Detection of *E. coli*, *Salmonella spp.*, and *Listeria Monocytogenes* in Retail Chicken Meat and Chicken Giblets Samples Using Multiplex PCR in Baghdad City

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Abstract

Contamination of chicken meat is an important public health problem and food of poultry origin is one of the most common sources of bacterial pathogens. *Escherichia coli*, *Salmonella spp.* and *Listeria monocytogenes* are considered among the most important pathogens which can be spread through meat and meat products consumption. Rapid methods for identification and detection of these dominant foodborne pathogens are still required. This study was conducted to determine the prevalence of these pathogens contamination of imported frozen chicken meat from different origin and locally frozen chicken (leg, breast and giblets) collected over a 6-month period between November and April 2016 from different markets in Baghdad, Iraq. Multiplex polymerase chain reaction (m-PCR) assay for the specific detection of the dominant foodborne bacterial pathogens, including *Escherichia coli, Salmonella spp.* and *Listeria monocytogenes* in chicken meat was used. The obtained results indicated that a large percentage of chicken samples 45% was positive for *E. coli*, followed *Salmonella* 17%, and *L. monocytogenes* 11%. The occurrence of *E. coli* was highest in giblets 26 (52%) followed by leg 22(44%) and breast 19(38%) samples. The occurrence of *S. spp.* in leg and giblets were 9 (18%), whilst in giblets 8(16%) samples. On other hand the occurrence of *L. monocytogenes* in giblets was 7 (14%), reduced to 5 (10%) samples in leg and breast. imported frozen chicken meat samples were the most frequently contaminated by *E. coli* of origin Turkish 63% and Brazilian 53%, followed by Iranian 40%, and while locally frozen chicken samples were the least frequently contaminated 33%. Samples of origin Turkish and Ukrainian the most frequently contaminated by *Salmonella* 27and 20% respectively, and *L. monocytogenes* 17%, and whilst locally frozen chicken samples were the least frequently contaminated 13 and 7% respectively. Multiplex PCR was found to be a very sensitive test that allowed rapid and reliable identification of these bacteria.

Keywords

Multiplex PCR; Chicken meat; *Escherichia coli; Salmonella spp.*, *Listeria monocytogenes*.

Article Info

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Introduction

Poultry meat is one of the most popular food products worldwide. Several nutritional factors such as high level of protein and low fat content and favorable content of unsaturated fatty acids contribute to the
popularity of poultry meat, of which sensory, dietary and economic factors are important (Abd El-Aziz, 2013). Outbreaks and Foodborne pathogens pose a significant threat to human public health, leading to a substantial economic burden both in developed and less developed countries (Chen et al., 2012).

More than 250 known foodborne diseases could be caused by food contaminated with bacteria, viruses, parasites, and toxins, which continue to be a public health problem in the world (Ahmed et al., 2014). Of these, bacteria cause a large proportion (approximately 90%) of all foodborne illnesses. The bacterial pathogens that are most likely to be found in commonly slaughtered livestock (cattle, sheep, and swine) and poultry (chicken and turkey) (Food Safety and Inspection Service, 1999). Chicken meats comprise about the two-thirds of the total production in the world (Mead, 2000). Meat and poultry carcasses and their parts are frequently contaminated with pathogens which reach the carcasses from intestinal tract or from fecal material on feet and feathers (Dinçer and Baysa, 2004).

In Iraq and many countries, foodborne pathogens and microbial food safety indicators that are prevalent in poultry, especially, Salmonella, Listeria monocytogenes, and Escherichia coli, have been reported (Kupradit et al., 2013, Rodpai et al., 2013,Saeed et al., 2013, Zhao et al., 2014, Adwan et al., 2015, AL Jobori and Aboodi, 2015,AL-Jobori et al., 2015, AL-Jobori and AL-Bakri, 2015,Nguyen et al., 2015, Hassan and Hama Saleh,2016).To minimize the prevalence of foodborne diseases and reduce microbial contamination in food supplies, effectively monitoring the occurrence and distribution of bacterial pathogens in food is essential.

The most common tools of standard methods used for pathogen detection are cultural based method, immunological based method, and molecular based methods (Lazcka et al., 2007). Classical cultural methods for the detection of Salmonella spp., Listeria monocytogenes and Escherichia coli are time-consuming and costly. Including step of pre-enrichment and isolation of presumptive colonies of bacteria on solid media, and final confirmation by biochemical and/or serological identification have been applied to detect foodborne pathogens (Lazcka et al. 2007, Xu et al. 2012). These methods are introducing sampling and enumeration errors, as these pathogenic bacteria occur in low numbers. In fact, the low-throughput of these traditional methods does not allow rapid screening of large numbers of food samples for the presence of one or more pathogens (Abubakar et al. 2007). To overcome these limitations, multiplex polymerase chain reaction (m-PCR), real-time PCR, and oligonucleotide arrays have been applied to detect multiple pathogens simultaneously (You et al., 2008; Severgnini et al., 2011). During the last few years, international standards have been agreed the use of PCR-based detection of foodborne pathogens and legislations are implementing new types of analyses as the accepted official methods. For example, European regulation EC 2073/2005 allows the use of alternative detection methods based on certified analyses of international standards (EC, 2005).

