STUDY PROTOCOL

REVISED Drivers of Resistance in Uganda and Malawi (DRUM): a protocol for the evaluation of One-Health drivers of Extended Spectrum Beta Lactamase (ESBL) resistance in Low-Middle Income Countries (LMICs) [version 2; peer review: 1 approved, 1 approved with reservations]

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Abstract

In sub-Saharan Africa (sSA), there is high morbidity and mortality from

severe bacterial infection and this is compounded by antimicrobial resistance, in particular, resistance to 3rd-generation cephalosporins. This resistance is typically mediated by extended-spectrum beta lactamases (ESBLs). To interrupt ESBL transmission it will be important to investigate how human behaviour, water, sanitation, and hygiene (WASH) practices, environmental contamination, and antibiotic usage in both urban and rural settings interact to contribute to transmission of ESBL E. coli and ESBL K. pneumoniae between humans, animals, and the environment.
Here we present the protocol for the Drivers of Resistance in Uganda and Malawi (DRUM) Consortium, in which we will collect demographic, geospatial, clinical, animal husbandry and WASH data from a total of 400 households in Uganda and Malawi. Longitudinal human, animal and environmental sampling at each household will be used to isolate ESBL E. coli and ESBL K. pneumoniae. This will be complimented by a Risks, Attitudes, Norms, Abilities and Self-Regulation (RANAS) survey and structured observations to understand the contextual and psychosocial drivers of regional WASH practices.
Bacterial isolates and plate sweeps will be further characterised using a mixture of short-,long-read and metagenomic whole-genome sequencing. These datasets will be integrated into agent-based models to describe the transmission of EBSL resistance in Uganda and Malawi and allow us to inform the design of interventions for interrupting transmission of ESBL-bacteria.

Keywords
Antimicrobial Resistance, Africa, One Health, Environment

This article is included in the Malawi-Liverpool Wellcome Trust Clinical Research Programme gateway.

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Any reports and responses or comments on the article can be found at the end of the article.

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REVISED **Amendments from Version 1**

We have expanded on specific sections of the microbiological methods and made amendments in line with the reviewers' responses. In particular, these include updated information about the sites selected alongside further details of the chromogenic methods, genomic analysis and the inclusion of collection SOPs to improve the reproducibility of the protocol.

Any further responses from the reviewers can be found at the end of the article

Introduction

Antimicrobial resistance (AMR) is a huge and complex global public health problem[1]. It is a threat to health that reflects both the interconnectedness of humans, animals and the environment and humanity's dependence on antimicrobials[2]. In sub-Saharan Africa (sSA), there is a high incidence of severe bacterial infection, frequently inadequate health system infrastructure to diagnose and treat bacterial disease, and widespread and uncontrolled availability of antimicrobials, which drives antibiotic use (ABU) in both human and animal sectors[3,4]. There is also inadequate water, sanitation and hygiene (WASH) infrastructure to mitigate spread of environmentally dependent bacteria between humans, animals, and the environment[5]. This situation favours the transmission of AMR-bacteria, but the relative contribution of these different factors is uncertain.

The 3rd-generation cephalosporin (3GC) ceftriaxone is frequently the antimicrobial agent of choice for the empirical management of sepsis across much of sSA[6,7]. 3GC resistant (3GC-R) enteric bacteria have rapidly emerged, largely due to acquisition of genes encoding extended-spectrum beta lactamase (ESBL)-producing enzymes, resulting in infections that are frequently locally untreatable, due to unavailability of carbapenems or other reserve antibiotics[8]. ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* are key examples of this. As low-income countries (LIC) in Africa have poor access to watch and reserve agents[7,9], it is critical to define the relative importance of different transmission routes of ESBL-producing enteric bacteria in order to develop interventions that will interrupt pathogen transmission and ultimately prevent drug resistant infections (DRI).

Uganda and Malawi are LIC with a high burden of disease from sepsis and malaria, high prevalence of HIV, poorly regulated antimicrobial markets, and inadequate WASH infrastructure[5,10–14]. Here, we present the protocol developed by the Drivers of Resistance in Uganda and Malawi (DRUM) Consortium. DRUM will work in urban, peri-urban, and rural settings in Uganda and Malawi and focus on the isolation and genomic characterisation of ESBL producing *E. coli* (ESBL-E) and *K. pneumoniae* (ESBL-K). These bacteria were selected as they belong to the same family and often share AMR phenotypes, however *E. coli* is typically considered to be both community-acquired and nosocomial, whereas *K. pneumoniae* is more often judged to be the archetypal nosocomial AMR pathogen[15].

We will take an interdisciplinary, One-Health approach to assess how human behaviour, WASH practices, environmental contamination with ESBL bacteria, and ABU in urban and rural locations within Uganda and Malawi contribute to the transmission of ESBL-E and ESBL-K between humans, animals, and the environment and how this transmission relates to strains isolated from the blood of humans with drug-resistant infection (DRI). We will collect demographic, geospatial, WASH, longitudinal clinical and molecular microbiological data, and integrate these data into agent-based models designed to estimate the impact of putative interventions on interrupting transmission of ESBL bacteria.

Aim

In order to determine the critical points at which efforts to interrupt human AMR acquisition are likely to have the greatest impact in Eastern Africa and beyond, we hypothesise that the household is a key setting in which ESBL enteric bacteria are transmitted. We therefore aim to identify risk factors for and infer drivers of ESBL-E and ESBL-K transmission in Uganda and Malawi at the household level. This is summarised in Figure 1, created following a stakeholder meeting in Uganda in 2018 by Design Without Borders.

Site selection

DRUM consortium members identified sites representing urban, peri-urban, and rural settings to enable variations in WASH behaviours, animal husbandry practices, ABU, and household and broader environmental contamination with ESBL-producing bacteria to be contrasted. Additionally, sites were considered based on perceived acceptability of research within the communities and existing research capacity. Therefore, in Malawi, Ndirande (urban) and Chikwawa (rural) were selected because of the opportunity to utilize data from previous studies (i.e. detailed censuses) and prior research engagement, and Chileka (peri-urban) was selected due to local prior knowledge. We sought to achieve a comparable mixture in Uganda with varied socioeconomic status in Kampala (urban) and Hoima District (peri-urban and rural). Within these sites, recruitment polygons were drawn from local administrative wards (Figure 2).

Malawian site descriptions

Healthcare is free at the point of delivery in Uganda and Malawi, and this should be assumed unless otherwise stated.

Site 1: Ndirande, Blantyre, Malawi (Urban)

Ndirande is a large urban settlement with high-density housing 4 km from the geographical centre of Blantyre, the second largest city of Malawi[16,17] and where 15% (109,164) of the Blantyre population resides[18]. Healthcare is provided by one large, government Health Centre (Ndirande Health Centre) and by the tertiary referral hospital for the Southern region, Queen Elizabeth Central Hospital (QECH), 2–6 km away[16,19]. HIV prevalence in adults aged 15–65 is 18% and there is a high burden of typhoid and tuberculosis[20,21]. According to the Malawian Health Management Information System report, malaria accounts for about 34% of all outpatient visits and 18% of

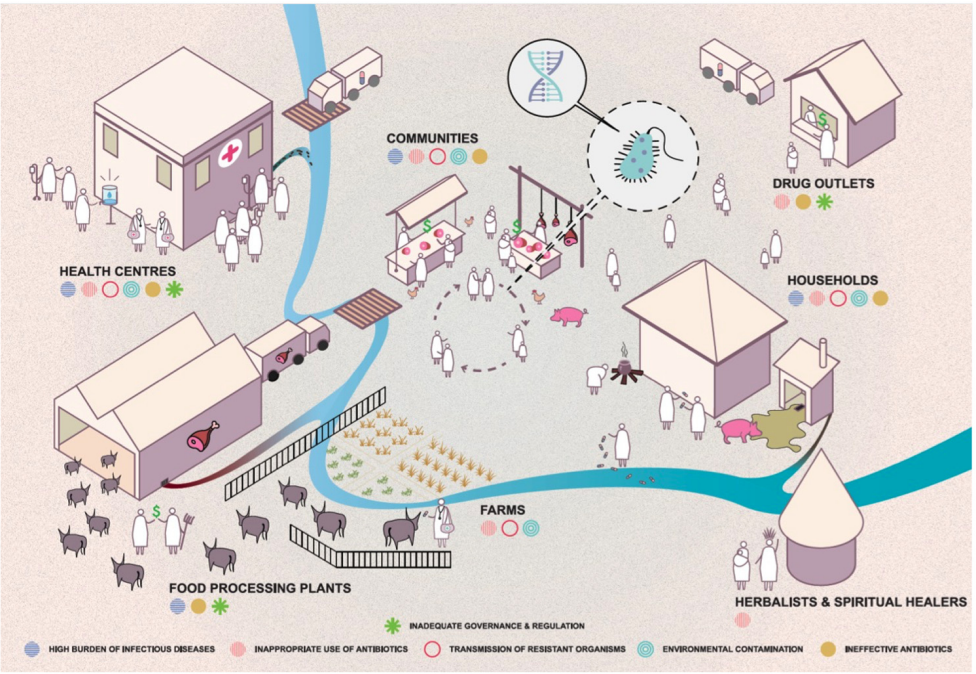

Figure 1. Hypothetical model of related behaviours and the movement of AMR-bacteria in Uganda and Malawi. The schematic situates the household at the heart of the model, in which humans act in response to their environment within which bacteria are evolving in response to selective pressures around them.

