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Detection of Multidrug Resistance in *Salmonella* sp. Isolated from Diarrhoea of Bovine Calves in Mhow, Indore

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ABSTRACT

The research was conducted during July, 2021 to April, 2022 in the Department of Veterinary Microbiology, Veterinary College, Mhow, Madhya Pradesh, India, with the objective of multidrug resistance in *Salmonella sp.* isolated from diarrhoea of bovine calves in Mhow, Indore, India. A total of 250 faecal samples were collected from diarrheic bovine calves and transported to the laboratory for immediate processing. The fecal sample was inoculated in buffered peptone water (BPW) as a pre-enrichment and incubated at 37°C for 16 hrs. After 16 hrs. Rappaport Vassiliadis (RV) broth were inoculated from this BPW with the help of inoculation loop and again incubated at 37°C for 18 hrs. followed by culture on MacConkey agar, XLD agar and BG agar. On MacConkey agar *Salmonella* shows colorless colony. Typical colonies of Salmonella grown on XLD-agar has a slightly transparent zone of reddish color and a black center. While on the BG agar colonies of the *Salmonella* showing pinkish white in color. Twenty-three *Salmonella* isolates were initially identified phenotypically and further confirmed by PCR targeting the *invA* gene. Antimicrobial susceptibility testing was conducted using 15 antibiotics. The isolates showed the highest sensitivity to co-trimoxazole, while the highest resistance was observed against enrofloxacin. Molecular analysis of six antibiotic resistance genes revealed that the *sul1* gene was the most prevalent among the isolates, followed by *blaCTX-M*, *blaSHV*, *aadA* and *blaTEM*. The *tetA* gene was not detected in any of the isolates. Additionally, hemolysis testing indicated that none of the *Salmonella* isolates exhibited hemolytic activity on blood agar.

KEYWORDS: *Salmonella* spp., calf diarrhoea, antibiotic resistance

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1. INTRODUCTION

In India, livestock is a cornerstone of the agricultural sector, significantly contributing to economic growth and rural livelihoods. According to the 20th Livestock Census, livestock contributes around 4.1% to India's GDP and nearly 25.6% to agricultural GDP, with about 16% of smallholder incomes derived from this sector (Jain et al., 2023). The dairy industry, in particular, relies heavily on the health and survival of calves-success of initiatives like the White Revolution depends on maintaining stable calf populations (Malik et al., 2012). Neonatal calves (under 3 months) experience the highest morbidity and mortality rates among all age cohorts, underscoring the urgent need for preventative interventions. Neonatal diarrhoea remains one of the most challenging syndromes to control; bacterial pathogens are responsible for over 50% of cases in young calves (Arero, 2021). Diarrhoeal illness often results from complex interactions among bacteria, viruses, and protozoa-with *E. coli* and *Salmonella* spp. being the most economically impactful pathogens (Acha et al., 2004, Malik et al., 2012). Co-infections are common, though single-agent infections also occur, with regional variability influenced by farm management, herd size, and geographic factors (Cho and Yoon, 2014).

Salmonella is a significant enteric pathogen implicated in neonatal calf diarrhoea, leading to high morbidity, mortality, and substantial economic losses in the livestock industry. Calves, particularly those under three months of age, are highly susceptible to *Salmonella* infections due to their immature immune systems and environmental stressors. The infection typically manifests as enteritis characterized by watery to bloody diarrhoea, fever, dehydration, and systemic illness. *Salmonella enterica* serovars such as Typhimurium and Dublin are most commonly associated with calf diarrhoea outbreaks. (Haque et al., 2022)

Accurate identification of the causative agents in outbreaks enables targeted strategies like vaccination and improved hygiene, as well as risk-factor identification (Izzo et al., 2011). *Salmonella enterica*, while often present in healthy adult cattle, can cause severe illness in calves during the first three months of life (Fossler et al., 2005). Misuse or incomplete administration of antimicrobials in treating calf salmonellosis has likely fostered the emergence of more resistant and virulent *Salmonella* strains.

Multidrug-resistant bacteria represent a growing global concern in ruminants, largely driven by the inappropriate and excessive use of antibiotics. Resistance genes can be transmitted between ruminants, humans, and the environment through various routes, including the consumption of contaminated animal products (meat, milk, and dairy by-products) and through direct or indirect contact (Abdalhamed et al., 2021). The rapid dissemination of antimicrobial resistance is primarily facilitated by mobile genetic elements such as plasmids, transposons, and integrons, which enable horizontal gene transfer among bacterial populations (Adefisoye and Okoh, 2016).

Although comprehensive microbial isolation and susceptibility testing can be cost-prohibitive, regular surveillance of antimicrobial resistance remains essential for guiding judicious antibiotic use. Contemporary insights into the prevalence and susceptibility of *Salmonella* in diarrheic calves are vital for informed clinical decisions. Thus, the present study was undertaken to isolate and identify *Salmonella* from diarrheic calves in the Mhow region of Indore, Madhya Pradesh, and to assess the antimicrobial resistance patterns.

