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Detection and Characterization of Extended-Spectrum β-lactamases in *Salmonella* Isolates of Meat, Milk and Human Clinical Samples from Different Districts of Chhattisgarh

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A B S T R A C T

Present study was undertaken to assess the hygiene quality of the meat, milk and also its prevalence on human population. A total of 330 samples including, chicken meat (n=98), Chevon (n=82), raw milk samples (n=90) and human urine (n=56) and stool samples (n=4) Samples were aseptically collected from different districts of Chhattisgarh. Samples were processed for isolation of *Salmonella* further subjected for the different biochemical tests and confirmed by serotyping. Confirmed isolates were tested for its antibiogram pattern against 13 different antibiotic discs. Isolates were examined for phenotypic method for Extended Spectrum β-lactamases (ESBLs) by double disc synergy test and molecular characterization was done by multiplex PCR method. A total of 14.2% (47/330) samples were positive for *Salmonella* spp. highest prevalence rate of 27.55% (27/98) were observed in chicken meat, followed by 15% (9/60) and 13.41% (11/82) samples were found positive for *Salmonella* in contrary none of the milk samples found positive for *Salmonella*. Serotyping reveals the presence of *S. typhiurium* and *S. enteritidis* serotype among the isolates. Isolates showed highest sensitivity (95.7%) against imipenem and however 89.2 % isolates were found resistant to oxytetracylin. Out of 47 isolates 2.12% isolates were found as ESBL positive by phenotypic and genotypic method.

Introduction

Food-borne pathogens are major source of illness and death thus leading to a huge expenditure of money on healthcare (Nagrajan *et al.*, 2018). The burden of food borne diseases is substantial: every year almost 1 in 10 people fall ill and 33 million of healthy life years are lost. Food borne diseases can be severe, especially for young children (WHO, 2018). Diarrheal diseases are the most common illnesses resulting from unsafe food, 550 million people falling ill each year, including 220 million children

under the age of 5 years (WHO, 2018). Salmonellosis is one of the major food borne pathogens affects humans causing foodborne illnesses worldwide with serious implications in ill developed countries (Forshell and Wierup, 2006). Outbreaks due to *Salmonella* have been associated with a wide variety of foods especially those of animal origin (Hernandez *et al.,* 2005) such as meat, chicken, egg, milk and sometimes vegetables in the food chain (Bouchrif *et al.,* 2009; Naugle *et al.,* 2006). *Salmonella* infections are largely classified into four clinical types (Bisi-Johnson and Obi, 2012); first, gastroenteritis caused by *Salmonella enterica* serovar Typhimurium; second, Bacteremia, osteomyelitis, reactive arthritis due to *Salmonella typhimurium* and *Salmonella enteritidis* infection; third, enteric fever caused by *Salmonella* Typhi and *Salmonella* Paratyphi and lastly, a carrier state in persons with previous infections (Owens and Warren, 2009; Klotchko and Wallace, 2009).

In developed countries antimicrobial drug resistance in non-typhoidal *salmonella* organisms is an almost inevitable consequence of the use of antimicrobial drugs in food producing animals. Such drugs may be used either therapeutically or prophylactically, or for growth promotion (feed additives). In developing countries contrast to the situation in developed countries such increases has been almost entirely associated with the use of antimicrobials in human medicine, both in hospitals and the community. Examples of increases in resistance in non-typhoidal *salmonellas* in developing countries, particularly in the Indian subcontinent, South East Asia, South and Central America and Africa, are exemplified by outbreaks caused by organisms such as *Salmonella* wien, *Salmonella* Typhimurium, *Salmonella* Johannesburg and *Salmonella* Oranienburg, all of which have caused numerous outbreaks of serious disease both in hospitals and the community over wide geographical areas (Rowe and Threlfall, 1984). *Salmonella* isolates harbouring Extended Spetrum β Lactamases (ESBLs) have emerged worldwide during the last decade. This has caused concern since cephalosporins are drugs of choice for the treatment of salmonellosis in children. Different bla_{SHV}, bla_{TEM}, bla_{CTX} and bla_{CMY} genes have been found to encode ESBL resistance in *Salmonella* (Tzouvelekis *et al.,* 2000; Mulvey *et al.,* 2003).

