

Genotypic and Phenotypic Characterization of *MDR Salmonella typhimurium* Isolated from various Sources in Maharashtra State

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(Received: 15 December 2022; Revised: 25 January 2023; Accepted: 31 January 2023; Published: 04 February 2023)

(Published by Research Trend)

ABSTRACT: *Salmonella* infections are still a paramount public health concern around the world. Humans can become infected with *Salmonella* by being exposed to it directly or indirectly through poultry meat, water, or soil. Even after having antibiotics to conquer it, multidrug resistance which makes it difficult to employ the treatments that are accessible. Hence, a comprehensive policy framework and integrative approach are needed for the prevention, control, and eradication of multidrug resistance in *Salmonella typhimurium*. The main goal of this study was to determine the ubiquity of multidrug resistance in *Salmonella Typhimurium* isolates from Maharashtra state. This study will define source and nature of development multidrug resistance in *Salmonella typhimurium*. This *in vitro* study was carried out at bacteriology laboratory of PWCOP in New Yavatmal. Samples were collected from various sources in the five regions of Maharashtra State. Various morphological, biochemical, cultural, and molecular approaches were used to obtain and confirm *Salmonella typhimurium* isolates. Antibiotic susceptibility testing of *Salmonella typhimurium* isolates was carried out using disk diffusion method. Total 300 samples were collected out of which 69 (23%) were positive for *Salmonella* spp. In addition, 48 (69.56 percent) of the 69 *Salmonella* spp. isolates tested positive for *Salmonella typhimurium*. The prevalence rate for *Salmonella typhimurium* in the samples from the three sources was Chicken meat: 27 (56.25%), roadside water: 15 (31.25%), and for roadside Soil: 6 (12.5%). Likewise prevalence of *Salmonella typhimurium* was found as Yavatmal: 9(18.75%), Parbhani: 7 (14.58%), Mumbai: 14 (29.16%) Ahmednagar: 7 (14.58%) and Pune were 11 (22.91%). Of total isolates studied (29.16%) were found to be multidrug resistant (MDR). Out of 14 MDR *Salmonella typhimurium* isolates examined, 12 (85.71%) had the most resistance to Chloramphenicol, 9 (64.28%) had the higher side resistance to Amoxicillin, and the lower resistance to Nalidixic acid. Further molecular confirmation of these MDR *Salmonella typhimurium* isolates was done with 16s rRNA gene amplification with universal primers. A total of 14 PCR showed the presence of *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{OXA} gene was determined in confirmed isolates. The presence of multidrug resistance in *Salmonella typhimurium* isolates revealed that unclean chicken meat contaminated water and soil posed a significant risk of human infection. The current study shows that MDR *Salmonella typhimurium* is common in Maharashtra, and it is recommended that antibiotics be used ethically and appropriately.

Keywords: *Salmonella typhimurium*, Multidrug resistance, Prevalence, chicken meat, Molecular characterization.

INTRODUCTION

Salmonella is one of the notably commonly isolated food-borne infections. It is an intercontinental public health problem that causes about 94 million foodborne illnesses and 0.15 million fatalities per year. To date, more than 2500 *Salmonella* serotypes have been described and more than 60% belong to the Subsp. *Salmonella enterica*. Enterica, which assign to source for the number of human *Salmonella* infections (Crump *et al.*, 2015) *Salmonella* infections caused by invasive serotypes are very commonly fatal, necessitating prompt and effective antibiotic treatment. The advent of multidrug-resistant (MDR) *salmonella* subtypes has a significant impact on the effectiveness of the antibiotic treatment, and a growing prevalence of MDR strains

may contribute to a rise in number and death rates of *Salmonella* infections (Shu *et al.*, 2015).

Animal consumable food stuffs and, increasingly crops debased with animal excrement are key factors of non-typhoidal *Salmonella typhimurium* in developed countries (Painter *et al.*, 2013) *Salmonella enterica* serovar *typhimurium* persistence on lettuce and parsley and in soils cultivated in fields treated with polluted manure composts or irrigation water (Islam *et al.*, 2017).

