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Isolation and antibiogram of *Escherichia coli* and *Salmonella* spp. isolated from diarrheic cow calves

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Abstract
Aim: The aim of the present work was to ascertain the role of *E. coli* and *Salmonella* spp. causing calf diarrhea, their biochemical profile and antibiotic sensitivity.
Materials and Methods: A study was conducted to isolate the bacterial spp. - *E. coli* and *Salmonella* spp. and to determine their antibiogram pattern. A total of 110 diarrheic fecal samples were collected from calves below 6 months age from different organized and unorganized farms in Shivamogga and surrounding three districts. Samples were collected directly from rectum using sterile cotton swabs and kept on ice and transported to the laboratory. The bacteria from the samples were isolated, biochemical profiling was performed and sensitivity towards antibiotics was determined by measuring the zone of inhibition on Muller Hinton agar.
Results: *E. coli* was isolated with the highest frequency of 95.45%, whereas *Salmonella* spp. was isolated at a low frequency of 7.27% from the diarrheic fecal samples. The result of antibiogram pattern of *E. coli* isolates showed that co-trimoxazole was the most sensitive antibiotic followed by ceftriaxone-tazobactum and ciprofloxacin. *E. coli* isolates showed highest resistant to metronidazole, nitrofurantoin, penicillin, streptomycin followed by polymyxin B and kanamycin. While *Salmonella* isolates were highly sensitive to ciprofloxacin, levofloxacin followed by co-trimoxazole and amoxicillin-sulbactum. However, the Salmonella isolates were resistant to metronidazole, cefixime, chloramphenicol followed by gentamicin, kanamycin and cefaperazone.
Conclusion: *E. coli* and *Salmonella* species isolated from calf diarrhea could be treated with co-trimoxazole and ciprofloxacin as they were found to be the most effective antibiotics.

Keywords: Calf diarrhea, isolation, biochemical profile, antibiogram, antibiotics

Introduction
Calf diarrhea is a multifactorial disease entity with a number of infectious and non-infectious factors. It is the major cause of morbidity and mortality in calves during the first three weeks of life, resulting in severe direct and indirect economic losses (Grove-White, 1998; Lorenz, 2004; Smith, 2009) [7, 11, 21]. The infectious diarrhea is caused by varied etiological agents such as bacterial, viral and parasitic agents. Previous studies show that the most important infectious agents were enterotoxigenic *E. coli*, *Salmonella* spp., *Rotavirus*, *Coronavirus* and *Cryptosporidium* spp. either singly or in combination (Steiner *et al*., 1997 and De la Fuente *et al*., 1999) [24, 5]. Among the bacteria, *E. coli* and *Salmonella* spp. are notable in diarrhea. Repeated exposure to antibacterial agents has not only promoted adaptive resistance, but also conferred decreased sensitivity to antibiotics in *E. coli* and *Salmonella* strains (Udaykar and Sharda, 2009) [25]. Therefore, the present study was carried out to find the association of *E. coli* and *Salmonella* spp. with the cases of calf diarrhea and to study their antibiogram.

Materials and Methods
Materials used
Sterile cotton swab - Hiculture Collecting Device was used for collection of diarrheic fecal samples. This device consists of sterile cotton swab in screw capped polypropylene tube with size 75 mm x 12 mm diameter. All the media including MacConkey agar, EMB agar, Mueller Hinton agar (MHA), Xylose lysine desoxycholate agar (XLD agar), Brilliant green agar (BGA agar), nutrient agar and broth, Simmons citrate agar, Triple sugar iron agar, 40% Urea, Voges-Proskauer's test medium, peptone water and glycerol were obtained from M/s Hi-media Laboratories Ltd., Mumbai. The reagents used were: Gram staining kit, Kovac's reagent, Methyl red indicator, Alpha naphthol, 40% KOH, Hydrogen peroxide and Oxidase discs.

The media and reagents employed for the identification of bacterial species from fecal swab were prepared as recommended by the manufacturer or as per the standard procedure, *i. e*., sterilized by autoclaving (Collee *et al*., 1989) [4].

