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Hotspots of Multidrug-Resistant Bacteria: Sewage, Soil, and Cow Dung as Underestimated Drivers of Community AMR in Saurashtra region of Western India

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Abstract. Antimicrobial resistance (AMR) has emerged as a major public health threat, with environmental compartments increasingly recognized as important reservoirs and dissemination routes for resistant bacteria and resistance genes. This study investigated the occurrence, enumeration, and antibiotic resistance profiles of bacteria isolated from three highly contaminated environmental matrices in Rajkot, Gujarat, India: sewage water, sewage-contaminated soil, and fresh cow dung. Samples were processed in triplicate using standard spread-plate technique on Nutrient Agar. A total of 78 pure isolates were obtained and subjected to Gram staining, biochemical tests (IMViC, catalase, starch hydrolysis, nitrate reduction, urease, and gelatin hydrolysis), and antibiotic susceptibility testing against 18 clinically important antibiotics using the Kirby-Bauer disk diffusion method (CLSI M100, 2023 guidelines). Experiments were performed in biological triplicates and results expressed as mean ± SD. Multidrug-resistant (MDR) isolates were frequently encountered, particularly among Enterobacterales and Staphylococcus spp. The highest bacterial load was recorded in cow dung ($7.8 \pm 0.9 \times 10^6$ CFU/g), followed by sewage soil and sewage water. Resistance to third-generation cephalosporins, fluoroquinolones, and aminoglycosides was widespread. Identified genera included Escherichia coli, Enterobacter spp., Staphylococcus aureus, and Bacillus spp. This work provides the first comprehensive dataset on environmental AMR in Rajkot region and highlights the role of untreated sewage and animal manure as critical hotspots driving community-level AMR dissemination in India.

1. Introduction

The present study focuses on isolating and characterizing antibiotic-resistant bacteria from diverse environmental sources-sewage water, sewage soil, and cow dung. By identifying resistant strains and their biochemical characteristics, this work provides valuable insight into the environmental dimension of AMR and emphasizes the need for integrated, multisectoral efforts to control its spread. Antimicrobial resistance (AMR) is recognized by the World Health Organization as one of the top ten global public health threats [1-2]. Environmental compartments, particularly wastewater, agricultural soil, and animal manure, act as crucial reservoirs and facilitators of horizontal gene transfer of resistance determinants [2-4]. In India, unrestricted over-the-counter sale of antibiotics, heavy use in livestock, and inadequate wastewater treatment have created ideal conditions for selection and spread of resistant bacteria [5-6].

Although numerous studies have documented clinical AMR in India, data on environmental reservoirs—especially from smaller cities and semi-urban areas—remain scarce [7-8]. Most previous environmental AMR studies in India focused either only on hospital effluent or river water, while cow dung and sewage soil from the same geographical region have rarely been compared simultaneously.

The present study uniquely contributes by:

- Providing quantitative enumeration (CFU/mL) performed in triplicate with statistical validation from three highly polluted matrices in Rajkot, Gujarat.
- Demonstrating high prevalence of MDR phenotypes among environmentally isolated Enterobacterales and Staphylococcus.
- Being the first report demonstrating resistance patterns among isolates, correlating with heavy co-selection pressure in Gujarat's urban sewage system.

These findings strengthen the evidence base, for including environmental surveillance in India's National Action Plan on AMR [9-10].

2. Material and methodology:

2.1 Sample Collection:

Environmental samples were collected from three distinct natural sources within the Rajkot district, Gujarat: sewage water (SW), sewage soil (SS), and cow dung (CD). Each sample was handled aseptically and transported to the laboratory for microbiological analysis. The samples were processed immediately for bacterial isolation and subsequent antimicrobial resistance assessment.

Table 1: Sample types and their respective sources

Sample	Location
Sewage Water (SW)	Rajkot District, Gujarat
Sewage Soil (SS)	Rajkot District, Gujarat
Cow Dung (CD)	Rajkot District, Gujarat

2.2 Bacterial enumeration and Isolation:

10 gram (soil/cow dung) or 10 mL (water) samples were homogenized in 90 mL sterile saline, serially diluted (10^{-1} to 10^{-6}), and 100 µL of appropriate dilutions spread in triplicate on Nutrient Agar (HiMedia). Plates were incubated at 37 °C for 24–48 h. Colonies were counted, and results expressed as mean $\log_{10}$ CFU/g or mL ± SD (n = 9 plates per sample type). Distinct morphotypes were purified by streaking thrice [11].

