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Isolation, identification, molecular characterization and antibiogram studies of *Salmonella* spp. isolated from calf diarrhea in and around Udaipur (Rajasthan)

Anju Chaudhary, Sudeep Solanki, Deepak Sharma, Rajesh Singathia, Karishma Rathore, Arpita Sain and Durga Devi

Abstract
Background: Calf diarrhoea has been a major threat to dairy farms and industry. It is a multi-etiology disease with *Salmonella spp.*as one of the predominant cause. Emergence of antimicrobial resistance is of serious concern because it has rendered the present day antibiotics less effective. The present study aims for isolation, molecular characterization and antimicrobial resistant profiling of *Salmonella spp.* from calf.
Method: A total of 100 fecal samples were collected from calves of cattle and buffalo of below 3 months of age. Sterile fecal swabs (Hi-media, India) were used for sample collection. All the samples were transported immediately to the laboratory under cold chain and were processed for isolation of *Salmonella spp.* using standard microbiological techniques. For isolation and identification of *Salmonella spp.* a loopful of fecal sample was enriched in Selenite broth and incubated at 42 °C for 12-14 hours. After that a loopful of selenite broth was inoculated and streaked separately onto selective agar plates as xylose Lysine Deoxycholate (XLD) agar, Brilliant Green agar (BGA), MacConkey's agar and incubated at 37 °C for 24hours. Partially identified cultures were suspended in normal saline and smeared over a slide. The smears were allowed to air dry followed by heat fixing and Gram's staining. All Gram negative culture was subjected to biochemical characterization by using various biochemical tests such as catalase, oxidation, oxidation-fermentation, Indole, methyl –red, Voges-Poskauer, citrate test, TSI and urease test. Molecular characterization by Genomic DNA isolation using heat treatment method All the biochemically confirmed isolates for *Salmonella* spp. Where subjected to PCR for the presence of *inv*A gene (284bp) genus-specific primer (F-GTGAAATTATCGCCACGTTCGGGCAA) (R-TCATCGCACCGTCAAAGGAACC) (Borges *et al.*, 2017) [4] were used to amplify *Salmonella* strain.
Results: Only 3% (3/100) of them were found positive for *invA* (universal stress protein) gene. The nucleotide sequences flanking the gene encoding the *invA* and was found to be highly specific for *Salmonella*. The antibiogram of *Salmonella* isolates by Kirby Bauer disc diffusion method revealed highest resistance against azithromycin, streptomycin and tetracycline and lowest against ciprofloxacin and gentamicin. All three isolates were MDR. Hence, surveillance based study and search for an alternative to antibiotics is needed to address the global issue of the antimicrobial resistance.

Keywords: Calf diarrhea, Multidrug-resistant (MDR) *Salmonella*, *inv*A gene, Antibiogram

Introduction
The success of replacement calf and heifer production is critical to the future of dairy industry. Young animals have a higher death rate than adults, which has a major impact on the cattle industry's finances (Muktar *et al*., 2015) [10]. Calf diarrhoea is caused mostly by viruses such as bovine coronavirus, bovine rotavirus group A and bovine viral diarrhoea virus, bacteria such as *Salmonella* spp., *Escherichia coli*, and *Clostridium perfringens* type C (Bhat *et al.*, 2013) [3]. *Salmonella* are Gram-negative, rod-shaped bacteria that belong to the Enterobacteriaceae family and are facultative anaerobes. *Salmonella enterica* and *Salmonella bongori* are two wide species of the genus *Salmonella*, based on differences in their 16S rRNA sequence analysis (Mezal *et al*., 2014) [9].
The scouring calf loses fluids, dehydrates quickly, loses electrolytes, and develops acidosis. Infectious pathogens may cause initial damage to the intestine, but dehydration, acidosis, and electrolyte loss are the most common causes of scours death (Radostits *et al.*, 2007) [13]. Reduced weight gain and development, increased time to first calving, and reduced milk production in the first lactation are all long-term effects of NCD in dairy heifers, all of which result in significant economic losses to the livestock sector, as well as excessive antibiotic use,

which could lead to antibiotic resistance (Afema *et al.*, 2019) [1]. Antimicrobial resistance (AMR) in food animal production systems is a global concern, as AMR infections can result in increased animal morbidity and death, as well as public health repercussions. Many factors can contribute to antimicrobial resistance, including changes in bacterial cell wall permeability or target sites, enzymatic drug modifications, and energy-dependent removal of antimicrobials via membrane-bound efflux pumps (Chen *et al*., 2005) [5].