Collection of samples

A total of 110 diarrheic fecal samples were collected from calves below 6 months age from different organized and unorganized farms in Shivamogga and surrounding three districts *viz*., Davangere, Chikmagalur and Chithradurga. Samples were collected directly from rectum using sterile cotton swabs and kept on ice and transported to the laboratory. Collected samples were subjected for bacterial culture and further the bacterial isolates were subjected to antimicrobial susceptibility assay. The present research work was conducted in the Department of Veterinary Microbiology, Veterinary College, Shivamogga, a constituent institute under Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka state.

Sample Processing

For bacterial isolation and identification, rectal swabs were inoculated in to buffered peptone water (BPW). Further they were inoculated in to differential enrichment media and selective media.

Isolation and identification of *E. coli* from fecal samples

Isolation of *E. coli* was conducted following standard procedures described in Quinn *et al*. (2002) [17]. Each swab was inoculated in to a test tube containing 1 ml of sterile buffered peptone water (BPW) broth. It was incubated at 37 ºC for 18-24 hours. After 24 hours, the test tubes were checked for turbidity or sedimentation or pellet formation.

Selective plating was done by streaking a loop full of broth culture on to a sterile MacConkey agar plate, which selectively grows members of the *Enterobacteriaceae* and permit differentiation of enteric bacteria, and was incubated at 37 ºC for 18-24 hours. Then, the plates were checked for growth of bacterial colonies. Colonies showing characteristic lactose fermenting (having pink colonies) were repeatedly streaked on MacConkey agar plate until pure colonies were obtained (Plate1A). The selected pink colonies were inoculated further on to Eosin methylene blue (EMB) agar and incubated at 37 ºC for 18-24 hours to visualize the metallic sheen growth. Colonies showing characteristic metallic sheen on EMB agar plates after the incubation were considered as presumptive of *E. coli* isolates (Plate1B). From each MacConkey agar plate, *E. coli* characteristic colonies were streaked on to nutrient agar for further confirmation by biochemical tests.

For the morphological identification, all the isolates were stained by Gram's stain to determine the cell morphology, gram reaction and purity of the isolates under the oil immersion objective (100x magnification). The smear was prepared on clean grease free microscopic glass slide by picking pink colonies grown on MacConkey agar plate. The smear was heat fixed, stained with Gram's method and observed under microscope to study morphological characteristics and staining reaction (Plate1C).

Suspicious colonies of *E. coli* were further sub cultured in nutrient agar for biochemical tests. The suspected pure colonies from nutrient agar were inoculated onto tryptone water broth for indole tests, MR-VP medium for methyl red and Voges proskauer tests, Simmon's citrate agar slant, TSI agar slant and urea agar slant and incubated at 37 ºC for 18-24 hours. Simultaneously, catalase and oxidase tests were performed. Isolates producing indole positive, methyl red positive, Voges-Proskauer negative, citrate negative, TSI positive (yellow slant and yellow butt), urease negative, catalase positive and oxidase negative results were identified as *E. coli* as described by Quinn *et al*. (2002) [17] (Plate 2A and 2B).

Isolation and identification of *Salmonella* from fecal samples

Salmonella species were isolated and identified according to the techniques recommended by the International Organization for Standardization (ISO, 2002) [9]. Accordingly, the detection of *Salmonella* spp. in fecal samples was accomplished in four stages *viz.,*

1. Non-Selective Pre-Enrichment
2. Selective Enrichment
3. Selective Plating out and Identification
4. Confirmation of Identity

Non-Selective Pre-Enrichment

Samples were pre-enriched in BPW in a ratio of 1ml of the sample to 9 ml of BPW (1:10) and incubated at 37 ºC for 18-24 hours (ISO, 2002) [9].

Selective Enrichment

The pre-enrichment broth after incubation was mixed and 0.1 ml of the broth was transferred aseptically into a tube containing 10 ml of Rappaport-Vassiliadis medium 44 (RV broth). The inoculated RV broth was incubated at 41.5 ºC ±1 ºC for 24±3 hours (ISO, 2002) [9].

