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ORIGINAL ARTICLE

Detection of Multidrug Resistance *Salmonella* spp. from Chicken Meat by Multiplex PCR and VITEK 2 system

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ABSTRACT

Salmonellae are highly pathogenic foodborne bacteria able to cause infection even at low doses. Infection by *Salmonella* from contaminated foods leads to gastrointestinal disease known as salmonellosis. Raw chicken can be a source of human infection if the meat products are not properly handled, stored or cooked. The aim of the present study was to investigate the prevalence of *Salmonella* Enteritidis and *Salmonella* Typhymurium serovars in retail raw chicken meat from retail market in Erbil, as well as, to assess their antimicrobial resistance.

A total of 100 raw retail chicken meat samples were collected and plating on Xylose Lysine Deoxycholate and Salmonella-Shigella agar media. In addition, the typical black colonies were identified and stored for further analysis. In addition, *Salmonella* spp. isolates recovered from the samples were identified and tested for antibiotics susceptibility by using VITEK 2 automated system. The multiplex polymerase chain reaction was employed to detect *Salmonella* Enteritidis and *Salmonella* Typhymurium in the meat samples.

Out of 100 raw chicken meat analysed samples, 5 (5%) were positive for *Salmonella* Typhimurium, while none of the samples were contaminated with *Salmonella* Enteritidis.

The resistance levels of the isolates against 12 different antimicrobial agent were tested: all the strains were suscebtible to Imipenem, Meropenem, and Ciprofloxacin. However, the resistance rates in the bacterial isolates were 20% each for Amikacin, Gentamicin, and Tobramycin, also 40% and 30% resistance for Minocycline, and Trimethoprim/Sulfamethoxazole. All isolates of the *Salmonella* Typhymurium were multiresistant to three or more antimicrobial agents. Preventive measure such as proper temperature control as well as proper handling of raw chicken meat in the market place are crucial to the minimization of any potential health hazard by this foodborne pathogen.

INTRODUCTION

Salmonellae are rod-like Gram-negative bacteria that cause salmonellosis, one of the most important foodborn illnesses worldwide. *Salmonella* is describe as highly pathogenic bacteria as their infective does is as low 108 to 10^4 cells (perreten, 2005). The intestinal epithelium infection known as salmonellosis is caused by genus *Salmonella* (Kamarenko *et al*, 2014; Yang *et al*, 2016; Ed-dra *et al*, 2017).

Salmonella typhi and *Salmonella paratyphi* are pathogenic exclusive for humans, causing systemic infections and typhoid fever, where as *Salmonella enterica* serovar Typhimurium cause gastroenteritidis (zhang *et al*, 2008).

Transmission of infection is generally considered to occur orally. Massive bacterial multiplication occurs within the gut and tissue invasion occurs rapidly (Barrow *et al*, 1987). Death has been reported to result from a combination of systemic salmonellosis and diarrhea (Hole, 1932). To date, more than 2500 *Salmonella* serotypes have been identified which half of them belongs to *Salmonella enterica* serovar Typhimurium. The main sources of transmission of *Salmonella* spp. are poultry, read-to-eat products, dairy products, fruits, and vegetables (Caponigro *et al*, 2010; Hur *et al*, 2012; Keithlin *et al*, 2015).

Traditionally, *S. enterica* serovar Typhimurium has been thought to be the prototypical broad-host-range serotype, since it is frequently associated with disease in numerous species, including humans, livestock, domestic fowl, rodent and birds (Rabsch *et al*, 2002). It was estimate that approximately 75% of human *Salmonella* infection cases were due to contaminated food products derived from beef, pork, poultry and eggs (Hald *et al*., 2004). Poultry often become infected through the consumption of contaminated feed, cross-contaminated in brooding houses, or during slaughter and processing (Doyle *et al*., 2006; Fratamico, 2003).

