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Case Series

Prudent use of Antibiotics in Animal Health Sector to Mitigate Antimicrobial Resistance: Case Series

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Abstract

This study investigates antimicrobial resistance (AMR) in bacteria causing mastitis in cattle, particularly focusing on the pathogens' resistance patterns to commonly used antibiotics for cattles in Rajasthan, India. As AMR poses a significant global health threat, the research underscores the urgent need for antibiotic susceptibility testing (AST) prior to antibiotic administration. The study identifies a troubling trend of indiscriminate antibiotic use due to their widespread availability without prescription, and a concerning lack of awareness among farmers about AMR. Addressing these issues, the study emphasizes educating farmers, promoting regular livestock vaccinations, and implementing AST in veterinary practices as critical steps toward reducing AMR. Detailed methodologies including milk sample collection, bacterial culturing, and AST via the Kirby-Bauer disc diffusion method are described. The findings reveal distinct resistance patterns in *Escherichia coli* and *Staphylococcus aureus* against multiple antibiotics, suggesting the necessity for alternative treatments post-AST. The study advocates for a One Health approach, integrating human, animal, and environmental health strategies to effectively manage and mitigate AMR, thereby enhancing the efficacy of antibiotic treatments and safeguarding public and animal health.

Keywords: Antimicrobial Resistance (AMR); Antibiotic Susceptibility Test (AST); Mastitis; Antibiotics; One Health; Public Health; Environmental Health

Introduction

The rise of antimicrobial resistance (AMR) presents a formidable challenge to public health, animal welfare, and environmental safety, necessitating a multifaceted approach to combat its spread [1]. Among the various contexts in which AMR has become a critical concern, bacterial infections causing mastitis in cattle stand out due to their impact on agricultural productivity and animal health [2]. Mastitis, a prevalent and costly disease in dairy cattle, is increasingly becoming difficult to manage due to the emergence of antibiotic-resistant pathogens [3]. This situation is

exacerbated by the misuse and overuse of antibiotics, facilitated by their widespread availability without prescription and the lack of proper guidance from healthcare professionals [1,3]. The current investigation explores the resistance patterns of key bacterial pathogens, such as *Escherichia coli* and *Staphylococcus aureus*, against commonly used antibiotics, underscoring the urgent need for judicious antibiotic use guided by antibiotic susceptibility testing (AST).

The usage of antibiotics in agriculture especially in livestock is a significant contributor to the occurrence and spread of antibiotic resistance [4]. The great concern arises as the types of antibiotics and their mode of action which are prescribed to cattle are somewhat closely related to the antibiotics that are prescribed to humans [5]. The major proportion of milk produced in India comes from crossbred cattle which are more susceptible to infectious diseases, especially mastitis. Mastitis is a disease in cattle in which inflammation of mammary glands (udder) occurs [6,7]. Mastitis is caused mainly by Staphylococcus aureus and by many other bacteria like *Streptococcus* spp., *Escherichia coli*, *Pseudomonas* spp., *Mycoplasma* spp, etc [8]. Mastitis causes a decrease in milk production in both quality and quantity wise, increased cost of treatment by giving antimicrobial therapy, labor, and premature culling of animals [9]. But now cattle are commonly given antibiotics to increase the milk yield and prevent or treat infections which have become a major concern of antibiotic resistance among cattle. These antibiotics have been used indiscriminately due to the lack of awareness about the standard antibiotic regimen and possible direct/indirect harm caused by antibiotics among the farmers who keep cattle [10]. Reasons behind AMR in animals could be overdose or underdose of antibiotics, direct marketing of antibiotics to the farmers, over-the-counter access by using an old prescription, no awareness about the antibiotic withdrawal period, etc. [11]. Additionally, antibacterial therapy commonly started before antibiotic susceptibility tests of pathogens shows one of the most important reasons for treatment failure. So to overcome this problem of antibiotic resistance we need to perform an antimicrobial susceptibility test (AST) / antibiotic susceptibility test (ABST). Antibiotic susceptibility testing is a lab procedure used to assess an antibiotic's efficacy against a specific bacterial strain. Various antibiotics are tested against the bacteria to find out which ones are most effective at treating the infection (WHO). It is essential as it helps doctors and veterinarians to choose the most effective antibiotic to treat the infection and to avoid the antibiotics that are unlikely to be effective due to resistance. Testing for antibiotic susceptibility can also assist in identifying the emergence of antibiotic resistance over time. Healthcare practitioners may recognize growing resistance trends and take measures to stop the spread of 16 antibiotic-resistant bacteria by tracking the susceptibility of bacterial strains to antibiotics [12]. The disc diffusion method or it is also known as Kirby-Bauer test is a type of AST. This test involves the method of evaluating antibiotic resistance. The efficacy of a particular antibiotic can be monitored by measuring the diameter of the zone of inhibition [13].

