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## Multiple-Antibiotic Resistance and Plasmid Profiles of *Salmonella enteritidis* Isolated from Retail Chicken Meats

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**Abstract:** A study was conducted to determine the prevalence, antibiotics resistant and plasmid patterns of *Salmonella enteritidis* on chicken meat samples. A total of 578 chicken meat samples were examined over a period of 2 years from different retail outlets of a residential area of Namakkal City, South India. *S. enteritidis* prevalence was recorded 92 of 578 (15.91%). Seasonal variations in the prevalence pattern were identified with, a higher prevalence during monsoon months (19.68%) followed by post-monsoon (17.61%) and premonsoon. Present finding revealed that *S. enteritidis* isolates recovered from retail raw meats are resistant to more than one antibiotics, including those commonly used antibiotics in poultry feed were erythromycin, ampicillin, kanamycin, cephalothin and tetracyclin. All the isolates exhibited Multiple Antibiotic Resistance (MAR) and more than one plasmid. The plasmid size ranged between 0.43 and 115 MDa. Prevalence of multiple antimicrobial resistance among these strains suggesting possible prior selection by use of antimicrobials in meat production.

**Key words:** Antibiotics, meat samples, *Salmonella enteritidis*, antibiotic resistance, plasmids

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### INTRODUCTION

The emergence of antibiotic-resistant *Salmonella* strains has become a major public health concern. *Salmonella* species are widely distributed in the environment that causes a diverse spectrum of diseases in human and animals. Nontyphoidal *Salmonella* species are among the foremost bacterial pathogens implicated in food-borne gastroenteritis worldwide (Foley *et al.*, 2006). The World Health Organization (WHO) has estimated that annually 1.3 billion cases of acute gastroenteritis or diarrhea due to non typhoid salmonellosis causing 3 million deaths. In India an estimated 4,00,000 children below 5 years age die each year due to diarrhoea (Sudershan *et al.*, 2009). *Salmonella* is most often transmitted to humans through the food chain, with over 95% of salmonellosis cases attributable to the consumption of undercooked or mishandled beef, chicken and eggs etc., (Foley *et al.*, 2006). A variety of antimicrobial agents have been used to treat the

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salmonellosis. An increasing rate of antimicrobial resistance in *Salmonella* has been reported in many developing and developed countries (Threlfall *et al.*, 1993). Briggs and Fratamsco (1999) reported that the frequency of resistance is presumably due to extensive use of antimicrobial agents in human and veterinary medicine. Furthermore, resistance to combinations of several classes of antimicrobials has led to the emergence of Multi-Drug Resistant (MDR) strains that may pass from food animals to humans (White *et al.*, 2001). The genes involved in resistance in *Salmonella* are often plasmid-born and therefore potentially transmissible to other pathogenic enteric microorganism with genetic factors which control antibiotic resistance. Spread of antibiotic resistance plasmids in *Salmonella* from chickens to human handlers (White *et al.*, 2001) or of antibiotic-resistant microorganisms from poultry to humans in various countries (Poppe *et al.*, 2002) has been reported.

Poultry in India started as a backyard activity and in the past three decades it has gone through a revolutionary change. Today India ranks 8th in broiler production in the world. Statistics of 2002 show that India produced 1.2 billion kg of broiler meat per annum which gave an annual per capita consumption of 1.2 kg (Asha, 2004). Chicken is considered to be the most widely produced in Namakkal (Latitude:11° 13' 48" N; Longitude: 78° 10' 12" E). Thus, the poultry industry is one of the major sources of economy. There are mass production centres in various parts of the country especially in Southern states, Tamil Nadu, Karnaka and Andhra Pradesh. Though the consumption has been promoted, no effective steps are taken to monitor the quality of chicken meat and no guidelines have been prescribed for the meat processing in retail markets. The present study has been taken up primarily to assess the level of *Salmonella* contamination in meat with special reference to *S. enteritidis*. The export of poultry product from this region is increasingly on the rise. The possibility for transfer of antibiotic resistance genes among humans, animals and the environment is a direct threat to public health. The incidence of antibiotic-resistant *Salmonella* may pose health risk to many populations in world.

