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Molecular Survey of Quinolone Resistance in *Salmonella* spp. Isolated From Poultry Products in Karaj, Iran

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Abstract

Background: Non-typhoidal salmonellae and serovars of *Escherichia coli*, *Listeria*, *Shigella*, and *Campylobacter* are among the most important food-borne bacterial agents.

Objective: The aim of the present study was molecular detection of quinolone resistance in *Salmonella* spp. isolated from poultry products in Karaj, Iran, using polymerase chain reaction (PCR).

Materials and Methods: Ninety samples of poultry products were collected from different brands and markets during September-December 2017. All samples were enriched in nutrient broth and Selenite F broth, and salmonellae were isolated by xylose lysine deoxycholate agar. The presence of specific genes of *qnr*A, *qnr*B, and *qnr*S was investigated employing PCR technique and subsequently, specific primers.

Results: None of the 30 egg yolk samples had bacterial growth in the culture medium. In total, 29 (48.33%) out of 60 raw chicken meat samples were determined to be contaminated with *Salmonella* using culture-based methods (i.e., 7 (35%) out of 20 drumstick, 15 (62.5%) out of 24 breast, and 7 (43.75%) out of 16 liver samples). In addition, the frequency of *qnr*A, *qnr*B, and *qnr*S genes in the samples was 10.34%, 68.96%, and 86.20%, respectively.

Conclusion: The results of this study showed a high frequency of *Salmonella* contamination and *qnr* genes in the contaminated samples.

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Background

Salmonella is considered as an important bacterial agent of zoonoses,[1] which causes one of the most prevalent food-borne illnesses, namely, salmonellosis.[2] The ability of *Salmonella* species to cause infection in human involves the attachment and colonization of columnar epithelial cells of the intestine and specialized microfold cells overlying the Peyer's patches.[3] In addition, gastroenteritis is the most frequent clinical symptom of non-typhoidal *Salmonella* infection.[4] The symptoms of salmonellosis include diarrhea, abdominal pain, as well as nausea and vomiting and the illness is generally self-limiting in healthy adults.[5] In severe cases, the infection may progress to septicemia and death, unless the person is promptly treated with appropriate antimicrobials. Further, individuals who are immunocompromised, that is, children, infants, and the elderly are most likely to require antimicrobial treatment.[3] Furthermore, *Salmonella* has a broad host range among mammalians, birds, ectotherms, and human. Although many *Salmonella* serovars are identified, the serovars from farms have a significant overlap with those causing illnesses in human.[6] Animal husbandry and aviculture lead to the colonization of this organism, thus food products with animal and especially poultry origin are viewed as the major sources of *Salmonella* infections.[7] Antimicrobial-resistant bacteria emerge from employing the antimicrobial drugs to treat and prevent diseases and to promote growth in large-scale animal production.[8] Quinolones, particularly fluoroquinolones, are commonly used for treating multi-drug resistant salmonellosis "in human and veterinary medicine" due to their broad-spectrum antimicrobial activity.[9,10] Moreover, point mutations in DNA gyrase and topoisomerase IV genes are directly related to quinolone resistance in *Enterobacteriaceae* through the changes in the target site called quinolone resistance-determining regions.[11] "In *Salmonella* species, these mutations are related to resistance to nalidixic acid and reduced susceptibility to fluoroquinolones such as that of ciprofloxacin."[12] Additionally, resistance to quinolones

is believed to be mediated only by this mechanism. However, the situation changed with the discovery of a variety of determinants related to the plasmid-mediated quinolone resistance (PMQR).[5,9] Currently, three mechanisms are recognized as PMQRs. The *qnr* genes with five different qnr families each with different numbers of alleles "(i.e., *qnrA1–7*, *qnrS1–4*, *qnrB1–31*, *qnrC*, and *qnrD*)", "a modified aminoglycoside acetyl-transferase gene (*aac(6′)-1b-cr*)", and a specific quinolone efflux pump (*qepA*) and multidrug resistance pumps including *oqxAB*.[12] PMQR-positive isolates represent a low-level of resistance to quinolones (i.e., only a small reduction in susceptibility to nalidixic acid). However, the ability to highlight pre-existing resistance mechanisms such as chromosomal mutations in the target regions of the quinolones that still allow the selection of resistant mutants to quinolone concentrations (therapeutic doses) emphasize the importance of studying these genes.[9]

