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Fecal Colonization of Extended-Spectrum Beta Lactamase-Producing *Salmonella* spp. in Broilers in Lorestan Province of Iran

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Abstract

Background: Poultry is considered as a major source of human contamination with non-typhoidal *Salmonella* species. Global concern regarding the emergence and dispersion of extended-spectrum beta-lactamase (ESBLs)-producing isolates in broilers has increased during recent years.

Objective: This study was proposed to evaluate the prevalence of *Salmonella* and the associated ESBLs in broilers in Lorestan province of Iran.

Materials and Methods: Five hundred fresh fecal samples of broilers were phenotypically screened for *Salmonella*. The isolates were confirmed molecularly using an *invA*-based polymerase chain reaction (PCR). Confirmatory combination disk method was applied for phenotypic detection of ESBLs among the isolates, followed by molecular identification of $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{SHV} genes in 3 single PCR assays among positive isolates. Chi-square test in SPSS software was used for the assessment of statistical relationships.

Results: Of the 95 *Salmonella* isolates detected using routine bacteriological methods, all were confirmed molecularly. They generated the expected 254-bp amplicon. Moreover, 13 isolates were phenotypically recognized as ESBL determinants, among which 9 and 4 harbored $bla_{CTX\text{-}M}$ and bla_{TEM}, respectively. No bla_{SHV} and co-existence of the genes were determined.

Conclusion: The threat imposed by dissemination of ESBL-producing non-typhoidal *Salmonella* spp. isolated from broilers was confirmed in the studied region. Continuous monitoring programs, application of biosecurity measures, and prudent prescription of antibiotics are warranted in order to prevent the introduction or dispersion of the ESBL-producing *Salmonella*.

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Background

Salmonella Species is one of the most important food-borne pathogens worldwide. Poultry, particularly broilers, is by far proved to be a major gut reservoir of the bacterium.[1] Therefore, inappropriate evisceration is a plausible way of carcass contamination with *Salmonella*. Besides, close occupational contact is also proposed as a way for acquisition of the infection.[2] Despite the remarkable improvement of hygienic practices in industrialized poultry rearing, slaughtering, postmortem processing, and handling in Iran, the public health threat imposed by food-borne pathogens still remains serious. Some domestic literature have documented high rates of contamination with zoonotic agents in foodstuffs, including broiler meat.[3-7]

Non-typhoidal *Salmonella* infections in human cause fever, acute gastroenteritis, nausea, and bacteremia.[8] Given than fluoroquinolones are not yet authorized for use in neonates and infants, as a susceptible age group for *Salmonella* infections, the choice of antibiotics is limited to cephalosporins in the cases of invasive infections.[9] Besides, cephalosporins are widely prescribed against other *Enterobacteriaceae* infections in public and veterinary sectors.[10]

A controversial issue in husbandry practice, particularly in developing countries, is the uncontrolled use of antibiotics such as prophylaxis and/or growth promoters.[11] The selective pressure of antibiotics has led to massive and dramatic emergence and dissemination of antimicrobial resistance among bacteria. This facilitates the propagation of resistance genes among normal microbiota as well as hampering the effectiveness of antibiotic chemotherapy or therapy failure against pathogenic bacteria.[12,13]

Beta-lactamases are the group of enzymes that

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inactivate beta-lactam antibiotics through the hydrolysis and disruption of beta-lactam ring. Extended-spectrum beta-lactamases (ESBLs), mostly encoded in *Enterobacteriaceae*, confer resistance against the first to the fourth generation of cephalosporins and aztreonam.[14,15] Because of their typical location on mobile genetic elements, the mobility of ESBLs is eased via horizontal transmission through plasmids or integrons.[16] Therefore, the dissemination of ESBLs may not only occurs within a genus but also among the close genera including members of *Enterobacteriaceae*.[12] ESBL-producing *Salmonella* spp. have emerged during recent years and triggered a drastic and global public health concern.[13,16,18-24] Despite the existence of various ESBLs, some of the most common genes encoded in Salmonella *spp*. are bla_{CTX-M}, bla_{TEM}, and bla_{SHV}.[13,16,18-27] An additional major consequence of the propagation of ESBLs, particularly bla_{CTX-M}, among bacteria is the render of co-resistance to other antibiotic classes through these genes.[28,29] The co-carriage or regulation of other antimicrobial genes with the same plasmids confers an evolutionary advantage for bacterial survival in an unfavorable drug environment.[17]

As very little is known about the epizootiology of ESBL-producing *Salmonella* spp. in food-producing animals in Iran, an attempt was made to provide a preliminary insight regarding the frequency and types of the most common ESBLs in *Salmonella* spp. isolated from broiler in Lorestan province in the west of the country. The results may be valuable in evaluating the public health hazard imposed in this premise and establishment of surveillance measures and controlling strategies against the infection.

