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KONSERVE-AB study: Protocol for Investigating Antimicrobial Resistance in Enterobacterales Across Human, Animal, and Environmental Samples Using a One Health Approach

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We use this protocol and it's working

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Abstract

Abstract

This protocol details the methodology for a cross-sectional study investigating antimicrobial resistance (AMR) patterns in *Enterobacterales* across human, animal, and environmental samples within Kisii, Kenya, emphasizing a One Health approach. The study aims to elucidate the prevalence of AMR in gastrointestinal pathogens, specifically *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp., and *Shigella* spp., in human stool, animal feces, drinking water, and soil samples. Specimens are meticulously collected from patients with gastrointestinal symptoms at Kisii Teaching and Referral Hospital, their surrounding environment, and animal sources within households. Sample collection adheres to stringent protocols to ensure accuracy and minimize contamination. Microbiological analysis involves culturing on selective media, followed by antimicrobial susceptibility testing using the disk diffusion method, based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. Molecular characterization of extended-spectrum beta-lactamase (ESBL) and carbapenemase-producing *Enterobacterales* is conducted using PCR and electrophoresis to detect key resistance genes, including blaTEM, blaOXA-48, blaCTX-M, and blaVIM. The study's objective is to investigate the transmission pathways of resistant strains within the human-animal-environment interface and to determine the association between resistance patterns in different sample types. The findings will contribute to understanding the local dynamics of AMR, informing more effective diagnostic, treatment, and preventive strategies in Kenya. Furthermore, it aims to support the integration of AMR surveillance into a One Health framework for future interventions.



Guidelines

- **Specimen Collection:** Ensure that all samples are collected in accordance with the specified procedures to minimize contamination and ensure proper handling. Use sterile equipment and containers at all stages of sample collection and transportation.
- **Laboratory Procedures:** All microbiological analyses must be carried out following the established standard operating procedures (SOPs) for culturing, isolating, and identifying pathogens. Use only approved media and reagents.
- **Antimicrobial Susceptibility Testing:** Ensure that all antimicrobial susceptibility testing is performed according to the Clinical and Laboratory Standards Institute (CLSI) 2024 guidelines, ensuring accuracy in resistance interpretation.
- **Molecular Characterization:** All PCR and electrophoresis procedures should be done under controlled conditions, ensuring proper handling and disposal of reagents and samples to avoid contamination and ensure safety.



Materials

	Category	Materials/Reagents
	Specimen Collection	Sterile specimen containers
		Transport bags
		Transport cooler with ice packs (for maintaining 4°C)
		Sterile water collection bottles (250 mL)
		Shovel or sterile spatula (for soil collection)
	Culture and Isolation	MacConkey (MAC) Agar
		Salmonella-Shigella (SS) Agar
		Selenite F broth (for enrichment)
		Buffered peptone water (for water sample enrichment)
		Sterile saline (for homogenizing animal stool)
		Gram staining reagents
		Triple Sugar Iron (TSI) Agar
		Motility test media



		Indole, Citrate utilization, and Urease test reagents
	Antimicrobial Susceptibility Testing (AST)	Mueller-Hinton Agar
		Sterile cotton swabs
		Antibiotic disks (CLSI 2024 guidelines):
		- Amoxicillin-clavulanate (AMC, 20/10 µg)
		- Ampicillin (AMP, 10 µg)
		- Trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg)
		- Imipenem (IPM, 10 µg)
		- Cefotaxime (CTX, 30 µg)
		- Ceftazidime (CAZ, 30 µg)
		- Amikacin (AK, 30 µg)
		- Ceftriaxone (CRO, 30 µg)
		- Piperacillin-tazobactam (TZP, 100/10 µg)
		- Kanamycin (KAN, 30 µg)
		- Cefepime (FEP, 30 µg)



		- Chloramphenicol (CHL, 30 µg)
		- Ciprofloxacin (CIP, 5 µg)
		Incubator (for 35°C incubation of plates)
		Caliper (for measuring inhibition zones)
	Molecular Characterization	Invitrogen [®] PureLink [®] Genomic DNA Mini Kit (for DNA extraction)
		Proteinase K (for cell lysis)
		RNase A (for RNA removal)
		Ethanol (for DNA binding)
		PureLink [®] Genomic Elution Buffer
		Thermo Scientific [®] DreamTaq PCR Master Mix (2X)
		Forward and reverse primers for ESBL and carbapenemase genes:
		- blaTEM , blaSHV , blaCTX-M , blaVIM , blaNDM , blaKPC , blaIMP , blaOXA-48
		Nuclease-free water
		Agarose (for gel electrophoresis)