Salmonella contamination in chicken carcasses can occurred due to contamination is supply chain starting from the process of production, distribution, seller, and handling by consumers. If this chain is not well controlled, it will result the increasing number of whole pathogenic bacteria, include *Salmonella* (Malorny *et al*, 2013).

Meat contributes on important proportion in the human diet. It is classified into red and white meat depending on the concentration of myoglobin, a substance in the muscle fiber of animals that is responsible for the meat color (Mancini and Hunt, 2005).

Poultry meat is one of the most popular food products worldwide. Several nutrition factors such as high level of protein and low fat content and favorable content of unsaturated fatty acids contribute to the popularity meat, of which sensory, dietary and economic factors are important (Mulder, 1999).

Salmonella generally grows on media that has water activity above 0.94 and pH 4.1-9.0 with the optimum pH 7.0-7.5. *Salmonella* can grow in a temperature of 5.2-43 with an optimum temperature of 35-3 ºC and will die on media with salt content above 9% (FSA, 2007). *Salmonella* tend to tolerate low temperature for long periods of time (Almashhadany, 2008; Shafini *et al*, 2017).

Several food animals such as poultry and cattle have been reported to serve as reservoirs for multidrug-resistant *Salmonella* (Yoke. Kqueen *et al*, 2008; Arsland and Eyi, 2010; Thung *et al*, 2016).

Increased public awareness related to health and the economic impact of foodborn illness demands specific techniques for detection of these food borne pathogens. For *Salmonella* Typhimurium and *Salmonella* Enteritidis primers targeting the STM4495 and SEN1392 genes, coding for a putative cytoplasmic protein, which are sensitive and specific for the detection of these serotypes (Lui *et al*, 2012).

The target of the STM gene is the gene responsible for fimbrial biosynthesis (Clavijo *et al*, 2006). Long polar fimbrial is absent in all Salmonella strains, specific to *Salmonella* Typhimutium , long polar fimbrial from *Salmonella* Typhimutium mediated adhesion and is required for full virulence. Fimbrial is generally responsible for the initial adhesion of *Salmonella* bacteria to eukariotic cells (Malorny *et al*, 2003).

The bacteriological method recommended for the diagnosis of *Salmonella* spp. is labor intensive and requires 4 to 5 days to obtain presumptive positive or negative results, therefore the development of rapid tests is essential for diagnosis and the control of *Salmonella* spp. (Alcocer & Oliveira, 2003).

Several techniques for the detection of *Salmonella* have been developed such as serology test, selective culture medium, and enzyme linked immunosorbent assay (Eriksson and Aspan, 2007). However, these method have several limitations because of low sensitivity and specificity (Kim, 2006).

The polymerase chain reaction (PCR) is a rapid method for detection and identification of pathogens such as Salmonella. These molecular methods, such as multiplex PCR are highly sensitive, very specific, fast and reproducible (Kim *et al*, 2006).

The emergence of antimicrobial-resistant bacterial pathogens has become a major public health concern. The use of antimicrobials in any venue, including disease treatment and growth promotion in domestic livestock, can potentially lead to widespread dissemination of antimicrobial-resistant bacteria (Gomez-Lus, 1998; Tollefson, *et al*, 1997; Witte, 1998). There is also the hazard of therapeutic failure due to the increasing incidence of antimicrobial resistance among *Salmonella* species (Straley *et al*, 2006; White *et al*, 2001). Therefore the objective of this study to conduct a survey of chicken meat using multiplex PCR assays for screening to determine the presence of *S. enterica*. The *S. enterica* isolates were characterized to determine their serovars and antibiotic resistance profiles. In this study, we monitored the presence of *S. enterica* serovars including *S.* Typhimurium and *S.* Enteritidis with particular reference to pathogenic potential and profiles for epidemiological analysis and risk assessment.

MATERIAL AND METHOD

Collection of Samples: A total of 100 sample of raw broiler chicken were collected from various retail markets in Erbil, Iraq from October 2017 to January 2018. samples were collected in a sterile plastic bag, placed into a cold storage box with ice packs and transported to the laboratory. Samples were analysed immediately upon arrival to the laboratory.

