Global Tricycle Surveillance

ESBL *E.coli*

WHO integrated global surveillance on ESBL–producing *E. coli* using a "One Health" approach: Implementation and opportunities

World Health Organization

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Foreword

Antimicrobial resistance (AMR) is a threat to global public health, which involves the human, food chain, and environment sectors. The Global Action Plan on AMR (GAP-AMR) adopted by WHO Member States addresses the importance of a cross-sectoral "One Health" approach to contain this problem. One of the overarching requirements of the GAP-AMR is for all Member States to develop National Action Plans (NAPs) on AMR with due consideration to achieving five strategic objectives, including AMR surveillance systems grounded in the One Health approach.

AMR is a complex and challenging One Health issue. In order to establish a national programme on integrated surveillance, countries need key elements including holistic collaboration, involvement of key sectors and subsectors, laboratory capacity to culture, isolate, identify, and characterize the pathogens involved, and human and financial resources.

This protocol is a simple and feasible approach providing countries the opportunity to increase capacities to build national integrated surveillance systems for AMR starting with a simple indicator: frequency rates of extended-spectrum beta-lactamase producing *Escherichia coli* (ESBL-Ec). The stepwise approach adopted in the protocol allows countries to gradually include other cities and provinces, sectors and subsectors, and bacterial pathogens.

The AMR information collected through this protocol will enable monitoring of this indicator at the national, regional and global level. The data can also be combined with information on the use and consumption of antimicrobials in human and animal sectors that countries are collecting using WHO and OIE guidance. This will strengthen understandings of the magnitude of AMR, and facilitate the development of containment strategies. Importantly, this protocol will increase multisectoral collaboration for AMR surveillance, promoted and supported by the Tripartite Collaboration established by the Food and Agriculture Organization (FAO), World Organisation for Animal Health (OIE), and WHO.

Executive summary

Antimicrobial resistance (AMR) has emerged as a key public health challenge for the years ahead, with major economic consequences, particularly in low- and middle-income countries (LMICs). The United Nations (UN) advocates a global, holistic, "One Health" approach to the problem, involving its specialized agencies. The Tripartite plus agreement,[1] for example, links together the World Health Organization (WHO), World Organisation for Animal Health (OIE), Food and Agriculture Organization (FAO), and United Nations Environment Programme (UNEP) for action against AMR. An efficient, robust, and multisectoral surveillance system is a central tool to steer this action and assess its effectiveness. However, such a foundational surveillance system is still lacking.

For this reason, the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) and the WHO Surveillance Prevention and Control of AMR have developed the Tricycle protocol for global surveillance on extended-spectrum beta-lactamase producing Escherichia coli (ESBL-Ec), which describes the implementation of a simplified, integrated, trans-sectoral surveillance system for bacterial resistance to antibiotics. This integrated surveillance protocol uses a One Health approach that is built upon principles of WHO surveillance tools, such as the AGISAR guidance on integrated surveillance of AMR in foodborne bacteria,[2] and the Global Antimicrobial Resistance Surveillance System (GLASS).[3] The surveillance focuses on the single key indicator that these two programmes recommended: the frequency rates of ESBL-Ec. ESBL-Ec is also used as an indicator for resistance development in gram-negative bacteria in hospitalized humans by the European Antimicrobial Resistance Surveillance Network (EARS-Net),[4] supported by the European Centre for Disease Prevention and Control (ECDC). The proposed name is "Tricycle", after its three-wheeled namesake, to demonstrate the idea that it will simultaneously address three aspects of bacterial resistance (human health, food chain (animals), and the environment) as a One Health approach, in a simple and feasible manner designed to provide robust and valid statistically-based surveillance outcomes using minimal resources.

In addition to addressing the three sectors impacted by AMR (humans, animals, and the environment), Tricycle is also a three-level process that includes:

(i) A core surveillance protocol, the Tricycle surveillance itself;

(ii) Links with other UN proposed surveillance systems in the field of AMR;[i] and

(iii) Opportunities to add satellite surveillance and research project protocols on AMR.

Indeed, the implementation of the Tricycle core protocol in any country may be a unique opportunity to elaborate and implement additional exploratory analyses as satellite national, regional, or global projects, depending on whether additional specific resources are available.

In the core part of the ESBL-Ec Tricycle protocol, ESBL-Ec is determined yearly under identical and controlled conditions in humans, the food chain, and the environment. The protocol comprises epidemiological and microbiological procedures specifically designed to be conducted in a harmonized manner in all countries, even those with limited resources. Based on this protocol, countries can build on this approach, and develop a more comprehensive and enhanced integrated surveillance system on AMR. In addition, an epidemiologically representative subset of the bacterial isolates is available for molecular/genomic characterization, to explore the occurrence and paths of dissemination of ESBL-Ec at the global level.

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i The Global Antimicrobial Resistance Surveillance System (GLASS) from WHO, and surveillance systems that deal with antibiotic usage, as developed by WHO for humans and OIE for animals.

Acknowledgements

Abbreviations

AGISAR	Advisory Group on Integrated Surveillance of Antimicrobial Resistance
AMR	Antimicrobial resistance
AMC	Antimicrobial consumption
AMU	Antimicrobial use
AST	Antimicrobial susceptibility testing
BSI	Bloodstream infections
CDT	Combination disk diffusion test
CFU	Colony forming units
CI	Confidence interval
CLSI	Clinical & Laboratory Standards Institute
CTX	Cefotaxime
CPE	Carbapenem-producing Enterobacteriaceae
CRE	Carbapenemase-resistant Enterobacterales
DDST	Double disk synergy test
DTU	Technical University of Denmark
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ENA	European Nucleotide Archive
ESBL	Extended-spectrum beta-lactamase
ESBL-Ec	Extended-spectrum beta-lactamase producing *Escherichia coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
GLASS	Global Antimicrobial Resistance Surveillance System
HIA	Heart infusion agar
IPC	Infection prevention and control
LMIC	Low- and middle-income countries
MIC	Minimum inhibitory concentration
MLST	Multilocus Sequence Type
MOH	Ministry of Health
MTA	Material transfer agreement

NAP	National Action Plan
NCBI	National Center for Biotechnology Information
NCSU	North Carolina State University
NRL	National Reference Laboratory
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
RIVM	Dutch National Institute for Public Health and the Environment
RRL	Regional Reference Laboratory
SNP	Single-nucleotide polymorphism
TBX	Tryptone Bile X-glucuronide Agar
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UN	United Nations
UNEP	United Nations Environment Programme
WP	Work package
WGS	Whole genome sequencing
WHO	World Health Organization
WHO CC	WHO Collaborating Centre
WHO CO	WHO Country Office
WHO-ERC	WHO Research Ethics Review Committee
WHO RO	WHO Regional Office
WWTP	Wastewater treatment plant

List of tables and figures

01 The Tricycle protocol for WHO integrated global surveillance on ESBL–Ec

1.1 Aim

The aim of the Tricycle protocol is to provide Member States with a common, simplified, and integrated multisectoral surveillance system designed to detect, and then estimate, the prevalence/quantity of a microorganism indicator, *E. coli,* producing a specific resistance mechanism conferring resistance to third-generation cephalosporins (ESBL) in three key sectors, namely: (i) humans (hospital and community); (ii) the food chain (animals); and (iii) the environment. A subset of strains from each sector will be saved for genomic analysis. Repeated annually, this protocol will provide a common set of metrics to follow over time, thereby supporting and expanding national integrated surveillance systems for AMR in Member States. In addition, these data and genomic analyses will facilitate the monitoring of prevalence trends at regional and global levels.

The simple and standardized methodology to isolate and monitor ESBL-Ec in the three sectors has been developed by an international group of experts gathered by WHO with the collaboration of OIE and FAO, and has been tested in six countries[ii] with the help of various academic and non-profit organizations (Acknowledgments).

The comprehensive and combined data gathered by each sector will be analysed to characterize the current patterns and evolution of ESBL-Ec global emergence and dissemination. The information gained from the molecular characterization of the isolates will help to better understand the occurrence and paths of dissemination of AMR. The methodology will also allow for the detection of trends in the prevalence of ESBL-Ec in each of the three sectors and, over time, to enable assessment of the effect of interventions implemented to control AMR. Tricycle provides countries a longitudinal surveillance system to assess the effects of interventions applied as part of national action plans (NAPs).

In addition, once sufficient data are collected, the outputs of Tricycle may be used to identify and characterize specific risk factors associated with variations in trends in ESBL-Ec numbers within the three sectors nationally, regionally, and globally.

ii Ghana, Pakistan, Malaysia, Indonesia, Madagascar, and Senegal.

1.2 Rationale for choice of ESBL–Ec as indicator

The key characteristic of Tricycle is that it focuses on a single key sentinel or indicator organism, namely ESBL-Ec. It is acknowledged that this indicator does not represent the overall global problem of AMR, and there are many other aspects of AMR than this single microorganism and resistance trait. However, the existing literature shows: (i) great variations in the rates of ESBL-Ec colonization in humans in and between countries, and prevalence trends over time;[5] (ii) variable prevalence among farm animals, with indications that some of the human morbidity linked to ESBL-Ec is due to antibiotic usage in the food chain,[6] and the presence of ESBL-Ec in the environment;[7] (iii) interventions leading to a decreased exposure to antibiotics in animals[8] or humans[9] have been followed by a decrease in ESBL-Ec occurrence rates; and (iv) ESBLs confer resistance to critically-important antimicrobial drugs. Therefore, notwithstanding the fact that the comparison of these studies is sometimes difficult as different sampling schemes and methods have been used in the past, ESBL-Ec is a relevant and representative proxy for the magnitude and trends of the global AMR problem. Furthermore, infections with ESBL-Ec result in severe morbidity and mortality in humans, with substantial health costs and disease burdens.[10]

Another important feature that supports the choice of ESBL-Ec as the single indicator is that these bacteria represent a turning point in the evolution of AMR. They are resistant to most beta-lactam antibiotics, with the result that affected patients often must be treated with drugs of last resort such as carbapenems or colistin. Access to these drugs may be lacking in some settings. However, the use of these latter antimicrobials has greatly increased over the last few years, promoting, in turn, the selection of carbapenem-resistant and even colistin-resistant *Enterobacterales*, against which none of the current antibiotics are readily effective.[11] This may result in treatment failure.[12] However, solid epidemiological data on the associated health burden are still lacking.[13]

Although it will not cover the full scope of the AMR problem, the outputs of Tricycle will provide some specific and valuable insights into the respective roles of the three sectors, human health, the food chain, and the environment, in the development and spread of ESBL-Ec. This understanding is needed to design and implement effective control plans. The laboratory protocols involved can also be used in any country, thereby expanding the scope of AMR surveillance and increasing laboratory capacity, wherever resources are available. Therefore, the ESBL-Ec Tricycle surveillance protocol is a unique opportunity to implement and strengthen multisectoral surveillance and control of AMR.

1.3 Structure and organization of Tricycle

The protocol, depicted in Figure 1.1 and Figure 1.2, is composed of eight work packages (WPs), including six "core" WPs (WP1 to WP6), and two ancillary WPs that provide links with other UN supported AMR surveillance programmes (WP7 and WP8):

1. WP1: Human (hospital and community)

2. WP2: Food chain

3. WP3: Environment

4. WP4: Molecular characterization

5. WP5: Epidemiology design and analysis

6. WP6: Management and coordination

7. WP7: Linkage with GLASS

8. WP8: Links with antimicrobial consumption and use surveillance systems

Figure 1.1 ESBL–Ec Tricycle structure

WP6: Management
- Country level
- Regional level
- Global level

WP8:
Links with AMC/U surveillance systems

WP1: Surveillance in humans
- Hospitals: bloodstream infections
- Community (carriage): pregnant women

WP7:
Linkage with GLASS

WP5:
Epidemiology design and analysis

WP2: Surveillance in the food chain
- Chicken caeca from the live bird open markets in major cities

WP4:
Molecular characterization

WP3: Surveillance in the environment
- River (upstream)
- River (downstream)
- Animal slaughter wastewater
- Human communal wastewater

Figure 1.2 Satellite approaches to enhance integrated surveillance on AMR around GLASS and Tricycle[iii]

Residues of antimicrobials

Advanced EPI

CPE

Core Tricycle

Food chain

Human

Environment

GLASS

Other FB pathogens

Other approaches

WGS

iii CPE: carbapenem-producing Enterobacteriaceae; FB: foodborne; WGS: whole genome sequencing; EPI: epidemiology.

02 Work packages: Generalities

Separate, but harmonized, protocols are available for human blood and faecal specimens, poultry caecal contents, and environmental samples for WP1 (human), WP2 (food chain), and WP3 (environment), respectively. The sampling schemes for each of the human, food chain, and environmental sectors were designed independently to provide valid estimates of ESBL-Ec sample-level prevalence (both human and food sectors) and microbial load (ESBL-Ec numbers/sample – environment only). The precision of each estimate varied depending on the sector, sample size, and expected prevalence in each population. Selective media form the basis of the sample-level estimates across all three sectors. Environmental prevalence estimates are derived from the ratio of colonies counted on antibiotic-containing versus antibiotic-free media, and the prevalence estimates for environment refer to the bacterial population of the waste or water sample, instead of the patient or animal sample.

The epidemiological design of the sampling protocols was neither intended nor sufficient for attribution of dissemination from one sector to another, such as from animals to humans and vice versa. Repeated over time, however, trends in ESBL-Ec resistance in each sector will provide strong evidence for the magnitude of selection pressure applied in each sector and, in combination with molecular characterization (WP4), may provide evidence for directionality of flows of resistance elements among sectors. The epidemiological protocols for location selection, patient, animal, and environmental sample selection, and data analysis, described in WP1 to WP3, support the overall study objectives, and include reporting standards. Separately, there is a protocol for the molecular characterization of ESBL-Ec isolates (WP4). Additional objectives, grouped under the wording of "satellite projects", may be undertaken by Member States as additions (not replacements) to those of Tricycle to address additional questions. For instance, questionnaires regarding antibiotic usage and the presence of residues also may be developed in the corresponding satellite projects.

The core Tricycle protocols (WP1 to WP6) will be rolled out globally by Member States as part of GLASS.

03 Tricycle activities at the three geographic levels

The ESBL-Ec Tricycle surveillance protocol involves activities on three WHO geographic levels: national, regional, and global. Any support to implement Tricycle at the national level will be raised by the national authorities through the WHO country office (WHO CO), who will then coordinate with the other two levels.

3.1 National level

Successful implementation of the Tricycle surveillance protocol in Member States requires official long-term commitment and support. It is recommended to start the implementation in the largest city/capital city in country, and expand over time based on available resources. A national core group should be established to coordinate and monitor the different activities in each WP, aligning with other salient activities related to the AMR NAP. The core group should be multidisciplinary and integrated, comprised of individuals from each of the human, food chain, and environment WPs, and supported by the WHO CO, at least during the implementation phase. The WHO CO AMR focal point will be the contact point for the WHO regional and global levels for all matters concerning Tricycle implementation, as well as for the NAP. The WHO CO focal point will be responsible for identifying country needs, and coordinating the support provided through the regional and global level of WHO.

3.2 Regional level

The WHO AMR regional focal points will support, facilitate, and link the activities from the national level to the global level. The WHO AMR regional focal points, together with the WHO COs, will monitor the country activities, and link with the global level to provide support in the form of information and training, such as during the implementation phase, and later requests for extension or satellite activities.

3.3 Global level

WHO will coordinate the implementation and monitoring of surveillance at the global level. Early on, and later upon request through the WHO CO, WHO will support countries with assessment visits and/or training in order to support sampling sites' selection, methodology, and data collection and analysis.

04 Methodology by work package

4.1 Work package 1: ESBL-Ec in humans

In order to isolate, confirm, and characterize ESBL-Ec in humans, two populations will be under surveillance. WP1 will obtain data from: (i) hospitals, to calculate the proportion of ESBL-Ec strains among all *E. coli* isolated from patients with bloodstream infections, as documented by positive blood cultures (based on GLASS system); and (ii) the community, to calculate the prevalence of faecal ESBL-Ec colonization in healthy pregnant women approaching/at delivery. Countries participating in the GLASS system should include hospital surveillance sites.

Depending on the types of hospital metadata that are made available, rates for *E. coli* and ESBL-Ec bacteraemia/septicaemia can be estimated either directly for all admissions, or as a proportional morbidity among only those patients with blood culture performed.

4.1.1 Hospitalized patients

The microbiological analysis will be conducted for *E. coli* isolated from patients with bacteraemia (i.e. positive blood culture).

Rationale: (i) *E. coli* isolated from blood culture is usually considered a cause of infection, not a contaminant; (ii) *E. coli* is among the most frequent *Enterobacteral* pathogen in blood culture; and (iii) in every country there is usually at least one hospital equipped with a laboratory which performs and analyses blood cultures.

4.1.1.1 Surveillance sites

The largest hospital in the capital city (same as WP2 and WP3) of each participating country will be selected as the first-choice surveillance site. If this institution is not adequate to obtain the expected number of positive *E. coli* blood cultures during a one-year period (see the sample size numbers below), then one to several other hospitals (from the public or private sector) will be added. Notably, results from a single hospital, chosen as described here, will not give a representative estimate for the country as a whole. However, the comparative value of the rates obtained will come from the fact that the chosen hospital from each country will be selected following the same criteria, which will indicate: (i) year to year variations in the chosen hospital in each country; and (ii) differences in rates observed between similar hospitals from different countries.

Hospital-based cases of *E. coli* bacteraemia represent the most extreme outcomes for infection with this organism. These may represent two very different types of events: (i) community-related infections when the positive blood culture is drawn within 48 hours after admission; or (ii) hospital-acquired infections when they are drawn thereafter.

4.1.1.2 Sub-populations under surveillance

From the microbiology laboratory information system,[iv] two sub-populations of hospitalized patients will be under surveillance in order to produce denominators:

(i) All patients detected with bacteraemia: All those with a positive blood culture (first isolate per patient per year) will be included. Patients with contaminated blood cultures will not be included. A blood culture will be considered contaminated if one or more of the following organisms is found in a series of blood culture specimens: coagulase-negative *Staphylococcus* spp.; *Micrococcus* spp.; alpha-hemolytic viridans group streptococci; *Propionibacterium acnes; Corynebacterium* spp. (diphtheroid); or *Bacillus* spp. If there has been only one blood culture taken from a patient (i.e. not a series), and this blood culture is positive with any of the above-mentioned organisms, it will also be considered as contaminated, and the patient will not be counted among those with bacteraemia.[14]

(ii) All patients from whom blood samples for haemoculture were taken (i.e. includes both positive and negative samples). For patients with multiple serial samples, a negative for only the first sample will be included in the denominator. Two types of metrics will be calculated: isolate-based and sample-based.

For all patients with positive *E. coli* blood cultures, the time between admission and the drawing of the first positive blood culture will be recorded in order to sort patients with community- or hospital-acquired infections according to the above-mentioned definition.

4.1.1.3 Metrics

Two types of metrics will be used.

Isolate-based metrics

The major indicator will be the proportion of *E. coli*-positive blood culture samples with growth of ESBL-Ec.

Numerator: Number of blood culture samples with growth of ESBL-Ec during the period of surveillance.

