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Original article

MOLECULAR TYPING OF CEPHALOSPORIN RESISTANT SEROVARS OF *SALMONELLA ENTERICA* FROM POULTRY AND FARM ANIMALS

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Summary

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In this study, the susceptibility to cephalosporins of 74 *Salmonella* isolates, including 16 isolates from farm animals and 58 isolates from poultry was assessed by the disc diffusion method. ESBL production was evaluated by combined disc diffusion method (CDDM) and double disc synergy test (DDST). The genetic relatedness of isolates was investigated by the Rep-PCR method. The highest prevalence of resistance was observed against cefotaxime (27%) and the least – against cefixime (4%). None of the isolates was ESBL positive. The Rep-PCR generated 54 reproducible fingerprint patterns for all isolates and grouped them in four clusters and six singletons. Due to public health risk of cross contamination, it is important to have sufficient information on the occurrence of these resistant isolates.

Key words: cephalosporin resistance, ESBL, Rep-PCR, *Salmonella*

INTRODUCTION

There is a dramatic increase in the incidence of *Salmonella* species worldwide. *Salmonella* has been mostly reported in poultry products and animals, while poultry meat and eggs are considered to be the main source of human infection (Anderson *et al.*, 2010).

Typing of bacteria can be used to determine whether isolates collected from different sources are related or not. Many typing methods have been applied in epidemiological studies. Phenotyping methods, such as serotyping and phage typing, and genotyping methods, such as pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and repetitive extragenic palindromic PCR (Rep-PCR), are some of the generally

used methods for recognition and characterisation of *Salmonella* strains (Hyeon *et al.*, 2013).

Traditional methods such as serotyping do not always give enough information for the epidemiological investigation. Furthermore, custom serotyping is mostly not sensitive enough to comply with the level of discrimination required for foodborne disease prevalence investigations, and it cannot be used to conclude phylogenetic relatedness (Bourdon *et al.*, 2011).

Molecular typing tools are helpful in identifying the feasible ways of transfer, origin, and traceability of the pathogens. Rep-PCR and PFGE are extensively used to identify the genetic relationship among *Salmonella* isolates (Budiati *et al.*, 2016). PFGE brings up the gold standard procedure for *Salmonella* molecular typing but is expensive, laborious and lengthy. Clearly, an effective way should be inexpensive, feasible, reproducible, quick and able to distinction genetically unrelated strains with the same phenotype (Szabó, 2014).

The increase of multi-drug resistant (MDR) isolates is a worldwide problem. Extended-spectrum beta-lactamase (ESBL) producing isolates which are resistant to cephalosporins (third and fourth generation) have beem globally reported. These enzymes have been detected in many species of *Enterobacteriaceae*, including different *S. entrica* serovars (Djeffal *et al.*, 2017).

The ESBL-producing *Salmonella* species might be transferred to humans from animals via contact or usage of contaminated food, and make it difficult to treat (Qiao *et al.*, 2017).

In the present study, we aimed to evaluate the serotype distribution and molecular subtyping of cephalosporin-resistant *Salmonella* isolates from chickens and calves. These isolates were detected for ESBL production. The genetic relationship among isolates was determined.

MATERIALS AND METHODS

Bacterial isolates

In this study, a total of 74 *Salmonella* isolates from poultry (58 isolates from broiler carcasses and poultry-related samples) and farm animals (16 isolates from aborted and septicaemic cases in sheep and calves) were used. These isolates were recovered from 2014 to 2016 from different farms in eastern provinces of Iran. They have already serotyped and preserved at –20 °C in nutrient broth with 15% glycerol.

Screening for cephalosporin resistant isolates

The disc-diffusion method was used to determine the bacterial susceptibility of the isolates to cephalosporins and interpreted according to CLSI guidelines (CLSI M100-S27). The following antibiotic discs (Mast group, UK) were tested: cefixime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg) and cefpodoxime (10 µg). If the isolates exhibited resistant properties, they were further subjected to confirmatory phenotypic ESBL detection test (CLSI, 2017).

