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Research Article

MOLECULAR CHARACTERIZATION OF VIRULENCE AND ANTIBIOTICS RESISTANCE GENES AND GENETIC DIVERSITY OF *SALMONELLA ENTERITIDIS* FROM RAW CHICKEN MEAT IN DUHOK CITY, IRAQ

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ABSTRACT: *Salmonella enteritidis* (*S. enteritidis*) is considered a major cause of human salmonellosis and the infection is acquired through the consumption of contaminated meat and meat products with this pathogen. The objective of this study was to investigate the presence of virulence and resistance-associated genes in *S. enteritidis* isolates from local and imported raw chicken meat and to study their genetic diversity using enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) profile analysis. The results of the current study showed that the prevalence of virulence genes was high and the isolates harbored more than five class of virulence-associated genes and their frequency were different among the isolates. All the isolates showed 100% positivity for the *invite* and *hilA* genes. Genes including *lpfA*, *avrA*, *sopE2*, *spvB*, *sifA* and *flaK* were in 91%, 82%, 82%, 82%, 73% and 54.5% respectively. The *sipA* was only present in one isolate. The susceptibly test showed 100% sensitivity to chloramphenicol, norfloxacin, ceftriaxone, and cefotaxime and all the isolates were 100% resistant to ampicillin. However, two of the isolates showed resistance to gentamycin and trimethoprim with sulphamethoxazole. The isolates were found to harbor the *dfrA1* for trimethoprim, *blaTEM* for ampicillin, and the resistance rate was 100%. However, none of the *S. enteritidis* isolates were found to contain the resistance genes specific to fluoroquinolone, erythromycin, chloramphenicol, cefotaxime, and carbapenems (*NDM* and *OXA*) resistance. ERIC-PCR allowed the grouping of the isolates into 5 different ERIC patterns including ET1-ET5. It can be concluded that the detection of the high frequency of virulence genes and MDR suggests the high virulence potential of *S. enteritidis* isolates which could be of major public health concern.

Key words: *Salmonella enteritidis,* Virulence, Resistance, ERIC-PCR, Duhok.

INTRODUCTION

Salmonella species are the major food-borne pathogens associated with both typhoidal and non-typhoidal salmonellosis worldwide (Card *et al.* 2016, Crump *et al.* 2004). Human *Salmonella* infections are mostly associated with food of animal origin, such as chickens, eggs, meat, and dairy items (Steve Yan *et al.* 2004). *S. enteritidis* and *S. typhimurium* are responsible for the majority of human salmonellosis (EUSR 2014). In many countries, *S. enteritidis* emerged during the 1980s and it was a major cause of *Salmonella* outbreaks related to raw or undercooked chicken and eggs.

Nowadays, *S. enteritidis* remains the most confined serovar in African, Asian, European, and Latin American nations and the second common serovar in North America and Oceania, accounting for 43.5% of all *Salmonella* species (Hendriksen *et al.* 2011). Salmonellosis is characterized by diarrhea, fever, headache, abdominal pain, nausea, and vomiting (CDC 2007).

Chicken is considered a reservoir of *S. enteritidis* and studies have identified poultry and chicken products as the major source of disease in humans. *S. enteritidis* infection mostly occurs via the consumption of food such as contaminated chicken meat and eggs (Little *et al*. 2008, Patrick *et al*. 2004). In Iraq after 1991, the high incidence of typhoid fever is directly related to improper or poor sanitation and hygiene, consumption and utilization of polluted water, overcrowding, and social unrest. A significant number of people have been affected annually with a mortality rate of 10-20%, mainly resulting

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from inappropriate sewage disposal into the river bodies and limited access to fresh water. *Salmonella* spp. considered one of the most common causes of diarrhea in Iraq alongside *Shigella* spp., *Campylobacter,* and enterotoxigenic *Escherichia coli* (Abdulhaleem *et al*. 2019).

The advancement of food animal production particularly, broiler chicken production, is directly based on the use of antimicrobials as growth promoters (Costa *et al*. 2017). Antimicrobials are also utilized for treatment purposes in broiler chickens (Gadde *et al*. 2018). Globally, antimicrobial resistance is developed as a result of extended use of various types of antibiotics causing serious public health problems (Davison *et al*. 2000). Moreover, the acquisition of antibiotic resistance by pathogenic bacteria may lead to more severe infections (Heidary *et al*. 2014). Antibiotic resistance can occur via mobile genetic elements such as plasmids, transposons, integron, and insertion sequence (IS) components (Partridge *et al*. 2018). Due to their zoonotic nature, there are conceivable outcomes for the transmission of Multi-Drug-Resistance (MDR) Salmonella from chicken to humans through the food chain (Jajere 2019, Mthembu *et al*. 2019). In *Salmonella* spp. antibiotic resistance genes have been widely detected and various types of resistant genes have been investigated in previous studies (Alam *et al*. 2020, Jaja *et al*. 2019, Si *et al*. 2020). *S. enteritidis* pathogenicity has been related to genes encoding different virulence factors and these genes can be present also in genetic mobile elements such as transposons, plasmids, and bacteriophages (Van Asten and Van Dijk 2005). Virulence genes may involve in the pathogenesis through adherence to the host cell, resistance to antimicrobials, and overcoming the host defense mechanism (Van Asten and Van Dijk 2005). Virulence genes including *invA*, *hilA*, *sipA*, *sipD*, *avrA*, *sopA*, *sopB*, *sopD*, and *sopE2* are found in SPI-1 allowing *S. enteritidis* to attack phagocytic and non-phagocytic cells. The genes *ssaR* and *sifA*, are located in SPI-2, which allows the intracellular survival and replication of *Salmonella* spp. in the host cells. Other virulence genes, such as *flgK*, *fljB*, *and flgL*, encode flagella-associated proteins and have been shown to play an important role in the invasiveness of *S. enteritidis*. Furthermore, plasmid-associated *spv* (RABCD) contains five genes commonly associated with some serotypes, and one significant role of the *spv* operon is to potentiate the systemic spread of the pathogen (Andrews-Polymenis *et al*. 2010, Shah *et al*. 2011a). Genotyping approaches have been used for the typing of *S. enteritidis* strains such as Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), Agarose gel

Table 1. Details of oligonucleotide primers used for detection of virulence associated genes of *S. enteritidis* isolates.