Denominator: Number of blood culture samples with growth of *E. coli* and tested for ESBL during the same period.

Sample-based metrics

The major indicator will be the frequency of patients with growth of ESBL-Ec.

Numerator: Number of sampled patients for haemoculture with growth of ESBL-Ec during the period of surveillance.

Denominators: Denominator 1: Total number of patients with blood samples for haemoculture taken during the same period.

Denominator 2: Total number of patients with positive blood cultures for any bacteria during the same period.

Denominator 3: Total number of patients with positive blood cultures where *E. coli* was isolated during the same period.

4.1.1.4 Sample size

Assuming 15% of blood samples are positive for bacteraemia,[v,15,16] and *E. coli* is isolated in 15% of positive blood cultures, with an unknown proportion of ESBL-Ec among *E. coli* isolates (expected to be between 25% and 75%), there is a need to identify hospitals performing at least 5,000 blood cultures per year. This is in order to acquire approximately 100 patients with a blood culture testing positive for *E. coli*.[17]

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iv Use of WHONET is highly recommended to record, collect, and analyse the information.

v The 15% proportion has been chosen as representative of what is observed in many EU countries according to the ECDC AMR surveillance system (https://ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc). Large variations in this proportion may exist in different countries. For instance, rates around 75% of *E. coli* isolated from blood cultures in India are reported as non-susceptible to third-generation cephalosporins in the WHO-GLASS 2017-2018 report.

Table 4.1 Number of blood cultures

NUMBER OF BLOOD CULTURES ESTIMATED AS A REPRESENTATIVE NUMBER (PROVIDING 100 *E. COLI* PER HOSPITAL/YEAR TESTED FOR ESBL)		
Number of blood cultures per year (# of sampled patients)	5000	
Estimate of blood cultures positive for any bacterium (%)	15	5000*15% = 750 isolates
Estimate of *E. coli* isolates in positive blood culture (%)	15	750*15% = 113 *E. coli* isolates
All of these *E. coli* isolates (113) would be tested to estimate the % of ESBL-Ec.		

4.1.1.5 Epidemiological estimation of samples

Based on sample size calculations for estimates of single proportions, and assuming an infinite population size (N), the precision of the estimates of the proportion of positive blood cultures (n=750) with *E. coli* ranges between 1-7% for an apparent prevalence of 1-50% of *E. coli* among all cases of bacteraemia, respectively (at 15%, precision is ± 3.0%). The relationship between estimate and precision is symmetrical in reverse from 50-99%. For the prevalence of ESBL-Ec among *E. coli* isolates (n=100), the precision of the estimates ranges from 2-20% for apparent prevalence of 1-50%, respectively (at 15%, the precision is ± 8.2%).

An absolute precision of 10%, 7.5%, and 5% can be achieved in any span of expected prevalence proportions, as shown below in Figure 4.1 inside the green line at a sample size of 100. Additional estimates of precision achievable at larger sample sizes on the y-axis can similarly be determined easily.

Figure 4.1 Sample size calculation based on absolute precision
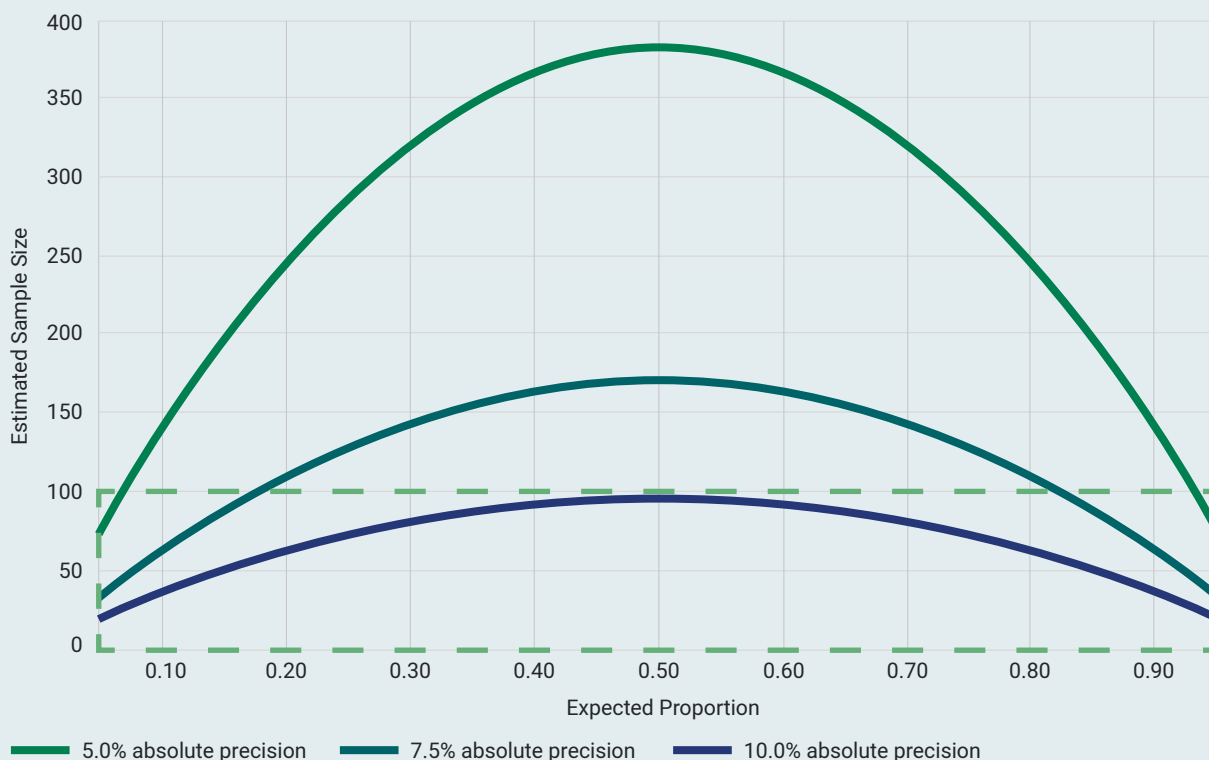
Figure created by Statulator beta: www.statulator.com

Prevalence estimates (expressed as a proportion) for WP1 will be estimated as follows using WP1 hospital bloodstream analysis as an example:

Prevalence of ESBL-Ec phenotype among *E. coli* bloodstream infections:

$$P_{(ESBL\text{-}Ec\ Blood)} = y_{(ESBL\text{-}Ec\ Blood)}/n_{(Ec\ Blood)}$$

Where:

$P_{(ESBL\text{-}Ec\ Blood)}$ = Proportion of bloodstream *E. coli* exhibiting presumptive ESBL phenotype;

$y_{(ESBL\text{-}Ec\ Blood)}$ = Number of bloodstream *E. coli* exhibiting presumptive ESBL phenotype;

$n_{(Ec\ Blood)}$ = Number of bloodstream *E. coli* isolated in one year at large referral hospital and tested for ESBL phenotype.

Depending on the prevalence, approximate or exact 95% confidence intervals (CI) may be calculated. For the purposes of choosing the most robust method to avoid CIs that span either 0 or 1, an exact method is most robust for sample sizes that exceed 100. Using either standard statistical software packages (e.g. R, SAS, Stata) or else an online calculator, such as is found at https://epitools.ausvet.com.au/ciproportion, the 95% Clopper-Pearson exact CI for the prevalence estimate of 15 ESBL-Ec out of 100 *E. coli* is: 15% (8.6% - 23.5%). Note that the exact method does not yield symmetrical CIs as do some approximate methods.

Similar approaches are taken with:

Prevalence of *E. coli* among all laboratory confirmed bloodstream infections:

$$P_{(E.\ coli\ Blood)} = y_{(E.coli\ Blood)}/n_{(Bloodstream\ infections)}$$

Where:

$P_{(E.\ coli\ Blood)}$ = Proportion of bloodstream infections attributed to *E. coli;*

$y_{(E.\ coli\ Blood)}$ = Number of bloodstream infections attributed to *E. coli;*

$n_{(Bloodstream\ infections)}$ = Number of laboratory-confirmed bloodstream infections in one year at large referral hospital.

For the example sample sizes given earlier, if 15% of bloodstream infections are *E. coli,* then 113/750 or 15.1% is the attributable fraction (95% CI: 12.6%-17.8%).

For ESBL-Ec prevalence at the sample/host level, the formulas are easily adapted from above, and point estimates for ESBL-Ec among pregnant women (n=100) and chicken caeca (n=240) can readily be determined by using the number of ESBL-Ec positive samples as the numerator, and the number of faecal/caecal samples collected as the denominator. For the chicken samples, in consultation with an epidemiologist and/or a statistician, the estimate and precision may need to be adjusted for effects of within-flock clustering and temporal dependencies, based on how the samples were acquired.

4.1.1.6 Clinical subjects and sample specifications[vi]

Sample specification should include all the following for inclusion in the study:

(i) All patients attending the participating hospitals during the surveillance period, for which a blood culture has been taken, as recorded in the microbiology laboratory, will be included.

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vi The surveillance period will usually be one year. However, the time needed to reach the required number of samples for Tricycle surveillance may be shorter than one year in hospitals where a large number of *E. coli* blood culture isolates are observed.

(ii) The minimum data set for data collection will comprise at least all the variables included in a typical data set for a standard configuration of a WHONET laboratory, and may include other optional variables according to specific needs of the participating hospital (see Chart 4.1). The variables considered mandatory will include the specimen date, admission date, date of birth, and gender. These will ensure that a standard approach according to GLASS specifications is followed in order to determine the origin of infection (community versus hospital), and that data can be stratified (i.e. by age and gender of patient).

(iii) At the microbiological level, only the first *E. coli* isolate after admission will be included. Patients with repeated admissions will be included as separate cases.

(iv) Time between admission and the drawing of the first positive blood culture will be recorded in all patients with ESBL-Ec positive blood culture in order to differentiate those with community-acquired (<48 hrs) from those with hospital-acquired episodes.

Important note: Tricycle utilizes results obtained from blood samples taken strictly on a routine basis for patient care. Absolutely no additional blood samples will be taken. The surveillance will be an opportunity to disseminate WHO guidelines on drawing blood and best practices in phlebotomy,[18] improve diagnostic stewardship for bacteraemia, equip laboratories with WHONET, and implement an important component of the GLASS WHO surveillance system.

4.1.1.7 Main limitations

Testing for ESBL-Ec in hospitalized patients will not give representative country estimates, but will give estimates for the largest hospital of the capital, which can then be compared year after year to study trends. Comparison between similar hospitals (e.g. tertiary-care hospitals of similar size in terms of bed capacity, similar catchment populations, and systematic testing of suspected bloodstream infections) from different countries may be carefully considered, but limitations of this approach must be kept in mind (e.g. the hospitals, not the countries, are being compared).

The quality of the results will depend on whether hospitals perform systematic testing of suspected bloodstream infections. This is often not the case, particularly in developing countries, where selection bias therefore is likely to occur, resulting in an overestimation of the proportion of ESBL-Ec bloodstream infections (e.g. if only more severe cases are tested).

4.1.2 Community (healthy) subjects

4.1.2.1 Subpopulation under surveillance

Healthy pregnant women seen at pre- and peri-natal healthcare settings or during delivery will be targeted as community representatives (healthy carriers). In some centres, for practical reasons, participation of pregnant women at their last routine visit before delivery instead of at delivery may be proposed. The main concern for ESBL-Ec faecal carriage in pregnancy is the increased risk of transmission of these resistant strains to newborn babies, which subsequently may contribute to neonatal sepsis. ESBL-Ec carriage may also increase the risk of transmission to healthcare providers and other hospitalized patients. To ensure these risks are minimized, before the beginning of the study, the Ministry of Health (MOH) will ensure that the participating clinics and hospitals apply the infection prevention and control (IPC) measures as defined in the national IPC guidelines.

4.1.2.2 Surveillance site

The largest maternity health facility from the capital city (same city as WP2 and WP3) will be chosen for surveillance as a first choice.

4.1.2.3 Type of Samples

For routine visits, a stool sample or rectal swab[vii] will be requested from pregnant women. Such samples can also be collected during the delivery.[viii]

Ethical rules in each country will be followed in order to obtain informed consent from the women. If possible, the number of women that decline to participate will be recorded.

4.1.2.4 Number of samples and periodicity of surveillance

Given that approximately 100% of healthy humans are *E. coli* carriers, and that the expected rates of ESBL-Ec carriage will vary greatly between regions, it is proposed that 100 samples are collected on a yearly basis[ix] (100 distributed in the 12 months). The precision of the estimates for ESBL-Ec carriage within each subject will be similar to that of the blood culture approach described above. Note that the estimates themselves depict different things: (i) for the blood culture results, the estimated proportion and 95% CIs will reflect the prevalence of ESBL phenotype among all *E. coli* isolated in one year at a large referral hospital in each country, whereas (ii) the results from selective culture of stool samples from healthy pregnant women will estimate the prevalence of ESBL-Ec at the sample level (presence/absence), and infer their presence in the host as a whole. In other words, the results from testing applied to positive *E. coli* bacteraemia isolates will reflect a bacterial-level prevalence of ESBL phenotype, whereas the results of selective culturing of stool will reflect a sample/host level prevalence of ESBL-Ec.

4.1.2.5 Patient inclusion and informed consent

Participation is on a voluntary basis. To allow for relatively unbiased results, and to obtain samples during the entire year, the appointed maternity health facility should collect samples from at least the first nine women attending the facility each month who meet the eligibility criteria.

Eligibility criteria (inclusion and exclusion) should be specified so that only healthy pregnant women meeting the inclusion criteria are asked to participate. Eligibility should be restricted to the following recommended criteria, with the national coordinator of the ESBL-Ec Tricycle surveillance clearly determining the eligibility criteria at the beginning of implementation:

(i)   Proposed inclusion criteria for healthy pregnant women seen at pre- and peri-natal healthcare settings are:

- Over the legal age of consent;
- Able and willing to provide consent;
- Able and willing to provide a stool sample or rectal swab for testing.

(ii)   Proposed exclusion criteria for healthy pregnant women seen at pre- and peri-natal healthcare settings are:

- Do not give their informed consent to participate in the study;
- Documented use of antibiotic in the previous three months;
- Hospitalization in the previous three months.

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vii According to best accepted local practices for detection of microbiologic intestinal carriage.
viii According to the best accepted local practices.
ix The rates of carriage are estimated to range from 25% to 50% according to literature and to preliminary results from the implementation phase of Tricycle.

Patients are received at the clinic as per local procedures. Eligible pregnant women will be identified during registration, and informed about the project by appointed personnel. Depending on the national policies, it might be necessary to ask patients for oral or written consent before participation in this surveillance. It is advisable that the informed consent is obtained by someone who has no direct impact on patient care, due to power balances. For example, if the treating health professional requests consent, the patient may feel obliged to accept. Also, this is not an intervention study, so no experimental changes will be made to the care regime.

Patients should be provided with the following information:

- Purpose of the surveillance;
- Voluntary nature of participation;
- Procedures and protocols;
- Risks and benefits of participation;
- Confidentiality elements;
- How results will be shared.

4.1.2.6 Microbiological requirements for ESBL-Ec detection and confirmation

Samples will be collected, stored, and shipped to the appointed clinical laboratory according to the best accepted local practices for detection of microbiologic intestinal carriage.

To culture, detect, and confirm the presence of ESBL-Ec, the sample will be analysed following microbiological procedures described in Annex 1.

As part of the Tricycle activities, the laboratory will periodically transmit the results aggregated by clinics to the national coordinator. The coordinator will ensure that the results are analysed periodically, and shared with the participating clinics so that the IPC and neonatal sepsis guidelines are revised based on the evidence.

Antibiotic treatments aiming to decolonize patients carrying ESBL-Ec are not recommended. However, the result of the screening may be used to adapt the therapy in case the newborn develops neonatal sepsis. Countries are invited to set up a system to ensure that treating clinicians are informed if treated pregnant women are identified as carrying ESBL-Ec. This mechanism should be discussed at the beginning of the implementation, and set up according to the best accepted local practices in terms of data security.

4.1.2.7 Minimum standards of information when sampling

The minimum standards for data collection among healthy pregnant women will comprise at least all the variables included in a typical data set for the standard configuration of a WHONET laboratory (see Chart 4.1). In addition, data collection may include optional variables according to the specific needs of the participating healthcare setting or country. The variables considered mandatory are the specimen date, admission date, and age of the mother. In order to exclude carriage acquired from the hospital, only specimens obtained within 48 hours after admission in hospitalized women will be acceptable (when sample is collected during the delivery).

4.1.2.8 Main limitations

This approach does not give an estimate for community ESBL-Ec carriage in the population, as it specifically targets an age group and sex category, excluding males as well as younger girls and older women. For example, older men and women may be at higher risk of ESBL-Ec carriage. However, we believe this is the best compromise between study feasibility, and reliability of the estimates that are generated, and we think that community carriage among pregnant women remains an indicator of great interest. Another limitation refers to the refusal rate: If it is too high, there is a risk that selection bias occurs, as women who decline to participate may substantially differ in the risk of ESBL-Ec, as compared to those who agree to take part in the study (e.g. they participated because they have a stronger health-seeking behaviour, and may therefore be at lower risk of having ESBL-Ec).

4.1.3 Ethical committee

Tricycle is designed for public health surveillance purposes; it is nonexperimental, does not collect sensitive information, and does not introduce any interventions.

This protocol has been reviewed and approved by the WHO Research Ethics Review Committee (WHO-ERC).

Before implementing the ESBL-Ec Tricycle surveillance, the national coordinator must seek human subjects review from the relevant national ethical/legal review boards or committees per national and institutional policies. Review of WP1 of the protocol should be sought, when possible, as public health surveillance rather than research. Where countries implement the protocol with support from WHO, site specific studies are to be submitted to the WHO-ERC for review.

4.2 Work package 2: ESBL–Ec in the food chain

4.2.1 Food animal samples

Poultry (chicken) was selected as the host species for several reasons: (i) it is the most common and most frequently consumed; (ii) it is reared in virtually every country of the world in conditions ranging from highly intensive to medium intensive/pastoral to backyard/household; (iii) there are few, if any, religious aversions to the animal or its meat products being reared and consumed; (iv) it is readily available to acquire samples; and (v) will harmonize nicely in countries with existing integrated surveillance systems where chicken caecal samples are routinely collected for analysis of *E. coli, Salmonella,* and *Campylobacter*.

4.2.2 Sampling locations

Considering the diversity in poultry production and sales in developing countries, a wet market in the capital city was determined to be the most likely common source to acquire samples. If there is no such market in the capital city, the next largest city should be considered. If wet markets are not common in a given country, samples may be collected from an alternative poultry slaughter facility, ensuring that samples taken are from poultry intended to be sold for domestic consumption only. In all cases, it is important to select chickens that are as epidemiologically independent of one another as is feasible. This means they are likely to have arisen from different primary sources (i.e. farms). This necessitates sampling at a frequency that ensures any one flock[x,6] has 'cleared' before sampling another bird. This can be accomplished by walking through the wet market and sampling at multiple stalls, or else (in a more traditional slaughter plant) waiting until the equivalent of a shipment/load from a single source has passed.