2. MATERIALS AND METHODS

2.1. Study area

The present study was conducted from July, 2021 to May, 2022 in Mhow, located in Madhya Pradesh, India, at a latitude of 22.552437°N and a longitude of 75.756531°E. Mhow experiences a subtropical climate, with an average annual temperature of 25.1°C and an annual rainfall of approximately 1126 mm. The region supported a significant number of both organized and unorganized dairy farms, which played a vital role in supplying milk and dairy products to the local population and surrounding areas. These farms rear a variety of animals, including indigenous breeds and crossbred cattle, depending on the scale and purpose of production.

The animals included in this investigation were bovine calves under four months of age exhibiting clinical signs of diarrhoea, along with symptoms of systemic illness such as impaired suckling reflex, poor appetite, fever, and dehydration. A total of 250 faecal samples were collected from calves displaying these clinical signs. Rectal swabs were taken directly from diarrhoeic calves using sterile techniques, placed in sterile test tubes, and promptly transported to the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Mhow, for further processing and analysis.

2.2. Isolation of Salmonella spp.

2.2.1. Pre-enrichment and selective enrichment

The Fecal samples was inoculated in buffered peptone water (BPW) as a pre-enrichment and incubated at 37°C for 16 hrs. After 16 hrs. Rappaport Vassiliadis (RV) broth were inoculated from this BPW with the help of inoculation loop and again incubated at 37°C for 18 hrs. A loop full of inoculum from RV broth was plated out on MacConkey agar (MAC), Xylose lysine deoxycholate (XLD) agar and Brilliant green (BG) agar and incubated at 37°C for 18–

24 hrs. After incubation, the plates were examined for the presence of typical and *Salmonella* suspected colonies. On MAC agar, *Salmonella* appears as colorless colonies due to its inability to ferment lactose, whereas on XLD agar, *Salmonella* forms colonies with a transparent reddish zone and a characteristic black center. On BG agar colonies of the *Salmonella* were pinkish in colour. All the suspected colonies of the *Salmonella* picked and transferred on NA slants and stored at 4°C for further biochemical confirmation (Collee et al., 1996).

2.3. DNA extraction

The isolates were inoculated in Brain heart infusion broth (Hi Media, Mumbai) and incubating at 37°C for 12–18 hrs. Extraction of DNA was done using readymade kit (GenElute™ Bacterial Genomic DNA Kit, Sigma) as per the manufacturer's instructions. The extracted DNA was stored at -20°C till further use.

2.4. Molecular identification of Salmonella spp. using polymerase chain reaction (PCR)

Salmonella isolates identified phenotypically as above were confirmed genotypically by PCR using primers for genus specific gene *invA* (Table 1). DNA amplification was accomplished in a total 25 µl mixture volume, including 2 µL of DNA template, 12.5 µl of Master mix (SIGMA life science), 1 µl of forward and reverse primers (20 pmol μL^{-1}), and 8.5 µL PCR-grade nuclease free water (SIGMA life science). Samples were subjected to initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation (94°C, 30 s), annealing (59°C, 60 s), and elongation (72°C, 90 sec). These cycles were followed by a final elongation step (7 min at 72°C).

Table 1: Thermocyclic conditions for *invA* gene of *Salmonella*

Sl. No.	Steps		invA	No. of cycles
1.	Initial denaturation	Temp.	94°C	1
		Time	5 min	
2.	Denaturation	Temp.	94°C	35
		Time	30 sec	
3.	Annealing	Temp.	59°C	
		Time	60 sec	
4.	Extension	Temp.	72°C	
		Time	90 sec	
5	Final extension	Temp.	72°C	1
		Time	7 min	

2.5. Antimicrobial susceptibility tests

Isolates were screened for resistance to antibiotics by a disk diffusion method. Bacteria were suspended in saline to the density of a McFarland No. 2 standard, diluted 1:20, and streaked by the method of Bauer et al. (1966) on Mueller-Hinton agar and incubated for 24 hrs at 37°C. Characterization of strains as sensitive, intermediate or resistant was based on the manufacturer's instructions (Hi-media). The different antibiotics like Ampicillin (AMS) 10 µg, Cefotaxime (CTX) 30 µg, Enrofloxacin (Ex) 10 µg, Gentamycin (G) 10 µg, Azethromycin (AZM) 15 µg, Ceftrixone (Ci) 10 µg, Ciprofloxacin (CIP) 5 µg, Colistin (CL) 10 µg, Co-trimoxazole (COT) 25 µg, Ceftazidime plus Clav (CAC) 30 µg, Aztreonam (AT) 30 µg, Nitrofurantoin (NF) 100 µg, Streptomycin (S) 10 µg, Sulphadiazine (SZ) 100 µg, Tetracycline (TE) 10 µg were used in this investigation.