Gene	Primer name	Functions	Sequence (5'—3´)	Size	References
lpfA	lpfA_F lpfA_R	Fimbria	CTTTCGCTGCTGAATCTGGT CAGTGTTAACAGAAACCAGT	250	(Baumler and Heffro 1995)
InvA	lpfA_R InvA-R	Invasion	ACAGTGCTCGTTTACGACCTGAAT AGACGACTGGTACTGATCGATAAT	284	(Bhatta *et al*. 2007)
hilA	hilA_F hilA_R	Invasion	CTGCCGCAGTGTTAAGGATA CTGTCGCCTTAATCGCATGT	497	(Guo *et al*. 2000)
avrA	avrA_F avrA_R	Effector protein	GTTATGGACGGAACGACATCGG ATTCTGCTTCCCGCCGCC	385	(Prager *et al*. 2003)
sipA	sipA_F sipA_R	Effector protein	ATGGTTACAAGTGTAAGGACTCAG ACGCTGCATGTGCAAGCCATC	2055	(Shah *et al*. 2011b)
sopE2	sopE2_F sopE2_R	Effector protein	TACTACCATCAGGAGG GAATGTTTTATGTGACGCAG	995	(Raffatellu *et al*. 2005)
sifA	sifA_F sifA_R	Effector protein	ATGCCGATTACTATAGGCAATGG TTATAAAAAACAACATAAACAGCCG	1011	(Hur *et al*. 2011)
flgK	flgK_F flgK_R	Flagella associated	ATGTCCAGCTTGATTAATCAC GCGAATATTCAATAACGCATC	1659	(Shah *etal*. 2011b)
spvB	spvB_F spvB_R	Plasmid-virulence	CGGTTATAGAAGAGCTCCTGT CCGGTATACGACTCTGTGATC	349	(Rychlík *et al*. 2008)

electrophoresis, and Pulsed-Field Gel Electrophoresis (PFGE). Moreover, the techniques have been shown to efficiently discriminate the *S. enteritidis* strain (Aktas *et al*. 2007, Rivoal *et al*. 2009). Due to an increase in the consumption of poultry meat of local and imported origin in the area, this study, therefore, aimed to evaluate the potential virulence of *S. enteritidis* isolates from local and imported chickens by screening the nine associated virulence genes with different functions. In addition, to characterize antibiotic resistance patterns by screening the 13 resistance genes from different antimicrobial groups and analyzing the genetic diversity of isolates using ERIC-PCR to determine the genetic relationship between the isolates from local and imported origin.

MATERIALS AND METHODS

Bacterial isolates

Salmonella enteritidis isolates (n=11) incorporated in this study were isolated from 250 raw chicken meat samples including 100 local chickens, 100 imported chickens, and 50 liver (25 local and 25 imported) in their previous study by Taib and Jakhsi (2019). These isolates were detected in imported chicken (n=2), local chicken meat (n=8), and liver from local chicken (n=1). The isolates were then characterized using phenotypic characterization based on colony morphologies on XLD agar and biochemical characters including urease negative, positive citrate unitization test, non-lactose fermenter, and positive H2S production on triple sugar iron (TSI) agar. The detection of *Salmonella* was confirmed using molecular methods based on the detection of specific *Salmonella* spp. *16S rRNA* gene and sequencing. Primer 6SF1 (F: TGTTGTGGTTAATAACC GCA) and primer 16SIII (R: CACAAATCCATCT CTGGA) were used to amplify the partial sequence of *16S rRNA* gene (572 bp) specific for the genus *Salmonella* according to the previously described method by (Nyabundi *et al.* 2017). Serotyping of the isolates was performed at Central Public Health Laboratory in Baghdad. The details of isolation and detection have been described by Taib and Jakhsi (2019). The bacterial isolates were stored in glycerol (50%) at -20^0 C.

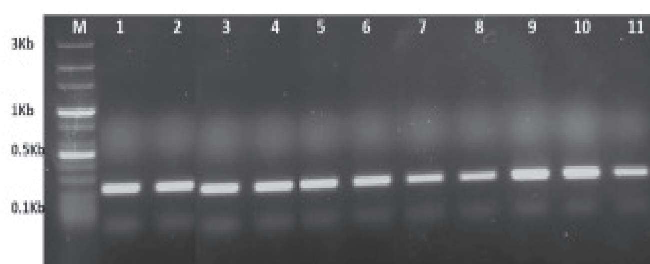

Fig. 1. Detection of *invA* gene (284 bp) in *S. enteritidis* isolates from raw chicken meat. Lanes 1-11 indicate the number of the corresponding strains; Lane M is DNA marker.

DNA preparation

Salmonella enteritidis isolates were recovered from frozen glycerol (50%) stocks and grown overnight in brain heart infusion broth (BHIB) at 37°C. A loopful of overnight broth culture was then streaked onto xylose lysine desoxycholate (XLD) and incubated at 37°C for 24 hr. DNA was extracted according to previously described methods (Abdulrahman 2021, Aranda *et al.* 2004). Briefly, a loopful of bacterial colonies was removed from XLD plates and resuspended 500 µl of deionized double distilled water. The suspension was mixed very well and boiled for 10 min. Cell debris was then removed by centrifugation at 13,000 xg for 1 min and the supernatant was used as the DNA template for PCR reactions.

Detection of *Salmonella enteritidis* virulence-associated genes

The confirmed *S. enteritidis* isolates were screened for the genes that play a key role in its virulence *invA*, *hilA*, *sipA*, *sopE2*, *sifA*, *flgK*, *spvB*, *lpfA*, and *avrA*. Individual PCR reactions using annealing temperatures of 60°C, 62°C, 53°C, 56°C, 52°C, 53°C, 60°C, 55°C, and 60°C were used, respectively. The details of primers including primer pairs used, product sizes, and references are described in Table1. A total reaction volume of 20 µl was used and each reaction consisted of a 10 µl ready-to-use master mix (Ruby Taq Master®, Jena Bioscience, Thuringia, Germany), *2* µl of each primer at a concentration of 10 pmol µl-1, 3 µl of the DNA template and 3 µl of dH2O. The amplification was performed in a GeneAmp PCR System 9700 Thermo Cycler (Applied Biosystems). PCR conditions were carried out according to the details mentioned in Table1. The PCR products were visualized on 1% agarose gel with Prime Safe Dye (GeNet Bio, Korea).

