Salmonella in Indian ready-to-cook poultry: antibiotic resistance and molecular characterization

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Abstract

The availability and popularity of processed, ready-to-cook (RTC) poultry products are increasing in India. Though fresh poultry is known to be contaminated with *Salmonella*, the prevalence of this foodborne pathogen in RTC poultry products is not reported. Eighty-seven chilled and frozen RTC poultry samples of 4 different brands obtained from supermarkets and departmental stores in Mumbai were analyzed for the presence of *Salmonella*. The prevalence of *Salmonella* was higher (51%) in chilled RTC samples as compared to the frozen RTC samples (5%). The frozen RTC samples of one brand were free from *Salmonella*. *S.* Typhimurium (75.2%) was the most prevalent serovar, followed by *S.* Enteritidis (23%) and *S.* Weltevreden (1.7%). A high percentage (81.4%) of the isolates were found to be resistant to 5 or more antibiotics and class 1 integron, which has been shown to confer multi-drug resistance, was detected in 69.9% of the isolates. Multiple antibiotic resistance index of isolates was high (0.6) indicating the indiscriminate use of antibiotics during poultry farming. High genetic diversity was observed among the *Salmonella* serovars based on Pulsed Field Gel Electrophoresis profiles. Results showed the presence of multi-drug resistant *Salmonella* serovars in processed, chilled RTC poultry products marketed in Mumbai, India.

Introduction

Salmonella is one of the most important foodborne pathogens. Poultry meat, eggs and foods of animal origin are important sources of human *Salmonella* infections.[1,2] The ubiquitous distribution of *Salmonella* in the natural environment,[3] and its prevalence in many foods have made inspection a mandatory requirement world-wide.[4-6] *Salmonella* is often transmitted to humans through the food chain, with over 95% of salmonellosis cases attributable to the consumption of undercooked or mishandled pork, poultry and eggs.[6-8] A multistate outbreak of *Salmonella* Heidelberg infections linked to foster farm brand chicken has been reported.[9]

Emergence of multi drug resistant *Salmonella* has been reported worldwide and it is a major public health concern.[6,10-12] The antimicrobial resistance is frequently associated with integrons, transposons, and plasmids, which are involved in horizontal transfer of antibiotic resistance genes among bacteria and increase in the overall resistance gene pool.[11,13,14] Integrons are genetic elements able to capture, integrate and rearrange open reading frames (ORFs) embedded in variable regions of genes cassette units and convert them to functional genes by ensuring their correct expression.[15] Integrons do not transfer themselves; instead facilitate transmission of antibiotic resistance genes via transposons or conjugative plasmids.[16] Class 1 integron, the most common integron located on *Salmonella* genomic island 1 (SGI 1), has been detected in different *Salmonella* serovars such as *S.* Typhimurium, *S.* Bareily, *S.* Oslo, and *S.* Newport in several countries.[4,10,13] There are reports on multi-drug resistant *Salmonella* isolated from India.[4,13,17] However, incidence of *Salmonella* in RTC food samples in India is not well documented. Moreover there are very few studies on molecular characterization of these *Salmonella* isolates.[4,18]

Fresh poultry slaughtered in local shops is generally preferred by consumers in India. However, due to changes in life style and modernization, fresh chilled and frozen RTC poultry products has become readily available in retail high end shops and supermarkets in major cities. But, there are few studies on the presence of *Salmonella* in these RTC products from India.[18] The aim of present study was to i) screen branded RTC poultry products from Mumbai for the presence of *Salmonella,* ii) study the antimicrobial resistance profile of *Salmonella* isolates and presence of class 1 integron in MDR strains and iii) characterize these isolates by Pulsed Field Gel Electrophoresis (PFGE).

Materials and Methods

Sampling

Forty eight chilled and thirty nine frozen RTC poultry samples of four different brands were obtained from various supermarkets and departmental stores in Mumbai (Supplementary Table S1). The chilled RTC samples included mixed boneless chicken, leg cut, precut, soup pieces, kheema, assorted cut pieces, lollipops or drumsticks. The frozen RTC samples were comprised of sausages, kheema, cutlet, nuggets, tandoori chicken nuggets, tandoori chicken tikka, chicken samosa, salami slices, sheekh kebab, burger patty, lollipops, and spring roll. The frozen RTC samples contained ingredients such as flour, onion, water, spices and condiments (coriander leaves, garlic, ginger, red chili powder, coriander powder, curry powder, turmeric powder, green chili, kasoori methi, edible vegetable oils, soya bean granules, iodized salt, and acidity regulators-INS 330, 452). The samples were brought in ice and analyzed immediately.