Salmonellosis is a clinical infection caused by *Salmonella* bacterium. (CDC, *Salmonella*, 2015) Diarrheic conditions, fever, stomach pains, and vomiting-nausea are the most commonly seen symptoms. Symptoms often appear 12 to 36 hours after exposure and continue two to seven days. Dehydration

can be seen as a consequence of more critical disease (CDC, *Salmonella*, 2015). The upper age people, children, and people with depressed immune systems are very likely to acquire serious disease (CDC, 2008) *Salmonella* strains can cause typhoid fever or paratyphoid fever (Hald *et al.*, 2013).

Enteric NTS had been tentatively to cause more than 93 million diarrheal diseases and 0.15 million demises worldwide in 2006. In the Center for Health Indicators and Assessment Global Burden of Disease 2010 research, enteric non-typhoidal *salmonella* was expected to account for 4.8 million disabilities adjusted life-years and 81,300 deaths. Non-typhoidal *Salmonella* was expected to cause 3.4 million invasive infections and 0.6 million deaths in 2010; 57% of these diseases and fatalities occurred in African countries (Ao *et al.*, 2010; GBD, 2017).

Typhoidal *salmonella*, non-typhoidal *salmonella* has seen a growing prevalence of antimicrobial resistance in recent decades (Akeeb *et al.*, 2001). Fluoroquinolones and chloramphenicol susceptible *salmonella* strains in India are endemic in nature (Jesudason *et al.*, 1992). *Salmonella paratyphi* infections are increased with high number of MDR resistance, out of total isolates more than 45% were MDR strains (Chandel *et al.*, 2000). In Tamil Nadu prevalence of MDR *S. typhimurium* were more than 40% (Singh *et al.*, 2012). In Uttar Pradesh rate higher than previous one that is 45% (Arjunan *et al.*, 2010). The Multi Drug Resistance phenotype in *Salmonella typhimurium* first appeared in the beginning of 1980s in the UK, where it was strongly related with a kind of phage known as form that cannot be changed (White *et al.*, 2001). Isolates from UK resistant to given antibiotics chloramphenicol, ampicillin, streptomycin, sulfonamides, and tetracycline, a resistance subtype generally referred to as R-type ACSSuT. By the 1990s, this subtype had been reported from many other European countries, as well as from the US, Canada, Israel, Turkey, and Japan (Crump *et al.*, 2015). Recently reported outbreak of MDR *Salmonella typhimurium* shows resistant to more than nine higher antibiotics including nalidixic acid at 18 to 20% (Villar *et al.*, 1999). Endemic regions of Pakistan show prevalence of chloramphenicol, ampicillin, trimethoprim, sulphamethoxazole MDR *S. typhimurium* (Kwai *et al.*, 2000).

Extended spectrum beta-lactamases (ESBL) is a type of catalyst or biochemical generated by certain bacteria. Some antibiotics fail to treat bacterial infections because of ESBL enzymes. The existence of genes associated with tetracycline, tet (A), tet(B) and beta-lactam resistance (*bla* SHV, *bla* CMY) has been determined. ESBL-producing *S. typhimurium* strains exhibited resistance mechanisms linked to aminoglycosides, quinolones, sulfonamides, beta-lactam resistance (Bouchrif *et al.*, 2009). A SHV-producing *Salmonella serovar Typhimurium* strain isolated in Canada in 2000 was the first to produce ESBL. Two years later, the first CMY-generating *Salmonella* subtype was found during a mild sudden start of *Salmonella serovar Newport* in Alberta,

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Canada. Several studies have now been published on the prevalence of CMY and ESBL enzymes in *salmonella* isolates obtained from human, animal, and retail meat outlets in Canada and the US (Crump *et al.*, 2015).