This paper delves into the necessity of integrating AST in veterinary practices to ensure effective treatment strategies and mitigate the advancement of AMR. It highlights the critical need for increased awareness among farmers about AMR and the importance of regular livestock vaccinations as preventive measures. Furthermore, the research emphasizes a holistic One Health approach, recognizing the interconnectedness of human, animal, and environmental health in addressing AMR challenges. By detailing the methodologies employed for isolating, identifying, and assessing the antibiotic susceptibility of pathogens, this study not only provides valuable insights into current resistance trends but also proposes actionable strategies to enhance treatment efficacy and reduce the overall AMR burden in the community.

Through meticulous analysis and a comprehensive understanding of the AMR landscape, this study aims to inform and refine veterinary practices, promote responsible antibiotic usage, and encourage preventive measures such as vaccination, thus contributing significantly to the global fight against AMR.

Materials and Methods

Sample collection

A total of 7 milk samples from the cases of clinical mastitis in cattle from district Jhunjhunu, Rajasthan were received at CSIR-IGIB, New Delhi in January 2023. To avoid any sort of contamination in the samples strict precautions were taken while sampling of milk from the cows. Dirt was brushed away from the udder and teats and appropriately washed. Several streams of milk were discarded. All the quarters were dipped in effective pre milking teat disinfectant for around 30 sec. All the teats were dried with the help of a sterile cloth towel. The end of the teats of each quarter

was cleaned with 70% ethanol. 20 ml milk samples were then collected in vials. Seven milk samples were collected from different cattle. Then samples were stored in a -20°C fridge when received at CSIR-Institute of Genomics and Integrative Biology (IGIB), New Delhi.

Antibiotic residues detection

All the milk samples procured were estimated for the antibiotic residues in them by HPLC.

Materials used

For HPLC, a gradient mobile phase was used where 2 solutions A and B were used as per the variable concentrations. Solution A was formed by mixing 0.3% Formic Acid in Water, and solution B was prepared using 0.3% Formic Acid in Acetonitrile (Table 1). The injection volume was 30 µL having a column temperature of 30°C.

S. No.	Time	A (%)	B (%)	Flow (ml/min)
1	2	90.00	10.00	0.3ml/min
2	2.01	35.00	65.00	0.3ml/min
3	8	35.00	65.00	0.3ml/min
4	8.01	5.00	95.00	0.3ml/min
5	20	5.00	95.00	0.3ml/min

Table 1: Composition of the mobile phase.

Sample preparation- We took 1 ml sample of milk and made a volume up to 20 ml using acetonitrile. Centrifuged and collected the upper layer and filtered for injecting into HPLC.

Standards and their preparation- A set of standards was used (Table 2). For the preparation of the standards, we took 20 mg standard from each antibiotic in a 200 ml volumetric flask and made up the volume to 200 ml using acetonitrile.

S. No.	Antibiotic Standard
1	Ampicillin sodium
2	Vancomycin Hydrochloride
3	Trimethoprim BP
4	Streptomycin Sulphate IP
5	Oxytetracycline Hydrochloride
6	Ceftriaxone Sodium USP
7	Erythromycin Stearate
8	Levofloxacin Hemihydrate
9	Doxycycline
10	Oxacillin
11	Tetracycline HCL

Table 2: Antibiotics Standard used for HPLC.

Isolation of bacteria and its identification

Preparation of brain heart infusion media and plate preparation

The Brain Heart Infusion (BHI) media, crucial for bacterial growth, was prepared following a specific composition that included yeast extract, agar, sodium chloride, and tryptone, adjusted to a final pH of 7.5 ± 0.2.

Ingredients	Gms/Litre
HM infusion powder	12.500
BHI powder	5.00
Proteose peptone	10.00
Dextrose (Glucose)	2.000
Agar	15.00
Sodium chloride	5.00
Disodium phosphate	2.500
Final pH (at 25°C)	7.4 ± 0.2

Table 3: Composition of BHI media (himedia).

For bacterial growth, BHI media was carefully poured into petri plates under sterile conditions within a laminar flow hood. The parafilm was put around all the Petri plates and placed in the incubator in an inverted position for incubation for around 16-18 hrs at 35°C. The milk samples were streaked onto the media and incubated, resulting in the growth of bacterial colonies.

Broth preparation, pellet extraction, DNA isolation, and quantification

Subsequent steps involved the enrichment of isolated bacteria in BHI broth, followed by centrifugation to extract bacterial pellets for DNA isolation using the QIAamp DNA mini kit. This process involved several stages of lysis and precipitation to isolate high-quality DNA, which was then quantified using a nanodrop spectrophotometer.

Broth preparation

Broth preparation (enrichment of isolated bacteria) was done for the DNA isolation of the bacteria that were growing on LB agar plates (colony 1 and colony 4). We took 3.7g BHI broth for the preparation of 100 ml of LB broth. The broth-containing flask was sterilized in the autoclave at 121°C for 15 minutes.

Pellet extraction

The pellet was extracted from the broth by centrifugation for DNA isolation. To further proceed, the supernatant was discarded. After centrifugation, the pellets were suspended in TE buffer.

DNA isolation

DNA isolation of both the samples, colony 1 and colony 4 was done via QIAamp® DNA mini kit. The general principle of DNA isolation involves mainly three types of lysis-cell wall lysis, cell membrane lysis, and nuclear membrane lysis either physically, chemically, or enzymatically to obtain the highly intact DNA into solution. All this is followed by protein precipitation and then DNA precipitation.