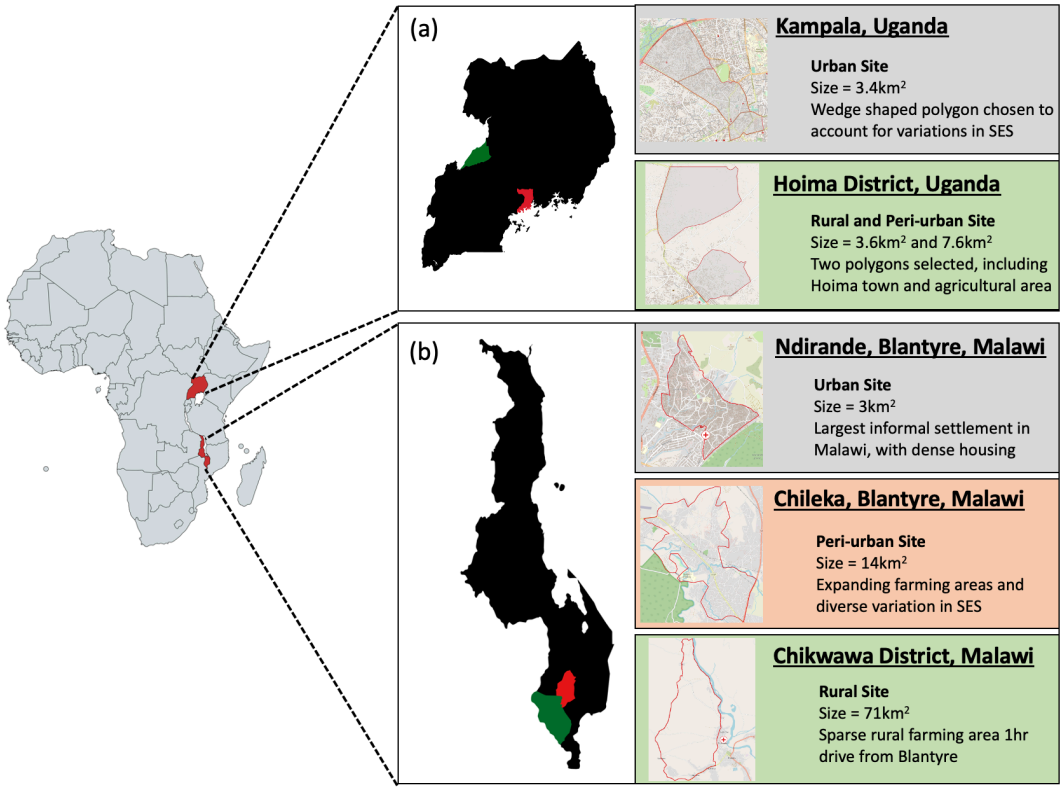

Figure 2. Diagram of DRUM study sites. (**a**) We selected two geographic areas within Uganda including Kampala (red) and Hoima District (green). From these areas, polygons were created that mapped an urban setting (Kampala) and peri-urban/rural setting (Hoima District). (**b**) We selected sites in two regions within southern Malawi including Blantyre (red) and Chikwawa District (green). Polygons were created and mapped for urban (Ndirande) and peri-urban (Chileka) settings within Blantyre and a rural setting within Chikwawa District.

all hospital deaths in Malawi[22]. The study polygon is 3 km^2, and our initial survey in April-May 2019 identified 8 secondary schools, 46 primary (or nursery) schools, 52 places of worship, 15 markets, 1 farm and 9 pharmacies within it.

Site 2: Chileka, Blantyre, Malawi (Peri-Urban)

Chileka is a peri-urban administrative ward on the northern outskirts of Blantyre city. Healthcare is provided by a government Health Centre (Chileka Health Centre), a small local private hospital (Mtengo-Umodzi) or admission to QECH 10–16 km away. The study polygon is 14 km^2, and our initial survey in April-May 2019 identified 3 secondary schools, 20 primary (or nursery) schools, 14 places of worship, 4 large farms and 6 pharmacies within it.

Site 3: Chikwawa, Malawi (Rural)

Chikwawa is a large district with a population of ~450,000, situated in the southern Shire valley and its border is 50 km from Blantyre[23]. It is a rural area, including a mixture of subsistence and large-scale sugar farming, and given its low-lying situation is historically prone to flooding[24]. Healthcare is provided by Chikwawa District hospital, 14 health centres and 26 community health care worker outposts[23]. We identified a 71 km^2 study polygon readily accessible from Blantyre by road, including villages engaged in research activity on the edge of Chikwawa town. Our survey in April-May 2019 identified 2 secondary schools, 9 primary (or nursery) schools, 29 places of worship, 3 markets, 11 farms and 1 pharmacy within the polygon.

Ugandan site descriptions

Site 4: Kampala, Uganda (Urban)

Kampala, the capital and largest city of Uganda has a metropolitan area population of 3.3 million people. Adult HIV prevalence is 6.9%[25]. In 2020, Uganda had the 3rd highest global burden of malaria cases and deaths (5.4%) and the 5th highest level of deaths (3.5%)[12]. The sampling frame comprises of 3 contiguous areas drawn in wedge shape (measuring 3.4 km^2 x 2.7 km^2 x 1 km^2) with a spectrum of population density areas. These areas were loosely stratified relative to each other as being of low, medium or high socioeconomic status based on local knowledge. The smallest polygon closest to the centre is considered low, whilst the one furthest from the centre as medium and the middle one as high socioeconomic status.

Site 5: Hoima, Uganda (Rural and Peri-urban)

Hoima, in the Western Region of Uganda, is the main municipal, administrative, and commercial centre of Hoima District and has a population of 122,700 people[26]. HIV prevalence among adults aged 15–64yrs in the Mid-West Region of Uganda where Hoima is located is 5.7%[25]. The sampling frame comprises of two non-contiguous polygons of 3.6 km^2 and 7.6 km^2, the former incorporating Hoima town (peri-urban) and the latter (rural) being a few kilometres away from Hoima town and which has more animal and human cohabitation.

Methods

Household selection process

As DRUM will investigate AMR transmission at the household level, we chose a spatial design based on the "inhibitory with close pairs" approach[27]. This enables us to distribute primary sampling sites across the study area evenly, avoiding systematic biases that may occur when sampling on a regular grid. Secondly, "close-pair" points are added to the design to allow localised comparison of sample sites and therefore measurement of close-range correlation in AMR status. Thus, seventy percent of households will be sampled at a minimum inhibitory distance (MID) from all other points[28]. Using one inhibitory point at a time, the rest of the points, called close pairs, are randomly selected within a circle with a pre-determined close-pairs radius (CPR). The minimum distance for our design is 100 meters and the radius for each close pair is 30 meters. These values were chosen based on results from a spatial investigation of enteric pathogen *Salmonella* Typhi in Blantyre that showed a spatial correlation up to approximately 150 meters[29].

Depending on the richness of existing geospatial data within each study area, we will implement different versions of the algorithm in each area. In Ndirande (Malawi), where all households had previously been geolocated, direct random sampling of households subject to the spatial constraints above is possible[16]. In Hoima (Uganda), where OpenStreetMap (OSM) data appears complete, OSM-derived building locations can be chosen to identify potential households. In Chikwawa (Malawi), WorldPop population density rasters allow us to preferentially (though not exclusively) propose sampling sites in high population density areas thus avoiding field teams visiting vacant sites (www.worldpop.org/). In Kampala (Uganda) and Chileka (Malawi), apparent uniformity of the population density across the study area allows a simple spatially uniform proposal to be used. Two practical site-specific considerations are necessary. Firstly, for Chileka, the MID and CPR must be doubled due to the sparse population. In Kampala, the availability of a marked socioeconomic gradient within the study region allows stratification of the population by socioeconomic status, with households randomised within strata, but respecting our spatial design constraints across strata borders.

Proposed sampling locations are then translated into households by the data collection field teams. For instances where either no suitable household exists at the location or in the event that a household declines to participate in the study, a random direction is selected by the field team, and the closest consenting household in that direction is chosen.

Recruitment of households

We aim to enrol up to 100 households in each of the five sites. Households will be grouped into either "intensive" or "sparse", with 15 intensive households pre-selected at random within each polygon, and all others allocated as sparse (Figure 3). Intensive households will undergo extensive WASH observations at the first and last visit, whereas "sparse" households will not undergo any WASH observations (Figure 3).

All households will be followed up at 3–4 time points over a period up to 6 months to provide longitudinal microbiological and WASH data. Household recruitment will be staggered over 12 months to assess seasonality of transmission of ESBL-bacteria. At each visit, households will be asked to respond to

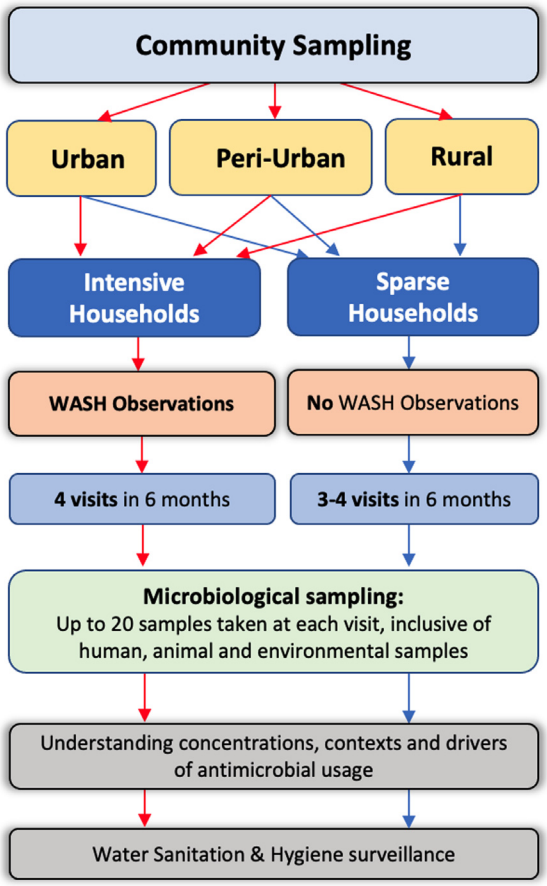

Figure 3. DRUM household study design.

questionnaires to provide information at the individual and household level on ABU, health seeking behaviour and WASH behavioural practices. Microbiological sampling will be undertaken to determine the presence of ESBL *E. coli* and ESBL *K. pneumoniae* from human, animal and environmental samples.