Antimicrobial susceptibility test of *S. enteritidis* isolates

The Kirby-Bauer-disk diffusion method was used to carry out sensitivity testing of *S. enteritidis* isolates. In this study, according to the Clinical and Laboratory Standards Institute (CLSI 2015), various types of antibiotics from different classes were selected: gentamicin (10 µg), amoxicillin (10 µg), trimethoprim/ sulphamethoxazole (25 µg), ampicillin (25 µg), chloramphenicol (30 µg), doxycycline (30 µg), oxytetracycline (30 µg), norfloxacin (10 µg), ciprofloxacin

(5 µg), cefotaxime (30 µg), ceftriaxone (30 µg) and cefpodoxime (10 µg). Briefly, pure colonies from overnight XLD cultures were selected and the suspension of 0.5 McFarland turbidity standards was prepared in 5 ml of autoclaved nutrient broth. Uniform lawn growth was prepared on Mueller-Hinton agar plates using a sterile cotton swab. Antibiotic discs were then applied and incubated at 37 °C for 24 h. the results were interpreted based on the size of the inhibition zone according to CLSI (CLSI 2015).

Detection of antibiotics resistance genes in *Salmonella enteritidis* isolates

The isolates were screened by individual PCR for the detection of the 13 resistance genes encoding resistance for different categories of antimicrobials. These genes include *aadA1* for streptomycin, *tetA* for tetracycline, *dfrA1* for trimethoprim, *qnrA* for fluoroquinolone, *aac(3)-IV* for gentamicin, *sul1* for sulfonamide, *ereA* for erythromycin, *cmlA* for chloramphenicol, *blaTEM* for ampicillin,*blaSHV* for cephalothin, *blaCTX-M* for cefotaxime, and *blaNDM*, *blaOXA*for carbapenems resistance. The details of genes and the primers targeting the antimicrobial resistance genes are presented in Table 2.

Genetic diversity using ERIC-PCR based method

ERIC-PCR was performed on *S. enteritidis* isolates the primer ERIC-PCR (ERIC1R: 5'ATGTAAGCTCCT GGGGATTCAC-3', ERIC2: 5'-AAGTAAGTGACTGG GGTGAGCG-3') (Versalovic *et al.* 1991). The reactions were performed according to Bakhshi *et al.* (2018). A total reaction of 25 µL was used and each reaction consisted of 12.5 µl ready-to-use master mixes (Ruby Taq Master®, Jena Bioscience, Thuringia, Germany), 2 µl of each primer at the concentration of 10 pmol µl-1, 5 µl of the DNA template and 3.5 µl of dH2O. The amplification was performed in a GeneAmp PCR System

Table 2. Details of oligonucleotides primers used for detection of antibiotic resistance genes of *S. enteritidis* isolates.

Antimicrobial Group	Antibiotic Resistance	Resistance Gene	Primer Sequence	Size	References
Aminoglycosides	Streptomycin	*aadA1*	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	447	(Randall *et al*. 2004)
Aminoglycosides	Gentamycin	*aac(3)-IV*	CTTCAGGATGGCAAGTTGGT TCATCTCGTTCTCCGCTCAT	286	(Van *et al*. 2008)
Tetracyclines	Tetracycline	*tetA*	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	577	(Randall *et al*. 2004)
Folate pathway inhibitors	Trimethoprim	dfrA1	GGAGTGCCAAAGGTGAACAGC GAGGCGAAGTCTTGGGTAAAAAC	367	(Van *et al*. 2008)
Quinolones	Fluoroquinolones	*qnrA*	TCAGCAAGAGGATTTCTCA GGCAGCACTATTACTCCCA	627	(Wang *et al*. 2006)
Sulfonamides	Sulphamethoxazole	*sull*	TTCGGCATTCTGAATCTCAC ATGATCTAACCCTCGGTCTC	822	(Van *et al*. 2008)
Macrolide	Erythromycin	*ereA*	GCCGGTGCTCATGAACTTGAG CGACTCTATTCGATCAGAGGC	419	(Van *et al*. 2008)
Phenicols	Chloramphenicol	*cmlA*	CCGCCACGGTGTTGTTGTTATC CACCTTGCCTGCCCATCATTAG	698	(Van *et al*. 2008)
Beta lactam	Ampicillin	*TEM*	TCCGCTCATGAGACAATAACC ATAATACCGCACCACATAGCAG	296	(Doosti *et al*. 2015)
Beta lactam	Cephalothin	*SHV*	TACCATGAGCGATAACAGCG GATTTGCTGATTTCGCTCGG	451	(Doosti *et al*. 2015)
Beta lactam	Cefotaxime	*CTX-M*	TCTTCCAGAATAAGGAATCCC CCGTTTCCGCTATTACAAAC	909	(Stürenburg *et al*. 2004)
Beta lactam	Carbapenems	*OXA*	GCAGCGCCAGTGCATCAAC CCGCATCAAATGCCATAAGTG	198	(Van *et al*. 2008)
Beta lactam	Carbapenems	*NDM*	CTTCCAACGGTTTGATCGTC TTGGCATAAGTCGCAATCC	280	(Rathinasabapathi *et al*. 2015)

9700 Thermo Cycler (Applied Biosystems). PCR reactions were performed using the following conditions: initial denaturation of 94 °C for 5 min and 35 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 5 min followed by a final extension of 72 °C for 10 min. The PCR products were visualized on 1% agarose gel with Prime Safe Dye (GeNet Bio, Korea) for 70 V for 10 min and later 80 V for 1 hr. ERIC-PCR results were analyzed using the GelJ gel (v. 2.0) analysis package. The dendrogram was constructed with the Unweighted pair group method with arithmetic averages (UMGMA) method using the DICE similarity coefficient and a position tolerance of 1.0 (Heras *et al*. 2015).