Selective Plating out and Identification

MacConkey agar plate and two selective media *viz.,* XLD agar, BGA agar plates were used for selective plating and identification purpose. A loop-full of inoculum from the RV broth was transferred aseptically and streaked onto MacConkey agar plate and incubated at 37 ºC for 16-18 hours. The pale colonies showing non-lactose fermentation (NLF) (Plate 3A) were then streaked onto the surface of XLD agar and BGA agar separately. The plates were incubated at 37 ºC±1 ºC for 18-24 hours. After proper incubation, the plates were examined for the presence of suspected *Salmonella* colonies, which appear black centered colonies on XLD agar (Plate 3B) and pinkish white or red colonies on BGA agar (Plate 3C). Black centered colonies and pinkish white or red colonies were repeatedly streaked on to the fresh XLD and BGA agar plates, respectively until pure colonies obtained and which were then picked up for sub culturing on to nutrient agar for further confirmation of *Salmonella* isolates by biochemical tests.

For the morphological identification, all the isolates were stained by Gram's stain to determine the cell morphology, gram reaction and purity of the isolates under the oil immersion objective (100x magnification). The smear was prepared on clean grease free microscopic glass slide by picking pale coloured colonies grown on MacConkey agar plate. The smear was heat fixed stained with Gram's method and observed under microscope to study morphological characteristics and staining reaction (Plate 3D).

Confirmation of Identity

Suspicious colonies were further sub cultured in nutrient media for biochemical tests. Confirmation was done by using biochemical test according to ISO, 2002 [9]. The suspected pure colonies from nutrient agar were inoculated onto trypone water broth for indole tests, MR-VP medium for methyl red and Voges proskauer tests, TSI agar slant, Simmon's citrate agar slant, urea agar slant and into 0.5 ml of normal saline for ONPG test. Simultaneously, catalase and oxidase tests were performed. Isolates producing indole negative, methyl red positive, Voges-Proskauer negative, citrate positive, red (alkaline) slant, yellow (acid) butt, H_2S positive/negative in TSI, urease negative, catalase positive and oxidase negative, ONPG negative results were identified as *Salmonella* spp. (Plate 4A and 4B).

Preservation of pure culture

After phenotypic identification by biochemical tests, pure cultures of *E. coli* and *Salmonella* isolates were streaked onto slants of nutrient agar and preserved at 4 ºC for further study. Alternatively, cultures were also preserved in sterile nutrient-glycerol broth vials at -20 ºC until further use.

Antibiotic Sensitivity Test (ABST)

Antibiogram assay was performed following the standard disc diffusion method. Inoculum from pure culture on nutrient agar was picked and added into 2 ml of Nutrient broth and mixed properly. This was incubated at 37 ºC for 24 hours. The bacterial inoculum was adjusted to 0.5 on McFarland scale and it was spread onto the Mueller Hinton agar plates using sterile swab. Three Mueller-Hinton Agar (MHA) plates were used for each sample. The antibiotic discs were applied aseptically onto the agar surface with the distance between centers of at least 30 mm apart and examined after incubation for 12-24 hours at 37 ºC as per the standard procedure for disc diffusion method as described by Bauer *et al*. (1966) [2]. The zone of inhibition was then measured using antibiotic zone scale and expressed in millimeters. Sensitivity/resistance was assessed by comparing the values of the zone of inhibition obtained for each antibiotic disc against the standard chart provided with the discs. The interpretation was done in accordance to the performance standards for antimicrobial susceptibility tests, Clinical Laboratory Standard Institute (CLSI, 2020) [2]. Antibiotic discs used in the present study and the results of their sensitivity and resistance are shown in the Table 1.

Table 1: Antimicrobial susceptibility pattern of *E. coli* and *Salmonella* isolates