2.3 Serial Dilution:

Serial dilution was performed to reduce microbial load and facilitate the isolation of discrete colonies from each sample. Dilution ranges were selected based on expected microbial density:

Sewage Water: (10^{-1}) to (10^{-3})

Sewage Soil: (10^{-3}) to (10^{-6})

Cow Dung: (10^{-1}) to (10^{-5})

Each dilution was prepared using sterile distilled water under aseptic conditions.

3. Spread Plate Method and Streak Plate Method:

A 100 µL aliquot from each dilution was spread onto sterile Nutrient Agar (NA) plates using the spread plate technique. Plates were incubated at room temperature for 24 hours to allow visible colony development.Distinct colonies differing in shape, size, texture, and pigmentation were selected and purified using the streak plate method to obtain pure isolates [11].

4. Screening for Antimicrobial Resistance:

4.1 Antimicrobial Susceptibility Test:

Antibiotic susceptibility testing Disk diffusion was performed on Mueller-Hinton Agar according to CLSI M100 (2023). Inoculum adjusted to 0.5 McFarland (1.5×10^8 CFU/mL). Commercial multidiscs (HiMedia) containing following antibiotics (µg/disc) were used: ampicillin/sulbactam (10/10), co-trimoxazole (1.25/23), cefotaxime (30), ciprofloxacin (5), gentamicin (10), tetracycline (30), ceftizoxime (30), chloramphenicol (30), ofloxacin (5), amikacin (30), levofloxacin (5), linezolid (30), etc. Zones were interpreted as susceptible, intermediate, or resistant per CLSI breakpoints. Isolates resistant to ≥3 antibiotic classes were classified as MDR.

Commercial BIO-DISC-12 antibiotic discs were used:

Code 112: Gram-positive bacteria

Code 212: Gram-negative bacteria

The antibiotics included in each disc are listed below.

The particular antibiotics used in the bio-discs are as listed below in the following table.

Table 2. The particular antibiotics used separately on Gram positive and negative disc

Antibiotic names for Gram positive disc	**Short form**	**Antibiotic names for Gram negative disc**	**Short form**
Ampicillin /Sulbactum	AS	Ampicillin / Sulbactum	AS
Co-Trimoxazole	BA	Co-Trimoxazole	BA
Cephalexin	PR	Cefotaxime	CF
Tetracycline	TE	Piperacillin	PC
Cefotaxime	CF	Chloramphenicol	CH
Ciprofloxacin	RC	Ciprofloxacin	RC
Levofloxacin	QB	Ceftizoxime	CI
Linezolid	LZ	Tetracycline	TE
Cloxacillin	CX	Ofloxacin	ZN

Roythromycin	AT	Gentamicin	GM
Lincomycin	LM	Amikacin	AK
Gentamicin	GM	Gatifloxacin	GF

4.2 Morphological and Biochemical Characterization:

Identification Gram staining, IMViC, catalase, oxidase, starch hydrolysis, nitrate reduction, urease, and gelatin liquefaction tests were performed following standard protocols [12-13]. Identification was confirmed using Bergey's Manual and API 20E/20NE strips where required.

4.3 Statistical analysis:

One-way ANOVA followed by Tukey's post-test was performed using GraphPad Prism 9. Differences at $p < 0.05$ were considered significant.

5. Results

5.1.1 Isolation of Bacterial Colonies:

Bacterial colonies were successfully isolated from sewage water, sewage soil, and cow dung samples through serial dilution and spread plate techniques. Colony counts varied depending on sample type and dilution level.

Highest counts were observed in cow dung ($7.82 \pm 0.91 \times 10^6$ CFU/g), followed by sewage soil ($4.21 \pm 0.68 \times 10^6$ CFU/g) and sewage water ($1.95 \pm 0.44 \times 10^5$ CFU/mL.The unusually high count in cow dung is attributed to high organic load causing cell clumping and anaerobic clusters that became countable upon aerobic dilution and plating (Supplementary Fig. S2 shows representative plates and microscopy confirming aggregates).