RESULTS AND DISCUSSION

Virulence-associated genes in *S. enteritidis* isolate

The isolates used in this study were examined for the presence of a variety of virulence genes associated with the pathogenesis of *S. enteritidis.* The results showed that the isolates harbored at least more than five class of virulence-associated genes and their frequency were different among the isolates (Table 3). All of the *S. enteritidis* isolates (11/11) were found to harbor the *invA* (Fig. 1) and *hilA* genes. Both genes were reported in the genus *Salmonella* and almost 100% of the *S. enteritidis* were isolated globally. This could be due to their importance in cell invasion and therefore, can be used as target genes used for the detection of *Salmonella* species (Borges *et al*. 2013, Campioni *et al*. 2012, Siddiky *et al*. 2021). The fimbrial gene *lpfA* which involves *Salmonella* pathogenicity through promoting bacterial binding to the intestinal epithelium was detected in 10 isolates. The high frequency of *lpfA* has been reported in *S. enteritidis* previously at the rate of 100% (Siddiky *et al*. 2021), 99% (83/84) (Borges *et al*. 2013), and also in different serovars (Borsoi *et al*. 2009, Mendonça *et al.* 2020, Webber *et al.* 2019). The *avrA* and *sopE2* were determined in 82% (9/11) of the isolates. This is in agreement with previous studies in which both genes were found a high frequency of isolates (100% and 99%) (Borges *et al.* 2013, Hopkins and Threlfall 2004, Siddiky *et al.* 2021). However, the low frequency of *avrA* and *sopE2* in *S. enteritidis* was also recorded previously by Liu *et al*. (2012). Variation in the occurrence of *arvA* and *sopE2* may be due to the recombination that occurs commonly in the location of these genes (Hopkins and Threlfall 2004). Besides, the study suggested that the high rate of *avrA* gene was present in serovars that mostly cause salmonellosis (Ben-Barak *et al*. 2006). The *spvB* is another virulence gene of the genus *Salmonella* which is associated with the virulence plasmid and is responsible for the maintenance and bacterial survival within the cell (Webber *et al*. 2019). Gene *spvB* was found in 82% (9/11) of the *S. enteritidis* isolates in this study. However, *S. heidelberg* was found at a low rate and was detected only in 0.79% of the isolates. It has been shown that the *spvB* is more common in *S. enteritidis*. On the other hand, *spvB* was not detected in both serovars including *S. enteritidis* and *S. typhimurium* isolated from chicken meat (Tarabees *et*

Table 3. Antimicrobial sensitivity test of *S. enteritidis* (n=11) isolates.

Antibiotics	Antimicrobial susceptibility profile					
	Sensetive		Intermediate		Resistant	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Gentamycin (CN)	9	82	0	0	2	18
Amoxicillin (AX)	4	36	3	27	4	36
Trimethoprim/sulphamethoxazole (SXT)	9	82	0	0	2	18
Ampicillin (AMP)	0	0	0	0	11	100
Chloramphenicol (C)	11	100	0	0	0	0
Doxycycline (DO)	9	82	0	0	2	18
Oxytetracycline	6	55	0	0	5	45
Norfloxacin (NOR)	11	100	0	0	0	0
Ciprofloxacin (CIP)	2	18	6	55	3	27
Ceftriaxone (CRO)	11	100	0	0	0	0
Cefotaxime (CTX)	11	100	0	0	0	0
Cefpodoxime	9	82	0	0	2	18

Table 4. Resistance genes in *S. enteritidis* (n=11) isolates.

Sample No.	Resistance genes													R Patterns
	aadA1	*tetA*	*dfrA1*	*NDM*	*qnrA*	*aac(3)-IV*	*sull*	*ereA*	*cmlA*	*TEM*	*SHV*	*CTX-M*	*Oxa*	
S1	-	-	+	-	-	-	+	-	-	+	-	-	-	I
S2	-	-	+	-	-	-	+	-	-	+	-	-	-	I
S3	-	+	+	-	-	+	-	-	-	+	+	-	-	IIa
S4	-	+	+	-	-	+	-	-	-	+	-	-	-	IIb
S5	-	+	+	-	-	+	-	-	-	+	-	-	-	IIb
S6	+	+	+	-	-	-	-	-	-	+	-	-	-	IIIa
S7	+	-	+	-	-	-	-	-	-	+	-	-	-	IIIb
S8	+	-	+	-	-	-	-	-	-	+	-	-	-	IIIb
S9	-	-	+	-	-	-	-	-	-	+	-	-	-	IV
S10	-	-	+	-	-	-	-	-	-	+	-	-	-	IV
S11	-	-	+	-	-	-	-	-	-	+	-	-	-	IV
No. of resistant strains	3	4	11	0	0	3	0	0	0	11	1	0	0	

al. 2017). The *sifA* found in SPI-2 allows *Salmonella* species to survive and replicate in the host cells and it was present in73% (8/11) of the isolates included in the current study. Similar results have been recorded by other studies and they found that all the *S. enteritidis* isolates possessed the *sifA* (Campioni *et al.* 2012, Tarabees *et al.* 2017). The *flgK* gene was found in 54.5% (6/11) of the isolates and the *sipA* was only present in one of the isolates. However, a higher frequency of both genes has been detected in *S. enteritidis* (Campioni *et al.* 2012). None of the isolates were found to be negative for all the examined virulence genes. Similar observations were also reported previously in many countries, worldwide. Variations in the frequency

