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Haritha Routhu
Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Corresponding Author:
Haritha Routhu
Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana, India

Antimicrobial susceptibility and molecular characterization of resistance genes in *Salmonella* isolated from quail samples

Haritha Routhu

Abstract
A total of 30 *Salmonella* isolates isolated from Quail meat, egg shell and cloacal swabs in different parts of Greater Hyderabad Municipal Corporation, India were selected for testing antibiotic resistance/susceptibility against ten selected antibiotics and specific antibiotic resistant genes. The *Salmonella* isolates from quail samples were highly resistant to gentamycin (70.0%) followed by streptomycin (53.33%), sulphamethoxazole (46.67), tetracycline (30.0%), ampicillin (26.67%), amikacin and ciprofloxacin (16.67%), nalidixic acid (13.33%), chloramphenicol (10.0%) and least with ceftriaxone (6.67%). The *Salmonella* isolates were highly susceptible to ceftriaxone (93.33%) followed by nalidixic acid (83.33%), amikacin and chloramphenicol 80% each, ciprofloxacin (76.67%), ampicillin (66.67%), tetracycline (50.0%), streptomycin and sulphamethoxazole (36.67%) and least with gentamicin (23.33%). Out of 9 tetracycline resistant isolates 5(55.5%) and 3(33.3%) harboured tet A and B genes respectively. Out of 8 ampicillin resistant isolates 5(62.5%) had blatem-1 gene and out of 16 streptomycin resistant isolates 5(31.2%) had str A gene. Out of 14 sulfamethoxazole resistant *Salmonella* isolates from quails, 5(35.7%) and 8(57.1%) harboured sul 1 and sul 2 genes respectively whereas out of 21 gentamicin resistant isolates 13(61.9%) had ant (3") 1 gene.

Keywords: *Salmonella*, antibiogram, antibiotic genes, Quail meat, egg shells and cloacal swabs

1. Introduction
Quail (*Coturnix japonica*) is a new addition in the poultry industry in India and number of quail farms is increasing day by day due to its easy management, faster growth rate, high nutritional value of meat and egg. In India, quail rearing started in 1974 at Izatnagar, when Central Avian Research Institute, introduced improved germplasm of domesticated quail varieties from Japan (Premavalli *et al*.,2015) [1]. Quail eggs contain 13% proteins compared to 11% in chicken eggs. It is tasty and has high nutritional value and low-fat content (Mir *et al*.,2015) [2]. Unlike chicken eggs, quail eggs do not cause allergies or diathesis (Miranda, 2008) [3]. However, some of these factors have also favored the entrance and dissemination of avian pathogens, such as *Salmonella* spp.(Van Hoorebeke *et al*.,2011) [4] an enteropathogenic bacteria which is prevalent among quails. The genus *Salmonella*, is a facultative intracellular pathogen that is capable of causing different disease syndromes in a wide range of hosts. To date, more than 2,541 serovars of *Salmonella* have been described (National *Salmonella* Reference Laboratory, Galway, Ireland), with new serovars being identified every year (Premavalli *et al*., 2015) [1].
Also the resistance of *Salmonella* to multiple antibiotics (EFSA, 2013) [5], makes the study of the antibiotic susceptibility profile and its ecology of this zoonotic pathogen a great priority. The widespread misapplication and overuse of antimicrobial agents in food animal production have contributed to the development of antimicrobial resistant pathogens that has emerged as a major public health implication (Antune *et al*., 2016) [6]. Virulence gene encodes products that aid the organisms to interact with the host cells (Latasa, 2012) [7] contributing numerous virulence genes that are incriminated in the pathogenesis of salmonellosis (Ammar *et al*.,2016) [8]. These genes are clustered within *Salmonella* pathogenicity islands SPI-1 to SPI-21 and participate in the adhesion and invasion of the pathogen to the host as inv gene or help in the pathogen survival within the host like mgtC5 gene (Oliveira, 2003) [9]. Therefore, sound management practices are vitally important in preventing and controlling disease (Ferket, 2007) [10]. According to a reports by Wambugu ATC (2013) [11] and Dozier *et al*. (2010) [12] on quail production and management there are no approved medications and disease preventive vaccines in the market.

Quails are more resistant to infectious diseases as compared to chickens although few infectious diseases are encountered in quails. But the advancement of quail production is being hampered by some managemental factors, fatal infectious, noninfectious and parasitic diseases (Barnes, 1987) [13]. Therefore, the present study was set to determine the antimicrobial resistance profiles of *Salmonella* isolates obtained from quails in and around Greater Hyderabad Municipal Corporation and the genes responsible for specified antibiotic resistance.

