31.5 (13.0-79.9)	0.693	29.5 (3.8-72.8)
ART regimen: EFV/3TC/TDF	110/117 (94%)	8/9 (89%)	17/18 (94%)	1.000	135/144 (94%)
ART status					
Current CPT [†]	98/141 (70%)	5/12 (42%)	7/18 (39%)	0.328	110/171 (64%)
Healthcare exposure last 4wk					
Antibiotics	60/225 (27%)	0/100 (0%)	0/100 (0%)	<0.001	60/425 (14%)
Hospitalised	18/225 (8%)	1/100 (1%)	0/100 (0%)	0.001	19/425 (4%)
Tobacco/alcohol use					
Never tobacco	196/225 (87%)	93/100 (93%)	90/100 (90%)	0.929	379/425 (89%)
Ex tobacco	17/225 (8%)	6/100 (6%)	2/100 (2%)	0.180	25/425 (6%)
Current tobacco	12/225 (5%)	1/100 (1%)	8/100 (8%)	0.070	21/425 (5%)
Current alcohol	51/225 (23%)	16/100 (16%)	18/100 (18%)	0.502	85/425 (20%)
Education					
Primary incomplete or complete	97/225 (43%)	50/100 (50%)	42/100 (42%)	0.739	189/425 (44%)
Some secondary education	47/225 (21%)	18/100 (18%)	30/100 (30%)	0.238	95/425 (22%)
Secondary school complete	48/225 (21%)	16/100 (16%)	19/100 (19%)	0.677	83/425 (20%)
No formal schooling	16/225 (7%)	13/100 (13%)	4/100 (4%)	0.094	33/425 (8%)
College or higher	17/225 (8%)	3/100 (3%)	5/100 (5%)	0.346	25/425 (6%)
Employment					
Unemployed	82/225 (36%)	34/100 (34%)	32/100 (32%)	0.866	148/425 (35%)
Self-employed	56/225 (25%)	32/100 (32%)	35/100 (35%)	0.325	123/425 (29%)
Currently employed	65/225 (29%)	26/100 (26%)	18/100 (18%)	0.269	109/425 (26%)
Student	21/225 (9%)	6/100 (6%)	15/100 (15%)	0.153	42/425 (10%)
Retired	1/225 (0%)	2/100 (2%)	0/100 (0%)	0.280	3/425 (1%)

Toilet facilities					
Pit latrine with slab +/- foot rest	104/225 (46%)	25/100 (25%)	35/100 (35%)	0.039	164/425 (39%)
Pit latrine with slab and cover +/- foot rest	45/225 (20%)	19/100 (19%)	55/100 (55%)	<0.001	119/425 (28%)
Hanging toilet/latrine	59/225 (26%)	48/100 (48%)	9/100 (9%)	<0.001	116/425 (27%)
Flush Toilet (any type)	14/225 (6%)	5/100 (5%)	1/100 (1%)	0.118	20/425 (5%)
No toilet	2/225 (1%)	2/100 (2%)	0/100 (0%)	0.533	4/425 (1%)
Composting toilet	1/225 (0%)	1/100 (1%)	0/100 (0%)	0.720	2/425 (0%)
Main water source					
Public tap/standpipe	51/225 (23%)	8/100 (8%)	66/100 (66%)	<0.001	125/425 (29%)
Piped outside dwelling	69/225 (31%)	37/100 (37%)	9/100 (9%)	<0.001	115/425 (27%)
Tube well/borehole	64/225 (28%)	35/100 (35%)	15/100 (15%)	0.032	114/425 (27%)
Piped into dwelling	30/225 (13%)	11/100 (11%)	7/100 (7%)	0.353	48/425 (11%)
Unprotected well/spring	5/225 (2%)	6/100 (6%)	2/100 (2%)	0.181	13/425 (3%)
Surface water (including rainwater collection)	4/225 (2%)	2/100 (2%)	0/100 (0%)	0.556	6/425 (1%)
Tube well with powered pump	2/225 (1%)	1/100 (1%)	1/100 (1%)	1.000	4/425 (1%)
Treat water with chlorine	19/225 (8%)	5/100 (5%)	0/100 (0%)	0.004	24/425 (6%)
No. household members					
Children	2.0 (1.0-3.0)	2.0 (1.0-3.0)	2.0 (1.0-3.0)	0.395	2.0 (1.0-3.0)
Adults	2.0 (2.0-3.0)	3.0 (2.0-4.0)	2.0 (2.0-4.0)	0.907	3.0 (2.0-4.0)
Electricity					
Electricity available in house	119/225 (53%)	41/100 (41%)	58/100 (58%)	0.357	218/425 (51%)
Main cooking fuel					
Charcoal	161/225 (72%)	63/100 (63%)	88/100 (88%)	0.291	312/425 (73%)
Wood	61/225 (27%)	35/100 (35%)	11/100 (11%)	0.004	107/425 (25%)
Electricity	3/225 (1%)	2/100 (2%)	1/100 (1%)	0.869	6/425 (1%)
Animals at home?					
Any animal	71/225 (32%)	43/100 (43%)	15/100 (15%)	0.004	129/425 (30%)
Poultry	46/71 (65%)	34/43 (79%)	10/15 (67%)	0.800	90/129 (70%)
Dogs	18/71 (25%)	11/43 (26%)	9/15 (60%)	0.201	38/129 (29%)
Other	11/71 (15%)	9/43 (21%)	5/15 (33%)	0.413	25/129 (19%)
Goats	12/71 (17%)	7/43 (16%)	1/15 (7%)	0.830	20/129 (16%)
Cattle	2/71 (3%)	3/43 (7%)	0/15 (0%)	0.406	5/129 (4%)

