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Detection of ESBL and ACBL-producing *Salmonella enterica* in Cattle from Different Districts of South Bengal

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ABSTRACT

Background: *Salmonella* spp. is a pathogenic bacterium that can cause infection in animals and human beings. Drug-resistant Salmonellae make this infection more deadly. In this background, this study was undertaken to investigate the prevalence and antimicrobial resistance pattern of ESBL / ACBL-producing *Salmonella enterica* strains, obtained from bovine faecal samples from different districts of West Bengal.

Methods: A total of 170 bovine faecal samples were aseptically collected from Purba Barddhaman, Birbhum and North 24 Paraganas in this study. Standard procedures were followed to identify *Salmonella enterica* isolates followed by their detailed characterization and *in-vitro* antibiogram.

Result: A total of 35 (20.59%) *Salmonella* spp. isolates were recovered after detailed morphological and biochemical characterization which were also confirmed by PCR. Higher prevalence was recorded in cross-breed jerseys (88.57%) and younger cattle within 2 yrs. of age (24.53%). A total of 18 *Salmonella* spp. (51.24%) isolates were detected as ESBL and 29 *Salmonella* spp. (82.86%) isolates were detected as ACBL producers both phenotypically. In molecular characterization, the *bla*AmpC (91.43%) gene was the highest followed by other genes. No TEM gene was detected in this study. Antibiogram of all the ESBL-producing *Salmonella* spp., isolates showed high-level resistance (58-92%) to various antibiotics like cefotaxime, doxycycline, ampicillin/cloxacillin, tetracycline, enrofloxacin etc. Antibiotics like azithromycin, chloramphenicol, HL gentamicin *etc*. were sensitive to these isolates.

Key words: ACBL, Antibiogram, Cattle, ESBL, Faeces, Salmonellosis.

INTRODUCTION

Livestock is a source of subsidiary income for many families in India especially the resource-poor who maintain few heads of animals. Cows and buffaloes if in milk will provide regular income to the livestock farmers through the sale of milk (BAHS, 2014). Thus, the animal husbandry sector plays an important role in a country's national economy and socio-economic development. Out of the 535.78 million livestock populations in India, cattle represent over 35.97% (192.49 million) of the animals, the 2nd highest in the World. In West Bengal, out of a total 37.4 million livestock population, cattle represent over 50.80 per cent (19 million) of the animals [Annual Report 2018-19, DAHD].

Salmonellosis in cattle is one of the main zoonotic diseases worldwide. *Salmonella* is a genus of gram-negative, facultative anaerobic bacteria that belong to the family of *Enterobacteriaceae*. *Salmonella enterica* is an important public health pathogen causing foodborne zoonoses. *Salmonella* infections cause gastrointestinal symptoms predominantly with generalist serotypes and are often characterized by high morbidity but low mortality (Hoelzer *et al.,* 2011). *Salmonella* spp. can produce ESBLs and can show resistance to extended-spectrum cephalosporins due to the action of the plasmid AmpC gene (Rodríguez *et al.,* 2009).

Salmonella organisms carried zoonotic importance. It is the most common food-borne pathogenic bacteria in humans as well as in animals (Cummings *et al.,* 2010). Foodborne infections are the most common form (Scallan *et al.,* 2011; Cummings *et al.,* 2012) of Salmonellosis caused by undercooked foods and unpasteurized dairy products. Thus, the drug-resistant *Salmonella* spp. can be a matter of great concern in starting a foodborne zoonosis. In this context, the present study was aimed at the prevalence study of salmonellosis in cattle, identification of the pathogen and detailed characterization, detection of

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antibiotic resistance (ESBL) properties in the positive strains and *in-vitro* drug resistance study of ESBL-producing *Salmonella* spp isolates.

MATERIALS AND METHODS

A total of 170 faecal samples (135 non-descript and 35 crossbreed Jersey cows) were collected from Indigenous or Deshi and Crossbred Jersey cattle during the period January to July 2022. All these animals were reared on free-range systems with no history of disease outbreaks. All the samples were collected on a random basis from these apparently healthy cattle from Purba Barddhaman, Birbhum and North 24 Parganas districts of West Bengal (Table 1). The faecal swab samples were aseptically collected directly from animals and were kept in sterile containers with peptone water (HiMedia, India) under ice cover for transport.