Bacterial species isolated	S/R	Amoxicillin	Amoxyclav	AMS*	Cefaperazone	Cefixime	Ceftriaxone	CIT#	Cephalexin	Chloramphenicol	Ciprofloxacin	Co-trimoxazole	Doxycycline	Gentamicin	Kanamycin	Levofloxacin	Metronidazole	Nitrofurantoin	Penicillin	Polymyxin B	Streptomycin	Tetracycline
Escherichia coli	S	27.2	30.7	31.9	55.9	13.8	61.5	64.1	20.4	27.2	63.8	67	14.9	47.8	6.79	59.8	0	0	0	7	0	16.9
	R	40.7	37.1	35.9	11.9	53.9	6.38	3.7	47.5	40.6	3.93	4	52.8	19.9	61.1	7.9	100	100	100	61.1	100	50.8
Salmonella spp.	S	42	41.2	87.5	12.4	0	25	50	25	0	100	87.5	62.5	12.4	12.4	100	0	75	37.4	0	37.5	62.5
	R	57.9	58.7	12.4	87.5	100	75	50	75	100	0	12.4	37.4	87.5	87.5	0	100	25	62.5	100	62.4	37.4

Results and Discussion

In the present study, *E. coli* was isolated with the highest frequency of 95.45% from the diarrheic fecal samples (105 out of 110). However, *Salmonella* spp. was isolated at a low frequency with 7.27% from the diarrheic fecal samples (8 out of 110).

These findings agree with the results of Younis *et al*. (2009) [27] who reported *E. coli* as predominant isolate with 45.25% (20 out of 193) followed by *Salmonella* spp. with 4.09% (9 out of 193) in calf diarrheic cases. The findings of the present study is also in accordance with that of Nasr *et al*. (2014) [16] who reported similar prevalence of *E. coli*, followed by *Salmonella* and others in pathogenic bacteria associated enteritis in lambs in Behera provinces of Egypt.

Similar to the present study, several other studies conducted by many researchers *viz*., Joon and Kaura (1993) [10], El-Seedy *et al*. (2016) [6], Ashraf *et al*. (2017) [1], Manickam and Ponnusamy (2017) [13], Mona *et al*. (2020) [14], Sharma *et al*. (2015) [20], Rahn *et al*. (1992) [18] worldwide revealed the predominance of *E. coli* over the other bacterial species in causing diarrhea in calves.

Contrasting results were reported by Reynolds *et al*. (1986) [19] where in 12% of the diarrheic fecal samples were positive for *Salmonella* spp. and 3% samples were positive for *E. coli* K99. Hoque and Samad, (1996) [8] isolated *Salmonella* (9.61%) from calves and they have not isolated *E. coli* from the diarrheic calves.

In vitro antibiotic resistance pattern of the *E. coli* isolates was determined by disc diffusion method described by Bauer *et al*. (1966) [2]. The *E. coli* and *Salmonella* spp. positive samples were subjected to antimicrobial susceptibility test to study the susceptibility or resistance patterns. In our study the result of antibiogram pattern of *E. coli* isolates when tested against 21 commonly used antimicrobial agents reflected varying sensitivity. The highest sensitivity was observed for co-trimoxazole (67%), followed by ceftriaxone + tazobactum (64.09%), ciprofloxacin (63.89%), ceftriaxone (61.45%), levofloxacin (59.88%), cefaperazone (55.88%) and gentamicin (47.87%). Results also showed that all the 105 isolates were resistant to metronidazole, nitrofurantoin, penicillin and streptomycin (100% resistance) followed by polymyxin B (61.11%), kanamycin (61.04%), cefixime (53.98%), doxycycline (52.89%), tetracycline (50.85%), cephalexin (47.46%), amoxycillin (40.7%**)** and chloramphenicol (40.67%). Our findings were similar to the findings of Sruthy (2019) [23] who reported that all the 41 isolates were resistant to metronidazole (100% resistance) followed by penicillin (82.93%), ceftazidime (80.49%), amoxicillin + clavulanic acid (80.49%), furazolidone (75.61%), ceftriaxone (70.74%), amoxycillin (68.30%) and amoxicillin + sulbactam (56.10%) (Plate 5A).

Eight *Salmonella* isolates were found sensitive to ciprofloxacin and levofloxacin (100%) followed by co-trimoxazole (87.5%), amoxycillin +sulbactum (87.5%), nitrofurantoin (75%), tetracycline (62.5%) and doxycycline (62.5%). Results also showed that all the eight isolates were resistant to metronidazole, cefixime and chloramphenicol (100% resistance) followed by gentamicin (87.5%), kanamycin (87.5%), cefaperazone (87.5%), ceftriaxone (75%), cephalexin (75%), penicillin (62.5%) and streptomycin (62.5%) (Plate 5B).