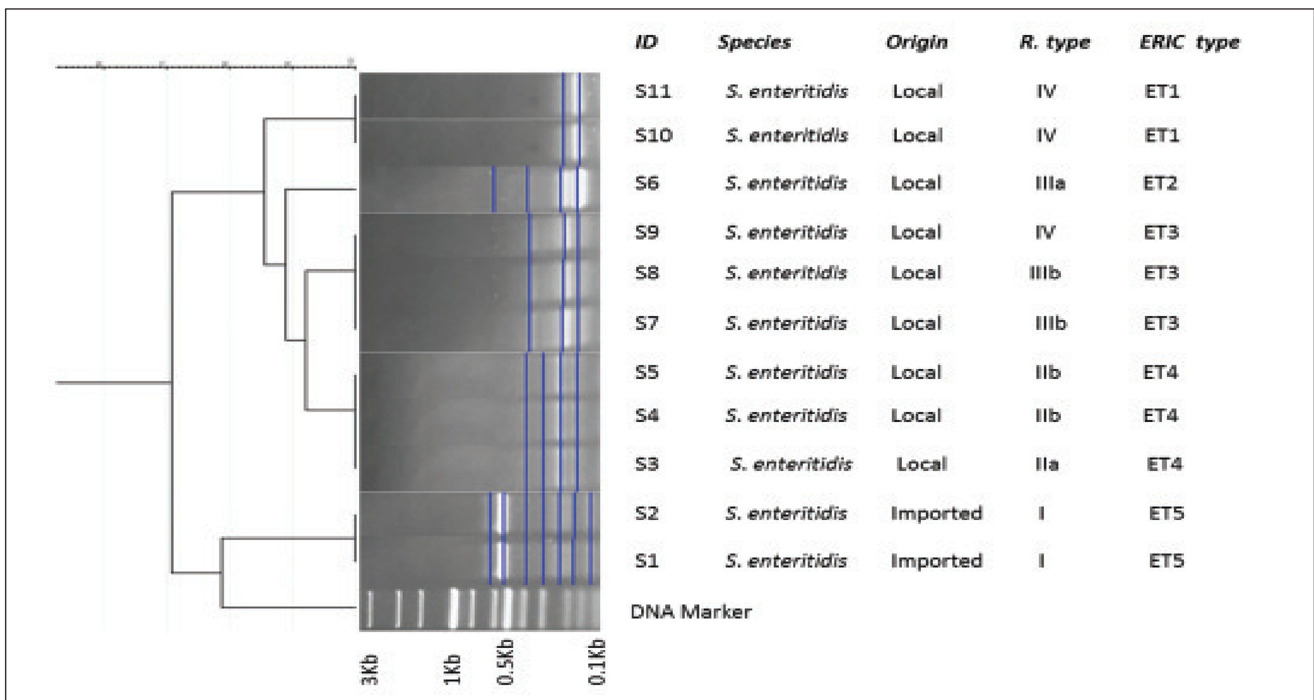

Fig. 2. Dendrogram generated from ERIC-PCR banding pattern of eleven *S. enteritidis* isolates from raw chicken of local and imported origin. [The similarity analysis was performed using Dice coefficient and UPGMA method in GelJ software (v.2.0)]

of virulence-associated genes in *S. enteritidis* isolates from the chicken were also reported (Borges *et al*. 2013). Likewise, almost all strains (97.6%) harbored all 13 virulence genes investigated in *S. enteritidis* isolated from food and humans (Campioni *et al*. 2012). Similar results have been mentioned in *Salmonella heidelberg* isolated from chicken carcasses (Webber *et al*. 2019). It has also been shown that 86.1% of *Salmonella* strain isolates from retail duck meat carried seven classes of virulence-associated genes (Chen *et al*. 2020). Overall, the results suggest the pathogenic potential of *S. enteritidis* isolates from local and imported chicken meat because they were found at high frequency and each isolate harbored at least five of the genes essential for the pathogenesis of this pathogen. Therefore, hygienic precautions should be taken regarding the processing and storage of meat to prevent infection in humans.

Antimicrobial susceptibility testing

The antimicrobial resistance profiles of the isolates to 12 antimicrobial agents using the disc diffusion technique are presented in Table 3. The isolates showed variations in the antibiotics used in this study. The 11 isolates were 100% sensitive to chloramphenicol, norfloxacin, ceftriaxone, and cefotaxime. Nine isolates (82%) were sensitive to gentamycin, trimethoprim/ sulphamethoxazole, doxycycline, and cefpodoxime. Six isolates were sensitive to oxytetracycline. On the other hand, all 11 isolates were 100% resistant to ampicillin. However, two of the isolates showed resistance to the following antibiotic including gentamycin, trimethoprim/sulphamethoxazole, doxycycline, and cefpodoxime. The isolates showed resistance to ciprofloxacin, amoxicillin, and oxytetracycline at the rate of 27%, 36%, and 36%, respectively.

Antibiotic susceptibility testing has been performed to examine if there is an increase in the number of resistant *Salmonella-causing* food-borne diseases and has been also used as typing method (Oliveira *et al*. 2007). Antibiotic resistance in *S. enteritidis* has been considered low when compared to *S. typhimurium* isolates. Therefore, attention should be given to the case of repeated isolation of *S. enteritidis*, especially multi-resistant strains (Oliveira *et al*. 2007). The antimicrobial susceptibly profiles of the isolates using the disc diffusion technique showed variation toward the 12 antibiotics used in this study. Several studies have been conducted to investigate the susceptibility of a variety of antibiotics and results also showed variation in antibiotic sensitivity (Bhatta *et al*. 2007, Guo *et al*. 2000, Mendonça *et al*. 2020). According to the susceptibility test, 4 isolates were classified as multi-drug resistant (MDR) strains because they were found resistant to at least four antibiotics used in this study. The results were in agreement with the previous study (Bhatta *et al*. 2007, Siddiky *et al*. 2021).