Note:

ART = Antiretroviral therapy, CPT = Co-trimoxazole preventative therapy, EFV: Efavirenz, 3TC: Lamivudine, TDF: Tenofovir. Numeric values are median (IQR)) unless otherwise stated. P-values are to assess for differenced across the three groups: Fisher's exact test across the groups for categorical variable, and Kruskal-Wallace test for continuous variables.

* ART status includes HIV reactive only as denominator

† Missing CPT data for two participants.

5.4.2 Exposures during the study period

Exposures to antimicrobials and hospitalisation of the cohort are shown in Figure 5.3 and Table 5.2. Antimicrobial-unexposed inpatients (Arm 2 participants) had a shorter length of hospital stay than participants with sepsis (Arm 1 participants): median (IQR) 2 (2-7) versus 5 (2-10) days, $p = 0.002$ by Kruskal-Wallis test. Five of the 100 Arm 2 participants were taking co-trimoxazole preventative therapy (CPT) at baseline, 18 received further courses of antimicrobials during the study period, and two were started on TB therapy. Some participants received combinations of these therapies, so in total 23% (23/100) Arm 2 participants received an antibacterial during the study period, mostly within 30 days following enrollment (Figure 5.3), and most commonly ceftriaxone (Table 5.2).

Both antimicrobial exposure and hospitalisation were unusual in the community cohort; 7% (7/100) community (Arm 3) participants were taking CPT and one received a 5-day course of amoxicillin meaning that 8% (8/100) Arm 3 participants received an antibacterial during the study period. In addition one Arm 3 participant was hospitalised for 1 day in the study period. No Arm 3 participant received any TB therapy, and no Arm 2 or 3 participants received any antimalarial or antifungal therapy during the study period.

The most commonly received antibacterial by Arm 1 participants - those with sepsis - (apart from co-trimoxazole and TB therapy) was ceftriaxone by some distance with 998 participant-days of exposure in 189 participants during the study period, and a median 5 (IQR 3-7) day course. Ciprofloxacin and amoxicillin were also commonly received, with 61 participants receiving 398 participant-days of exposure to ciprofloxacin with a median 7 (IQR 5-7) day course, and 39 participants receiving 235 participant-days of exposure to amoxicillin with a median 5 (IQR 5-7) day course. Because of the chronic nature of the therapy, the greatest exposure (in terms of participant-days) were to co-trimoxazole and TB therapy, by an order of magnitude (Table 5.2).

