RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2024.55.1.14-24

Antimicrobial resistance profile of *Salmonella* Typhimurium isolated from meat samples of Marathwada region, Maharashtra, India[#]

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Citation: Chilkewar, D.D., Waghamare, R.N., Ghorpade, P.B., Rawool, D.B., Khose K.K., and Rasve R.L. 2024. Antimicrobial Resistance Profile of *Salmonella Typhimurium* isolated from meat samples of Marathwada region, Maharashtra, India**.** *J. Vet. Anim. Sci.* **55**(1):14-24
DOI: https://doi.org/10.51966/jvas.2024.55.1.14-24

Received: 29.08.2023 *Accepted: 27.12.2023* *Published:31.03.2024*

Abstract

The current study was planned to assess the antimicrobial resistance profile of Salmonella spp*. isolated from meat samples collected from the Marathwada region. A total of 180 meat samples comprising 60 each of chicken, chevon and meat products were collected from 3 districts of Marathwada. The isolation of Salmonella spp. was carried out as per IS-5887 (Part 3): 1999 protocol and confirmed by morphological characteristics, biochemical reactions and PCR (invA gene) assay. A total of 5 isolates with an occurrence rate of 2.78 per cent were recovered from the 180 samples analysed, with an occurrence rate of 0, 3.33 and 5 per cent, respectively, from chicken, chevon and meat products. All isolates were serotyped as Salmonella Typhimurium by identifying the spy gene by PCR assay. Antimicrobial susceptibility testing revealed higher resistance against Erythromycin (100%), Amoxy-clav (80%), Ceftazidime (80%), Ciprofloxacin (80%), Amoxicillin/ Sulbactam (60%), Chloramphenicol (40%) and Amikacin (40%), while sensitivity was recorded against Tetracycline (100%), Co-Trimoxazole (100%), Ampicillin/Sulbactam (80%) and Gentamicin (80%). The average multiple antibiotic resistance (MAR) index of Salmonella Typhimurium isolates was 0.372. Genotypic resistance pattern revealed that all isolates were carrying the* bla_{TEM} *gene (100%), while none of the isolates carried tetA and Sul1 gene, which correlated with phenotypic AST results. The study concluded that the occurrence of multiple antibiotic-resistant Salmonella Typhimurium in meat could pose a high risk to human health; therefore, continuous monitoring of*

[#]*Part of M.V. Sc thesis submitted to Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra*

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multidrug resistance Salmonella spp. in animal origin food supply chain is essential to control the outbreaks.

Keywords: *Salmonella typhimurium, antimicrobial resistance, PCR assay, bla$_{TEM}$ gene and food supply chain.*

Amongst all animal-origin food items, consumption of meat is increasing in India (Waghamare *et al*., 2021). Meat plays an important role in a balanced ration, as it is high in nutritive value with both essential amino acids and macro and micronutrients (Varijakshapanicker *et al.,* 2019). The high nutritive value and moisture content make the meat a good vehicle for the growth of various microorganisms (Maharjan *et al.,* 2006).

The genus Salmonella is divided into two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is divided into six subspecies (*Salmonella enterica* subsp. *entrica, Salmonella enterica* subsp. *salamae, Salmonella enterica* subsp. *arizonae, Salmonella enterica* subsp. *diarizonae, Salmonella enterica* subsp. *houtenae and Salmonella enterica* subsp. *indica*), and each of these has several serovars or serotypes (Jacobsen *et al.,* 2011). More than 2600 serotypes are known of which almost 1500 belong to the subspecies *enterica* (Anukampa *et al.*, 2017).

Salmonellosis is one of the major foodborne diseases in the world and it is estimated that 93.8 million cases of gastroenteritis due to *Salmonella* species occur globally each year, with 155,000 deaths (Heredia *et al*., 2018). The disease is transmitted to humans usually by consumption of undercooked meat, poultry, dairy products and other cross-contaminated foods (Samad *et al*., 2018). The clinical signs of salmonellosis include gastroenteritis, nausea, vomiting, and abdominal pain (Vidayanti *et al*., 2021).