For the DNA extraction first the samples were thawed before usage and centrifugation was done for 5 min at 14000 rpm. The bacterial pellet was then put in a 1.5 ml microcentrifuge tube. 20 µl proteinase K and 180 µl buffer ATL (lysozyme) were added to this tube. Incubation at 56°C was done in the heating block for around 2 hrs till the bacterial pellet completely got lysed. Now, buffer AL was added to the tube, and vortexed. Incubation was done for 10 min at 70°C. Then, 200 µl of absolute ethanol was added, and vortexed. After this, it was pipetted and transferred onto a mini spin column (in a 2 ml collection tube). Centrifugation was done for 1 min at 7000 rpm. 500 µl buffer AW1 was added after the spin column was put in a new 2 ml collection tube. Again, for 1 min centrifugation was done at 7000 rpm. Then, in a new 2 ml collection tube the spin column was placed and this time 500 µl buffer AW2 was added. Centrifugation for 3 min was done at 10000 rpm. The mini spin column was put in a new 1.5 µl microcentrifuge tube, and an AE buffer of 200 µl was put in and incubated for 1 min at room temperature. Lastly, centrifugation for 1 min at 10000 rpm was done to elute the DNA. The purity of dsDNA was checked by a nanodrop spectrophotometer**.**

Agarose gel electrophoresis

The quality of the isolated genomic DNA (gDNA) was assessed through agarose gel electrophoresis, ensuring the DNA was intact and suitable for further analysis. For this the gel casting plates open ends were sealed by keeping the casting tray tightened with clamps. The comb was then placed properly on the casting plate after wiping it with 70% ethanol. 50 ml of 1x TAE was taken into which 0.8% agarose was added and boiled in the microwave until the agarose dissolved fully and a clear mixture was obtained. Then EtBr (visualization agent) was added (6 µl/100 ml) when the temperature reached around 50°C. Subsequently, after that the solution was poured into the gel mould and allowed to set until it gets solidified. After solidification, the comb was removed, and the casted gel was put in the electrophoresis unit having well towards the cathode and immersed with 1x TAE to a depth of about 1cm. 100 ng of both DNA samples were mixed properly with 6x loading dye. The mixed DNA samples were loaded into the wells on the agarose gel. A 1 kb ladder was loaded in the first well. The gel was then run at 90V for 1 hour and visualization of DNA bands was done using a gel documentation system.

16S rRNA gene PCR and sequencing

For microbial identification, 16S rRNA gene sequencing was conducted after the PCR amplification of the DNA template. After PCR, the gel electrophoresis is done for resolution, purification of the amplified gene product using the Qiagen Oiaquik kit, and sequencing. (Table 4). For the PCR, 2 different sets of universal primers Forward primers and reverse primers were used for the 16S rRNA gene. The sequence of the primers are as follows

- 16S rRNA forward primer- 5'-AGAGTTTGATCCTGGCTCAG-3'
- 16S rRNA reverse primer - 5'-TACGGCTACCTTGTTACGACTT-3'

A 0.2 ml PCR tube was taken into which 5.6 µl NFW was added. Then 1 µl taq buffer E was added. After this, 0.5 µl dNTPs, and 0.5 µl of DMSO were added and then 0.4 µl of $MgCl_2$ was added. After this, 0.4 µl of each forward and reverse primer were added. 1 µl of DNA template was then added afterward. At last, 0.2 µl Taq DNA pol was added to the reaction. All the steps were repeated for both samples. The PCR tubes were placed in a thermal cycler. The cycling conditions were initial denaturation at 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 40 sec, then annealing was set

at 59°C for 30s, and extension at 72°C for 10min. 1.5% agarose gel electrophoresis was then run to check bands of 1.5 Kb 16S rRNA gene after amplification.

After isolating PCR products from the gel concentration of the product was checked by a nanodrop spectrophotometer. Purification of amplified gene product from the agarose gel was done by using a Qiagen Qiaquik Gel Extraction kit with some minor modifications. The amplified band from 16S rRNA PCR was cut out with a clean sharp scalpel from the 1.5% agarose gel. One volume of gel was added to three volumes of the buffer QG (100 mg gel ~100 µl) followed by incubation for 10 minutes at 50°C. The tube was time to time vortexed after every 2-3 minutes which helped in dissolving the gel. 1 ml of isopropanol was poured into the sample and mixed thoroughly. The sample was applied to the QIAquick spin column for binding of the 16S rRNA gene and followed by centrifugation at 8000 rpm for 1 minute. QIAquick spin column was put back into the same tube after throwing flow through. In the QIAquick spin column, 500 µl of buffer QG was added to them, and then centrifugation was done for 1 minute at 8000 rpm, and then flow-through was discarded. Washing buffer PE was added 750 µl and then again centrifuged for 1 minute, flow-through was disposed off. The QIAquick spin column was transferred onto a fresh and clean 1.5 ml microcentrifuge tube. The gene was eluted after adding 50 µl EB buffer and incubated at room temperature for 5 minutes and after this centrifugation was done for 1 minute at 12,000 RPM. Eluted DNA was stored at -20°C.