2. Materials and method

2.1 Antibiotic Sensitivity/Resistance test

A total of 30 *Salmonella* isolates obtained from Quail samples (meat, egg shells and cloacal swabs) were subjected to antimicrobial susceptibility/resistance using the disc diffusion assay with Muller-Hinton (MH) agar and in accordance with CLSI recommendations. The antibiotics tested were Tetracycline (30µg), Ampicillin (10µg), Streptomycin (10µg), Chloramphenicol (30µg), Gentamicin (10µg), Sulphamethoxazole (100µg), Amikacin (30µg), Ciprofloxacin (5µg), Ceftriaxone (30µg) and Nalidixic acid (30µg).

MH broth was inoculated with five colonies of the isolate and tubes were incubated at 37 ^{0}C for 2-8 hours until achieving a turbidity equivalent to 0.5 on the Mac Farland scale. After turbidity adjustment, a sterile swab was introduced, pressed against the tube well in order to remove any excess liquid and then seeded on the surface of a petri dish containing MH agar, rotating atleast twice. Using sterile forceps seven discs impregnated with antimicrobials were placed at equal distances from each other on the surface of inoculated agar plate. Subsequently the plate was inverted and incubated at 37 ^{0}C for 24 hours. Disc readings were performed after incubation and the diameter of inhibition halos was measured with the aid of a ruler. The interpretation was made as per the zone size interpretation chart provided by manufacturer of discs.

2.2 Detection of genes responsible for specific antibiotic resistance

For detecting antimicrobial-resistant genes, targeting specific genes of tetracyclines (tet A and B), sulfonamides (sul1and 2), streptomycin (strA), Gentamycin ant (3")-la and Ampicillin (blatem-1) are screened by PCR with their respective primers shown in Table 1.

Table 1: Details of primers used for antibiotic resistance genes in *Salmonella* isolates

Antibiotic	Gene	Oligonucleotides primers	Fragment size (bp)	Annealing temp.(C)	Reference
Tetracycline	tet(A)	(F) GCTACATCCTGCTTGCCTTC (R) CATAGATCGCCGTGAAGAGG	210	55/58	Ng *et al*. (2001) [14]
	tet(B)	(F) TTGGTTAGGGGCAAGTTTTG (R) GTAATGGGCCAATAACACCG	659	55	Ng *et al*. (2001) [14]
Sulfamethoxazole	sul1	(F) TTTCCTGACCCTGCGCTCTAT (R) GTGCGGACGTAGTCAGCGCCA	793	55	Ma *et al.* 2007) [15]
	sul 2	(F) CCTGTTTCGTCCGACACAGA (R)GAAGCGCAGCCGCAATTCAT	667	55	Ma *et al* (2007) [15]
Streptomycin	strA	(F)CCAATCGCAGATAGAAGGC (R)CTTGGTGATAACGGCAATTC	548	58	Aarestrup *et al*. (2003) [16]
Ampicillin	*blatem*	(F) CATTTCCGTGTCGCCCTTAT (R) TCCATAGTTGCCTGACTCCC	793	55	Randall *et al*. (2004) [17]
Gentamycin	Ant (3")-la	F:GTGGATGGCGGCCTGAAGCC R:ATTGCCCAGTCGGCAGCG	526	58	Bacci *et al*. (2012) [18]

The DNA was extracted using Phenol Chloroform method as per method described by Cocolin *et al.* (1998) [19] with little modification. Five µl of the bacterial lysate or 20ng of purified DNA, 2.5 µl of 10x assay buffer for Taq polymerase containing 1.5 mM $MgCl_2$, 1 µl of 25µM each dNTP mix, 2 µl each of forward and reverse primer (4pmol) and 0.9U/µl of Taq DNA polymerase made up to 25 µl using molecular grade water. Routinely, master mix was set up and 20 µl each was distributed to the PCR tubes, to which 5 µl of the template was added.

The cycling conditions used were Initial denaturation at 95 ºC for 5 min, followed by 35 cycles each of denaturation at 95 ºC for 1 min with annealing temperatures specified in Table 1. for 80 sec and extension at 72 ºC for 45 sec with a final extension period of 7 min at 72 ºC was found to be optimum for obtaining the desired PCR amplicons. The PCR products were stored at -20 ºC until further use.

