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

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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 blaTEM 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

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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 (Varjakshapanicker 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. enterica, 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 \( \beta \)-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
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°C ± 1°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°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°C ± 1°C for 24 h, for the development of colonies of *Salmonella* spp. The suspected colonies were subcultured on Nutrient Agar and incubated at 37°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), Amoxicillin/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 calculated 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<sub>TEM</sub>*, *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 MgCl₂ (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

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Primer</th>
<th>Target</th>
<th>Primer sequence (5' - 3')</th>
<th>Product Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1      | *invA* | Invasion-associated protein | F: TCGTGACTCGGCAATGCGATA  
R: GCCAGGGCGACGGCATATTCAAT | 423 | Nair et al. (2015) |
| 2      | *Sdf* I | *Salmonella enterica* serotype Enteritidis | F: TGTGTTTTATCTGATGCAAGG  
R: TGAACTACGTT CCTTCTTG | 304 | De Freitas et al. (2010) |
| 3      | *Spy*  | *Salmonella enterica* serotype Typhimurium | F: TTGTTCACCTTTTACCCTGAA  
R: CCTGACAGCGTTAGATATT | 401 | De Freitas et al. (2010) |
| 4      | *bla<sub>TEM</sub>* | Broad Spectrum β-lactamase | F: ATGAGTTAATCAATTCCG  
R: CTGACAGTTACCAATGGTTA | 867 | Bhattacharjee et al. (2007) |
| 5      | *tetA* | Tetracycline | F: GCTACATCTCGGCTTGCTTC  
R: CATAGATCGGCTGTGAGAGG | 210 | Fonseca et al. (2006) |
| 6      | *Sul1* | Sulphonamide | F: TTTCCTGACCTGCGGCTCTAT  
R: GTGCGGACGTAGTCCGCACCA | 425 | Nair et al. (2015) |

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

<table>
<thead>
<tr>
<th>PCR steps</th>
<th>PCR conditions</th>
<th><em>invA</em></th>
<th><em>Spy</em></th>
<th><em>Sdf</em></th>
<th><em>bla&lt;sub&gt;TEM&lt;/sub&gt;</em></th>
<th><em>tetA</em></th>
<th><em>Sul1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>Temperature (°C)</td>
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<td>94</td>
<td>94</td>
<td>95</td>
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<tr>
<td></td>
<td>Time (min.)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Denaturation</td>
<td>Temperature (°C)</td>
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<td>94</td>
<td>94</td>
<td>95</td>
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<tr>
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<td>Time (sec.)</td>
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<td>30</td>
<td>30</td>
<td>60</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Annealing</td>
<td>Temperature (°C)</td>
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<td>57</td>
<td>57</td>
<td>55</td>
<td>64</td>
<td>56</td>
</tr>
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<td>60</td>
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<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>Temperature (°C)</td>
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<td>72</td>
<td>72</td>
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</tr>
<tr>
<td></td>
<td>Time (sec.)</td>
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<td>90</td>
<td>60</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Final Extension</td>
<td>Temperature (°C)</td>
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<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
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</tr>
<tr>
<td></td>
<td>Time (min.)</td>
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<td>10</td>
<td>10</td>
<td>7</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Number of cycles</td>
<td></td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>40</td>
<td>35</td>
</tr>
</tbody>
</table>
Polymerase Chain Reaction for \( \text{bla}_\text{TEM} \), \( \text{tetA} \) and \( \text{Sul1} \) 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.
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;
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 characterised genotypically by PCR targeting *bla*<sub>TEM</sub> 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*<sub>TEM</sub> 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*<sub>TEM</sub>, *tetA* and *Sul1* genes in *Salmonella* Typhimurium. Similar to the results of our study, a high resistance rate to *bla*<sub>TEM</sub> was reported by Ghazaei (2018), who reported the presence of the *bla*<sub>TEM</sub> 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*
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 Sul1 gene.

The detection of a high resistance rate to β-lactam antibiotics (80%) and the presence of the blaTEM 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 blaTEM 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.

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Conflict of interest

The authors declare no conflict of interest.

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