Participant eligibility

Eligibility will be considered at the level of the household and individuals. Households will be required to exist within the boundaries of the study polygon and be able to provide a minimum of 12 samples at the baseline visit, inclusive of a minimum of 2 human stool samples from household members. Individuals will be required to speak either the predominant local language (Chichewa in Malawi or Luganda or Runyoro in Uganda) or English to provide informed consent, and not have confirmed or suspected acute bacterial infection at the time of recruitment.

Data collection

Case Report Forms (CRFs)

Study CRFs have been designed by an interdisciplinary working group of the DRUM consortium that included specialists in human health, animal health, food, water and environmental microbiology, WASH & Environmental health and medical anthropology. Questions were selected from pre-tested tools evaluating regional demographics, human and animal health, WASH infrastructure and behavioural practices, humans and animal ABU determinants and environmental exposures[29,30]. These questions were inputted into CRFs that were tailored to the resident population, structured into either individual or household level, thematically separated into key drivers of AMR and translated into local languages (Table 1).

At the baseline visit, these CRFs will be completed to provide information at the individual and household level on human health, ABU, socioeconomic status, health seeking behaviour, structural and behavioural WASH practices and animal husbandry (Extended data). At each follow-up visit, changes to human health, household practices and antibiotic exposure will be assessed (Extended data).

Longitudinal microbiological sampling

The consortium was asked to consider priorities for household sampling at the kick-off meeting at Liverpool School of Tropical Medicine (LSTM), UK (23/09/2018). We decided to

Table 1. DRUM CRF themes and data capture.

	Individual Level Data	Household Level Data
Demographic	· Participant Demographics	· Household Demographics · Socio-Economic Information · Household Head Information
Health	· Health Status and Comorbidities · Regular Medication Use · Recent Illness	· Household Health Seeking Behaviour
Exposure Risk	· Healthcare Exposure · Travel and Residency · Health Seeking Behaviour	· Visitors into the household
Antibiotic Usage	· Antibiotic Usage	· Household Experience of Illness and Antibiotics
WASH	· Hand-Washing Data	· Household WASH Infrastructure · Toileting Behaviour · Waste Management · Water Usage and Management · Washing and Bathing Practices · Food Preparation and Hygiene Information. · Hand-Washing Data
Environmental		· Household Infrastructure · Household Environment
Animal		· Household Animal Husbandry · Animal Health and Disease Prevention · Drug Usage in Household Animals (including antibiotics)

focus on areas identified as hand-contact zones or where food handling occurred and also to include broader environmental sites that we hypothesise serve as important reservoirs of ESBL-producing organisms. We established a consensus opinion for the microbiological sampling strategy based on a maximum of 20 samples per visit, inclusive of human and animal stool samples and environmental samples (see Figure 4).

Field sampling methods

Human stool will be self-collected in a 30 mL stool pot by participants. Animal stool samples will either be collected using rectal/ cloacal swabs (for poultry) or taken directly from the ground. Food samples will be placed in sterile Whirl-Pak® bags, and all water samples will be collected using sterile 500 ml Nalgene® BPA-free, polypropylene bottles.

Household environmental sampling will be informed by the WASH observations to determine high-risk areas of environmental contamination. Environmental contact-surface samples and clothing samples will be collected with 3M™ Sponge-Sticks containing 10 ml sterile buffered peptone water (BPW) broth, and floor samples will be collected with the use of boot socks. Outer drain samples (defined as water in motion either in a constructed drain (dug or built) or moving on a surface) will be collected in a 30 ml universal container from within the household compound. Detailed descriptions of the sampling processes are included in the study Standard Operating Procedures (SOPs) in the Extended material.

All samples will be issued a unique identification code, labelled, stored in ice chests at 2–8°C in the dark and transported to the laboratory, for processing within 24 hours, where possible.

Microbiological Methodology

Human stool, animal stool and environmental swabs will not require pre-processing steps. BPW (Oxiod) will be directly added to the sample upon reception. Water and food samples will be pre-processed as follows:

- Water samples will be filtered through a sterile 0.45 µm cellulose-ester gridded membrane (VWR™) using a vacuum-based manifold, before adding to universal containers with 20 ml of BPW (Oxiod). In river water samples, a second sample will be processed in parallel, and the filter membrane will be stored at -80°C without the addition of BPW.
- Fruit (whole) will have enough BPW (Oxiod) added to the Whirl-Pak® bag to cover before being manually

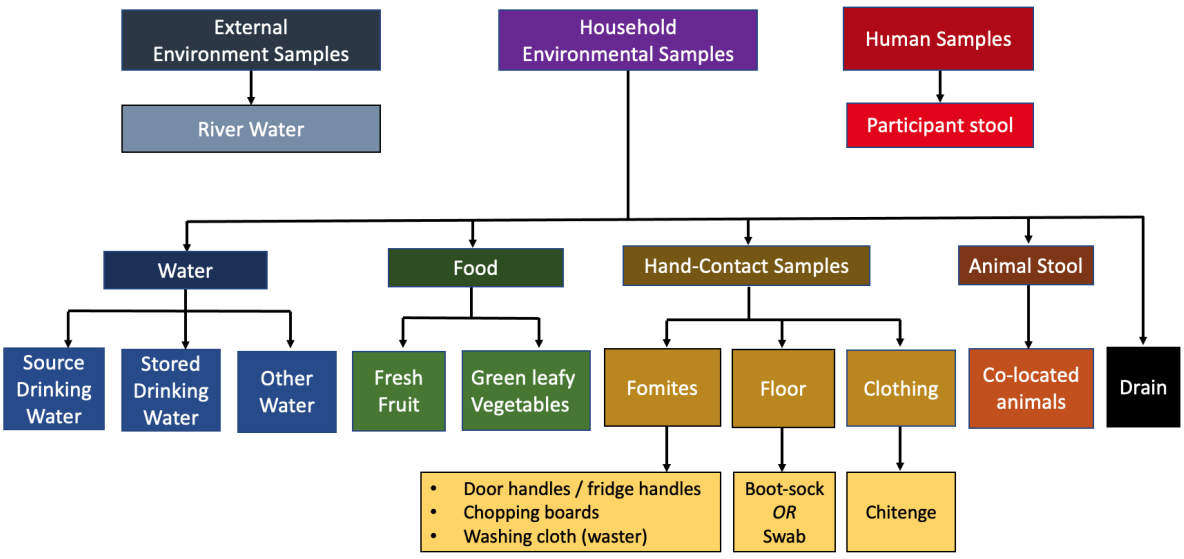

Figure 4. DRUM microbiological sampling frame, used at household visits. Samples are inclusive of human stool, animal stool, and a selection of household environment and the broader external environment.

stomached for a period of 30 sec to 3 min. The fruit will then be aseptically removed from the bag prior to incubation.

- Green leafy vegetables will be weighed and have nine times the weight of the food added in BPW (Oxiod) to obtain a sample-to-diluent weight ratio of 1:9, before being manually stomached for 30 sec to 3 min. Vegetables will be left inside the Whirl-Pac® bag while incubated.

Consistent with practice at the UK Health Security Agency Food Water and Environment (FWE) Microbiology Services, all samples will initially be cultured in enrichment broth (BPW) to improve the recovery of Gram-negative organisms. The volumes of BPW (Oxiod) added will depend on sample type and will be determined by either the manufacturer's advice (3M™ Swab-Samplers), expert opinion and SOPs from FWE (3M™ Sponge-Sticks, water filtration methods, food processing methods), previous local experience (stool processing) or from pre-testing and optimisation in the piloting phase of the study (river water processing, drain sample processing, boot socks) (see Extended material).

Once the enrichment broth (BPW) has been added, all samples will be placed in an aerobic incubator at 37 ± 1°C for 18–24 hours. After incubation a 1.8 ml aliquot of the culture BPW will be stored at -80°C, and a 1µl loop of the remaining sample will be plated onto ESBL CHROMagar™ chromogenic agar (CHROMagar™, France). Plates will be placed in an aerobic incubator at 37 ± 1°C for 18–24 hrs and read for growth of ESBL bacteria, via the presence of either pink, blue or white colonies. Pink colonies and (indole positive) white colonies will be categorised as ESBL *E. coli* while blue colonies will undergo speciation for *K. pneumoniae*, using high resolution melt-curve (HRM) PCR (QIAGEN, USA), to identify ESBL *K. pneumoniae* isolates[31]. ESBL status will be inferred by chromogenic methods alone and not formally confirmed by phenotype, however isolates growing on ESBL media will be whole genome sequenced. A plate sweep will be taken from each ESBL CHROMagar™ plate that has positive growth of ESBL colonies. ESBL isolates and plate sweeps will be stored at -80°C.

Samples will be stored at intervals during the microbiological processing to facilitate subsequent whole genome sequencing (WGS), including aliquots of the original sample (shotgun metagenomics); samples pre-enriched with BPW (limited-diversity metagenomics via mSWEEP/mGEMS), CHROMagar™ plate sweeps (limited-diversity metagenomics) and single colony picks (short-read and long-read sequencing)[32].

DNA extraction, Library preparation and Sequencing

DNA will be extracted from all ESBL-positive isolates, plate sweeps and pre-enriched BPW ESBL positive samples using the QIASymphony DSP Virus/Pathogen mini-kit® on the QIASymphony® (QIAGEN, USA) automated DNA extraction platform or manually extracted using the DNeasy® blood and tissue kit (QIAGEN, USA).