2.5. Identification of antibiotic resistance genes

All *Salmonella* isolates were analysed for six antibiotic resistance genes *blaTEM* (betalactams), *blaCTX-M* (Cephalosporin), *blaSHV* (betalactams), *tetA* (tetracycline), *sul1* (sulfonamides) and *aadA* (streptomycin) by PCR. Several PCR protocols were applied to investigate the target genes of the *Salmonella* isolates. Lists of primers used and the amplified products were presented in Tables 2. All reactions were carried out on a PCR thermocycler (Applied Biosystems). Amplification of DNA was conducted in a total volume of 25 µl, including 2 µL of DNA template, 12.5 µl of Master mix (SIGMA life science), 1 µl of forward and reverse primers (20 pmol μl^{-1}), and 8.5 µl PCR-grade nuclease free water (SIGMA life science). The PCR were carried out as per specific cyclic conditions with different genes as per the table 3. The amplified products were separated by electrophoresis through 1.5% agarose (wt/vol), stained with 0.5 µg ml^{-1} ethidium bromide, visualized under UV illumination, imaged with a GelDoc 1000 fluorescent imaging system (Uvitech).

3. RESULTS AND DISCUSSION

Neonatal calf diarrhoea remained a major challenge for the livestock industry, contributing to considerable economic losses. The risk was highest during the first month of life, gradually decreasing as the calf matures (Garcia et al., 2000). Despite decades of research, it continued to be the leading cause of mortality in neonatal calves (Uhde et al., 2008). The complex pathophysiology of infectious diarrhoea in calves not only results in direct losses due to calf deaths but also leads to indirect costs associated with preventive interventions, treatment, and reduced growth performance in surviving animals (Radostits et al., 2007).

Salmonella showed lactose non fermenting pale colourless, smooth, transparent, raised colonies on the MacConkey agar (Plate 1). Typical colonies of *Salmonella* grown on Xylose lysine deoxycholate agar (XLD) had slightly transparent zone of reddish colour and a black centre (Plate 2). While on

Table 2: Details of primers used for PCR reactions

Target gene	Primer Sequence	Product size (bp)	Reference
blaCTX-M	F: GTGCAGTACCAGTAAAGTTATGG R: CGCAATATCATTGGTGGTGCC	538	Adesoji et al., 2015
blaSHV	F: TCGGGCCGCGTAGGCATGAT R: AGCAGGGCGACAATCCCGCG	628	Gundran et al., 2020
blaTEM	F: TTGGGTGCACGAGTGGGTTA R: TAATTGTTGCCGGGAAGCTA	506	Gundran et al., 2020
Sul1	F: TTCGGCATTCTGAATCTCAC R: ATGATCTAACCCTCGGTCTC	822	Shehata et al., 2016
tetA	F: GTCAAACCCAACATACCCC R: GAAGGCAAGCAGGATGTAG	887	Van et al., 2008
aadA	F: TGATTTGCTGGTTACGGTGAC R: CCGTACGCATACTGGCTTTGC	284	Van et al., 2008

Table 3: Thermocyclic conditions for different AMR genes

S.No.	Step	*blaTEM*	*blaCTX-M*	*blaSHV*	*sul1*	*tetA*	*aadA*
1.	Initial denaturation	95°C	95°C	95°C	94°C	94°C	95°C
2.	Denaturation	94°C	96°C	94°C	94°C	94°C	94°C
3.	Annealing	60°C	52°C	64°C	54°C	60°C	58°C
4.	Extension	72°C	72°C	72°C	72°C	72°C	72°C
5.	Final Extension	72°C	72°C	72°C	72°C	72°C	72°C

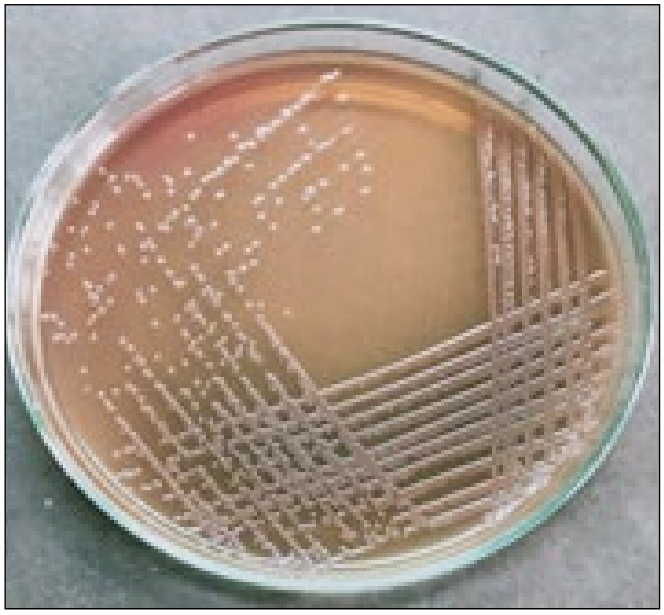
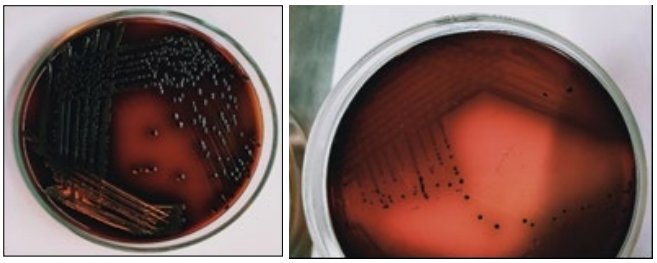