Resistance-associated genes in *S. enteritidis*

To determine the presence of 13 resistance genes used in the current study, *S. enteritidis* isolates were subjected to PCR using individual reactions for each gene. The results were presented in Table 4. Overall, all isolates were found to harbor the *dfrA1* for trimethoprim, *blaTEM* for ampicillin with a 100% resistance rate. Only one isolate was found to contain the resistance gene *blaSHV* specific for cephalothin. The results showed the presence of genes encoding *sul1* specific for sulfonamide in two isolates and *aadA1* specific for streptomycin and *aac(3)-IV* for gentamicin in three isolates, while four isolates were found to harbor *tetA* gene specific for tetracycline resistance. On the other hand, none of the *S. enteritidis* isolates were found to contain the resistant genes specific to fluoroquinolone, erythromycin, chloramphenicol cefotaxime, and carbapenems (*NDM* and *OXA*) resistance. Few isolates showed the presence of four or more resistance genes and they were considered MDR isolates and the resistance profile were different among the isolates. Three isolates showed the presence of resistance genes specific to tetracycline, trimethoprim, gentamicin, and ampicillin. However, the other three isolates shared identical resistance profiles as they were found positive for the resistance genes specific to streptomycin, trimethoprim, and ampicillin. The results determined 4 different resistance patterns including types I, II (IIa and IIb), III (IIIa and IIIb), and IV as shown in Table 4. However, high chloramphenicol resistance (33.3%) was found in the previous study (Si *et al*. 2020), which may be due to previous long-term and frequent use of chloramphenicol in the broiler industry or the vertical transmission of genetic elements (Sin *et al*. 2020). In the current study, variation was detected in the occurrence of genes encoding resistance in this pathogen. Similar results were also been recorded previously (Alam *et al*. 2020, Jaja *et al*. 2019, Si *et al.* 2020). Few isolates (local isolates) showed the presence of four or more resistance genes and they were considered MDR isolates and the resistance profile were different among these isolates. Three isolates showed the presence of resistance genes specific to tetracycline, trimethoprim, gentamicin, and ampicillin. However, the other three isolates shared identical resistance profiles as they were found positive for the resistance genes specific to streptomycin, trimethoprim, and ampicillin. The results determined 4 different resistance patterns including types

I, II (IIa and IIb), and III (IIIa and IIIb). Similarly, it has been shown that 97.1% to 77.1% of the isolated *Salmonella* showed resistance against commonly used antibiotics including tetracycline, ampicillin, streptomycin, and chloramphenicol (Alam *et al*. 2020). The high prevalence of multidrug-resistant (MDR) *S. enterica* isolates was also determined in another study (Jaja *et al.* 2019). Detection of MDR *S. enteritidis* in raw chicken meat in this study could be of great public health concern as it may transmit to humans through the consumption of chicken meat (local and imported). It has been mentioned that several factors including strong selective pressure resulting from the indiscriminate use of antibiotics may cause the development of antibiotic resistance in *Salmonella* species (Alam *et al*. 2020) along with horizontal gene transfer.

Genetic diversity using ERIC-PCR analysis

Genotyping methods *e.g.* ERIC-PCR, agarose gel electrophoresis, PFGE have been used widely for the study of genetic diversity and/or for the typing of bacteria including *Salmonella* species. Moreover, the techniques have been shown to efficiently discriminate *S. enteritidis* strains (Aktas *et al*. 2007, Rivoal *et al*. 2009). ERIC-PCR fingerprinting was used to generate a genotypic profile and phylogenetic analysis of *S. enteritidis* isolates as shown in Fig. 2. The results of ERIC-PCR identified five different ERIC patterns among the isolates which were classified into 5 ET groups including ET1-ET5 (Fig. 2). The ERIC-PCR banding patterns have indicated 2 to 7 bands ranging from 600 bp to 150 bp. The similarity ranged from 80% to 100%. The results showed the correlation between ERIC and resistance types (Fig. 2 and Table 4). The results demonstrated a low level of genetic diversity. It has been revealed that *S. enteritidis* isolates showed low levels of genetic diversity in different studies because the isolates were frequently grouped in one or few fingerprinting patterns (Oliveira *et al*. 2007). There was a correlation between ERIC and resistance type. For example isolates from imported chicken were identical and shared the same resistance profile and ERIC types indicating that those may come from the same origin. The *S.* Enteritidis isolates recovered from the local raw chicken meat also shared identical and/ or similar resistance patterns and ERIC types. Different ERIC and PEGE types were also identified among the *S. enteritidis* as mentioned in the previous study by Campioni *et al.* (2012). However, most of the studies were focused on the study of virulence and resistance genes without comparing their genetic diversity using ERIC-PCR. Therefore, in the future large number of isolates from different food sources and clinical samples need to be included to study their genetic correlation in more detail.

CONCLUSION

Overall, the results suggest the virulence potential of *S. enteritidis* isolates from local and imported chicken meat because at least five of the virulence genes essential for the pathogenesis were found in each of the isolates. The detection of pathogenic MDR *S. enteritidis* could be of great public health concern. Consequently, food safety programs and avoidance of random use of antibiotics are needed to decrease the human health risk associated with salmonellosis and to reduce the development of antimicrobial resistance in this pathogen. In the future, *S. enteritidis* from clinical and food sources should be examined using genotyping methods to analyze the relationship between isolates from different sources in Duhok city.

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REFERENCES

Abdulhaleem N, Garba B, Younis H, Mahmuda A, Hamat RA *et al.* (2019) Current trend on the economic and public health significance of salmonellosis in Iraq. Adv. Anim Vet Sci 7: 484-491.

Abdulrahman RF (2021) Virulence potential, antimicrobial susceptibility and phylogenetic analysis of *Corynebacterium pseudotuberculosis* isolated from caseous lymphadenitis in sheep and goats in Duhok city, Iraq. Adv Anim Vet Sci 9(6): 919-925.

Aktas Z, Day M, Kayacan CB, Diren S, Threlfall EJ (2007) Molecular characterization of *Salmonella typhimurium* and *Salmonella enteritidis* by plasmid analysis and pulsed-field gel electrophoresis. Int J Antimicrob Agents 30: 541-545.

Alam SB, Mahmud M, Akter R, Hasan M, Sobur A *et al.* (2020) Molecular detection of multidrug resistant *Salmonella* species isolated from broiler farm in Bangladesh. Pathogens 9: 1-12.

Andrews-Polymenis HL, Bäumler AJ, McCormick BA, Fang

FC (2010) Taming the elephant: *Salmonella* biology, pathogenesis, and prevention. Infect Immun 78: 2356-2369.

Aranda KRS, Fagundes-Neto U, Scaletsky ICA (2004) Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic *Escherichia coli* and *Shigella spp*. J Clin Microbiol 42: 5849-5853.

Bakhshi B, Afshari N, Fallah F (2018) Enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis as a reliable evidence for suspected *Shigella* spp. outbreaks. Brazilian J Microbiol 49: 529-533.