## MATERIALS AND METHODS

### Sample Collection and Processing

Chicken meat samples were purchased from five retail chicken outlets in Namakkal, Southern India. A total of 578 samples were obtained once in every 2 weeks for period of 2 year from June 2003 to May 2005. The samples were collected aseptically in pre-sterilized (box) and transported in a thermo-cool box to the Microbiology Laboratory, K.S. Rangasamy College of Arts and Science (Tiruchengode, Namakkal District, Tamilnadu, India) for immediate processing upon arrival. The samples were stored at 4°C if it will be processed within 1h. Twenty-five gram of chicken sample was aseptically weighed, minced into small pieces and transferred to 225 mL of sterile Buffered Pepton Water (BPW) (Hi-Media laboratories, Mumbai, India) for isolation of *Salmonella*. The sample was homogenized in a sterile blender for 2 min. The homogenate was taken up and thereby transferred into a sterile wide mouth, screw capped jar and incubated for 60 min at room temperature. After this pre-enrichment step, 1 mL from this homogenate was transferred into 10 mL of Tetrathionate broth (Hi-Media) and incubated at 37°C for 24 h (Vongsawasdi *et al.*, 2008).

### Isolation of *Salmonella*

A loopful from the Tetrathionate broth culture was streaked onto MacConkey agar, Xylose lysine deoxycholate agar, Bismuth sulphate agar and hektoen enteric agar

(Himedia, Bombay) and incubated at 37°C for 24-48 h. The plates were observed for typical *Salmonella*-like colonies and three isolated colonies per plate were picked and subjected to primary biochemical screening which involved reactions on indole production in tryptone broth, carbon utilization test in Simmon's citrate medium, triple sugar iron agar and urea splitting ability in Christesen's urea agar (Himedia, Bombay). Cultures that matched typical reactions of *Salmonella* in preliminary screening were further subjected to carbohydrate utilization involving glucose, lactose, sucrose, manitol, salicin, dulcitol and melibiose fermentation test which differentiate *S. enteritidis* and *S. typhimurium* (Cox and Williams, 1976) and further confirmed by slide agglutination test using commercial polyvalent O antisera (King Institute of India, Tamilnadu, India).

#### **Antibiotic Sensitivity Testing**

Resistance of all the 92 isolates of *S. enteritidis* to antibiotics was tested using the disc diffusion susceptibility test (Bauer *et al.*, 1966). The cultures were grown in peptone water broth, the density compared with McFerland standard 0.5. The antibiotic tested were Amikacin (Ak-30 µg), ampicillin (A-10 µg) erythromycin (E-15 µg) cephalothin (Ch-30 µg), tetracyclin (T-30 µg), cotrimoxazole (Co-23.75 µg), chloramphenicol (C-30 µg), gentamycin (G-10 µg) ciprofloxacin (Cf-5 µg), amoxicillin (Ac-30 µg), kanamycin (K-30 µg) and nalidixic acid (Na-30 µg). Multiple antibiotic resistance index was calculated according to method of Krumperman (1983). The MAR index is  $a/b$  where a number of resistant antibiotics, b total number of antibiotics exposed. The MAR index values greater than 0.2 indicate high risk source of contamination where antibiotics are often used. The MAR index values less than 0.2 indicate a strain from sources where antibiotics are seldom or never used.

