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Research Article

Prevalence Study and Resistance Profiles of Multi-Drug Resistant *Salmonella* Obtained from Poultry Across Mumbai Region

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Abstract

The present study was undertaken to investigate the prevalence of multi-drug resistant *Salmonellae* in raw chicken liver and egg yolks in 3 major regions of Mumbai, namely western suburbs, central and harbor areas. A total of 115 Salmonella isolates were identified and studied. Biochemical reactions-based identification was employed to determine and confirm identity of isolates. Slight differences in colony characteristics and H2S production on Xylose Lysine Deoxycholate agar medium was observed. Overall, the isolates were found to be most sensitive to Ceftriaxone, Ciprofloxacin and Levofloxacin as per microbroth dilution technique. Chicken liver isolates showed 100% resistance against Azithromycin and egg yolk isolates showed 100% resistance against Azithromycin, Erythromycin, Amoxicillin and Nitrofurantoin. MDR *Salmonella* isolates were found to be widespread in poultry samples across various parts of Mumbai region. This study can be used to benefit the public at large since there is a very high number of poultry consumers in Mumbai.

Keywords: Multi-Drug Resistant Salmonella Species; Chicken Liver; Egg Yolk; Mumbai

Introduction

Not only do *Salmonella* spp. cause several infections in humans [1], but also cause diseases in mammals, birds, cold-blooded animals [2]. In humans, salmonellosis is caused by *Salmonella enteritidis* and *Salmonella typhimurium* and is one of the most common causes of food poisoning [3]. In fowls, it is caused by *S. gallinarum* and *S.pullorum* [4]. *Salmonella* infections also lead to Enteric fever (typhoid) and acute gastroenteritis [5]. Consumption of fresh or processed meat and poultry, eggs, and fresh produce contaminated with *Salmonella* leads to salmonellosis [6].

Poultry products are widely consumed around the world due to their high protein and low-fat content [7]. However, *Salmonella* outbreaks have challenged the poultry industry [8]. In poultry, Salmonella colonization is determined by factors like age of the birds, overcrowding within the bird habitats, stress or illness in the birds, infectious dose, gut health, nature of the Salmonella serovar and genetically transmissible elements [6]. Birds infected with Salmonella spread the bacterium to other birds via feces, food and water sources [9]. Transmission of Salmonella into the eggs may occur within the ovary [10] and hatchlings may acquire the bacterium and pass it to other chicks via feces. In commercial markets, chances of spread of Salmonella is increased, due to unhygienic practices and improper handling by vendors [9].

Most of these infections do not require antimicrobial treatment as they are self-limiting. However, bacteremia can cause widespread dissemination of Salmonella in blood. Complications like meningitis arise in children and patients with low immunity. Antibiotic therapy then becomes necessary [1]. However rampant and indiscriminate use of antibiotics has led to the emergence of multi-drug resistant strains [11]. The aim of this study was thus to understand the prevalence of multi-drug resistant *Salmonella* strains found in raw chicken liver and eggs obtained from poultry vendors across Mumbai region. This study was conducted to elucidate the drug resistance pattern and extent of spread of multi-drug resistant (MDR) Salmonella, which could potentially put the consumers' health at risk.

Methods and Materials

Collection of samples: The research design for this study involved the segregation of poultry consumers of Mumbai into 3 distinct groups since Mumbai has 3 main regions- the western suburbs, the central and the harbor areas. The collection of samples was randomly designed in such a way that well known populous regions of Mumbai, at considerable distances, can be covered for the present study. 4 towns each from western, central and harbour lines were included. The areas under present study are named as follows: vasai, borivali, santacruz, lower parel, matunga, sion, ghatkopar, Kurla, chembur, wadala, vashi, panvel. Raw chicken liver (25g) and fresh eggs were collected from poultry shops across Mumbai region. A total of 24 samples (12 eggs, 12 chicken livers) were collected from all the mentioned areas across Mumbai were collected . Samples were investigated without further delay.

Pre-enrichment, enrichment and isolation on media: The egg shells were first wiped and sterilized with 70% alcohol and then cracked open under laminar air flow. The yolks and whites were separated carefully and the yolks were individually inoculated into 50mL of sterile Buffered Peptone Water (BPW). The chicken liver was minced well using sterile scalpels and then inoculated in 50mL BPW. After 24hrs pre-enrichment at 37°C on shaker conditions, 5mL from each of the 24 samples were re-inoculated into 45mL of *Salmonella* Selection Broth (HiMedia) and kept at 37°C for 24hrs. Brilliant Green (0.07g/L) was added into SS broth to ensure inhibition of growth of Gram-positive organisms. After enrichment, loopful of samples were streaked on Xylose Lysine Deoxycholate media (HiMedia) by hexagonal method and kept at 37°C for 24hrs. This process was done in triplicates for all 24 samples. Colonies were later repeatedly streaked on fresh medium till pure (axenic) isolates were obtained.