Material and Methods

Sample collection

Fecal samples of neonatal calves of cattle and buffalo were collected from in and around college of veterinary and animal science, Navania Vallabhnagar, Udaipur (Rajasthan). Basic information about the dairy farm and management was taken. A total of 100 faecal samples were collected from calves of cattle and buffalo of below 3 months of age. Sterile fecal swabs (Hi-media, India) were used for sample collection. All the samples were transported immediately to the laboratory under cold chain and were processed for isolation of *Salmonella spp.* using standard microbiological techniques.

Bacterial isolation

For isolation of *Salmonella spp.* a loopful of fecal sample was enriched in Selenite broth and incubated at 42 °C for 12-14 hours. After that a loopful of selenite broth was inoculated and streaked separately onto selective agar plates as xylose Lysine Deoxycholate (XLD) agar, Brilliant Green agar (BGA), MacConkey's agar and incubated at 37 ℃ for 24hours.

Identification of Salmonella *spp.*

Gram's staining

Partially identified cultures were suspended in normal saline and smeared over a slide. The smears were allowed to air dry followed by heat fixing and Gram's staining.

Culture and Biochemical characters

All Gram-negative culture was subjected to biochemical characterization by using various biochemical tests such as catalase, oxidation, oxidation-fermentation, Indole, methyl – red, Voges-Poskauer, citrate test, TSI and urease test.

Molecular characterization

DNA extraction

Genomic DNA isolation was carried out by heat treatment method as described (Dashti *et al.*, 2009) [6]. *Salmonella* colony was inoculated in BHI broth was incubated at 37 ℃ overnight. After incubation streaked on nutrient agar plate with the help of sterile loop and incubated for 24 hours. Isolated colonies of bacteria were used and placed in a test tube containing 1 ml of nuclease free water and kept in water bath for 10 minutes at 100℃ and then centrifuged at 1000 rpm for five minutes. Supernatant was collected in Eppendorf tube and store at -20 ℃ till further use.

PCR confirmation. All the biochemically confirmed isolates for *Salmonella spp.* Where subjected to PCR for the presence of *inv*A gene (284bp) genus-specific primer (F-GTGAAATTATCGCCACGTTCGGGCAA) (R-TCATCGCACCGTCAAAGGAACC) (Borges *et al.*, 2017) [4] were used to amplify *Salmonella* strain.

PCR steps

PCR steps	Temperature	Duration
Initial denaturation	95 ℃	5 min
Denaturation	95 ℃	1min
Annealing	55 ℃	1 min
Extension	72 ℃	1 min
Elongation	72 ℃	5 min

Antimicrobial resistance profiling

The detailed antibiogram of *Salmonella* isolates was determined by against 8 antibiotics belonging to different classes Sulphonamides, Beta-lactam, Aminoglycosides, Tetracycline, Macrolids and Quinolones group by Kirby Bauer disc diffusion method (CLSI, 2018). The antibiotics disc used were chloramphenicol, gentamicin, ampicillin, cotrimaxazole, tetracycline, streptomycin, azithromycin, and ciprofloxacin (Table 1).

The *Salmonella* isolates were grown in 5 ml LB broth (BD, BBL Difco, USA) at 37 °C for 8 hr to develop turbidity of 0.5 McFarland standards. Uniform bacterial lawn was prepared on sterile Mueller Hinton Agar (MHA BD, BBL Difco, USA) plates surface with the help of cotton swab using aseptic technique. The antibiotic discs were placed on MHA with adequate spacing between each other with sterilized forceps. The discs were gently pressed to ensure full contact with the medium. The plates were then incubated overnight at 37 °C. Next day, the total diameter of the zone of inhibition was recorded in mm. The result was interpreted as per standard guidelines (CLSI, 2018).