Extracted DNA will be shipped to the Wellcome Sanger Institute (WSI, UK) under export licences issued following signature of Access and Benefit Sharing agreements in accordance with the Nagoya protocol.

All DNA samples will be quantified with Biotium Accuclear® Ultra high sensitivity dsDNA quantitative kit (Bioitium, USA) using Mosquito LV liquid platform (SPT LabTech, UK), Agilent Bravo WS (Agilent Technologies, USA) and BMG FLUOstar Omega plate reader (BMG LabTech, Germany)

and cherrypicked to 200ng / 120ul using Tecan liquid handling platform (Tecan, Switzerland). Cherrypicked plates will be sheared to 450bp using a Covaris LE220 (Covaris, USA) instrument and samples purified using Agencourt AMPure XP SPRI beads Beckman Coulter, Inc., USA) on Agilent Bravo WS. Libraries will be constructed using the NEB Ultra II custom kit (New England Biolabs, USA)

DNA from single colony pick isolates will be whole genome sequenced on the Illumina X10 platform (Illumina Inc, California, USA) to produce 150bp paired end short reads. Preliminary analysis of these short-read WGS data will inform the identification of clusters from which representative isolates will be selected for long read sequencing on the MinION platform (Oxford Nanopore Technologies, UK) in order to generate hybrid, improved draft assemblies, and thus characterise mobile genetic elements (MGEs). Finally, shotgun metagenomic and limited diversity metagenomic (plate sweeps) sequencing will be performed on up to 420 pre-enriched BPW samples and 1500 ESBL selected plate sweep samples on the Illumina HiSeq 4000 platform (Illumina Inc, California, USA) to investigate within host microbial diversity and AMR gene pool or "resistome".

WASH Evaluations

Household WASH, environmental health and food safety evaluations

Each recruited household will be asked to engage with a range of qualitative and quantitative data collection methods to gain an understanding of the contextual and psychosocial elements of their household, individual and habitual WASH practices as outlined in IBM-WASH[33]. Questions will be asked of household members at the baseline assessment (combined with the household and individual CRFs), and a checklist and sanitation inspection form will be completed by a member of the study team at each visit to evaluate WASH infrastructure. Lastly, a household plan will be completed at baseline to contextualise the household infrastructure where specific activities take place (including perceived high-risk areas) and aid in analysis.

WASH practices will be assessed via checklist and structured observations at households and identified for "intensive surveillance", at both the baseline and fourth visit. Observations will be undertaken on 3 consecutive days, for a period of 6 hours per day, with two morning sessions (6am-12pm) and one afternoon session (12pm-6pm) to describe WASH practice over the period of a day. The focus on early sessions has been chosen due to previous studies illustrating that key WASH activities occurred mainly in the mornings[34]. Observations will be recorded by research staff and summarised in a structured format for content analysis to enable the identification of critical control points around WASH behaviours for faecal and environmental exposure.

Understanding WASH behavioural drivers

Psychosocial drivers of WASH practices will be explored using the Risks, Attitudes, Norms, Abilities and Self-Regulation (RANAS) Model, undertaken at up to 100 households in each region[35,36]. The RANAS questionnaire design will be informed by the structured observations in intensive households and focus on hand hygiene, food preparation, waste management, water usage and environmental exposure. The RANAS survey will be conducted with 2 household members in each household, and where possible, will be directed to the household head (e.g. father) and one household worker (household staff member). RANAS data will be analysed using an ANOVA mean comparison to determine the differences between doer and non-doer contextual and psychosocial factors for potential targeted behaviours. The data from this survey will be used to inform potential behaviour change techniques which could be used to tackle high risk transmission areas identified in the agent-based model.

Assessment of broader environmental exposure

Transect walks of each region will be undertaken using an integrated approach to the collection and evaluation of environmental, WASH and microbiological data to understand the wider context in which household members are living. Based on the principles of the SaniPath method, walks will be undertaken with community leaders using walking interviews, while collecting video footage and photographs and geolocating walk routes and sampling sites. Reference will be made to specific Shit Flow Diagrams (SFD), where available, which visually describe excreta flow in urban and rural settings, and data will be mapped to provide a spatial outline of potential pathways for faecal exposure[37–39]. Wherever feasible, longitudinal data will be collected on study sites to assess the effects of seasonality. This novel adaption of the SaniPath tool will enable us to integrate environmental AMR data into urban and rural WASH exposure pathways.

Observations and structured checklists will be completed at 10 public and institutional settings within each of the five sites (n=50). This will be complemented by Focus Group Discussions (FGDs) and In-depth interviews (IDIs) with key informants (heads of household, primary caregivers, school children, market vendors, etc.) to explore perceptions of barriers and challenges to WASH posed by circumstances of daily life.

Spatial analysis and integration of datasets into an agent-based model

The initial approach will be to determine variables that have strong associations with ESBL status using model-based statistical analysis[40]. Spatial and temporal correlations will be accounted for using both geostatistical and agent-based modelling techniques to increase the precision of our inference, and hence insight into the main demographic and environmental drivers of transmission and carriage. Geostatistical models will initially be used as a phenomenological way of detecting such associations in the quantitative elements of our data[41]. Findings from our qualitative components will then be used to inform the structure of an agent-based model. This will allow us to test different systems models of social and behavioural features of the population that may contribute to ESBL emergence, transmission, and colonisation/decolonisation of individuals[41].

Data management and analysis

In Uganda CRF data and laboratory data will be collected using REDCap (version 10.0.25). In Malawi CRF data will be collected using tablets with Open Data Kit software (ODK, 1.4.10) and laboratory data will be collected using Teleform Data Capture software (10.7). Initial transcription (where needed) and data cleaning will be performed within the local data centres in Uganda and Malawi, close to the data collection context. These data will then be pulled nightly from the local data centres to the University of Lancaster (UoL), UK and formalised into an SQL database to facilitate full record linking with RANAS and WASH study data, extract query construction, and final quality assurance (Figure 5). All data will be securely stored with restricted access to the study PIs and database administrators at Malawi-Liverpool Wellcome Trust (MLW, Malawi), IDI (Infectious Diseases Institute, Uganda) and Lancaster, and shared where required with specific members of the DRUM project team using a secured instance of Dataverse hosted on UoL servers.

Community engagement and involvement

Prior to study initiation and at regular intervals throughout the study, programme-wide community engagement and involvement will be held at study sites in Uganda and Malawi, including the convening of community advisory groups and meetings with the local leadership, district health offices and district executive councils. Findings will be shared with participants, communities and local government, including key stakeholders such as the Malawian Ministry of Health AMR technical working group, the University of Malawi and the Uganda National One Health Platform's national AMR Sub-committee of the One Health Technical Working Group within Makerere University College of Health Sciences (CHS) and College of Veterinary, Animal resources and Biosecurity (COVAB).

Ethics statement, regulatory approvals and governance

The protocol, participant information sheets, consent forms and data collection tools have been approved by the LSTM Research and Ethics Committee (REC, #18-090), College of Medicine REC, Malawi (#P.11/18/2541), Institutional Animal Care and Use Committee (IACUC), Uganda (Ref: SVARREC/18/2018), Joint Clinical Research Centre (JCRC) REC, Uganda (#JC3818) and Uganda National Council for Science and Technology (UNCST, #HS489ES).

In addition, administrative permissions have been granted from community leaders and support obtained from local community advisory groups. Sensitizations of study areas will be conducted prior to initiation and full informed written consent will be obtained from all participants recruited into the study, in their local language when required.

Study status

In Uganda and Malawi, household recruitment and follow-ups have been completed in line with this protocol. Observational, CRF and microbiological data have been collected, cleaned, and integrated into the SQL database. RANAS questionaries and transect walks have been undertaken, and genomic and spatial analysis is underway. The available data has been fed into agent-based models, which are underdoing iterative developments and optimisation.

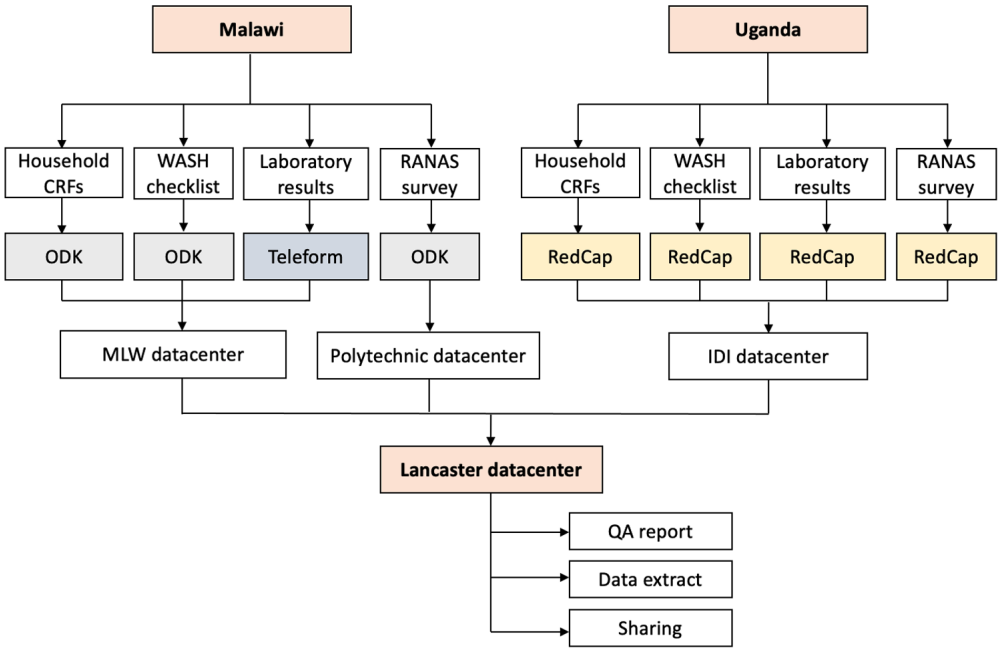

Figure 5. DRUM data management workflow.