Identification of selected isolates: Presumptive *Salmonella* isolates were subjected to tests like oxidase, catalase, sugar fermentation, indole, methyl red, Voges Proskauer, citrate utilization, TSI, urease, nitrate reduction, lysine decarboxylase, ornithine decarboxylase, malonate and gelatinase. The sugars employed for the test included glucose, lactose, maltose, sucrose, xylose, mannitol. After 24hrs incubation at 37°C, only the confirmed *Salmonella* isolates were subjected to antibiotic resistance screening.

Antibiotic resistance screening: As with any antimicrobial susceptibility testing, we utilized Mueller Hinton broth for all isolates. The antibiotics were procured from pharmacy/distributors after written approval by a practising doctor, since antibiotics are not given without prescription. Ceftriaxone and Gentamicin were available as sterile injectables, rest all were available as sterile powders. 1mg/ml was prepared as stock for all and kept under storage at 4.C as per manufacturer's instructions. A combination of guidelines with modifications were used. Research papers employing US FDA, NARMS by CDC, CLSI and ECOFF were used as reference [12,13] MDR phenotype was estimated as being resistant to 3 or more than 3 antibiotics at 10 μg/mL. 100μL of 10^6 cfu/mL of all the confirmed Salmonella isolates were inoculated into sterile 96 well microtiter plates containing 100μL antibiotics at a final concentration of 10μg/mL. For this screening, 12 antibiotics were employed namely Amoxicillin, Azithromycin, Ciprofloxacin, Ceftriaxone, Chloramphenicol, Erythromycin, Gentamicin, Levofloxacin, Nitrofurantoin, Tetracycline, Trimethoprim-Sulfamethoxazole and Ofloxacin. The plates were incubated at 37°C for 24hrs following which growth was checked using Resazurin assay. 5μL of Resazurin dye was added into each well and positive growth was indicated by a change in Resazurin dye colour from blue to bright pink.

Resistance-tolerance-sensitivity profiles: Variations or changes in Resazurin dye colour after incubation were also recorded as it indicated varying degrees of resistance-tolerance-sensitivity pattern. Optical densities were compared with positive (media with respective isolate, without antibiotics) and negative controls (media with saline) to assess each pattern. Prominent blue was considered as sensitive, light purple-pink as higher tolerance, dark purple as lower tolerance and bright pink-pinkish red as resistant.

Multiple Antibiotic Resistance (MAR) indices: Multiple antibiotic resistance index or MAR index was calculated as a/b, where a is the number of antibiotics against which resistance was observed and b is the total number of antibiotics employed for the present study. The MAR index of more than 0.2 is considered as a health hazard due to possibility of high contamination from multiple sources.

Results and Discussion

Poultry is one of the major sources of dietary protein. There are many poultry consumers in Mumbai and this study was aimed at determining the safety of poultry consumption, owing to the widespread dissemination of multi-drug resistant organisms in recent years. The areas undertaken for study are heavily populated and that correlates with a large consumer base of poultry. Poultry consumers usually buy fresh eggs and chicken liver from local poultry vendors on a regular basis due to multiple reasons like easy accessibility and/or affordability, as opposed to buying poultry products from shelves of air conditioned super marts. Poultry shops are available everywhere and the hygiene and sanitation levels observed within poultry shops has always been a questionable issue as many poultry vendors do not maintain even the minimalistic personal hygiene practices and often keep diseased chickens in unclean cages with limited ventilation. Fecal contamination and unhygienic environment lead to the spread of pathogenic organisms within the fowls and its consumption without proper cooking can lead to multiple diseases in the consumers. Excessive use of antibiotics in animal feed is also one of the prime reasons of antibiotic resistance. Thus, from the view of food safety and quality, it is necessary to understand the prevalence of food pathogens and their levels of antibiotic resistance.

Our primary focus was to identify *Salmonella* as the prevalent genera and to understand the extent of dissemination of Salmonella species across Mumbai. Species wise distribution of the isolates was not the focus of the present study. We did not identify individual species, and similar approaches have been carried out in the recent past [12,15-17]. 115 isolates confirmed to be *Salmonella* were chosen and used for further study. They were grouped into 6 groups based upon colony variations (table 1). Identification was performed according to Bergey's Manual of Determinative Bacteriology (Group 5: Family Enterobacteriaceae Lactose negative flowchart) was used for the study. Out of the 115 *Salmonella* isolates obtained, 71 (61.73%) were obtained from chicken liver, 44 (38.26%) from egg yolks.