Baumler AJ, Heffron F (1995) Identification and sequence analysis of lpfABCDE, a putative fimbrial operon of *Salmonella typhimurium*. J Bacteriol 177: 2087-2097.

Ben-Barak Z, Streckel W, Yarona S, Cohenc S, Prager R, Tschape H (2006) The expression of the virulence-associated effector protein gene *avrA* is dependent on a *Salmonella enterica*-specific regulatory function. Int J Med Microbiol 296: 25-38.

Bhatta DR, Bangtrakulnonth A, Tishyadhigama P, Saroj SD, Bandekar JR *et al.* (2007) Serotyping, PCR, phage-typing and antibiotic sensitivity testing of *Salmonella* serovars isolated from urban drinking water supply systems of Nepal. Lett Appl Microbiol 44: 588-594.

Borges KA, Furian TQ, Borsoi A, Moraes HLS *et al*. (2013) Detection of virulence-associated genes in *Salmonella enteritidis* isolates from chicken in south of Brazil. Pesqui Vet Bras 33: 1416-1422.

Borsoi A, Santin E, Santos LR, Salle CTP, Moraes HLS, Nascimento VP (2009) Inoculation of newly hatched broiler chicks with two Brazilian isolates of *Salmonella heidelberg* strains with different virulence gene profiles, antimicrobial resistance, and pulsed field gel electrophoresis patterns to intestinal changes evaluation. Poult Sci 88: 750-758.

Campioni F, Moratto Bergamini AM, Falcão JP (2012) Genetic diversity, virulence genes and antimicrobial resistance of *Salmonella enteritidis* isolated from food and humans over a 24-year period in Brazil. Food Microbiol 32: 254-264.

Card R, Vaughan K, Bagnall M, Spiropoulos J, Cooley W *et al*. (2016) Virulence characterisation of *Salmonella enterica* isolates of differing antimicrobial resistance recovered from UK livestock and imported meat samples. Front Microbiol 7.

Centers for Disease Control and Prevention (CDC) (2007) *Salmonella* serotype *enteritidis* infections among workers producing poultry vaccine-Maine, November-December 2006. MMWR Morb Mortal Wkly Rep 31: 877-879.

Chen Z, Bai J, Wang S, Zhang X, Zhan Z *et al.* (2020) Prevalence, antimicrobial resistance, virulence genes and genetic diversity of *Salmonella* isolated from retail duck meat in southern China. Microorganisms 8: 1-12.

CLSI (2015) Informational supplement susceptibility testing; Twentyfifth performance standards for antimicrobial, in: Clinical and Laboratory Standards Institute. Wayne, PA, USA.

Costa MC, Bessegatto JA, Alfieri AA, Weese JS, Filho JAB, Oba A (2017) Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. PLoS One 12: e0171642.

Crump JA, Luby SP, Mintz ED (2004) The global burden of typhoid fever. Bull World Health Organ 82: 346-353.

Davison HC, Woolhouse MEJ, Low JC (2000) What is antibiotic resistance and how can we measure it? Trends Microbiol 8: 554-559.

Doosti A, Pourabbas M, Arshi A, Chehelgerdi M, Kabiri H (2015) TEM and SHV genes in *Klebsiella pneumoniae* isolated from cockroaches and their antimicrobial resistance pattern. Osong Public Heal Res Perspect 6: 3-8.

Gadde UD, Oh S, Lillehoj HS, Lillehoj EP (2018) Antibiotic growth promoters virginiamycin and bacitracin methylene disalicylate alter the chicken intestinal metabolome. Sci Rep 8(1): 3592. DOI: 10.1038/s41598-018-22004-6.

Guo X, Chen J, Beuchat LR, Brackett RE (2000) PCR detection of *Salmonella enterica* serotype Montevideo in and on raw tomatoes using primers derived from hilA. Appl Environ Microbiol 66: 5248-5252.

Heidary M, Momtaz H, Madani M (2014) Characterization of diarrheagenic antimicrobial resistant *Escherichia coli* isolated from pediatric patients in Tehran, Iran. Iran Red Crescent Med J 16: e12329.

Hendriksen RS, Vieira AR, Karlsmose S, Lo Fo Wong DMA *et al*. (2011) Global monitoring of *Salmonella* serovar distribution from the world health organization global foodborne infections network country data bank: Results of quality assured laboratories from 2001 to 2007. Foodborne Pathog Dis 8(8): 887-900. DOI: 10.1089/fpd.2010.0787.

Heras J, Domínguez C, Mata E, Pascual V, Lozano C *et al.* (2015) GelJ - a tool for analyzing DNA fingerprint gel images. BMC Bioinformatics 16: 1-8.

Hopkins EJ, Threlfall KL (2004) Frequency and polymorphism

of sopE in isolates of *Salmonella enterica* belonging to the ten most prevalent serovars in England and Wales. J Med Microbiol 53: 539-543.

Hur J, Choi YY, Park JH, Jeon BW, Lee HS *et al*. (2011) Antimicrobial resistance, virulence-associated genes, and pulsed-field gel electrophoresis profiles of *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolated from piglets with diarrhea in Korea. Can J Vet Res 75: 49-56.

Jaja IF, Bhembe NL, Green E, Oguttu J, Muchenje V (2019) Molecular characterisation of antibiotic-resistant *Salmonella enterica* isolates recovered from meat in South Africa. Acta Trop 190: 129-136.

Jajere SM (2019) A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and adaptation and antimicrobial resistance including multidrug resistance. Vet World 12: 504-521.

Little CL, Rhoades JR, Hucklesby L, Greenwood M, Surman-Lee S *et al*. (2008) Survey of *Salmonella* contamination of raw shell eggs used in food service premises in the United Kingdom, 2005 through 2006. J Food Prot 71: 19-26.

Liu Y, Yang Y, Liao X, Li L, Lei C *et al*. (2012) Antimicrobial resistance, resistance genes and virulence genes in *Salmonella* isolates from chicken. J Anim Vet Adv 11: 4423-4427.

Mendonça EP, Melo RT, Oliveira MRM, Monteiro GP, Peres PABM *et al.* (2020) Characteristics of virulence, resistance and genetic diversity of strains of *Salmonella infantis* isolated from broiler chicken in Brazil. Pesqui. Vet Bras 40: 29-38.