Isolation and Identification of *Salmonella* spp: A 25 gram portion of each sample was aseptically transferred to 225 ml of buffered pepton water (Oxoid, England) and homogenized for 2 min by stomacher lab-Blender (Bagmixer 400, Interscience, Paris, France). The homogenized sample was incubated at 37 ºC for 24 h and constituted the preenrichment stage of the isolation. Subsequently, 0.1 ml of this preenrichment culture was added to 100 ml of selenite cystine broth (Oxoid, England) as selective enrichment media and incubated for 24 h at 37 C , After incubation, a loopful of the enriched cultures was streaked onto Xylose Lysine Deoxycholate (XLD; Oxoid, England and Salmonella-Shigella agar (SSA; Oxoid, England) plates using simultaneously two different selective agar plates. Following incubation, the red with black center on XLD and the colorless colonies with a black center on Salmonella-Shigella agar were counted as suspected Salmonella spp. The presumptive *Salmonella* colonies were confirmed and identified by using VITEK 2 automated system (bio Merieux).

Antimicrobial susceptibility test: Susceptibility to antimicrobial agents was tested using the VITEK 2 system for the following 12 antimicrobial agents:

Ticacillin, Piperacillin, Piperacillin / Tazobactam, Ceftazidime, Cefepime, Aztreonam, Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Minocycline, Trimethoprim / Sulfamethoxazole.

DNA extraction: Bacterial DNA was extracted from *Salmonella* Typhimurium isolates subcultured on nutrient agar plates using DNA extraction kit (geneaid/Korea) according to manufacture's instructions. The DNA extracted samples were stored at -20 ºC for further use as template for PCR. From this 4 µl of template DNA were used directly for the PCR.

Multiplex PCR: The multiplex PCR was standardized for detecting the pathogens under study simultaneously in a single reaction tube containing the two primer sets for these organisms i.e., *sen* and *stm* primers for *Salmonella* Typhimurium and *Salmonella* Enteritidis (Table1). The total of 25 µl PCR master mix reaction volume was performed containing 4µl of genomic DNA, 12.5 µl of 2X GoTaqGreen Master Mix (Promega, USA) and 1.5µl was added for each of the forward and reverse primer for both genes.

PCR amplification was performed as follows: Initial denaturation of 7 min followed by 35 cycles of final denaturation at 95 ºC for 2 min at 72 ºC and one cycle of final denaturation at 72 ºC for 10 min (Table 2).

The PCR products were separated in a 2 percent agarose gel and stained with Safe dye (GenetBio/Korea). The gel was visualised under UV transiluminator (Synegene/UK).

Table 1. The specific sequence oligonucleotide for *stm* and *sen* genes

Organisms	(Gene name)	DNA sequence of the primer	Suspected size of PCR product (bp)
*S.*Typhimurium	*stm*	F 5' GGTGGCAAGGGAATGAA 3' R 3' CGCAGCGTAAAGCAACT 5'	915 bp
S. Enteritidis	*sen*	F 5' GCCACTGTCTGATGCTCTTG 3' R 3' GAAAGGCTCCGTGGTTAGT 5'	656 bp

Table 2. Performance PCR using the thermal cycling conditions for *stm* and *sen* genes.

Step	Temperature °C	Time	Number of cycles
Initial denaturation	94°C	7 min	1
Denaturation	95°C	1min	35
Annealing	60°C	40sec	
Primer Extension	72°C	2min	
Final extension	72°C	10min	1

RESULTS

Out of 100 raw chicken meat samples were analysed and the presumptive Salmonella colonies were confirmed and identified by the VITEK 2 automated system (Fig. 1 and 2). The multiplex PCR results revealed that 5/100 (5%) of the samples were found to harbor *Salmonella* Typhymurium, meanwhile none of the samples was contaminated with *Salmonella* Enteritidis (Fig. 3).