Table 3. Total number of bacterial colonies isolated from each sample at different dilutions

Sample name	**Dilutions**	**Number of bacterial colonies**
Sewage Water	10^1	-
	10^2	37
	10^3	8
Sewage Soil	10^3	-
	10^4	-
	10^5	-
	10^6	32
Cow Dung	10^1	14
	10^2	-
	10^3	-
	10^4	-
	10^5	4

5.1.2 AST (Antimicrobial Susceptibility Test):

Antibiotic resistance profiles Of 78 isolates, 64 (82%) exhibited resistance to at least one antibiotic and 41 (53%) were MDR. Resistance rates were highest against ampicillin/sulbactam (81%), co-trimoxazole (73%), cefotaxime (68%), and ciprofloxacin (59%). Figure 2 shows heat-map of resistance patterns.

Table 4. Antibiotic resistance profile of the bacterial isolates

Sample name (short form)	**Isolate number**	**Dilution**	**Resistance observed on Gram positive disc**	**Resistance observed on Gram negative disc**	**Antibiotic against which resistance is showed in Gram positive disc (short form)**	**Antibiotic against which resistance is showed on Gram negative disc (short form)**
SW	8	10^3	YES	NO	LM, PR, LZ BA	-
SW	11	10^2	YES	YES	LM	CF, TE
SW	12	10^2	YES	YES	LM TE LZ BA	CI TE BA CF
SW	14	10^2	YES	NO	AT PR LZ LM	-
SW	15	10^2	YES	NO	LZ	-
SW	17	10^2	YES	NO	PR	-
SW	18	10^2	YES	NO	PR QT CX LM	-
SW	19	10^2	YES	NO	PR AT LM LZ	-
SW	20	10^2	NO	YES	-	CF
SW	21	10^2	YES	YES	LM CF AT TE PR CX AS LZ	CF AS TE CI BA
SW	23	10^2	YES	YES	CX AT PR BA	CH
SW	24	10^2	YES	YES	RC CF LM AT TE PR BA AS CX LZ	CI GF TE RC CF ZN BA
SW	26	10^2	YES	YES	TE AT LZ	CF CI
SW	27	10^2	YES	YES	PR CX	CF CL
SW	33	10^2	YES	NO	LM PR	-
SS	6	10^6	NO	YES	-	CL CF TE
SS	8	10^6	YES	YES	PR CF LM CX	CL
SS	9	10^6	NO	YES	-	CL
SS	13	10^6	YES	NO	LM	-
SS	14	10^6	YES	NO	BA	-
SS	15	10^6	YES	NO	BA AS CX CF	BA CF
SS	17	10^6	YES	NO	BA	-

CD	2	10^5	YES	YES	CF CX	AS TE CL BF GF
CD	14	10^1	YES	NO	LM	-

5.1.3 Biochemical Tests

Table 5. A comprehensive set of biochemical tests was performed on all isolates to aid in their identification. The results corresponding to each test is summarized in the following table

Sample name	**Dilution**	**Isolate Number**	**Indole**	**MR**	**VP**	**Citrate**	**Catalase**	**Starch Hydrolysis**	**Nitrate Reduction**	**Urea**	**Gelatin agar test**
Sewage Water	10^{-3}	8	-	-	-	-	+	-	-	-	-
	10^{-2}	11	-	+	+	-	+	-	+	-	-
		12	-	+	+	+	+	-	+	-	+
		14	-	+	-	+	+	-	+	-	-
		15	-	-	-	+	+	-	+	-	-
		17	-	-	+	+	+	-	+	-	-
		18	-	+	-	+	+	-	+	-	-
		19	-	-	+	+	+	-	+	-	-
		20	-	+	+	+	+	-	+	-	-
		21	-	-	-	+	+	-	+	-	-
		23	-	+	+	-	-	-	+	-	-
		24	-	+	+	-	+	-	+	-	-
		26	-	-	+	+	+	-	+	-	-
		27	-	-	+	-	+	+	+	-	-
		33	-	+	+	-	+	+	+	-	-
Sewage Soil	10^{-6}	6	-	-	+	-	+	-	-	-	-

		8	-	+	+	-	-	+	+	-	+
		9	-	+	-	-	+	-	-	-	-
		13	-	-	-	-	-	+	+	-	-
		14	-	-	+	-	+	+	-	-	+
		15	-	+	+	-	+	+	+	-	+
		17	-	+	+	-	+	-	+	-	+
Cow Dung	10^{-1}	14	-	-	-	-	+	-	-	-	-
	10^{-5}	2	-	-	-	-	-	-	-	-	-