Previous studies by Wani *et al*. (2013) [26] where they used 23 *E. coli* isolates isolated from of 286 fecal samples of calves and subjected it to antimicrobial susceptibility test using ten

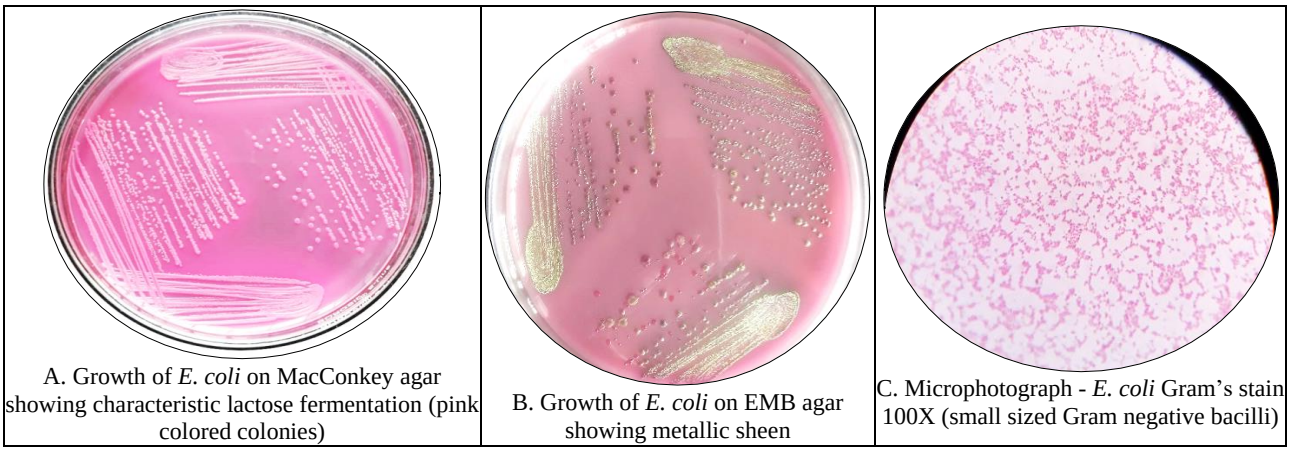
antimicrobial agents that were used to treat calf diarrhea. High susceptibility was exhibited to amikacin (100%), followed by gentamicin (83%), enrofloxacin (74%), ciprofloxacin (74%), norfloxacin (70%), streptomycin (61%), chloramphenicol (57%), oxytetracycline (57%), cefotaxime (56%) and ceftriaxone (56%). Most of the isolates were resistant to co-trimoxazole (91%), followed by ampicillin (78%), cephalexin (74%) and co-amoxiclav (amoxicillin +clavulanic acid) (74%). The *Salmonellae* isolates were sensitive to all antibiotic disc tested except ampicillin, co-trimoxazole, oxytetracycline and streptomycin. One isolate of *S. Enteritidis* was MDR and it was sensitive only to the streptomycin.

Contrary to our study Manickam and Ponnusamy (2017) [13] performed antimicrobial susceptibility testing by using disc diffusion method and found bacterial isolates sensitive to amikacin (55%), ceftriaxone (55%), ciprofloxacin (69%), kanamycin (81.5%) and nalidixic acid (75.5%). Similarly, majority of the bacterial isolates showed resistance to ampicillin (75%), amoxycillin (62%), ceftriaxone (45%), chloramphenicol (68%), gentamicin (50%), streptomycin (65%) and tetracycline (74%).