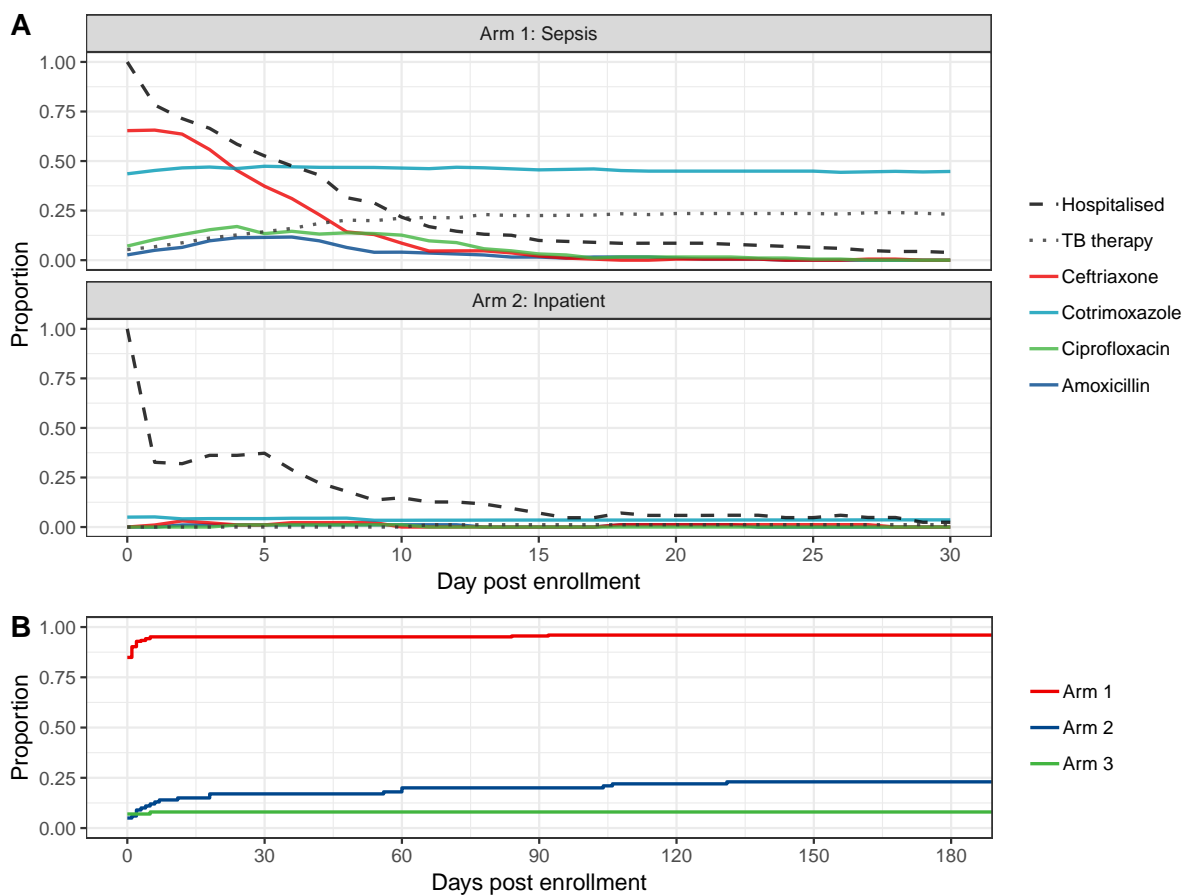


Figure 5.3: Hospital and antibacterial exposure of participants expressed as (A) proportion of Arm 1 and Arm 2 participant who are hospitalised and/or exposed to the most commonly received antibacterials on any given day and (B) cumulative proportion of participants who have been exposed to any antibacterial over the study period.