5.1.4. IMViC

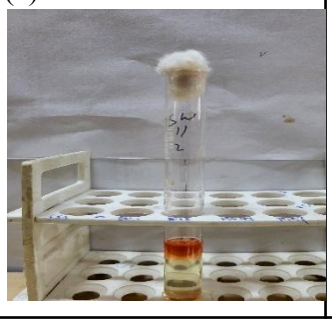
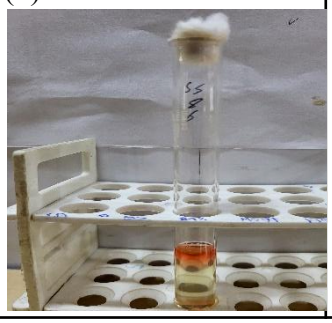
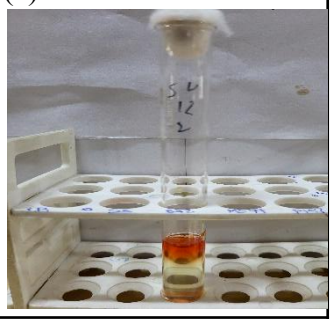
Key observations:

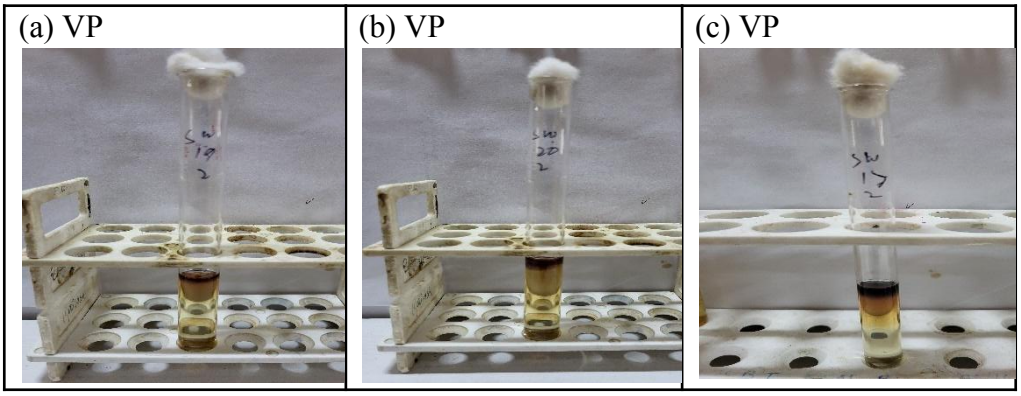
5.1.4.1 Indole Test:

All isolates were negative.

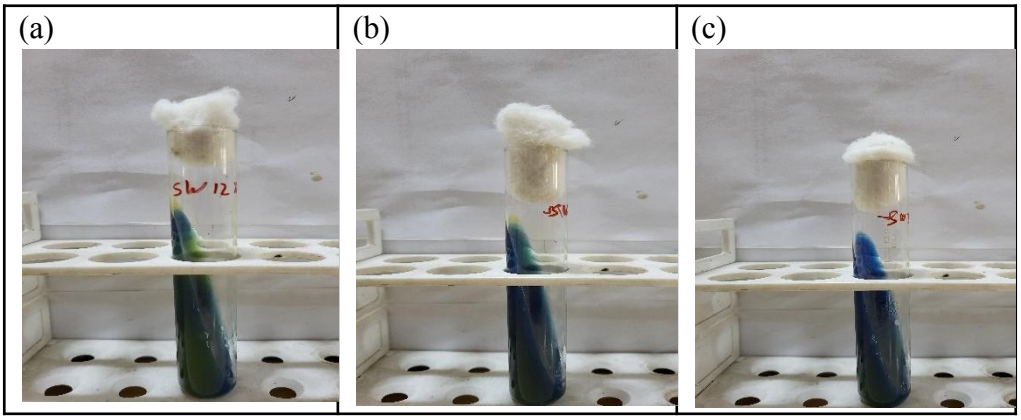
5.1.4.2 Methyl-Red Test and Voges-Proskauer:

Several isolates showed positive results for either mixed acid fermentation or acetoin production.