Srivani *et al.* (2017) [22] revealed that upon antimicrobial susceptibility testing, 69.81% of the Shiga-Toxin Producing *Escherichia coli* (STEC) isolates were resistant to three or more antimicrobial agents. Among the STEC isolates, highest percentage of antimicrobial resistance was observed for tetracycline (63.21%), followed by ampicillin (48.11%), aztreonam (36.79%), cefotaxime (31.13%), ceftazidime (31.13%), streptomycin (31.13%), nalidixic acid (29.25%), sulfisoxazole (28.30%), cotrimoxazole (26.42%), amoxicillin +clavulanic acid (20.75%), piperacillin +tazobactem (18.87%), meropenem (17.92%), kanamycin (12.26%), nitrofurantoin (12.26%), ciprofloxacin (4.72), chloramphenicol (3.77%) and gentamycin (3.77%) while lowest % of 0.94 was observed for imipenem antibiotics. Malik *et al.* (2013) [12] also observed that the *E. coli* isolated from diarrheic calves in Uttar Pradesh, India showed highest antimicrobial resistance to the tetracycline and ampicillin whereas, highest susceptibility was observed for gentamicin. The *E. coli* isolates from calves in Bangladesh (Mushtaq *et al.*, 2013) [15] were showing highest sensitivity to chloramphenicol and gentamicin which, corroborate with the findings of the present study.

In this study, the high prevalence of multidrug-resistant bacteria observed may be the cause for treatment failures and may result in economic losses for the dairy farmers. With the continuing emergence of antibiotic resistance, it is imperative that actions should be taken to prolong the effectiveness of existing antibiotics while maintaining the levels of food animal production. Multidrug resistance showed by these isolates in this study is alarming. This may be due to indiscriminate use of antibiotics in clinical practice. This study detected that most of the isolates were susceptible to ciprofloxacin, levofloxacin, ceftriaxone-tazobactum, co-trimaxazole, cefaperazone, gentamicin and amoxycillin-sulbactum antibiotics, because these were seldomly used in the treatment of calf diarrhea cases in the study area.

Hence, present investigation emphasizes on judicious selection of antibiotics or antimicrobial agents, preferably after *in vitro* antimicrobial susceptibility testing and using such antimicrobials at an adequate dose for sufficient duration for effective treatment and control of calf diarrhea caused by *E. coli* and *Salmonella*.