Table 5.2: Antimicrobial and hospital exposure stratified by arm

Exposure	Number exposed			Exposure (person-days)			Median (IQR) exposure length (days)		
	Arm 1	Arm 2	Arm 3	Arm 1	Arm 2	Arm 3	Arm 1	Arm 2	Arm 3
Total At Risk	225	100	100	33797	14336	21983	-	-	-
Exposures									
Hospitalised	225	100	1	1727	500	1	5 (2-10)	2 (2-7)	1 (1-1)
Cotrimoxazole	110	6	7	14447	549	1388	180 (27-190)	86 (6-177)	190 (183-206)
TB therapy	52	2	0	6843	291	0	178 (58-180)	146 (133-158)	-
Ceftriaxone	183	7	0	997	26	0	5 (3-7)	3 (2-4)	-
Ciprofloxacin	61	2	0	398	12	0	7 (5-7)	6 (6-6)	-
Amoxicillin	38	3	1	235	21	5	7 (5-7)	5 (5-8)	5 (5-5)
Metronidazole	24	2	0	148	10	0	6 (2-7)	5 (5-5)	-
Fluconazole	27	0	0	118	0	0	3 (2-5)	-	-
Aciclovir	2	0	0	47	0	0	24 (16-31)	-	-
Co-amoxiclav	10	2	0	40	12	0	5 (2-5)	6 (6-6)	-
Erythromycin	5	0	0	38	0	0	7 (5-11)	-	-
Doxycycline	7	0	0	34	0	0	3 (2-6)	-	-
Artesunate	11	0	0	25	0	0	2 (2-3)	-	-
LA	7	0	0	19	0	0	3 (2-3)	-	-
Streptomycin	2	0	0	16	0	0	8 (7-9)	-	-
Gentamicin	4	0	0	15	0	0	4 (3-5)	-	-
Amphotericin	2	0	0	8	0	0	4 (4-4)	-	-
Azithromycin	2	2	0	7	12	0	4 (3-4)	6 (6-6)	-
Penicillin	2	0	0	5	0	0	2 (2-3)	-	-
Flucloxacillin	2	0	0	5	0	0	2 (2-3)	-	-
Chloramphenicol	1	0	0	1	0	0	1 (1-1)	-	-
Quinine	1	0	0	1	0	0	1 (1-1)	-	-

Note:

TB = tuberculosis, LA =lumefantrine artemether. Median exposure length includes only those exposed. Total at risk shows the total number of participants and participant-days of follow up included in the study.

Table 5.3: ESBL carriage stratified by arm and visit

Visit	Arm 1 (Sepsis)		Arm 2 (Inpatient)		Arm 3 (Community)	
	n	Any ESBL	n	Any ESBL	n	Any ESBL
Day 0	222	109 (49%)	99	41 (41%)	99	28 (28%)
Day 7	162	127 (78%)	63	32 (51%)	-	-
Day 28	148	106 (72%)	71	37 (52%)	92	29 (32%)
Day 90	126	71 (56%)	60	29 (48%)	-	-
Day 180	127	61 (48%)	65	29 (45%)	83	24 (29%)

5.4.3 ESBL-E colonisation

ESBL-E colonisation prevalence as a function of time across the three arms of the study is shown in Table 5.3 and Figure 5.4. Baseline colonisation prevalence was high in all groups, and higher in hospitalised participants than community members: 49% (95% CI 42-56%) in Arm 1 participants, 41% (95% CI 32-52%) in Arm 2 and 28% (95% 20-38%) in Arm 3. Both hospitalised groups showed a rise in colonisation prevalence following admission, though this is much more marked in Arm 1 participants: by the day 7 visit 78% (95% CI 71-84%) of Arm 1 participants were colonised compared to 51% (38-64%) of Arm 2 participants. By the end of the study period the prevalence had fallen back to baseline levels in both groups.

In total, 723/1417 (51%) of samples grew at least one ESBL-E; 1032 organisms were grown from the 723 samples, with a median 1 (IQR [1-2]) ESBL-E per sample. The most commonly isolated organism as identified by the API system was *E. coli* (n = 686) followed by *Klebsiella pneumoniae* (n = 245, Figure 5.5). Antimicrobial sensitivity testing was carried out on the first 694/1032 (67%) organisms; meropenam and amikacin sensitivity was near universal (680/694 [98%] and 679/694 [98%] of isolates respectively), but cotrimoxazole sensitivity very unusual (19/694 [3%] of isolates), with intermediate proportions of gentamicin (327/694 [47%]) and ciprofloxacin (237/694 [34%]) sensitivity. The antimicrobial to which the greatest proportion of isolates were sensitive was chloramphenicol (462/694 [67%] of isolates).