#### **Isolation of Plasmid DNA**

A single bacterial colony was transferred into a conical flask containing Luria Broth (LB) medium amended with the appropriate antibiotics and incubated at 37°C overnight with mild shaking (120 rpm). After incubation, 1 mL of the culture was taken and centrifuged (Sigma, Germany) at 12,000 rpm for 30 sec at 4°C. The supernatant was removed and the pellet was dried. The pellet was resuspended in 150 µL of Tris- EDTA buffer [10 mM Tris chloride (pH 8), 1mM EDTA (pH 8)] solution by vigorous vortexing. Two hundred microliter of NaOH-SDS (0.2 M NaOH, 1% SDS) solution and 150 µL of 3M potassium acetate (pH 4.8) were then added and vortexed for 10 sec. It was centrifuged again at 12000 rpm for 5 min at 4°C and the supernatant was precipitated with 600 µL of ice cold ethanol (Birnbom and Doly, 1979). A 15 µL plasmid DNA was loaded on to a 1.0% agarose gel containing 0.5 µg mL<sup>-1</sup> ethidium bromide and electrophoresed in TBE (Tris Boric acid EDTA) buffer. The plasmid DNA were visualized by placing the gel on a UV (300 nm) transilluminator and the gel recorded using the Alfa Digital documentation imaging system (Alfa Innotech Corporation, USA).

#### **Statistical Analysis**

Two way ANOVA was used in the statistical analysis to evaluate the data. Correlation factors were calculated based on the average of replication and treatment. Degree of freedom was analyzed based on the replication and treatment. The p-value at 5% level of was calculated based on the standard errors comparisons. The method was followed according to the procedure described by Gomez and Gomez (1984).

## RESULTS

We isolated 92 strains of *S. enteritidis* from a total of 578 health chicken samples during the period two years. The incidence of *S. enteritidis* contamination was high in the seasons of monsoon and post monsoon (Table 1). The samples were replicated three times. The statistical result revealed that the incidence of *Salmonella* contamination was found to highest during monsoon (19.68%) followed by post-monsoon (17.61%) and pre-monsoon seasons (10.10%). However, no significant differences were observed between the years. On the other hands, a strong Critical Differences (CD) was observed between the seasons at 5% level ( $p = 0.05$ ).

All the isolates further confirmed agglutinated with the antiserum O and D based on which it was inferred that the isolates are *S. enteritidis*. The highest percentage of antibiotics resistance was found against erythromycin, ampicillin, kanamycin, cephalothin and tetracycline with 91.30, 84.78, 81.52, 71.17 and 70.65% resistance, respectively. Less than 50% of isolates were resistant against nalidixic acid, amoxicillin, co-trimoxazole, chloramphenicol, amikacin and gentamycin with the resistances of 46.73, 32.60, 29.34, 20.65, 15.21, 5.73, 5.96 and 3.26%, respectively (Fig. 1).

Eleven MAR patterns were observed among 92 *S. enteritidis* (Table 2). Ten isolates have the MAR frequency of 0.2-0.3 and were resistant against three antibiotics AEK. They exhibited a plasmid with molecular weight of 83 MDa. Eleven isolates were resistant to four antibiotics (AEChNa) with a MAR frequency of 0.31-0.4 and exhibited plasmid with molecular weight 95, 7.5, 3.7 and 0.43. Twenty nine isolates showed resistance towards five to six antibiotics and exhibited three resistance patterns (AEACHT, AEChTKand AEChTAcK) (Table 2) with three plasmid profile of molecular weight 90, 7.0, 3.95, 0.41 MDa, 101, 7.5, 4.1 0.46 MDa and 101, 7.5, 4.2, 0.46 MDa.

Twenty four isolates with a MAR frequency of 0.51-0.6 were resistant to seven antibiotics (AEChTCAcK, AEChTCoKNa and AEChCoCKNa) (Table 2) and exhibited three plasmid profiles. They had a molecular weight 115, 83, 7.8, 3.9, 0.41 MDa; 115, 1.9 MDa and

Table 1: No. of samples collected in individual season and percentage of *Salmonella enteritidis* contamination in different seasons

Seasons	No. of samples collected	Positive for <i>S. enteritidis</i>	Percentage of incidence
Pre-monsoon	192	20	10.10
Monsoon	193	38	19.68
Post-monsoon	193	34	17.61