Qiet. *al.*, (2019) studied pattern of multidrug resistance in *Salmonella* species PFGE banding methods and found that levofloxacin, ceftriaxone, and ampicillin/sulbactam all had lower sensitivity rates than fosfomycin (78.2%), which had the highest sensitivity rate (97.5%). There was a 60.9 and 50.61% resistance to piperacillin and ciprofloxacin, respectively.

Heir *et al.* (2002) gave important details on the relative importance, geographic distribution, and characteristics of antibiotic resistance of several *S. typhimurium* clones that cause human salmonellosis.

As per (Raseala *et al.*, 2020) Soil, manure, irrigation water and freshwater stream samples shared five serovars, which indicated circulation of ESBL-producing *Salmonella* spp. Confirmed with PCR studies.

MATERIAL AND METHODS

Sampling and isolation of bacteria: Samples from various sources related chicken meat, roadside water, roadside soil from five regions of Maharashtra state were collected. All the samples were examined after collection. The sample (25 g) was mixed thoroughly and incubated at 37 °C for 19-24 hours in 225 mL of buffered peptone water (BPW). This inoculated BPW were then transferred to 10 mL of selected proliferative agar media (Boukharouba *et al.*, 2022). *Salmonella* spp. was specifically identified by Brilliant Green Agar (BGA), Bismuth Sulphite Agar (BSA), Xylose Lysine Deoxycholate agar (XLD), Hektoen enteric agar (HEA) and further incubated at 37 °C for 24–48 h and isolated prominent colonies of *Salmonella* species. For water and soil samples were processed with normal saline (Rajaei *et al.*, 2021).

Biochemical and microbiological analysis: Bacteriological analysis and biochemical characterization of collected samples were carried out as per (FDA, 2007) Bacteriological Analytical Manual. and Various biochemical identification tests such as Gram staining's, triplate sugar iron (TSI) agar test, urease test, lysine iron agar (LIA), mannitol motility (MM) test, citrate test, motility-indole-ornithine (MIO) agar test and oxidative fermentative test (OF), were performed and confirmation of *Salmonella typhimurium* was done (Colello *et al.*, 2018)

Identification of MDR *Salmonella typhimurium*: The isolated *Salmonella* were subject to 7 selected antibiotics. Antimicrobial susceptibility testing was performed according to CL Standards Institute formerly known as (National Committee for Clinical Laboratory Standards). MICs were observed by disc diffusion method for antimicrobial agents which were commonly, preferably used in animal and humans (EUCAST, 2000) (HiMedia Lab, Pvt. Ltd. Mumbai, India): Gentamycin (10 µg), Amoxicillin (30 µg), Enrofloxacin (10 µg), Chloramphenicol (30 µg), Cefotaxime (30 µg), Tetracycline (30 µg), and Nalidixic

acid (30 µg), and The isolates were categorized as resistance (R), intermediate (I), or susceptible (S) based on their inhibitory zone (S).

Molecular identification of *Salmonella typhimurium*:

The Polymerase chain reactions for *Salmonella typhimurium* were carried out as per standard protocols by sambrook and russel also as per methods described by references using 16S rRNA method (Hellberg *et al.*, 2012) with primers F5'AGAGTTTGATCCCTGGCTCAG3', R5'GTACGGCTACCTTACGAC3' with MDR *Salmonella typhimurium* genomic DNA extracted by boiling method with expected Amplicon size 1503bp. PCR cycling conditions used was 95°C cycled for 5 min., 1 min. at 95°C, 56°C for 1 min., 72°C for 1 min., 72°C for 5min. Agarose gel electrophoresis was performed to confirmed Amplicon size on gel.

Molecular characterization of ESBL resistance: The Polymerase chain reactions for ESBL genes that are *bla*_{CTX-M}, *bla*_{OXA}, *bla*_{TEM} and *bla*_{SHV} was performed as per standard protocols by sambrook and russel also as per methods described by reference (Filip *et al.*, 2004) using primers (Table 1) with plasmid DNA extracted with Hi-media miniprep plasmid DNA extraction kit. PCR cycling conditions used for all set of primers was initial cycle for 5 min. at 95°C, 95°C for 1 min., 56°C annealing for 1 min., 72°C for 1min., 72°C for 5min. Agarose gel electrophoresis was performed to confirmed Amplicon size on gel (Lee *et al.*, 2012).