Materials and Methods

Study area and sampling

The surveillance of non-typhoidal *Salmonella* serovars was done to evaluate the hygienic quality of meat (Chicken and Chevon) and milk, however human urine and stool samples were also collected to assess prevalence of organism in the community. A total of 330 samples including chicken meat (n=98), Chevon (n=82), raw milk samples (n=90) and human urine (n=56) and stool samples (n=4) Samples were aseptically collected from Jagdalpur, Dantewada, Kanker and Kondagaon districts of Chhattisgarh (Table 1). Meat samples were collected from local meat shop and slaughter houses. Raw milk samples were collected from hotels and restaurants and Human stool and urine samples were collected from human hospital and from private clinics of above said districts. All the samples were collected aseptically and transported to the laboratory under refrigerated condition for analysis within 4-6 hrs.

Isolation of *Salmonella*

Isolation of *Salmonella* from milk and meat samples was performed as per the standard procedure described by ISO 6579: (2002)

with slight modifications (ISO, 2002). Briefly 25gm/ml of food sample were taken in 225 ml of Buffered peptone water (BPW) (Himedia, India) and incubated overnight at 37°C, after that 1ml of the culture from pre-enrichment was taken and incubated in tube containing 9ml of Tetra Thionate (TT) broth (Himedia, India) for selective enrichment and incubated at 37°C for 24h, further loop full culture from selective enrichment broth was streaked on to Brillient Green Agar (BGA) (Himedia, India) and Bismuth Sulfide Agar (BSA) (Himedia, India).

Isolation from human urine and stool samples were performed as per the method outlined by Nesa *et al.,* (2011) and Singh *et al.,* (2011), respectively. According to the protocols each samples were enriched in 1:10 ratio in freshly prepared Nutrient Broth (NB) and incubated at 37°C for 24 h and loopful culture from enrichment were streaked on BGA and BSA. Colonies appearing moderately large moist colorless surrounded by pink background on BGA and typical black colony surrounded by brownish-black zone with metallic sheen on BSA were considered as a presumptive *Salmonella*. The presumptive *Salmonella* Colonies (3-4 colonies/plates) were further subjected to biochemical characterization.

Identification of *Salmonella*

Biochemical characterization

The presumptive colonies of *Salmonella* were further subjected to biochemical tests viz., triple sugar iron (TSI), Urease broth, indole, methyl red, Voges-Proskauer and Citrate test (IMViC) as per the procedure described by Agarwal *et al.,* (2003). The colonies showing negative urease test with (-+-+) IMViC pattern were further inoculated on TSI slants and colonies producing alkaline slant (pink) and acidic butt (yellow) with or without H2S production (blackening) were considered as a biochemically confirmed *Salmonella* isolates.

Serotyping of *Salmonella* isolates

All *Salmonella* isolates were further submitted to the National Salmonella and Escherichia Centre, Central Research Institute, Kasauli (Himachal Pradesh) for serotyping.

Antimicrobial susceptibility pattern

All *Salmonella* isolates were tested for their antimicrobial susceptibility pattern by disc diffusion method as described earlier by Bauer and Kirby (1966). Isolates were tested against 11 commercial antibiotic disc (Himedia, India) on Mueller Hinton Agar plates (MHA) (Himedia, India). Antibiotic discs including Oxytetracycline (30mcg), Cephalexin (30mcg), Ciprofloxacin (5mcg), Gentamicin (30mcg), Cefotaxime (10mcg), Ampicillin (10mcg), Ceftazidime (30mcg), aztreonam (30mcg), Imipenem (10mcg), Cefixime (5mcg), and Meropenem (10mcg) were used in the study. The diameter of the zones of complete inhibition was measured and compared with the zone size interpretation chart provided by the supplier and were graded as sensitive, intermediate and resistant. The multiple antibiotic resistance (MAR) index was also calculated for all *Salmonella* isolates following the protocol described by (Krumperman, 1983) by applying formula a/b where "a" is the number of antibiotics to which an isolate was resistant and "b" is the number of antibiotics to which the isolates were exposed.