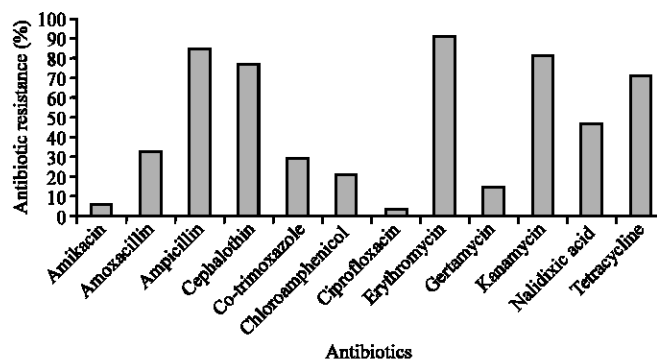


Fig. 1: *Salmonella enteritidis* resistant percentage of different antibiotics

Table 2: The MAR value patterns and plasmid profiles of *S. enteritidis* isolated from chicken sample

Sample No.	Total No. of isolates	MAR index value	MAR patterns	Plasmid profiles (MDa)
1	10	0.2-0.3	AEK	83
2	11	0.31-0.4	AEChNa	95,7.5,3.7,0.43
3	29	0.41-0.5	AEACHT	90,7.0,3.95,0.41
			AEChTK	101,4.2,1.93,0.41
4	24	0.51-0.6	AEChTAcK	101,7.5,4.2,0.46
			AEChTCAcK	115,83,7.8,3.9,0.41
			AEChTCoKNa	115,1.9
5	7	0.61-0.7	AEChCoCKNa	115,53,22,4.2,0.53
			AEChTCoCfKNa	115,3.9
6	5	0.71-0.8	AEChTCGAcKNa	89,7.0,3.9,0.41
7	6	0.91-1.0	AkAEChTCoCGAcKNa	101,60,7.5,3.95,1.8,0.46

MAR (Multiple antibiotic resistance) index is a/b where a is number of resistant antibiotics, b is total number of antibiotics exposed. Amikacin (Ak-30 µg), Ampicillin (A-10 µg), Erythromycin (E-15 µg), Cephalothin (Ch-30 µg), Tetracyclin (T-30 µg), Co-trimoxazole (Co-23.75 µg), Chloramphenicol (C-30 µg), Gentamycin (G-10 µg) and Ciprofloxacin (Cf-5 µg). Amoxicillin (Ac-30 µg), Kanamycin (K-30 µg) and Nalidixic acid (Na-30 µg)