Results and Discussion

Isolation and identification of *Salmonella spp.* following Gram's staining the smear revealed gram-negative pink rods of different shape and size. The cultural characters of the isolated Salmonellae appeared on MCA agar gave pale, colorless, smooth and transparent (non-lactose fermenter) colony, on BGA isolated produce pale pink color colonies against a pinkish background and on XLD agar produced with transparent zone of reddish color with black center (fig.1 and Fig.3). Application of different biochemical tests revealed the following results; negative oxidase reaction, negative urea hydrolysis, positive reaction on TSI agar, negative Indole reaction, positive MR reaction, negative VP reaction and positive on Simmon's Citrate agar (fig.2).

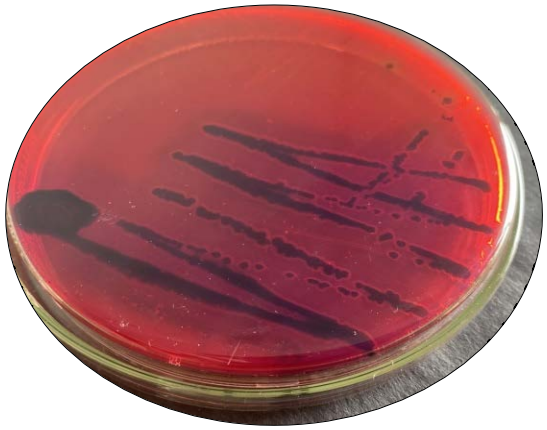

Fig 1: Growth of test culture on xylose lysine Deoxycholate agar

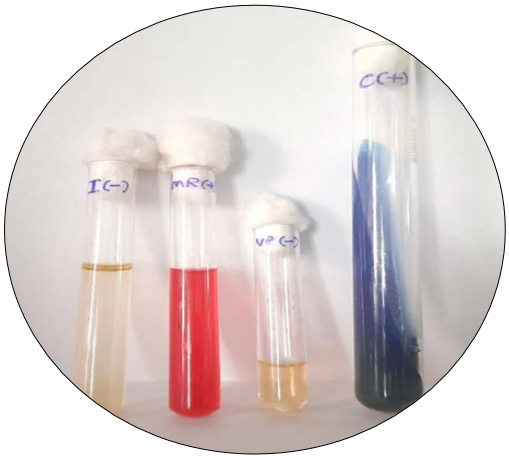

Fig 2: IMVIC Test of isolates

Fig 3: Growth of Salmonella on BGA agar

Molecular characterization

The PCR results with *Salmonella* isolated from diarrheic calf of *invA* gene were detected in three strain of *Salmonella.* (Fig.4)

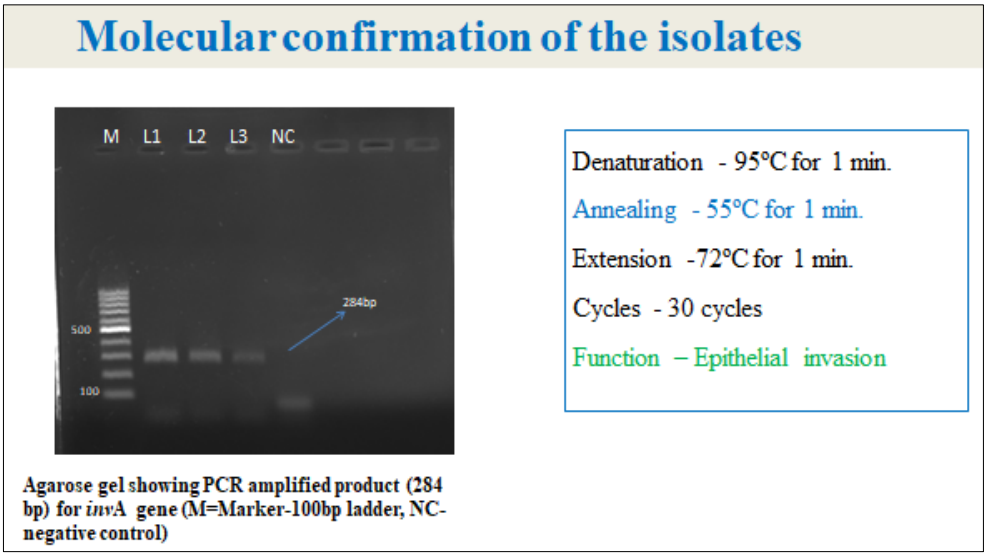

Fig 4: Molecular Confirmation of Salmonella by invA gene.