The antibiotic susceptibility test of Typhimurium revealed *Salmonella* that all isolates were resistant to Ticacillin, Piperacillin, Piperacillin/Tazobactam, Ceftazidime, Cefepime, Aztreonam. In addition, resistance to Minocycline, Trimethoprim/Sulfamethoxazole, Amikacin, Gentamicin, Tobramycin were 40%, 40%, 20%, 20%, 20% respectively, while intermediate resistant to Gentamicin and Tobramycin were 20%. All the isolates were susceptible to Imipenem, Meropenem, Ciprofloxacin (Table 3).

Observation from the presence study indicated that all *Salmonella* Typhymurium isolates were multi-drug resistant (MRD) isolates, which showed resistance to three antibiotics or more.

Table 3. Antimicrobial susceptibility test for *Salmonella* Typhimurium isolates

No.	Antimicrobial Agents	No. Isolates				
		1	2	3	4	5
1	Ticacillin	R	R	R	R	R
2	Piperacillin	R	R	R	R	R
3	Piperacillin/Tazobactam	R	R	R	R	R
4	Ceftazidime	R	R	R	R	S
5	Cefepime	R	R	R	R	S
6	Aztreonam	R	R	R	R	S
7	Imipenem	S	S	S	S	S
8	Meropenem	S	S	S	S	S
9	Amikacin	R	S	S	S	S
10	Gentamicin	R	I	S	S	S
11	Tobramycin	R	I	S	S	S
12	Ciprofloxacin	S	S	S	S	S
13	Minocycline	R	R	S	S	S
14	Trimethoprim/Sulfamethoxazole	R	R	S	S	S

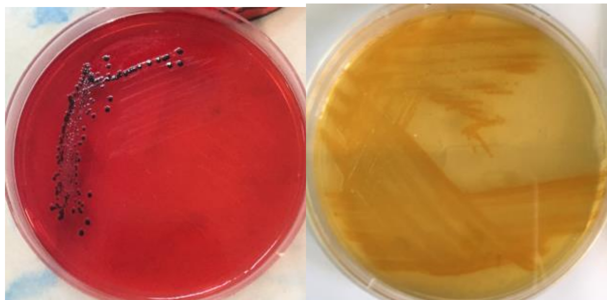

Figure 1. Growth of *Salmonella* Typhymurium on Xylose Lysine Deoxycholate (XLD) and MacConky agar.

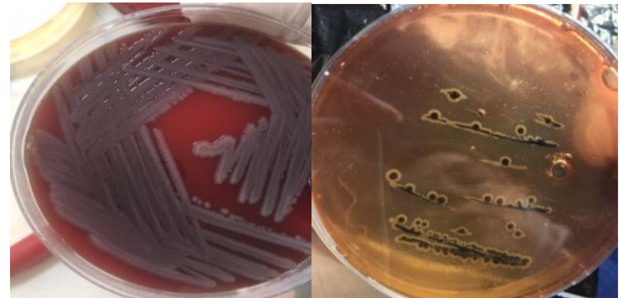

Figure 2. Growth of *Salmonella* Typhymurium on Blood and Salmonella-Shigella agar (SSA).

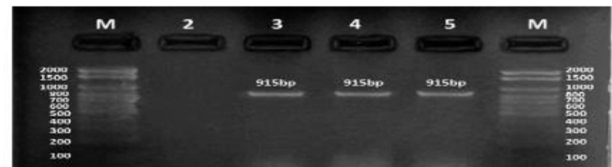

Figure 3. Multiplex PCR specific for *Salmonella* Typhimurium identification. Lane: 1-6 ladder of 1kb, lane 2: Negative control, Lane 3-5: represent *stm* (915 bp) gene.

DISCUSION

In our study, the incidence of *Salmonella* Typhimurium in raw chicken meat is 5%. Lower results reported by Arsinan and Eyi who isolated *S.* Typhimurium at rate of 26.7%.