Salmonella contamination in meat may occur at several stages of the food supply chain viz. production, processing, distribution, retailing and also preparation and handling by consumers (Abd El-Aziz, 2013). The development of antimicrobial resistance in *Salmonella* and antibiotic residues in meat is of great concern to public health in India (Waghamare *et al.,* 2018). A key contributor to the emergence of multidrug-resistant *Salmonella* is the administration of antimicrobial agents to food animals and poultry with the purpose of growth promotion, and prophylactic and therapeutic use (Kim *et al*., 2012). Irrational and inappropriate use of antimicrobial agents has provided favourable conditions for microorganisms to develop resistance, spread and persist in the environment (WHO, 2011).

Many factors are responsible for the development of antimicrobial resistance amongst bacteria, viz. change in permeability of bacterial cells, enzymatic drug modification and removal of antimicrobials by membrane-bound efflux pumps (Chen *et al*., 2004). Antibiotic resistance is frequently linked to genetic alterations encoded by chromosomal and plasmid genes. These genes are mainly located on integrons, plasmids and transposons, which are mobile genetic elements (Thong *et al*., 2011). In recent years, resistance in non-typhoidal *Salmonella* isolates to □-lactam, Tetracycline and Sulphonamides has increased dramatically (Eguale *et al.,* 2017; Waghamare *et al.,* 2018 and Mathews *et al*., 2021). Thus, the present study was carried out to evaluate the antimicrobial resistance profile of *Salmonella* spp. isolated from meat and meat products.

Materials and methods

Sample collection

In the present study, a total of 180 meat samples, comprising 60 each of chicken, chevon and meat products were collected from randomly selected 12 different talukas of 3 selected districts namely Aurangabad, Parbhani and Hingoli of Marathwada region of Maharashtra, India (Figure01). All the samples were collected aseptically; using sterilized polypropylene bags and brought to the laboratory under cold chain as per standard methods.

Isolation, identification and serotyping of Salmonella spp.