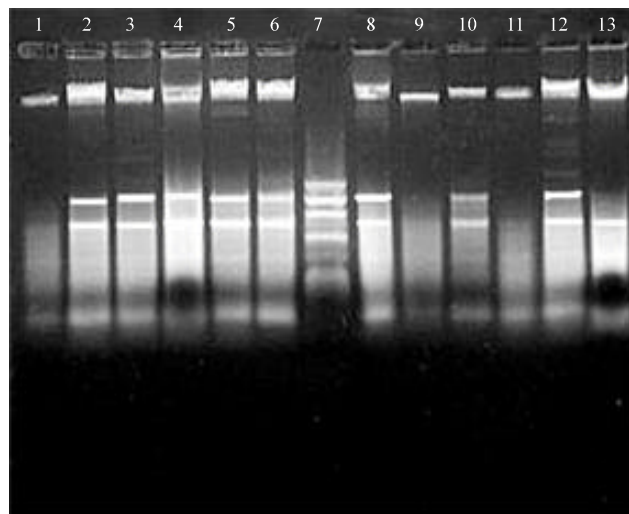


Fig. 2: Plasmid profiles of *Salmonella enteritidis* isolates from chicken meat samples. Lane 1: Mwt. 83 MDa (AEK), Lane 2: SE 46 with Mwt. 95, 7.5, 3.7 and 0.43 MDa (AEChNa), Lane 3: SE 78 with Mwt. 90, 7, 3.95 and 0.41 MDa (AEACHT), Lane 4: SE 51 with Mwt. 101, 7.5, 4.2, 0.2 and 0.46 MDa (AEChTAcK), Lane 5 and 6: SE 45 and 38 with Mwt. 101, 4.2, 1.93 and 0.41 MDa, (AEChTK), Lane 7: Molecular weight Marker 15.3, 6.3, 4.4, 2.9 and 1.5 MDa, Lane 8: SE 43 with Mwt. 115, 7.8, 3.9 and 0.41 MDa (AEChTCAcK), Lane 9: SE 70 with Mwt. 115 Mda, Lane 10: SE 28 with Mwt. 115, 53, 22, 4.21 and 0.53 (AEChCoKNa), Lane 11: SE 15 with Mwt. 89, 7, 3.9 and 0.41 MDa (AEChTCGAcKNa), Lane 12: SE90 with Mwt. 101, 60, 7.5, 3.95, 1.8 and 0.46 MDa (AkAEChTCoCGAcKNa) and Lane 13: SE 3 with Mwt. 115, 3.9 and 0.46 MDa (AEChTCoCfKNa)

115, 53, 22, 4.21, 0.53 MDa (Fig. 2). Seven isolates were with in the MAR frequency of 0.61 to 0.7. They were resistance towards eight antibiotics (AEChTCoCfK Na) and exhibited one plasmid profile of molecular weight 115, 3.9 and 0.46 MDa. Five isolates which fell with in the MAR frequency of 0.71-0.8, showed resistance against nine antibiotics (AEChTCGAcKNa) with plasmid profile molecular weight of 89, 7.0, 3.9 and 0.41 MDa. Six

isolates were within the MAR frequency of 0.91-1.0 and exhibited resistance against 11 antibiotics (AkaEChTCoCGAcKNa), the plasmid profile was with molecular weight 101, 60, 7.5, 3.95, 1.8 and 0.46 MDa.

## DISCUSSION

Present results showed that all the *S. enteritidis* from chicken samples were positive for melibiose fermentation. The similar confirmation were made by Kauffman (1966) and Ewing (1986), who have isolated and identified *S. enteritidis* from chicken samples based on melibiose fermentation. Chicken samples are more commonly contaminated with *S. enteritidis* and *S. typhimurium* (Herikstad *et al.*, 2002). Both these *S. enteritidis* are differentiated based on the ability to ferment sugar melibiose at 37°C in 18 to 24 h.

Salmonellosis is considered as one of the most widespread food borne infection in industrialized as well as developing countries even though the incidence seems to vary among countries. The prevalence level in the present investigation observed that *Salmonella* infection was high in the months of monsoon and post monsoon (Table 1). It may be noted that an average consumer in India is not very aware of the consequences of food-poisoning and often the producers and retailers take advantage of the situation. Similar results reported by Suresh *et al.* (2006) from Coimbatore, South India reported survival of *Salmonella* on eggshells results revealed seasonal variations in the prevalence pattern of *S. enteritidis* were identified with, a higher prevalence during monsoon months followed by post-monsoon and pre-monsoon. Among the isolates revealed high prevalence of multiple antimicrobial resistances in commonly used antibiotics ampicillin, neomycin, polymyxin-B and tetracycline. Studies in other countries have reported on the prevalence of *Salmonella* in poultry, with incidence percentage ranging from 13.7 to 66% (Fuzihara *et al.*, 2000; Duffy *et al.*, 1999). Number of other authors from different countries has reported different prevalence rates of *Salmonella* in poultry and poultry product. In United Kingdom 22.8% (Plummer *et al.*, 1995), in Malaysia 35.5% (Rusul *et al.*, 1996), in Spain 35.8% (Dominguez *et al.*, 2002), in Belgium 36.7% (Uyttendaele *et al.*, 1998) and 66% in Thailand (Jerngklinchan *et al.*, 1994) have been reported. These results indicate wide spread contamination of poultry products with *S. enteritidis*, regardless of the retail outlets and their potential as a risk factor for human health. Thornley *et al.* (2002) investigated that the incidence of non-typhoidal cases affected largest proportion in late summer and early autumn, peaking in March (13.1%) and smallest proportion were reported in winter with lowest incidence in July (4.9%). This report is from New Zealand which may vary in India.