Plate 1: Pale colonies of *Salmonella* on MacConkey agar

the Brilliant green (BG) agar colonies of the *Salmonella* were pinkish in colour (Plate 3). After confirming to be Gram negative rods (Plate 4), total 23 suspected isolates of the *Salmonella* were picked and confirmed by biochemical test.

Salmonella isolates identified phenotypically as above were confirmed genotypically by PCR using primers for genus



Front view Reverse view

Plate 2: *Salmonella* colonies showing transparent zone of reddish colour and a black centre on Xylose lysine deoxycholate agar

specific gene *invA* (Plate 5). The gene *invA* was genus specific as well as represents virulence. The *invA* gene was also used as a target for identification of *Salmonella* species by Fahmy et al. (2017) and Chaudhary et al. (2022).

In comparison to our data i.e. 9.2% incidence, the lower percent detection of *Salmonella* viz. 7.7%, 1%, 7, 7%, 9.1%, 1.04%, 3.79%, 6.2% and 2.71% by Abunna et al. (2017), El-Tawab et al. (2017), Fahmy et al. (2017), Khalifa et al. (2017), Refai et al. (2017), Lei et al. (2020), Merera et al. (2020) and de Vasconcelos et al. (2021).

However, higher percent detection viz. 20.95%, 36.6%, 14.5% and 15% by Abdulridha and Ibrahim (2018), Kadam (2018), Saleh et al. (2019) and Arbab et al. (2021). While Haggag et al. (2016) and Adeladlew (2020) recorded 11.43%

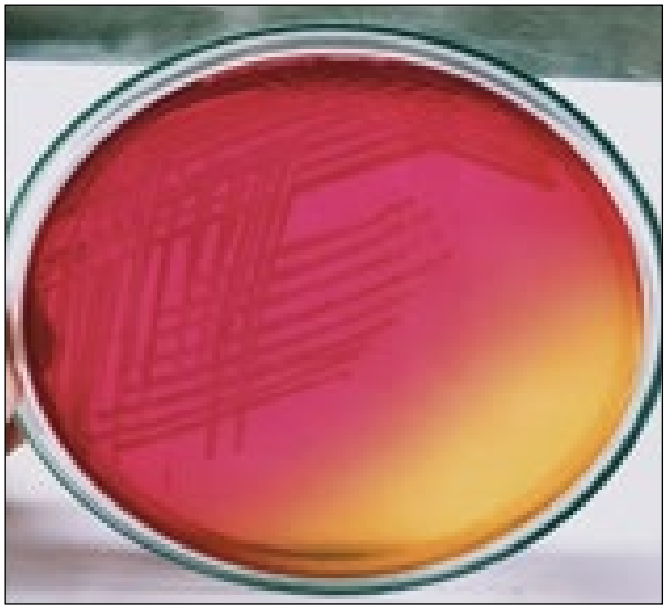


Plate 3: Pinkish colour colonies of *Salmonella* on Brilliant green agar

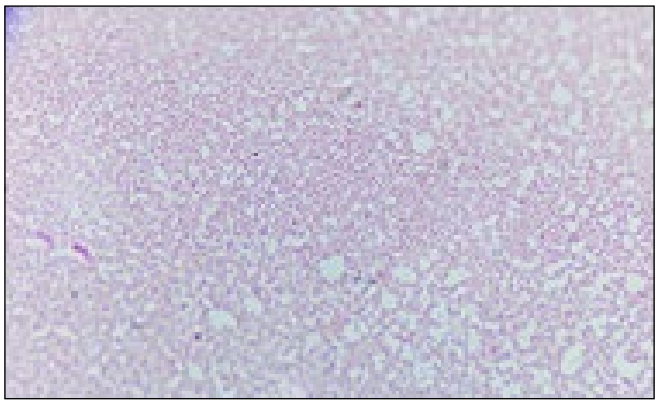


Plate 4: Gram negative rods of *Salmonella*

and 11.42% incidence which was very close to our data.