Detection of ESBL producing isolates

The isolate with a zone of inhibition diameter of ≤17 mm for aztreonam, ≤17 mm for Ceftazidime, and ≤22 mm for cefotaxime in disc diffusion susceptibility testing were considered further for screening for ESBL phenotypes.

Double disc synergy test

The phenotypic confirmatory test for ESBL producers was performed as per Clinical Laboratory Standard Institute (CLSI, 2012) guidelines. For this purpose, 5 antibiotics cefotaxime (10µg), ceftazidime (30µg), aztreonam (30µg), cefotaxime- clavulanic acid (30/10µg), amoxicillin-clavulanic acid (30µg) were used. Discs were placed 25 mm apart on a MHA plate inoculated with 0.5 McFarland suspension of the test isolate. Plates were incubated at 35°C for 18 hrs. After incubation the zone diameter around each of disc was measured.

A difference of ≥ 5mm between the zone diameters of either of the cephalosporin discs and their respective cephalosporin/ clavulanic acid discs were considered as positive phenotypic confirmatory test for ESBL producers.

Molecular characterization of ESBL producing isolates

Isolates which showing positive ESBL production in double disc synergy test were further subjected to test the presence of *bla* genes. Extraction of bacterial genomic DNA was performed by snap chill method, briefly, a single colony was inoculated into NB medium and was incubated at 37°C overnight. Then, 1.5 ml of the bacterial culture was centrifuged at 8000 rpm and the pellet was washed three times in PBS and eluted in 100 µl of Nuclease Free Water (NFW), kept in to boiling water bath for 10 minutes, after that pellet is immediately chilled in ice.

Detection of the gene sequence coding for the TEM, SHV, CTX-M enzymes were performed by the multiplex PCR as previously described with slight modifications (Apaka *et al.,* 2010) (Table 2).

Results and Discussion

A total of the 330 samples including 180 meat samples (98 chicken meats and 82 chevon), 90 milk sample and 60 human urine and stool samples were screened for isolation of *Salmonella* spp. Forty seven (14.2 %) samples were found positive by culture and biochemical tests. Out of 47 isolates, 27 (57.4 %) were from chicken, 11(23.4%) from chevon meat and 9 (19.1%) from human urine and stool samples. District wise highest prevalence was observed in Dantewada district (17.7%) followed by Kondagaon (16.5%), Jagdalpur (12.6%) and Kanker (9.2%) (Table 1). The prevalence of *Salmonella* in chicken meat was observed 27.6 % which is in agreement with the report of Moon (2011) and Bharathy *et al.,* (2015) However lower prevalence rate were also reported by several workers (Kumar and Lakhera, 2013 and Saad *et al.,* 2011). The significantly higher prevalence of 56% was reported in chicken meat by Ramya *et al.,* (2012).

In chevon, 13.4% prevalence of *Salmonella* was observed which is closely related with the findings of Panda *et al.,* (2012) and Ahmad *et al.,* (2013) who reported 10-13.9 % prevalence (Table 1). However lower prevalence rates varied between 2% (Zubir *et al.,* 2012) to 9% (Naik *et al.,* 2015*)* were reported by others. On contrary, Moon (2011) reported higher prevalence rate of 38.3%.

In human urine and stool samples, 15% prevalence of *Salmonella* was observed (Table 1). Nesa *et al.,* (2011) and Warjri *et al.,* (2015) reported 16.1% prevalence rate in stool samples which corroborate with the present findings. However, Babu *et al.,* (2010) and Singh *et al.,* (2011) reported lower prevalence rate. On the contrary, higher prevalence rate were reported by Chiu and Ou (1996) and Murugkar *et al.,* (2005).