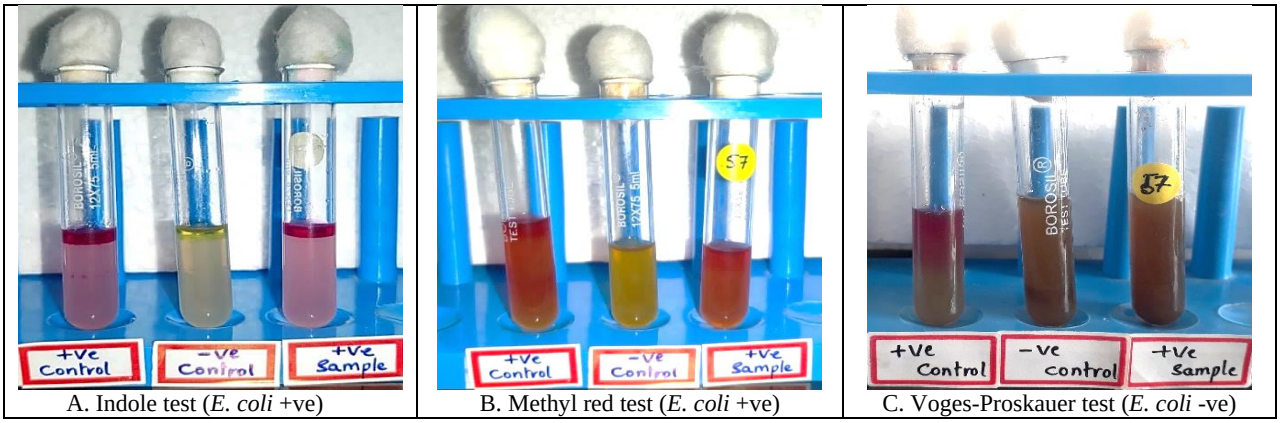


A. Growth of *E. coli* on MacConkey agar showing characteristic lactose fermentation (pink colored colonies)

B. Growth of *E. coli* on EMB agar showing metallic sheen

C. Microphotograph - *E. coli* Gram's stain 100X (small sized Gram negative bacilli)

Plate 2A: Panel of biochemical tests used for the identification of *E. coli*

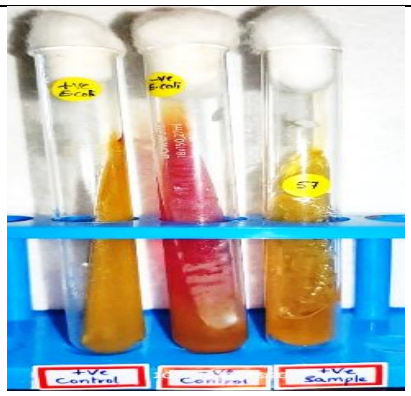


A. Indole test (*E. coli* +ve)

B. Methyl red test (*E. coli* +ve)

C. Voges-Proskauer test (*E. coli* -ve)

D. Citrate test (*E. coli* -ve)



E. Growth on TSI slant (Y slant/Y butt)

F. Urease test (*E. coli* -ve)

Plate 2B: Biochemical tests for the identification of *E. coli*

A. Oxidase test (*E. coli*–ve)

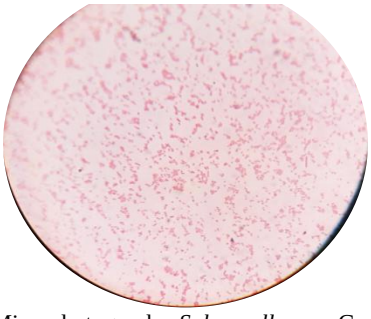
B. Catalase test (*E. coli* +ve)

Plate 3: Identification of *Salmonella* spp. by cultural characteristics and morphology

A. Growth of *Salmonella* spp. on MacConkey agar showing characteristic non-lactose fermentation (pale coloured colonies)

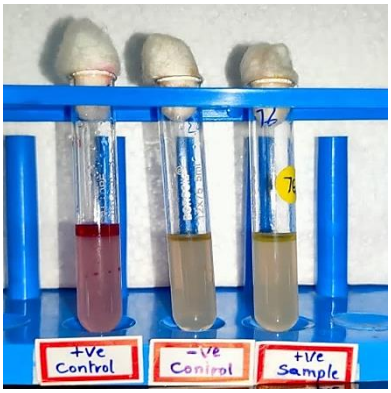
B. Growth of *Salmonella* spp. on XLD agar showing black centered colonies

C. Growth of *Salmonella* spp. on BGA agar showing pinkish-white colonies

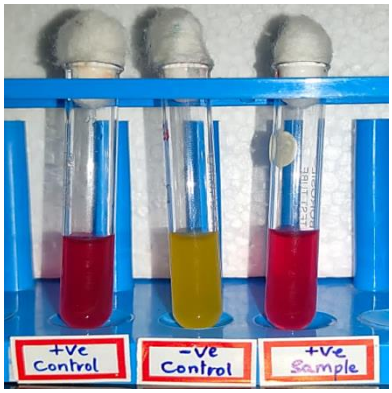


D. Microphotograph - *Salmonella* spp. Gram's stain 100X (small sized Gram-negative bacilli)

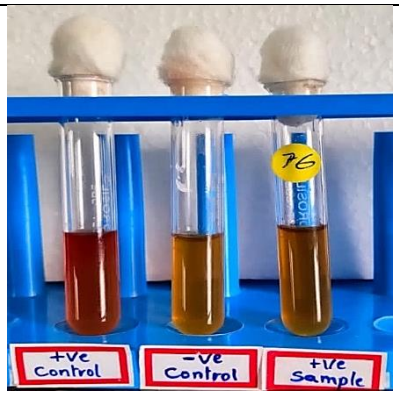
Plate 4A: Panel of biochemical tests used for the identification of *Salmonella* spp.