All the swabs were enriched into Selenite F broth (HiMedia, India) at 37°C for 24 hours and were streaked into Xylose Lysine Deoxycholate (XLD) Agar (HiMedia, India) and Salmonella Shigella agar (SSA, HiMedia, India) separately and incubated at 37°C, for approx. 16-18hrs.

Table 1: Details of sample collection from different districts of West Bengal and positivity (%) of ESBL and ACBL genes.

Districts of collection	No. of samples collected	No. of *Salmonella enterica* isolated (%)	ESBL gene positivity	ACBL gene positivity
Purba Barddhaman	52	12 (23.07)	9	10
North 24 Paraganas	67	18 (26.87)	14	17
Birbhum	51	05 (9.80)	4	5
Total	170	35 (20.59)	26	32

Table 2: Details of PCR conditions followed in this study.

Target gene	Primer details and reaction mixtures (25µl)	PCR conditions
Salmonella *16S rRNA* (1428 bp) (Pradhap *et al.,* 2011)	F: AGAGTTTGATCMTGGCTCAG R: TACGGYTACCTTGTTACGACTT 5 µl DNA samples, 20 µl of PCR mixture containing 1.9 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 15 pmole of each primer and 0.5U of Taq DNA polymerase, 10× Taq buffer, 0.05% Tween 20	Initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 92°C for 45s, annealing at 50°C for 15s and elongation at 72°C for 2 minutes and final extension at 72°C for 2 mins.
*bla*SHV genes (392 bp) (Colom *et al.* 2003)	F: AGGATTGACTGCCTTTTTG R: ATTTGCTGATTTCGCTCG 5× Taq buffer (Promega, USA), 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 picomol of each primer, 1U Taq DNA polymerase and 5µl DNA template	Initial denaturation of 3mins at 94°C, then 35 cycles of 94°C for 30 secs, 54°C for 30 secs (annealing), 72°C for 1min (extension) and final extension of 10 mins at 72°C
*bla*CTX-M gene (540 bp) (Weill *et al.* 2004)	F: CAATGTGCAGCACCAAGTAA R: CGCGATATATCGTTGGTGGTTGGTG 5 µl DNA templates, 50 pmol of each primer, 200 mM deoxynucleoside triphosphate, 1U Taq DNA polymerase (Promega, USA), 2 mM $MgC1_2$ and 10% dimethyl sulfoxide	Initial denaturation at 94°C followed by 30s of denaturation at 94°C (10 mins), 30s of annealing at 53°C, 1min of extension at 72°C for 35 cycles and 10 minutes of final extension at 72°C
*bla*TEM genes (Bert *et al.* 2002)	F: ATGAGTATTCAACATTTCCG R: CGTACAGTTACCAATGCTTA 5 µl of DNA templates, 20 picomol of each primer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1U Taq polymerase and 5× Taq buffer (Promega, USA)	Initially at 95°C for 5 mins, followed by 35 cycles of 1min at 95°C (denaturation), 1 min at 55°C (annealing) and 1min at 72°C (extension) with the final extension at 72°C for 10 mins
*bla*AmpC genes (Féria *et al.* 2002)	F: CCCCGCTTATAGAGCAACAA R: TCAATGGTCGACTTCACACC 1U of Taq DNA Polymerase (Promega, USA), 2 mM $MgC1_2$, 200 mM of each deoxynucleoside triphosphates, 100 pmol of each primer, 10% dimethyl sulfoxide and 5 µl of DNA templates	Initial denaturation step of 5 mins at 94°C; followed by 30 cycles of amplification consisting of 30s of denaturation at 94°C, 30s of annealing at 57°C, 1 min of elongation at 72°C and 10 min of final extension at 72°C

The tentatively positive colonies were preserved on separate nutrient agar (HiMedia, India) slants for subsequent biochemical characterization (Quinn *et al.,* 1994). The tentatively positive isolates were checked morphologically by Gram's method of staining and biochemically by different tests as per methods described by Quinn *et al.* (1994) with some modifications.