Ingredient	Stock Concentration	Working Concentration	For 10 µl Working Reaction
NFW	-	-	5.6
Taq buffer E	10X	1X	1
dNTPs	10 mM	0.5 mM	0.5
$MgCl_2$	25 mM	1.0 mM	0.4
16S rRNA FP	100 mM	10 mM	0.4
16S rRNA RP	100 mM	10 mM	0.4
DMSO	-	1X	0.5
Taq DNA polymerase	5000 U/ml	1U	0.2
DNA template	-	-	1

Table 4: This table outlines the components of the Master mix used for 16S rRNA gene PCR, specifying their stock and working concentrations, along with the volumes needed for a 10 µl reaction.

16s RNA sequencing

The sequencing of the 16S rRNA gene allows the identification of bacteria by determining the linear order of the PCR product amplified. DNA sequencing was done by Sanger sequencing, also called the di-deoxy sequence method or chain terminating method given by Sanger and Nicholson. It begins with the separation of two strands of DNA by denaturation. After denaturation, single-stranded DNA is obtained that is attached to oligonucleotide primers and extended using a mixture of dNTPs to form a double-stranded structure. In addition to this mixture, ddNTPs are also added which do not possess a hydroxyl group at the position of 3' of the nucleotide. Due to the presence of ddNTP, polynucleotide chain progression stops. As both dNTP and ddNTP have a uniform probability of joining with the sequence, each sequence terminates at different lengths. The sequencing results were analyzed using a bioinformatic tool (NCBI- Nucleotide BLAST) to identify the bacterial species present

Antibiotic Susceptibility Testing of the isolated bacteria

Mueller-Hinton agar media was prepared for antibiotic susceptibility testing, which is essential for determining the antimicrobial resistance patterns of the isolated bacteria.

Preparation of MH agar media

Mueller-Hinton agar media was prepared for performing antibiotic susceptibility tests. The media-containing flask was put

in the autoclave for 15 minutes at 121°C for sterilization. Mueller-Hinton agar is chosen over other media as it is somewhat loose in nature and antibiotics diffuse in a better way in this media and a better zone of inhibition is obtained from this.

Ingredients	Gms/litre
Starch	1.5
Agar	17
Beef, infusion from	300
Casein acid hydrolysate	17.5
Final pH (at 25°C)	7.5 ± 0.2

Table 5: Composition of Mueller-Hinton agar media.

MH agar plate preparation

Mueller-Hinton agar media was poured into Petri plates and streaking of bacterial colonies. After solidification, one sterile swab was taken and dipped into autoclaved water, and then, samples 1 and 4 were taken and bacterial colonies were picked from this culture plate and swabbed over the MH agar plate.

Kirby-Bauer disc diffusion assay

Different antibiotic disks were placed over MH agar plates to observe the resistance of bacteria that are swabbed over against the antibiotics. Antibiotic discs are made up of high-quality Whatmann paper of 6 mm impregnated with antibacterial agents of known concentrations. All of the first, sample 2 plates, number i, number ii, and number iii were taken, and five antibiotic discs were placed on each plate (E 15, TE 30, O 30, GEN 10, CTR 30; AK 30, CX 30, NX 30, AMP10, COX 1; and IC10/10, TEI 30, CFM 5, P 10, CPD 10) with the help of sterile forceps. Then, sample 4 plates, number i, number ii, and number iii were taken and five antibiotic discs were placed on each plate (AK 30, CX 30, NX 30, AMP 10, COX 1; C 30, AZM 30, RO 30, CFM 5, CTR 30; and GEN 10, OF 5, CN 30, MRP 10, P 10). After 18-20 hours of incubation, plates were taken out from the incubator, and zones of inhibition of all the plates were observed. All zones of inhibition were measured with the help of a ruler.

Results

The analysis of the samples yielded valuable results. There were no Antibiotic residues detected above the threshold levels but the isolated bacteria from the samples were found to gain resistance against some antibiotics.

Antibiotic residues in milk samples

All the test samples were run on HPLC for the presence of antibiotic residues and after coalescing the HPLC data it was seen that all the samples were seen below the detection level. (Supplementary data 1).

S. No.	Antibiotic Standard	Area of Standard	Area of Test
1	Ampicillin sodium	7535537	Bdl*
2	Vancomycin Hydrochloride	546973	Bdl
3	Trimethoprim BP	92605942	Bdl
4	Streptomycin Sulphate IP	4766	Bdl
5	Oxytetracycline Hydro-chloride	83557	Bdl
6	Ceftriaxone Sodium USP	10858630	Bdl
7	Erythromycin Stearate	84235820	Bdl
8	Levofloxacin Hemihydrate	6471023	Bdl
9	Doxycycline	457481738	Bdl
10	Oxacillin	3605148	Bdl
11	Tetracycline HCL	454598323	Bdl
* Bdl- Below detection level			

Table 6: Standard antibiotics peak area obtained by HPLC.