5.4.4 Associations of ESBL colonisation

I then used logistic regression to explore associations of ESBL-E colonisation at baseline. Of the 420 participants with an available enrollment stool culture result, 42% (178/420) cultured at least one ESBL-E. Univariable and multivariable associations of colonisation at enrollment are shown in 5.4. In univariable analysis HIV infection, ART and CPT are associated with ESBL-E colonisation, but this seems to be largely mediated by CPT as the HIV and CPT associations largely disappear on multivariable modelling but the effect of CPT is still apparent (aOR 2.3 [95% CI 1.0 - 5.5]). Hospitalisation within the 4 weeks prior to admission was

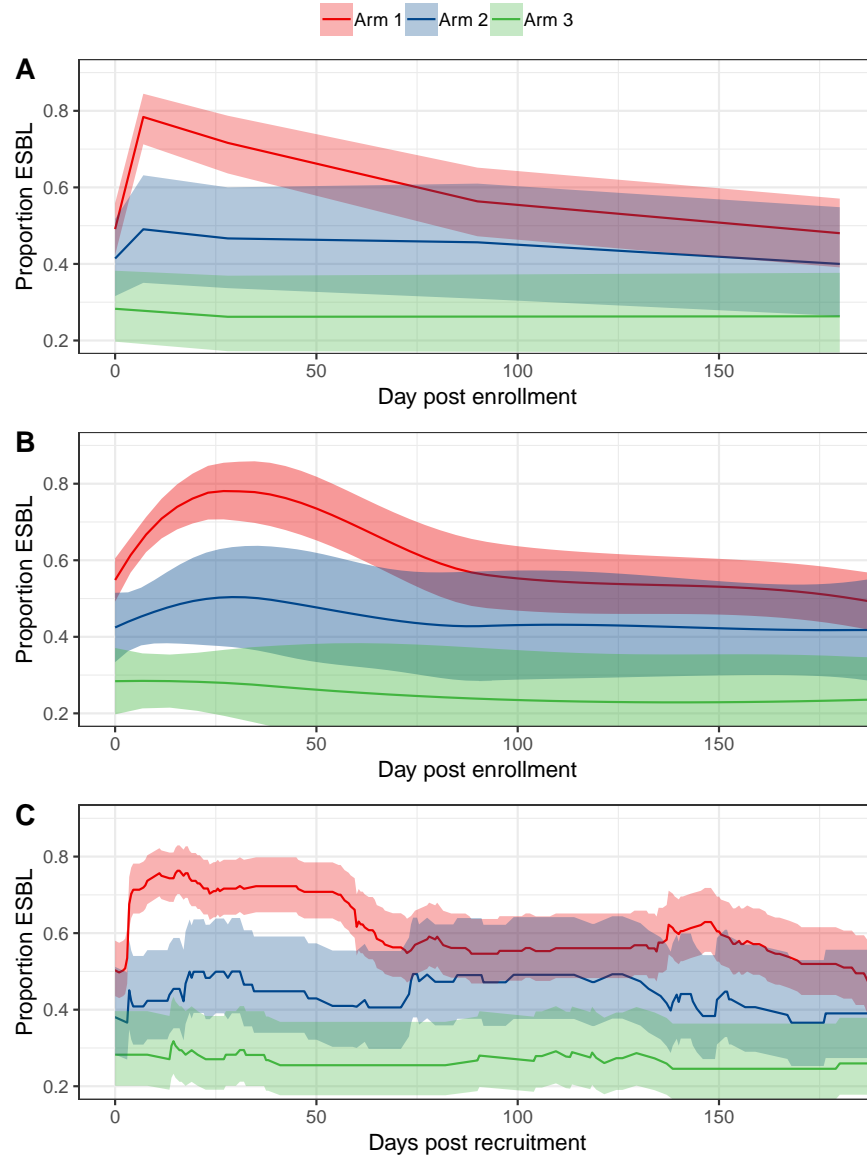


Figure 5.4: ESBL carriage prevalence as a function of time visualised in a number of different ways. In each case participants from Arm 2 are censored on first antimicrobial exposure and Arm 3 are censored on first antimicrobial exposure or hospitalisation. Top (A) prevalence at each visit plotted at ostensible visit time; however, the visits are in fact distributed in time themselves so the middle plot (B) is an attempt to show this by fitting a nonparametric smoothed LOESS regression line with a local linear regression. However the confidence intervals in this method are too narrow because they assume independence of the measurements, which are in fact clustered within patients. The bottom panel (C) is an estimate of the proportion of ESBL-colonised participants from the Aalen-Johansen estimate, which is a generalisation of the Kaplan-Meier curve. This takes into account the nonindependence of the measurements, but does not take into account the interval-censored nature of the data, and transitions to and from the ESBL colonised state are hence assumed to happen halfway between measurements. The best estimate of state occupancy to account for all these difficulties requires the fitting of a Markov model: see chapter xx.