Materials and Methods

A total of 500 fecal samples were collected aseptically and immediately after slaughtering broilers from all over Lorestan province, in the west of Iran. The sample from each bird was separately collected in sterile glass bottles and chilled until submission to the laboratory within three to five hours. The location, age, and seasonal variables regarding the samples are depicted in Table 1.

The samples were screened for *Salmonella* using conventional microbiological procedure.[30] Five grams of each fecal sample was homogenized in 90 mL Buffered Peptone Water (BPW, Merck, Germany) and incubated at 37°C for 18-24 hours. Further, 25 µL of the pre-enrichment medium was inoculated into 10 mL of Rappaport Vassiliadis Enrichment (RV, Merck, Germany) broth and incubated at 41.5-42°C for 15-18 hours. A loopful of the enrichment medium was streaked onto Xylose Lysine Deoxycholate (XLD, Merck, Germany) agar. The incubation time was 24 hours at 37°C. Finally, a presumptive colony of *Salmonella* on XLD agar (red colony with black center) from an individual plate was purified on MacConkey (MAC, Merck, Germany) agar and identified biochemically based on Gram staining and Urea, TSI, and IMViC reactions.

DNA of the *Salmonella* isolates was extracted from the overnight culture of the bacteria in Luria Bertani (LB, Merck, Germany) broth using boiling method. Molecular identification of the isolates was accomplished by screening the *invA* gene, based on the primer pair and thermal condition introduced by Rahn et al in 1992 (Table 2).[31] The final volume of the reaction master mix was 25

Table 1. Characterization of the Samples in the Present Study

Slaughter Code	Location	Number of Samples	Season (Warm Versus Cold)	Age	Number of *Salmonella* spp. Isolates	ESBL Determinants
A	Khorramabad	25	Summer	56	5	1
A	Khorramabad	25	Fall	44	6	0
B	Khorramabad	25	Summer	45	4	0
B	Khorramabad	25	Fall	60	7	1
C	Khorramabad	25	Summer	60	7	2
C	Khorramabad	25	Fall	65	10	3
D	Borujerd	25	Summer	50	3	0
D	Borujerd	25	Fall	42	2	0
E	Borujerd	25	Summer	60	6	1
E	Borujerd	25	Fall	46	3	0
F	Kuhdasht	25	Summer	56	2	0
F	Kuhdasht	25	Fall	45	4	0
G	Dorud	25	Summer	50	4	0
G	Dorud	25	Fall	56	8	2
H	Aligudarz	25	Summer	54	6	0
H	Aligudarz	25	Fall	48	4	2
I	Delfan	25	Summer	55	3	0
I	Delfan	25	Fall	45	4	0
J	Aleshtar	25	Summer	45	1	0
J	Aleshtar	25	Fall	50	7	1

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Table 2. Characterization of Primers Used in the Present Study

Primer	Sequence (5′→3′)	Amplified Gene	Product Size (bp)	Reference
ST139	GTGAAATTATCGCCACGTTCGGGCAA	*InvA*	284	31
ST141	TCATCGCACCGTCAAAGGGAACC			
TEM-F	ATCAGCAATAAACCAGC	bla_{TEM}	516	33
TEM-R	CCCCGAAGAACGTTTTC			
CTX-M-F	TTTGCGATGTGCAGTACCAGTAA	bla_{CTX-M}	544	34
CTX-M-R	CGATATCGTTGGTGGTGCCATA			
SHV-F	AGGATTGACTGCCTTTTTG	bla_{SHV}	392	35
SHV-R	ATTTGCTGATTTCGCTCG			

µL containing 12.5 µL of 2X ready-to-use PCR master mix (CinnaGen, Iran), 0.7 µL of each primer, 50 ng (2 µL) of template DNA, and 9.1 µL of deionized distilled water. *Salmonella typhimurium* ATCC 1730 and DNA-free master mix were used as positive and negative controls, respectively. The PCR products were electrophoresed in 1.2% agarose gel (CinnaGen, Iran) stained with DNA safe stain (CinnaGen, Iran).