Bacterial isolation and identification from milk samples

Colonies growth

The Preparation of Brain Heart Infusion (BHI) media was done to grow colonies of bacteria from the collected milk samples. Out of 7 plates, only 1 plate showed bacterial growth over BHI agar media, because other plates were of high serial dilution.

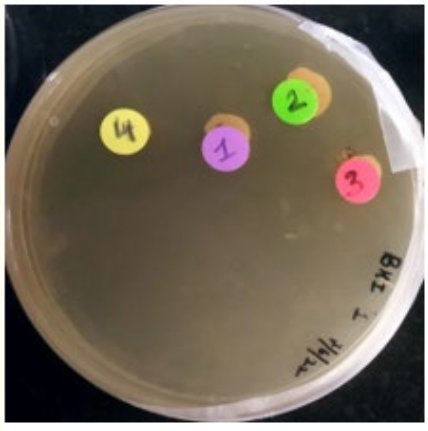
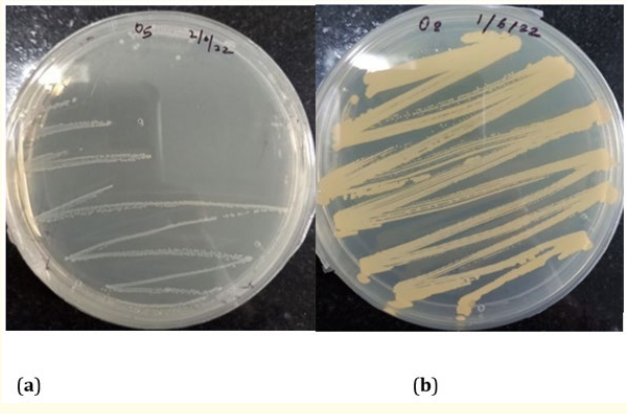


Figure 1. Colonies obtained on the mother culture plate.

S. No.	Colony
1	*E. coli*
2	*S. aureus*
3	*E. coli*
4	*S. aureus*

Table 7: List of colonies obtained on the mother culture plate.

After obtaining 4 colony growths on the mother culture plate the individual subcultures were done and there were only 2 different bacteria obtained.



(a) (b)

Figure 2: (a) Sub-culture plate of E. coli; (b) and S. aureus on LB agar plates of streaked samples.

DNA isolation and quantification

The bacterial colonies were then isolated from the plates and after following the protocol of DNA extraction the DNA was run on agarose gel electrophoresis. The concentration of the genomic DNA range was around 20-25 ng/µl. The 260/280 ratio was ~ 1.8; showing that isolated DNA was maximal pure and free of protein and RNA contamination.

S. No	Sample ID	Concentration (ng/µl)	Ratio (260/280)
1.	Sample no 1 (S1)	20.3	1.83
2.	Sample no 4 (S2)	25.1	1.79

Table 8: Obtained concentration of the samples.

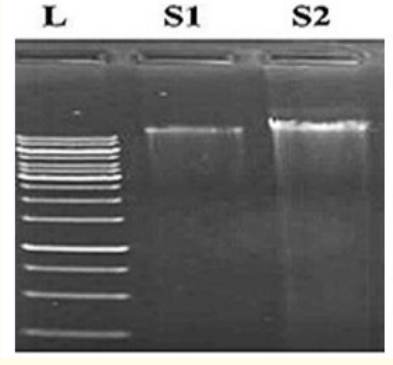


Figure 3: Genomic DNA: S1 and S2, Ladder: L (1kb).

16s RNA sequencing

The 16S rRNA gene was amplified at an optimized PCR reaction. The PCR product was then run on 1.5% agarose gel and then the products were purified from the gel using the Qiagen Qiaquik Gel Extraction kit. Later the concentration was measured using a Nanodrop spectrophotometer and then used for sequencing.

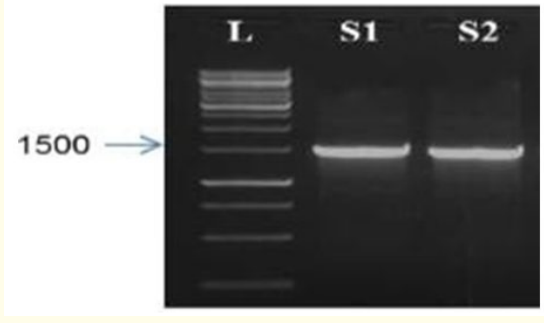


Figure 4: PCR amplified 16S rRNA gene.

The Sanger sequencing yields the sequence of the bacterial 16sRNA PCR products using the ABI-PRISM 377 DNA sequencer (Supplementary data 2). When the sequence of both the samples was acquired, the BLAST of the 16SrRNA gene of sample no. 2 was carried out which yielded a 99.8% identity match with *Escherichia coli* 16S ribosomal RNA (EHEC StrainATCC43895) while the result of 16S rRNA gene blast for sample no.4 yields 99.26% of similarity with *Staphylococcus aureus* 16S rRNA gene (strain HAR1).