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Isolation of *Salmonella*

The isolation of *Salmonella* was carried out as per US-FDA Bacteriological analytical manual 8th edition.[19] Microbiological media and antibiotic disc were from HiMedia Laboratories, Mumbai, India. Twenty five grams of poultry meat were homogenized in 225 mL lactose broth and incubated at 37°C for 24 h. After the initial pre-enrichment step, the samples were further enriched in Tetrathionate broth and Rappaport-Vassiliadis medium at 43°C for 24 h. A loopful culture from each of these media was streaked on Bismuth Sulfite Agar (BSA), Xylose lysine Deoxycholate Agar (XLDA), and Hektoen Enteric Agar (HEA) and plates were incubated at 35°C for 24 h. After pre-enrichment, enrichment and plating on selective agar plates, typical *Salmonella* were isolated, and identified by biochemical tests like glucose test by Triple sugar iron agar (TSI), lysine decarboxylase test by Lysine Iron Agar (LIA), Urease test (Urea broth), IMViC test.[19] Isolates were serotyped at the National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, India.

Antimicrobial susceptibility test

Salmonella isolates were screened for antibiotic sensitivity using 15 different antibiotics by agar diffusion method as described by Clinical and Laboratory Standards Institute (CLSI).[20] *Salmonella* isolates were grown in Mueller-Hinton broth (HiMedia) overnight to prepare inoculum in order to achieve colony suspension to match with McFarland standard 0.5. The culture suspensions were evenly spread on Mueller-Hinton Agar (HiMedia) and antibiotic discs were placed on agar surface followed by further incubation at 37°C for 24 h. Antibiotic resistance profiles were assigned according to CLSI as resistant (R), intermediate (I), or sensitive (S) after measuring average zone diameter.[5] The type and concentration of antibiotics in disc were as follows, Ampicillin (AMP) 10 µg; Chloramphenicol (CHL) 30 µg; Streptomycin (STR) 25 µg; Cephalothin (CEP) 30 µg; Nalidixic acid (NAL) 30 µg; Ciprofloxacin (CIP) 10 µg; Ceftriaxone (CTR) 30 µg; Sulfamethizole (SMZ) 300 µg; Enrofloxacin (EFX) 10 µg; Chlortetracycline (CTC) 30 µg; Kanamycin (KAN) 30 µg; Oxytetracycline (OTC) 30 µg; Ofloxacin (OFX) 2 µg; Trimethoprim (TMP) 30 µg; and Tetracycline (TET) 30 µg (HiMedia). Multiple antibiotic resistance (MAR) index is calculated as the ratio of number of resistant antibiotics to which organism is resistant to total number of antibiotics to which organism is exposed.[6]

Molecular characterization of *Salmonella*

Salmonella isolates were tested for the presence of *inv*A gene by PCR amplification using the primers as previously described by Chiu *et al.*[21] The Integron region was PCR amplified from MDR isolates using class 1 integron specific primers (CSL1 and CSR1) as previously described by Khan *et al.*[13]

PFGE was performed as per the Pulse Net USA protocol with 50 U of XbaI (Bangalore Genei, Bangalore, India) at 37°C.[22] PFGE was carried out with Gene Navigator System (Amersham Biosciences, Sweden) in 1% agarose gel [Seakem® Gold Agar (Lonza, Rockland, USA)] in 0.5 X Tris-Borate EDTA buffer at 9°C. Pulse times ramped from 5 to 120 s during a 25 h run at 160 V. Lambda ladder PFGE marker (New England Biolabs, Ipswich, MS, USA) was used as molecular weight standard. The gels were stained with ethidium bromide. The bands were analyzed visually and the 0 and 1 matrix (binary matrix) was developed based on the presence or absence of particular size band on the gel in all the samples. The matrix was analyzed using FREETREE software (Version 0.9.1.50, Folia Biologica, 2001). Strains differing by one band were considered as different pulsed field profiles (PFPs). The relatedness of the isolates was analyzed using Nei and Li/Dice distance similarity calculations and neighbor joining as the tree building algorithm. The output tree was visualized using the Tree View software (Version 1.5.2, Roderic D. M. 2005).