Subsequently, confirmatory combination disk method was applied for phenotypic detection of ESBL determinants. The ESBL determinants were interpreted as an increase of ≥5 mm in the diameter of the growth inhibitory zone related to ceftazidime/clavulanic acid and cefotaxime/clavulanic acid disks in comparison with ceftazidime and cefotaxime disks alone.[32] *Klebsiella pneumoniae* ATCC 700603 was used as a quality control organism.

Molecular detection of bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes was performed in three single PCR assays based on the primer pairs and thermal protocol represented in Table 2. The reaction mixture in each PCR assay contained 12.5 µL of 2X ready-to-use PCR master mix (CinnaGen, Iran), 50 ng (2 µL) of the template DNA, and 0.4 µM of each primer in the final volume of 25 µL. The used positive controls were *E. coli* PTCC 1533 and *K. pneumonia* RTCC 1248. Besides, DNA-free master mix was used as negative control. The visualization of PCR amplicons was the same as the previous assay.

The statistical association of the frequency of *Salmonella* spp. and ESBL determinants with the variables including location, age and season were analyzed in SPSS software (version 21.0) using Chi-square test. A *P* value of less than 0.05 was considered statistically significant.

Results

In general, 95 *Salmonella* spp. were isolated and identified using routine bacteriological methods, leading to an overall frequency of 19%, which is represented in Table 1 in details. All of the isolates generated the expected 284-bp amplicon in *invA*-based PCR, molecularly confirming them as *Salmonella* (Figure 1). Moreover, 13 isolates (13.68%) were recognized as ESBL determinants using phenotypic method (Figure 2). bla_{CTX-M} and bla_{TEM} genes were manifested in 9 (9.47%) and 4 (4.21%) isolates, producing a 554-bp and a 516-bp PCR product, respectively (Figures 3 and 4). No bla_{SHV}-harboring isolate was recognized among the isolates. The coexistence of the screened ESBL genes was not observed in any of the isolates. The frequency of ESBL-producing *Salmonella* spp. is illustrated in Table 1.

Likewise, the statistical association of the frequency of *Salmonella* spp. with season (*P*=0.049), age (*P*=0.034), and geographical location (*P*=0.037) was observed. In addition, the statistical association of the frequency of ESBL genes with season (*P*=0.041), age (*P*=0.038), and geographical location (*P*=0.024) was also observed.

Discussion

Non-typhoidal *Salmonella* spp., as zoonotic bacteria, is commonly isolated from poultry. In fact, poultry

Figure 1. Gel Electrophoresis of *invA* Gene. M: 100-bp DNA ladder; PC: Positive Control; NC: Negative Control; Lanes 1-9: 285-bp amplicon in field samples.

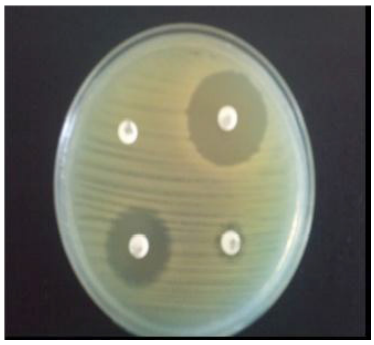

Figure 2. Phenotypic Confirmatory Disk Diffusion Test: An Indicative of ESBL Production in *Salmonella* isolates. A ≥ 5 mm increase in the diameter of inhibition zones related to ceftazidime/clavulanic acid and cefotaxime/clavulanic acid disks in comparison with ceftazidime and cefotaxime disks.

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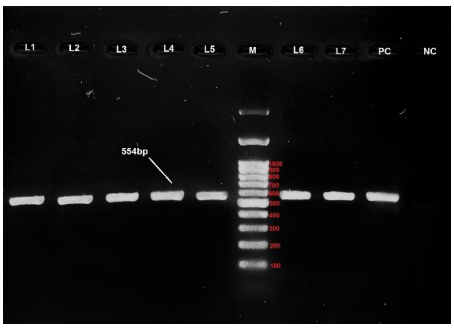

Figure 3. Gel Electrophoresis of bla_{CTX-M} Gene. L1-L7: 554-bp amplicon in field samples; M: 100-bp DNA ladder; PC: Positive Control; NC: Negative Control.