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x Flock: all poultry of the same health status kept on the same premises or in the same enclosure.

4.2.3 Animal subject and sample

The intact caecum (plus its contents) should be collected from a recently slaughtered bird from the wet market or other harvest facility. Only one bird per vendor/stand should be collected so as to minimize the likelihood the birds come from the same farm/source. Multiple vendors in different parts of the market should be selected. The final decision on how to best select the chickens (site options, characteristics) will be made locally based on how the poultry market works in the country and city. The intact caeca should be obtained by clipping at the ileocaecal junction and caecal-colon junction, and placing each entire caeca plus its contents in a separate, sterile, container (e.g. Whirl-pak® or another suitable sterile plastic sample bag). The caeca should immediately be deposited into an ice-filled cooler, transported to the laboratory, and maintained at 4°C for analysis within 48 hours.

4.2.4 Number of animal market/slaughter sites, number of samples per site, and temporal frequency of sampling

A target number of 240 caecal samples per year will be collected. Participants should target 20 samples per month per year, collected from poultry vendors/stands in the wet market or at another harvest facility (Table 2).

Table 4.2 Site options to be selected by countries to collect the poultry samples

SITE OPTIONS	CHARACTERISTICS	POULTRY TYPE
Wet market	Many sources, farm source often unknown	Backyard/bicycle birds, commercial farms, cooperative farms, etc.
Slaughterhouse[xi]	Fewer sources, origin of birds better known	Intensively raised chickens

The target number of 240 caeca is based similarly upon the assumptions for human samples (i.e. precision at 15% sample-level prevalence). However, the numbers are expected to be easier to acquire given costs and other logistical barriers to human-subject research. European Food Safety Authority[19] (EFSA) recommendations for surveillance at slaughter in EU Member States aim for approximately 170 isolates of each targeted bacterium for AMR phenotypic testing. Sources of dependent clustering within the Tricycle protocol include unavoidable/undetected source clustering at wet markets and slaughterhouses, and repeated monthly sampling over a one-year period. Applying the same criteria as for WP1, and adjusting the sample size needed due to likely temporal and source clustering (estimated intracluster correlation coefficient (ICC) = 0.15), yields inflated needs for sampling at each level of precision (Figure 4.2). If we assume 15% prevalence at the caecal level, the sample size needed for absolute precision of 0.07, with clustering (ICC = 0.15 and cluster size of 10) by source repeating monthly over a one-year period, would be 235 birds, versus 100 with no clustering. The red, blue, and yellow lines lying within the green box (less than or equal to 240 samples) reflect reliable estimates for 5%, 7.5%, and 10% absolute precision, respectively, at varying expected prevalence of ESBL-Ec.

In addition, the proposed sample size (n=240) is expected to exceed EFSA technical specifications, providing sufficient power to detect a 15% or greater difference in prevalence where resistance is widespread with ± 8% accuracy. The primary objective of sampling at animal markets is to estimate varying trends in ESBL-Ec prevalence over time. Differences can be explored temporally, or across regional or country-level boundaries. Coordination or domestic consultation with national/local animal health specialists can facilitate sampling at regular intervals.

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xi Ensure the samples taken are from poultry intended to be sold for domestic consumption only (i.e. not exported).

Figure 4.2 Sample size calculation based on absolute precision
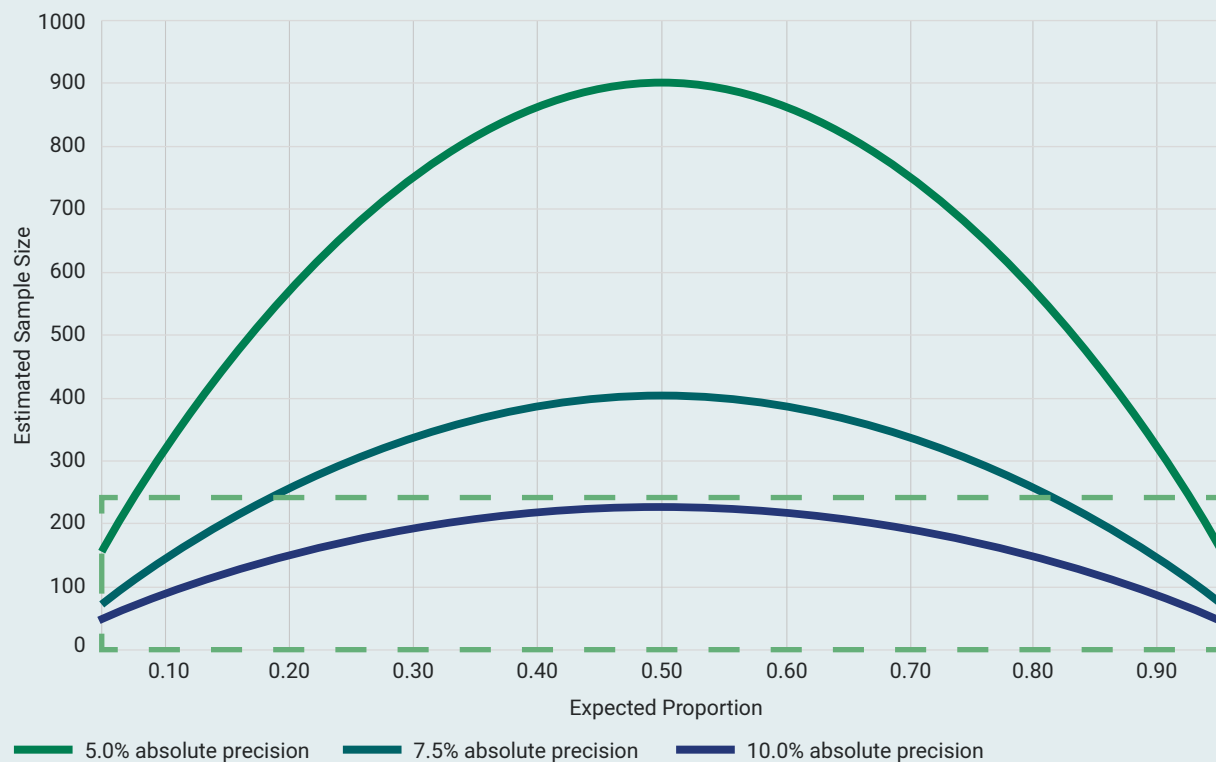
Figure created by Statulator beta: www.statulator.com

4.2.5 Microbiological requirements for ESBL–Ec detection and confirmation

The samples will be analysed following the microbiological procedures described in Annex 2 to culture, detect, and confirm ESBL-Ec.

Antimicrobial susceptibility testing (AST) for a list of antimicrobials, as suggested by the WHO Integrated Surveillance of AMR in Foodborne Bacteria,[2] should be conducted on all presumptive ESBL-Ec isolates to confirm the ESBL phenotype following Clinical & Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards (as described in Annex 2), when the laboratory conditions and capacity are in place. These isolates will be stored to confirm the presence of ESBL genes via polymerase chain reaction (PCR) tests and possibly whole genome sequencing (WGS) in a later stage.

4.2.6 Main limitations

Though the farm of origin should be asked, it is possible that in some countries this information is not available. In this case, the risk is that chickens have been raised in similar conditions, and the proportion of ESBL-Ec may therefore reflect the situation in one or a few specific suppliers, rather than overall. This is particularly true in countries where few farms supply many vendors. In countries where the poultry industry is not developed, and where many small-scale suppliers exist, this is unlikely to be a problem.

4.3 Work package 3: ESBL-Ec in the environment

4.3.1 Environmental samples

The environmental component of Tricycle proposes to detect and quantify ESBL-Ec in hotspot sources, as well as in rivers receiving wastewater from these sources. Sources include human sewage, and wastewater influenced by animal waste from wet markets or slaughterhouses. Ambient rivers will be sampled both upstream and downstream of the cities/communities. The downstream sample is intended to represent ESBL-Ec presence and concentrations as a result of inputs from the urban community, such as sewage discharges and waste from the wet market. The upstream site does not necessarily represent a site without human or animal influents, but serves to compare the results of the downstream sample due to sources in the city. It is proposed to also detect and quantify total *E. coli* in the samples. The total *E. coli* counts are used to calculate the proportion (percentage) of total *E. coli* that are ESBL producers.

Please note that attribution of the total quantities of ESBL-Ec, or of *E. coli* in general to animal or human sources would additionally require precise hydrological flow data.

4.3.2 Site selection

4.3.2.1 Country level

Within one given country, two cities should be identified:

(i) Major (capital) city where the analytical laboratories are located. This city should be identical to the city with the hospital, healthcare facility, and the wet markets (WP1 and WP2).

(ii) A sentinel city of about 100 000 inhabitants, in the proximity of the capital city (not more than 2-4 hours required for transport of samples).

The sentinel city is included to enable comparisons between cities of similar size among different countries. If no suitable sentinel city can be identified, the number of samples in the capital city should be doubled (then, two locations for each sample type as described below should be included in the single city studied).

4.3.2.2 City level

Within each city, four environmental sampling sites should be identified (Figure 4.3):

(i) River (upstream): Ambient surface water upstream of the city is representative of pre-city impacts and other upstream activities in the catchment. It serves as a background sample to be able to detect the influence of the city.

(ii) River (downstream): Ambient surface water downstream of the city is representative of city impacts, including human and animal faecal waste discharges.

(iii) Animal slaughter wastewater draining from a wet market where poultry is slaughtered or from a poultry slaughter facility. This sample serves as a representative source of poultry faecal material, or if other animals are also slaughtered in the wet market, of food animal faecal material.

(iv) Human communal wastewater: Influent urban wastewater from sewerage (so-called "sanitary sewerage"), urban human waste drainage flowing into a wastewater treatment plant, or major sewers (sanitary sewers) collecting human wastewater directly where no wastewater treatment occurs. Such sewage or wastewater should represent mainly human faecal material.

Figure 4.3 Environmental sample locations
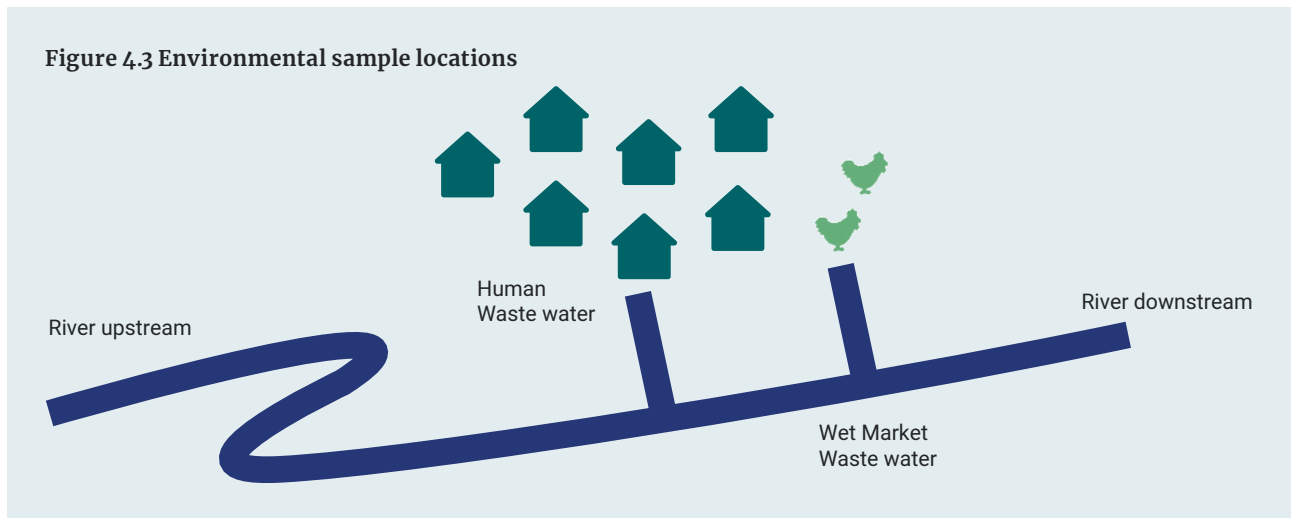
If an ambient river is not available for sampling sites, sampling of two other surface waters near the city should be considered: (i) one that is protected from human or animal waste, such as a drinking water reservoir; and (ii) another that is impacted by human and/or animal wastes, such as a pond that is surrounded by latrines or other human faecal waste sources, and/or allows food animal access to the water (for animal watering). These would serve as the substitutes for up- and down-stream samples from rivers.

Suggestions and considerations for extension of the basic Tricycle protocol to sampling of drinking water and hospital wastewater are given 4.8.4 and 4.8.5.

4.3.2.3 Key points for selection of sampling sites

(i) Selection of sampling sites for WP3 should be aligned with the human and food chain WPs (i.e. animal markets and hospitals should be located in the same city).

(ii) Sampling locations for human wastewater should be chosen such that a large population discharges faecal matter into the site investigated, such as detailed in the guidance on polio environmental surveillance.[20]

(iii) In case the country participates in poliovirus environmental sampling in the WHO global polio environmental surveillance system for polio eradication, alignment of the sampling locations for human wastewater with the sampling locations of the environmental polio surveillance system for the polio eradication campaign is strongly suggested.

(iv) Human and animal wastewater should be taken from locations that discharge into the investigated river, between the upstream and downstream location. Human and animal wastewater samples should be collected before the wastewater enters the river.

(v) If no river flows through the city, analyses in human and animal wastewater can still be performed if suitable sampling locations can be found (see above).

(vi) If the city is located at the seashore or a similar large coastal marine area, downstream samples can be taken at the river mouth discharge into the ocean or large lake, ideally during low tide.

(vii) Site selection should be performed with the assistance of all authorities and specialists who can assist in the identification of the most representative sampling sites for the types of samples to be collected. These include sanitation specialists (for information on human wastewater streams that are representative of a large population). Consideration should be given to avoiding, if possible, human and animal sanitary waste sources that are diluted significantly with storm water, as occurs in combined human sewage and storm drain systems. If the human and animal waste sources are known to be significantly diluted with storm water, this should be noted.

(viii) For consistency between sampling rounds, sampling locations (GPS codes) should be stored.

4.3.3 Sampling frequency

Eight to 12 sampling events should be distributed over the whole year in each city, including sampling in each of the major seasons.

4.3.4 Numbers of environmental samples

2 cities x 4 sample types x 8-12 rounds/year (ideally 1 per month) = 64-96 samples. If no sentinel city can be found: 1 city x 8 samples x 8-12 rounds/year = 64-96 samples.

4.3.5 Microbiological requirements for ESBL–Ec detection and confirmation

The samples will be analysed following the microbiological procedures to culture, detect, quantify, and confirm *E. coli* and ESBL-Ec as described in Annex 3.

Given the likelihood that a large proportion of samples will be ESBL positive, and that samples will contain many individual – and often unrelated – isolates, a random subset of five individual presumptive ESBL-Ec isolates will be kept from each sample. These isolates will be further characterized, and AST should be conducted on a random subset of presumptive ESBL-Ec isolates using standard methods as applied to samples from WP1 and WP2.

4.3.6 Analysis parameters

- Concentration of ESBL-Ec;
- Concentration of *E. coli;*
- Ratio of ESBL-Ec over *E. coli.*

4.3.7 Metadata

- Sampling locations and types, sample condition, and sample storage/handling (sampling questionnaire for data management in section 4.5.3);
- Information on sanitation status of the investigated cities.

4.3.8 Main limitations

To be able to compare the situation between hospital and community, it would have been interesting to add sampling sites downstream and upstream of the largest hospital in the capital. However, we decided to keep the study protocol as simple as possible, and focus on ESBL-Ec in the community. However, countries with capacity to also test environment close to the hospital are encouraged to do so.

4.4 Work package 4: Molecular characterization

All the ESBL-Ec isolates obtained from the human, food chain, and environmental specimens will be stored as explained in Annex 8. Periodically, a subset of those isolates will undergo further typing and molecular analysis. This may include multiplex quantitative PCR to detect the most common ESBL genotypes, and/or ultimately WGS to have more comprehensive information. When the National Reference Laboratory (NRL) in the country lacks the capacity to perform these steps, the NRL should collect the isolates from the three WPs, store them, and then forward them onward for transfer to another laboratory in consultation with the country, regional, and global WHO team. The country can request support for molecular testing from a Regional Reference Laboratory (RRL) or WHO Collaborating Centre (WHO CC).

Collaboration and sharing of isolates with RRLs or WHO CCs should be governed by material transfer agreements (MTAs) signed by all parties. Subsequently, DNA will be extracted and WGS applied using a recommended analytical platform for detection of AMR genes and cluster analysis. Countries are strongly encouraged to share open-access genomes and associated metadata through the National Center for Biotechnology Information (NCBI)/European Nucleotide Archive (ENA) for further future analysis to support the global effort to combat AMR in the respective country, RRL, or WHO CC.

The results of the WGS analysis can determine the multilocus sequence type (MLST), core gene MLST (cgMLST), virulence genotype, plasmid type, acquired AMR genes, and chromosomal point mutations, among other relevant properties, which all contribute to the understanding of the epidemiology of the pathogens. In addition, a genomic phylogenetic analysis based on single-nucleotide polymorphisms (SNP) and whole genome (wg)/core genome (cg) MLST (wg/cg MLST or extended MLST) will allow a nuanced picture of the clonality and further spread of the pathogens. The added value of using WGS typing is completeness, costs, and the ability to do a rapid re-analysis. Thus, using a species-independent technology such as WGS allows the identification of all relevant epidemiological data as well as relatedness among isolates. This enables the occurrence, emergence, and evolution of strains and/or their resistance genes, among others, to be tracked globally, which is indeed the ultimate goal of Tricycle (Figure 4.4). Laboratory protocols for preparing samples for sequence analysis are available online.[21,22]

Figure 4.4 Added value of sequencing analysis of the isolates from the three work packages

Integrated Surveillance of AMR: ESBL-Ec Tricycle surveillance

Sequencing analysis of isolates

| Species ID | Sub-type | AMR | Plasmids | Virulence | Phylogeny |

Antimicrobial Susceptibility Test ⟷ Whole Genome Sequencing

Phenotype

Genotype

Conceptual added value in WGS:
- More discriminatory methodology
- Better understanding of AMR
- Possibility for rapid re-analysis
- Easy data sharing
- Electronic data storage
- Direct comparison of data between sectors

4.4.1 Steps to be taken based on country capacity

4.4.1.1 Countries with no molecular technology in place

All presumptive ESBL-Ec isolates should be stored in leakproof cryogenic tubes or vials at -80°C in 1 mL volumes of Luria-Bertani (LB) broth, Trypticase soy broth, or equivalent media, including 15% sterile glycerol to prevent freezing damage. A purity control should be performed from each agar plate by collecting some remaining broth with a loop and streaking a new agar plate (e.g. blood agar), checking for purity after overnight incubation at the relevant temperature (e.g. 37°C). If the culture is not pure, all the cryogenic tubes made from that plate should be discarded. Results should be recorded for quality assurance purposes. All strain details and associated metadata should be recorded in a line list in Excel or equivalent for further data sharing (See Annex 9). Sensitive data (e.g. farm name, patient identifiers) should not be recorded in the metadata spreadsheet.