Conclusion

In settings where there is a high incidence of severe bacterial infection and inadequate WASH infrastructure, we will identify risk factors and infer drivers of ESBL-E and ESBL-K transmission in Uganda and Malawi at the household level.

This one-health study will also provide insights on how human behaviour, WASH practices, environmental contamination, and ABU in urban and rural locations within Malawi and Uganda contribute to the transmission of ESBL-E and ESBL-K between humans, animals, and the environment. By integrating this high-quality data into agent-based transmission models, we will be able to determine critical points at which efforts to interrupt human ESBL acquisition are likely to have the greatest impact in sSA and share this information with policymakers to co-produce future intervention strategies.

Data availability

Underlying data

No data are associated with this article.

Extended data

Zenodo: Case report forms (CRFs) used for the publication: Drivers of Resistance in Uganda and Malawi (DRUM): A protocol for the evaluation of One-Health drivers of Extended Spectrum Beta Lactamase (ESBL) resistance in Low-Middle Income Countries (LMICs), https://doi.org/10.5281/zenodo.7836247[42].

This project contains the following extended data:

- DRUM01 Participant Enrolement CRF V2.pdf
- DRUM02 Household Enrolement CRF V2.pdf
- DRUM03 Household WASH CRF V2.pdf
- DRUM04 Participant Follow-up CRF.pdf
- DRUM05 Household Follow-up CRF.pdf
- DRUM06 Human Stool Sample Collection CRF.pdf
- DRUM07 Animal Stool Sample Collection CRF.pdf
- DRUM08 Household Food Sample Collection CRF.pdf
- DRUM09 Household Environmental Sample Collection CRF.pdf
- DRUM10 Household Floor Sample Collection CRF.pdf
- DRUM11 Household Clothing Sample Collection CRF.pdf
- DRUM12 Household Water Sample Collection CRF.pdf
- DRUM13 River Water Sample Collection CRF.pdf
- DRUM14 Household Hand-Hygiene Audit CRF.pdf

Zenodo: Laboratory standard operating procedures (SOPs) used for the publication: Drivers of Resistance in Uganda and Malawi (DRUM): A protocol for the evaluation of One-Health drivers of Extended Spectrum Beta Lactamase (ESBL) resistance in Low-Middle Income Countries (LMICs), https://doi.org/10.5281/zenodo.7561679[43].

This project contains the following extended data:

- DRUM_Collection_SOP1 V2 Household human stool sampling.pdf
- DRUM_Collection_SOP2 V2 Household animal stool sampling.pdf
- DRUM_Collection_SOP3 V2 Household environmental sampling.pdf
- DRUM_Collection_SOP4 V2 River water sampling.pdf
- DRUM_SOP1_V2 Human and animal stool processing.pdf
- DRUM_SOP2_V2 Environmental sample processing .pdf
- DRUM_SOP3_V3 ESBL culture.pdf
- DRUM_SOP4_V2 K. pneumoniae identification.pdf
- DRUM_SOP5_V2 Storage.pdf

Acknowledgements

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http://www.doi.org/10.5281/zenodo.7561679

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Article summary:

This manuscript by Cocker *et al.* describes a study protocol to investigate the drivers of antimicrobial resistance in two sub-Saharan countries. The study protocol focuses on the AMR representative extended spectrum beta-lactamase (ESBL) producing organisms. The protocol is a comprehensive One Health study employing a variety of data and samples from 400 households in Uganda and Malawi, and interdisciplinary research approaches to pinpoint the drivers of resistance, from human behaviour, through antibiotic use, WASH practices, and genomic epidemiology investigation of human, animal and environmental carriage of ESBL producers.

The background and motivation for this study are clearly stated and well justified. The protocol is clearly presented and logically structured. The range of methods is described with very good level of detail. Overall, this study protocol is bound to generate multidisciplinary data of significant quality and quantity, which in turn will contribute to improved understanding of the drivers of AMR and unravel potential ways of tackling these.

I have the following comments addressing a few specific points relevant to article format “study protocol” requirement of “sufficient details of the methods provided to allow replication by others”:

One of the motivations for this study was the burden due to “severe bacterial infection” “compounded by antimicrobial resistance”. Please describe how your study of carriage (from healthy individuals) and environmental isolates will link to clinical isolates and understanding of resistance transmission to/from/within clinical settings.
Genomic analyses will be done predominantly on culture-selected ESBL producing organisms. Is AMR and multidrug resistance going to be established by phenotypic antimicrobial susceptibility testing of the isolates? If so, is AST going to be evaluated with the identified genomic

determinants of resistance? If not, why not? Please specify and/or add this in the protocol.

Further specific comments:

Introduction:

- Statement "The 3rd-generation cephalosporin (3GC) ceftriaxone is frequently the antimicrobial agent of first and last resort across much of sSA." Modify this statement so that you refer to 3rd generation cephalosporins as a class, rather than a single drug, or expand and explain why ceftriaxone is "of first and last resort across much of sSA" and support it with a reference.

- Add "genes encoding" in the statement "…due to acquisition of genes encoding extended-spectrum beta lactamase (ESBL)-producing enzymes…".

- What is the incidence of neonatal sepsis and malaria? What is the prevalence of HIV in these countries?

- Consider modifying this sentence to "…focus on isolation and genomic characterisation of ESBL producing *E. coli* (ESBL-E) and *K. pneumoniae* (ESBL-K)."

- Specify if in the statement "We will take an interdisciplinary, One-Health approach to assess how human behaviour, WASH practices, environmental contamination,…" you are referring to "environmental contamination by enteric bacteria" or other.

- In the final sentence "…on interrupting transmission." specify if you are referring to bacterial transmission, resistance transmission, or other.

Site selection:

- Why were there three sites defined in Malawi (sites 1, 2 and 3) but only two sites (sites 4 and 5) in Uganda? Was it due to the contiguous location of site 5 in Uganda? How is this going to be considered for future comparative data analysis (i.e. 100 households per site)?
- Consider using "animal husbandry practices" instead of "animal practices", or just "husbandry practices" as farming areas are mentioned in Fig. 2.

- Do you refer to "environmental contamination" (of household or premises?) in "contamination with ESBL-producing bacteria"? Please specify this.

- Was the socioeconomic status of the sites in Kampala and Blantyre comparable? Is there information on housing density comparison between the equivalent sites in Uganda and Malawi? Can you expand on this a bit more?

- Did you mean "Blantyre, the second largest city of Malawi"?

- What was the estimated population of Chileka?

- What was the estimated population of the urban study site in Kampala?

- Site 5: Hoima – check the subheading and the text in this paragraph. Was Hoima town area

defined as urban (subheading) or peri-urban (in the text)?

Methods:

- How was the “apparent uniformity of the population density across the study area” estimated in Kampala and Chileka?

- How was the data on socioeconomic gradient acquired for "In Kampala, the availability of a marked socioeconomic gradient within the study region”? Please use a reference if possible.

- In the statement “… and not have confirmed or suspected acute infection”, does this take HIV status into account?

- Figure 3 – please clarify if sampling procedure in Fig. 3 or in the main text is preferred, i.e. will 3-4 visits be done within 6 months (Fig) or 12 months (text); which is more representative of seasonality? Malawi has one rainy season, Uganda has two rainy seasons.

- In the statement “…sites that serve as important reservoirs of ESBL AMR.” – did you mean “ESBL-producing organisms”?

- Household environmental sampling – what size of surfaces were sampled and/or what exposure time was used for sampling (e.g. for boot socks)? Can you specify this please?

- Microbiological methodology order – to improve the flow of this section, specify pre-processing steps at first (e.g. filtration, centrifugation, homogenization etc.), then describe pre-enrichment (in BPW if needed), and then culture and isolation.

- Write out abbreviations in full at first use, e.g. buffered peptone water (BPW).

- Drain samples – specify if these are inner drains (e.g. in the kitchen or hand washing sinks) or outer drains (sewage, other).

- Manufacturers – manufacturers are written out for some consumables (e.g. VWR for water filtering, sampling bags) but not for other consumables. For consistency, decide on including all products and manufacturers or make these generic.

- Consistency in fruit and vegetable preparation for culture – what ratios and volumes of fruit and BPW will be used? Will peel of fruits be included for culture? What mass/weight of green leafy vegetables will be used?

- Terminology – choose either “massaged” or “manually stomached” if these refer to the same processes.

- Please check clarity of this sentence “Green leafy vegetables will be weighed and have nine times the weight of the food added in BPW to obtain a sample-to-diluent volume of 1:9”. Do you refer to weights or volumes? Did you mean the diluent (BPW) will have 9 times the weight/volume of the leafy vegetables?

- Storage of BPW cultures at -80°C – is any glycerol going to be added?

- Inoculation on CHROMagar – what volume of samples cultured in BPW will be plated on the selective agar? It was not specified what volume of BPW would be used for pre-enrichment.

- Plate sweeps – these are mentioned for the first time after ESBL isolates. What cultures and plates will be used for plate sweeps, the same as those for isolated ESBL producers?

- ESBL isolation – how many suspected ESBL colonies will be selected from each sample? A single colony of each ESBL-E and ESBL-K if present or multiple?

- Original sample storage for metagenomics – how will these be stored? E.g. will you store filtered and concentrated water, will you store swabs or swabs homogenized in PBS?

- It is not necessary to mention metagenomic tools (mSWEEP/mGEMS) in sample storage section, and it would be more suitable for sequencing data analysis section.

- DNA extraction and sequencing – how will ESBL-negative isolates be acquired and selected? This should be specified in the microbiology section.