Molecular confirmation of the *Salmonella* spp. isolates was done by PCR. Bacterial DNAs were extracted as per standard methods (Wani *et al.,* 2004). PCR detection of the *16S rRNA* gene as described by Pradhap *et al.* (2011) was followed for confirmation of the isolates. The primer sequences and predicted length of the PCR products are listed in Table 2.

Phenotypic detection of ESBL and ACBL production in *Salmonella enterica* isolates was done by disc diffusion method (Bauer *et al.,* 1966) following standard protocols (CLSI, 2021; Tan *et al.,* 2009).

Detection of the major beta-lactamases-producing genes in all *Salmonella enterica* strains were attempted by PCR as per Bert *et al.* (2002); Colom *et al.* (2003); Weill *et al.* (2004) and Féria *et al.* (2002). All the PCR primer details and PCR conditions are listed in Table 2. The PCR products were visualized by gel doc system (UVP, UK) after electrophoresis in 1.5% (w/v) agarose (SRL, India) gel containing ethidium bromide (0.5 µg/ml) (SRL, India).

In-vitro antibiotic sensitivity test of ESBL-producing *Salmonella enterica* isolates against 12 different antibiotics (Table 3) by the disc diffusion method as per Bauer *et al.,* (1966) and CLSI (2021).

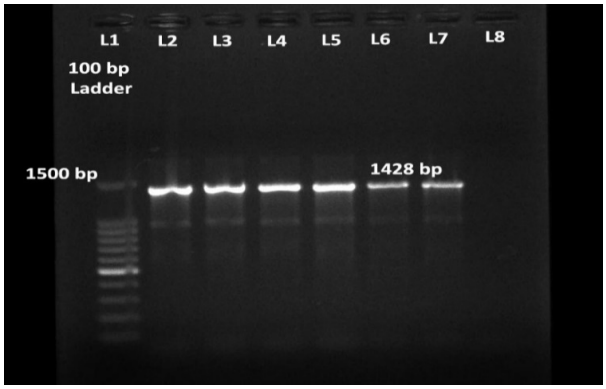
RESULTS AND DISCUSSION

A total of 170 faecal samples screened from cattle from different agroclimatic zones [Table 1] (119 samples from the Gangetic alluvial zone and the rest 51 samples from the Red and Undulating Laterite zone), yielded 35 *Salmonella* spp. isolates with typical characters (Quinn *et al.,* 1994). Scientists like Singh *et al.* (2018), Manishimwe *et al.* (2021), Ranjbar *et al.* (2020) and López-Martin *et al.* (2016) also reported similar positivity of salmonellosis from bovine faecal samples in their studies.

All the *Salmonella* spp. isolates showed standard results in all the biochemical tests such as catalase (+ve), oxidase (-ve), indole (-ve), methyl red (+ve), Voges-

Table 3: Antibiogram of ESBL-positive *Salmonella* spp. isolated (n=26).

Antimicrobials used	Sensitive		Intermediately sensitive		Resistant	
	No.	(%)	No.	(%)	No.	(%)
Enrofloxacin (10 µg)	0	0	3	11.53	23	88.46
Tetracycline (30 µg)	0	0	5	19.23	21	80.77
Co-trimoxazole (25 µg)	19	73.08	2	7.69	5	19.23
Cefotaxime (10 µg)	0	0	2	7.69	24	92.31
HL Gentamicin (120 µg)	21	80.77	5	19.23	0	0
Ampicillin/Cloxacillin (10 µg)	0	0	4	15.38	22	84.62
Amoxicillin/Clavulanic Acid (30/10 µg)	0	0	10	38.46	16	61.54
Doxycycline (30 µg)	0	0	4	15.38	22	84.62
Ciprofloxacin (5 µg)	0	0	11	42.31	15	57.69
Chloramphenicol (30 µg)	23	88.46	3	11.53	0	0
Azithromycin (15 µg)	24	92.31	2	7.69	0	0
Ceftriaxone/Tazobactam (30/10 µg)	18	69.23	8	30.77	0	0



[L1: 100 bp DNA Ladder, L2: Positive control, L3-L7: Test samples, L8: Uninoculated negative control].