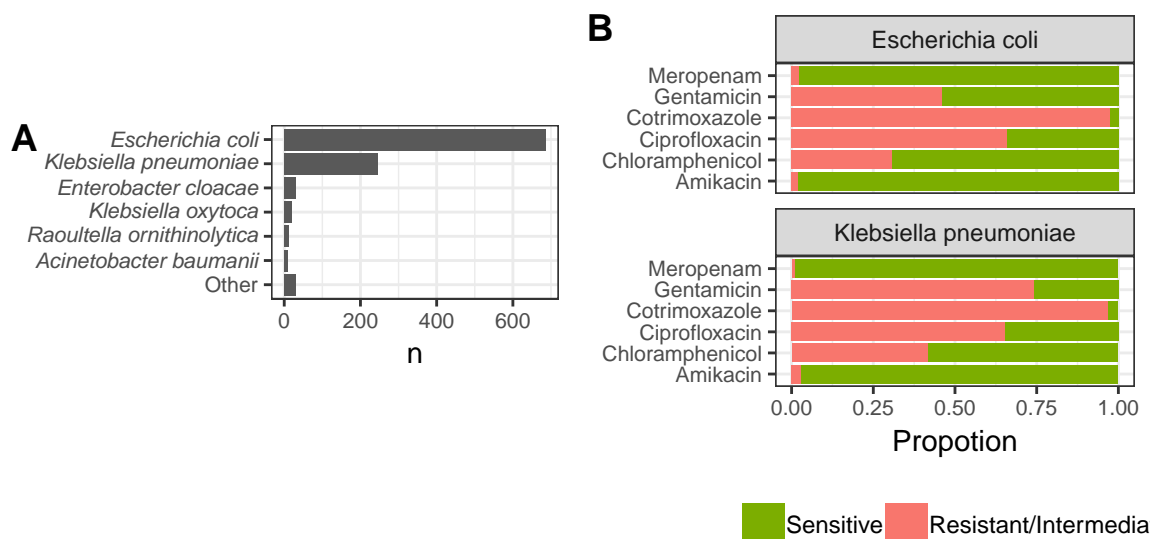


Figure 5.5: Species (A) and antimicrobial sensitivities (B) of cultured ESBL-E

strongly associated with ESBL-E colonisation on multivariable modelling, though with wide confidence intervals (aOR 5.9 [95% CI 1.8-27.0]), perhaps expected as it is a rare baseline exposure. Antimicrobial exposure was not, but with confidence intervals that contained a clinically relevant effect size (aOR 1.3 [95% CI 0.7 - 2.6]). ESBL-E colonisation was more likely with more adults in the household (aOR 1.2 [95% CI 1.0-1.4] per extra adult), with use of an unprotected water source (aOR 3.0 [95% CI 1.1 - 8.8]) and in the rainy season (aOR 2.2 [95% CI 1.4-3.4]); a constellation of variables that are consistent with a significant role of community faecal-oral transmission in the spread of ESBL-E.

To explore associations of acquisition of ESBL-E by the day 28 visit, I analysed only those participants who had no detectable ESBL-E at baseline, and an available follow up samples at 28 days \pm 14 days. These numbered 150 participants: 64 Arm 1, 37 Arm 2 and 49 Arm 3 participants, and 49% (73/77) of them had a detectable ESBL-E at day 28. Bivariable associations of ESBL-E acquisition with antimicrobial and hospital exposures are shown in Figure 5.6A, stratified by the length of exposure; all antibacterials (including TB therapy) showed an association with ESBL-E acquisition, with a suggestion of a dose-response effect, but confidence intervals were large in many cases. Antimalarials did not show this effect though here uncertainty in the estimates precludes drawing any firm conclusions, as it does for antifungals. These relationships are very likely confounded, so should be regarded with extreme caution; however, due to a small dataset size and collinearity, logistic regression modelling of ESBL-E acquisition (Figure 5.6B) produces such uncertain parameter estimates that no conclusions can be drawn. A better modelling strategy using continuous time Markov models was used to understand the drivers of ESBL-E carriage; this analysis is presented in

Table 5.4: Univariable and multivariable associations of ESBL colonisation at enrollment