Infections resulting from resistant strains may compromise treatment outcomes thus result in increased morbidity and mortality.[13] Therefore, to protect the public health, continuous monitoring and surveillance systems are necessary in order to prevent the spread of PMQR genes among *Salmonella* spp. that can be transmitted from poultry to humans.[7,14] *Salmonella* isolates are identified by colonial appearance, biochemical, serological (agglutination with specific antisera), and molecular methods. Molecular techniques have made the identification of many serotypes more rapid and precise compared to phenotypic techniques.

Objective

The current study sought molecular survey of quinolone resistance in *Salmonella* serotypes isolated from poultry products in Karaj, Iran due to the development of antibiotics resistance, the limitation of the published surveys about quinolone resistant salmonellae in Iran, especially in Karaj, and the existence of a large number of animal husbandry and avicultural practices in this city.

Materials and Methods

Sampling

Ninety poultry product samples including 60 samples of packed and unpacked raw chicken meat (i.e., drumstick = 20, breast = 24, and liver = 16) and 30 egg samples were purchased from different brands and markets during September-December, 2017.

Enrichment

The samples were cut into very small pieces under sterile conditions. Then, one g of each sample was transferred to a selective medium (selenite F) and incubated at 37°C for 20 hours. Next, the eggshells were disinfected with iodine and alcohol (70%), then, one mL of the yolks was injected to selenite F broth and incubated at 37°C for 20 hours. After the incubation, a loop full of each selenite F broth was streaked onto xylose lysine deoxycholate agar (XLD) and incubated at 37°C for 24 hours. All phases were performed in a biosafety cabinet. In addition, suspensions were prepared from *Salmonella* colonies in XLD agar and used for the extraction of bacterial DNA. Finally, the presence of *qnr* genes was evaluated by multiplex polymerase chain reaction (PCR) technique and specific primers (cinnagen).

DNA Extraction

DNA of the samples was extracted using the boiling method and then subjected to PCR. The quantification and analysis of the extracted DNA were performed applying 1.2% agarose gel electrophoresis. The results were visualized by gel documentation (E-Gel imager, UPV, Taiwan).

Multiplex PCR

Multiplex PCR was optimized using the primer sets described in Table 1. Only the *Salmonella* culture-positive samples were tested by PCR technique. Further, the PCR protocol was performed in the reaction mixture with a total volume of 25 µL, containing 2.5 µL of 10X PCR buffer, 0.7 µL of dNTPs (10 mM), 1 µL of $MgCl_2$, 1 µL (20 pmol) of each of the forward and reverse *qnr* primers, 1 µL of the template (DNA), 17.3 µL of sterile water, and 0.5 µL of Taq DNA polymerase (5 unit/µL). Furthermore, PCR amplification included primary denaturation at 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for one minute, annealing at 52°C for one minute (The thermal gradient setting was used for annealing the temperature optimization for resistance genes primers), and extension at 72°C for one minute. The final stage was an extension at 72°C for 7 minutes. Moreover, PCR

Table 1. The PCR Primers Used in This Survey

Primer	Sequence	Expected Amplicon Size (bp)	Reference
*qnr*A	F 5′-ATT TCT CAC GCC AGG ATT TG-3′ R 5′-GAT CGG CAA AGG TTA GGT CA-3′	516 bp	12
*qnr*B	F 5′-GTT GGC GAA AAA ATT GAC AGA A-3′ R 5′-ACT CCG AAT TGG TCA GAT CG-3′	526 bp	11
*qnr*S	F 5′-ACG ACA TTC GTC AAC TGC AA-3′ R 5′-TTA ATT GGC ACC CTG TAG GC-3′	417 bp	12

