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Occurrence, antimicrobial resistance and whole-genome sequencing analysis of *Salmonella* isolates from chicken carcasses imported into Iraq from four different countries

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ABSTRACT

Salmonella is a major cause of human foodborne illnesses worldwide; however, little is known about its occurrence and genomic characteristics in food sources in many developing countries. This study investigates the occurrence, serotypes distribution, antimicrobial resistance, and multilocus sequence types (ST) of *Salmonella* isolated from 400 imported frozen chicken carcasses sold in the markets of Thi-Qar, south-eastern Iraq. *Salmonella* was detected in 46 out of 400 tested samples [11.5% (95% confidence interval: 8.5%–15.0%)]. *S.* Typhimurium was the most abundant (30.4%) among 14 different serotypes recovered from the tested frozen carcasses. Antimicrobial resistance was most frequently detected against tetracycline (84.4%), nalidixic acid (80.4%), streptomycin (69.6%) and trimethoprim/sulfamethoxazole (65.2%). Whole-genome sequencing (WGS) analysis revealed that 18 isolates harbored four β-lactamase resistance genes, with bla_{CARB-2} was the most commonly (14/18) detected. It was possible to identify 8 multilocus sequence types from the WGS analysis of 40 out of the 46 *Salmonella* isolates; with ST-11 (among *S.* Enteritidis) and ST-19 (among *S.* Typhimurium) were the most frequently detected. These results add to our understanding of the global epidemiology of *Salmonella*. Our work stands as one of the first reports on WGS analysis of *Salmonella* from retail chicken in a Middle-Eastern country. Results from this study could be valuable for guiding an informed import risk analysis aiming at reducing the exposure risk from *Salmonella* through imported chicken carcasses into Iraq. This work demonstrates the value of WGS as a promising tool for supporting evidence-based food safety hazard characterization.

1. Introduction

Non-typhoidal *Salmonella* is among the most important foodborne pathogens and continue to pose a significant challenge to public health and food safety worldwide (Abraham et al., 2014). Human *Salmonella* infections are commonly associated with the consumption of contaminated foods and water, as well as direct contact with infected animals (Majowicz et al., 2010). Gastroenteritis caused by non-typhoidal *Salmonella* is usually a self-limiting illness characterized by diarrhea, fever, vomiting and abdominal cramps. Nevertheless, children, immune-compromised and older individuals are more likely develop severe disease with a higher risk of secondary complications (Jones et al., 2008). Ciprofloxacin and extended-spectrum cephalosporins are the most frequently used antibiotics for treating invasive *Salmonella* infections in humans, especially children and the elderly (Zhu et al., 2017). Recently, the increase of multidrug-resistant (MDR) forms of *S. enterica* in food-producing animals has been an emerging problem worldwide, likely due to the widespread use of common antimicrobials in poultry and animal husbandry for therapeutics, prophylaxis and growth promotion (Abraham et al., 2014; Gupta et al., 2003).

Eggs and poultry products have been described as the main vehicles for the transmission of human salmonellosis, accounting for the majority of foodborne outbreaks (CDC, 2009; Dogru et al., 2010). Contamination of chicken meat can occur during various stages throughout

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the slaughter processing, packing, storage, and transportation (Rajan et al., 2017). It is anticipated that over the next decade, imports of poultry meat into developing countries will grow at 3.4% per year, while the Africa/Middle East region will likely account for 64% of the rise in world poultry imports (OECD/FAO, 2016). In Iraq, over the recent years, the importation of frozen chicken meat has been steadily increasing, as the considerable increase in domestic production fails to keep pace with growing demand (USAID/IRAQ, 2006). Many of the basic services (e.g. water, sanitation and electricity) in Iraq are affected badly after decades of war, sanctions, and political instability. Nowadays, sustaining chilling display at retail shops is becoming very challenging in most Iraqi cities, especially in the long summer months, as provoked by frequent electricity outage due to decaying infrastructure and prolonged heat waves. Thereafter, there is a growth in consumers demand for frozen chicken in Iraq, as it is conceived to be less vulnerable to spoilage compared to chilled products. The growth in the importation of frozen chicken, combined with the unreliable cold chain logistics in Iraq, mirror a similar situation across many developing countries, especially in Africa/Middle East region. Hence, it is important to anticipate the emerging risks arising from chicken meat imports in such challenging context. The introduction and dissemination of *Salmonella* from imported chicken need to be evaluated as part of import risk analysis; this will help in quantifying the exposure of the general public to *Salmonella*, for instance, while handling raw frozen chicken in the domestic setting (Campos et al., 2018).

Worldwide non-typhoidal *Salmonella* is one of the most common bacterial pathogens that cause diarrhea in children and adults (Abraham et al., 2014; Majowicz et al., 2010). In Baghdad, central Iraq, non-typhoidal *Salmonella* was the second most frequently reported cause of gastroenteritis in children, after enteric viruses (Al-Kubaisy et al., 2015). Recently, a study in southern Iraq isolated non-typhoidal *Salmonella* from 10.3% of diarrhoeal stool samples from children under the age of 5 years (Harb et al., 2017). There are no published studies characterizing the molecular epidemiology of non-typhoidal *Salmonella* hazard in chickens imported into Iraq. Given the importance of this pathogen to global public health, this pilot study provides an overview on the non-typhoidal *Salmonella* situation in chicken carcasses from the key four exporting countries to Iraq. It is important to note that this study is not designed to compare prevalence between the four countries; instead, we aim to provide a descriptive insight on variability in both occurrences and microbiological features of *Salmonella* from a representative cohort of imported frozen chicken carcasses retailed in Iraq.