Table 1: List of primers ESBL genes.

Primer Name	Primer Sequence	Amplicon size
BlaCTX-m F	ATGGTTAAATCACTGCGCCA	867bp
BlaCTX-m R	CAAACCGTCGGTGACGATT	
BlaTEM F	TCAGGTGGCACTTTTCGGGGAA	656bp
BlaTEM R	ACGCTCGTCGTTTGGTATGGCT	
BlaOXA F	ACGCAACTGGTCCAGAACCTTG	1510bp
BlaOXA R	AAAAGCCAGCCTTTCATGATATA	
BlaSHV F	ACGGTAACTGATGCCGTATT	11000bp
BlaSHV R	GGCGGCATAACGCGCGCC	

RESULT AND DISCUSSION

Prevalence of *Salmonella typhimurium*: Out of total 300 samples 69 (23%) were positive for *Salmonella* spp. And out of 69 salmonella spp. isolates 48 (69.56%) isolates were positive for *Salmonella typhimurium*. The growth positive rate in the samples of the three sources for *Salmonella typhimurium* was for Chicken meat: 27

(56.25%), roadside Water: 15 (31.25%), and for roadside Soil: 6 (12.5%). Likewise prevalence of *Salmonella typhimurium* was found as Yavatmal: 9 (18.75%), Parbhani 7 (14.58%), Mumbai 14 (29.16%) Ahmednagar 7 (14.58%) and Pune were 11 (22.91%) (Fig. 1).

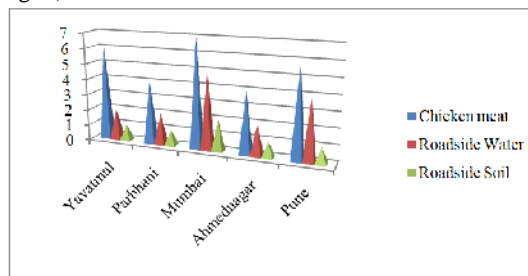


Fig. 1. Prevalence of *Salmonella typhimurium* region wise & source wise.

Colony characteristics of *Salmonella typhimurium*:

Salmonella typhimurium generally showed black colonies with a metallic sheen around them on BSA because to hydrogen sulphide generation and sulfite conversion to black ferric sulphide, on BGA showed Good growth; red-colored colonies, on HEA grown with black color colonies and on XLD also showed black color colonies (Fig. 2).

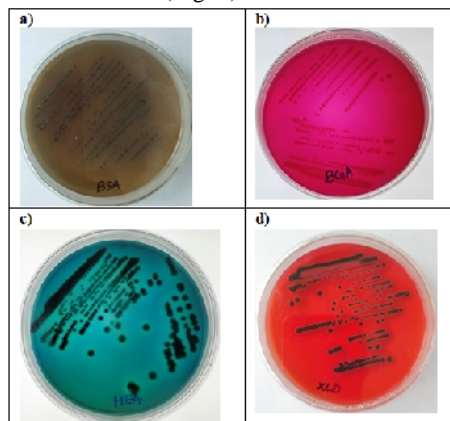


Fig. 2. Colony characteristics of *Salmonella typhimurium* on different agar media
a) BSA b) BGA c) HEA d) XLD

Biochemical and microbiological characteristics of *Salmonella typhimurium*:

Biochemical and microbiological characteristics of *Salmonella typhimurium* were done by conventional as well as using Himedia kit. The observations were showed in (Fig. 3) with values in (Table 2).