Antibacterial drug resistance profiling of the *Salmonella* isolates

Antibiotic susceptibility pattern of the 3 *Salmonella* isolates were determined by Kirby Bauer disc diffusion method against 8 different antibiotics (fig. 3). The overall resistance to azithromycin (100%), streptomycin (100%), tetracycline (100%) and ampicillin (66.6%). Moderate resistant to gentamicin (66.6%) and ciprofloxacin (66.6%) (Table 2). There was wide spread multidrug resistance among the *Salmonella* isolates, All of isolates were multi-drug resistant i.e. resistant against 3 or more antibiotics (Fig.5)

Neonatal calf mortality

Neonatal diseases and mortality among cattle and buffalo is

the major cause of economic losses in the livestock sector (Singh *et al.*, 2009) [15]. High morbidity and mortality during neonatal period is primarily due to diarrhea (Svensson *et al.*, 2006) [16]. *Salomonella* is considered as the most common bacterial cause of neonatal diarrhea. In the present study, all the 3 diarrheic samples were positive for *Salmonella spp.* A similar finding was obtained by Nair *et al*., (2015) [11] in India reported 2.5% prevalence, whereas 3% prevalence, reported in and around CVAS, Navania, Udaipur (Rajasthan). The differences in the prevalence rates of *Salmonella spp.* among diarrhoeic calves may be attributed to the geographical locations of the farm, management practice and hygienic measures (El-Seedy *et al.*, 2016) [8]. Therefore, it is imperative to study the prevalence of enter pathogens and related risk factors which can modulate the occurrence of diarrhoea among calves.

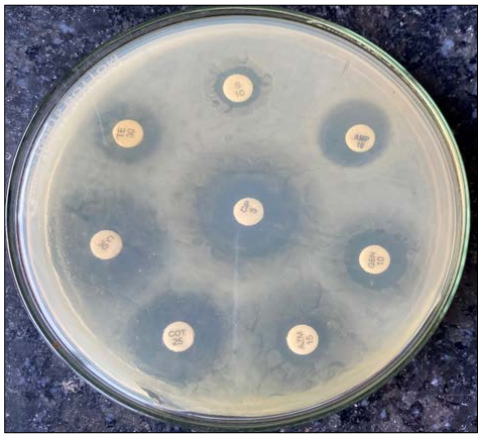

Fig 5: ABST of *Salmonella* Spp.