		SYBR [®] Safe DNA Gel Stain
		100–1,000 bp GeneRuler DNA Ladder (for size determination)
		Electrophoresis equipment (for DNA separation)
	Safety and PPE Supplies	Non-latex disposable gloves (for PCR and molecular procedures)
		Lab coats (to be worn at all times in the laboratory)
		Face masks (to prevent contamination)
		Safety goggles (for protection against splashes)
		Sterile instruments (for all procedures)
		Proper waste disposal containers (for contaminated gloves, specimens, and consumables)

Safety warnings

- **Contamination Risk:** Strictly adhere to sterile techniques to prevent cross-contamination between samples, especially during specimen collection, transportation, and microbial isolation.
- **Handling Hazardous Materials:** Wear appropriate personal protective equipment (PPE) at all times while handling reagents, samples, and laboratory waste. Follow all material safety data sheet (MSDS) guidelines for chemicals used.

Ethics statement

Experiments involving human, animal, water, and soil samples must be conducted in accordance with internationally accepted ethical standards. Prior approval from an Institutional Review Board (IRB) or equivalent ethics committee(s) for human subjects research, and an Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s) for animal research, must be obtained before conducting these experiments. Ethical approval must also be granted for the collection and handling of environmental samples (water and soil) to ensure compliance with local regulations and best practices. The ethics approval should explicitly cover all aspects of sample collection, handling, and analysis. If approval has been obtained, please include the name of the IRB, IACUC, or equivalent ethics committee, along with any relevant permit numbers in all published papers or conference proceedings or any forms of communication or research using this protocol. For human participants, written informed consent must be obtained prior to sample collection, with participants being fully informed of the purpose of the study and any potential risks. For animal research, the study must adhere to ethical guidelines regarding the humane treatment and use of animals, with particular attention to minimizing suffering and distress.

Before start

- **Pre-Start Information:** Ensure all necessary materials, including sterile specimen containers, transport coolers, and laboratory reagents, are readily available before initiating sample collection. All personnel involved in the study should be trained on the procedures and safety protocols.
- **Preparation of Laboratory:** Make sure that all laboratory equipment is calibrated and in proper working condition. Ensure that PCR machines, incubators, and autoclaves are fully functional before beginning molecular and microbiological testing.

A. Specimen Collection

- 1 Provide the patient with a sterile container for stool collection.
- 2 Instruct the patient to collect a fresh stool sample in the provided container. Ensure the sample is free from urine or water contamination.
- 3 Label the specimen with the study ID, collection date, and time.
- 4 Transport the stool sample to the laboratory within 1 hour of collection.
- 5 Place the sample in a transport bag to prevent contamination during transport.
- 6 Transport the sample to the laboratory immediately within 1 hour.
- 7 Collect 5g of less than 12-hour-old animal stool using sterile equipment (e.g., scoops or spatulas) from domestic animals within the patient's household.
- 8 Place the collected stool in a sterile container immediately.
- 9 Label the specimen with the study ID, collection date, and time.
- 10 Transport the sample to the laboratory at 4°C within 2 hours of collection.
- 11 Place the sample in a transport cooler with ice packs to maintain the required temperature (4°C).
- 12 Transport the sample to the laboratory within 2 hours.



- 13 Collect 250 mL of water aseptically from a water source such as pots, jerricans, or dispensers where the patient drinks water.
- 14 Label the water sample with the study ID, collection date, and time.
- 15 Transport the sample to the laboratory within 2 hours at 4°C.
- 16 Store the sample in a cooler with ice packs to maintain the temperature at 4°C during transport.
- 17 Ensure the sample is transported to the laboratory within 2 hours.
- 18 Collect 5g of soil from the upper 5 cm of the soil surface in frequently contacted areas near the patient's household.
- 19 Place the soil sample in a sterile container to avoid contamination.
- 20 Label the soil sample with the study ID, collection date, and time.
- 21 Transport the sample to the laboratory at room temperature within 2 hours.
- 22 Ensure the sample is placed in a labeled, leak-proof container.
- 23 Transport the sample to the laboratory at room temperature within 2 hours.

B. Culture and Isolation

- 24 Aliquot the stool sample into two portions:
 - Portion 1: Directly culture on MacConkey (MAC) Agar and incubate at 37°C for 18–24 hours.
 - Portion 2: Enrich in Selenite F broth at 37°C for 6–8 hours, then culture on Salmonella-Shigella (SS) Agar and incubate at 37°C for 18–24 hours.