4.4.1.2 Countries with capacity to characterize ESBL-Ec

ESBL-Ec isolates should be characterized following standardized protocols, and stored to be sent to the WHO CC for sequencing following the above description.

4.4.1.3 Countries with capacity to characterize ESBL-Ec and perform WGS

ESBL-Ec isolates should be characterized as indicated above. DNA extraction, library preparation, and WGS should be performed according to established protocols from sequencing platform providers. Several DNA-extraction protocols have been made available online.[21,22]

4.4.2 Data sharing

The genomes produced for ESBL-Ec with associated metadata (if possible) should be shared in a public domain such as NCBI or ENA. However, restricted access might be needed due to sensitivity or political/ethical reasons. The NCBI and ENA can both receive either assembled genomes (e.g. fasta) or raw reads (e.g. fastq). Thus, the genomes should be submitted to NBCI or ENA using the following options:

(i) NCBI: https://www.ncbi.nlm.nih.gov/genbank/genomesubmit/

(ii) ENA: https://www.ebi.ac.uk/ena/submit

(iii) CGE ENA uploader. Please note that the COMPARE uploader will upload sequence to ENA under COMPARE's ENA account, but there is an easy alternative to the direct submissions to NCBI and ENA: https://compare.cbs.dtu.dk/ https://www.ebi.ac.uk/ena/submit

4.4.3 Isolate selection to be characterized genetically

A subset of the bacterial strains of human, food chain, and environmental origin that were isolated will be further characterized based on WGS and bioinformatics tools. The subset of isolates will be selected, defined by their availability date, source, geographical distribution, and potential additional analysis (see strain and metadata lists).

4.5 Work package 5: Epidemiology

4.5.1 Minimum sample sizes and sampling strategy per domain

See description in each work package.

4.5.2 Protocol for site selection

Site selection for human, animal, and environmental sampling will be carried out using a stepwise approach, based on the availability of the following historical data:

• Historical (minimum one year) ESBL-Ec patient address data (by city suburb) – liaise with hospital authorities;

• List of wet markets/slaughterhouses in the city (geolocated to street or suburb) – liaise with animal health specialists;

• Wastewater and poultry or wet market effluent network maps, or river/water bodies networks – obtained with the assistance of local, regional, and national authorities and their specialists.

4.5.3 Data management

The minimum set of information to be associated with each specimen collected for the three WPs is provided in Chart 4.1.

Data may be digitalized using information technology systems already adopted and implemented at the hospital and/or laboratory level.

Alternatively, a data configuration for Tricycle surveillance has been created in the WHONET software (http://www.whonet.org). The configuration allows collecting the data as defined in Chart 4.1 for each of the three WPs, and extracting them in the aggregated format needed for analysis at the national level. The same format is used for data transmission to GLASS.

Chart 4.1 Metadata suggested to be collected by sector

GENERAL		
Country name		
City name		
Human	Bloodstream sample 1. Patient ID 2. Hospital name 3. Date of admission 4. Date of sampling 5. ID specimen number 6. Sex 7. Age 8. Isolate number 9. ESBL screening result 10. Isolate ID confirmation 11. AST results	Faecal sample 1. Patient ID 2. Clinic/hospital name 3. Date of sampling 4. Age 5. ID specimen number 6. Isolate number 7. ESBL screening result 8. Isolate ID confirmation 9. AST results
Food chain	1. Market or slaughterhouse name 2. Specimen number 3. Production type 4. Estimated bird description (chick, market weight or age days, spent hen)	5. Sample collection date 6. ESBL screening result 7. Isolate ID confirmation 8. AST results
Environment	1. Sample site and source type (upstream and downstream surface water, poultry waste, and urban human sewage or drainage) 2. Sample number 3. Collection round number 4. Sample type	5. Sample collection date 6. ESBL screening result 7. Isolate ID confirmation 8. *E. coli* concentration 9. ESBL-Ec concentration 10. ESBL-Ec ratio

WHONET software is perfectly suited for the purposes of Tricycle, is readily available at no cost to end users, and is fully supported by WHO. In addition, training courses and online manuals have been, and will continue to be, supported and made available.

As data on human specimens may contain information identifiable at the patient level, it is important that paper-based and electronic data are stored safely, with only authorized personnel able to obtain access to them. The means of storage may vary depending on the resources of the location, but the national coordinator should ensure that safe storage is achieved at the laboratory and hospital/clinic level, and that it is in accordance with the ethical and data safety regulations in that country.

For the purpose of Tricycle, data on individual or batches of samples (including metadata, Chart 4.1) and microbiological endpoints (by isolate) should be extracted, aggregated, and shared with the appointed epidemiologist. WHONET software allows the data to be exported in a common, aggregated, file format.

Aggregated data can then be imported into the Tricycle module that will be specifically created for ESBL-Ec Tricycle surveillance in the GLASS IT platform.[xii] The module will also allow for standard statistical analyses, suited to data representative of the binomial probability distribution (events/trials) to provide crude and model-adjusted estimates of prevalence.

Sampling of hospitals, pre-/peri-natal care facilities, wet markets, and surface and wastewater sources has previously been described in WPs 1-3. These are likely to be convenience samples insofar as access to the sampling sites is facilitated, and cooperation is maximized. Importantly, attempts to maximize the representativeness of the sampling sites in each country will pay dividends in the form of generalizability and the application of statistical inference. A true statistical sampling scheme would involve the construction of a sampling frame (identifying all possible sites meeting inclusion criteria), and random sampling from that list. Decision makers in each country need to weigh the added value achieved from such an approach versus the risks in sampling a site that is inaccessible, distant, or non-cooperative. Being able to achieve maximum internal study validity, with the potential to repeatedly sample over many years, and monitor trends in resistance prevalence, warrants consideration.

When analysing the data, the main limitations of the data collected should be discussed and openly disclosed to avoid misleading interpretations of the results. National authorities should clearly explain that sampling locations (i.e. cities, healthcare structures, wet markets/slaughterhouses, rivers) and target populations (i.e. pregnant women as the host group for ESBL-Ec circulation in the community, and chickens as the host species for the food-producing animal populations) were selected to support study feasibility, and not because they play a specific role in the emergence, maintenance, and dissemination of AMR in the country. National authorities must take all appropriate measures to avoid stigmatization of the studied population or the sampling locations.

The core elements of Tricycle do not support efforts to infer causation among the sectors representing the three WPs. Decades of sampling in support of integrated AMR surveillance and monitoring in highly developed programs such as Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN),[23] the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP),[24] the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS),[25] and others, have not yielded data suited to such pursuits, largely owing to the extremely complex and ecological nature of the problem, and the ubiquity of the indicator organisms being studied. If such objectives are determined to be the goal of Member States, other study designs beyond the scope of Tricycle should be utilized.

An epidemiologist in each country will support and coordinate all activities, including collection, reviewing, and verification of the metadata in the three main WPs. The epidemiologist will provide support for analysis of the information, and report to the national coordinating group about the analysis of the surveillance data, utilizing appropriate statistical methods to evaluate and interpret the data. The epidemiologist will visit and assess the activities in the sectors where the surveillance sampling is taking place.

xii https://www.who.int/glass/it-platform/en/

4.6 Work package 6: Management and strategy

4.6.1 Implementation of Tricycle in Member States

Each country selected to participate in ESBL-Ec Tricycle surveillance will, through the national authorities, designate five people as focal points to lead the implementation of the surveillance in the three main sampling sectors. This will be the national coordinating group for the implementation of ESBL-Ec Tricycle surveillance. This group will be trained, and they will also coordinate the national activities of this surveillance system as follows:

Each country should include:

(i) **Microbiologist(s)** that can be the same or (preferably) different people, depending on the resources of the country, from the human, food chain, and environment sectors;

(ii) **Epidemiologist** with an epidemiology background and expertise, who will lead and provide advice and support in the implementation of the whole surveillance system;

(iii) **National coordinator** with a public health background, who will coordinate and monitor the implementation of the surveillance, and will be the link between the national authorities and the AMR national focal point, as well as the WHO focal points in the three levels.

4.7 Links with other UN supported AMR surveillance programmes

4.7.1 Work package 7: Linkage with GLASS

Tricycle is considered a special project under the GLASS umbrella.

GLASS is the major WHO tool for AMR surveillance, for which AMR data have been collected from participating countries since 2017. As of 30 January 2020, 87 countries were enrolled in GLASS. The relationship between GLASS and Tricycle is described in paragraph 4.4.2 of Section 4 (GLASS developments) of the GLASS Report 2017-2018.[15] The pilot phase of implementation of Tricycle in the various WHO regions is described in Section 5 (Updates from WHO Regional Offices on AMR surveillance activities) of the same document.

Of note, the rate of ESBL-Ec in human blood culture is a common indicator between GLASS and Tricycle. Moreover, Tricycle applies the GLASS methodology to obtain the ESBL-Ec metrics from hospitalized patients.

To optimize resources, it is highly recommended for countries implementing Tricycle to select surveillance site(s) to obtain ESBL-Ec data from hospitalized patients from the surveillance sites already participating in the national AMR surveillance system.

Data from Tricycle will be entered in a specific module in the GLASS IT platform, and supported by WHONET.

4.7.2 Work package 8: Links with antimicrobial consumption and use surveillance systems in humans (WHO), animals (OIE), and plants (FAO)

Surveillance of antimicrobial consumption (AMC) and use (AMU) in humans is integrated in GLASS,[xiii,15] and will be available through the GLASS IT platform. These data will be used as necessary to analyse their relationship with Tricycle results.[26]

AMU surveillance is also being carried out in animals and plants, with ongoing collaborations between the OIE, FAO, and WHO through the Tripartite Collaboration to improve a comprehensive understanding of AMR across sectors, and to promote the One Health approach to AMR control.[27]

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xiii Paragraph 4.2.1 of the GLASS 2017-2018 implementation report.

4.8 Satellite projects

Only the satellite projects that have been presented so far are described in this section. It is expected, however, that other satellite projects will be included in the future, after their design has been discussed and their funding secured in some countries at least.

4.8.1 Usage of antimicrobials

OIE and WHO have developed methodologies for monitoring consumption/use of antimicrobials in the animal and human sectors, respectively. In 2015, OIE launched AMU data collection of veterinary antimicrobial agents, and sent a call for data to its 182 Member Countries. In 2016, WHO launched its surveillance program, and sent a call for data in an initial set of countries.

We propose that the participating countries will collect data on antimicrobial consumption/use for the whole country in humans and animals, following the WHO[xiv] and OIE[28] methodologies, respectively. The focus will be on antimicrobials for systemic use, whereas topical use will be excluded. Data include sales of antimicrobial medicines per year at the country level. Collection for both sectors will be coordinated at the country level. Countries will jointly analyse data on antimicrobial consumption/use in humans and animals and produce a joint national report on consumption of antimicrobials in both sectors. In addition, participating countries will submit their AMU data to the OIE and WHO databases as part of the respective regular global AMR/AMU surveillance activities.

4.8.2 Advanced epidemiological studies

Some countries have aimed at linking the Tricycle protocol to advanced epidemiological studies. This combination is not part of the core Tricycle protocol, and can be seen as satellite projects if funds are locally available.

4.8.3 Residues

As an alternative measure for investigating antibiotic usage at local scale, analysis of residues of antibiotics could be suggested for a subset of the samples. Communal sewage samples and wet market sewage samples can provide a snapshot of antibiotics resulting mainly from human and animal usage, across a larger population. However, such analyses are of limited value for antibiotics with high chemical instability, such as third-generation cephalosporins, and require detailed information on the size of the underlying population and the hydrology of the system. In addition, surface water samples can inform on the concentrations of the more stable antibiotics in the actual environment. Countries participating in ESBL-Ec Tricycle surveillance could consider the inclusion of the analysis of residues of antimicrobials in the samples collected in WP3.

In order to preserve water samples for later analysis of residues at a specialized laboratory, the sample should be frozen for storage within eight hours after sampling. For frozen storage, 500 mL plastic laboratory bottles (e.g. polypropylene bottles) should be used, and storage temperature should be -20°C until shipment to a specialized laboratory. Shipment should be made on dry ice.

4.8.4 Drinking water

If countries are interested in additional drinking water analysis, the same protocol can be used to determine ESBL-Ec and *E. coli* in drinking water samples.

*   Drinking water analysis can be performed at the point of use (i.e. households), or at the point of production (drinking water production sites), depending on the interests of the country.

*   Countries with an interest in drinking water analysis are encouraged to align sampling with their institutions responsible for drinking water quality.[29]

4.8.5 Hospital wastewater

Hospital wastewater can contain higher concentrations of ESBL-Ec that partially stem from clinical patients. However, hospital wastewater does not necessarily represent a major source of all ESBL-Ec as compared to communal wastewater. Therefore, and in order to keep the protocol simple, hospital wastewater has not been included in the core Tricycle protocol.

Upon interest, countries might consider additionally sampling hospital wastewater as a fifth sample type, and analyse this sample with the same method as used for human wastewater.

05 Annexes

5.1 Annex 1. Work package 1 – ESBL–Ec in human samples: Microbiology procedure

5.1.1 Materials and methods

This Annex describes a culture-based method for the detection of ESBL-Ec in samples from WP1, including blood culture samples from patients with *E. coli* bacteraemia and faecal samples from pregnant women.

Table 5.1 Summary of human samples analysis

TARGET ORGANISMS AND BACTERIOLOGIC CULTURE MEDIA	
Target organisms	*E. coli* and ESBL-Ec
E. coli selective and enriched media	**For blood culture:** Culture media to isolate and identify *E. coli* following the laboratory standard operating procedure in the hospital. **For faecal sample:** • MacConkey supplemented with cefotaxime (CTX) 4 µg/mL • Blood agar plate
Identification of *E. coli* and ESBL-Ec from blood culture bottle	**Pre-analytical phase:** The pre-analytical procedures are not described here because no blood culture will be obtained in relation to testing for the protocol. Only results of blood cultures drawn from patients within the context of their normal care will be used.[30] **Analytical phase:** The handling of the blood culture in the laboratory (incubation, detection of positivity). Identification of *E. coli* in positive blood culture will be done according to the routine methods in use in the laboratory in the hospital selected for bacterial identification (Annex 6). *E. coli* from positive blood culture will be sub-cultured on MacConkey agar supplemented with CTX (4 µg/mL) in case the laboratory is not confirming ESBL as routine. The inoculated plates will be incubated at 37°C for 18-24 hours. After the incubation process, any presumptive positive growth from up to three colonies will be confirmed as *E. coli* using the colony purification (one time) and identification procedure in use in the laboratory. Any confirmed *E. coli* colonies will be: (i) Tested for ESBL phenotype following EUCAST or CLSI recommendations (such as double disk synergy test (DDST), combination disk diffusion test (CDT), broth microdilution including automated commercial systems). (ii) If ESBL-Ec is confirmed (see Annex 4), store ESBL-Ec isolates at -80°C for potential further testing. **Post-analytical phase:** Results and metadata of the sample (if available) will be entered in WHONET.

TARGET ORGANISMS AND BACTERIOLOGIC CULTURE MEDIA	
Community settings: Faecal sample from pregnant women	Faecal samples or faecal swabs will be collected in healthy pregnant women during (or close to) delivery. Information to be collected could include (see Chart 4.1): Date of sampling and date of birth of the pregnant woman. Patients will not be asked if they have been exposed to previous antimicrobial therapy. The information will only be recorded if it is traced on a written document. Swab in transport media such as Cary Blair or Amies is the best option for transporting and preserving the sample.
	Pre-analytical phase: Transport to the laboratory will be as rapid as possible, and the date of arrival to the laboratory recorded. If faecal swabs are used, swabs with a preservative procedure and its materials should be used.
	Analytical phase: The sample information will be entered in WHONET as specimen type, date of collection, ID, etc. The faecal sample or faecal swabs will be streaked on MacConkey agar (reconstituted from commercial powder) supplemented with CTX 4µg/mL.
	The inoculated plates will be incubated at 37°C for 18-24 hours.
	Up to three lactose-positive colonies will be confirmed as *E. coli* using the colony isolate purification and identification procedure in use in the laboratory (Annex 6).
	E. coli isolates will be:
	(i) Tested for ESBL confirmation following EUCAST or CLSI recommendations (DDST, CDT, or broth microdilution, including commercial automated systems) (Annex 4).
	(ii) If CTX-resistant ESBL-Ec is confirmed, store ESBL-Ec isolates at -80°C for potential further testing (see Annex 8 for methods).
	Post-analytical phase: Identification and antimicrobial susceptibility results will be entered in WHONET.
QUALITY CONTROL/QUALITY ASSURANCE – CONTROL STRAINS OF ESBL & NON-ESBL *E. COLI* & NON-*E. COLI*	
E. coli strain (non-ESBL producer)	Type strain: *E. coli* ATCC 25922
Non-*E. coli* species and strain	Type species and strain: *Klebsiella pneumoniae* BAA 1706
ESBL-Ec strain	Type species and strain: *E. coli* NC11
ESBL non-*E. coli* strain	Type species and strain: *Klebsiella pneumoniae* ATCC 700603
Quality control/quality assurance use procedures for *E. coli* and ESBL *E. coli* and non-*E. coli* strains	Specific instructions will be provided on how positive control species and strains will be used and handled to support good quality control/quality assurance in all field sample analyses in Annexes 5-7.

5.2 Annex 2. Work package 2 – ESBL–Ec in a food chain sample: Microbiology procedure

5.2.1 Materials and methods

This protocol describes a culture-based monitoring method for the direct detection of ESBL-Ec in poultry samples.

Table 5.2 Summary of poultry samples analysis

TARGET ORGANISMS AND BACTERIOLOGIC CULTURE MEDIA	
Target organisms	ESBL *E. coli*
ESBL-Ec selective medium	MacConkey agar supplemented with CTX 4 µg/mL
FOOD CHAIN SAMPLING	
Market settings: Live or recently slaughtered bird bought in the live (wet) market, or fresh caecal samples bought in the market or selected from another harvest facility. Note: Caecal samples at the market should not appear to be dried out or otherwise contaminated with feathers, debris, dirt, etc. Note: If it is possible, information on the market and vendor will be collected.	
Sample transportation and storage should be kept at between 2°C and 8°C. Samples should arrive at the laboratory within 48 hours after sampling. Bacteriological analysis at the laboratory should be initiated as soon as possible after receipt at the laboratory, preferably within eight hours after sampling.	
ANALYSES	
Market: Poultry caecal sample	Culture medium: MacConkey supplemented with CTX at 4 µg/mL
Purification and isolation of presumptive target organisms	Each type of lactose-positive (typically pink) colony on the MacConkey plus CTX plate will be selected for bacterial identification to confirm *E. coli* (Annex 6).
Confirmation of presumptive *E. coli*	Conventional or automated biochemical identification methods established in the laboratory and used in routine practice and with quality assurance/quality control, including colony isolate identification, selection, purification, and then culture confirmation of identity as *E. coli* (see Annex 6-7). All isolates confirmed as *E. coli* will be stored, preferably at -80°C.
Confirmation as ESBL	As per CLSI or EUCAST standards (see Annex 4): • Disk diffusion: DDST, CDT. • Broth micro dilution including automated commercial systems.
QUALITY CONTROL/QUALITY ASSURANCE – CONTROL STRAINS OF ESBL & NON-ESBL *E. COLI* & NON-*E. COLI* COLIFORMS	
E. coli strains	Type strain: *E. coli* ATCC 25922
Non-*E. coli* species and strain	Type species and strain: *Klebsiella pneumoniae* ATCC BAA 1706
ESBL-Ec strain	Type species and strain: *E. coli* NC11
ESBL non-*E. coli* strain	Type species and strain: *Klebsiella pneumoniae* ATCC 700603
Quality assurance/quality control use procedures for *E. coli* and ESBL *E. coli* and non-*E. coli* strains	Specific instructions will be provided on how positive control species and strains will be used and handled to support good quality assurance/quality control in all field sample analyses in Annexes 5-7.