Isolation of *Salmonella* spp. was

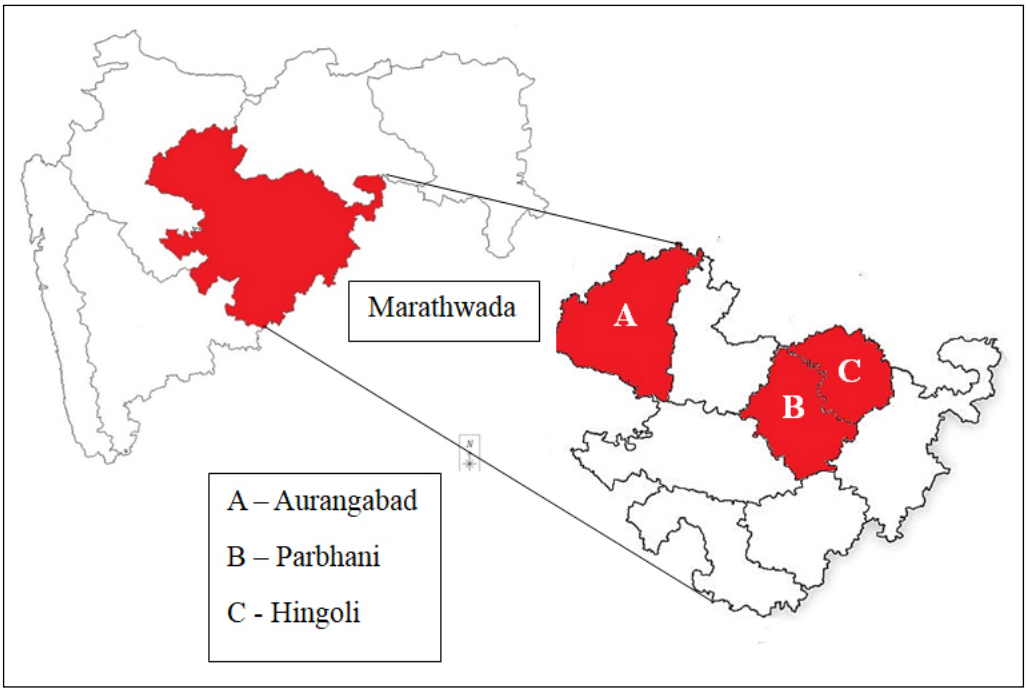

Fig.1. Districts selected for sample collection from Marathwada region, Maharashtra

carried out as per IS-5887 (Part 3): 1999. Under aseptic conditions, 25 g of each sample was weighed into a sterilized polypropylene bag and mixed with 225 mL of buffered peptone water (BPW) and then incubated at 37^{0}C ± 1^{0}C for 24 h for pre-enrichment. Then, 0.1 mL of pre-enrichment water (BPW) was suspended in 10 ml of Rappaport Vassiliadis (RV) Medium for enrichment and incubated at 42^{0}C for 24 h. After incubation, a loop-full of inoculum from RV Medium was streaked on Xylose Lysine Deoxycholate (XLD) Agar and Brilliant Green Sulpha Agar (BGSA) plates by four quadrant streaking method. The inoculated plates were incubated at 37^{0}C ± 1^{0}C for 24 h, for the development of colonies of *Salmonella* spp. The suspected colonies were subcultured on Nutrient Agar and incubated at 37^{0}C for 16-20 h. The bacterial isolates were identified using cultural, morphological and biochemical characteristics (BAM, 2007). The isolates were then further confirmed as *Salmonella* by polymerase chain reaction (PCR) targeting the *invA* gene (Nair *et al.*, 2015). Further, positive isolates were serotyped by PCR targeting serovar-specific gene *spy* and *sdf* I for *Salmonella* Typhimurium and *Salmonella* Enteritidis, respectively (De Freitas *et al.*, 2010). For PCR, extraction of DNA from urease-negative isolates was carried out by using Gsure® Bacterial Genomic DNA Isolation Kit (GCC BIOTECH). The details of PCR primers and cycling conditions used in the present study are mentioned in Table 1 and Table 2.

Antimicrobial susceptibility test

The *Salmonella* spp. isolates were tested for their susceptibility by disk diffusion method against the panel of 14 antimicrobials, namely Ampicillin/Sulbactam (10/10 µg), Amoxycillin/Sulbactam (30/15 µg), Amoxyclav (30 µg), Tetracycline (30 µg), Enrofloxacin (10 µg), Ceftazidime (30 µg), Levofloxacin (5 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Gentamicin (10 µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Co-Trimoxazole (25 µg) and Nalidixic Acid (30 µg). The zone of inhibition was measured in mm and interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2021).

Multiple Antibiotic Resistance (MAR) Index

The MAR index of individual isolates was calculated according to the method of

Krumperman, (1983). The MAR index of each isolate was carried out by dividing the number of antibiotics to which the isolates were resistant to the total number of antibiotics to which the isolate was exposed (Krumperman, 1983).

Molecular characterisation of antimicrobial resistance genes in Salmonella spp. by PCR

The bacterial genomic DNA template was extracted from the isolates by a heat boiling method (Anejo-Okopi *et al.,* 2016). All the positive *Salmonella* spp. isolates were screened for the presence of antimicrobial resistance genes viz., bla_{TEM}, *tetA and Sul1* as per the procedures described by Bhattacharjee *et al.* (2007), Fonseca *et al.* (2006) and Ma *et al.* (2007), respectively. Primers used are listed in Table 1. The cycling conditions for PCR are mentioned in Table 2.