Fig 1: PCR amplification of the specific *16S rRNA* gene (1428 bp) of *Salmonella* spp. strains.

Proskauer (-ve), citrate utilization tests (+ve) and urease (-ve) [Quinn *et al.,* 1994; Samanta *et al.,* 2014]. Similarly, Ranjbar *et al.* (2020) and Adel *et al.* (2021) showed similar typical results.

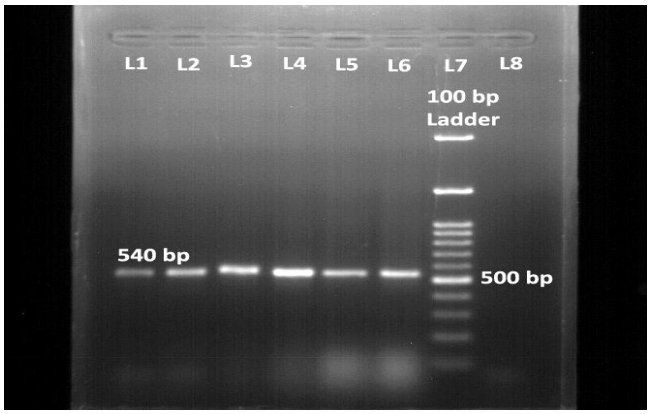
Molecular conformation of the *Salmonella* spp. isolates showed the presence of the *16S rRNA* gene (1428 bp) [Fig 1] specific for this genus in all 35 samples, thus confirmed as *Salmonella enterica*. Ranjbar *et al.* (2020) and Doosti *et al.,* (2017) also confirmed *Salmonella* spp. by detection of the *16S rRNA* gene in their studies.

The prevalence of Salmonellosis in bovine faecal samples was quite significantly high (20.6%) which almost, matches the 23.2% positivity of *Salmonella* spp. from beef calve samples by Obaidat (2020) and 18.1% positivity of *Salmonella* spp., was reported by El-Seedy *et al.* (2016) from the diarrheal calf samples. A prevalence of 3-9.6% *Salmonella* spp. from diarrhoeal cattle was observed by Murugkar *et al.* (2005); Sudhanthirakodi (2016). and Sharma and Joshi (2020) from different states of India.

The prevalence of *Salmonella enterica* strains among the faecal samples showed a higher positivity in Cross Breed jersey cows (88.57%), younger cattle (up to 2 years of age) [24.53%], male cattle (42.86%) and from the Gangetic Alluvial zone samples (25.21%). These were also reported by Nuzhat *et al.* (2020), Olaogun *et al.* (2016), Khan *et al.* (2021), Ibrahim *et al.* (2023) and Banerjee *et al.* (2019).

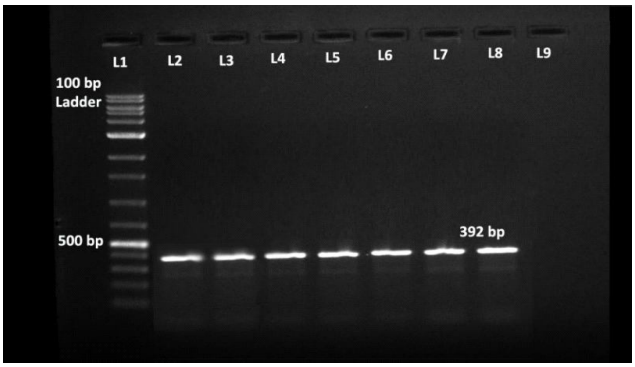
Eighteen (51.42%) and 29 (82.86%) *Salmonella* spp. isolates were found to be positive phenotypic ESBL and ACBL production respectively (Drieux *et al.,* 2008). Adel *et al.* (2021) showed 42% and Salvia *et al.* (2022) revealed 72% ESBL and ACBL positivity phenotypically in earlier studies which almost match with these results.