Table.1 Prevalence of *Salmonella* spp. in chicken meat, chevon and human urine and stool samples in different districts of Chhattisgarh

S. N	Districts	Chicken meat		Chevon		Milk		Human urine and stool samples		Total
		No. of samples analysed	No. of samples positive (%)	No. of samples analysed	No. of samples positive (%)	No. of samples analysed	No. of samples positive (%)	No. of samples analysed	No. of samples positive (%)	
01.	**Jagdalpur**	25	8 (32%)	25	0 (0.00%)	30	0 (0.00%)	15	4 (26.66%)	12 (12.63%)
02.	**Dantewada**	25	11 (44%)	25	3 (12%)	20	0 (0.00%)	15	1 (6.66%)	15 (17.64%)
03.	**Kondagaon**	25	6 (24%)	25	7 (28%)	20	0 (0.00%)	15	1 (6.66%)	14 (16.47%)
04.	**Kanker**	23	2 (8.69%)	7	1 (4%)	20	0 (0.00%)	15	3 (20%)	6 (9.23%)
	Total	**98**	**27** (*27.55%)*	**82**	**11 (13.41%)**	**90**	**0 (0.00%)**	**60**	**9 (15%)**	**47 (14.24%)**

Table.2 Detail of the primers used in multiplex PCR

Gene	Primer sequence	Length	References
***bla*$_{SHV}$**	5'- ATG CGT TAT ATT CGC CTG TG - 3' 5'- TGC TTT GTT ATT CGG GCC AA - 3'	747-bp	(Paterson *et al.*, 2003)
b*la*$_{TEM}$	5' – TCG CCG CAT ACA CTA TTC TCA GAA TGA - 3' 5' – ACG CTC ACC GGC TCC AGA TTT AT – 3'	445-bp	(Apaka *et al.*, 2010)
***bla*$_{CTX-M}$**	5' – ATG TGC AGY ACC AGT AAR GTK ATG GC – 3' 5' – TGG GTR AAR TAR GTS ACC AGA AYC AGC GG-3'	593-bp	(Boyd *et al.*, 2004)

Table.3 Prevalence of resistant isolates of *Salmonella* spp. and *E. coli* in chicken meat, chevon and human urine and stool sample

S.N.	Antibiotic disc	No. of resistant isolates (%)		
		Chicken (n=27)	Chevon (n=11)	Human urine and stool (n=9)
1.	**Ampicillin (AMP)**	4 (14.81%)	1 (9.09%)	3 (33.33%)
2.	**Oxytetracycline (O)**	24 (88.88%)	11 (100%)	4 (44.44%)
3.	**Gentamicin (GEN)**	1 (3.70%)	1 (9.09%)	0 (0.00%)
4.	**Ciprofloxacin (CIP)**	0 (0.00%)	0 (0.00%)	3(33.33%)
5.	**Cephalexin(CN)**	3 (11.11%)	1 (9.0%)	3 (33.33%)
6.	**Ceftazidime (CAZ)**	0 (0.00%)	0 (0.00%)	1 (11.11%)
7.	**Cefixime (CFM)**	2 (7.40%)	0 (0.00%)	3 (33.33%)
8.	**Cefotaxime (CTX)**	2 (7.40%)	0 (0.00%)	1 (11.11%)
9.	**Aztreonam (AT)**	0 (0.00%)	0 (0.00%)	1 (11.11%)
10.	**Meropenem (MRP)**	1 (3.70%)	0 (0.00%)	1 (11.11%)
11.	**Imipenem (IPM)**	1 (3.70%)	0 (0.00%)	0 (0.00%)