Mthembu TP, Zishiri OT, El Zowalaty ME (2019) Molecular detection of multidrug-resistant *Salmonella* isolated from livestock production systems in South Africa. Infect Drug Resist 14: 3537-3548.

Nyabundi D, Onkoba N, Kimathi R, Nyachieo A, Juma G *et al*. (2017) Molecular characterization and antibiotic resistance profiles of *Salmonella* isolated from fecal matter of domestic animals and animal products in Nairobi. Trop Dis Travel Med Vaccines 3: 1-7.

Oliveira FA, de Frazzon APG, Brandelli A, Tondo EC (2007) Use of PCR-ribotyping, RAPD, and antimicrobial resistance for typing of *Salmonella enteritidis* involved in food-borne outbreaks in Southern Brazil. J Infect DevCont 1: 170-176.

Partridge SR, Kwong SM, Firth N, Jensen SO (2018) Mobile genetic elements associated with antimicrobial resistance. Clini Microbiol Rev 31: e00088-17.

Patrick ME, Adcock PM, Gomez TM, Altekruse SF, Holland BH *et al*. (2004) *Salmonella enteritidis* infections, United States, 1985-1999. Emerg Infect Dis 10(1):1-7. DOI: 10.3201/eid1001.020572.

Prager R, Rabsch W, Streckel W, Voigt W, Tietze E, Tschäpe H (2003) Molecular properties of *Salmonella enterica* serotype Paratyphi B distinguish between its systemic and its enteric pathovars. J Clin Microbiol 41: 4270-4278.

Raffatellu M, Wilson RP, Chessa D, Andrews-Polymenis H *et al*. (2005) SipA, SopA, SopB, SopD, and SopE2 contribute to *Salmonella enterica* serotype Typhimurium invasion of epithelial cells. Infect Immun 73: 146-154.

Randall LP, Cooles SW, Osborn,MK, Piddock LJV, Woodward MJ (2004) Antibiotic resistance genes, integrons and multiple antibiotic resistance in thirty-five serotypes of *Salmonella enterica* isolated from humans and animals in the UK. J Antimicrob Chemother 53: 208-216.

Rathinasabapathi P, Hiremath DS, Arunraj R, Parani M (2015) Molecular detection of New Delhi metallo-beta-lactamase-1 (NDM-1) positive bacteria from environmental and drinking water samples by loop mediated isothermal amplification of bla NDM-1. Indian J Microbiol 55: 400-405.

Rivoal K, Protais J, Quéguiner S, Boscher E, Chidaine B *et al*. (2009) Use of pulsed-field gel electrophoresis to characterize the heterogeneity and clonality of *Salmonella* serotype Enteritidis, Typhimurium and Infantis isolates obtained from whole liquid eggs. Int J Food Microbiol 192: 180-186.

Rychlík I, Hradecka H, Malcova M (2008) *Salmonella enterica* serovar Typhimurium typing by prophage-specific PCR. Microbiology 154: 1384-1389.

Shah DH, Zhou X, Addwebi T, Davis MA, Call DR (2011a) *In vitro* and *in vivo* pathogenicity of *Salmonella enteritidis* clinical strains isolated from North America. Arch Microbiol 19: :811-821.

Shah DH, Zhou X, Addwebi T, Davis MA, Orfe L *et al*. (2011b) Cell invasion of poultry-associated *Salmonella enterica serovar enteritidis* isolates is associated with pathogenicity, motility and proteins secreted by the type III secretion system. Microbiology 157: 1428-1445.

Siddiky NA, Sarker MS, Khan MSR, Begum R, Kabir ME *et al*. (2021) Virulence and antimicrobial resistance profiles of *Salmonella enterica* serovars isolated from chicken at wet markets in Dhaka, Bangladesh. Microorganisms 9: 952.

Si M, Yoon S, Kim YB, Noh EB, Seo KW, Lee YJ (2020) Molecular characteristics of antimicrobial resistance

determinants and integrons in *Salmonella* isolated from chicken meat in Korea. J Appl Poul Res 29: 502-514.

Steve Yan S, Pendrak ML, Abela-Ridder B, Punderson JW, Fedorko DP, Foley SL (2004) An overview of *Salmonella* typing: Public health perspectives. Clin Appl Immunol Rev 4: 189-204.

Stürenburg E, Lang M, Horstkotte MA, Laufs R, Mack D (2004) Evaluation of the MicroScan ESBL plus confirmation panel for detection of extended-spectrum β-lactamases in clinical isolates of oxyimino-cephalosporin-resistant Gram-negative bacteria. J Antimicrob Chemother 54: 870-875.

Taib GA, Jakhsi NSA (2019) Isolation and identification of *Salmonella* from whole chicken samples by conventional culture and molecular based methods. Bas J Vet Res 18: 148-157.

Tarabees R, Elsayed MSA, Shawish R, Basiouni S, Shehata AA (2017) Isolation and characterization of *Salmonella enteritidis* and *Salmonella typhimurium* from chicken meat in Egypt. J Infect Dev Ctries 11: 314-319.

The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA J 2014/12(2): 3547.

Van Asten AJAM, Van Dijk JE (2005) Distribution of "classic" virulence factors among *Salmonella* spp. FEMS Immunol Med Microbiol 44: 251-259.

Van TTH, Chin J, Chapman T, Tran LT, Coloe PJ (2008) Safety of raw meat and shellfish in Vietnam: An analysis of *Escherichia coli* isolations for antibiotic resistance and virulence genes. Int J Food Microbiol 124: 217-223.

Versalovic J, Koeuth T, Lupski R (1991) Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. Nucleic Acids Res 19: 6823-6831.

Wang MG, Tran JH, Jacoby GA, Zhang YY, Wang F, Hoopner DC (2006) Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli*. Chinese J Infect Chemother 6: 217-221.

Webber B, Borges KA, Furian TQ, Rizzo NN, Tondo EC *et al*. (2019) Detection of virulence genes in *Salmonella heidelberg* isolated from chicken carcasses. Rev Inst Med Trop Sao Paulo 61: 1-7.

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