ESBL detection

The isolates that were resistant to the third and fourth generation of cephalosporins were selected for detection of ESBL producers by double disc synergy test (DDST) and combined disc diffusion method (CDDM), recommended by the CLSI (2017). For the DDST, the centered disc was amoxicillin-clavulanic acid (30/10 µg); four others discs were placed

within a 20 mm radius of the first one, cefixime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg) and cefpodoxime (10 µg). Any distortion or increase in the zone towards the disc of amoxicillin-clavulanic acid was considered as positive for the ESBL production. For the CDDM, cefpodoxime (10 µg) alone and in combination with clavulanic acid (10/1 µg) were used. The isolates were considered positive for ESBL production when the inhibition zones around any cephalosporin with clavulanic acid were ≥5 mm than the corresponding disc without clavulanic acid. *Klebsiella pneumonia* (ATCC 700603) was used as positive control in this study.

Rep-PCR fingerprinting

Salmonella isolates were grown on nutrient agar and kept at 37 ^{o}C overnight. Genomic DNA was extracted using the boiling method (Ahmed & Dablool, 2017). Primer sequences were REP1 (5-IIIGCGCCGICATCAGGC-3) and REP2 (5-ACGTCTTATCAGGCCTAC-3) (Martín-Lozano *et al.*, 2002). PCR was performed in a 25 µL reaction volume containing: 12.5 µL of PCR 2× Master Mix (Parstous, Iran) containing Taq DNA Polymerase, reaction buffer, dNTPs mixture, protein stabiliser, and the convenience for use was optimised by adding sediment for electrophoresis and 2× solution of loading dye,1 µL of each primer (2 µM), 2 µL of DNA template and 7.5 µL of nuclease-free water. PCR cycles were as followed: initial denaturation at 95 °C for 5 min; followed by 30 cycles of denaturation at 90 °C for 30 s, annealing at 45 °C for 1 min, and extension at 65 °C for 1 min with a final extension at 65 °C for 4 min.Amplification was performed using the MJ mini thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were run in 2% agarose, stained with ethidium bromide and images were taken.

DNA Fingerprint analysis

Gel images were analysed using the GelJ software (version 2.0). The software created customised reports, including a dendrogram, electrophoregrams, virtual gel images, and scatter plot. The Rep fingerprinting were transformed into binary code depending on the presence or absence of each band. A DNA fingerprint profile of each lane was measured pairwise against all other lanes. Patterns with noticeable differences were considered singleton and strains that showed more than 80% similarity were put into a cluster. Dendrogram were created according to distance matrices and the unweighted pair-group method with arithmetic (UPGMA) mean. The banding patterns were compared using dice coefficients with a 1.5% band tolerance.

RESULTS

ESBL-producing

The numbers of 74 isolates belonged to eleven serovars (Table 1). The most predominant serotypes among poultry isolates were Enteritidis (33 isolates) and serotype Tsevie with 4 isolates was the most prevalent among farm animal isolates. Our results showed that among 74 isolates, 7 (9.45%), 3 (4%), 8 (10.8%) and 20 (27%) were resistant against cefpodoxime, cefixime, ceftazidime and cefotaxime, respectively.

According to resistance phenotype of isolates, six patterns were observed (Table 2). Resistance to cefotaxime alone was the commonest pattern observed among isolates (including 6 isolates of *S.* Enteritidis and 3 isolates of *S.* Typhimurium*)*. The

results of ESBL detection by using two phenotypic methods, double disc synergy test (DDST) and combined double disk method (CDDM) were negative for all cephalosporin resistant isolates.

Rep-PCR

The results showed 56 fingerprint patterns for the 74 *Salmonella* isolates (Fig. 1). The multiple DNA bands obtained were between 150 bp and 1800 bp. Three common bands, 150 bp, 400 bp and 600 bp were observed among isolates. According to dendrogram, isolates were put into four different clusters with less than 80 % similarity and six singletons.

The cluster D was the largest cluster with 42 isolates (56.7%), which were from poultry. The cluster C, with 19 isolates (25.6%), included seven isolates from farm animals and 12 isolates from poultry. The cluster B contained 5 isolates from farm animals and cluster A, was the smallest one with 2 isolates from poultry with 88% similarity. Moreover, four isolates from farm animals and two isolates from poultry were detected as singletons.

Among resistant isolates, 8 and 13 isolates were grouped in cluster C and cluster D, respectively. Furthermore, one of the singletons from poultry was resistant to more than two cephalosporins. One of these resistant isolates which were obtained from farm animals was identified as serovar Sandiego. The other resistant isolates from poultry (12 isolates) were identified as Enteritidis (7 isolates), Typhimurium (3 isolates) and two isolates were nontypeable.