Variable	Univariable		Multivariable	
	OR (95% CI)	p-value	aOR (95% CI)	p-value
Demographics				
Age (per year)	1.00 (0.99-1.02)	0.709	1.00 (0.98-1.02)	0.898
Male sex (vs female)	1.23 (0.84-1.82)	0.287	1.42 (0.93-2.19)	0.106
Study Arm				
Arm 2 (vs 1)	0.73 (0.45-1.18)	0.203	1.57 (0.84-2.96)	0.157
Arm 3 (vs 1)	0.41 (0.24-0.68)	0.001	0.91 (0.45-1.84)	0.801
HIV status				
HIV+ (vs HIV-)	1.68 (1.09-2.59)	0.018	1.16 (0.46-2.84)	0.750
HIV unknown (vs HIV-)	0.71 (0.40-1.24)	0.229	1.09 (0.55-2.18)	0.798
CPT (vs none)	2.46 (1.58-3.86)	<0.001	2.29 (0.98-5.54)	0.060
ART (vs none)	1.99 (1.32-3.00)	0.001	1.06 (0.35-3.17)	0.918
Exposures last month				
Hospitalisation	7.87 (2.57-34.22)	0.001	5.90 (1.78-26.94)	0.008
Antibiotics*	2.14 (1.27-3.67)	0.005	1.34 (0.71-2.57)	0.368
Household size				
Children (per 1)	1.00 (0.87-1.14)	0.979	0.98 (0.84-1.14)	0.793
Adults (per 1)	1.14 (0.99-1.31)	0.064	1.19 (1.02-1.40)	0.026
Keep animals (vs not)	1.33 (0.88-2.03)	0.176	1.16 (0.73-1.85)	0.527
WaSH behaviour				
Flushing toilet (vs not)	1.38 (0.55-3.44)	0.481	0.94 (0.34-2.55)	0.908
Unprotected water source	2.43 (0.96-6.64)	0.068	2.98 (1.08-8.78)	0.039
Treat water (vs not)	1.16 (0.50-2.66)	0.725	0.94 (0.37-2.34)	0.900
Season				
Rainy season (vs. dry)	2.05 (1.38-3.06)	<0.001	2.17 (1.38-3.44)	0.001

Note:

CPT = Cotrimoxazole preventative therapy, ART = antiretroviral therapy, WaSH = Water, sanitation and hygiene. Entries in bold are those for which 95% confidence intervals do not cross 1.

* Antibiotics includes TB therapy but excludes CPT.

Chapter xx.