A. Indole test (*Salmonella* spp. –ve)



B. Methyl red test (*Salmonella* spp. +ve)



C. Voges-Proskauer test (*Salmonella* spp. –ve)

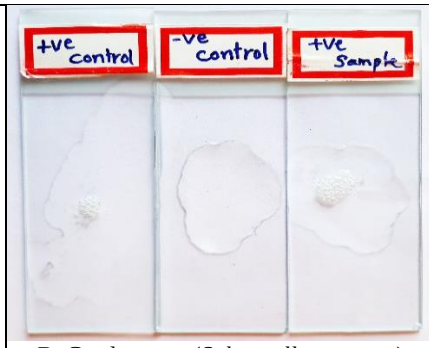
D. Citrate test (*Salmonella* spp. +ve)

E. Growth on TSI slant (*Salmonella* spp. R slant/ Y butt, H_2S)

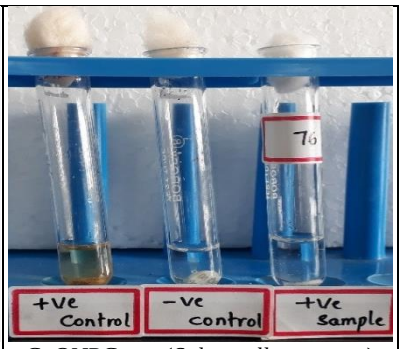
F. Urease test (*Salmonella* spp. –ve)

Plate 4B: Biochemical tests for the identification of *Salmonella* spp.

A. Oxidase test (*Salmonella* spp. –ve)

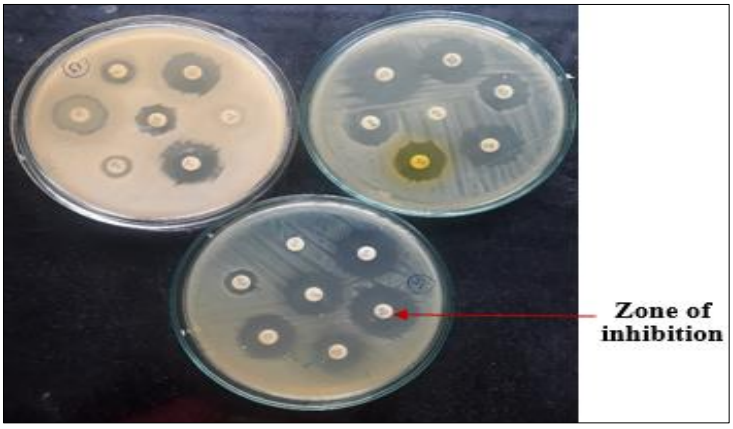


B. Catalase test (*Salmonella* spp. +ve)

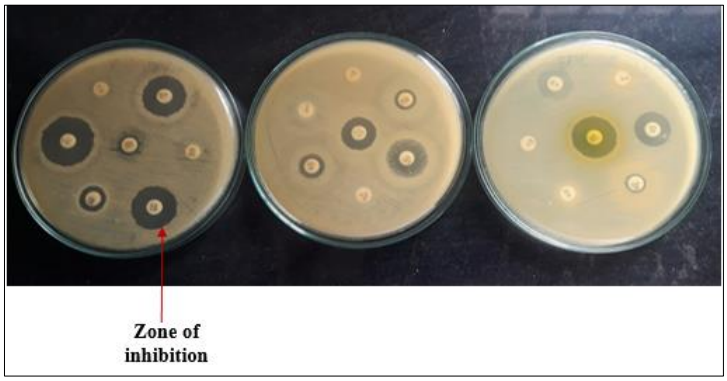


C. ONPG test (*Salmonella* spp. –ve)

Plate 5: Antimicrobial susceptibility pattern of bacterial isolates



A. Antimicrobial susceptibility pattern of *E. coli* isolate



B. Antimicrobial susceptibility pattern of *Salmonella* isolate

Conclusion

In our study, *E. coli* was the major bacterial agent associated with calf diarrhea with the prevalence rate 95.45% (105/110) followed by *Salmonella* with 7.27% (8/110). The most effective antibiotic against *E. coli* isolated from fecal samples of calves was found to be co-trimoxazole followed by ceftriaxone-tazobactum combination and ciprofloxacin. Metronidazole, nitrofurantoin, penicillin and streptomycin followed by polymyxin B and kanamycin were found to be least effective to *E. coli* isolates. While ciprofloxacin and levofloxacin are found to be most effective towards *Salmonella* isolates isolated from calf diarrhea followed by co-trimoxazole and amoxicillin-sulbactum. Metronidazole, cefixime and chloramphenicol were least effective to *Salmonella* isolates.

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