Table 5.3 Summary of method by day

DAY	POULTRY CAECAL SAMPLE[31]
Day 1	Collection of samples in the selected wet (live) bird market or other harvest facility.
	A sterile loop will be used to collect approximately 10 µg of caecal contents. The loop will be directly inoculated onto MacConkey agar plate plus 4 µg/mL CTX (MAC-CTX plates), by streaking for isolation and then incubated at 37°C for 18-24 hours.
	Register the sample and information in WHONET.
Day 2	Presumptive ESBL-Ec colonies will be coloured red or pink on the MAC-CTX plates.
	Sub-culture up to three colonies by re-streaking onto MAC-CTX plates.
	Incubate at 37°C for 18-24 hours.
Day 3	Select one of the sub-cultured isolates for bacterial identification (Annex 6-7). If the first isolate is not identified as *E. coli,* a second and eventually third isolate should be tested.
	Once confirmed, store presumptive ESBL-Ec isolates at -80°C for potential further testing (Annex 8).
	AST is highly recommended where the capabilities and conditions are in place in the laboratory.

5.2.2 Procedure for isolation of ESBL–Ec from caecal samples

Receipt of samples

(i) Samples should arrive in the laboratory within 48 hours of initial sample collection. Samples arriving >48 hours after initial collection should be discarded. Also, samples which have not been stored appropriately (i.e. between 2°C and 8°C) during transportation and/or storage, should be discarded. Samples with damaged packaging should be discarded as well.

(ii) The stored samples shall be kept at a constant temperature between 2°C and 8°C until bacteriological examination at the laboratory. This should be initiated as soon as possible after receipt at the laboratory, preferably within eight hours.

(iii) It is not recommended to have samples arriving close to weekends or holidays.

Sample analysis

(i) Inoculate one loop-full (10 µL loop) of caecal content using a single streak onto a MacConkey agar plate containing 4 µg/mL CTX. From this streak, further apply two successive streaks using a 1 µL loop to ensure bacteria separation that will yield single colonies (see Figure 5.1). The plates are then incubated for 18-22 hours at 37 ± 0.5°C.

(ii) Based on colony morphology and appearance – presumptive ESBL-Ec colonies are purple/red/pink on the MAC-CTX (Figure 5.2) – sub-culture up to three colonies by re-streaking onto MAC-CTX. Incubate the plates overnight at 37 ± 0.5°C.

(iii) Re-streak the colonies from the CTX MacConkey agar plates to blood agar plates. Incubate the plates for 18-22 hours at 37 ± 0.5°C.

(iv) Select one of these presumptive *E. coli* colonies for species verification/ identification (see Annexes 6-7 for procedure). If the first isolate is not identified as *E. coli,* a second and eventually third isolate should be tested to obtain a confirmed *E. coli* isolate.

(v) Confirm as ESBL-Ec by following the procedure in Annex 4. AST is highly recommended where the capabilities and conditions are in place in the laboratory.

(vi) The isolate that is identified as *E. coli* and confirmed as ESBL-producing should be stored according to Annex 8 (Figure 5.3).

5.2.3 Figures

Figure 5.1 Presumptive *E. coli* on MacConkey agar supplemented with 4μg/mL CTX

**Figure 5.2 Plating on selective plates to detect presumptive ESBL-Ec
(*E. coli* colonies are typically pink to red in colour)**
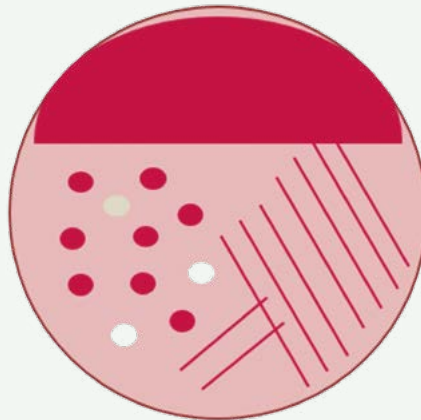
Figure 5.3 Workflow for identification of presumptive ESBL-Ec from caecal content

Indole Test

+ reaction

1. ESBL AST
2. SAVE ISOLATE IN STORAGE
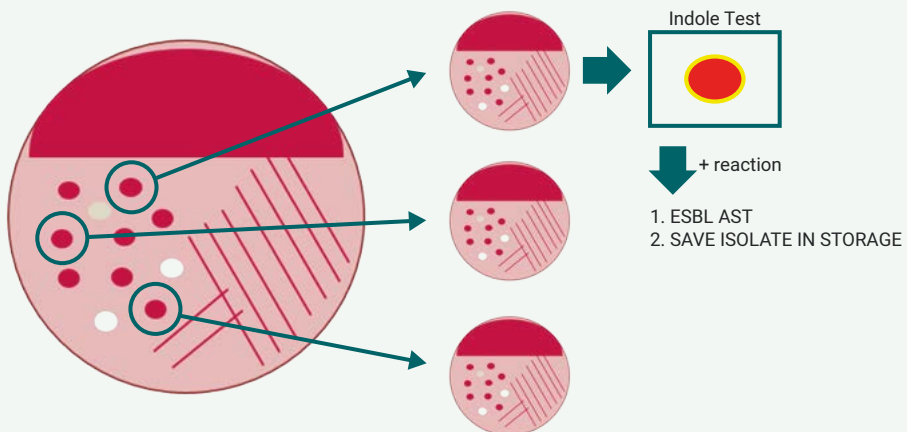
5.3 Annex 3. Work package 3 – ESBL–Ec in water and wastewater: Microbiology procedure

5.3.1 Materials and methods

A culture-based monitoring method is used for the direct detection and enumeration of ESBL-Ec in environmental samples. The focus is on hotspot sources of ESBL-Ec (human wastewater, and wastewater influenced by animal faecal waste), and river water impacted by these sources. A summary of elements to analyse these bacteria in such samples is shown in Table 5.4, including a list of the representative culture media and methods, sampling sites, and analysis strategies for the quantification and characterization of resulting assay data and bacterial isolates.

Table 5.4 Summary of environmental samples analysis

TARGET ORGANISMS AND BACTERIOLOGIC CULTURE MEDIA			
Target Organisms	A) *E. coli* B) Resistance mechanism: ESBL-Ec		
E. coli selective medium	Tryptone Bile X-glucuronide Agar (TBX)		
ESBL-Ec selective medium	TBX supplemented with CTX at 4 µg/mL (TBX-CTX)		
ENVIRONMENTAL SAMPLING SITES			
Community sewage from major collector sewers of human wastes, preferably from a sanitary sewer not appreciably diluted by storm water, or influent from a Wastewater Treatment Plant (WWTP)	Wastewater from city wet market or from poultry slaughterhouse	River water upstream of the city	River water downstream from the city
ANALYSES			
Quantitative analysis	Quantitative analysis by spread plate or membrane filter methods, yielding: (i) Concentrations of *E. coli* (colony forming units (CFU) /100 mL) (ii) Concentrations of ESBL-Ec (CFU /100 mL) (iii) Proportions of total *E. coli* that are ESBL-resistant (ESBL-Ec CFU divided by total *E. coli* CFU)		
Purification and isolation of presumptive target organisms	Select five representative colonies from TBX + CTX plates (with characteristic blue or blue-green *E. coli* colony colour), and streak to purification. To obtain pure isolates, colonies should be streaked initially on the same TBX medium from which the original colony was selected, and then a representative colony from this plate should be streaked onto blood agar or any nutrient agar plate available.		
Identification of presumptive *E. coli*	Conventional or automated (commercial) biochemical identification methods used as routine in the laboratory (see Annexes 6-7 for procedure). All isolates confirmed as *E. coli* will be stored (see Annex 8).		
Confirmation as ESBL	Confirm as ESBL-Ec by following the procedure in Annex 4. AST is highly recommended where the capabilities and conditions are in place in the laboratory.		
QUALITY CONTROL/QUALITY ASSURANCE – CONTROL STRAINS OF ESBL & NON-ESBL *E. COLI* & NON-*E. COLI* COLIFORMS			
E. coli strain	Type strain: *E. coli* ATCC 25922		
Non-*E. coli* species and strain	Type species and strain: *Klebsiella pneumoniae* ATCC BAA 1706		
ESBL-Ec strain	Type species and strain: *E. coli* NC11		
ESBL non-*E. coli* strain	Type species and strain: *Klebsiella pneumoniae* ATCC 700603		
Quality control/quality assurance use procedures for *E. coli* and ESBL *E. coli* and non-*E. coli* strains	Specific instructions will be provided on how positive control species and strains will be used and handled to support good quality assurance/quality control in all field sample analyses in Annexes 5-7.		

Table 5.5 Summary of the method by day

DAY	OBJECTIVE
Day 1	Sample collection, transport to, and cold storage in the laboratory.
	If testing of samples for antibiotic residues is intended: Frozen storage of an aliquot of 500 mL in plastic bottles at -20°C.
Day 2	Spread plate or membrane filtration method. All samples are analysed on TBX agar for total *E. coli* counts, and on TBX agar with 4 µg/mL CTX for ESBL-Ec counts, and further isolation/ characterization of ESBL.
Day 3	Counting presumptive total *E. coli* bacteria on TBX agar and presumptive ESBL-Ec bacteria on TBX agar with 4 µg/mL CTX.
	For purification, sub-culture five presumptive positive ESBL-Ec bacteria from TBX agar with 4 µg/mL CTX (possible pause point: storage of plates in refrigerator until next step).
Day 4	Second step of purification of presumptive positive ESBL-Ec bacterial isolates on the same media from which they were initially isolated, or on blood agar or any nutrient agar plate available (possible pause point: storage of plates in refrigerator until next step).
Day 5 and following	Identification of bacterial species (Annex 6), and ESBL confirmation (Annex 4).
	Storage of confirmed ESBL-Ec under appropriate conditions (Annex 8).

5.3.2 Sampling

Materials

Sampling pole with sampling jar (sterile glass or sterile plastic bottle), wipes, alcohol, funnel, insulated cooling box, sampling sheet, camera, thermometer, pH meter or pH strips.

Procedures

Collect samples wearing gloves to minimize infection risks. If possible, collect composite samples (24-hour mixed samples) by use of a flow proportional sampling device, if available.

Otherwise, take three point grab samples with at least five minutes between each sample. Record sample details (see sample sheet) and take a photograph of the sample and sampling site. To prevent carry-over contamination from first sampling point, rinse the sample apparatus a few times with the water sample from the subsequent sampling site, and pour the sample back at a place distant from the sampling location, prior to sample collection. For taking the actual sample, take a sample from beneath the surface (preferably at 20-30 cm depth if possible). Sub-surface samples from shallower depth can be taken if the water being sampled is too shallow for sampling from a 20-30 cm depth. Transfer the sample using the funnel to the sample bottle, and close the bottle. Disinfect the outside of the bottle and sampling apparatus with alcohol. Complete the sampling sheet with details on the sampling location and sampling time. During transport to the laboratory, store sample between 2°C and 8°C (in insulated cooling box) and analyse within 24 hours. More guidance can be found in the guideline ISO 19458:2007.[32]

Choice of spread plating versus membrane filtration

Samples with expected high concentrations of *E. coli* and ESBL-Ec, such as urban sewage and wet market animal wastewater samples, can be analysed by spread plating, both directly and after serial 10-fold dilution. Samples of surface water, both upstream and downstream, can be expected to have low to moderate *E. coli* and ESBL-Ec concentrations in many cities. Because such samples may not provide sufficient countable concentrations of *E. coli* and ESBL-Ec colonies to be detectable on spread plates, it is recommended to analyse these samples by membrane filtration. With this method, bacteria from larger volumes of water can be gathered on one filter, followed by placing the filter on agar plates of TBX agar (for *E. coli)* and TBX agar with 4 µg/mL CTX (for ESBL-Ec). This method is more sensitive than spread plating because of the larger sample volume analysed. When a membrane filtration apparatus is available, this method is preferable and should be used for all sample types. If no membrane filtration apparatus is available, spread plating can be performed as an alternative.

5.3.3 Recommended agars for Tricycle analysis

In Tricycle, two types of agar plates are used for each sample:

(i)   For the quantification of *E. coli:* TBX agar.

(ii)   For the quantification and isolation of ESBL-producing *E. coli:* TBX agar with 4 µg/mL CTX (TBX-CTX).

For each sample and for each agar, three to four 10-fold dilutions of a sample should be tested. This results in 2 agars x 4 dilutions = 8 determinations per sample.

5.3.4 Serial dilutions

Purpose

For enumeration, plates should contain a countable number of bacteria (10-100 colonies). However, the concentrations of ESBL-Ec and *E. coli* in water can vary greatly depending on the amount of faecal contamination and the amount of non-contaminated water, such as inputs from rain (e.g. storm water) and upland spring sources, and therefore, cannot be precisely predicted. In order to achieve plates with countable numbers of colonies, three to four different 10-fold dilutions and/or volumes of each sample have to be analysed. Suitable dilutions will avoid confluent bacterial growth and yield 10-100 well isolated colonies. In general, plates with <100 colonies (membrane filters) or <250 colonies (spread plate technique) are countable. Usable dilutions are sample- and medium-specific, with more dilutions needed for more highly contaminated samples and for *E. coli* quantification without CTX. Fewer dilutions need to be made for samples that are expected to be less contaminated, and for ESBL-Ec quantification, because the concentrations of these ESBL-Ec bacteria are lower than total *E. coli* bacteria. For instance, the concentrations of *E. coli* in raw sewage or similar urban wastewaters are high, typically between 10^6 to 10^8 colonies per 100 mL, while the concentrations of ESBL-Ec are lower by 10 to 100-fold, typically between 10^4 to 10^7 per 100 mL. Suitable dilutions should be determined in an initial test with several different decimal dilutions. A suggestion for dilutions of samples that can be applied during initial testing can be found below (under 5.3.6.7).

5.3.5 Materials

Sterile diluent, such as phosphate buffer or phosphate-buffered saline (PBS), in suitable volumes (e.g. 9 mL or 45 mL), larger volume sterile pipettes and/or graduated cylinders (for larger volumes), sterile pipette tips or smaller volume sterile pipettes (for smaller volumes), and vortex mixer for dilution tubes.

5.3.6 Procedures

Remove samples from 4°C storage, mix well to re-suspend sedimented matter, and dilute serially 10-fold using diluent to get in the range of countable colonies.

(i)   First, mix the sample well by swirling vigorously 25 times or by using a vortex mixer.

(ii)   Then remove 1 mL of sample and add to 9 mL of diluent. Mix the sample dilution well.

(iii)   Then discard the pipette used to make this dilution and use a new, sterile pipette to remove 1 mL from this dilution to make the next dilution using these same methods.

(iv)   Repeat these steps until all desired serial dilutions are made.

The procedure for making serial 10-fold dilutions is outlined in the figure below:

Figure 5.4 Procedure for making serial 10–fold dilutions
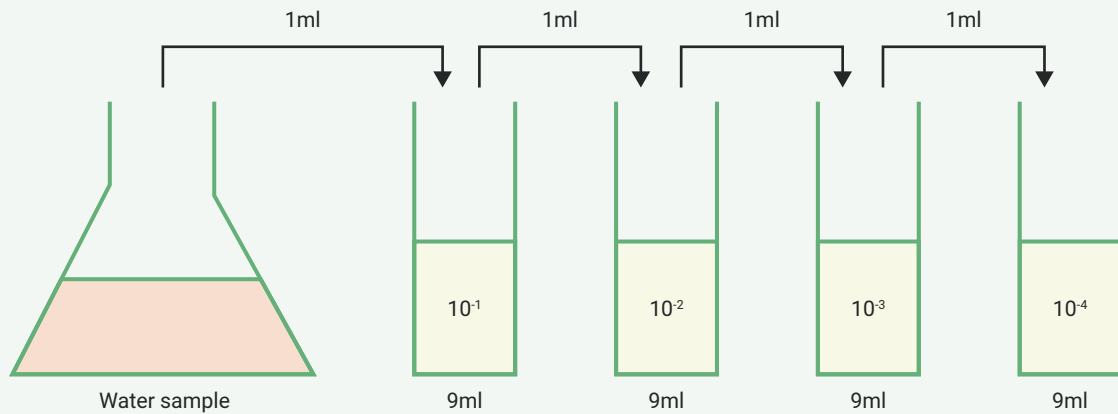
5.3.6.1 Membrane filtration

Materials

Membrane filtration apparatus with vacuum, sterile membrane filters (0.45 μm pore size and 47 mm diameter, gridded), sterile forceps, sterile pipettes for 1-20 mL, sterile pipette tips, agar media (3-4 TBX plates, and 3-4 TBX plates with 4 μg/ mL CTX for each sample), sterile bottles or large graduated tubes for making serial dilutions, and sterile graduated cylinders to measure volumes of up to 100 mL.