5.5 Discussion

5.5.1 Limitations

5.6 Conclusions and further work

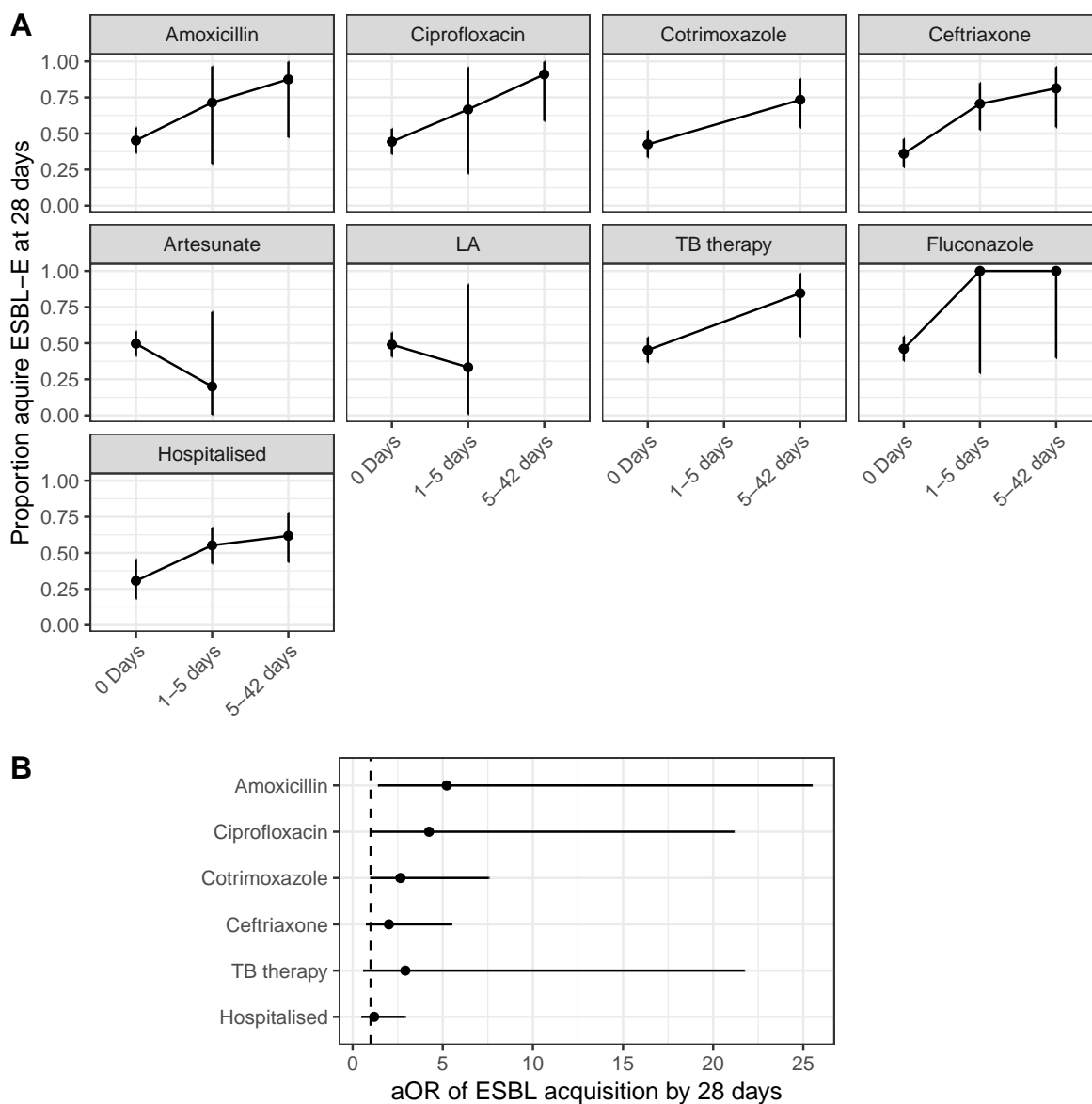


Figure 5.6: Association of ESBL-E acquisition by 28 days. Bivariable (A) and multivariable (B) associations of antimicrobial and hospital exposure with acquisition of ESBL-E by 28 days. A: These plots show the proportion of participants who have no detectable ESBL-E baseline but who do at 28 days, as a function of various exposures. All antibacterials and hospitalisation show an association between exposure and ESBL-E acquisition, with a suggestion of a dose-response relationship, though confidence intervals are wide in many cases. Antimalarials show no apparent relationship though, as with fluconazole, the wide confidence intervals make it difficult to draw any conclusions. The results from logistic regression to predict ESBL-E acquisition are shown in (B); collinearity and small dataset size means that confidence intervals are so large as to make the model useless. A better approach to modelling ESBL-E acquisition, Markov modelling, is shown in Chapter xx.

Chapter 6

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

Placeholder

6.1 Chapter overview

6.2 Methods

6.2.1 Bioinformatic pipeline

6.2.2 Global *E. coli* collection

6.2.3 Statistical analysis

6.3 Results

6.3.1 Samples and quality control

6.3.2 Phylogroup, MLST and core genome phylogeny of study isolates

6.3.3 Study isolates in a global context

6.3.4 Antimicrobial resistance determinants

6.3.4.1 β -lactam resistance

6.3.4.2 Quinolone resistance

6.3.4.3 Aminoglycoside resistance

6.3.4.4 Chloramphenicol, co-trimoxazole, tetracycline and other resistance determinants

6.3.4.5 Clustering and lineage association of AMR determinants

6.3.5 Plasmid replicons

6.3.6 Testing metadata associations: SNP distance, hierBAPS sequence clusters and ESBL-clusters

6.3.6.1 Hierarchical BAPS clustering of core gene pseudosequences

6.3.6.2 ESBL-clusters

6.3.6.3 Assessing for healthcare-associated lineages

6.3.6.4 Assessing for within-patient conservation of lineage or MGE

6.4 Discussion

Chapter 7

Gut mucosal carriage of ESBL-E in Blantyre, Malawi

References