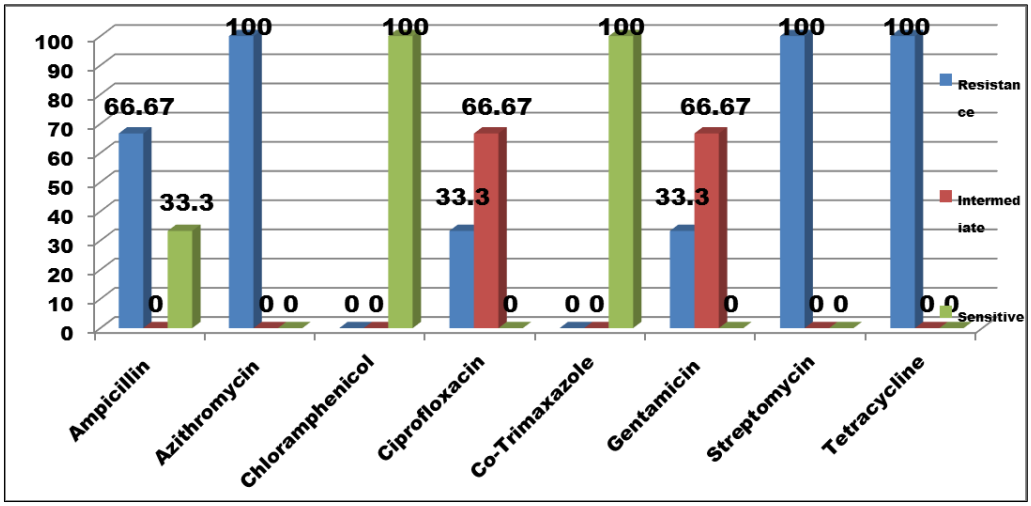

Fig 6: Antibiogram of Salmonella isolated from Calf Diarrhea.

Table 1: Normal indicator of Antibiogram for antibiotics.

S. No	Antimicrobial agent	Symbol	Disc content	Sensitive	Intermediate	Resistant
1	Ampicillin	Amp	10mcg	17	14-16	13
2	Azithromycin	AZM	15mcg	18	14-17	13
3	Chloramphenicol	C	30mcg	18	13-17	12
4	Ciprofloxacin	CIP	05mcg	21	16-20	15
5	Co-trimaxazole	CO-T	25mcg	16	14-15	10
6	Gentamicin	GEN	10mcg	15	12-14	11
7	Streptomycin	S	10mcg	15	12-14	11
8	Tetracycline	TE	30mcg	19	15-18	14

Table 2: Antibiogram of *Salmonella spp isolates*

S. No	Antibiotics	Symbol	Total isolates	Resistance	Intermediate	Sensitive
1	Ampicillin	AMP	3	2(66.7%)	0(0.0%)	1(33.3%)
2	Azithromycin	AZM	3	3(100%)	0(0.0%)	0(0.0%)
3	Chloramphenicol	C	3	0(0.0%)	0(0.0%)	3(100%)
4	Ciprofloxacin	CIP	3	1(33.3%)	2(66.7%)	0(0.0%)
5	Co-trimaxazole	COT	3	0(0.0%)	0(0.0%)	3(100%)
6	Gentamicin	GEN	3	1(33.3%)	2(66.7%)	0(0.0%)
7	Streptomycin	S	3	3(100%)	0(0.0%)	0(0.0%)
8	Tetracycline	TE	3	3(100%)	0(0.0%)	0(0.0%)

Molecular confirmation by *inv*A gene

Three isolates strain of *Salmonella* spp. gave positive result for *invA* gene.

Antimicrobial resistance

Extensive use of antibiotics in animal husbandry and veterinary practices at sub-therapeutic levels for growth promotion, prophylaxis and treatment had led to the development of antibiotic resistance in bacteria (Wileman *et al.*, 2009) [18]. Antimicrobial resistance is an increasing global threat to both human and animal health (Tadesse *et al.*, 2012) [17]. In the present study, the antibiogram pattern was recorded against 08 different antibiotics revealed 100% (3/3) were MDR. The overall resistance was maximum (100%) against

tetracycline streptomycin, azithromycin followed by ampicillin (66.6%) and ciprofloxacin, gentamicin each (33.3). One possible reason for such a widespread resistance may be intrinsic or acquired through spontaneous mutations (de novo), or may occur due to horizontal gene transfer from donor bacteria, phages, or free DNA (Dodd, 2012) [7]. Due to horizontal gene transfer even nonpathogenic microbial species that harbor resistant genes serve as an ecological reservoir for pathogenic bacteria (Salyers and Shoemaker, 2006) [14]. These mechanisms include the uptake of naked DNA and mobile genetic elements such as plasmids, transposons, integrons, gene cassettes, and bacteriophages (Nwosu, 2001) [12].

It can be concluded from the present study that; in diarrhoeic calves harvested MDR *Salmonella* which was around College of veterinary and animal science Navania, Udaipur (Rajasthan). The increasing trends of multiple drug resistance among *Salmonella* isolates from neonatal calves are of utmost concern. The large number of calves dies during pre-weaning age causing heavy loss to livestock production and in turn to economy. Hence, surveillance-based study, antimicrobial resistance profiling and search for an alternative to antibiotics is the need of hour to address the global issue of the antimicrobial resistance.