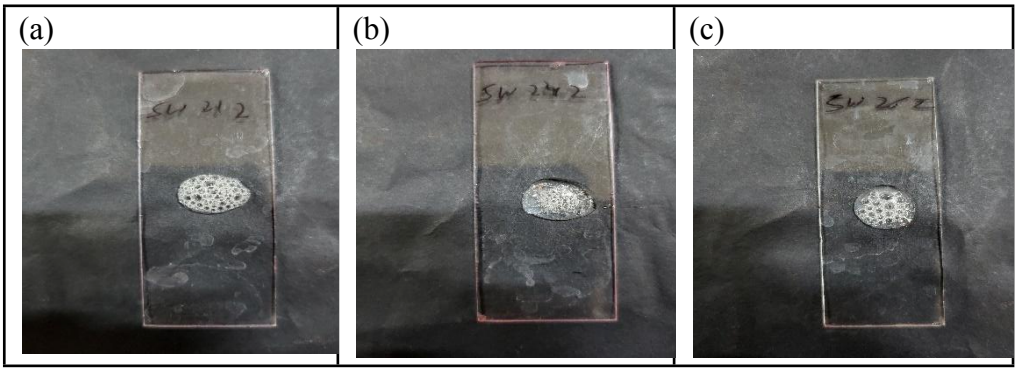
(a) MR	(b) MR	(c)MR
		



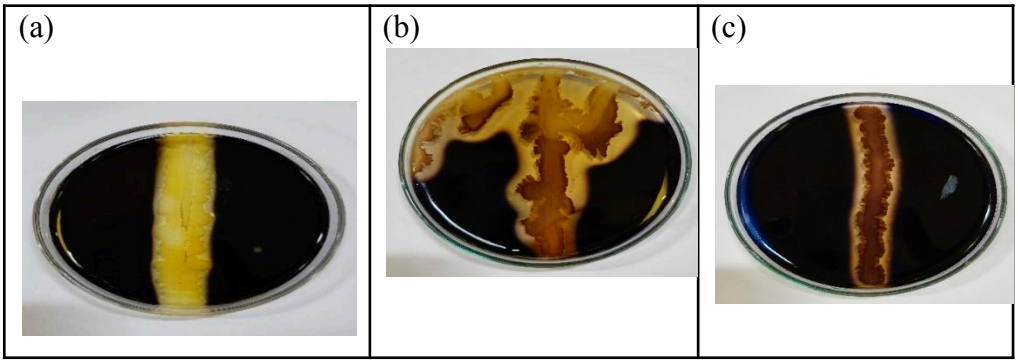
5.1.4.3 Citrate Utilization:
Many isolates utilized citrate as a carbon source.



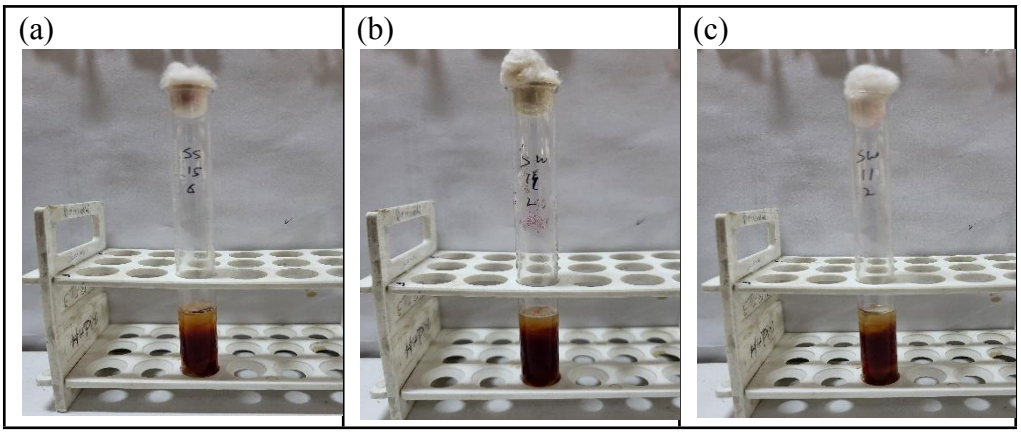
5.1.4.4 Catalase Test:
Most isolates were positive, indicating aerobic metabolism.