Polymerase Chain Reaction for *invA*, *spy* and *sdf* I gene was performed in 25 µL reaction volume containing 2.5 µL 10X buffer, 1 µL $MgCl_2$ (50mM), 1 µL dNTP mix (10mM), 1 µL of each forward and reverse primer (10 pmol), 1 µL of Taq DNA Polymerase (1U/µL), 4 µL of DNA template and 13.5 µL of molecular biology grade water used to make the desired volume.

Table 1. Details of primers used for PCR

Sl. No	Primer	Target	Primer sequence (5'- 3')	Product Size (bp)	Reference
1	*invA*	Invasion-associated protein	**F:** TCGTGACTCGCGTAAATGGCGATA **R:** GCAGGCGCACGCCATAATCAATAA	423	Nair *et al.* (2015)
2	*Sdf* I	*Salmonella enterica* serotype Enteritidis	**F:** TGTGTTTTATCTGATGCAAGAGG **R:** TGAACTACGTTCGTTCTTCTGG	304	De Freitas *et al.* (2010)
3	*Spy*	*Salmonella enterica* serotype Typhimurium	**F:** TTGTTCACTTTTTACCCCTGAA **R:** CCCTGACAGCCGTTAGATATT	401	De Freitas *et al.* (2010)
4	bla_{TEM}	Broad Spectrum β-lactamase	**F:** ATGAGTATTCAACATTTCCG **R:** CTGACAGTTACCAATGCTTA	867	Bhattacharjee *et al.* (2007)
5	*tetA*	Tetracycline	**F:** GCTACATCCTGCTTGCCTTC **R:** CATAGATCGCCGTGAAGAGG	210	Fonseca *et al.* (2006)
6	*Sul1*	Sulphonamide	**F:** TTTCCTGACCCTGCGCTCTAT **R:** GTGCGGACGTAGTCAGCGCCA	425	Nair *et al.* (2015)

Table 2. Details of PCR cycling conditions used for different primers

PCR steps	PCR conditions	Target genes					
		invA	***Spy***	***Sdf* I**	bla_{TEM}	***tetA***	***sul1***
Initial Denaturation	Temperature (^{0}C)	94	94	94	95	95	94
	Time (min.)	5	5	5	5	5	3
Denaturation	Temperature (^{0}C)	94	94	94	95	95	94
	Time (sec.)	30	30	30	60	60	30
Annealing	Temperature (^{0}C)	56	57	57	55	64	56
	Time (sec.)	60	60	60	60	30	35
Extension	Temperature (^{0}C)	72	72	72	72	72	72
	Time (sec)	90	90	90	60	30	45
Final Extension	Temperature (^{0}C)	72	72	72	72	72	72
	Time (min.)	10	10	10	7	10	5
Number of cycles		35	35	35	35	40	35

Polymerase Chain Reaction for bla_{TEM}, *tetA* and *Sul1* gene was performed in 25 µl volume containing 12.5 µl of 2x PCR master mix (HiMedia Laboratories Pvt. Ltd., Mumbai), 1.5 µl of each primer (10pmol/µl) (Eurofins Genomics India Pvt. Ltd., Bangalore), 2 µl of genomic DNA and 7.5µl molecular biology grade water (HiMedia Laboratories Pvt. Ltd., Mumbai) to make the desired volume.

Statistical analysis

The data was recorded by following standard procedures and protocol. The results obtained were subjected to statistical analysis by using the software "WASP-Web Agree Stat Package-2.0" developed at ICAR research complex, Goa for Completely Randomised Design and Microsoft Excel for prevalence and Multiple antibiotic Resistance (MAR) index.