Agarose gel (1.5%) was prepared by boiling agarose in an appropriate volume of 1X TAE buffer. After cooling for about 3 min, ethidium bromide (Biogene, USA) was added to the agarose solution to a final concentration of 0.5µg/ml. The molten agarose was then poured into the tray and the comb was fitted into the slots on the tray. The tray was kept undisturbed till the gel had solidified. The comb was then taken out carefully and the tray containing the gel was then placed in a submarine horizontal electrophoresis unit filled with 1xTAE buffer upto a level of 1mm above the gel surface. About 5 µl of each PCR product was mixed with 2 µl of bromophenol blue (6x) loading dye and loaded into each well. Electrophoresis was performed at 5 V/cm and the mobility was monitored by the migration of the dye. After sufficient migration, the gels were observed under UV transilluminator to visualize the bands. The PCR product size was determined by comparing with a standard molecular weight marker and was photographed by the gel documentation system.

3. Results and Discussion

The antibiotic resistance/susceptibility of *Salmonella* isolates from Quail samples was presented in table 2.

The *Salmonella* isolates were highly resistant to gentamycin (70.0%) followed by streptomycin (53.33%), sulphamethoxazole (46.67), tetracycline (30.0%), ampicillin (26.67%), amikacin and ciprofloxacin (16.67%), nalidixic acid (13.33%), chloramphenicol (10.0%) and least with ceftriaxone (6.67%).

The *Salmonella* isolates were highly susceptible to ceftriaxone (93.33%) followed by nalidixic acid (83.33%), amikacin and chloramphenicol (80%), ciprofloxacin (76.67%), ampicillin (66.67%), tetracycline (50.0%), streptomycin and sulphamethoxazole (36.67%) and least with gentamicin (23.33%).

The resistance of *Salmonella* isolates against tetracycline in present study was 30%, which was lower than the resistance of 90% and 86.5% reported by Jahan *et al*. (2018) [20] and Bacci *et al*. (2012) [18] respectively. The resistance of *Salmonella* isolates against ampicillin was 26.67% in present study, which was lower than the resistance of 81.1% reported by Bacci *et al*. (2012) [18].

The resistance of *Salmonella* isolates from Quail samples against nalidixic acid in present study was 13.33%, which was lower than the resistance (81%) reported by Fashae *et al*. (2010) [21] in *Salmonella* isolates from Poultry, whereas the susceptibility from Quail samples against nalidixic acid was 83.33% in the present study, which was lower than susceptibility (94.4%) reported by Garba *et al*. (2017) [22] in *Salmonella* isolates from Poultry.

Table 2: Antibiotic resistance pattern of *Salmonella* isolates from quail samples

Antibiotics	Susceptible (%)	Intermediate (%)	Resistant (%)
Tetracycline (TE) (30 µg)	15(50.00)	6(20.00)	9(30.00)
Ampicillin (AMP) (10 µg)	20(66.67)	2(6.67)	8(26.67)
Streptomycin (S) (10 µg)	11(36.67)	3(10.00)	16(53.33)
Chloramphenicol(C)(30 µg)	24(80.00)	3(10.00)	3(10.00)
Gentamicin (GEN) (10 µg)	7(23.33)	2(6.67)	21(70.00)
Sulphamethoxazole(SXT)(100µg)	11(36.66)	5(16.67)	14(46.67)
Amikacin (AK) (30 µg)	24(80.00)	1(3.33)	5(16.67)
Ciprofloxacin (CIP) (5 µg)	23(76.67)	2(6.67)	5(16.67)
Ceftriaxone (CTR) (30 µg)	28(93.33)	0	2(6.67)
Nalidixic Acid (NA) (30 µg)	25(83.33)	1(3.33)	4(13.33)

The *Salmonella* isolates from quail samples were highly susceptible to chloramphenicol (80.0%) in the present study, which was almost similar to the susceptibility of 82.35% reported by Ammar *et al*. (2016) [8].

The susceptibility of *Salmonella* isolates for ciprofloxacin in present study was 76.67%, which was lower than the susceptibility of 100% reported by Jahan *et al*. (2018) [20] and Ramya *et al*. (2013) [23]. Intermediate resistance/susceptibility in present study was 6.67%, whereas zero intermediate was reported by Jahan *et al*. (2018) [20]. Zero percent resistance against ciprofloxacin was reported by Jahan *et al*. (2018) [20], which was lower than resistance (16.67%) in the present study.

The resistance of *Salmonella* isolates from Quail samples against ceftriaxone in present study was 6.67%, which was higher than the resistance of 5.88% reported by Ammar *et al*.(2016) [8] from poultry samples, whereas the susceptibility was 93.33% in the present study from quail, which was higher than the susceptibility of 66% reported by Elkenany *et al*.(2019)[24] and lower than susceptibility of 100% reported by Kim *et al*.(2016)[25] for the isolates from poultry samples.