ABST profile

The ABST was performed based on the Kirby Bauer method and after placing the disc on the plates, all the plates were observed after 18-20 hours of incubation. Zones were clearly visible around the antibiotics. The measurement of the zone of inhibition of both plates was done.

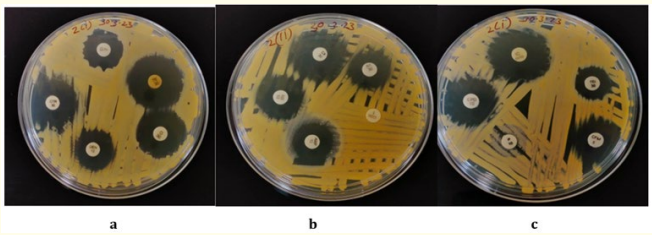


Figure 5: a). Plate 1 – *E.coli* (E15, TE30, O30, GEN10, CTR30) b). Plate 2 – *E. coli* (COX1, AK30, AMP 10, NX 10, CX 30) c). Plate 3 – *E.coli* (P10, CPD10, TEI 30, CFM5, IC 10/10)

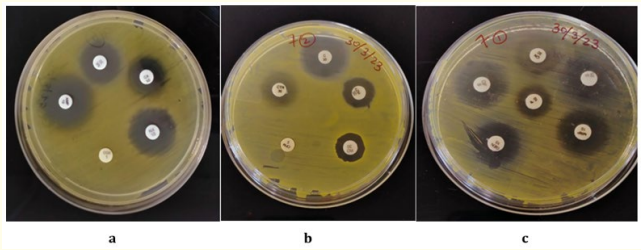


Figure 6: a). Plate 1- S.aureus (COX1, CX30, NX 10, AK30, AMP10) b). Plate 2- S.aureus (C30, AZM30, RO30, CFM5, CTR30) c). Plate 3- S.aureus (P10, GEN10, OF5, MRP10, S25, CN30).

The diameter of the zones of inhibition around the antibiotics was measured to the nearest whole mm by the ruler. This is recommended by the National Committee for Clinical Laboratory Standards (Performance Standards for Antimicrobial Susceptibility Testing, 31st edition). The sensitivity status of bacteria is divided into 3 categories: Susceptible (S) - this is a type of status in which the antibiotic dose is therapeutically effective against the pathogenic bacteria. Intermediate (I) - this type of status indicates the response of the bacteria against the drug is reduced. Resistant (R) - this is a status of therapeutic failure in which bacteria show no response to the given antibiotic. These all were determined according to the guidelines of the Clinical and Laboratory Standards Institute [CLSI].

The given tables (Table 9,10) show the standard measurements compared with experimental values for the determination of sensitivity and resistance status by the Kirby-Bauer method:

ANTIBIOTIC USED (all 3 plates)	R (in mm)	I (in mm)	S (in mm)	Zone of inhibition (in mm)	S/I/R
Amikacin (AK 30)	≤14	15-16	≥17	20	S
Ampicillin (AMP 10)	≤13	14-15	≥17	23	S
Norfloxacin (NX 10)	≤12	13-16	≥17	20	S
Gentamicin (GEN 10)	≤12	13-14	≥15	18	S
Cloxacillin (COX 1)	≤12	13-17	≥18	0	R
Ceftriaxone (CTR 30)	≤24	25-29	≥30	22	R
Imipenem (IC 10/10)	≤19	20-22	≥23	26	S
Tetracycline (TE 30)	≤14	15-18	≥19	27	S
Oxytetracycline (O 30)	≤18	19-24	≥25	23	I
Erythromycin (E 15)					

Cefixime (CFM 15)					
Teicoplanin (TEI 30)					
Penicillin G (P 10)					
Chloramphenicol (C 30)					
Cefpodoxime (CPD 10)					
Cefoxitin (CX 30)					

Table 9: *E. coli* (SAMPLE 1).

Reference: *Escherichia coli* ATCC®* 25922.

Antibiotic used (all 3 plates)	R (in mm)	I (in mm)	S (in mm)	Zone of inhibition (in mm)	S/I/R
Amikacin (AK 30)	≤14	15-16	≥17	19	S
Ampicillin (AMP 10)	≤28	-	≥29	24	R
Norfloxacin (NX 10)	≤12	13-16	≥17	20	S
Gentamicin (GEN 10)	≤12	13-14	≥15	17	S
Cloxacillin (COX 1)	≤18	19-29	≥30	0	R
Ceftriaxone (CTR 30)	≤31	32-36	≥37	10	R
Ofloxacin (OF 5)	≤14	15-17	≥18	21	S
Penicillin G (P 10)	≤28	-	≥29	12	R
Azithromycin (AZM 30)	≤13	14-17	≥18	15	I
Roxithromycin (RO 30)	≤22	23-29	≥30	13	R
Chloramphenicol (C 30)	≤12	12-17	≥18	24	S
Cefixime (CFM 5)				0	
Cefoxitin (CX 30)				12	
Norfloxacin (NX 30)				20	
Chloramphenicol (C 30)				24	
Meropenem (M 10)					
Streptomycin (S 25)					

Table 10: *S. aureus* (SAMPLE 4).