Procedure

Let agar plates reach room temperature (take out of fridge 30 minutes before filtration). Autoclaved membrane filter apparatus is recommended, either glass or plastic. For non-autoclaved membrane filtration set-ups, such as steel units, disinfect by adding alcohol, flaming, and cooling down with 100 mL sterile water (without filter) or allowing to stand at ambient temperature until the outside of the filter funnel is cool to the touch. To filter a sample, use flat-blade sterile forceps (sterilized by alcohol flaming) to aseptically place a 0.45 μm filter, grid side up, onto the porous filter base (without trapping air below the filter), and then place funnel back onto the filter base. If you need to filter <20 mL of sample, first add >20 mL of sterile diluent into the filter. Starting with the greatest sample dilution (i.e. the dilution with the lowest concentration of bacteria), pipette (or pour from a bottle) the amount of sample needed onto the filter (taking care to not touch the pipette tip onto the filter surface, with the vacuum valve closed). Apply vacuum until the complete sample volume is filtered. Do not continue to apply vacuum once no liquid is left on the membrane. Then rinse the interior walls of the filter funnel with sterile diluent and also vacuum filter. Remove the funnel (avoid touching the inner part of the funnel), and place the membrane filter (same side up) onto an agar plate. Filter successively the next most diluted sample volumes through the filter funnel without sterilization between different sample dilutions. Obtain a new sterile filter funnel set-up, or sterilize the filter funnel set-up by flaming with alcohol upon filtering a new sample. More guidance on membrane filtration can be found in ISO guideline 8199:2018.[33]

The steps of the membrane filter procedure are shown in the figure below:

Figure 5.5 Steps of membrane filter procedure

Membrane filter on a filter support → Water sample filtered through membrane filter (0.45μm) → Membrane filter removed and placed in plate containing the appropriate medium → Incubation for 24 hours → Typical colonies
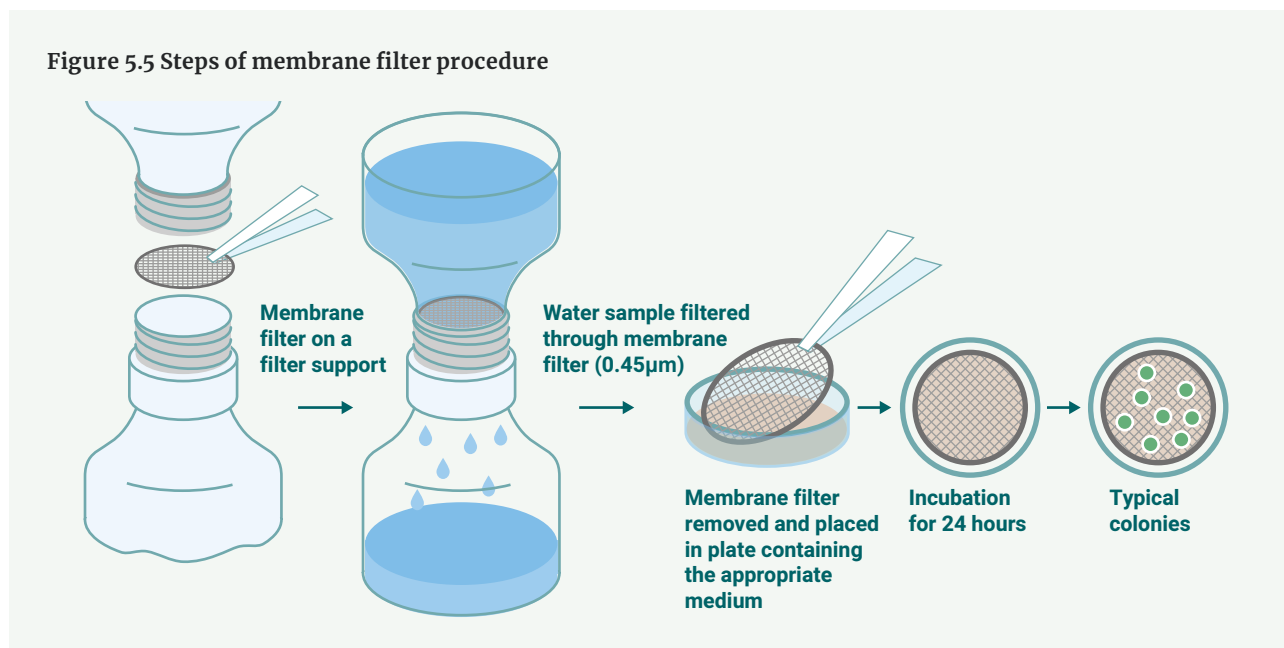
5.3.6.2 Direct one-step spread plate method

Materials

Serial dilutions, L-shaped hockey stick cell spreaders ("Drigalski spatulas"), a micropipette, sterile micropipette tips, and relevant agar media.

Procedure

Pre-warm the plates at 35°C to 55°C for 30 minutes to initially dry them. Pipette 100 mL of sample at an appropriate dilution onto the pre-warmed agar medium, and spread by using the sterile cell spreader and rotating the plate. Spread sample uniformly across the whole plate to achieve even uniform distribution of microbes. For each sample dilution, use a new sterile pipette and spreader (or alcohol flame a reusable spreader) to repeat these steps.

Plate each sample on all agar culture media at appropriate dilutions. Once complete, agar plates are inverted and incubated at 35°C to 37°C for 18-24 hours. More guidance on the spread plate technique can be found in ISO guideline 8199:2018.[33]

A diagram of the spread plate procedure is shown below as a guide:

Figure 5.6 Spread plate procedure

Surface colonies

Sample is pipetted onto surface of agar plate (0.1 ml or less) → Sample is spread evenly over surface of agar using sterile glass spreader → Incubation → Typical spread-plate results
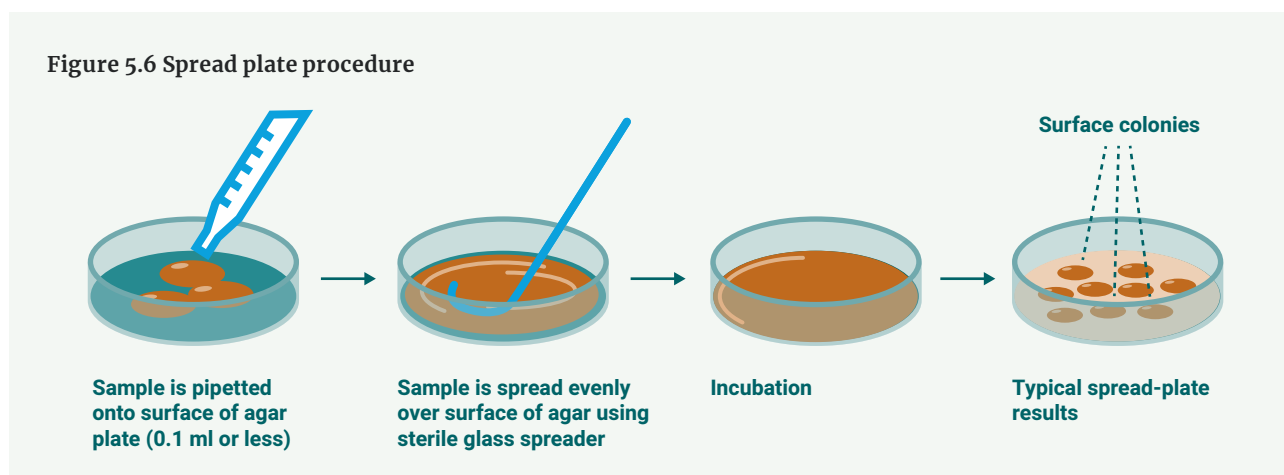
5.3.6.3 Quality control for membrane filtration and spread plating

Procedure

On each day that plating is performed, also include positive and negative controls on each medium plated. The negative control is composed of 100 µL of sterile diluent filtered or streaked onto the agar plates. The negative control should be filtered as the first sample, to ensure sterility of the complete system, and should be inoculated on TBX plates (i.e. a plate non-selective for ESBL). The positive controls include *E. coli* strains and non-*E. coli* strains that are CTX sensitive and resistant. Plate all positive and negative control strains on all media.

The quantitative positive control includes an ESBL-Ec strain from previously prepared stocks at known dilutions (concentrations) that are stored frozen with glycerol. Each day a filtration or spread plating of a sample is performed, thaw one vial of the quantitative positive control strain, and filter or spread plate on TBX agar with CTX. Record the counts of that strain, and evaluate whether the counts are within the expected mean ± 2x the standard deviation (see 5.3.6.8). Last, a range of commercial quantitative controls exist for non-resistant *E. coli,* several of which can be transported without cold chains. Countries are encouraged to also include an externally prepared quantitative control.

5.3.6.4 Enumeration (determination of bacterial concentrations, CFU/100 mL)

Procedure

After overnight incubation, observe and count all target bacterial colonies with the correct colour (blue or blue-green, Table 5.6) on the TBX agar plates with and without CTX having countable colonies. If available, use a colony counter. Mark the colonies that were counted on the outside surface of the plate with a pen. For each TBX and TBX/CTX plate, record the total number of colonies as CFUs for presumptive *E. coli* or ESBL-Ec. Store raw bacterial counts on a sheet including the sample, dilution, agar plate type, and count (see "Sample record sheet" attached for an example). Take photos of plates with countable colonies for future reference if there are questions or concerns about colony appearance and countability. Manufacturer's description of colony appearance assumes incubation at 37°C for 18-24 hours.

Table 5.6 Description of microorganism appearance

AGAR TYPE	MICROORGANISM	APPEARANCE
TBX agar with or without CTX	*E. coli*	Blue or blue-green colonies
	Non-*E. coli*	White or off-white (cream) colonies (not to be counted)

Calculate CFU concentration as CFU/100 mL from plates with counts <200 (spread plating) or <100 (membrane filtration). Per sample and medium, add up raw counts for all these countable plates, and divide this total number of colonies by the total undiluted sample volumes they represent per counted plate (including plates with zero colonies at a sample dilution that also had some plates with visible colonies at the same sample dilution). Calculate the 95% CI estimate by taking the square root of the total colony count per sample for each medium, multiplying this number by 1.96, and adding it to (for upper 95% confidence limit (CL)) and subtracting it from (for lower 95% CL) the total colony count. Then, for each number of colonies per sample on each medium, divide the total number of colonies by the total volume (in mL) of undiluted sample they represent, to calculate CFU per mL. Then, multiply these numbers by 100 to express the concentration as CFU/100 mL. Correct the raw counts by the proportion of confirmed ESBL-Ec as determined through species confirmation and AST testing. More guidance on the calculation of concentrations can be found in ISO guideline 8199:2018.[33]

Table 5.7 Example calculation

	DILUTION	EQUIVALENT VOLUME OF UNDILUTED SAMPLE	VOLUME FILTERED	PRESUMPTIVE ESBL-EC COUNTED ON PLATE	COUNTS VALID FOR ENUMERATION	TAKEN TO SPECIES AND ESBL CONFIRMATION	ESBL AND SPECIES CONFIRMED COLONIES
	10^{-3}	0.001	3 mL	0	-		
	10^{-2}	0.01	1 mL	8	8	8	6
	10^{-2}	0.01	3 mL	31	31	2	2
	10^{-1}	0.1	1 mL	110	-		
Total		3 mL* 0.01+1 mL*0.01=0.04 mL			8+31=39	10	8

Concentration = total count / total volume * ratio confirmed * 100 mL

Concentration = 39/ 0.04 mL * (8/10) * 100 mL = 78 000 CFU/100 mL = 4.9 $\log_{10}$ CFU/100 mL

95% CI = 2*sqrt (39) / 0.04 mL * (8/10) * 100 mL = 24 980 CFU/100 mL

Upper and lower confidence limit values:

Normal scale: Lower value 78 000-24 980 = 53 020, higher value 78 000+24 980 = 131 020

Log scale: Lower value $\log_{10}$ (53 020) = 4.7 $\log_{10}$ CFU/100 mL, higher value $\log_{10}$ (131 020) = 5.1 $\log_{10}$ CFU/100 mL

The resulting data can, for example, be collected per sampling location and plotted (it can be most easily visualized on a log scale as box-and-whisker plots, as shown below for a hypothetical example). (Most statistical software can generate box-and-whisker plots from a series of sample concentrations. Some free statistical software available online can produce box-and-whisker plots, such as:

- http://www.alcula.com/calculators/statistics/box-plot/

- https://www.meta-chart.com/box-and-whisker#/data; and

- https://www.easycalculation.com/statistics/box-plot-grapher.php.

On these plots, the line through the box is the median concentration, the upper and lower edges (tops and bottoms) of the box are the 75th percentile and 25th percentile values, and the whiskers span all of the sample values recorded (in some statistical software programs, they represent the upper and lower 95% CLs of the sample values)).

Figure 5.7 Box–and–whisker plots of sample concentrations
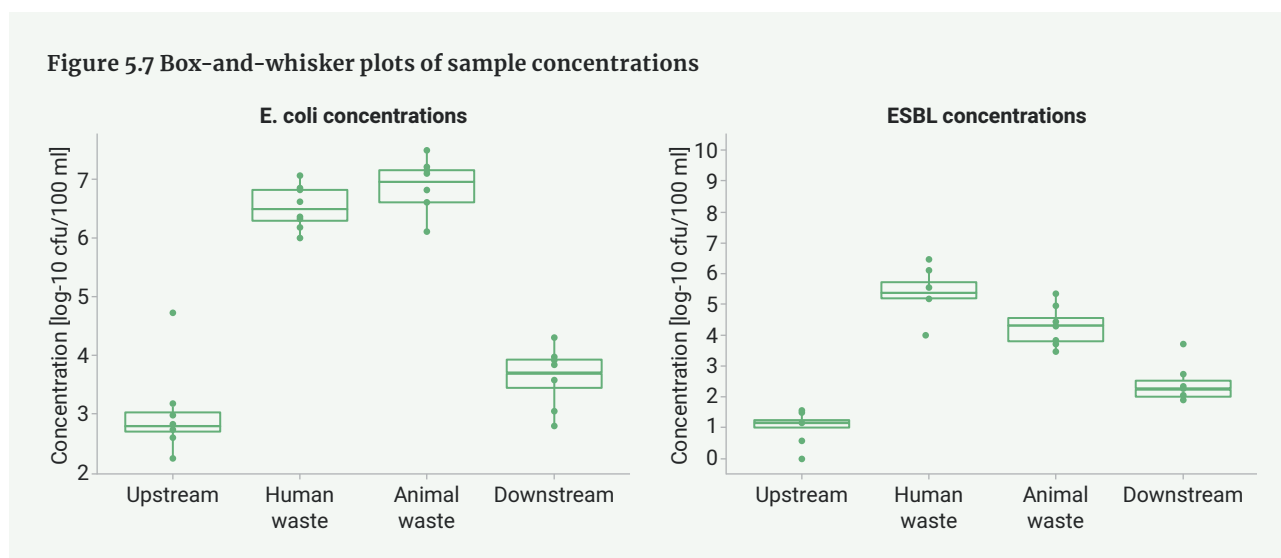
5.3.6.5 Calculations for proportions of presumptive ESBL-Ec

Procedure

For each type of sample, calculate proportions of total presumptive *E. coli* on each medium that are presumptive ESBL-Ec. To make these calculations, divide the presumptive ESBL-positive bacterial concentration for each sample (from TBX plates with CTX) by the total *E. coli* concentrations (from TBX plates) for each sample. Do not use concentrations in log units, but in 'normal' (arithmetic) units to determine the proportion. This result is the decimal fraction of ESBL bacteria among all *E. coli* bacteria. To express this as a percentage, multiply the number by 100.

Example for calculation:

ESBL concentration: 78 000 CFU/100 mL (4.9 log CFU/100 mL)

E. coli concentration: 830 000 CFU / 100 mL (5.9 log CFU/100 mL)

ESBL percentage: 78 000 / 830 000 * 100 = 9.4%

5.3.6.6 Isolation of colonies, species and ESBL confirmation, and storage

Procedure

For confirmation of ESBL-Ec, select five isolated colonies from the TBX plates with CTX from each sample for re-streaking on TBX/CTX (or, if there are fewer than five total colonies, select all colonies). Repeat re-streaking initially on TBX agar, and then from this re-streaked TBX onto blood agar (or an alternative agar medium such as tryptic soy agar (TSA) or LB agar, if blood agar is not available) to achieve colony purity. Proceed to species confirmation and ESBL confirmation according to the relevant annexes, and to colony storage (this primarily involves a re-streak on a non-selective medium, such as blood agar or TSA) (Annexes 5-7).

5.3.6.7 Suggested volumes for initial testing of expected concentrations at sampling sites

As stated above, initial tests should be performed on each sample location, to establish suitable dilutions of samples to be used during the rest of the sampling campaign.

Depending on the sample nature, the table below contains suggested volumes for this initial testing. For the actual investigations, this table should be adjusted by choosing four different volumes of water that are closest to the concentrations found in the pre-testing, and likely to generate plates with 10-100 colonies. The choice of dilutions should further be monitored throughout the project. If changes in the measured concentrations are observed during the measurement campaign (e.g. changes associated with a change from dry to wet season), and a sample site does not generate plates with 10-100 colonies for the dilutions used, the range of dilutions should be adjusted in the next round of sampling. For example, if dilutions -2, -3, -4 and -5 had been used previously, but there are >100 colonies in all plates, including in the -5 dilutions, then dilutions -4, -5, -6, and -7 should be tested in the next sampling round. The range of dilutions tested does not have to be constant during the whole measurement campaign. At any date, though, it is important to include three to four different volumes or dilutions in each analysis, such that at least one plate with 10-100 colonies is achieved, and the concentrations can be quantified, even if the sample shows differences from the previous measurements.

Table 5.8 Membrane filtration – samples, sample volumes, and sample dilutions for initial testing of expected *E. coli* and ESBL–Ec concentrations at different sampling sites

MEMBRANE FILTRATION METHOD	ESBL-Ec				*E. coli*			
Sewage collection site or WWTP influent								
Decimal dilution	0	-1	-2	-3	-1	-2	-3	-4
Volume [mL] for filtration	1*	1	1	1	1	1	1	1
Slaughterhouse/wet market waste								
Decimal dilution	0	0	-1	-2	-2	-3	-4	-5
Volume [mL] for filtration	1*	1	1	1	1	1	1	1
River upstream								
Decimal dilution	0	0	0	0	0	0	0	-1
Volume [mL] for filtration	100	100	10	1*	100	10	1	1
River downstream								
Decimal dilution	0	0	0	-1	0	0	-1	-2
Volume [mL] for filtration	100	10	1*	1	10	1	1	1

*Remember, when filtration volumes are small (<10 ml), first add 10-20 mL of sterile diluent to the filter. Funnel and filter before adding the sample.

Table 5.9 Spread plating – samples, sample volumes, and sample dilutions for initial testing of expected *E. coli* and ESBL–Ec concentrations at different sampling sites

SPREAD PLATE METHOD	ESBL-Ec				*E. coli*			
Sewage collection site or WWTP influent								
Decimal dilution	0	-1	-2	-3	-1	-2	-3	-4
Volume [mL] for filtration	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Slaughterhouse/wet market waste								
Decimal dilution	0	-1	-2	-3	-1	-2	-3	-4
Volume [mL] for filtration	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
River upstream								
Decimal dilution	0	0	0	0	0	0	0	-1
Volume [mL] for filtration	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
River downstream								
Decimal dilution	0	0	0	-1	0	0	-1	-2
Volume [mL] for filtration	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

5.3.6.8 Preparation of a quantitative positive control

Purpose

In addition to the positive control strains, a quantitative positive control is used in order to evaluate the quantitative aspect of the protocol. For this purpose, aliquots of an ESBL strain are prepared and frozen in glycerol as detailed below. Each time a sample is handled, one aliquot of the frozen quantitative control is thawed and filtered, and the results are compared with previous results.

Materials

- Glycerol
- ESBL-producing control strain (on agar plate or in glycerol stock)
- TSA plates or other non-selective plate (e.g. LB agar plate)
- TBX plates with CTX (4 mg/L)
- 9 mL sterile buffered peptone water or other growth medium (LB)
- 9 mL sterile diluent (phosphate buffered saline)
- Vortex mixer
- Pipettes for 1 mL
- Sterile loops
- Sterile sample tubes for colony storage (glass or plastic)

Preparations

(i) Dilute 40 mL glycerol with 60 mL deionized water, and autoclave.

Day 1

(ii) Inoculate one loop of the ESBL-producing control strain on a TSA plate by streak plating. Incubate at 37°C overnight.

Day 2

(iii) At the end of day 2, pick an isolated colony of the ESBL-producing control strain grown on the TSA plate, and inoculate 9 mL (or a volume between 2 mL and 50 mL) of growth medium. Inoculate at 37°C overnight.