Results and discussion

Occurrence rate of Salmonella serotypes in meat

A total of 5 (2.78%) isolates were confirmed as *Salmonella* spp. by biochemical reactions and *invA* gene PCR (Fig. 2). The occurrence of *Salmonella* spp. in different districts viz. Aurangabad, Parbhani and Hingoli were observed as 2.22, 1.67 and 4 per cent, respectively. The occurrence rate observed for chicken, chevon and meat products was 0, 5 and 3.33 per cent, respectively. No significant difference was observed between various categories of meat samples collected and locations of sampling. The negligible occurrence of Salmonella spp. in chicken samples documented in the present study was not in agreement with the previous studies conducted (Thung *et al.,* 2016 and Abd-Elghany *et al.,* 2015), wherein the prevalence of *Salmonella* spp. in chicken was observed as 20.80 and 34, respectively (Thung *et al.,* 2016 and Abd-Elghany *et al.,* 2015). The occurrence rate of five in the chevon samples was in agreement with Maharjan *et al., (*2006) and Makwana *et al., (*2015), who reported a prevalence of 3.3 and 3.57 per cent, respectively. The difference in occurrence rate might be attributed to differences in hygienic and sanitary practices adopted by the butchers. The results obtained with meat products were consistent with the findings of Osman and Abdallah, (2018), who reported an occurrence of four per cent in different types of meat products. The contamination of meat products could be due to poor hygienic practices in handling meat and cross-contamination at the production level (NagmEldin *et al.,* 2018).

All *invA* gene-positive isolates were found to carry *spy* gene (Fig. 3) and serotyped as *Salmonella* Typhimurium (100%). Several research workers isolated *Salmonella* Typhimurium from meat and meat products,

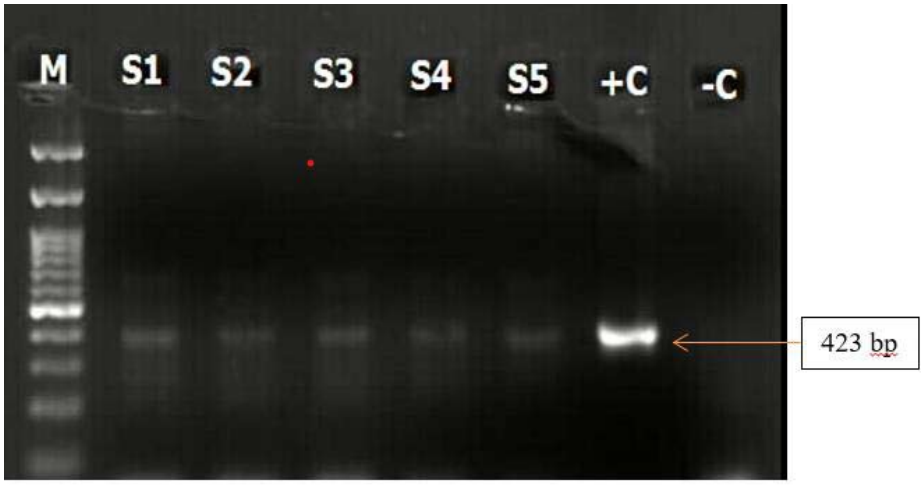

Fig. 2. Agarose gel electrophoresed image of the PCR amplicons of *invA* gene of *Salmonella* spp.