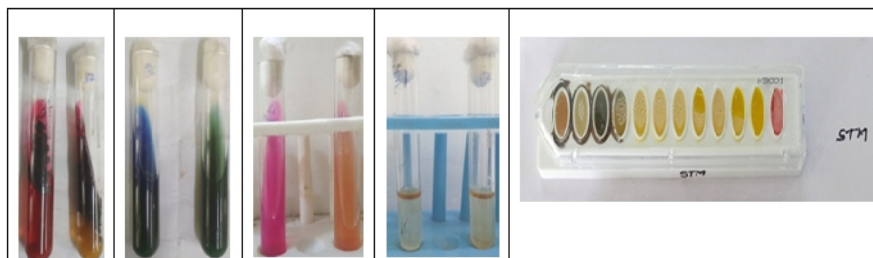


Fig. 3. Biochemical and microbiological characteristics of *Salmonella typhimurium*.

Table 2: Biochemical test results.

Test	Indole	Methyl red	Voges Proskauer's	Citrate Utilization	Glucose	Motility	Catalase	Lactose	Oxidase	Mannitol	TSI	Sucrose
Result	-	+	-	+	+	+	+	-	+	+	+	+

Antibiotics resistance pattern of *Salmonella typhimurium*: Out of total isolates studied (29.16%) were found to be multidrug resistant (MDR). Among all the tested antibiotics out of 14 MDR *Salmonella typhimurium*, 12 (85.71%) isolates exhibited maximum resistance against Chloramphenicol and 9 (64.28%) of isolates have exhibited resistance against Amoxicillin and lowest against Nalidixic acid (Fig. 4, 5).

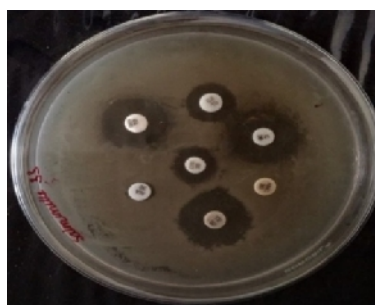


Fig. 4.Antibiotics resistance pattern of *Salmonella typhimurium* by disc diffusion method.

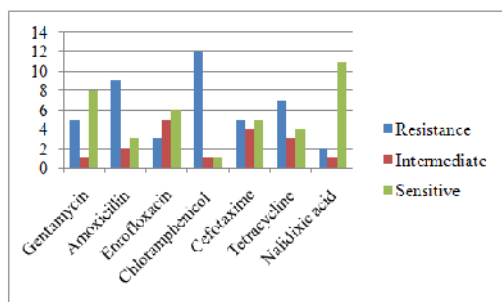


Fig. 5. Antibiotics resistance pattern of 14 MDR *Salmonella typhimurium* isolates against 7 Antibiotics.

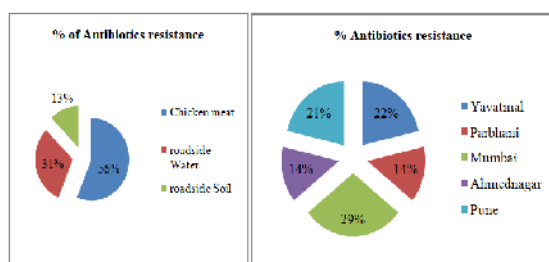


Fig. 6. Antibiotics resistance pattern of 14 MDR *Salmonella typhimurium* isolates source wise & Region wise.

Molecular identification of 14 MDR *Salmonella typhimurium* isolates: All 14 MDR *Salmonella typhimurium* isolates were further confirmed by PCR with desired Amplicon size 1503bp band on 1% agarose against 1kb DNA marker (Fig. 8).

Molecular characterization of ESBL resistance gene: The Polymerase chain reactions for ESBL genes that are *bla*_{CTX-M}, *bla*_{OXA}, *bla*_{TEM} and *bla*_{SHV} was performed for All 14 MDR *Salmonella typhimurium* Gaikwad *et al.*, *Biological Forum – An International Journal* 15(2): 66-72(2023)

isolates and desired amplifications bands were obtained on 1% agarose gel as 867bp, 1510bp, 656bp and 1100bp respectively [Fig. 9a-d].