Table.4 MAR index for *Salmonella* isolate from various district of Chhattisgarh

S.N	District	MAR index for *Salmonella* isolates											Total isolates
		0.00	0.09	0.18	0.27	0.36	0.45	0.54	0.63	0.72	0.81	0.90	
1.	Jagdalpur	1	4	6	-	-	1	-	-	-	-	-	12
2.	Dantewada	-	13	-	1	-	-	-	-	1	-	-	15
3.	Kanker	2	2	-	2	-	-	-	-	-	-	-	6
4.	Kondagaon	-	11	1	1	1	-	-	-	-	-	-	14
	Total	3	30	7	4	1	1	-	-	1	-	-	47

In the present study no *Salmonella* spp. was isolated from raw milk. Differences in the prevalence rate of *Salmonella* isolates with the previous study may be attributed by the multiple factors, such as geographic and seasonal variation, variations in sampling procedure and sample size, animal management practices, hygienic conditions during production and processing of meat and meat products or due to differences in the sensitivity and specificity of different isolation methods used.

Isolates were confirmed by serotyping and found they belongs to *S. enteritidis* and *S. typhimurium* serotypes which both are the most common serotype isolated from food borne outbreak. Our findings show resemblance with the reports of Vose *et al.,* 2010.

All the 47 *Salmonella* isolates were tested for their antibiotic sensitivity against 11 antibiotics by disc diffusion method and it was found that 95.7% isolates were found sensitive to imipenem. Whereas 91.5% isolates were sensitive for aztreonam and ceftazidime. However, 89.4% isolates were sensitive to gentamicin and ciprofloxacin. Variable sensitivity of 85.1%, 82.9%, 80.9 %, 78.7%, 74.5 % were detected for meropenem, cefixime, ampicillin and cephalexin, cefotaxime respectively. On contrary 82.9% isolates were highly resistant against oxytetracycline (Table 3). Similar type of pattern was reported by Naik *et al.,* (2015) who reported that *Salmonella* isolates were 100% sensitive to ciprofloxacin while 96.87%, 96.87% and 93.75% were sensitive to gentamicin, imipenem, and ceftazidime, respectively. Varying degree of sensitivity was found against cefixime (81.25%), cephalexin (78.12%), ampicillin (75%) and cefotaxime (59.37%).

Highest MAR index for *Salmonella* isolates were 0.72 (1 isolate) followed by 0.45 (1 isolate), 0.36 (1 isolate), 0.27 (4 isolates), 0.18 (7 isolates) and 0.09 (30 isolates). However 0.00 MAR index were recorded in 3 isolates (Table 4). The prevalence of MAR *Salmonella* spp. was also reported by others (Krumperman, 1983, Jaulkar *et al.*, 2011 and Naik *et al.,* 2015). The pathogens with higher indices of MAR in foods of animal origin may possibly be introduced from the environment. The wide use and abuse of antibiotic in mass production of live stock has promoted the emergence of and maintains the prevalence of MAR *E.coli* and *Salmonella* spp. in the faecal environment of these animals.

The isolates showed resistance against cefotaxime, Ceftazidime and Aztreonam were further tested for ESBL production. Among 47 isolates of *Salmonella* spp. only 1 (2.12%) isolate was found ESBL producer which was recovered from urine sample. Similar findings were also reported by Irajian *et al.,* 2009 and Parvin *et al.,* 2015.

Molecular characterisation of the isolates for the detection of ESBL genes (*bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M)}$ were performed by using multiplex PCR. Out of 47 *Salmonella* isolates One *Salmonella* isolate 2.12% expressed the presence of *bla*$_{TEM}$ gene. Similar type of result was also reported by Apaka *et al.*, (2010) highest prevalence of *bla*$_{TEM}$ gene than *bla*$_{CTX-M}$ and lowest prevalence of *bla*$_{SHV}$ gene. The genus *Salmonella* is not common hospital flora, and ESBL production with multiple antibiotic resistances is rarely associated with this organism. Constant antibiotic pressure can select multidrug-resistance and ESBL-producing bacteria, enabling their transmission among hospitalized patients. We cannot exclude the possibility that this mechanism of resistance was due to selective pressure, despite the evidence that very few patients had been exposed to the third-generation cephalosporins before isolation of the SI-ESBL

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