Isolates from chicken liver	Isolates from egg yolk	Colony characteristics
7	3	2-3 mm colonies with overall smooth appearance, blackish grey appearance
21	14	Jet black slightly raised colonies 1-2mm
-	7	Jet black flat colonies with glossy appearance
6	13	Jet black and flat 1-2mm colonies, no glossy appearance
18	-	H_2S produced mostly in the center of the colonies
19	7	Jet black and flat 3-4mm colonies, no glossy appearance

Table 1: The above table shows the colony characteristics and bifurcation of *Salmonella* isolates obtained from chicken liver and egg yolk.

Biochemical tests were employed to confirm the genera of the isolates as *Salmonella*. All the Salmonella isolates were subjected to antibiotic sensitivity screening against 12 antibiotics namely Amoxicillin, Azithromycin, Ciprofloxacin, Ceftriaxone, Chloramphenicol, Erythromycin, Gentamicin, Levofloxacin, Nitrofurantoin, Tetracycline, Trimethoprim-Sulfamethoxazole and Ofloxacin using microbroth dilution technique. Since there were variations in the concentrations of antibiotics used in multiple research papers, we decided to set the concentration of tested antibiotics as 10 μg/mL. The rationale to keep constant concentration of antibiotics was to make our research more uniform and understand the patterns of resistance of Salmonellae when challenged with same concentration of different classes of antibiotics.

Amongst the 71 chicken liver isolates (Table 2), 100% resistance to Azithromycin was observed. The isolates were prominently sensitive to only Ceftriaxone (90.14%), Levofloxacin (80.28%) and Ciprofloxacin (87.32%). Thus, out of 12 antibiotics, only 3 were found to be effective against the chicken liver isolates. However, resistance towards these were also observed in some isolates. Isolates also showed a very high resistance pattern against Trimethoprim (90.14%), Erythromycin (90.14%) and Nitrofurantoin (91.54%). 19 isolates (26.76%) showed higher tolerance for Tetracycline. 15 isolates (21.12%) and 13 isolates (18.30%) showed higher tolerance against Amoxicillin and Gentamicin respectively.

Antibiotics	Number of resistant organisms	Number of sensitive organisms	Number of organisms with higher tolerance.	Number of organisms having lower tolerance
Ceftriaxone	4 (5.63%)	64 (90.14%)	-	3(4.22%)
Trimethoprim	64 (90.14%)	2(2.81%)	3(4.22%)	2(2.81%)
Tetracycline	45(63.38%)	4(5.63%)	19(26.76%)	3(4.22%)
Chloramphenicol	57(80.28%)	3(4.22%)	-	11(15.49%)
Ofloxacin	35(49.29%)	30(42.25%)	2(2.81%)	4(5.63%)
Azithromycin	71 (100%)	-	-	-
Amoxicillin	45(63.38%)	3(4.22%)	15(21.12%)	8(11.26%)
Levofloxacin	3(4.22%)	57(80.28%)	6(8.45%)	5(7.04%)
Erythromycin	64(90.14%)	1(1.40%)	6(8.45%)	-
Gentamicin	49(69.01%)	4(5.63%)	13(18.30%)	5(7.04%)
Nitrofurantoin	65(91.54%)	1(1.40%)	5(7.04%)	-
Ciprofloxacin	4(5.63%)	62(87.32%)	-	5(7.04%)

Table 2: The above table shows the efficacy of individual antibiotics against 71 chicken liver *Salmonella* isolates. Ceftriaxone, Levofloxacin, and Ciprofloxacin were the most efficient amongst other antibiotics.

Amongst 44 egg yolk isolates (Table 3), all isolates showed 100% resistance against Azithromycin, Amoxicillin, Erythromycin and Nitrofurantoin. 32 isolates (72.72%), 33 isolates (75%) and 34 isolates (77.27%) showed resistance against Chloramphenicol, Tetracycline and Gentamicin respectively. 23 isolates (52.27%) and 25 isolates (56.81%) showed resistance against Ofloxacin and Ciprofloxacin respectively. Sensitivity towards Ceftriaxone and Levofloxacin was recorded the highest as 36 isolates (81.81%) were sensitive towards Ceftriaxone and 21 isolates (47.72%) towards Levofloxacin. Thus, in the case of egg yolk isolates, only 2 out of 12 antibiotics showed highest efficacy.