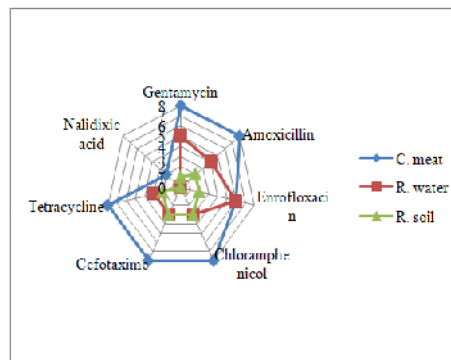


Fig. 7. Resistance pattern of different Antibiotics among 14 MDR *Salmonella typhimurium* isolates from various sources.

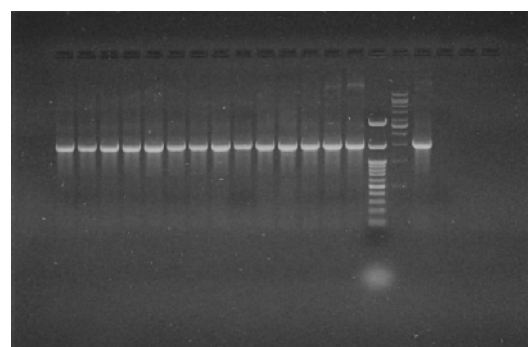


Fig. 8. Molecular identification of 14 MDR *Salmonella typhimurium* isolates.

In most of the people *Salmonella typhimurium* causes diarrhea, pyrexia, and septicemia. *Salmonella* serovars that aren't typhoid are nonetheless a risk to human health, and chickens meat, water, soil could be sources of these bacterial infections in the environment (Abouzeed *et al.*, 2000).

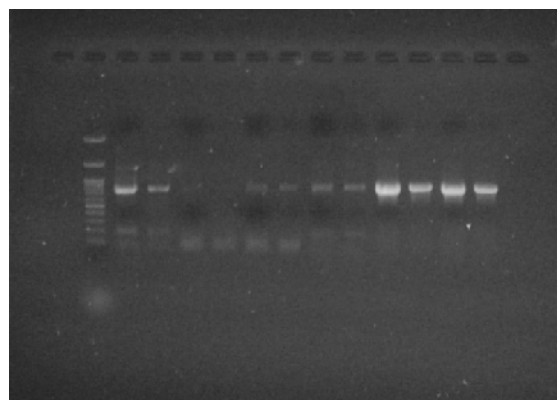


Fig. 9a. Molecular characterization of CTXm gene.

As per (Akoachere *et al.*, 2009), *S. typhimurium* was isolated from samples with a prevalence of 32.6%. Chicken meat isolates (40%) showed highest *Salmonella* prevalence in northern India (Jaishree *et al.*, 2019). As our findings were out of total 300 samples 69 (23%) were positive for *Salmonella* spp. And out of 69 salmonella spp. isolates 48 (69.56%) isolates were positive for *Salmonella typhimurium*.

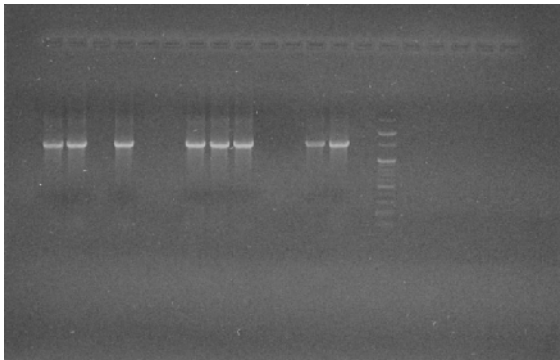


Fig. 9b. Molecular characterization of OXA gene.