The highest sensitivity among *Salmonella* isolates was attributed towards antibiotic co-trimoxazole (73.91%), highest intermediate sensitivity against cefotaxime (26.06%) and highest resistance against enrofloxacin (100%) (Figure 1). Similar to our study, similar antibiotics against *Salmonella* were also reported by other researchers but found the different type of sensitivity patterns. Abdullah et al. (2013) found ampicillin was 100% resistant while azithromycin, gentamycin and ciprofloxacin were found sensitive against the *Salmonella*. Haggag et al. (2016) tested gentamicin, cefotaxime and oxytetracycline and reported varying degree of resistance. Sohidullah et al. (2016) reported sensitivity against ciprofloxacin, gentamicin and streptomycin while azithromycin and tetracycline found resistant. Khalifa et al. (2017) recorded enrofloxacin and trimethoprim and sulpha combination to be sensitive while gentamicin, cefotaxime, tetracycline and streptomycin

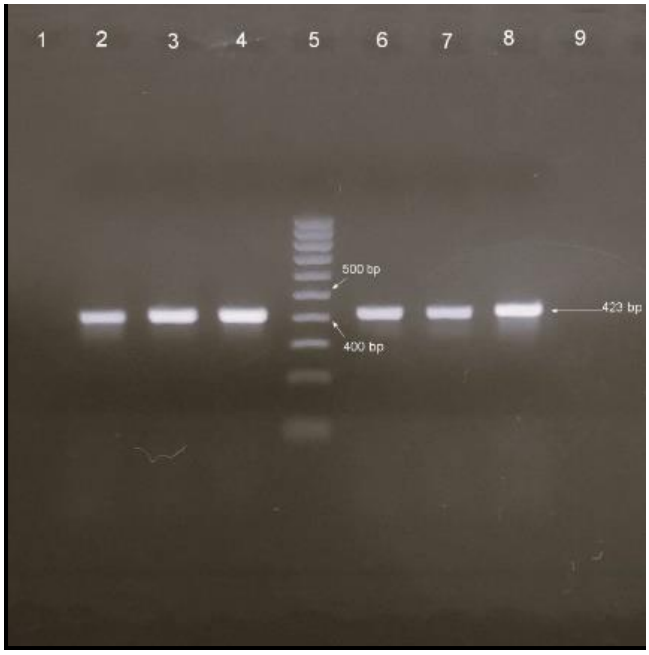


Plate 5: Results of PCR showing *invA* gene of *Salmonella*; Lane 5: 100 bp ladder; Lane 2: Positive control; Lane 1: Negative control; Lane 3,4,6,7 and 8: Positive samples (Size 423 bp); Lane 9: Negative sample

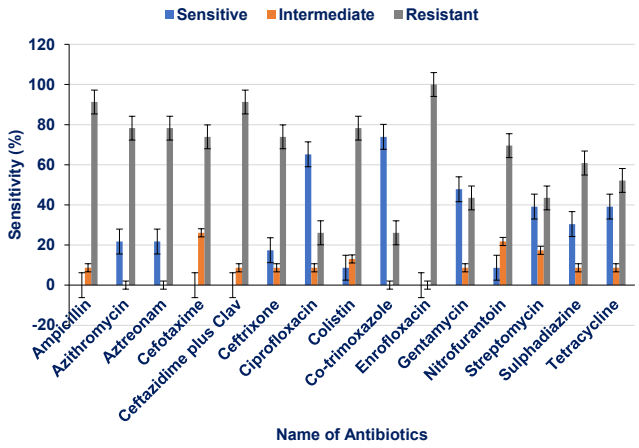


Figure 1: Sensitivity of *Salmonella* isolates to different antimicrobial agents

were resistant. Abunna et al. (2017) tested ampicillin, gentamicin, streptomycin, ciprofloxacin, tetracycline and trimethoprim and reported 39.3%, 0%, 10.7%, 0%, 96.4% and 10.7% resistance, respectively. These variations could be attributed to several factors, including geographical differences, variations in antimicrobial usage practices, differences in animal husbandry and management systems, and the selective pressure exerted by antibiotic overuse or misuse. Additionally, the genetic diversity among *Salmonella* strains and the presence of resistance genes or plasmids could significantly influence their antimicrobial susceptibility profiles.

All *Salmonella* isolates in this study exhibited multidrug resistance (MDR), with resistance observed against 6 to 15 antibacterial agents simultaneously. The highest proportion of isolates (26.09%) were resistant to seven different antibiotics, while two isolates demonstrated resistance to all 15 tested antimicrobials (Table 4 and Figure 2). Similar patterns of multidrug resistance in *Salmonella* have been reported by several researchers, including Anjum et al. (2011), Sohidullah et al. (2013), Haggag et al. (2016), Manickam and Ponnusamy (2017), Khalifa et al. (2017), Rahman et al. (2018), Abdulridha and Ibrahim (2018), McMillan et al. (2019), Abunna et al. (2020), Hua et al. (2020), Ranjbar et al. (2020), and Merera et al. (2020). These findings collectively underscored the growing threat of antimicrobial resistance in *Salmonella* isolates from animal sources.