The resistance against amikacin in present study was 16.67% from Quail samples, which was lower than the resistance of 73% reported by Miko *et al*. (2005) [26] and zero percent resistance reported by Sudhanthirakodi *et al*. (2016) [27] and Zhao *et al*. (2003) [28] in *Salmonella* isolated from poultry samples. The susceptibility for amikacin was 80% from quails in the present study, which was higher compared to the susceptibility 37.5% reported by Mir *et al*. (2015) [2] in poultry isolates.

The resistance against gentamicin in present study was 70%, which was higher than the resistance 27% reported by Bacci *et al*. (2012) [18]. The resistance was 46.67% against sulfamethoxazole in present study, which was lower than the resistance 89.2% reported by Bacci *et al*. (2012) [18].

The susceptibility for streptomycin in the present study from Quail samples was 36.67%, which was almost similar to the susceptibility of 35.29% reported by Ammar *et al*. (2016) [8] for the isolates from poultry samples whereas Fashae *et al*. (2010) [21] reported 23% resistance for the isolates from poultry samples, which was lower than the resistance observed in the present study (53.33%).

Antibiotic resistant genes in *Salmonella* isolates from quail for different antibiotics are presented in table 3.

The prevalence of tetracycline resistant *Salmonella* isolates that harboured tet A gene in present study was 55.5%, which was lower than the prevalence of 86.5% reported by Bacci *et al*. (2012) [18]. The *Salmonella* isolates that harboured tet B gene of tetracycline observed in present study was 33.3%, which was higher than the prevalence of 27% reported by Bacci *et al*. (2012) [18]. Other genes responsible for tetracycline resistant such as tet(C), tet(D), tet(G) and tet(H) have been reported in non-typhoidal *Salmonella* from clinical or retail meat isolates (Alcaine *et al*., 2007 and McDermott *et al*., 2016) [29, 30].

The prevalence of ampicillin drug resistant gene blatem-1 has been frequently demonstrated in *Salmonella*, which is extended spectrum beta lactamases (Oghenevo *et al*., 2016) [31]. The prevalence of ampicillin resistant blatem-1 gene observed in *Salmonella* isolates in the present study from quails was 62.5%, which was higher than the prevalence of 20% reported by Vuthy *et al*. (2017) [32] for *Salmonella* isolates from poultry.

Aminoglycoside phosphotransferases are involved in resistance development and are encoded by the genes strA, strB, aph(3)-Ib and aph(6)-Id, which provide resistance to streptomycin (Alcaine *et al*., 2007) [29].The prevalence of streptomycin resistant *Salmonella* isolates from quails that harboured str A gene in present study was 31.2%, which was lower than the prevalence (90%) reported by Vuthy *et al*. (2017) [32]. The prevalence of gentamicin resistant *Salmonella* isolates that harboured ant(3")1a gene in present study from quail was 61.9%, which was lower than the prevalence (67.5%) reported by Bacci *et al*. (2012) [18].

Table 3: Antibiotic resistant genes in *Salmonella* isolates from quail for different antibiotics

Antibiotics	Genes	No. tested	No. positive	%
Tetracycline	tet A	9	5	55.5
	tet B		3	33.3
Ampicillin	blatem-1	8	5	62.5
Streptomycin	str A	16	5	31.2
Gentamicin	ant(3")la	21	13	61.9
Sulfamethoxazole	sul 1	14	5	35.7
	sul 2		8	57.1

The sulfonamide group resistance in *Salmonella* is due to the presence of common sul genes i.e sul1, sul2 and sul3, which causes the expression of dihydropteroate synthetase that cannot be inhibited by sulfonamides. The genes are present in integrons, *Salmonella* genomic islands or transferrable plasmids (Alcaine *et al*., 2007) and Huovinen *et al*., 1995) [29, 33]. The prevalence of sulfamethoxazole resistant *Salmonella* isolates that harboured sul 1 and sul 2 gene in present study from quail was 35.7% and 57.1% respectively. The prevalence of sulfamethoxazole resistant *Salmonella* isolates that harboured sul 1 gene in present study from quail was 35.7%, which was lower than the prevalence of 44% reported by Maka *et al*. (2015) [34] for *Salmonella* isolates from poultry.

The prevalence of sulfamethoxazole resistant *Salmonella* isolates that harboured sul 2 gene in present study from quail was 57.1%, which was higher than the prevalence of 53% reported by Vuthy *et al*. (2017) [32] for *Salmonella* isolates from poultry.

4. Conclusion

The relatively high, varying occurrence and multi-drug resistant *Salmonella* in Quails in the current study necessitates the need to implement interventions to minimize cross contaminations at all stages of production and processing levels. Proper prevention, control and sanitary management practices are the best guarantees against *Salmonella* infections.

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