Reference: *S. aureus* ATCC®* 25923.

The results of the ABST were worth noting. Both the isolated bacterial species are seen to possess resistance against commonly used antibiotics. *E. coli* depicted resistance against 2 antibiotics Cloxacillin and Ceftriaxone while showed intermediate resistance against Oxacillin. Cloxacillin and Oxacillin come under the "Access" category both of which are narrow-spectrum antibiotics. Interestingly, Ceftriaxone is a Broad-spectrum 3rd generation cephalosporin that comes under the "Watch" category of the WHO AWaRe classification [14]. Thus, depicting the narrowing of treatment options after seeing bacterial resistance moving to last-resort antibiotics. *S. aureus* was seen to possess more resistance to antibiotics as compared to *E. coli* (Figure 7,8). Ampicillin,

Cloxacillin, and Penicillin come under the "Access" category which are totally resistant, Then comes the "Watch" category antibiotics which should be used cautiously, and under this category, Macrolides like Roxithromycin, and Ceftriaxone are seen to possess resistance while azithromycin has Intermediate resistance. The important takeaway message after looking at such a spectrum is that there is a rising trend of antibiotics resistant from "Access" to the "Watch" category antibiotics and if this trend continues then soon we will be seeing bacteria being resistant to "Reserve" or last resort antibiotics.

According to recent data published by our group, it has been confirmed that veterinarians in Jhunjhunu most frequently prescribe cephalosporins, with Ceftriaxone being the most commonly prescribed among them. Our research indicates that bacteria acquired in this region have developed resistance to Ceftriaxone, which aligns with our previous findings regarding prescription patterns [15].

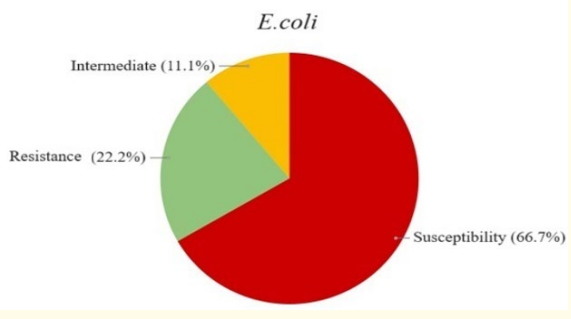


Figure 7: Depicts 66.7 % sensitivity, 11.1 % intermediate susceptibility, and 22.2 % resistance to *E.coli* against the antibiotics used.

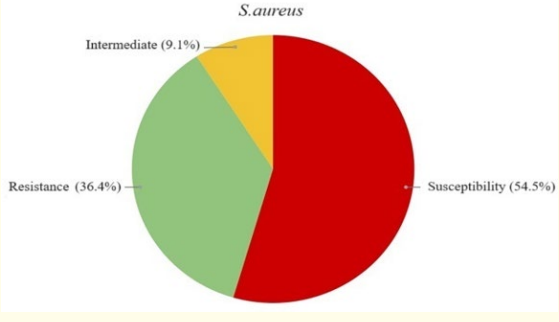


Figure 8: Depicts 54.5 % sensitivity, 9.1 % intermediate susceptibility, and 36.4 % resistance to *S. aureus* against the antibiotics used.

Discussion

In this investigation, we delve into the resistance patterns of bacteria causing mastitis against the antibiotics commonly in use today, highlighting the pressing global health concern of antimicrobial resistance (AMR). The study emphasizes the critical need for increased awareness about AMR and the implementation of antibiotic susceptibility testing (AST) to guide treatment. Given the escalating reports of AMR in pathogens responsible for mastitis in cattle-a condition that can be effectively managed with careful antibiotic application—the research advocates for the execution of AST before prescribing antibiotics. This proactive approach aims to significantly decrease AMR instances, aiding veterinarians in administering more precise and restrained antibiotic treatments. By emphasizing early and appropriate interventions for mastitis, the study suggests a pathway to mitigate the severity of this infectious condition and potentially prevent further infections, aligning with recommendations for reducing antibiotic misuse and enhancing treatment efficacy in veterinary practice.

The widespread availability of antibiotics without prescription and insufficient guidance from local healthcare providers have heightened concerns regarding the misuse of these critical medications [16]. This easy access enables individuals, especially those without direct veterinary consultation, to obtain antibiotics, risking both underuse and overuse [17]. A fundamental solution to this challenge is the education of farmers on antimicrobial resistance (AMR) and antibiotic susceptibility testing (AST). Observations indicate a significant gap in farmers' awareness regarding AMR and its current implications, underscoring the urgent need for education at the grassroots level [18].

Moreover, the regular vaccination of livestock plays a crucial role in animal health management, offering a viable alternative to antibiotic use. Vaccinations not only prevent primary infections but also bolster immunity against secondary infections, thereby reducing the reliance on antibiotics and mitigating the AMR burden. Unfortunately, the lapse in timely vaccinations among farmers contributes to the rising levels of AMR [19]. Promoting consistent vaccination practices is essential for disease prevention in livestock, thereby reducing the societal AMR burden. This study was initiated in response to these concerns, aiming to decrease the AMR load within the community through increased awareness and vaccination efforts, as highlighted by Heymann D.L. in 2016 [20].