Table 4: Multiple drug resistance in *Salmonella* isolates

Sl. No.	No. of antibiotics	No. of resistant isolates	Percent of resistant isolates (N=23)
1.	6	3	13.04
2.	7	6	26.09
3.	8	4	17.39
4.	11	2	8.70
5.	12	1	4.35
6.	13	1	4.35
7.	14	4	17.39
8.	15	2	8.70

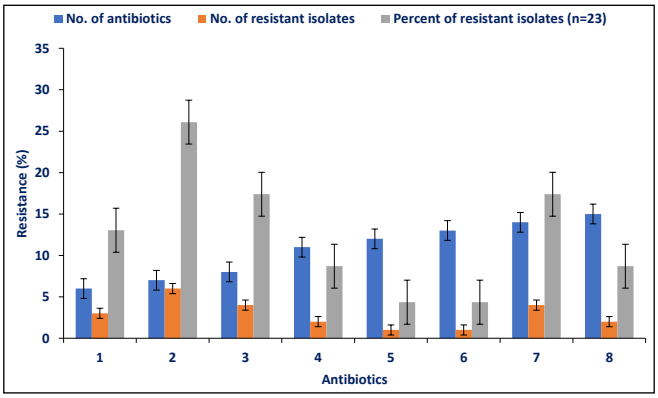


Figure 2: Multiple drug resistance in *Salmonella* isolates

The Salmonella isolates in this study were tested for haemolytic activity on 5% sheep blood agar. None of the 23 isolates exhibited haemolysis on the blood agar plates. In contrast, previous studies by Manickam and Ponnusamy (2017) and Abdulridha and Ibrahim (2018) reported haemolytic activity in Salmonella isolates. This discrepancy might be attributed to several factors, including differences in Salmonella serovars, genetic variations among isolates, or host-specific adaptations.

For molecular characterization of antibiotic resistance, twenty *Salmonella* isolates were randomly selected and screened for the presence of selected antibiotic resistance genes (ARGs) using polymerase chain reaction (PCR) with published primers.

Detection of the *blaTEM* gene was performed following the protocol described by Gundran et al. (2020). A specific amplicon of 506 bp was considered indicative of a positive result, with only 10% (2/20) of the isolates testing positive (Plate 6). Similarly, PCR amplification for the *blaSHV* gene, also as per Gundran et al. (2020), produced the expected 628 bp amplicon in 20% (4/20) of the isolates (Plate 7). The *blaCTX-M* gene was detected using primers described by Adesoji et al. (2015), where an amplicon size of 538 bp confirmed positivity; this gene was also present in 20% (4/20) of the tested isolates (Plate 8). PCR screening for the *sul1* gene was conducted using published primers isolates (Plate 9), representing the highest detection rate among the ARGs tested. For the *tetA* gene, primers described by Van et al. (2008) were used, targeting an expected product of 887 bp. None of the tested isolates showed amplification for this gene, indicating its absence in all samples. Lastly, detection of the *aadA* gene, also using primers from Van et al. (2008), revealed a 284 bp product in 20% (4/20) of the isolates (Plate 10). These findings suggested the presence of diverse resistance determinants among the *Salmonella* isolates, with sul1 being the most prevalent, followed by blaSHV, blaCTX-M, and aadA, while blaTEM was less common and tetA was undetected in the current study population.

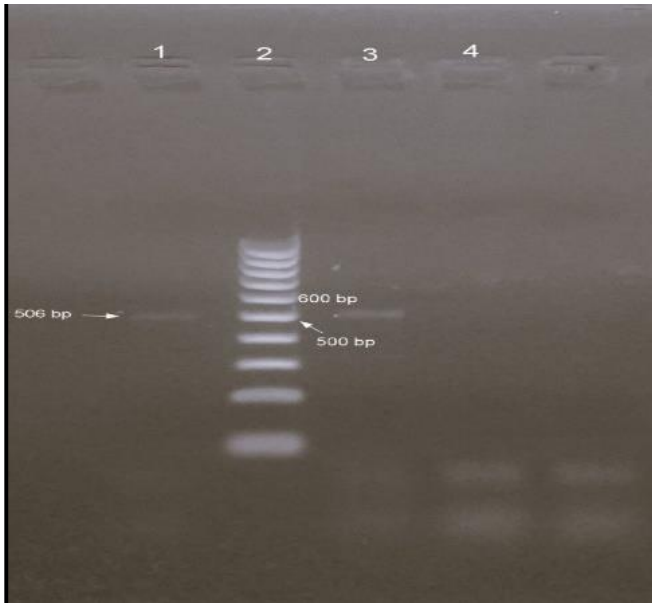


Plate 6: Results of PCR showing *blaTEM* gene of *Salmonella*; Lane 2: 100 bp ladder; Lane 1: Positive control; Lane 3: Positive sample (Size 506 bp); Lane 4: Negative sample