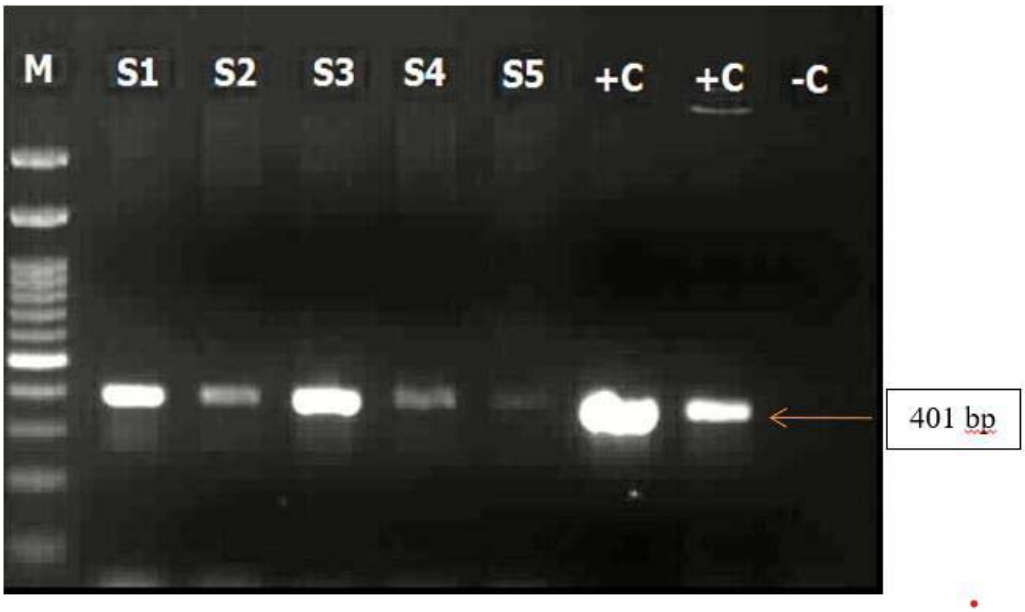

Fig. 3. Agarose gel electrophoresed image of PCR amplified product of *Spy* gene of *Salmonella* Typhimurium

but the occurrence of *Salmonella* Typhimurium (100%) in the current study is higher than in the previous reports (Abd El-Aziz, 2013 and Makwana *et al.,* 2015). The frequency and distribution of *Salmonella* serotypes from meat and meat products varies geographically. The presence of *Salmonella* Typhimurium in meat and meat products is an indication of improper handling. As this serotype affects both humans and animals, the findings of the present study are of great public health concern.

***Phenotypic antimicrobial resistance pattern of Salmonella* Typhimurium**

The resistance pattern of all *Salmonella* Typhimurium isolates was tested against the panel of 14 commonly used antimicrobial agents. The higher resistance was observed against Erythromycin (100%), followed by Amoxy-clav (80%), Ceftazidime (80%), Ciprofloxacin (80%), Amoxicillin/Sulbactam (60%), Chloramphenicol (40%) and Amikacin (40%). All isolates were found to be sensitive to Tetracycline (100%), Co-Trimoxazole (100%), Ampicillin/ Sulbactam (80%) and Gentamicin (80%). The isolates showed intermediate susceptibility to Enrofloxacin (80%), Levofloxacin (80%) and Nalidixic Acid (80%). The emergence of multi-drug-resistant *Salmonella* is a growing problem and has become a serious health hazard worldwide (Kim *et al.,* 2012). The practice of using antimicrobial agents for growth promotion, prophylaxis or therapeutics in livestock decreases their efficiency and is found to be an important factor for the emergence of multi-drug resistant *Salmonella* (Waghamare *et al.,* 2018). In the present study, higher resistance patterns observed against Erythromycin, Amoxy-clav and Amoxicillin/Sulbactam were in agreement with the findings of Naik *et al., (*2015) and Mthembu *et al. (*2019). The resistance patterns for Ciprofloxacin (80%) and Ceftazidime (80%) were in contrast with the results documented by Waghamare *et al. (*2018) and Thai *et al.* (2012). These observations were however in agreement with the findings of Thai *et al.* (2012) and Akond *et al.* (2012), wherein, the resistance reported were 37.3 and 58 per cent, respectively. Velez, *et al.* (2017) reported 100% resistance against Amikacin and Gentamicin, which was in contrast with the current results (Velez *et al*., 2017). The results of the present study regarding Ampicillin/Sulbactam and Tetracycline were not in agreement with Mthembu *et al.* (2019) who observed resistance of 64 and 63 per cent, respectively (Ahmed *et al.,* 2016). The difference in susceptibility and resistance pattern to Co – Trimoxazole, Enrofloxacin, Levofloxacin and Nalidixic Acid was observed by several researchers (Waghamare *et al.,* 2018; Thong *et al.,* 2011;

Akond *et al.,* 2012; Adesiji *et al.,* 2014). The high resistance rate detected in *Salmonella* spp. against Erythromycin, Amoxyclav, Ceftazidime and Ciprofloxacin might be associated with the use of these antibiotics in food animals (Ferede *et al.,* 2015).