Results and Discussion

Prevalence of *Salmonella*

High percentage of chilled RTC poultry samples (53% of brand 1 and 50% of brand 2) were positive for *Salmonella* as compared to frozen RTC samples (5% samples of brand 4). The samples from brand 3 were free from *Salmonella* (Table 1). Present investigation shows both fresh chilled and processed frozen RTC poultry products are contaminated with *Salmonella*. Poultry meat and egg are established as a major source of contamination by *Salmonella*.[6,22] Presence of *Salmonella* in fresh poultry has been well reported worldwide.[6,8] In the present study, samples were taken from very diverse products. The comparison between these products with respect to *Salmonella* incidence is difficult, but irrespective of the products, high incidence of *Salmonella* was found in all the samples except one. It was found that minimally processed, fresh RTC poultry samples (mixed boneless, soup pieces, mixed boneless, precut, drumstick and leg cut) and also processed fresh/frozen RTC poultry samples (from brand 1 lollipops and kheema) were contaminated with *S.* Typhimurium and *S.* Enteritidis. The fresh RTC poultry (brand 1 and 2) comprised of both processed and un-processed poultry products. Therefore, there is a high level of the *Salmonella* incidence in these products (Table 1 and Supplementary Table S1). Out of 113 *Salmonella* isolates, 75.2% were *S.* Typhimurium, 23% were *S.* Enteritidis and 1.7% were *S.* Weltevreden (Table 1). Thirty four percent of *Salmonella* positive samples were contaminated with at least 2 serovars (Table 1 and Supplementary Table S1). *S.* Typhimurium and *S.* Enteritidis were the most frequently reported serovars associated with human foodborne illnesses and poultry industry in India.[6,8] Also, *S.* Typhimurium is one of the most commonly detected serovars from animals used for food and retail meat in the USA.[10]

Antibiotic resistance

More than 80% of *Salmonella* isolates

Table 1. Isolation of *Salmonella* spp. from different brands of poultry.

Brand	Total samples	Sample type	Sample positive for *Salmonella* (%)	Serotypes identified (number of isolates)
1	34	Chilled RTC[a]	52.9	*S.* Typhimurium (58) *S.* Enteritidis (12) *S.* Weltevreden (2)
2	14	Chilled RTC[a]	50	*S.* Typhimurium (23) *S.* Enteritidis (12)
3	20	Frozen RTC[b]	ND[c]	ND[c]
4	19	Frozen RTC[b]	5.2	*S.* Typhimurium (4) *S.* Enteritidis (2)

[a]Chilled RTC poultry samples comprises of raw meat only. [b]Frozen RTC poultry samples contained ingredients such as flour, onion, water, spices and condiments. [c]ND stands for Not detected.

were multi-drug resistant (MDR) with resistance to 5 or more different antibiotics. *S.* Typhimurium (17 isolates), *S.* Enteritidis (11 isolates) and *S.* Weltevreden (2 isolates) with MAR index from 0.5333 to 0.6 were resistant to more than 8 antibiotics belonging to different class of antibiotics (Table 2). Multidrug resistance has been reported in a number of serovars of *Salmonella* from different foods.[13,17] *Salmonella* isolates from brand 1 and brand 2 were resistant to 9 different antibiotics; whereas, *Salmonella* isolates of brand 4 were resistant to 4 antibiotics (Table 2). Ninety percent of *S.* Typhimurium isolates were resistant to NAL, CTC, KAN, OTC and TET. All *S.* Enteritidis isolates were resistant to KAN and OTC, while more than 80% of isolates were resistant to NAL and CTC. Both the *S.* Weltevreden isolates were resistant to AMP, NAL, SMZ, CTC, KAN, OTC, and TMP (Table S2). The sensitivity pattern indicated that all isolates were sensitive to CHL, STR, CIP, CTR, EFX, and OFX (Supplementary Table S2).