Day 3

(iv) After one night of growth at 37°C, there are approximately 1×10^9 CFU per mL in the broth, and the broth should appear visibly turbid.

(v) Prepare 10-fold serial dilutions: Take 1 mL of the overnight culture into 9 mL diluent and vortex (this is dilution 10^{-1}). Repeat 4x (yielding dilutions 10^{-2}, 10^{-3}, 10^{-4}).

(vi) Add 0.3 mL of dilution 10^{-4} to 29.7 mL sterile glycerol, and mix very well (this is a further dilution of 100x and thus a 10^{-6} dilution).

(vii) Add 0.1 mL of dilution 10^{-6} that was prepared with glycerol to 0.9 mL sterile glycerol and mix very well (this is a 10^{-7} dilution).

(viii) Spread 100 µL of the 10^{-6} dilution on a TBX plate with CTX. Repeat two times (total three plates).

(ix) Spread 100 µL of the 10^{-7} dilution on a TBX plate with CTX. Repeat two times (total three plates).

(x) Inoculate the TBX/CTX plates overnight at 37°C.

(xi) Keep the 50 mL of the two dilutions 10^{-6} and 10^{-7} in the fridge. Also keep the 10^{-4} serial dilution in the fridge.

Day 4

(xii) Count the colonies on the TBX/CTX plates.

(xiii) Prepare a further dilution of the dilution 10-6, such that 100 μL contain between 100 and 120 colonies, as shown in the next three examples.

(xiv) For example, if the 10-6 dilution contained between 100 and 120 colonies, use this dilution directly in the next step.

(xv) If the 10-6 dilution contained more than 120 colonies/100 μL, look at the counts from the 10-7 dilution. If the 10-7 dilution contained 10-100 colonies, dilute the 10-6 dilution with sterile 40% glycerol to reach 100 colonies per 100 μL. For example, if the 10-7 dilution contained 80 colonies, dilute the 10-6 dilution 8x (5 mL 10-6 dilution + 35 mL sterile 40% glycerol) to achieve about 100 colonies/100 μL.

(xvi) If the 10-6 dilution contained between 10 and 100 colonies, use the 10-4 dilution to prepare an appropriate dilution containing 80-100 colonies/μL by diluting with sterile 40% glycerol. For example, if the 10-6 dilution contained 50 colonies, dilute the 10-4 dilution 50x (1 mL 10-4 dilution + 49 mL sterile 40% glycerol).

(xvii) For the dilution that contains 80-100 colonies per 100 μL, prepare portions of 500 μL (>20 portions) in cryogenic tubes for colony storage. Freeze at -80°C. (If only a -20°C freezer or a blood bank freezer is available, the counts of the positive control might decline over time more quickly than at -80°C. You might have to repeat the preparation of positive controls more frequently if counts decline to numbers <10).

Next week

Day 1

(xviii) Select four random tubes and thaw.

(xix) With the first random tube, perform membrane filtration (or spread plating) according to the standard protocol on TBX plates (by using 100 μL from the random tube), and on TBX/CTX plates (each using 100 μL from the random tube), resulting in one inoculated TBX plate and one TBX/CTX plate. Repeat for the other three random tubes. In total, for all four random tubes, this gives four TBX plates, and four TBX/CTX plates. Incubate according to standard protocol.

Day 2

(xx) Count colonies per plate. Determine mean and standard deviation of the four counts per plate as previously described.

Each day of performing a membrane filtration / spread plating of a sample

(xxi) Thaw one random tube.

(xxii) Perform membrane filtration or spread plating for 100 μL of the thawed tube on TBX and TBX/CTX.

Next day

(xxiii) Determine the counts of the quantitative positive control. Determine whether the counts lie within the range of the mean ± 2x standard deviation. If the counts are larger than mean + 2x standard deviation or smaller than mean – 2x standard deviation, but within mean ± 3x standard deviations, investigate whether there was a reason for the deviating counts (i.e. control tubes stored for a long time, and counts started to decline in earlier measurements). If counts are not within the range of mean ± 3x standard deviations, reject the measurement of all samples run that day.

5.3.7 Satellite projects: Analysis of drinking water

Countries with an interest in drinking water analysis as a satellite project are encouraged to align sampling with their institutions responsible for drinking water quality.[29] Drinking water analysis can be performed at the point of use (i.e. households), or at the point of production (drinking water production sites, such as water treatment plants), depending on the interests of the country.

For drinking water analysis, the same protocol as described here can be applied. However, in this case, the sample volume to be used in membrane filtration for ESBL-Ec should be increased to 500 mL.

Sample record sheet

Sampling

- Date:
- Time:
- Name of sampling person:
- Contact details (address of institute):
- City:
- Sample nature:

	Tick if yes
Human wastewater: Community sewage or WWTP	☐
Animal wastewater: Wet market waste or slaughterhouses	☐
River upstream city	☐
River downstream city	☐
Other (please describe)	

- Sample type:

	Tick if yes
Human wastewater: Influent of wastewater treatment plant	☐
Human wastewater: Open sewer line, drain, or channel	☐
Human wastewater: Can the sample also contain storm water?	☐
Animal wastewater: Wet market wastewater	☐
Animal wastewater: Slaughterhouse wastewater	☐
River	☐
Other (please describe)	

- GPS coordinates:[xv]

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xv Such as available from Google maps by right-clicking on a location, in latitude longitude format (e.g. 52.118096, 5.188670 for Bilthoven). If no GPS data available, provide other location data, such as street address.

- Sample flow:

	Tick if yes
5 – rapid flow	☐
4 – moderate flow	☐
3 – slow flow	☐
2 – very slow flow	☐
1 – stagnant	☐
Other (please describe)	

- Sample colour:

	Tick if yes
Blackish	☐
Brownish	☐
Greenish or purple	☐
Close to transparent	☐
Other (please describe)	

- Air temperature at the time of sampling:
- Temperature of the water sample at the time of sampling:
- pH of sample at time of sampling:
- Season:

	Tick if yes
Spring	☐
Summer	☐
Autumn	☐
Winter	☐
Wet	☐
Dry	☐

- Storage temperature of sample during transport / storage: _____°C
- Photo:

Sample handling sheet

- Date of sample handling:
- Start time of sample handling:
- Duration of sample transport time:
- Duration of sample storage time in the laboratory:

Quality assurance: Media and reagents

Media and reagents	Date of preparation / lot number	Exp. Date
Diluent: (specify which one is used)		
0.45 µm filters:		
Control ESBL:		
Control *E. coli:*		
Agar 1: TBX		
Agar 2: TBX with CTX 4 µg/mL		
Blood agar, TSA, or LB agar		
Glycerol for bacteria frozen storage		
CTX (specify source, form (powder or liquid concentrate) and storage condition)		

- Preparation of serial dilutions:
- Volume of diluent:
- Volume of sample:
- Range of dilutions prepared:

Isolation – ESBL producing *E. coli*

- Agar medium: TBX with CTX 4 µg/mL
- Date filtration / spread plating
- Date counting / re-streaking

Volumes

	Dilution	Volume filtered (or spread)	Presumptive colonies counted (estimated count)	Presumptive colonies re-streaked
1				
2				
3				
4				
Total				

Positive / negative controls

	Concentration	Volume filtered (or spread)	Colonies counted
1 Blank			
2 Quantitative control			

- Incubation temperature:
- Start incubation:
- Start reading plates:

Enumeration – *E. coli*

- Agar medium: TBX
- Date filtration/ spread plating:
- Date counting / re-streaking:

Volumes

	Dilution	Volume filtered (or spread)	Presumptive colonies counted (estimated count)
1			
2			
3			
4			
Total			

Positive / negative controls

	Concentration	Volume filtered (or spread)	Colonies counted
1 Blank			
2 Quantitative control			

- Incubation temperature:
- Start incubation:
- Start reading plates:

Confirmation and storage

Date of sampling	Agar: TBX with CTX	Colony code	Storage details (location)	Date of species confirmation	Result of species confirmation: *E. coli* y/n	Date of ESBL phenotype confirmation	Result of species confirmation: ESBL y/n

- Species confirmation method:

- Phenotype confirmation method:

Calculation of concentrations of *E. coli* and ESBL-Ec

Sample	Target (ESBL-Ec or *E. coli)*	Agar medium (specify TBX or TBX + CTX)	Countable colonies (counts from plates with <100 colonies for membrane filtration or <200 colonies for spread plating)	Z_{tot}=Sum countable colonies	Volumes used (volumes from plates with <100 colonies for membrane filtration or <200 colonies for spread plating) [mL]	V_{tot}= Sum all volumes (as undiluted sample volumes for all dilutions used)	a=n colonies picked for confirmation	b=n colonies confirmed	Concentration [CFU/ reporting volume = Z_{tot}/ V_{tot} * $V_{reporting}$ * b/a]

Sample	Target (ESBL-Ec or *E. coli)*	Agar medium	Concentration [CFU/ reporting volume]	Concentration [log CFU/ reporting volume]

Calculation of concentrations:

$$C = Z / V_{tot} * V_{rep}$$

C = concentration

Z = total count of countable colonies (CFU on plates with <100/<200 colonies)

V_{tot}= total undiluted volume plated on the plates used for the total count of countable colonies

V_{rep}= reporting volume (100 mL)

a = number of colonies taken to species / ESBL confirmation

b = number of colonies confirmed as *E. coli* / ESBL

Calculation of % ESBL-Ec:

% ESBL-Ec of total *E. coli* = Concentration ESBL-Ec/Concentration *E. coli)*100

Calculation of % ESBL over *E. coli*

Sample	Concentration *E. coli*	Concentration ESBL-Ec	%: ESBL-Ec / *E. coli*

5.4 Annex 4. ESBL confirmatory test

5.4.1 Phenotypic confirmation methods

Phenotypic confirmation of ESBL on the *E. coli* isolates follow the CLSI[34] and EUCAST[35] standards. For a complete explanation of the principles and methods, it is highly recommended to review both CLSI and EUCAST documents.

For the uses of this protocol, this Annex is referring the phenotypic methods that can be affordable for most low-resource settings. Those methods are: (i) CDT included in both CLSI and EUCAST standards; and (ii) DDST, included in the EUCAST standard only.

The principle of the methods is based in the capacity of clavulanic acid to inhibit ESBL, and the synergy that is produced in combination with CTX and the combination with ceftazidime. It is very important to highlight that for both methods, CDT and DDST, testing should include both third-generation cephalosporins, CTX (or ceftriaxone), and ceftazidime.

Combination disk diffusion test (CDT)

This method uses the cephalosporin discs, CTX and ceftazidime alone, and combination discs CTX/clavulanic acid and ceftazidime/clavulanic acid. Per isolate, four discs (the two cephalosporins and the two combination discs) are used in a Mueller Hinton plate with a 0.5 MacFarland solution of the isolate to be tested. The incubation time is 16-18 hours.

The interpretation of the results is based in the zone size of each cephalosporin alone, compared with the discs containing the combination of the cephalosporin and clavulanic acid. If the zone diameter of the disc with the combination for any or both cephalosporins is higher or equal to 5 mm (≥5 mm), the result is interpreted as positive for ESBL (see Figure 5.8).

Figure 5.8 Combination disk diffusion test
CTX=cefotaxime CTX/CA= cefotaxime/clavulanic acid

CAZ=ceftazidime CAZ/CA= ceftazidime/clavulanic acid
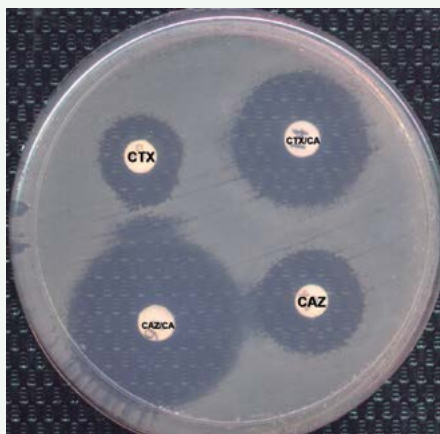
CTX=18mm
CTX/CA=28mm
28-18=10=ESBL + AADF

CAZ=22mm
CTX/CA=37mm
37-22=15=ESBL + AADF

Double disk synergy test (DDST)

This method uses the cephalosporin discs and clavulanic disc alone. The principle is applying the cephalosporin discs next to the clavulanic disc with a distance of 20mm centre to centre. The interaction between each cephalosporin and the clavulanic acid will produce a synergy effect if ESBL is produced by the isolate. If no synergy effect is observed, the zone diameters are not affected. Synergy effect is produced because clavulanic acid inhibits the ESBL, and the third-generation cephalosporin can kill the bacteria (see Figure 5.9).

Figure 5.9 Double disk synergy test
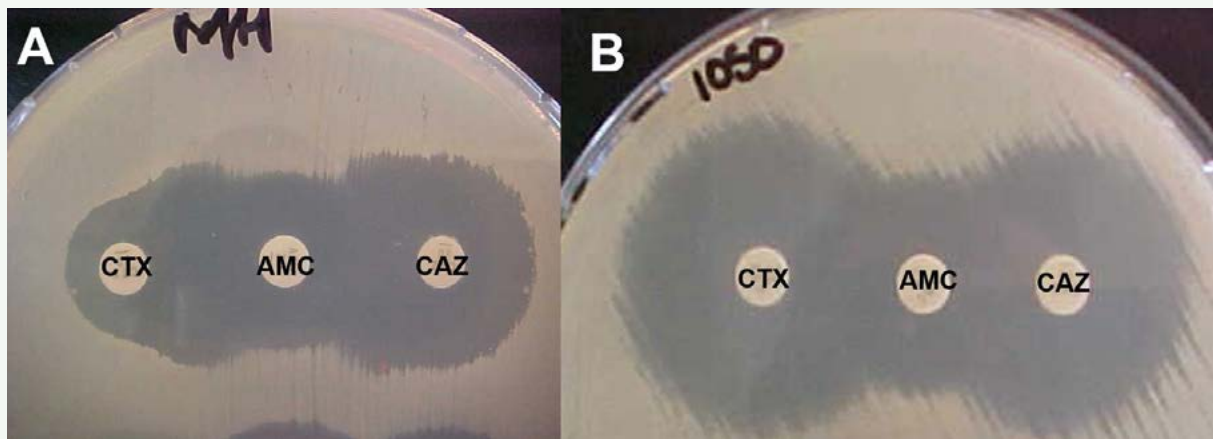
CTX=cefotaxime, AMC=amoxicillin/clavulanic acid, CAZ=ceftazidime.

A) CTX and CAZ are showing synergy with AMC. This synergy effect shows that AMC is inhibiting the ESBL and the two cephalosporins show more activity increasing the inhibition zone.

B) A synergy effect between the cephalosporins, CTX and CAZ is not observed, zone diameters are not affected, indicating no production of ESBL.

NOTE: For a complete explanation of the methods and principles, it is highly recommended to review both standards, CLSI and EUCAST, for the detection and confirmation of ESBL.

Other methods

In laboratories with more existing capacity, and where automated systems for identification and AST are being used, the ESBL confirmation is included in the panels for Enterobacterales. For laboratories using automated systems, broth microdilution method, or gradient test, the result is based on minimum inhibitory concentration (MIC). The parameters to be used in the confirmation of these methods are the same as CDT, and the interpretation is based on the MIC ratio between the third-generation cephalosporin and the same third-generation cephalosporin combined with clavulanic acid. An MIC ratio ≥ 8 using the methods indicated above is interpreted as ESBL-positive.

5.4.2 Quality control

Table 5.10 Quality control

REFERENCE STRAIN	RESULT
Klebsiella pneumoniae ATCC 700603	Positive control
Escherichia coli ATCC 25922	Negative control

5.5 Annex 5. Reference material for quality control of the microbiological methodologies

Countries should include a set of reference strains for quality control of the media, using MacConkey, Mueller Hinton, and TBX, and for the biochemical tests to identify and confirm *E. coli.* For the methodologies of this protocol, reference strains have been selected to use for quality control (Table 5.11).

Table 5.11 Reference strains for quality control

MICROORGANISM	REFERENCE NUMBER	DESCRIPTION
Escherichia coli	ATCC 25922	Negative control: Non-ESBL-producing *E. coli*
Klebsiella pneumoniae	ATCC 700603	Positive control: Non-*E. coli* ESBL-producer
Klebsiella pneumoniae	ATCC BAA 1706	Negative control: Non-*E. coli* non ESBL-producer
NCSU *E. coli*	*E. coli* NC11	Positive control: *E. coli* ESBL-producer

5.6 Annex 6. Biochemical identification of ESBL–Ec isolates

After isolation of presumptive *E. coli* on MacConkey or TBX agar, the isolates need to be confirmed as *E. coli.* Different methodologies exist for identification of *E. coli,* and all have advantages and disadvantages in regard to specificity, costs, robustness, and/or level of equipment needed. However, by combining methods, relatively economical approaches exist, depending on the equipment and other resources available in the laboratory performing the verification. The different *E. coli* identification tests presented in this document are what have been found to be cost-effective, robust, and practical, and will work also in laboratories that might have limited access to more advanced equipment, such as commercial strips with biochemical tests, automated systems, matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectrometry or DNA sequencing.

Besides having an appropriate verification method, it is important to train the staff in recognizing typical *E. coli* on MacConkey or TBX plates, both in regard to appearance, morphology, and its characteristic smell. The staff should also be aware of contaminants, as such an occurrence interferes with further testing.

Presumptive *E. coli* isolated on MacConkey (and other selective media such as TBX) are all lactose fermenters. Non-lactose fermenting E. coli at 37°C will not be tested by this method. Among the presumptive *E. coli* isolated on the MacConkey agar from blood culture bottles, rectal swabs, faeces, caecal samples, and environmental samples of wastewater and water, most isolates will be *E. coli.* However, a small fraction may be false positives. Therefore, it is necessary to perform a verification of presumptive ESBL-producing *E. coli* isolates to distinguish *E. coli* from other coliform bacterial species. A biochemical test can be easily used to differentiate *E. coli* from other bacteria capable of growing on MacConkey agar or TBX. One such test is the indole test.

An indole test is used to determine the ability of an organism to split the amino acid tryptophan to form the compound indole. Tryptophan is hydrolysed by tryptophanase to produce three possible end products – one of which is indole. Indole production is detected by Kovac's or Ehrlich's reagent, which contains 4 (p)-dimethylamino benzaldehyde, which reacts with indole to produce a red- to red-violet coloured compound.

5.7 Annex 7. Media, reagents, and quality control

Each laboratory must ensure adequate control of the media and reagents it uses. Quality control includes the selection of authentic or other verified reagents, the preparation of media according to approved formulations or specific manufacturer's instructions, and the use of well-characterized reference strains to check prepared media.[35] Water used to prepare media and reagents must be high-quality distilled, deionized, or similar reagent-grade water, and must be protected from deterioration in quality during storage and use. Water for microbiological use should be at least Grade 2. The ISO standards for reagent grade water are as follows:[36]

Table 5.12 International Organization for Standardization (ISO) – ISO 3696:1987: Specifications for water for analytical laboratory use

ISO 3696:1987	GRADE 1	GRADE 2	GRADE 3
pH value (25°C)	-	-	5.0-7.0
Conductivity, min. µS/cm (25°C)	0.1	1	5
Oxidizable matter Oxygen content, max. mg/l	-	0.08	0.4
Absorbance, max. absorbance units, 254 nm and 1 cm optical path length	0.001	0.01	-
Residue post-evaporation, max. (110°C) mg/kg	-	1	2
Silica, max. mg/l	0.01	0.02	-

5.7.1 Quality control of media

A summary of considerations for quality control of media, methods, and sources of quality control strains follows.