Molecular detection of ESBL genes revealed that 26(74.29%) *Salmonella* spp. isolates were positive for at least one of the *bla*CTX-M (Fig 2) and *bla*SHV (Fig 3) genes. No *bla*TEM gene was detected in this study. Again 32 (91.43%) isolates showed the presence of the *bla*AmpC gene (Fig 4) which was the highest among all genes (Worku *et al.,* 2022) (Table 1). Only 8 isolates showed the presence of both genes. Similarly, Adel *et al.* (2021) observed the prevalence of the *bla*CTX-M (32.4%) and the *bla*SHV-12 (14.7%) genes in *Salmonella enterica* isolates which



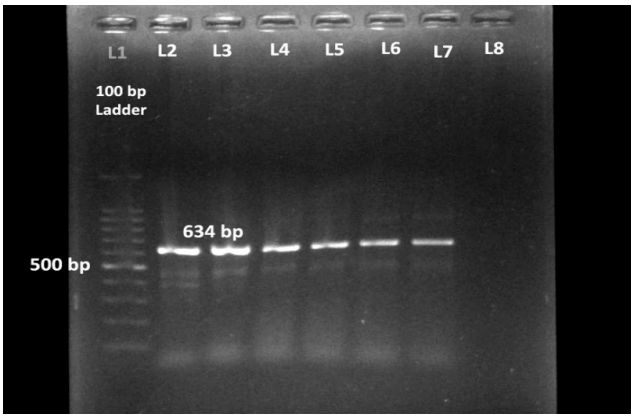
[L1-L5: Test samples, L6: Positive control, L7: 100 bp Ladder, L8: Uninoculated Negative control].

Fig 2: Detection of the *bla*CTX-M gene (540 bp) in bacterial isolates by PCR.



[L1: 100 bp Ladder, L2: Positive control, L3-L8: Test samples, L9: Uninoculated Negative control].

Fig 3: Molecular detection of the *bla*SHV gene (392 bp) in bacterial isolates by PCR.



[L1: 100 bp Ladder, L2: Positive control, L3-L7: Test samples, L8: Uninoculated Negative control].

Fig 4: PCR detection of the *bla*AmpC gene (634 bp) in bacterial isolates.

almost matches the current findings. Ahmed *et al.* (2014), and Banerjee *et al.* (2019) also reported 52-70% ACBL positivity in *Salmonella* spp. isolates in their studies.

In vitro antibiogram *of* the ESBL-producing *Salmonella* spp. isolates (n=26) showed high-level resistance to most of the antibiotics like cefotaxime, enrofloxacin, ampicillin/ cloxacillin, doxycycline etc. (Akinyemi *et al.,* 2017, Kolhe *et al.,* 2022) whereas few antibiotics like azithromycin, chloramphenicol, HL gentamicin, etc. were comparatively sensitive to them (Table 3). Scientists like Khan *et al.* (2021), Adzitey *et al.* (2020) and Ahmed *et al.* (2014) reported similar findings in their studies and thus confirm the present report. The antibiotic resistance pattern observed in this report partially matches the antibiotic usage patterns on those animals as per the data collected from those areas. The antimicrobial resistance is quite an alarming phenomenon now worldwide due to the human-animal interaction and transferrable nature of antimicrobial resistance.

CONCLUSION

A total of 35 (20.59%) *Salmonella* spp. isolates were isolated from bovine faecal samples within which crossbreed jersey cattle (88.57%) were mostly affected. Eighteen (51%) *Salmonella* spp. isolates were detected as ESBL producers and 29 isolates were detected as ACBL producers with the presence of the beta-lactamases-producing genes viz. *bla*AmpC (91.43%) followed by *bla*CTXM (55.28%) and *bla*SHV (42.86%). Antibiogram of all the ESBL-producing *Salmonella* spp., isolates showed high-level resistance (60-95%) to most of the drugs. A few antibiotics like azithromycin, chloramphenicol, HL gentamicin, co-trimoxazole and ceftriaxone/tazobactam were sensitive to these isolates.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors. The authors are responsible for the accuracy and completeness of the information provided but do not accept any liability for any direct or indirect losses resulting from using this content.

Informed consent

This study does not involve an animal experiment, so the permission of the University Animal Ethics Committee is also not required.

Conflict of interest

The authors declare that there are no conflicts of interest among themselves regarding the publication of this article.

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