A total of 19 antibiotic resistance patterns were observed. The most predominant antibiotic pattern was NAL, SMZ, CTC, KAN, OTC, TMP, TET (29 isolates) followed by AMP, CEP, NAL, SMZ, CTC, KAN, OTC, TMP, TET (16 isolates) (Table 2). Multiple antibiotic resistance (MAR) index of both *S.* Typhimurium and *S.* Enteritidis was 0.6 while that of *S.* Weltevreden was 0.53 (Table 2). The high MAR index indicates indiscriminate use of antibiotics in poultry farming for growth promotion, prophylaxis as well as therapeutic purposes. The injudicious use of antibiotics in poultry which has increased the emergence and maintenance of MAR bacteria in the environment has been reported.[1,6]

Molecular characterization

All the *Salmonella* serovars isolated from poultry were *inv*A positive. The *inv*A gene is located on *Salmonella* Pathogenicity Island 1 (SPI-1), which is essential for the invasion of host's epithelial cells by *Salmonella*. This gene is highly conserved in *Salmonella* serotypes and has been used as a potential target for *Salmonella* detection.[23] In a study of 630 different strains of *Salmonella enterica*, 99.4% of strains were found to harbour the *inv*A gene.[24] Whereas another study reported the presence of *inv*A gene in *Salmonella* is not universal, as during their study out of 35 tested strains of *Salmonella*, 33 harboured *sse*L as well as *invA* virulence genes, however two strains (*Salmonella* Molade and *Salmonella* München), did not harbour the *inv*A virulence gene.[25]

Seventy percent of the *Salmonella* isolates carried the class 1 integron. Class 1 integron specific PCR detected double amplicons (1 kb and 1.2 kb) in 53.9% isolates (Supplementary Figure S1). The rest of the class 1 integron positive isolates showed multiple bands. These results were consistent with earlier studies.[11,26-28] Integrons may carry antibiotic resistance gene cassettes, which confer resistance to antimicrobials.[13] In the present study, 82% of the integron positive isolates were MDR. But interestingly, 18% of the integron positive isolates were non-MDR (resistant to less than 5 antibiotics). Ampicillin and

Table 2. Antibiotic resistance pattern of *Salmonella* spp. isolated from poultry.

S. no	Antibiotic pattern[a]	MAR[b] index	Serovars (number of isolates)		
			Brand 1	Brand 2	Brand 4
1	AMP, CEP, NAL, SMZ, CTC, KAN, OTC, TMP, TET	0.6	*S.* Typhimurium (5); *S.* Enteritidis (7)	*S.* Typhimurium (4)	
2	AMP, CEP, NAL, SMZ, CTC, KAN, OTC,TET	0.5333	*S.* Enteritidis (3)		
3	AMP, CEP, NAL, SMZ, KAN, OTC, TMP, TET	0.5333	*S.* Typhimurium (5)		
4	AMP, NAL, SMZ, CTC, KAN, OTC, TMP, TET	0.5333	*S.* Typhimurium (3); *S.* Weltevreden (2)		
5	CEP, NAL, SMZ, CTC, KAN, OTC,TMP, TET	0.5333		*S.* Enteritidis (1)	
6	NAL, SMZ, CTC, KAN, OTC, TMP, TET	0.4666	*S.* Typhimurium (18); *S.* Enteritidis (4)	*S.* Typhimurium (7)	
7	NAL, CTC, KAN, OTC, TMP, TET	0.4		*S.* Typhimurium (1)	
8	NAL, SMZ, KAN, OTC, TMP, TET	0.4		*S.* Enteritidis (3)	
9	NAL, SMZ, CTC, KAN, OTC, TET	0.4	*S.* Typhimurium (10)		
10	AMP, NAL, CTC, KAN, OTC, TET	0.4	*S.* Typhimurium (5)	*S.* Typhimurium (1)	
11	NAL, SMZ, KAN, OTC, TMP	0.3333	*S.* Typhimurium (1)		
12	AMP, CTC, KAN, OTC, TET	0.3333		*S.* Typhimurium (1); *S.* Enteritidis (1)	
13	NAL, CTC, KAN, OTC, TET	0.3333		*S.* Typhimurium (3); *S.* Enteritidis (2)	
14	NAL, CTC, OTC, TET	0.2666			*S.* Typhimurium (4) *S.* Enteritidis (2)
15	CTC, KAN, OTC, TET	0.2666		*S.* Typhimurium (3); *S.* Enteritidis (3)	
16	CTC, OTC, TET	0.2		*S.* Typhimurium (2)	
17	AMP, NAL, KAN	0.2	*S.* Typhimurium (1)		
18	NAL, KAN	0.1333	*S.* Typhimurium (2)		
19	NAL	0.0666	*S.* Typhimurium (4)		