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References

1. Afema JA, Davis MA, Sischo WM. Antimicrobial use policy change in pre-weaned dairy calves and its impact on antimicrobial resistance in commensal *Escherichia coli*: A cross sectional and ecological study. BMC microbiology. 2019;19(1):1-14.
2. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 1966;45(4):493-6.
3. Bhat SA, Juyal PD, Singla LD. Bovine cryptosporidiosis: brief review of its distribution in India. Trends Parasitol. Research. 2013;2(2):5-13.
4. Borges KA, Furian TQ, de Souza SN, Menezes R, Salle CTP, de Souza Moraes HL, *et al*. Phenotypic and molecular characterization of *Salmonella* Enteritidis SE86 isolated from poultry and salmonellosis outbreaks. Foodborne pathogens and disease. 2017;14(12):742-754.
5. Chen S, Zhao S, McDermott PF, Schroeder CM, White DG, Meng J. A DNA microarray for identification of virulence and antimicrobial resistance genes in *Salmonella* serovars and *Escherichia coli*. Molecular and cellular probes. 2005;19(3):195-201.
6. Clinical and Laboratory Standards Institute. 2018. Performance standards for antimicrobial disk susceptibility tests, 13th ed. CLSI standard M02. Clinical and Laboratory Standards Institute, Wayne, PA.
7. Dashti AA, Jadaon MM, Abdulsamad AM, Dashti HM. Heat treatment of bacteria: a simple method of DNA extraction for molecular techniques. Kuwait Medical Journal. 2009;41(2):117-122.
8. Dodd MC. Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. Journal of Environmental Monitoring. 2012;14(7):1754-1771.
9. El-Seedy FR, Abed AH, Yanni HA, Abd El-Rahman SAA. Prevalence of *Salmonella* and *E. coli* in neonatal diarrhoeic calves. Beni-Suef University journal of basic and applied sciences. 2016;5(1):45-51.
10. Mezal EH, Sabol A, Khan MA, Ali N, Stefanova R, Khan AA. Isolation and molecular characterization of *Salmonella enterica* serovar Enteritidis from poultry house and clinical samples during 2010. Food microbiology. 2014; 38:67-74.
11. Muktar Y, Mamo G, Tesfaye B, Belina D. A review on major bacterial causes of calf diarrhoea and its diagnostic method. Journal of Veterinary Medicine and Animal Health. 2015;7(5):173-185.
12. Nair A, Rawool DB, Doijad S, Poharkar K, Mohan V, Barbuddhe SB, *et al*. Biofilm formation and genetic diversity of *Salmonella* isolates recovered from clinical, food, poultry and environmental sources. Infection, Genetics and Evolution. 2015; 36:424-433.
13. Nwosu VC. Antibiotic resistance with particular reference to soil microorganisms. Research in Microbiology. 2001;152(5):421-430.
14. Radostits OM, Gay CC, Hinchcliff KW, Constable PD. Veterinary Medicine: A textbook of diseases of cattle, horses, sheep, pigs and goats.10th Ed., Elsevier Scientific Publications, Saunders, 2007, p 2160.
15. Salyers A, Shoemaker NB. Reservoirs of antibiotic resistance genes. Animal biotechnology. 2006;17(2):137-146.
16. Singh DD, Kumar M, Choudhary PK, Singh HN. Neonatal calf mortality-An overview. Intas Polivet. 2009;10(2):165-169.
17. Svensson C, Linder A, Olsson SO. Mortality in Swedish dairy calves and replacement heifers. Journal of dairy science. 2006;89(12):4769-4777.
18. Tadesse DA, Zhao S, Tong E, Ayers S, Singh A, Bartholomew MJ, *et al*. Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950–2002. Emerging infectious diseases. 2012;*18*(5):741.
19. Wileman BW, Thomson DU, Reinhardt CD, Renter DG. Analysis of modern technologies commonly used in beef cattle production: Conventional beef production versus nonconventional production using meta-analysis. Journal of animal science. 2009;87(10):3418-3426.