This result was in contrast with findings of other previous study, who reported that none of the samples was positive for . *S* Typhimurium and 4.3 percent of raw chicken meat samples were positive for *S.* Enteritids (Anju *et al*, 2014).

Variations in sampling methods and culture techniques used to detect the pathogen in individual studies (ray, 2004; Uyttendaele *et al*,, 1998). Moreover the reasons for difference in the distribution of *Salmonella* serotypes are complex, as they may be associated with seasonal or demographic factors or the prevalence of a particular species in different product types (Ziprin, 1994). Also, this could be due to the geographical variation such as climate and feed (Pui *et al*, 2011).

Salmonellae invade and destroy specialized epithelial cells in the host intestine, and migrate to the mesenteric lymph nodes, where they encounter, subsequently survive and replicate within macrophage (Groisman *et al*, 1999).

Salmonella and other microorganisms have the ability to survive and multiply in internal organs, especially the liver and heart as these areas provide sites where bacterial multiplication can occur without exposure to host defense mechanisms (Gast, 2003). This probably explain the high incidence of *Salmonella* found in livers and hearts (Abd El-Aziz, 2013).

Accordingly, the virulence of bacteria is influenced by both antimicrobial resistance and the presence of virulence

genes (Huehn *et al*., 2010; Dong *et al*., 2014). The emergence of MDR strains of *Salmonella* are mainly based on the factors of genetic and biochemical mechanisms in order to enhance their survivability by preserving their drug resistance genes (Yang *et al*., 2010)

Some uses of antimicrobial agents may contribute to the emergence of resistant strains including use in agriculture both as growth-promoting substances in animal feeds and as prophylactics, also the widespread use of antibiotics as a treatment for disease. By overloading various environments with antibiotics, rapid development of drug resistance may result (Arslan and Eye, 2010; Thung *et al*, 2016).

In this study, MDR *Salmonella* isolates are prevalent in retail markets. Hence, more attention should be focused on the supervision and control of antimicrobial use, typically in the agriculture and human health care sectors in Erbil (Huehn *et al*, 2010; Dong *et al*, 2014). Multiple drug resistance transmitted genetically by plasmids among enteric bacteria is a problem in *Salmonella* infections. Susceptibility testing in an important adjunct to selecting a proper antibiotics (Jawetz *et al*, 2007). Liu , (2010) illustrated the specifically gene for identifying et al of *S*. Typhimurium using different genes and found that STM4495 gene was highly target genes between 13 comparison genes.

Molecular technique, such as PCR, are being used routinely in laboratories with bacteriological techniques to provide a more accurate diagnosis in a shorter period of time (Andrade *et al*, 2010). However, it is essential to invest in research efforts to improve or develop rapid test for the detection of *Salmonella* spp. in poultry meat to minimize the risks of consumers contracting salmonellosis (Eijkelka *et al*, 2009).

Poultry products have consistently been identified as important sources of *Salmonella* infection in humans, because of vertical transfer of infection from breeding hens to progeny is an important aspect of the epidemiology of *Salmonella* spp. infection within the poultry industry (Bae *et al*, 2013).

Implementation and maintenance of some control measures like good manufacturing practices (GMP) and hazard analysis and critical control point (HACCP), as well as further strengthening the education of food processors will be necessary, for reducing *Salmonella* contamination (Sallam *et al*, 2014).

CONCLUSION

In this research multiplex PCR has been carried out to identify *Salmonella* Typhimurium serovar from retail chicken meat. Chicken meat can be become a common vehicle for *Salmonella* transmission and if inadequate practice of food hygiene and sanitation, *Salmonella* infection is possible.

Multiple antibiotic resistant *Salmonella* should be regarded as an issue and the spread should be viewed as a challenge to combat the bacteria and conserving public health.

Moreover, the application of targeting *stm* and *sen* genes provides a valuable tool for the rapid identification of *Salmonella enterica* serovars Typhimurium and Enteritidis from food samples especially milk and dairy products.

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