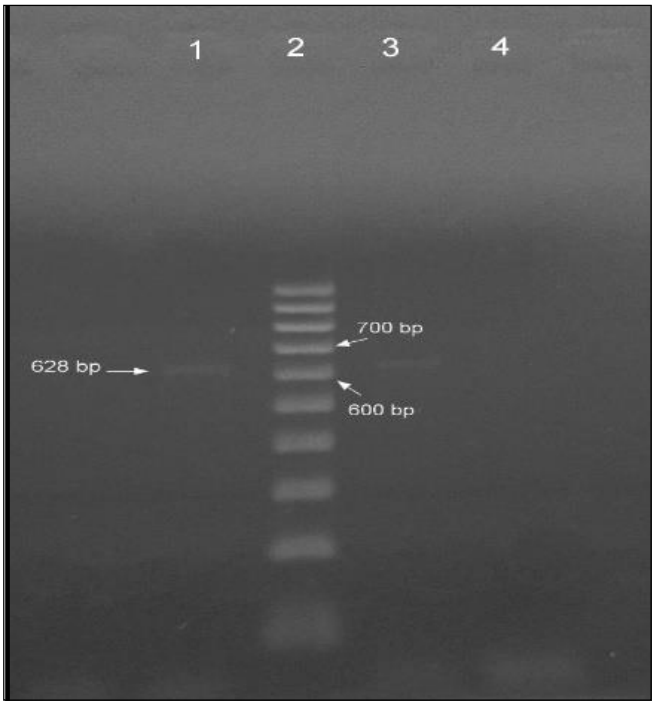


Plate 7: Results of PCR showing *blaSHV* gene of *Salmonella*; Lane 2: 100 bp ladder; Lane 1: Positive control; Lane 3: Positive sample (Size 628 bp); Lane 4: Negative sample

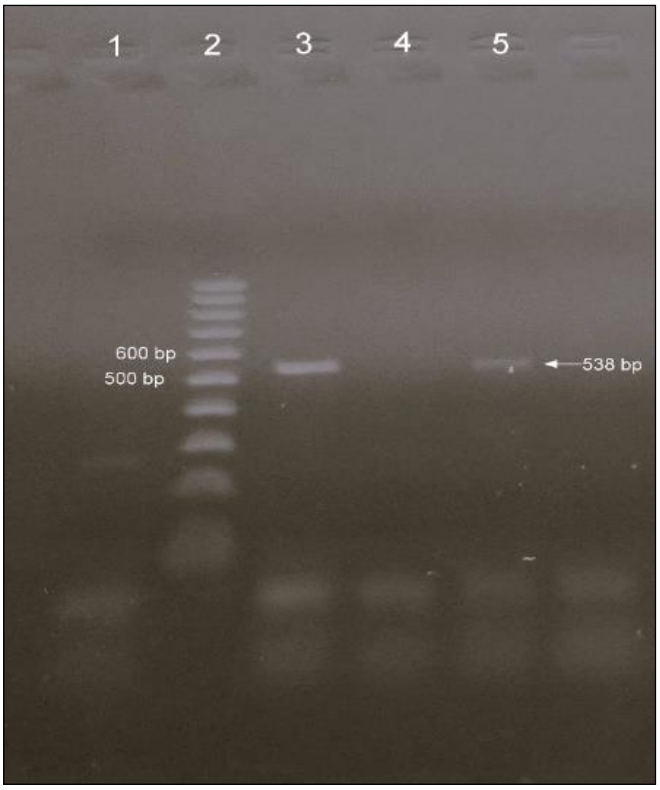


Plate 8: Results of PCR showing *blaCTX –M* gene of *Salmonella*; Lane 2: 100 bp ladder; Lane 3: Positive control; Lane 4: Negative control; Lane 5: Positive sample (Size 538 bp); Lane 1: Negative sample

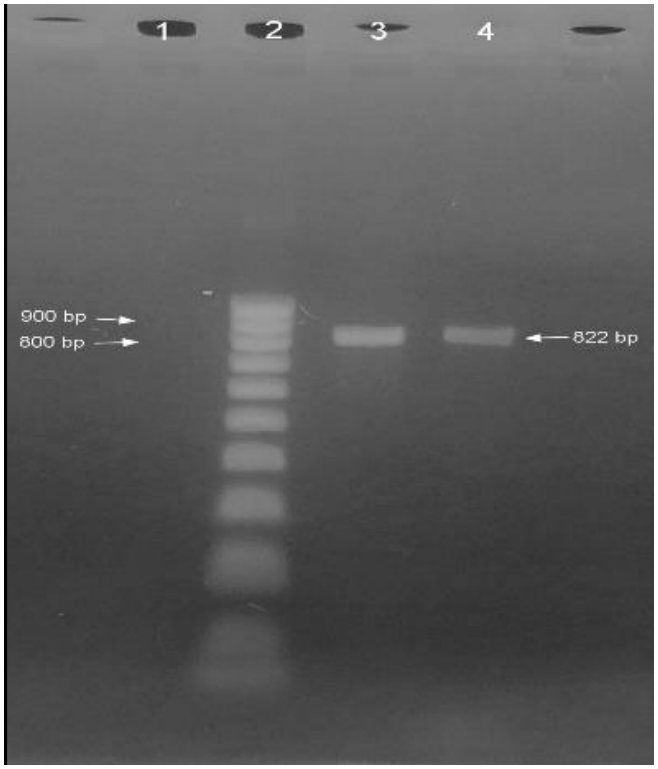


Plate 9: Results of PCR showing *sul1* gene of *Salmonella*; Lane 2: 100 bp ladder; Lane 3: Positive control; Lane 4: Positive sample (Size 822 bp); Lane 1: Negative control