Multiple Antibiotic Resistance (MAR) index of Salmonella Typhimurium

Multiple antibiotic resistance (MAR) index of 3 or more was observed for all *Salmonella* Typhimurium isolates (Fig. 4). The value of the MAR index of *Salmonella* Typhimurium isolates ranged from 0.21 to 0.43 with an average of 0.372. The MAR index shown in the present study was found in agreement with the study conducted by Talukder *et al.* (2021) and Mir *et al.* (2022), who reported that 100% of *Salmonella* isolates had a MAR index of more than 0.2.

Molecular detection of antimicrobial resistance pattern in Salmonella Typhimurium

All phenotypically antibiotic-resistant and sensitive *Salmonella* Typhimurium isolates were characterisation genotypically by PCR targeting bla_{TEM} for ESBL, *tetA for* tetracycline and *Sul1* gene for sulphonamide groups with expected amplicons of sizes 867 bp, 210 bp and 425 bp, respectively. All *Salmonella* Typhimurium isolates were found to carry only β-lactam resistance bla_{TEM} gene (Fig.5), whereas, none of the isolates were positive for *tetA* and *Sul1* gene.

Antimicrobial resistance in *Salmonella* spp. has become a growing concern for the global community. Bacteria can exchange resistance genes through transformation, transduction or conjugation (Thong *et al.,* 2011). The dissemination of antimicrobial resistance through mobile genetic elements such as plasmids, transposons and integrons gene cassette was well documented by Thong and Modarressi, (2011) reported the presence of $bla_{TEM,}$ *tetA* and *Sul1* genes in *Salmonella* Typhimurium. Similar to the results of our study, a high resistance rate to bla_{TEM} was reported by Ghazaei (2018), who reported the presence of the bla_{TEM} gene in 85 per cent of isolates. In the present study, a good correlation between phenotypic and genotypic results was obtained for β-lactam resistance among *Salmonella* Typhimurium isolates. The results of the *tetA*

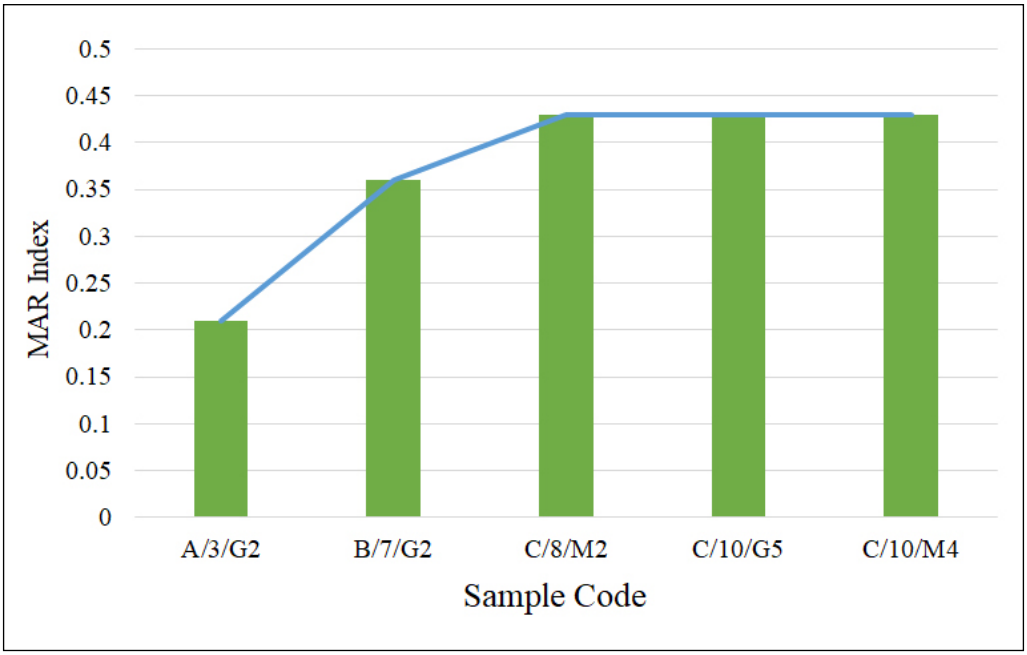

Fig. 4. Multiple antibiotic resistance index (MAR) of Salmonella *Typhimurium* isolates