Multiplex polymerase chain reaction (m-PCR) is a reaction that amplifies more than one target gene simultaneously by mixing multiple primer pairs. mPCR-based methods have been widely used and adapted for the rapid detection of single and multiple bacterial species, for example, Salmonella spp., Escherichia coli, and L.
monocytogenes in meat (Kupradit et al., 2013) in chicken meat (Zarei et al., 2013). Although mPCR can amplify multiple targets in a single tube, its detection capability is still restricted to only a few targets per assay due to the complexity of the amplification (Wang et al., 2007). For these reasons, typically only 2 (Radhika et al., 2014, AL Jobori and Aboodi, 2015), 3 (Xiao et al., 2014, Zhao et al., 2014, AL-Jobori et al., 2015, Adwan et al., 2015), 4 (Wei et al., 2013), or 5 (Chen et al., 2012) bacterial species are simultaneously detected using m-PCR. However, most research has not combined the detection of Escherichia coli, Salmonella spp. and L. monocytogenes together.

The gene specificity for all the bacteria of interest can be used in the m-PCR to detect each bacterial species. Therefore the objective of this study is to detect the prevalence of Salmonella spp., Escherichia coli, and L. monocytogenes, in frozen raw chicken meat (leg, breast and giblets) using multiplex PCR to take care during cooking and consumption of these products.

Materials and Methods

Collection of Samples

During a 6-month period between November and April 2016, a grand total of 150 random samples of frozen chicken meat cuts (leg, breast and giblets) were collected of five origins (Turkish, Iraqi, Iranian, Brazilian and Ukrainian) from different markets at Baghdad governorate.

The collected samples were transferred directly to the laboratory in an ice box with a minimum of delay, and samples were kept frozen until analysis. Thawing of samples occurred during overnight incubation in refrigerator (Roberts and Greenwood, 2003).

Preparation of Samples

The samples were cut into pieces and 25 grams of the examined samples were weighed aseptically into sterile blender container and 225 ml of nutrient broth were added. Each sample was then homogenized in the blender at 2000 rpm for 1-2 minutes to provide a food homogenate (APHA, 1992). The homogenate was incubated at 37°C for 24 h.

DNA Extraction

A volume of 1.5 ml of the post-enriched sample was centrifuged at 14,000 g for 1 min, DNA was extracted using Presto Nini g DNA Bacteria Kit according to manufacturers’ instructions (Geneaid, Korea). The extracted DNA was stored at -20°C until use.

Agarose Gel

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA. A 10 μl portion of the sample was analyzed by electrophoresis in agarose gel (2%), staining with ethidium bromide (Promega USA), and visualized in UV light. A DNA molecular weight standard 100 bp was analyzed alone with the samples (Wang et al., 1997).

PCR Primers

For m-PCR assay, three primer sets were selected. The primers sequences and their corresponding genes are shown in Table 1.
was added, together with 10 μl of nuclease free water, 5 μl master mix. PCR reactions were performed in a thermal cycler (Eppendorf, Mastercycler Gradient) under the following conditions: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, and extension at 72°C for 5 minutes. The final cycle included a 10-minute additional extension at 72°C. PCR products were observed following gel electrophoresis on 2% agarose gels (Promega, USA) using ethidium bromide staining (Promega, USA).

**Results and Discussion**

In the present study, the multiplex PCR-based method was used for detection of three food-borne pathogens after enrichment. The amplifications of the mPCR products were visualized in agarose gel and a 153 base pair fragment (uidR gene) was identified in the positive samples for the genus *E. coli*; a 284 base pair fragment (invA gene) for the samples belonging to the *S. spp.* and a 217 base pair fragment (LIS gene) for the samples belonging to the *L. monocytogenes*. The specificities of these genes (Table 1) were tested using the gDNA templates. Figure 1 shows the data from multiplex assay performed on the three tested pathogens. As shown, after enrichment, the method was able to detect the pathogens at the tested contamination levels in chicken meat (leg, breast and giblets) samples. The prevalence of *E. coli*, *Salmonella spp.* and *L. monocytogenes* in different chicken cuts were shown in Table 2 by using this method.

One or more of the bacterial pathogens were detected in 87 of 150 (58%) samples. In this study, uidR gene was present in 67 samples. The invA gene was present in 26 samples and LIS gene was found in 11 samples. A large percentage of chicken samples (45%) was positive for *E. coli*, followed *Salmonella* 17%, and *L. monocytogenes* 11% (Table 2). The occurrence of *E. coli* was highest in giblets 26 (52%) followed by leg 22 (44%) and breast 19 (38%) samples. The occurrence of *S. spp.* in leg and giblets were 9 (18%), whilst in giblets 8 (16%) samples. On other hand the occurrence of *L. monocytogenes* in giblets was 7 (14%), reduced to 5 (10%) samples in leg and breast (Table 2).