5.1.4.5 Starch Hydrolysis Test: Only a subset of isolates demonstrated amylase activity.



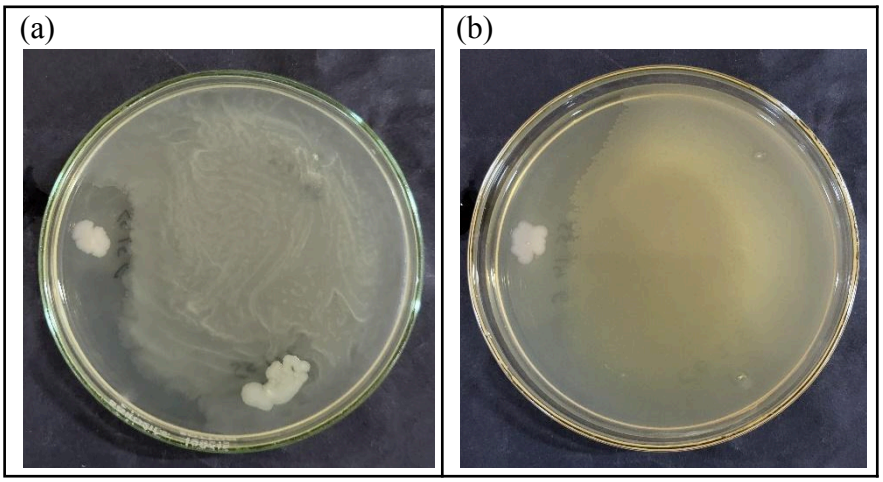
5.1.4.6 Nitrate Reduction Test:
These are some positive results of this test in which it shows the colour change in the medium.



5.1.4.7 Urea Broth Test: All isolates tested negative.

5.1.4.8 Gelatin Hydrolysis Test:

Some isolates produced gelatinase, indicating proteolytic enzyme activity.



5.2 Identification of Bacterial Isolates:

Based on morphological, biochemical, and Bergey's Manual characterization, isolates were identified as follows:

Table 6. Identified bacterial isolates based on Bergey's Manual

Sample name (short form)	**Colony number**	**Dilution**	**Bacteria name**
SW	8	10^3	*Staphylococcus aureus*
SW	11	10^2	*Escherichia coli*
SW	12	10^2	*Enterobacteraerogenes*
SW	14	10^2	*Escherichia coli*
SW	15	10^2	*Escherichia coli*
SW	17	10^2	*Enterobacteraerogenes*
SW	18	10^2	*Escherichia coli*
SW	19	10^2	*Enterobacteraerogenes*
SW	20	10^2	*Enterobacteraerogenes*
SW	21	10^2	*Enterobacteraerogenes*
SW	*23*	*10^2*	*Escherichia coli*
SW	24	10^2	*Enterobacteraerogenes*
SW	26	10^2	*Enterobacteraerogenes*
SW	27	10^2	*Enterobacteraerogenes*
SW	33	10^2	*Enterobacteraerogenes*
SS	6	10^6	*Enterobacteraerogenes*
SS	8	10^6	*Enterobacteraerogenes*
SS	9	10^6	*Escherichia coli*
SS	13	10^6	*Bacillus subtilis*
SS	14	10^6	*Bacillus subtilis*
SS	15	10^6	*Enterobacteraerogenes*
SS	17	10^6	*Enterobacteraerogenes*
CD	2	10^5	-
CD	14	**10^1**	*Staphylococcus aureus*

As observedmajor resistant species identified, included *Escherichiacoli, Enterobacter aerogenes, Staphylo coccusaureus, Bacillus subtilis.*

This indicates that AMR-associated bacterial species are present across sewage water, sewage soil, and cow dung environments.

6. Discussion

The emergence and global dissemination of antimicrobial resistance (AMR) is increasingly recognized as an ecological problem that transcends clinical boundaries [3-4]. Our study demonstrates that untreated urban sewage, sewage-contaminated soil, and fresh cow dung in Rajkot, Gujarat, harbour extraordinarily high densities of culturable bacteria (up to 7.8×10^6 CFU/g in cow dung), of which more than 80% exhibited phenotypic resistance to at least one clinically important antibiotic and 53% qualified as multidrug-resistant (MDR). These findings are consistent with recent nationwide surveys that have identified wastewater and livestock manure as the most critical environmental hotspots for AMR in India [7,14] .
The predominance of resistant Escherichia coli and Enterobacter spp. in all three matrices mirrors clinical surveillance data from Gujarat and neighbouring states, where third-generation cephalosporin and fluoroquinolone resistance in community-acquired infections now frequently exceeds 60–70% [15]. This striking overlap strongly suggests bidirectional exchange between environmental reservoirs and human pathogens, facilitated by contaminated water used for irrigation and bathing, direct contact with manure during farming, and consumption of vegetables grown on sewage-irrigated fields [16-17]. The isolation of linezolid-resistant *Staphylococcus aureus* from sewage water is particularly alarming because linezolid is a last-resort drug for MRSA and VRE infections, and environmental detection of linezolid resistance remains extremely rare globally [18].