Considerations for quality control of media

Each batch of medium prepared from individual ingredients, or each different manufacturer's lot number of dehydrated medium, should be tested for sterility, the ability to support growth of the target organism(s), and/or the ability to produce appropriate biochemical reactions, as appropriate.

1. Sterility

Incubate one tube or plate from each autoclaved or filter-sterilized batch of medium overnight at 35°C to 37°C, and examine it for bacterial contaminants (growth of bacteria).

2. Ability to support growth of the target organism(s)

Use at least one strain to test for ability of selective media to support growth of the target bacteria. Documentation should be made regarding whether this strain produces the appropriate biochemical reactions/colour on the test medium.

3. Ability to produce appropriate biochemical reactions

For selective media: Use at least one organism that will grow on the medium, and at least one organism that will not grow on the selective medium to test for the medium's ability to differentiate target organisms from competitors. If the medium is both selective and differential, it may be useful to include two organisms that will grow on the medium and produce different reactions (e.g. for MacConkey agar: a lactose-nonfermenting organism such as *Proteus mirabilis* ATCC® 12453, a lactose-fermenting organism such as *E. coli,* and *Staphylococcus aureus (S. aureus),* which should not grow).

For biochemical media: Use at least one organism that will produce a positive reaction, and at least one organism that will produce a negative reaction (e.g. for indole, an indole-positive organism such as *E. coli,* and an indole-negative organism such as *Proteus mirabilis* ATCC® 12453).

5.7.2 Media preparation

MacConkey agar and MacConkey agar supplemented with 4 µg/mL CTX[39]

(i) Weigh 25 g MacConkey agar (as per manufacturer instructions) powder, and add 500 mL distilled water.

(ii) Dissolve agar by shaking.

(iii) Sterilize the medium by autoclaving at 121˚C for 15 minutes.

(iv) Cool to 50˚C in water bath.

(v) *Add 400 µL CTX and mix well.

(vi) Stock concentration of CTX should be 5 mg/mL.

(vii) Final concentration should be 4 µg/mL (or 4 mg/L).

(viii) Pour agar into Petri plates (ca 20 mL per plate, to a uniform depth of 3–4 mm).

(ix) Leave lids ajar to dry the plates. The surface of the agar should be dry before use. No drops of water should be visible on the surface of the agar or inside the lid.

(x) Close lids and store at 4˚C for up to 2 weeks. If plates are stored for more than a few days, put them in a sealed plastic bag to prevent drying.

NOTE: *For MacConkey agar supplemented with CTX.

Cefotaxime stock solution preparation

Stock preparation: the CTX (Sigma Aldrich) powder used to prepare stock concentration of 5 mg/mL.

Steps:

(i) Weigh 0.1 g (equal to 100 mg) on wax paper or aluminium foil using proper weighing balance.

(ii) Transfer weighed powder into 50 mL conical tube and add 20 mL of molecular grade water (DNA/RNA free water). If molecular grade water is not available, then use sterilized deionized water to dilute the antibiotic.

(iii) Mix well, and make sure the antibiotic powder is dissolved completely.

(iv) This will give us stock concentration of 5 mg/mL.

Formula

Formula to calculate how much stock needs to be added for the volume of media that is prepared in the laboratory.

Volume to be added in prepared media = volume of media/dilution factor

Dilution factor = stock concentration/final concentration in the plate

Example

1. Prepare 500 mL of MacConkey agar with 4 µg/mL final concentration of CTX antibiotic.

Steps

(i) First calculate dilution factor.

Dilution factor = stock concentration/final concentration in the plate

= 5000 µg/mL (stock 5 mg/mL is equal to 5000 µg/mL)/4 µg/mL

= 1250 is our dilution factor

(ii) Once the dilution factor is calculated, calculate the amount of antibiotic stock that needs to be added to the desired amount of media to get a final concentration of 4 µg/mL.

Volume to be added in prepared media = volume of media/dilution factor

Volume to be added = 500 mL/1250

 = 0.4 mL (400 µL) of stock (5 mg/mL) CTX antibiotic needs to be added to the 500 mL MacConkey agar before pouring into the plates to get final concentration of 4 µg/mL.

Table 5.13 Selective supplements

COMPOSITION	MG/ML
CTX sodium salt stock solution prepared in molecular grade (preferred) or distilled (alternative) water	5

It is important to take the activity of the drug into account to ensure that 5 mg/mL active compound is used. Aliquots of aqueous CTX stock solution (concentration 5 mg/mL) can be stored at −20°C.

Example: If the manufacturer has given an activity of 50%, 10 mg/mL should be prepared, as the active concentration will then be 5 mg/L.

Expected quality control outcomes

Figure 5.10 Expected quality control outcomes

	MacConkey Agar	MacConkey Agar with 4 µg/mL Cefotaxime
ATCC 25922 Non ESBL Strain		
ESBL + Control Strain		

5.7.3 Mueller Hinton agar (For ESBL confirmation and antimicrobial susceptibility testing)

Table 5.14 Mueller Hinton agar

COMPOSITION	G/L
Beef Extract	2.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH 7.3 ± 0.1 @ 25°C	
Reagent Water	1 liter

(i)   Dissolve Mueller Hinton (MH) agar in reagent water according to manufacturer's instructions.

(ii) Sterilize the medium by autoclaving at 121°C for 15 minutes.

(iii) Cool to 50°C, and pour into Petri plates (to a uniform depth of 3–4 mm).

(iv) Leave lids ajar for about 20 minutes so that the surface of the agar will dry.

(v)   Close lids and store at 4°C for up to one month. If plates are to be stored for more than a few days, put them in a sealed plastic bag to prevent drying.

Saline solution (needed for AST)

(i)   Weigh 8.5 g sodium chloride.

(ii) Dissolve in 1000 mL reagent water (heat if necessary).

(iii) Adjust pH to 7.0.

(iv) Autoclave at 121°C for 20 min.

(v)   Dispense the solution into tubes at 4 mL per tube.

5.7.4 Reagents

Indole Test preparation (needed for biochemical identification)

Table 5.15 Indole test preparation – tryptone broth

COMPOSITION TRYPTONE BROTH	PER LITRE
Tryptone	10 g
Sodium chloride	5 g
Distilled Water	1000 mL

(i) Dissolve ingredients in 1L of distilled water.

(ii) Sterilize the medium by autoclaving at 121°C for 15 minutes.

(iii) Cool to room temperature.

(iv) Dispense 4 mL per tube.

(v) Store the tubes in the refrigerator at 4°C to 10°C.

Table 5.16 Indole test preparation – Kovac's reagent

COMPOSITION KOVAC'S REAGENT[xvi]	
p-Dimethylaminobenzaldehyde (DMAB)	10 g
Hydrochloric Acid, 37%	50 mL
Amyl Alcohol	150 mL

(i) Dissolve DMAB in the amyl alcohol.

(ii) Slowly add the acid to the DMAB-alcohol mixture.

(iii) Filter the solution.

(iv) Store in the refrigerator at 4°C to 10°C.

A positive indole test gives a red or red-violet colour (see below).

Figure 5.11 Indole reaction

Positive Indole Reaction

Negative Indole Reaction

xvi Because it is a toxic reagent, it is highly recommended to get a commercial preparation of Kovac's reagent.

5.8 Annex 8. Isolate storage

5.8.1 Storage of bacterial isolates

It is often necessary for isolates to be examined at a time period after the analysis of the sample from which the culture was obtained. For example, it is sometimes appropriate to refer back to an isolate for epidemiological purposes (e.g. to learn if a new case/patient is infected with the same strain of a pathogen as an individual who had an earlier case of disease). Another example would be a situation where a laboratory chooses to screen a number of isolates at one time each year for additional antimicrobial agents (e.g. for beta-lactamase production). This practice would assist in the detection of emerging characteristics in known pathogens. Sometimes isolates need to be sent to reference laboratories for confirmation and/or further testing, and must be stored prior to packing and shipping. Selection of a storage method depends on the length of time the organisms are to be held, and the laboratory equipment and facilities available. Short-term storage may be accomplished with transport media, refrigeration (for days to weeks), freezing (for as long as months to years), or, in some cases (and for some pathogens) at room temperature (for days to weeks) on simple media plus mineral oil to prevent drying.

Long-term storage of *E. coli*

E. coli isolates may be stored indefinitely if they are maintained frozen at -70°C or below; these temperatures can be achieved in an "ultralow freezer" (-70°C) or a liquid nitrogen freezer (-196°C). Storage of isolates at -20°C is not recommended, because some organisms will lose viability over time at that temperature.[37]

Frozen storage

(i) Inoculate a TSA or heart infusion agar (HIA) slant (or other non-inhibitory salt-containing growth medium), and incubate at 35°C to 37°C.

(ii) Harvest cells from the slant and make a suspension in the freezing medium.

(iii) Freezing medium is prepared with tryptic soy broth (TSB) with sterile glycerol in a proportion 2:1.

(iv) Dispense the suspension into cryogenic vials (freezing vials specially designed for use at very low temperatures). Store at -20°C, or preferably at -70°C.

(v) Caution: Glass ampoules should never be used for freezing because they can explode upon removal from the freezer.

Glycerol solution preparation

Prepare a solution of 30% glycerol (v/v) by mixing 30 mL of glycerol with 70 L of water. Transfer the solution to a screw-cap glass bottle, and sterilize by autoclaving at 121°C for 15 minutes. Loosen the cap during autoclaving.

(i) Aliquot 500 µL of sterile 30% glycerol into sterile 2 mL microfuge tubes.

(ii) Add 500 µL of bacterial culture to the tube, and mix with the glycerol using a vortex mixer.

(iii) Label the tube with the organism name, strain, date, etc.

(iv) Place the tube in the freezer, and record its location. Store at -20°C, or preferably at -80°C.

Recovery of isolates from long-term storage

To recover an isolate from frozen storage, remove the frozen cultures from the freezer, and place them quickly on dry ice or into an alcohol and dry-ice bath, if possible. Transfer to a laboratory safety cabinet or a clean area if a cabinet is not available. Using a sterile loop, scrape the top-most portion of the culture, and transfer to a growth medium, being careful not to contaminate the top or inside of the vial. Re-close the vial before the contents completely thaw, and return the vial to the freezer, with careful technique. Transfers can be successfully made from the same vial several times. Incubate 18–24 hours at 35°C to 37°C. Perform at least one sub-culture before using the isolate to inoculate a test.

To recover lyophilized specimens of *E. coli* or other bacteria, rehydrate the cells by adding sterile water to the container in which they are present using aseptic technique. Then, inoculate a tube of non-selective broth (e.g. TSB or heart infusion broth), and incubate the suspension overnight. Sub-culture the broth to a non-selective growth medium (e.g. TSA or HIA), and incubate 18–24 hours at 35° C to 37°C.

5.8.2 Isolate shipping guide

For additional characterization, such as genomic sequencing, isolates should be prepared following the below instructions, as per WHO procedures:[38]

Prior to shipping, it is important to examine each isolate to ensure purity and detect potential contamination issues. If no typical growth is observed, the sample is presumed to be negative and can be discarded. However, it is imperative to complete the log sheet, as this documentation is as important for negative as for positive samples. If typical growth is observed, then swab the growth or use a sterile inoculating loop to transfer the growth into TSB with 15% glycerol mixture, and freeze at -80°C. Repeat procedure for each isolate. Ship all isolates on dry ice to the centre where the molecular characterization will be done. Laboratories should keep duplicates of strains within their culture collections for later use, or in the case that shipped isolate integrity is lost.

5.9 Annex 9. Whole genome sequencing: Data to be included to perform sequencing analysis of ESBL–Ec isolates from countries

Mandatory information prior to initiation									
Optional information									
Example information									
1	Optional	Mandatory	Mandatory	Optional	Mandatory	Mandatory	Mandatory	Mandatory	Optional
	Kingdom	Genus	Species (serovar for *Salmonella)*	Subspecific genetic lineage (e.g. MLST, serovar, biotype)	Original strain name (number) Banned characters	Strain supplier	Date of isolation	Country	Pathotype
	Bacteria	*Salmonella*	*Choleraesuis*	*ST13*	*9924286_1*	*Zoonose lab*	*e.g. 05-Oct-1990 or Oct-1990 or 1990*	*Denmark*	*VTEC*

2	Optional	Mandatory	Optional	Optional	Mandatory	Mandatory	Mandatory	Mandatory
	Type of Pathogen	Host	Host disease	Other isolate information	Availability of data	ENA/NCBI date (No release, type: None)	Uploaded to SRA	Gram differential
	Environmental food or other pathogen	*Human*	*Blood infection*		*Restricted*	*e.g. 05-Oct-1990 or Oct-1990 or 1990*	*Yes*	*Gram negative*

References

1. Guerra R. Tripartite Plus: Recent update and Memorandum of Understanding on Antimicrobial Resistance. 2018. https://www.who.int/antimicrobial-resistance/interagency-coordination-group/Tripartite_Plus_update_info_session_011018.pdf?ua=1.

2. Integrated surveillance of antimicrobial resistance in foodborne bacteria: application of a one health approach: World Health Organization, 2017.

3. Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2020: World Health Organization, 2020.

4. European Antimicrobial Resistance Surveillance Network (EARS-Net). https://www.ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/ears-net.

5. Woerther P-L, Burdet C, Chachaty E, Andremont A. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. *Clinical microbiology reviews* 2013; **26**(4): 744-58.

6. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017: European Food Safety Authority; European Centre for Disease Prevention and Control, 2019.

7. Mughini-Gras L, Dorado-García A, van Duijkeren E, et al. Attributable sources of community-acquired carriage of Escherichia coli containing β-lactam antibiotic resistance genes: a population-based modelling study. *The Lancet Planetary Health* 2019; **3**(8): e357-e69.

8. Høg BB, Bager F, Korsgaard HB, et al. DANMAP 2017-Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. 2018.

9. Schechner V, Temkin E, Harbarth S, Carmeli Y, Schwaber MJ. Epidemiological interpretation of studies examining the effect of antibiotic usage on resistance. *Clinical Microbiology Reviews* 2013; **26**(2): 289-307.

10. Temkin E, Fallach N, Almagor J, et al. Estimating the number of infections caused by antibiotic-resistant Escherichia coli and Klebsiella pneumoniae in 2014: a modelling study. *The Lancet Global Health* 2018; **6**(9): e969-e79.

11. Pitout JD, Nordmann P, Poirel L. Carbapenemase-producing Klebsiella pneumoniae, a key pathogen set for global nosocomial dominance. *Antimicrobial Agents and Chemotherapy* 2015; **59**(10): 5873-84.

12. Xu L, Sun X, Ma X. Systematic review and meta-analysis of mortality of patients infected with carbapenem-resistant Klebsiella pneumoniae. *Annals of Clinical Microbiology and Antimicrobials 2017; **16**(1): 18*.

13. Malchione MD, Torres LM, Hartley DM, Koch M, Goodman J. Carbapenem and colistin resistance in Enterobacteriaceae in Southeast Asia: review and mapping of emerging and overlapping challenges. *International Journal of Antimicrobial Agents* 2019.

14. Kirn T, Weinstein M. Update on blood cultures: how to obtain, process, report, and interpret. *Clinical Microbiology and Infection* 2013; **19**(6): 513-20.

15. Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2017−2018: World Health Organization, 2019.

16. Data from the ECDC Surveillance Atlas – Antimicrobial resistance. https://www.ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc.

17. Reddy EA, Shaw AV, Crump JA. Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. *The Lancet infectious diseases* 2010; **10**(6): 417-32.

18. WHO guidelines on drawing blood: best practices in phlebotomy: World Health Organization, 2010.

19. Aerts M, Battisti A, Hendriksen R, et al. Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food: European Food Safety Authority, 2019.

20. Guidelines for environmental surveillance of poliovirus circulation: Geneva: World Health Organization, 2003.

21. Engage: Protocols and Training. https://www.engage-europe.eu/resources/protocols-and-training.

22. Protocols and SOPs. https://www.compare-europe.eu/library/protocols-and-sops.

23. MARAN reports. https://www.wur.nl/en/Research-Results/Research-Institutes/Bioveterinary-Research/In-the-spotlight/Antibiotic-resistance-2/MARAN-reports.htm.

24. DANMAP. https://www.danmap.org.

25. National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Centers for Disease Control and Prevention.

26. FAO/OIE/WHO Tripartite Collaboration on AMR.
    https://www.who.int/foodsafety/areas_work/antimicrobial-resistance/tripartite/en/.

27. UN Interagency Coordination Group on Antimicrobial Resistance (IACG) 2018.
    https://www.who.int/antimicrobial-resistance/interagency-coordination-group/en/.

28. Guidance for completing the OIE template for the collection of data on antimicrobial agents intended for use in animals: World Organisation for Animal Health, 2019.

29. Drinking-water quality regulation.
    https://www.who.int/water_sanitation_health/water-quality/regulation/en/.

30. Ombelet S, Barbé B, Affolabi D, et al. Best practices of blood cultures in low-and middle-income countries. *Frontiers in medicine* 2019; **6**.

31. Hasman H, Agersø Y, Hendriksen R, Cavaco L, Guerra-Roman B. Isolation of ESBL-, AmpC-and carbapenemase-producing *E. coli* from caecal samples. Laboratory Protocol. Lyngby, Denmark: DTU, 2017.

32. 19458: 2006—Water Quality—Sampling for Microbiological Analysis. Geneva, Switzerland: International Organization for Standardization.

33. Water quality-General guidance on the enumeration of micro-organisms by culture.
    Geneva, Switzerland: International Organization for Standardization 2018.
    https://www.iso.org/standard/64151.html

34. Weinstein MP, Lewis JS, Bobenchik AM, et al. CLSI M100-ED30: 2020 Performance Standards for Antimicrobial Susceptibility Testing: CLSI, 2020.

35. Giske CG, Martinez L, Cantón R. EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. Version 2.0 *European Committee on Antimicrobial Susceptibility Testing, Växjö, Sweden* 2017.

36. Water for analytical laboratory use–Specification and test methods. Geneva, Switzerland: International Organization for Standardization; 1987.

37. Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world: Haemophilus influenzae, Neisseria meningitidis, Streptococcus pneumoniae, Neisseria gonorrhoea, Salmonella serotype Typhi, Shigella, and Vibrio cholerae: World Health Organization, 2003.

38. Laboratory Biosafety Manual: World Health Organization, 2004.

39. Megan E. Jacob, Shivaramu Keelara, Awa Aidara-Kane, Jorge R. Matheu Alvarez, Paula J. Fedorka-Cray. Optimizing a Screening Protocol for Potential Extended-Spectrum β-Lactamase Escherichia coli on MacConkey Agar for Use in a Global Surveillance Program. Journal of Clinical Microbiology Aug 2020, 58 (9) e01039-19; DOI: 10.1128/JCM.01039-19

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