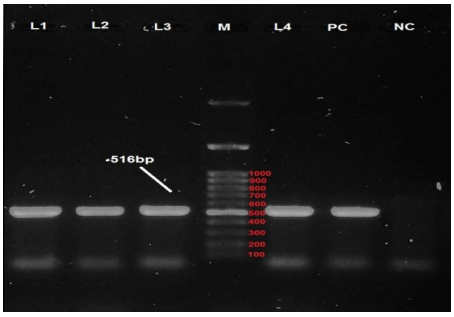

Figure 4. Gel Electrophoresis of bla_{TEM} Gene. L1-L4: 516-bp amplicon in field samples; M: 100-bp DNA ladder; PC: Positive Control; NC: Negative Control.

products are the main source of human contamination with *Salmonella*.[2,10,19,20,23-27] Following the introduction of the infection in a farm and subsequent colonization of the bacterium in the intestines, carcass contamination may occur during slaughtering process.[36] Therefore, biosecurity measures and sanitation practices in farms and post-slaughtering need to be more industrialized in Iran in order to reduce the contamination rates.[2] In the present study, the overall distribution of *Salmonella* infection among broilers in Lorestan province of Iran was 19%. In comparison, the frequency of *Salmonella* spp. infection in broiler farms of Sanandaj was reported to be 1.80%.[37] It was reported that 2.11% and 19.23% of the fecal samples collected from poultry flocks in the vicinity of Tehran and Mashhad were contaminated with *Salmonella* spp., respectively.[38,39] The prevalence of *Salmonella* spp. in broiler flocks of Ahvaz was 40%.[40] The prevalence rates of *Salmonella* in broilers were corroborated from Ecuador (16%) and Pichincha in Pakistan (15.9%).[24,27] Our results were notably lower compared to the reports from the northeastern Phatthalung in Thailand (22%) and Algeria (34.37%).[21,41] This implicates the better hygienic state of poultry farms in our local area than the mentioned districts. However, lower rates of *Salmonella* distribution were reported in broilers from some European Union members (Italy (9.2%), France (3.4%), Germany (2.7%), and Spain (1.02%).[42,43] The absence of strategies to control *Salmonella* in broiler farms and the overuse of antibiotics are the plausible explanations for the higher frequency of the infection in Iran, with emphasis on surrounding cities of the studied area, in comparison with European countries. Moreover, geographical conditions, sampling season, dietary regime and management measures, particularly at early life stages, are attributed to the incidence of gut colonization of *Salmonella* in broilers.[44] Additionally, it is proved that culturing fecal samples rather than rectal swab samples may enhance the probability of *Salmonella* detection.[21] The high distribution of *Salmonella* spp. in nature and its dispersion and survival in vectors, including rats, birds, and flies, may facilitate the bacterial dissemination to poultry. This is because warehouses in farms are usually not approparietely fenced to control the traffic of animals. This elevates the chance of oral acquisition of the infection.[45]

Based on the data gained in the present study, the lower the age is, the higher the rate of *Salmonella* infection would be. This coincides with the results documented from northeastern Algeria and Denmark.[41,46] This may be related to the immune status of the poultry, as the highest immunity is reached at advanced ages.[47] On the other hand, a seasonal trend of *Salmonella* prevalence among broilers was observed. A possible explanation for the higher frequency of the infection in cold seasons is due to higher rainfall rates, which may accelerate the dissemination of the bacteria. Besides, cold stress may impose alterations in common symbiont of the intestines. This may influence gut colonization and/or excretion of *Salmonella* spp.[48]

Moreover, our results underscored the potential role of broilers as reservoirs of ESBL determinants in the west of Iran, which confirms the previous report.[49] However, no ESBL-producing *Salmonella* spp. were detected in a previous domestic study.[23] Due to the potential propagation of ESBLs to other Gram-negative bacteria, this has been considered as a great economic loss in poultry industry in terms of high morbidity and mortality rates, decreased hatchability, and egg production.[27] On the other hand, this is a matter of concern due to the transmission of the trait of these genes to human through direct contact or food chain.[17] Although the frequency of ESBLs in non-typhoidal *Salmonella* spp. is lower in comparison with other members of *Enterobacteriaceae* including *E. coli* and *K. pneumonia*, their existence and dissemination may be considered as a treatment failure in the cases of acute salmonellosis in children.[9,50] The association of antibiotic-resistant *Salmonella* spp. originated from food animals with human disease has been confirmed previously.[51] Furthermore, the emergence of resistance in other Gram-negative bacteria in human following the acquisition of these genes has devastating complications in therapeutic trials in terms of eroding the antimicrobial chemotherapy. In addition to the existence of multidrug efflux pumps, the outer membrane permeability barriers in Gram-