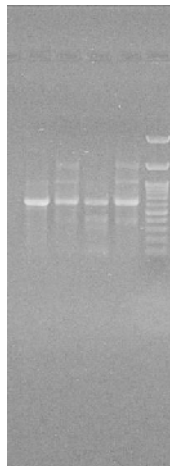


Fig. 9c. Molecular characterization of TEM gene.

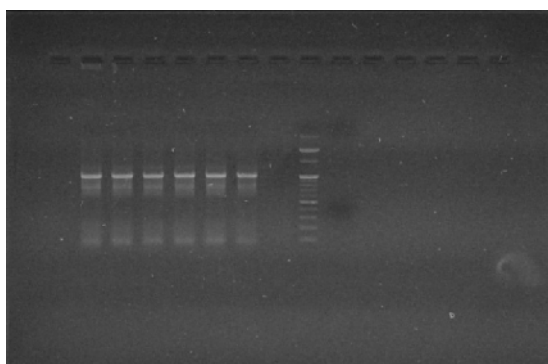


Fig. 9d. Molecular characterization of SHV gene.

As per this study (85.71%) of the isolates were resistance to Chloramphenicol which is as much synchronize to study performed by (Shrestha *et al.*, 2016) mentioned that the 98.8 % of the *Salmonella* spp. were susceptible to chloramphenicol.

Chicken samples shows more prevalence than water and soil, Mumbai region was more incident with *Salmonella typhimurium* than others this was similar to study by Samanta *et al.* (2014).

Salmonella spp. were isolated and confirmation with utilizing bacterial culture on *Salmonella* specific media and confirmatory assays such as 16S rRNA gene amplification. All results of these diagnostic procedures indicated that all isolates were *Salmonella* spp. (Hayder *et al.*, 2019). We have also got the desired amplification using 16s rRNA gene confirmation for *Salmonella typhimurium*.

Owing to the development of Extended Spectrum Beta-Lactamase (ESBL) and decreased tolerance to ciprofloxacin, resistance to cefotaxime and ceftriaxone has arisen resulting in failure to treat (Biendo *et al.*, 2003). ESBLs of type Cefotaximase (CTX-M) are the most common beta lactamase among Enterobacteriaceae including salmonellae. Our findings similarly confirm the ESBL resistance in *Salmonella typhimurium* (Ramachandran *et al.*, 2017).

This study correlates the finding of Waghmare *et al.* (2017); Rayanoothala *et al.* (2021) by considering the prevalence in chicken, water, and soil samples along with multidrug resistance in *Salmonella typhimurium*.

Our results for multidrug resistance genes *bla*_{CTX-M}, *bla*_{OXA}, *bla*_{TEM} and *bla*_{SHV} by polymeric chain reactions also confirmation of *Salmonella typhimurium* by 16s RNA are agreements with study performed by Ahmad *et al.* (2020).

CONCLUSIONS

The presence of multidrug resistance in *Salmonella typhimurium* isolates suggested that there is noticeable risk of infections to humans from unhygienic chicken meat also from roadside contaminated water and soil. From the present study it is clear that occurrence of MDR *Salmonella typhimurium* are high in the Maharashtra region and suggested to use the antibiotics ethically and appropriately.

FUTURE SCOPE

To investigate the determinants of resistance and comprehend the niches that have contributed or may in the future contribute to infections with antibiotic-resistant bacteria, there must be an interest in working on sampling the environmental resistomatics in these areas. Further studies on development of natural alternative remedies for combating MDR *Salmonella typhimurium* were needed.

Acknowledgments. This study was conducted at Pataldhamal Wadhvani College of Pharmacy, Yavatmal (M.S), 445001.

Conflict of Interest. None.

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How to cite this article: Rahul Prabhu Gaikwad, Madhuri A. Channawar, Deepak S. Mohale, Nitin I. Kochar and Anil V. Chandewar (2023). Genotypic and Phenotypic Characterization of *MDR Salmonella typhimurium* Isolated From various Sources in Maharashtra State. *Biological Forum – An International Journal*, 15(2): 66-72.