- 25 Homogenize 1g of animal stool in sterile saline.
- 26 Culture on MAC Agar and incubate at 37°C for 18–24 hours.
- 27 Enrich the second aliquot in Selenite F broth at 37°C for 6–8 hours, then culture on SS Agar and incubate at 37°C for 18–24 hours.
- 28 Filter 100 mL of drinking water through a 0.45 µm membrane filter.
- 29 Culture the filter on MAC Agar and incubate at 37°C for 18–24 hours.
- 30 Enrich a second aliquot in buffered peptone water at 37°C for 6–8 hours, then culture on SS Agar at 37°C for 18–24 hours.
- 31 Suspend 1g of soil in sterile saline, vortex, and allow it to settle.
- 32 Plate an aliquot of the supernatant on MAC Agar and incubate at 37°C for 18–24 hours.

5. Identification of Colonies

- 33 Describe colonies based on their morphological characteristics.
- 34 Identify the colonies by Gram staining and standard biochemical tests:
 - Triple Sugar Iron (TSI)
 - Motility
 - Indole
 - Citrate utilization
 - Urease tests.
- 35 **Table 1: Biochemical Reaction Patterns.**



A	B	C	D	E	F	G	H
Test	Isolate reaction Pattern [n (%)]	<i>E. coli</i> (ATCC 25922)	<i>K. pneumoniae</i> (ATCC 700603)				
MacConkey Agar	LF	NLF	NLF	LF+ mucoid	LF	LF+ mucoid	
SS Agar	Pink	Colorless + black	Colorless	Pink	Pink	Pink	
Gram Staining	GNR	GNR	GNR	GNR	GNR	GNR	
TSI Agar	Slant	A	K	K	A	A	A
Butt	A	A	A	A	A	A	
H₂S	-	+	-	-	-	-	
Indole	+	-	-	-	+	-	
Citrate	-	+	-	+	-	+	
Urease	-	-	-	+	-	+	
Motility	+	+	-	-	+	-	
Probabl	<i>Escherichia coli</i>	<i>Sal</i>	<i>Shi</i>	<i>Klebsiel</i>	—	—	

	A	B	C	D	E	F	G	H
e Pathogen			<i>monella</i> <i>spp.</i>	<i>gella</i> <i>spp.</i>	<i>la</i> <i>spp.</i>			

LF: Lactose Fermenter; NLF: Non-Lactose Fermenter; GNR: Gram Negative Rods; A: Acidic (Yellow); K: Alkaline (Red); H₂S: Hydrogen Sulfide Production; +: Positive; -: Negative.

C. Antimicrobial Susceptibility Testing (AST)

- 36 Preparation of Bacterial Suspensions:
 - Select 3-5 isolated colonies from a fresh culture.
 - Suspend colonies in 4-5 mL of normal saline.
 - Adjust the turbidity manually to the 0.5 McFarland standard using a visual comparison.
- 37 Inoculation of Mueller-Hinton Agar:
 - Dip a sterile cotton swab into the bacterial suspension.
 - Remove excess liquid by pressing the swab against the side of the tube.
 - Spread the suspension evenly onto Mueller-Hinton Agar plates using the lawn culture technique.
- 38 Antibiotic Testing:
 - Antibiotics were selected based on CLSI 2024 guidelines (CLSI, 2024) and placed on the inoculated agar plates using forceps.
- 39 The selected antibiotics were as follows:
 - Amoxicillin-clavulanate (AMC, 20/10 µg)
 - Ampicillin (AMP, 10 µg)
 - Trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg)
 - Imipenem (IPM, 10 µg)
 - Cefotaxime (CTX, 30 µg)
 - Ceftazidime (CAZ, 30 µg)
 - Amikacin (AK, 30 µg)
 - Ceftriaxone (CRO, 30 µg)
 - Piperacillin-tazobactam (TZP, 100/10 µg)
 - Kanamycin (KAN, 30 µg)
 - Cefepime (FEP, 30 µg)
 - Chloramphenicol (CHL, 30 µg)
 - Ciprofloxacin (CIP, 5 µg)

- 40 Incubate the plates at 35°C for 16–18 hours.
- 41 Measurement of Inhibition Zones:
- After incubation, measure the diameters of inhibition zones manually.
 - Classify isolates as susceptible, intermediate, or resistant based on CLSI 2024 guidelines (42).
- 42 Detection of Extended-Spectrum Beta-Lactamases (ESBLs):
- Use the double-disk synergy method with cefotaxime (30 µg), ceftazidime (30 µg), and their combinations with clavulanate (30/10 µg) (Silago et al., 2021).
 - Positive: A ≥5 mm increase in the inhibition zone around the clavulanate combination disk compared to individual antibiotic disks indicates ESBL production.
 - Negative: No significant increase in inhibition zone around the clavulanate disk.