[a]Ampicillin (AMP) 10 µg; Chloramphenicol (CHL) 30 µg; Streptomycin (STR) 25 µg; Cephalothin(CEP) 30 µg; Nalidixic acid (NAL) 30 µg; Ciprofloxacin(CIP) 10 µg; Ceftriaoxome (CTR) 30 µg; Sulfamethizole (SMZ) 300 µg; Enrofloxacin(EFX) 10 µg; Chlortetracycline (CTC) 30 µg; Kanamycin (KAN) 30 µg; Oxytetracycline (OTC) 30 µg; Ofloxacin(OFX) 2 µg; Trimethoprim(TMP) 30 µg and Tetracycline (TET) 30 µg. All the isolates were sensitive for Chloramphenicol (CHL) 30 µg; Streptomycin (STR) 25 µg; Ciprofloxacin (CIP) 10 µg; Ceftriaoxome (CTR) 30 µg; Enrofloxacin (EFX) 10 µg; and Ofloxacin (OFX) 2 µg. [b]MAR (multiple antibiotic resistance) = the ratio of number of resistant antibiotics to which organism is resistant to total number of antibiotics to which organism is exposed (15). Brand 3 was free of *Salmonella* contamination.

tetracycline resistance of these isolates may be due to presence of genes responsible for antibiotic resistance on class 1 integron.[29] However, resistance to NAL could be due to mutation in the target genes of proteins of these antibiotics.[14] Resistance to KAN, and OTC could be attributed to inhibition of protein synthesis.[26] It was found that 26 MDR strains of *Salmonella* lacked the class 1 integron. This could be due to the presence of antibiotic resistance genes elsewhere on the chromosome as reported earlier.[11,26]

Vast genetic diversity was observed among the *S.* Typhimurium and *S.* Enteritidis isolates. Eighty-five *S.* Typhimurium isolates were clustered into twenty different PFGE patterns (Supplementary Figure S2). The major cluster of *S.* Typhimurium comprised of 13 isolates from brand 2 and 4 samples (Supplementary Figure S2). Twenty six *S.* Enteritidis isolates were grouped into 5 PFGE patterns. Fifteen *S.* Enteritidis isolates from brand 1, 2 and 4 samples were clustered in 2 PFGE patterns (Supplementary Figure S3). The PFGE patterns clearly indicated that *Salmonella* spp. isolated from the poultry samples were of same clonal origin. For example, the PFGE pattern of *S.* Typhimurium isolate no 445 from brand 4 was same as that of *S.* Typhimurium isolate no 501 from brand 2. Also, PFGE patterns of *S.* Enteritidis isolates no 32,123 and 458 were same; however, these serotypes were isolated from brand 1, 2 and 4, respectively. Previous study on genetic diversity of *Salmonella* showed high diversity in *Salmonella* isolates obtained from different food samples.[30] However, there is no PFGE data bank like PulseNet (http://www.cdc.gov/pulsenet/) program in India. Therefore, it is very difficult to trace *Salmonella* isolated from different regions and tag them to the source of origin. Moreover, serovars isolated at different time periods of the year also showed same PFGE pattern, for example, *S.* Enteritidis isolate no 32 and 242 were from same brand but they were isolated in different months. These results indicate that same *Salmonella* serovars are present in poultry products from different brands. Similarity in PFGE patterns could be due to cross-contamination that most likely happened during processing and handling, which would account for the isolation of same organism at different stages of processing from different meat carcasses.[31] One of the sources of contamination could be the feed used in the poultry farms.[32,33]

Conclusions

Present study demonstrates the high incidence of *Salmonella* in poultry samples. We also observed high percentage of *Salmonella* isolates resistant to multiple antibiotics. Our study also reveals that, the modern food processing methods adopted by poultry industry are insufficient to produce microbiologically safe poultry products. We observed that minimally processed fresh chilled RTC poultry products and processed, frozen RTC poultry samples are contaminated with *S.* Typhimurium, *S.* Enteritidis and *S.* Weltevreden serovars. It may be due to poor processing practices followed by the poultry industry. Recently more stringent food laws are being implemented to improve the processed food quality in India.

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