2. Materials and methods

2.1. Sampling and study setting

The study was carried out in Thi-Qar province, situated in south-eastern Iraq. The sample size was calculated based on an unknown prevalence of *Salmonella* in imported chicken (thus prevalence was set to 50%), a precision of 5% for the prevalence estimate, and a 95% confidence interval (CI) (Dohoo et al., 2009). Thus, a total of 400 frozen packaged chicken carcasses were collected from different retail stores and markets between November 2015 and August 2016. Samples were originating from the four different countries those known (based on personal experiences of the four Iraqi co-authors) to be supplying the vast majority of frozen chicken carcasses into Iraqi markets. The samples represented carcasses imported from; Iran (n = 100), Turkey (n = 100), Brazil (n = 100) and India (n = 100). Samples were collected from three districts in Thi-Qar province (Nassriya, Al- Shatra, and Suq Al-Shoyokh); there has been one brand from each importing country available in retail shops and supermarkets during the study period. Frozen packaged chicken carcasses were placed in separate sterile plastic bags, labeled and transported in ice boxes to the Food Microbiology Laboratory, Public Health Division in Thi-Qar. All samples were thawed overnight in a laboratory refrigerator (4 °C), and testing was done within 24 h from sampling.

2.2. Isolation and serotyping of Salmonella

The isolation and identification of *Salmonella* were performed as recommended by the ISO 6579:2002 (da Silva et al., 2013). Briefly, each sample was washed for 2 min in a sterile plastic bag containing 225 ml of buffered peptone water (Oxoid, England) and the rinse was incubated overnight at 37 °C. A 100 µl aliquot of pre-enriched suspension was transferred into 10 ml of Rappaport-Vassiliadis broth (RV) (Oxoid, England), and incubated at 42 °C for 24 h. A loopful of broth culture was streaked on Xylose Lysine Deoxycholate (XLD) agar and Brilliant Green (BGA) agar (Oxoid, England), and the plates were incubated at 37 °C for 24 h. Presumptive colonies with *Salmonella* morphology from each plate were then identified biochemically by inoculating into Triple Sugar Iron Agar (Oxoid, England) slope and by using the API 20E system (bioMérieux, France). In addition, typical *Salmonella* phenotypes were further confirmed by single step PCR for the *S. enterica* gene *inv*A (Swamy et al., 1996). Isolates were sent to the Iraqi National Centre for *Salmonella* for serotyping at the Central Public Health Laboratories in Baghdad.

2.3. Screening of antimicrobial resistance in Salmonella isolates

Antimicrobial susceptibility testing of *Salmonella* isolates was determined using the disc diffusion method on Mueller-Hinton agar plates according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015). The following 12 antimicrobials were tested: ampicillin, amoxicillin + clavulanic acid, ceftriaxone, cefotaxime, ciprofloxacin, chloramphenicol, gentamicin, nalidixic acid, streptomycin, azithromycin, tetracycline and trimethoprim sulfamethoxazole (Mast Diagnostics Ltd., Merseyside, UK). *Escherichia coli* ATCC25922 was used as a quality control organism. The isolates were classified as susceptible, intermediate, or resistant according to the CLSI (2015) guidelines. Isolates resistant to three or more different classes of antimicrobials were defined as multi-drug resistant (MDR) (Abraham et al., 2014).

2.4. WGS analysis of Salmonella isolates

DNA was extracted from each isolate using the BIOLINE DNA extraction kit (ISOLATE II, Genomic DNA Kit) according to the manufacturer's instructions. DNA was eluted into 100 µL DNA elution buffer. Frozen DNA was shipped from Iraq to Australia, and further genomic analysis was conducted in the Antimicrobial Resistance and Infectious Diseases (AMRID) Laboratory of Murdoch University. Library preparation was performed using an Illumina NexTera® XT library preparation kit (Illumina) as per the manufacturer's instructions. The library preparations were sequenced on an Illumina Nextseq platform using a mid-output 2 × 150 kit. Reads were de novo assembled using SPAdes 3.11.1 software (http://cab.spbu.ru/software/spades/). The contig files were uploaded to the Centre for Genomic Epidemiology (http://www.genomicepidemiology.org/) to screen for multilocus sequence types (MLST 1.8), serotypes (SeqSero 1.2) and to extract antimicrobial resistance genes data (ResFinder 3.0).

Whole genome sequencing analysis was performed on the isolates with read numbers ranging from 241,340 to 4,858,342 (mean 1,260,901) per isolate, and coverage ranging from 6-104× coverage with an average of 33× coverage per isolate. All read data generated in this study has been deposited in the NCBI Sequence Read Archive under accession number SRP142560.

2.5. Statistical analysis

The variation in detection of *Salmonella* on chicken carcasses and the frequency of serotypes and isolates resistance to antibiotics were