The unusually high bacterial counts observed in fresh cow dung merit specific discussion. Although initial plate counts appeared anomalously elevated compared with published literature from temperate regions, triplicate experiments and microscopic examination confirmed extensive cell clumping and the presence of facultative anaerobes that only formed visible colonies after aerobic dilution and plating (Supplementary Fig. S2). High organic carbon and moisture content in fresh dung create micro-niches that protect bacterial aggregates from desiccation and antibiotic residues, thereby enhancing survival and potential horizontal transfer of resistance plasmids [19]. Moreover, the widespread practice of using untreated cattle manure as organic fertilizer in Gujarat agriculture creates a direct conduit for introducing MDR enteric bacteria and resistance genes into the food chain.

Co-selection by heavy metals and biocides probably contributes to the maintenance of antibiotic resistance in these environments. Sewage in Rajkot receives substantial industrial effluent containing zinc, copper, and chromium — metals known to co-select for resistance to β-lactams, fluoroquinolones, and aminoglycosides via shared efflux pumps and mobile genetic elements [20]. Although we did not quantify metal concentrations in the current study, future work incorporating metagenomic and resistome analyses is planned to confirm this mechanism.

Compared with earlier studies from India, our resistance rates for cefotaxime (68%) and ciprofloxacin (59%) in environmental isolates are significantly higher than those reported from the Ganga river basin (45–55%) [21]but comparable to hospital wastewater in Delhi and Hyderabad [22]. This indicates that smaller cities like Rajkot, despite having lower hospital bed density, can still generate extremely polluted environmental compartments

capable of driving regional AMR because of absent or dysfunctional sewage treatment infrastructure.

From a One Health perspective, our results underscore the urgent need to integrate environmental surveillance into India's National Action Plan on AMR. Current clinical surveillance systems (e.g., ICMR-AMR network) capture only the "tip of the iceberg". Routine monitoring of municipal sewage and livestock manure could serve as an early-warning system for emerging resistance traits before they dominate clinical settings [23-24].

7. Conclusion

This study provides robust, statistically validated evidence that untreated sewage and cattle manure in Rajkot, Gujarat, function as massive reservoirs of multidrug-resistant Enterobacterales, *Staphylococcus aureus*, and Bacillus spp. capable of resisting multiple classes of critically important antibiotics. The high prevalence of resistance to third-generation cephalosporins, fluoroquinolones, and last-resort agents such as linezolid in environmental isolates highlights the advanced stage of AMR pollution in urban and peri-urban ecosystems of western India. These contaminated matrices constitute direct exposure routes for farmers, waste handlers, and the general population through contaminated water and vegetables, thereby sustaining a vicious cycle of community-acquired resistant infections.

Immediate actions required include:

- Establishment of decentralized sewage treatment plants with tertiary disinfection,
- Regulation of antibiotic use in veterinary medicine and dairy farming,
- Inclusion of sewage and manure surveillance in the national AMR action plan,
- Community awareness programs on rational antibiotic use and safe manure management.

Without swift multi-sectoral intervention under the 'One Health framework', environmentally driven AMR will continue to erode the effectiveness of our remaining therapeutic options and push us closer to a post-antibiotic era. The data presented here should serve as a wake-up call for local and state authorities to treat environmental AMR not as a distant threat but as an ongoing public health emergency.

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Author Contributions:

Mohammadsakil Badi: Conceptualization, methodology, data collection, visualization, writing, and revision.

Meet Singhadiya: Conceptualization, methodology, data collection, visualization, writing, and revision.

IshitaRaninga: Data collection and literature review.

Debashis Banerjee: Supervision, conceptual guidance, and critical manuscript review.

All authors approved the final manuscript for submission.

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The authors declare no conflicts of interest.

Ethical Approval and Consent:

Not applicable. This study did not involve human or animal subjects.

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