In Nepal, the prevalence of *Salmonella* was found highest during the months of April and May (Maharjan *et al.*, 2006). The temperatures rise as high as 38°C during late Spring and early Summer in India. This might be due to the fact that prevalence of *Salmonella* is higher during hot dry and monsoon seasons (Mohanty *et al.*, 2006). *Salmonella enteritidis* showed resistance to a minimum of 3 antimicrobial agents to as many as 11 antimicrobial agents of the 12 drugs tested. Similar antibiotic resistance results were observed by Gebreves and Altier (2002). In the present study the highest number of isolates showed resistance against erythromycin (91.30%) followed by ampicillin (84.78%), kanamycin (81.52%), cephalothin (77.17%), tetracycline (70.65%) and nalidixic acid (46.73%), amoxicillin (32.60%), co-trimoxazole (29.34%), chloroamphenicol (20.65%), gentamycin (15.21%), amikacin (5.69%) and ciprofloxacin (3.26%).

Murugkar *et al.* (2005) reported that *Salmonella* isolated from diarrhoeal samples collected from adult patients who consumed chicken meat in Addis Ababa, 71.1% were resistance to tetracycline, 68.9% to ampicillin, 66.7% to cephalothin, (57.8%) to cotrimoxazole,

53.9% to kanamycin and 46.7% to chloroamphenicol. The high level of antimicrobial resistance observed in present study is probably due to indiscriminate and widespread use of the commonly available antimicrobials both in the veterinary and public health practices since in our countries people have easy access to various antimicrobial and purchase them without prescription (Poppe *et al.*, 1995; Lee *et al.*, 1993).

The findings observed in the present study indicate that the *Salmonella* strains exhibit multiple resistances up to eleven antimicrobials. *Salmonellae* are known to carry genes in their plasmids, which encode for drug resistance (R plasmids). It implies that wide spread use of antimicrobials in animals or human may cause an increase in the frequency of occurrence of bacterial resistant to other antimicrobials as the R. Plasmid may encode resistance to additional antimicrobials (Poppe *et al.*, 1995, 2002). Plasmid profiles were correlated with antibiotic resistance of *S. enteritidis* with MAR values. Seven MAR values (Table 2) and eleven plasmid profiles were observed (Fig. 2) In all the isolates of *S. enteritidis* more than one plasmid was found. The plasmid size ranged between 0.43 and 115 MDa. Similar findings were reported by Brown *et al.* (2003) in *Salmonella* and plasmid size was between 1.5 and 133 kDa. Eman and Mohamed (2008) reported that molecular sizes of *S. typhimurium* plasmids ranged between 3.8 and 90 kb while in *S. enteritidis* the molecular size ranged between 4 and 65 kb. The high molecular determinants. weight plasmids, 90 kb was observed in all tested *S. typhimurium* and the high molecular weight 65 kb was detected in all tested and *S. enteritidis*. The geographical location of isolates and antibiotics exposure of *Salmonella* sp. which vary the revealing patterns of plasmid may differ from country to country. The high prevalence of multidrug-resistant *Salmonella* in retail chicken meats reflects a reservoir of resistance in animals that can be transmitted to humans.

It was concluded that it is a first report from global Namakkal poultry centre, the prevalence of most frequently isolated serogroup of *S. enteritidis* present in retail chicken carcasses. *S. enteritidis* strains isolated from retail outlet exhibiting different plasmid patterns were observed. This high incidence of plasmids may reflect the prophylactic and therapeutic uses of antimicrobial agents and recommends that the plasmids analysis of *S. enteritidis* isolates could be useful for epidemiological study. Continuous monitoring and control methodologies, which should be applied in poultry farms and slaughterhouses and by retailers, for the control of spread and eradication of this pathogen, where possible, are strongly recommended. Efforts including critical control point programs in food production are needed to reduce the incidence of *Salmonella* in food. The judicious use of antibiotics, erythromycin, ampicillin, kanamycin, cephalosporin and tetracycline in food animals, is also critical to control the rapid spread of antimicrobial-resistance bacteria. Consumer-awareness efforts would protect public health from food borne salmonellosis.

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