PCR, polymerase chain reaction.

cycles were implemented in an applied biosystem thermal cycler. The amplified PCR products were stained with DNA Safe Stain (Cinnagen) and visualized using a UV transilluminator. Additionally, the length of the PCR amplicons was estimated by comparison with a 100-bp DNA ladder (Figure 1). Since a standard strain containing three resistance genes was unavailable, one of the positive samples isolated in XLD agar, which was positive by PCR using specific primers, was used as positive control. The presence of each of the three *qnr* genes in the above-mentioned strain was separately checked and confirmed using a single PCR method.

Data were analyzed using the SPSS software (version 16) and *t* test. *P* value <0.05 was considered statistically significant.

Results

Based on the results, none of the 30 egg yolk samples had bacterial growth in the culture medium. However, *Salmonella* contamination was detected in 48.33% (29/60) of raw chicken meat samples including 35% (7/20) of drumstick, 62.5% (15/24) of the breast, and 43.75% (7/16) of liver samples by culture-based methods. In addition, the frequency of *qnr*A, *qnr*B, and *qnr*S genes was 10.34%, 68.96%, and 86.20%, respectively, in 29 *Salmonella* culture-positive samples evaluated by the PCR technique. Further, *Salmonella* contamination rate of drumstick and breast unpacked samples was 45% (9/20) while it was 54% (13/24) in the packed samples (Table 2).

There was no statistically significant difference between the frequency of *Salmonella* contamination in different groups of the samples based on the type of raw chicken meat and packaging.

Discussion

As previously mentioned, *Salmonella* is regarded as one of the essential bacterial pathogens which lead to acute illness or mortality.[15] Although the rate of food contamination with *Campylobacter* spp. has nowadays increased, non-typhoidal salmonellae are still considered as the major cause of food-borne infections in some cases since salmonellae have several hosts and can contaminate animal and vegetative products. Furthermore, these pathogens can be transmitted to humans by the food chain.[6,7] Poultry products are the cheap source of animal proteins for all social and ethnic groups, therefore,

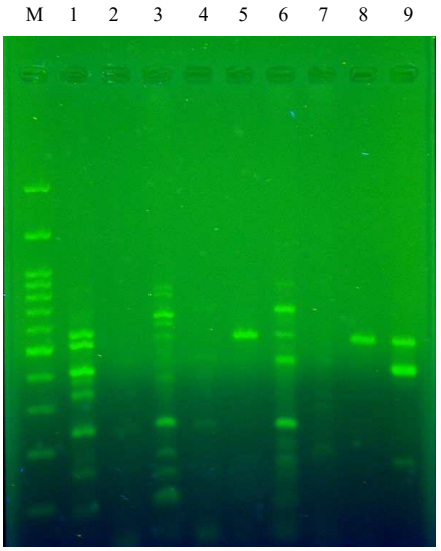

Figure 1. Agarose Gel Electrophoresis of Multiplex Polymerase Chain Reaction Products. M: 100bp DNA ladder; Lane 1: Positive control for 3 resistance genes of *qnr*A, *qnr*B, and *qnr*S; Lane 2: Negative control; Lanes 3, 5, and 6: *qnr*B positive samples (526 bp product); Lanes 8 and 9: *qnr*A positive samples (516 bp product); Lane 9: *qnr*S positive sample (417 bp product).

the transmission of *Salmonella* by consuming chicken meat should be considered very important.[2] Moreover, the survival and growth of *Salmonella* on the poultry products may be due to inappropriate thermal conditions during storage, poor hygiene in handling, or improper cooking techniques.[3] On the other hand, in the last 60 years, the increase of antimicrobial resistance has resulted in many difficulties in controlling the infections caused by *Salmonella* spp.[9] Therefore, the current study aimed to determine the frequency of *Salmonella* serotypes contamination in poultry products and *qnr* genes in *Salmonella* contaminated samples in Karaj, Iran.