D. Molecular Characterization of ESBL and Carbapenemase-Producing Enterobacterales

- 43 DNA Extraction:
- Use Invitrogen™ PureLink™ Genomic DNA Mini Kit.
 - Lyse cells with Proteinase K, remove RNA with RNase A, and bind DNA with ethanol on a spin column.
 - Elute DNA with PureLink™ Genomic Elution Buffer and store at -20°C.
- 44 PCR:
- Prepare a 25 µL reaction:
 - 12.5 µL Thermo Scientific™ DreamTaq PCR Master Mix (2X)
 - 1 µL each of forward and reverse primers
 - 2 µL DNA template
 - 8.5 µL nuclease-free water
 - Amplification cycle:
 - Initial denaturation: 94°C for 5 minutes
 - 30 cycles of:
 - Denaturation: 95°C for 30 seconds
 - Annealing: temperature based on primer details (see Table 2) for 30 seconds
 - Extension: 72°C for 30 seconds
 - Final extension: 72°C for 10 minutes.
- 45 Electrophoresis:
- Separate PCR products on a 1% agarose gel with SYBR™ Safe DNA Gel Stain.
 - Use a 100–1,000 bp GeneRuler DNA Ladder to determine the product size.
 - Run gel at appropriate voltage for 30–60 minutes and visualize the bands under UV light to confirm the presence of the targeted genes.

Table 2: List of primer sequences used for screening ESBL and carbapenemase-encoding genes.

	Gene name	Primer sequence	Annealing Temp	Fragment size(bp)	Reference
	blaTEM	F: AAACGCTGGTGAA AGTA R: AGCGATCTGTCTAT	46oC	822	(Mohamed et al., 2020)
	blaSHV	F: ATGCGTTATATTCG CCTGTG R: TGCTTTGTTATTCG GGCCAA	53oC	753	
	blaCTX-M-1	F: GGT TAA AAA ATC ACT GCG TC R: TTG GTG ACG ATT TTA GCC GC	55oC	850	
	blaCTX-M-9	F: ATG GTG ACA AAG AGA GTG CA R: CCC TTC GGC GAT GAT TCT C	53oC	850	
	blaCTX-M-8	F: TCGCGTTAAGCGG ATGATGC R: AACCCACGATGTG GGTAG	57oC	666	
	blaCTX-M-15	F: GTGATACCACTTCA CCTC R: AGTAAGTGACCAGA ATCAG	53oC	255	(Khalifa et al., 2021)



	blaVIM	F: GATGGTGTTCGGTC GCATA R: CGAATGCGCAGCA CCAG	53oC	390	(Poirel et al., 2011)
	blaND M	F: GGTTTGGCGATCTG GTTTTC R: CGGAATGGCTCATC ACGATC	53oC	621	
	blaKP C	F: CGTCTAGTTCTGCT GTCTTG R: CTTGTCATCCTTGT TAGGCG	53oC	798	
	blaIMP	F: GGAATAGAGTGGCT TAAYTCTC R: GGTTTAAYAAAACA ACCACC	52oC	232	
	blaOX A-48	F: GCGTGGTTAAGGAT GAACAC R: CATCAAGTTCAACC CAACCG	53oC	438	

- **Gene Name:** Targeted gene for ESBL and carbapenemase detection.
- **Primer Sequence (Forward/Reverse):** Nucleotide sequences used in PCR for amplifying the gene.
- **Annealing Temp:** Temperature for primer binding during PCR.
- **Fragment Size (bp):** Expected size of the PCR product in base pairs.
- **Reference:** Source of the primer sequences and methodology.

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- **Preparation and procedural details** for culture, isolation, and antimicrobial susceptibility testing are derived from standard laboratory procedures and relevant scientific literature.
- **ESBL detection methodology** is acknowledged from previous studies and established methods in the field.
- **DNA extraction and PCR methods** are based on commercially available products from reputable scientific suppliers.
- **Primer sequences** used for molecular characterization are based on established references in the field of antimicrobial resistance research.