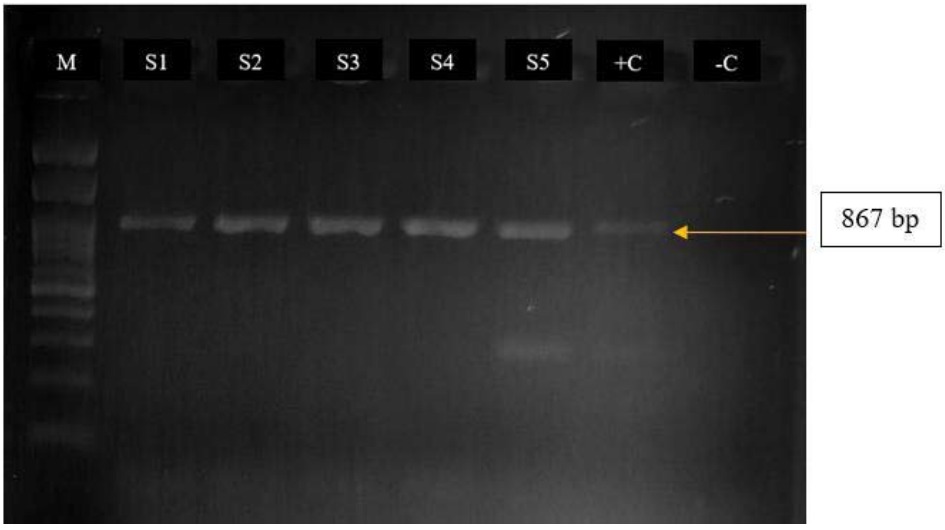

Fig. 5. Agarose gel showing PCR amplified product of bla_{TEM} gene of *Salmonella* Typhimurium

gene were found contradictory to the findings by Ahmed *et al*. (2016) and Gargano *et al*. (2021), who reported that 60 and 56 per cent of isolates, respectively harboured the *tetA* gene.

Observations of the present study on the *Sul1* gene were contradictory to the reports of Adesiji *et al.* (2014) and Ahmed *et al.* (2016) who had reported that 100 and 96.7 per cent of the *Salmonella* Typhimurium isolates, respectively, harboured *Sul* gene.

The detection of a high resistance rate to β-lactam antibiotics (80%) and the presence of the bla_{TEM} gene in *Salmonella* Typhimurium isolates could be due to the indiscriminate use of β-lactam drugs in the treatment of animals and poultry. A low level of resistance to tetracycline and sulphonamide with the absence of *tetA* and *Sul1* genes was highly appreciable and could be due to reduced use of these drugs at the farm level.

Conclusion

Multidrug-resistant *Salmonella* Typhimurium serotype with *invA* and *spy* virulence genes is a major pathogen detected in the present study, and it causes non-typhoidal infections in humans. The Salmonella isolates with high MAR index documented in the study are a major public health issue because they have acquired resistance to several antibiotics. *Salmonella* Typhimurium isolates with the bla_{TEM} gene, showing both phenotypic and genotypic resistance to β-lactam antibiotics indicated the extensive use of these antibiotics. The monitoring of multidrug resistance *Salmonella* spp. in food of animal origin is essential to control the outbreak of MDR *Salmonella* Typhimurium.

Acknowledgements

The authors are thankful to the College of Veterinary and Animal Sciences, Parbhani, (MAFSU, Nagpur) for providing the facilities needed for carrying out the research.

Conflict of interest

The authors declare no conflict of interest.

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