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negative bacteria drastically influence the distribution of resistance genes among them.[52] Considering the routes of dispersion of resistance genes among poultry farms, inefficient biosecurity measures and colonization of stock received from broiler breeder flocks should not be neglected.[45]

Despite the limited consumption of beta-lactams and cephalosporins in poultry husbandry in Iran, they may be used as feed additives. Although this may generate a selective pressure leading to the dissemination and maintenance of ESBLs, the persistence of ESBL determinants among intestinal microbiota of poultry is proved to occur even in the absence of selective antimicrobial pressure.[53,54] This merits further investigations to identify the exact mechanism of propagation and retention of ESBLs in the lack of selective pressure.[55] Until then, the moderate use of antibiotics is highly recommended to help the prevention of antibiotic resistance among bacteria.[25] Likewise, the pervasive prescription of antibiotics, particularly tetracyclines and fluoroquinolones, in poultry industry in Iran may direct the co-selection of ESBL determinants, harboring resistance genes against these drugs. Various studies have reported the co-carriage of ESBLs and quinolone/tetracycline resistance genes on an individual mobile genetic element.[56] The high prevalence of ESBL harboring bacteria in companion animals treated with enrofloxacin was also reported.[57]

Examining the trends and pattern of antimicrobial resistance among bacteria isolated from animals should be taken into account as an important step for sampling and assessing antimicrobial susceptibility in veterinary practice. The predominant ESBL gene detected in the present study was bla_{CTX-M}, followed by bla_{TEM}, with the proportion of 9.47% and 4.21%, respectively. While bla_{TEM} was the predominant ESBL gene detected in *Salmonella* spp. in pediatric patients in Iran,[58] bla_{CTX-M} group was observed to be the most prevalent ESBLs in a foreign study.[59] It should be noted that the prevalence rate of ESBL-producing *Salmonella* spp. in this study in under-estimated as other ESBLs or AmpC genes have not been assessed. Data related to the frequency of ESBLs in animal-originated *Salmonella* spp. in Iran are scarce. In line with our findings, bla_{CTX-M} was the predominant ESBL gene recovered from *Salmonella* in poultry,[24,41,60,61] while some others reported bla_{TEM} [25,62] and bla_{SHV} as the most abundant ESBL genes.[63,64] The bla_{SHV} gene, responsible for resistance to ampicillin, was not detected herein which coincides with the results revealed from Ecuador, northeastern Algeria, China, and northern Egypt.[24-26,41] In addition to the geographical variations, sample type and size may fundamentally affect the discrepancies of ESBL types among different studies. The dissemination mechanism of bla_{TEM}, and bla_{SHV} genes follows the epidemic pattern, limiting them to particular geographical regions. In contrast, bla_{CTX-M} obeys the allodemic pattern of dispersion, leading their acquisition by several clones of bacteria. Besides, the relationship between the high distribution of bla_{CTX-M} ESBLs in veterinary sector is manifested by effective seizure and distribution of these genes by plasmids and transposons and the low fitness costs imposed by them.[65] Finally, the same as ESBLs, class I integrons reside on plasmids and transfer conjugally. The high prevalence of class I integrons among ESBL-producing isolates of Gram-negative bacteria emphasizes the continuous stewardship programs to moderate or prevent further propagation of these elements.[66]

Conclusion

Briefly, the presence of ESBL-producing *Salmonella* spp. was demonstrated for the first time in broilers of Lorestan province of Iran. The potential dissemination of these genes to public health and veterinary sectors demands the application of regular monitoring and surveillance programs with emphasis on the prudent and judicious administration of antibiotics in veterinary husbandry. Moreover, complementary studies are needed to assess the potential role of animal products as a vehicle for transmission of ESBL determinants to human.

Authors' Contributions

EA: Conception and design, Data analysis and interpretation, Drafting the manuscript, Critical revision of the manuscript for important intellectual content and supervision. AH: Data acquisition, statistical analysis.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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