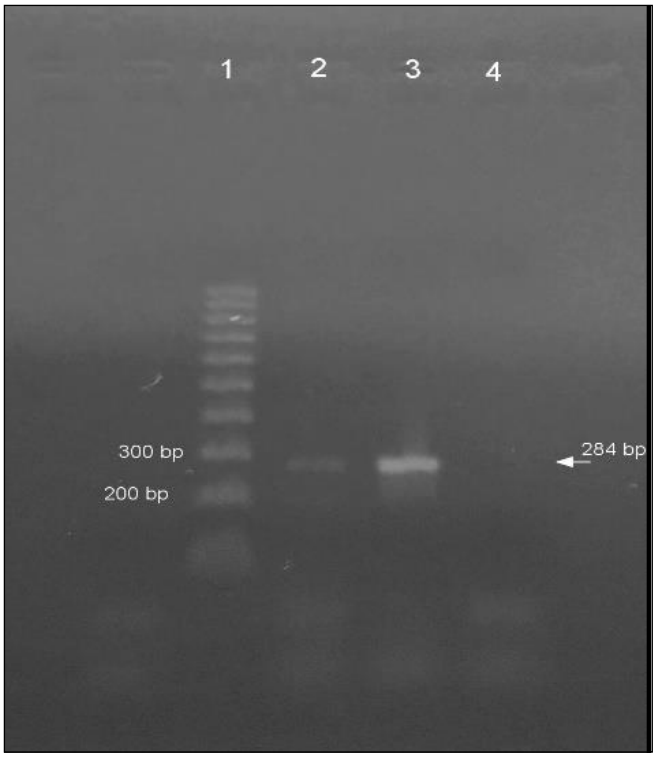


Plate 10: Results of PCR showing *aadA* gene of *Salmonella*; Lane 1: 100 bp ladder; Lane 2: Positive control; Lane 3: Positive sample (Size 284 bp); Lane 4: Negative sample

Among the six antibiotic resistance genes (ARGs) screened in the present study, the *sul1* gene was detected in the highest proportion of *Salmonella* isolates. These findings were consistent with global reports, although the prevalence of ARGs varies considerably across studies. For instance, Srisanga et al. (2017) reported detection rates of 40% for *blaTEM*, 13.9% for *sul1*, 12.5% for *aadA*, and 51.1% for *tetA*. Nahar and Rashid (2018) found *aadA1* (15.56%), *aadA2* (11.11%), *tetA* (8.89%), and *sul1* (17.78%) in their isolates. Yukawa et al. (2019) also detected *blaTEM*, *blaSHV*, *blaCTX-M*, *aadA*, and *tetA* in varying proportions. Similarly, Mthembu et al. (2019) reported *blaTEM* (14.4%), *tetA* (8%), and *sul2* (21%), while Arkali and Cetinkaya (2020) observed *sul1* in 58% and *blaTEM* in 20% of the isolates. Chakraborty et al. (2020) reported a higher prevalence of *blaTEM* (60%). Borah et al. (2021) detected *blaTEM* and *tetA* in 63.64% and 1.85% of isolates, respectively. Khalifa et al. (2021) found *blaTEM* in 73%, *sul1* in 100%, and *aadA* in 66.7% of isolates. Hope and Bright (2022) reported lower detection rates for *blaTEM* (5%), *blaCTX-M* (5.8%), *blaSHV* (9.6%), and *aadA* (17.4%). Parada et al. (2022) and Isoken (2022) reported *blaTEM* in 49% and 19.3% of *Salmonella* isolates, respectively. Significant discrepancies in the detection rates of antibiotic resistance genes (ARGs) in *Salmonella* spp. have been reported by various researchers. These variations could be attributed to multiple factors, including geographic differences in antimicrobial use policies and farming practices, which influenced the selective pressure on bacterial populations. The type and frequency of antibiotic administration in veterinary and agricultural settings, along with differences in antibiotic stewardship programs, directly impact the emergence and spread of resistance. Additionally, diagnostic capacity, and surveillance systems, as well as the genetic diversity of *Salmonella* strains and the presence of mobile genetic elements (e.g., plasmids and integrons), further contribute to variability in antibiotic resistance.

4. CONCLUSION

The variation in the prevalence of resistance genes among isolates reflected the influence of regional antimicrobial use practices, farm management systems, and genetic diversity of circulating strains.

5. ACKNOWLEDGEMENT

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7. REFERENCE

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