The subsequent steps of the study involved meticulous methodologies for isolating, identifying, and assessing the antibiotic susceptibility of bacterial cultures from mastitis-affected cattle. This included the collection and preparation of milk samples, bacterial culturing on Luria-Bertini agar media, DNA isolation, PCR amplification for 16S rRNA gene sequencing, and the Kirby-Bauer disc diffusion method for antibiotic susceptibility testing. The results obtained from these methods underscored the critical need for precise and judicious antibiotic use, guided by AST results to combat the escalating issue of AMR effectively.

Upon detailed analysis of table 9, the data explicitly indicates that *E. coli* strains exhibit resistance to the Access and Watch category of antibiotics. This resistance pattern significantly undermines the efficacy of these drugs in treating mastitis cases attributed to *E. coli*, thereby necessitating the exploration of alternative therapeutic options guided by rigorous antibiotic susceptibility testing (AST). Additionally, *E. coli*'s demonstrated intermediate susceptibility to oxytetracycline suggests a nuanced approach to its application, where its effectiveness is recognized yet tempered by the potential for developing resistance, highlighting the importance of judicious use to forestall the exacerbation of AMR.

Conversely, the findings from table 10 concerning *S. aureus* underscore a slightly increased trend of resistance to a spectrum of antibiotics such as Ampicillin, Cloxacillin, Roxithromycin, and Ceftriaxone. This resistance portends the likely failure of these antibiotics in effectively managing mastitis caused by *S. aureus*, pressing the need for alternative antibiotics post-AST. The intermediate susceptibility of *S. aureus* to azithromycin presents a potential, albeit limited, therapeutic window that must be navigated with caution to mitigate further development of AMR. Encouragingly, the susceptibility of S. aureus to Amikacin, Norfloxacin, Gentamicin, Ofloxacin, and Chloramphenicol illuminate viable pathways for effective treatment, offering hope in the battle against mastitis. One inference can be made from this result that doctors are prescribing directly broad-spectrum antibiotics to the animals rather than first going to the Access category or narrow-spectrum drugs.

This scenario underscores the broader imperative for heightened awareness and proactive measures against AMR, which looms as a formidable threat to public health, animal welfare, and environmental integrity. The One Health approach, recognizing the intricate interplay between human, animal, and environmental health, becomes ever more pertinent. In this interconnected framework, disturbances in one domain can precipitate far-reaching repercussions, underscoring the necessity for a unified, informed strategy to combat AMR and preserve the health of our planet and its diverse inhabitants [21]. In conclusion, the study's findings emphasize the importance of integrating AST in veterinary practices to refine antibiotic usage, alongside enhancing farmers' awareness about AMR and advocating for regular livestock vaccinations. These measures are pivotal in curbing the rise of AMR, ensuring both animal and human health are safeguarded against the backdrop of increasing antibiotic resistance.

Conclusions

The findings of this study bring insights into the patterns of antimicrobial resistance (AMR) among bacteria causing mastitis in cattle, emphasizing the need for a structured and informed approach to antibiotic usage in veterinary medicine. The widespread resistance of *Escherichia coli* and *Staphylococcus aureus* to "Watch" category antibiotics underscores the complexity of treating mastitis and highlights the potential consequences of unchecked antibiotic prescribing. By implementing antibiotic susceptibility testing (AST) prior to treatment, veterinarians can tailor antibiotic use more precisely, reducing the likelihood of resistance development.

Furthermore, this research illustrates the necessity for comprehensive educational programs aimed at farmers to enhance their understanding of AMR and its implications. Regular vaccination of livestock emerges as a vital preventive measure that can significantly decrease the reliance on antibiotics and, consequently, the AMR burden. The study's call for a One Health approach stresses the interconnectedness of human, animal, and environmental health, advocating for a unified strategy to combat AMR effectively. Ultimately, by integrating AST into routine veterinary practice, promoting education on AMR, and enforcing regular vaccination schedules, we can mitigate the spread of AMR. These measures not only protect animal health but also contribute to safeguarding public health and preserving environmental integrity, ensuring a sustainable approach to disease management in the face of escalating antibiotic resistance.

Author Contributions

V.S.D.: Data curation, Formal analysis, Methodology, Writing- review and editing; D.J.: Data curation, Methodology, Validation, Formal analysis, Writing- review and editing; B.U.R: Data curation, Formal analysis, Methodology, Validation, Writing - original draft; A.K.: Data curation, Methodology, Validation, Writing - review and editing; P.B.: Methodology, Validation, Project Administration; A.R.: Methodology, Validation, Project Administration, Writing - review and editing; A.K.: Methodology, Validation, Project Administration; V.P.S.: Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing - review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The animal study protocol was approved by the Institutional Animal Ethics Committee (protocol code IAEC/23/1/8).

Data Availability Statement

The data of this study can be retrieved from the websites of the respective institutes which are mentioned in the reference section.

Conflicts of Interest

The authors declare no conflicts of interest.

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