Antibiotics	Number of resistant organisms.	Number of sensitive organisms.	Number of organisms with higher tolerance.	Number of organisms having lower tolerance.
Ceftriaxone	1(2.27%)	36(81.81%)	1(2.27%)	6(8.45%)
Trimethoprim	41(93.18%)	-	-	3(6.81%)
Tetracycline	33(75%)	1(2.27%)	10(22.72%)	-
Chloramphenicol	32(72.72%)	10(22.72%)	-	2(4.54%)
Ofloxacin	23(52.27%)	7(15.90%)	1(2.27%)	13(29.54%)
Azithromycin	44 (100%)	-	-	-
Amoxicillin	44 (100%)	-	-	-
Levofloxacin	15(34.09%)	21(47.72%)	5(11.36%)	3(6.81%)
Erythromycin	44 (100%)	-	-	-
Gentamicin	34(77.27%)	1(2.27%)	9(20.45%)	-
Nitrofurantoin	44(100%)	-	-	-
Ciprofloxacin	25(56.81%)	-	-	17(38.63%)

Table 3: The above table shows the efficacy of individual antibiotics against 44 egg yolk *Salmonella* isolates. Ceftriaxone and Levofloxacin were efficient amongst other antibiotics.

The tolerance level study was undertaken to analyze the patterns of all those isolates which were neither resistant nor sensitive but showed signs of upcoming resistance. Often these patterns are not assessed or overlooked and our aim was to highlight the most minute resistance patterns of the *Salmonella* isolates so obtained. Subtle changes in the Resazurin dye assay and comparison of their optical densities helped us to demonstrate higher and lower tolerance levels with respect to all the 12 antibiotics tested. Out of all the 115 *Salmonella* isolates obtained (Table 4, figure 1), 29 isolates (25.21%) and 22 isolates (19.13%) revealed higher tolerance to Tetracycline and Gentamicin respectively. 22 isolates (19.13%) and 17 isolates (14.78%) revealed lower tolerance against Ciprofloxacin and Ofloxacin respectively.

Antibiotics	Total resistance	Total sensitivity	Total higher tolerance	Total lower tolerance
Ceftriaxone	5 (4.34%)	100 (86.95%)	1(0.86%)	9 (7.82%)
Trimethoprim	105 (91.30%)	2(1.73%)	3(2.60%)	5(4.34%)
Tetracycline	78 (67.82%)	5 (4.34%)	29 (25.21%)	3 (2.60%)
Chloramphenicol	89 (77.39%)	13 (11.30%)	-	13 (11.30%)
Ofloxacin	58 (50.43%)	37 (32.17%)	3(2.60%)	17 (14.78%)
Azithromycin	115 (100%)	-	-	-
Amoxicillin	89 (77.39%)	3(2.60%)	15 (13.04%)	8(6.95%)
Levofloxacin	18 (15.65%)	78 (67.82%)	11(9.56%)	8 (6.95%)
Erythromycin	108 (93.91%)	1(0.86%)	6 (5.21%)	-
Gentamicin	83 (72.17%)	5 (4.34%)	22 (19.13%)	5(4.34%)
Nitrofurantoin	109 (94.78%)	1(0.86%)	5 (4.34%)	-
Ciprofloxacin	29 (25.21%)	62 (53.91%)	-	22(19.13%)

Table 4: The above table shows the efficacy of individual antibiotics against all 115 *Salmonella* isolates. The parentheses indicate % of isolates out of 115 isolates for each parameter.

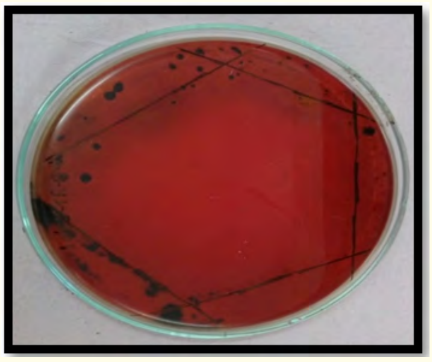

Figure 1: XLD plate showing hexagonal streak of a pure *Salmonella* spp. isolated from egg yolk.

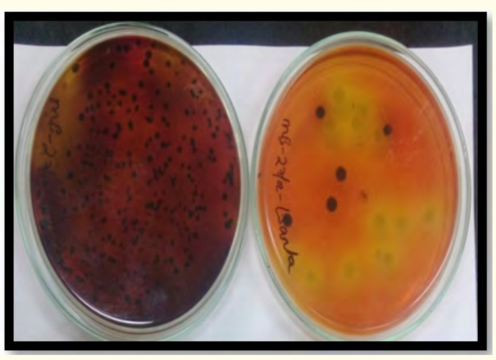

Figure 2: XLD plates showing mixed culture of *Salmonella* with other organisms from raw chicken liver after enrichments.