Table 1. Frequency of isolation of *Salmonella enterica* serotypes from poultry and farm animals

Salmonella serovars	Isolates from poultry	Isolates from farm animals
S.enterica ssp. enterica serovar Entritidis	33	1
S. enterica ssp. enterica serovar Typhimurium	14	0
S.enterica ssp. enterica serovar Infantis	1	0
S.enterica ssp. enterica serovar Colindal	2	0
S.enterica ssp. enterica serovar Sandiego	0	1
S. enterica ssp. enterica serovar Senftenberg	0	1
S.enterica ssp. enterica serovar Tsevie	0	4
S. enterica ssp. enterica serovar Texas	0	3
S. enterica ssp. enterica serovar Paratyphi B	0	1
S.enterica ssp. enterica serovar Dublin	0	1
S.enterica ssp. enterica serovar Saintpaul	0	1
Nontypeable	8	3

Table 2. Cephalosporin resistance patterns for *Salmonella* isolates

Resistance patterns	Number (%)
cefotaxime	9 (40.9)
cefexime, ceftazidime	2 (9.0)
cefpodoxime, cefotaxime, ceftazidime	3 (13.6)
cefotaxime, ceftazidime	3 (13.6)
cefpodoxime, cefotaxime	4 (18.1)
cefotaxime, cefexime, ceftazidime	1 (4.5)

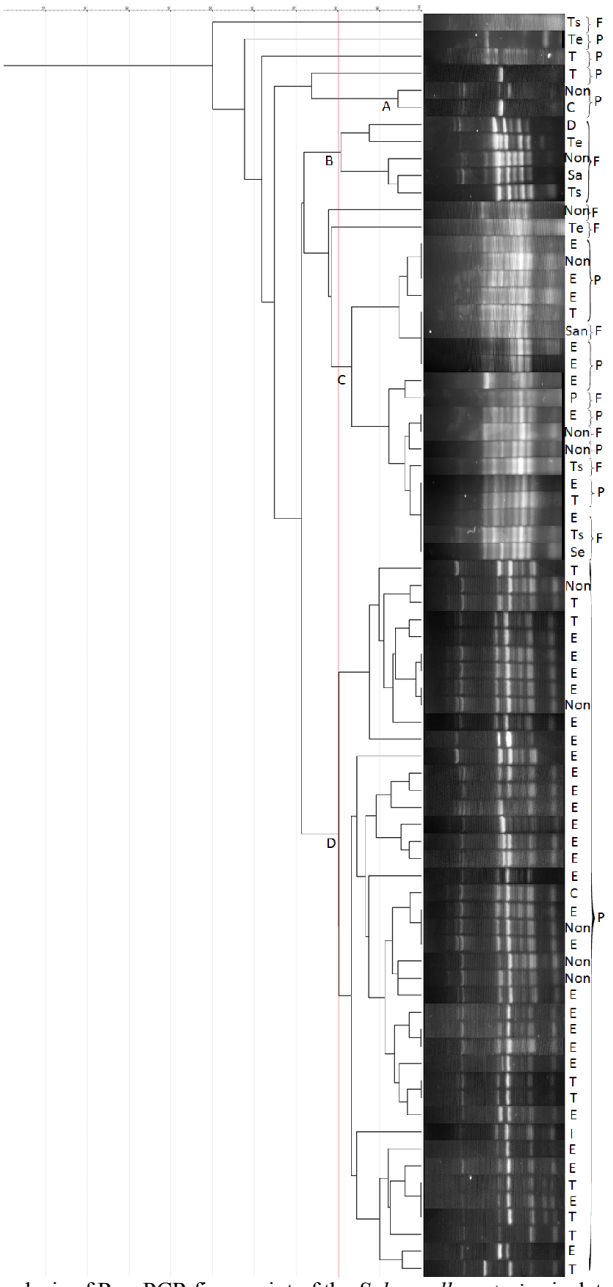

Fig. 1. Cluster analysis of Rep-PCR fingerprint of the *Salmonella enterica* isolates. E; Enteritidis; T; Typhymurium; Ts: Tsevie; Te: Texas; C: Colindal; I; Infantis; D: Dublin; San; Sandiego; Se; Senftenberg; P; Paratyphi B; Sa; Saintpaul; Non: Nontypeable. P: poultry isolates, F: farm animals isolates.