Nayebi et al reported a rate of 40% (16/40) and 23% (9/40) of *Salmonella* contamination in chicken products and egg samples, respectively, using PCR method.[1] Nikbakht et al found 0% contamination in 230 egg samples by culture-based methods.[16] Additionally, Zhao et al collected 1105 rectal swabs from chicken, duck, and pig farms and indicated that *Salmonella* contamination rate was 13.93% (154/1105) and the frequency of *qnr*A, *qnr*B, and *qnr*S genes was 73.37% (113/154), 64.28% (99/154), and 6.49% (10/154).[6] Li et al detected 20% (40/200) of retail chicken carcasses as *Salmonella* contaminated.[17] Similarly,

Table 2. The Frequency of *Salmonella* spp. Contamination in Packed and Unpacked Samples

	Drumstick Unpacked	Drumstick Packed	Breast Unpacked	Breast Packed	Liver Packed	Total
Number	11	9	9	15	16	60
Culture positive samples	4	3	5	10	7	29
*qnr*A	0	1	0	0	2	3
*qnr*B	2	2	2	8	5	19
*qnr*S	4	3	4	9	5	25

Nidaullah et al collected 182 samples including 30 poultry and 152 environmental samples and salmonellae were isolated from 161 out of 182 samples (88.46%).[2] High frequency of *Salmonella* contamination in some of the above-mentioned surveys could be due to the common source of contamination, namely, the epizootic outbreak of salmonellosis at sampling time in the farms, as well as the high sensitivity of molecular techniques compared to culture-based methods. In addition, Pirbul et al evaluated 129 isolates including those originated from the food of the animal sources (n = 39), environmental sources (14), as well as those from animals (9), and humans (67), namely, *qnr*S 53.33% (8/15), *qnr*B 40% (6/15), and *qnr*D 6.66%, (1/15).[9]

A total of 90 samples including 60 raw chicken meat (i.e., drumstick = 20, breast = 24, and liver = 16) and 30 egg samples were collected for the purpose of the study. None of the 30 egg yolk samples demonstrated bacterial growth in the culture media. Although using selenite F broth as the enrichment medium limited the growth of intestinal normal flora while promoting *Salmonella* growth, some degree of limitation of the growth and isolation of *Salmonella* was observed on XLD agar probably due to the overgrowth of the coliforms, particularly during the incubation time longer than 9 hours in selenite F broth. Therefore, a suitable incubation period can be helpful for *Salmonella* isolation. Further, the absence of contamination in egg yolk samples in this survey may be due to the number of the samples, different laboratory methods, improved sanitary conditions of the layer farms, and, as mentioned before, the epizootic outbreak of salmonellosis at the time of sampling in previous studies.[1,4,7,16]

As previously explained, the current study sought to detect quinolone resistance (*qnr*) genes (potential for resistance) in *Salmonella* isolates, while not investigating the resistant or multi-drug resistant phenotypes. Of course, using "Selenite F broth" and "XLD agar" prior to the molecular survey was necessary for enriching salmonellae and excluding other bacteria containing *qnr* genes.

In general, the findings of the current study confirmed the presence of *Salmonella* in 48.33% of the raw chicken meat samples and the frequency of resistance genes of *qnr*A, *qnr*B, and *qnr*S was 10.34%, 68.96%, and 86.20%, respectively. The results of this study and those of other similar studies emphasize the increased frequency of *qnr* genes which is a warning for the public health. These high resistance rates may be the result of the wide use of quinolone antibiotics in chicken farms and highlight the importance of restricted use of antibiotics based on in vitro susceptibility testing in poultry farming and treatment of human salmonellosis.

Authors' Contributions

All authors participated equally in this study.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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