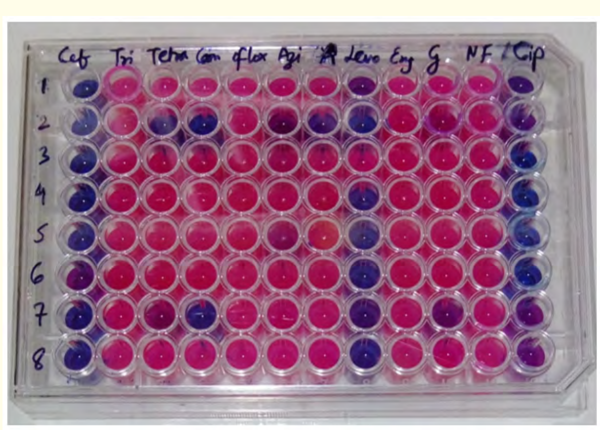

Figure 3: A microbroth AST plate result having resazurin dye variations, after 24hrs growth of chicken liver isolates with 10 mcg/mL of antibiotics: Abbreviations are as follows: cam- chlo-ramphenicol, NF- nitrofurantoin, cef- ceftriaxone, Tri- trimethoprim, cip- ciprofloxacin, G- gentamicin, Azi- azithromycin, ofl- ofloxacin, A- amoxicillin, Lev- levofloxacin, ery- erythromycin, tet- tetracycline. Prominent blue was considered as sensitive, light purple-pink as higher tolerance, dark purple as lower tolerance and bright pink-pinkish red as resistant.

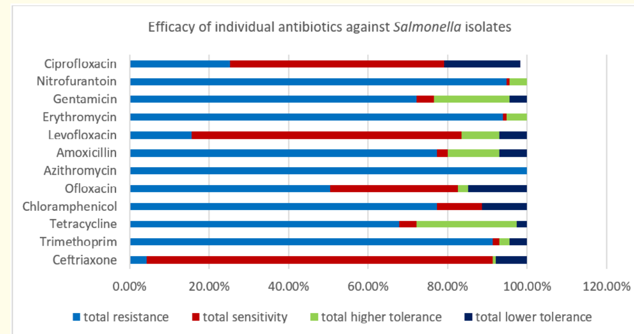

Figure 4: The above graph shows the overall efficacy of individual antibiotics against all 115 *Salmonella* isolates.

The resistance patterns and phenotypes of the *Salmonella* isolates were tabulated along with their MAR indices (supplementary information). Phenotypic resistance profile revealed 1 isolate, Salmonella isolate no. 25, that was resistant to all the 12 antimicrobials tested, thus having a MAR index of 1. A MAR index of 0.2 is regarded as high-risk contamination, but our study demonstrated MAR indices 0.25 – 1 as mentioned in the MDR supplementary information. It is worth observation that majority of the multi-drug resistant *Salmonella* isolates were obtained from chicken liver. Liver tends to be a hospitable environment for enteric bacteria. However, the antibiotic resistance recorded in the isolates was found to be very high.

With just 2-3 antibiotics showing efficacy, it is worth considering the speed at which these robust pathogens are gaining resistance. From the point of view of antibiotic classes, highest resistance has been recorded for macrolides i.e., Azithromycin, which inhibits protein synthesis. *Salmonella* isolates have demonstrated very high resistance against the Beta Lactams, Nitrofurans and Folic acid inhibitors. We observed marked variations in resistance patterns towards same members of fluoroquinolones- Ciprofloxacin, Levofloxacin, Ofloxacin. While we observed Ciprofloxacin and Levofloxacin to be the most efficient, we observed a good amount of resistance against Ofloxacin. As per multiple research findings, variations in resistance to individual members of fluoroquinolones depends on the presence of selective gyrA mutations by amino acid substitutions or presence of QRDR elements [18,19]. Resistance is also usually acquired due to acquisition of genetic elements or presence of antibiotic specific efflux pumps. Thus, this study can be useful for future in depth molecular analysis and devising essential steps targeting new sites or improving lead discovery for existing antibiotic target sites. A study like the present one undertaken, can help to plan necessary steps to protect the health of poultry consumers. Periodic epidemiological survey must be carried out to ensure food safety and check the emergence and existence of any novel antibiotic resistant species within the food chain [20,21].

Conflict of Interest

Authors declare no conflicting interests.

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