DISCUSSION

The incidence of cephalosporin resistant *Salmonella* from poultry and farm animals represents a serious threat to public health, complicating future programmes for treating human infections. In our study 22 from 74 *Salmonella* isolates were resistant to cephalosporin and all of these isolates were not ESBL producers. ESBL resistance has been reported for *Salmonella* isolates in many geographic areas (Szabó, 2014; Fernandes *et al.*, 2017; Yang *et al.*, 2017). However, resistance of *Salmonella* isolates to broad-spectrum cephalosporins remained low (Chon *et al.*, 2015; Lee *et al.*, 2016). In a study (Pokharel *et al.*, 2006), it was reported that 28 isolates of *Salmonella spp*. were cephalosporin resistant and only three of those were ESBL positive. Jazayeri Moghadas *et al.* (2009) reported only one ESBL positive *Salmonella* isolate, but Hamidian *et al.* (2009) documented three ESBL producing isolates. Tiong *et al.* (2010) reported that over 50% of 78 *Salmonella* isolates were cephalosporin resistant and only one was ESBL positive. In a Chinese study, the number of cephalosporin resistant isolates was 25 with three ESBL producers (Yu *et al.*, 2011).

Cephalosporins are the drug of choice for invasive *Salmonella* infections, thus, the incidence of cephalosporin-resistant isolates should emphasise the necessity of regular monitoring of the occurrence of ESBL-positive *Salmonella* strains.

In the present study, among the 22 *Salmonella* spp., nine isolates were resistant only to one cephalosporin and the others were resistant to more than one cephalosporin. From the six detected resistance profiles, resistance to cefotaxime was predominant and included nine isolates (6 *S.* Enteritidis and 3 *S.* Typhimurium). There was no ESBL positive among these cephalosporin resistant isolates using both confirmatory tests. These tests can accurately detect ESBL-producing bacteria.

The surveillance and subtyping of *Salmonella* serovars are necessary for epidemiological investigation of *Salmonella* outbreaks. In the current study, Rep-PCR has grouped the isolates into four clusters and six singletons. Dispersion of isolates in clusters according to their source showed that 72% of isolates from poultry were put into cluster D and 31% of isolates from farm animals were included in cluster B. The cluster C was contained 36.8% farm animal isolates and 63.1% of poultry isolates. As a result of our analyses, *Salmonella* isolates sharing similar serotypes can be distinguished by Rep primer. These results are in good agreement with prior reports indicating that Rep-PCR could discriminate *Salmonella* isolates of the same serotype via generating different profiles (Albufera *et al.*, 2009; Hyeon *et al.*, 2013; Hashemi & Baghbani-Arani, 2015).

Moreover, 73.5% of *Salmonella* Enteritidis and 71.4% of *S.* Typhimurium, being the commonest serovars among isolates, were classified into cluster D. On the other hand, it was showed that about 95% of resistant isolates were included in two clusters, C and D. All of these isolates except one were from poultry. Only one of 22 resistant isolates was known as a singleton. These results indicated the clonal relationship of cephalosporin resistant isolates. Rep-PCR showed a powerful method to discriminate isolates from different sources but different serovars were clustered together.

Albufera *et al.* (2009) observed that the Rep-PCR was able to differentiate human isolates from non-human isolates.

In addition, they reported that the Rep-PCR differentiated isolates with the same serovar in the same cluster. In the study conducted by Tiong *et al.* (2010), PFGE with 57 generated profiles for 78 *Salmonella* isolates, had more discrimination power than Rep-PCR that produced 35 profiles. In another report, 20 human and non-human *Salmonella* isolates were typed by using antimicrobial susceptibility profiling, Rep-PCR, PFGE and MLST, and Rep-PCR had higher diversity index (DI=0.81) than the other methods. However, the isolates number in this study was limited and therefore not relevant (Hyeon *et al.*, 2013). In a study by Ranieri *et al.* (2013) among five different typing methods, Rep-PCR has shown the highest diversity after PFGE, MLST and Ribotyping.

According to the results of Hashemi & Baghbani-Arani (2015) BOXAIR fingerprinting method produced4 9 patterns (DI =0.985), while REP generated 55 patterns (DI =0.991) and ERIC resulted in 48 fingerprints (DI= 0.983).

CONCLUSION

Cephalosporin-resistant *Salmonella* in this study could be attributed to the predominant serovars including Enteritidis and Typhimurium. Genetic relationship analysis using Rep-PCR successfully revealed the clonal relatedness of resistant isolates and differentiated the isolates on the basis of their origin. This technique could not discriminate different serotypes. However, due to public health risk of cross-contamination, it is important to have sufficient information on the occurrence of these resistant isolates.

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