- What library preparation kit will be used for Illumina sequencing?

- Numbers of samples for metagenomic sequencing are specified. How many isolates and sweeps will be whole genome sequenced on an Illumina platform? How many or up to how many isolates will be Nanopore sequenced?

- Metagenomic resistome of PBP pre-enriched samples will be investigated. Will you investigate the resistome of CHROMagar plate sweeps? If so, please specify this.

- Spatial analysis and integration of datasets into an agent-based model. **"**The initial approach will be to determine variables (as described above)" please specify in which paragraph are the variables mentioned. While biological sampling and microbiological analysis are described in a great level of detail, this section is less evolved. What software, computational tools will be used? What geostatistical models will be used? How will qualitative components be evaluated and chosen for ABM?

- As this manuscript is a study protocol and investigating "the transmission of ESBL-E and ESBL-K between humans, animals, and the environment" was one of the study objectives, it would be valuable to add a section on genomic epidemiological methods to investigate the transmission (although, this may be further detailed in publication of the study results).

Is the rationale for, and objectives of, the study clearly described?

Yes

Is the study design appropriate for the research question?

Yes

Are sufficient details of the methods provided to allow replication by others?

Partly

Are the datasets clearly presented in a useable and accessible format?
Not applicable

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: microbiology, bacterial pathogens, antimicrobial resistance, ESBL, TB, Streptococcus, WGS, genomics, East Africa

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Apr 2023

Derek Cocker

Reviewer 2: Article summary: I have the following comments addressing a few specific points relevant to article format "study protocol" requirement of "sufficient details of the methods provided to allow replication by others":

One of the motivations for this study was the burden due to "severe bacterial infection" "compounded by antimicrobial resistance". Please describe how your study of carriage (from healthy individuals) and environmental isolates will link to clinical isolates and understanding of resistance transmission to/from/within clinical settings. The study aims to understand the transmission of bacteria with known human pathogenic potential in the community. It will be conducted in two settings with robust capacity to undertake surveillance of severe bacterial infection in individuals from the study sites. MLW, Malawi has offered quality assured diagnostic microbiology services to patients presenting to Queen Elizabeth Central Hospital, Blantyre with suspected sepsis, an area including Ndirande and Chileka since 1998 and more recently to febrile patients presenting to Chikwawa district hospital through the FIEBRE study (Hopkins H et al, BMJ Open. 2020 Jul 21;10(7):e035632), which was contemporaneous to DRUM. IDI has secured grant funding to undertake bacteraemia surveillance in Uganda as it was a Fleming Fund inception site (https://www.flemingfund.org/countries/uganda/). The link to clinical samples will therefore be made through whole genome sequencing of isolates both from DRUM and from the archives of these clinical diagnostic services. We will not undertake resistance transmission within clinical settings as this is not a study of healthcare associated infection. **Genomic analyses will be done predominantly on culture-selected ESBL producing organisms. Is AMR and multidrug resistance going to be established by phenotypic antimicrobial susceptibility testing of the isolates? If so, is AST going to be evaluated with the identified genomic determinants of resistance? If not, why not? Please specify and/or add this in the protocol.** The genomic analysis will be undertaken on a combination of culture-selected ESBL producing organisms and enrichment broth. For isolates growing on ESBL-selective media, we will not formally confirm ESBL status, rather this will be inferred from growth on ESBL CHROMagar™ media. This decision was taken because we have previously validated ESBL CHROMagar™ media in our laboratory in Malawi for this purpose

(manuscript in preparation). During this validation exercise, (*J. Lewis, personal communication*) 663 ESBL Enterobacteriaceae isolates that had screened positive by ESBL CHROMagar methods had ESBL phenotype formally confirmed by combination disc testing, as per EUCAST guidance. Of the 663 bacterial isolates included in this validation exercise, ESBL production was formally confirmed phenotypically in 659/663 (99.4%). Specifically, 447/449 (99.55%) for ESBL-*E. coli* and 156/156 (100%) for ESBL-*K. pneumoniae*. Having established there was minimal to be gained from formal phenotypic confirmation and given our we plan to undertake molecular confirmation of ESBL status of isolates growing on 3rd-generation cephalosporin containing media (and therefore phenotypically resistant to 3rd-generation cephalosporins) by whole genome sequencing we decided against formal confirmation of ESBL status due to time and cost. Aside from ESBL screening, phenotypic antimicrobial susceptibility testing will not be undertaken, and will instead be inferred from the genotype.

We have noted the limitation that phenotypic confirmation of ESBL status will not be made, by amending the manuscript to clearly state that "*ESBL status will be inferred by chromogenic methods alone and not formally confirmed by phenotype, however isolates growing on ESBL media will be whole genome sequenced."* **Further specific comments: Introduction:**

- **Statement "The 3rd-generation cephalosporin (3GC) ceftriaxone is frequently the antimicrobial agent of first and last resort across much of sSA." Modify this statement so that you refer to 3rd generation cephalosporins as a class, rather than a single drug, or expand and explain why ceftriaxone is "of first and last resort across much of sSA" and support it with a reference.**

This comment was noted by Reviewer 1. We have now reworded the statement to read that *"The 3rd-generation cephalosporin (3GC) ceftriaxone is frequently the antimicrobial agent of choice for the empirical management of sepsis across much of sSA"* and referenced papers on its use in one of the study states (Malawi) and globally. References addition (Lester et al. Clin Infect Dis. 2020 Dec 3;71(9):e478–86) & Browne AJ et al. Lancet Planet Health. 2021 Dec;5(12):e893-e904)

- **Add "genes encoding" in the statement "…due to acquisition of genes encoding extended-spectrum beta lactamase (ESBL)-producing enzymes…".**

This wording has now been added into the manuscript, as follows: *"The 3rd-generation cephalosporin (3GC) ceftriaxone is frequently the antimicrobial agent of first choice for the empirical management of sepsis across much of sSA (6-7). 3GC resistant (3GC-R) enteric bacteria have rapidly emerged, largely due to acquisition of genes encoding extended-spectrum beta lactamase (ESBL)-producing enzymes, resulting in infections that are frequently locally untreatable, due to unavailability of carbapenems or other reserve antibiotics"*

- **What is the incidence of neonatal sepsis and malaria? What is the prevalence of HIV in these countries?**

The wording of this sentence has now been changed to reflect the burden of disease rather than incidence of sepsis and Malaria, as per comments by reviewer 1. We have amended the manuscript to state that there is a "high burden of disease from neonatal sepsis and malaria". The details of this are included in the references attached for maternal sepsis (*Seale AC et al. Maternal and early onset neonatal bacterial sepsis: burden and strategies for prevention in sub-Saharan Africa. Lancet Infect Dis. 2009 Jul;9(7):428–38*) and malaria (WHO. World Malaria report. Geneva, World Health Organization, Global Malaria Programme; 2021.). We have now included data on the burden of malaria in the site sections for Malawi *("According to the Malawian Health Management Information System report, malaria accounts for about 34% of all outpatient visits and 18% of all hospital deaths in Malawi (22)"*) and Uganda

(" In 2020, Uganda had the 3rd highest global burden of malaria cases and deaths (5.4%) and the 5th highest level of deaths (3.5%) (13)). In relation to the HIV prevelance in Malawi and Uganda, this has already been stated in the text, with associated references in the study site sections, as follows: (Malawi: "HIV prevalence in adults aged 15–65 is 18% and there is a high burden of typhoid and tuberculosis (17,18).". Kampala: "Adult HIV prevalence is 6.9% (21).", Hoima: "HIV prevalence among adults aged 15–64yrs in the Mid-West Region of Uganda where Hoima is located is 5.7% (21).")

- **Consider modifying this sentence to "…focus on isolation and genomic characterisation of ESBL producing *E. coli* (ESBL-E) and *K. pneumoniae* (ESBL-K)."**

Thank you for the comment. We have amended the wording of this statement thus; *"DRUM will work in urban, peri-urban, and rural settings in Uganda and Malawi and focus on the isolation and genomic characterisation of ESBL producing E. coli (ESBL-E) and K. pneumoniae (ESBL-K)."*

- **Specify if in the statement "We will take an interdisciplinary, One-Health approach to assess how human behaviour, WASH practices, environmental contamination,…" you are referring to "environmental contamination by enteric bacteria" or other.**

We have now amended to specify contamination with ESBL bacteria, as follows: *"We will take an interdisciplinary, One-Health approach to assess how human behaviour, WASH practices, environmental contamination with ESBL bacteria, and ABU in urban and rural locations within Uganda and Malawi contribute to the transmission of ESBL-E and ESBL-K between humans, animals, and the environment and how this transmission relates to strains isolated from the blood of humans with drug-resistant infection (DRI)."*

- **In the final sentence "…on interrupting transmission." specify if you are referring to bacterial transmission, resistance transmission, or other.**

We have amended to specify bacterial transmission, as follows: *"We will collect demographic, geospatial, WASH, longitudinal clinical and molecular microbiological data, and integrate these data into agent-based models designed to estimate the impact of putative interventions on interrupting transmission of ESBL bacteria."* **Site selection:**

- **Why were there three sites defined in Malawi (sites 1, 2 and 3) but only two sites (sites 4 and 5) in Uganda? Was it due to the contiguous location of site 5 in Uganda? How is this going to be considered for future comparative data analysis (i.e. 100 households per site)?**

Yes, it was indeed due to the characteristics of site 5 in Uganda, which had both peri-urban and rural characteristics, and peri-urban and rural polygons within Hoima were classified a priori. **Consider using "animal husbandry practices" instead of "animal practices", or just "husbandry practices" as farming areas are mentioned in Fig. 2.** This has been amended within the manuscript to animal husbandry practices, as follows: *"DRUM consortium members identified sites representing urban, peri-urban, and rural settings to enable variations in WASH behaviours, animal husbandry practices, ABU, and household and broader environmental contamination with ESBL-producing bacteria to be contrasted."*

- **Do you refer to "environmental contamination" (of household or premises?) in "contamination with ESBL-producing bacteria"? Please specify this.**

We have amended the wording to be more specific, including household and broader environmental contamination, as follows: *"DRUM consortium members identified sites representing urban, peri-urban, and rural settings to enable variations in WASH behaviours, animal husbandry practices, ABU, and household and broader environmental contamination*

with ESBL-producing bacteria to be contrasted."

- **Was the socioeconomic status of the sites in Kampala and Blantyre comparable? Is there information on housing density comparison between the equivalent sites in Uganda and Malawi? Can you expand on this a bit more?**

The socioeconomic status of Kampala and Blantyre is broadly comparable, but there will be variation between the households, which will be quantified in numerous ways in this study using the household questionnaires; details of which are included in the household CRFs (questions 15-22). We have highlighted in the text that socioeconomic status will be evaluated in the questionnaires. *"At the baseline visit, these CRFs will be completed to provide information at the individual and household level on human health, ABU, socioeconomic status, health seeking behaviour, structural and behavioural WASH practices and animal husbandry (Extended data)." & Table 1.* We do not have specific data on housing density at either site and this has not been included as a variable in our analyses.

- **Did you mean "Blantyre, the second largest city of Malawi"?**

Thank you for this comment. This has now been amended to make it clearer, as recommended. Now stating in the manuscript that Blantyre, the second largest city of Malawi. *"Ndirande is a large urban settlement with high-density housing 4 km from the geographical centre of Blantyre, the second largest city of Malawi (13,14)"*

- **What was the estimated population of Chileka?**

The estimated population of Chileka is 34,720. This figure has been obtained via verbal correspondence with the Blantyre District Health Office, as it is not available through published sources.

- **What was the estimated population of the urban study site in Kampala?**

The areas sampled comprise of a number of neighbourhoods within Rubaga Division of Kampala District. Rubaga has an estimated population of 427,300 (1). (1) https://www.citypopulation.de/en/uganda/central/admin/kampala/SC0479__rubaga/

- **Site 5: Hoima – check the subheading and the text in this paragraph. Was Hoima town area defined as urban (subheading) or peri-urban (in the text)?**

Hoima has two polygons, comprising of a peri-urban area and rural area. This has now been amended in the text and subheading for consistency. **Methods:**

- **How was the "apparent uniformity of the population density across the study area" estimated in Kampala and Chileka?**

The uniformity of population density was estimated through visual inspection of OpenStreetMap building location data plotted over satellite imagery of each area.

- **How was the data on socioeconomic gradient acquired for "In Kampala, the availability of a marked socioeconomic gradient within the study region"? Please use a reference if possible.**

No formal data describing socioeconomic metrics of these specific regions of Kampala exist. Socioeconomic gradients for areas included within the polygons were determined based on local expert opinion, via information obtained through verbal communication with local health teams and WASH organisations, and considered alongside broad geographic details included in the 2013 Uganda "slum settlement profile" (https://askyourgov.ug/).

- **In the statement "… and not have confirmed or suspected acute infection", does this take HIV status into account?**

To clarify, this is to reduce the chance that our participants have an active bacterial infection at recruitment and is not specifically related to HIV status. We have, therefore, amended

this in the text, as follows: *"and not have confirmed or suspected acute bacterial infection at the time of recruitment."*

- **Figure 3 – please clarify if sampling procedure in Fig. 3 or in the main text is preferred, i.e. will 3-4 visits be done within 6 months (Fig) or 12 months (text); which is more representative of seasonality? Malawi has one rainy season, Uganda has two rainy seasons.**

The follow-up for each household (and associated sampling) will occur at 3-4 visits over a 6-month period. This is reflected in Figure 2 and in the text. We will, however, stagger recruitment of the 100 households per region over a 12-month period (i.e. ~8-10 households per region, per month), and this will enable us to have baseline (and follow-up) data from houses that span the period of a year, enabling us to capture data in wet and dry seasons for both Malawi and Uganda.

- **In the statement "…sites that serve as important reservoirs of ESBL AMR." – did you mean "ESBL-producing organisms"?**

Thank you for this comment. We have reworded the manuscript, as follows: *"We decided to focus on areas identified as hand-contact zones or where food handling occurred and also to include broader environmental sites that we hypothesise serve as important reservoirs of ESBL-producing organisms."*

- **Household environmental sampling – what size of surfaces were sampled and/or what exposure time was used for sampling (e.g. for boot socks)? Can you specify this please?**

Thank you for this comment. We have now included the collection SOPs, which provide details of all environmental sampling methods inclusive of boot socks and household environmental swabbing in the extended material (https://doi.org/10.5281/zenodo.7561679). To answer the reviewer's specific questions, the size of the surface for household environmental swabs was 30cm^2 and boot socks were worn for 1 minute. These are described in detail in newly added SOPs.

- **Microbiological methodology order – to improve the flow of this section, specify pre-processing steps at first (e.g. filtration, centrifugation, homogenization etc.), then describe pre-enrichment (in BPW if needed), and then culture and isolation.**

The order of this section has now been changed to reflect this flow.

- **Write out abbreviations in full at first use, e.g. buffered peptone water (BPW).**

For reference, BPW was first mentioned in the field sampling section, so no amendments were made in relation to abbreviations for BPW. The rest of the document has been checked for abbreviation use, and we have added in the full title for SOP prior to its use. ANOVA and HIV remain in the manuscript text having not been expanded upon, which is consistent with other journal manuscripts.

- **Drain samples – specify if these are inner drains (e.g. in the kitchen or hand washing sinks) or outer drains (sewage, other).**

Thank you for this comment. We have now stated as "Outer drains" in the text to clarify their location. Also we have added collection SOPS to the protocol (https://doi.org/10.5281/zenodo.7561679), so these methods are further clarified.

- **Manufacturers – manufacturers are written out for some consumables (e.g. VWR for water filtering, sampling bags) but not for other consumables. For consistency, decide on including all products and manufacturers or make these**

generic.

We have now added the manufactures onto all products stated in the body of the text for consistency. Updates include BPW (Oxoid) and HRM-PCR (Qiagen).

- **Consistency in fruit and vegetable preparation for culture – what ratios and volumes of fruit and BPW will be used? Will peel of fruits be included for culture? What mass/weight of green leafy vegetables will be used?**

We feel this is adequately stated in the text and expanded on within the extended material and SOPs. The ratio of 1:9 sample-diluent only pertains to green leafy vegetables, whereas fruit will have enough BPW added to cover. Our focus for fruit pertains to the outside, where hand-contact or environmental contact with AMR bacteria can take place. In this regard a whole fruit is used. We have stated "whole" in the methods section for clarification, and also added the collection SOPs where it states that fruit are to be sampled whole (without peel removed for citrus samples). The mass of vegetables used can vary and is not a set amount. By using a consistent 1:9 sample-diluent approach this will standardise the processing between various vegetables recovered. These approached were designed in conjunction with specialist advice from the UK Health Security Agency Food Water and Environment (FWE) Microbiology Services.

- **Terminology – choose either "massaged" or "manually stomached" if these refer to the same processes.**

These refer to a similar process, so we have made this more consistent in the manuscript as follows: *"Fruit (whole) will have enough BPW added to the Whirl-Pak® bag to cover before being manually stomached for a period of 30 sec to 3 min."*

- **Please check clarity of this sentence "Green leafy vegetables will be weighed and have nine times the weight of the food added in BPW to obtain a sample-to-diluent volume of 1:9". Do you refer to weights or volumes? Did you mean the diluent (BPW) will have 9 times the weight/volume of the leafy vegetables?**

We have amended the wording to clarify this, as follows: *"Green leafy vegetables will be weighed and have nine times the weight of the food added in BPW (Oxiod) to obtain a sample-to-diluent weight ratio of 1:9"*

- **Storage of BPW cultures at -80°C – is any glycerol going to be added?**

For BPW (enrichment broth), these are stored at -80°C without the addition of glycerol. Glycerol is added to BPW stock, used for the storage of ESBL isolates. The details of this process are included within laboratory SOPs 1,2 and 3. We store the BPW directly (without glycerol), as DNA extraction will occur only once, whereas the isolates may need to be re-plated or used for future analysis.

- **Inoculation on CHROMagar – what volume of samples cultured in BPW will be plated on the selective agar? It was not specified what volume of BPW would be used for pre-enrichment.**

We have amended to state a "1µl loop" will be plated onto ESBL CHROMagar™. The details for this are also in the extended material under ESBL culture SOP.

- **Plate sweeps – these are mentioned for the first time after ESBL isolates. What cultures and plates will be used for plate sweeps, the same as those for isolated ESBL producers?**

To clarify, the plate sweeps will occur directly from the CHROMagar plates where there is growth of ESBL colonies (as per our SOPs in the extended material). We have now added an additional line to clarify this prior to the storage paragraph, as follows: *A plate sweep will be*

taken from each ESBL CHROMagar™ plate that has positive growth of ESBL colonies.

- **ESBL isolation – how many suspected ESBL colonies will be selected from each sample? A single colony of each ESBL-E and ESBL-K if present or multiple?**

Details of this are included in Section 4.4 of laboratory SOP3. A single pick will be taken of pink, blue and white colonies and white colonies/ blue colonies will undergo further additional testing. Most white colonies are not ESBL *E. coli*, but we know from local experience that this is a possibility, and so we test them to ensure that they do not represent false negatives in our phenotypic results. If however they were to be indole positive, we would store them as well. In this regard, we will take between 1-2x ESBL-E and 1x ESBL-K per plate.

- **Original sample storage for metagenomics – how will these be stored? E.g. will you store filtered and concentrated water, will you store swabs or swabs homogenized in PBS?**

The only direct sample that is stored unprocessed is human and animal stool (laboratory SOP 1, section 4.3). Duplicate river water (filtrate membranes) are also stored (laboratory SOP2, section 4.4.3) representing concentrates of the bacteria present in river water. No PBS is added to these membranes, as recommended by specialist advice from environmental scientists at CEH. For other sample types (i.e. environmental swabs, boot socks, food etc) only the BPW diluent will be stored. Details of these processes are included in the lab SOPs 1/2.

- **It is not necessary to mention metagenomic tools (mSWEEP/mGEMS) in sample storage section, and it would be more suitable for sequencing data analysis section.**

As there is a specific purpose of storage, and storage can impact on findings at subsequent metagenomic genomic analysis (i.e. diversity and relative abundance of species) we have retained this information here.

- **DNA extraction and sequencing – how will ESBL-negative isolates be acquired and selected? This should be specified in the microbiology section.**

As we are no longer planning on sequencing ESBL-negative isolates in the primary phase of our genomic analysis we have removed the line where this is stated in this protocol. As stated in our SOPS, aliquots of buffered peptone water, which are not under antimicrobial selection, will be stored at -80˚C, and if necessary, these will be regrown on MacConkey Agar to obtain ESBL-negative isolates at a later date. Any details of the methods employed in selection and regrowth of ESBL-negative isolates will be included in subsequent papers, should this be work be undertaken.

- **What library preparation kit will be used for Illumina sequencing?**

We have added details on library preparation, including kits used to the DNA extraction in the library preparation and sequencing section.

- **Numbers of samples for metagenomic sequencing are specified. How many isolates and sweeps will be whole genome sequenced on an Illumina platform? How many or up to how many isolates will be Nanopore sequenced?**

We have added the number of plate sweeps (1500) but are unable to specify the number of single colony pick isolates we will sequence on Illumina platform as we plan to sequence all ESBL positive isolates, and thus the final number is dependent on the ESBL positivity rate. Similarly, we are unable to specify the number of isolates to be sequenced on nanopore, as these will be representative of the major lineages we will identify and therefore dependent

on the diversity of the of isolate whole genomes which we do not know beforehand.

- **Metagenomic resistome of PBP pre-enriched samples will be investigated. Will you investigate the resistome of CHROMagar plate sweeps? If so, please specify this.**

Yes, and a line has been added to reflect this.

- **Spatial analysis and integration of datasets into an agent-based model. "The initial approach will be to determine variables (as described above)" please specify in which paragraph are the variables mentioned. While biological sampling and microbiological analysis are described in a great level of detail, this section is less evolved. What software, computational tools will be used? What geostatistical models will be used? How will qualitative components be evaluated and chosen for ABM?**

Given the scope of this protocol, the details of agent-based modelling methods, variable selection and spatial analysis will be included in further papers. In relation to the spatial analysis, we will use a Bernoulli geostatistical model to explore eventual spatial clustering of the prevalence of ESBL colonisation and determine which risk factors should be included in the ABM. The details of this are currently available online within the thesis of M Sammaro (https://eprints.lancs.ac.uk/id/eprint/183677/). In relation to the ABM algorithms, these have been described online at https://arxiv.org/abs/2208.11331. We have therefore included these references in the text of the manuscript, amended the text to remove the "as described above" comment, and not expanded this section.

- **As this manuscript is a study protocol and investigating "the transmission of ESBL-E and ESBL-K between humans, animals, and the environment" was one of the study objectives, it would be valuable to add a section on genomic epidemiological methods to investigate the transmission (although, this may be further detailed in publication of the study results).**

This will indeed be further detailed in publication of the study results.

Competing Interests: No competing interests were disclosed.

Reviewer Report 20 September 2022

https://doi.org/10.21956/wellcomeopenres.19440.r52055

Catrin E. Moore
[1] St George's, University of London, London, UK
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Overview:

The protocol describes an interdisciplinary study using a One Health approach which will

characterise *E. coli* and *K. pneumoniae* strains carrying the extended spectrum beta lactamase resistance in the environment, animals and being carried by people in 400 households in Uganda and Malawi. The study provides a baseline examining the consequences of human behaviour, WASH practices, environmental contamination, and antibiotic use using longitudinal clinical and molecular microbiological data, set in urban and rural locations in both countries, before the introduction of relevant interventions. The study aimed to identify risk factors for and infer drivers of ESBL-*E. coli* and ESBL-*K. pneumoniae* transmission in Uganda and Malawi at the household level.

The protocol is well written and all encompassing, describing a very holistic study to examine the drivers of antibiotic resistance in two low- and middle-income countries. I look forward to reading the results which will be published soon.

I have some specific questions, separated by sections in the protocol:

Introduction:

- Please explain what you mean or provide a reference when you say "The 3rd-generation cephalosporin (3GC) ceftriaxone is frequently the antimicrobial agent of first and last resort across much of sSA".

- You state that low-income countries in Africa have poor access to watch and reserve antibiotics, either provide a reference for this statement or explain this further with an additional sentence.

- What is a high incidence of sepsis and malaria? Please quantify.

Site descriptions:

- The site descriptions differ markedly between Malawi and Uganda, I would like to see a similar breakdown of the sites in Uganda as described in Malawi – the number of schools, farms, places of worship, hospitals and pharmacies etc.

Some clarification is required on the data collection questions on the Case Report Forms:

01: Please write CHAM (facility) in full the first time.
02: Please write CPPC in full the first time.
If a patient received more than one antibiotic, how was the formulation (oral, syrup etc) recorded?
03: Please add handwashing facilities (HWF) first time in the document.
13: How did you determine the cloudiness of the water colour to ensure robustness?

Is the rationale for, and objectives of, the study clearly described?

Yes

Is the study design appropriate for the research question?

Yes

Are sufficient details of the methods provided to allow replication by others?

Yes

Are the datasets clearly presented in a useable and accessible format?
Not applicable

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: microbiology, AMR, burden of AMR, antimicrobial use.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 17 Apr 2023

Derek Cocker

Reviewer 1: **I have some specific questions, separated by sections in the protocol**
Introduction:

- **Please explain what you mean or provide a reference when you say "The 3rd-generation cephalosporin (3GC) ceftriaxone is frequently the antimicrobial agent of first and last resort across much of sSA".**

Thank you for this comment. We mean that this is typically the agent used for empirical antimicrobial chemotherapy for individuals presented to hospital with features of severe infection, however if an isolate cultured from a clinical sample is resistant, there are typically no available agents that have broader spectrum (i.e. carbapenems). We have reworded the statement to read that *"The 3rd-generation cephalosporin (3GC) ceftriaxone is frequently the antimicrobial agent of choice for the empirical management of sepsis across much of sSA"* and referenced a paper on its use in one of the study states (Malawi) alongside global estimates. Reference additions (Lester et al. Clin Infect Dis. 2020 Dec 3;71(9):e478–86 & Browne AJ et al. Lancet Planet Health. 2021 Dec;5(12):e893-e904)

- **You state that low-income countries in Africa have poor access to watch and reserve antibiotics, either provide a reference for this statement or explain this further with an additional sentence.**

We have now cited in the manuscript recent data from Malawi, highlighting the precarity of antibiotic availability in rural settings, alongside global modelling data evidencing a lack of access. Reference additions (MacPherson EE et al. PLOS Glob Public Health. 2022 Jun;2(6):e0000314 and Browne AJ et al. Lancet Planet Health. 2021 Dec;5(12):e893-e904.)

- **What is a high incidence of sepsis and malaria? Please quantify.**

We have amended the manuscript to state that there is a "high burden of disease from neonatal sepsis and malaria". The details of this are included in the newly added references attached for sepsis (Lewis et al, Crit Care. 2019 Jun 11;23(1):212, Rudd KE et al. Lancet. 2020 Jan 18;395(10219):200-211 & *Seale AC et al. Lancet Infect Dis. 2009 Jul;9(7):428–38*), and malaria (WHO. World Malaria report. Geneva, World Health Organization, Global Malaria Programme; 2021.).

Site descriptions:

- **The site descriptions differ markedly between Malawi and Uganda, I would like to see a similar breakdown of the sites in Uganda as described in Malawi – the number of schools, farms, places of worship, hospitals and pharmacies etc.**

We regret that this level of detail could not be provided by the Uganda team prior to the

study, as Uganda was more severely affected by pandemic lockdowns and the team was unable to generate these data. The study has no capacity to generate these data now, and the situation is likely to have changed.

Some clarification is required on the data collection questions on the Case Report Forms:

01: Please write CHAM (facility) in full the first time. This has now been amended, and new versions of the CRFs are available online (https://doi.org/10.5281/zenodo.7836247). **02: Please write CPPC in full the first time. If a patient received more than one antibiotic, how was the formulation (oral, syrup etc) recorded?** This has now been amended, and new versions of the CRFs are available online (https://doi.org/10.5281/zenodo.7836247). For patients who received more than one antibiotic a separate entry was made for each antibiotic, inclusive of the formulation. This has now been made clearer on version 2 of the CRF, where is reminds the data collector to complete an entry for each antibiotic separately.

03: Please add handwashing facilities (HWF) first time in the document. This has now been amended, and new versions of the CRFs are available online (https://doi.org/10.5281/zenodo.7836247). **13: How did you determine the cloudiness of the water colour to ensure robustness?** Study staff will be trained using examples of where turbidity is considered clear, cloudy (mild), cloudy (moderate), cloudy (severe) for comparison. This assessment will then be made by the by study team at sample collection by visual inspection, and not measured formally by nephelometric turbidity units (NTU), therefore, these parameters are subjective. River water colour is determined in a similar way.

Competing Interests: No competing interests were disclosed.