The results given in figure 2 indicated that imported frozen chicken meat samples were the most frequently contaminated by *E. coli* of origin Turkish 63% and Brazilian 53%, followed by Iranian 40%, and while locally frozen chicken samples were the least frequently contaminated 33%. Samples of origin Turkish and Ukrainian the most frequently contaminated by *Salmonella* 27 and 20% respectively, and *L. monocytogenes* 17%, and whilst locally frozen chicken samples were the least frequently contaminated 13 and 7% respectively.

Contaminations of meat by bacteria have been linked to consumer health problems, as reported by outbreaks and recalls from marketplaces associated with contaminated products. Although classical microbiological methods offer reliable and standardized procedures for the detection of food-borne pathogens (e.g. ISO standards), they often including time consuming analyses that are not always compatible with the need for rapid results. The mPCR based assay was a rapid method and compatible with most methods used to ensure the safety of food products. Bacterial contaminations could be detected specifically depending on the selected primers and the process of determination was much shorter than standard microbiological methods. In this study, multiplex PCR as a rapid and cost-
effective method, was used for the detection of pathogens in chicken meat.

Triplex PCR method was successfully used to investigate the risk of potential foodborne bacterial contaminations in 150 samples collected from different markets at Baghdad governorate. Our data showed that poultry samples appear to be prominent reservoirs of *E. coli*, *Salmonella*, and *L. monocytogenes* (Table 2). Experimental results using specific primer sets showed that three target genes were successfully amplified without nonspecific bands in the triplex PCR, demonstrating the specificity of this method (Figure 1). The three genera of bacteria (*E. coli*, *Salmonella*, and *Listeria*) targeted in the current study have been associated with food borne infections and diseases as reported by others (Chen et al., 2012; Saeed et al., 2013; Zarei et al., 2013; Zhao et al., 2014, Adwan et al., 2015, Nguyen et al., 2015, Hassan and Saleh, 2016). The authors have previously reported that white cheese, meat, chicken, vegetative, ice cream and beverage samples were contaminated with this pathogens (AL-Jobori and Aboodi, 2015, AL-Jobori et al., 2015, AL-Jobori and AL-Bakri, 2015).

As shown in Table 2, a large percentage of chicken samples 45% was positive for *E. coli*, followed *Salmonella* 17%, and *L. monocytogenes* 11%. The incidence of *E. coli* and *L. monocytogenes* were more frequently in giblets than in leg and breast samples. The presence of *E. coli* in high numbers may be due to improper slaughtering techniques, contaminated surfaces and/or handling of the meat by infected food handlers (Nel et al., 2004). Also, the presence of these pathogens can be due to contamination taking place during the meat processing at slaughterhouse or to the retailers' poor handling of meat (Kagambéga et al., 2012). In the last decade, there has been a wide interest in the use of the multiplex PCR (mPCR) technique. mPCR approaches have been applied to detect different species of several bacteria, to differentiate closely related species and to recognize single species (Settanni and Corsetti, 2007). Most of foodborne pathogens have a zoonotic origin and have reservoirs in healthy food animals from which they spread to an increasing variety of foods. *E. coli*, *Salmonella* and *L. monocytogenes* are the most common and frequent pathogens responsible for food poisoning and food related infections. According to WHO, 25% of the diarrhea in foodborne illness is caused by food infected with *E. coli* (WHO, 2006). The results of the present study revealed that chicken meat can be easily contaminated by *E. coli*. Preparation of healthy chicken meat is very important. We recommend the PCR technique as an accurate, rapid, and safe method for inspection of chicken meat. Providing healthy food has been considered in many countries (Momtaz and Jamshidi, 2013).

*Salmonella* spp. is among the most important food borne pathogens in the world. Poultry and poultry products are usually causing human salmonellosis outbreaks. Chicken products are widely acknowledged to be a significant reservoir for *Salmonella*. They have frequently been incriminated as a source of *Salmonella* contamination and consequently thought to be major sources of the pathogen in humans (Baeumler et al., 2000). Furthermore, one of the commonest causes of *Salmonella* infection reported in humans has been through the handling of raw poultry carcasses and products, together with the consumption of undercooked poultry meat (Panisello et al., 2000). *Salmonella* spp. was detected 18% in leg and breast, and 16% in giblets samples (Table 2) which may
interpret due to that the defeathering process may spread microorganisms between carcasses or from the defeathering equipment contributing to an increase in the numbers of psychrotrophs and aerobe mesophiles on the carcasses. The evisceration process provides an opportunity for cross contamination from human, equipments and worker’s hands (Jackson et al., 2001). The leading source of contamination of carcasses by Salmonella is the evisceration step at the slaughterhouse (Bouchrif et al., 2009). As well as poor hygiene conditions, regarding the temperature of storage, the equipment and the employees’ personal hygiene. The cutting tables were seldom washed or disinfected before use. These benches could therefore be reservoirs from which Salmonella could spread to other equipment through flies or direct contact (Stevens et al., 2006). The extent of Salmonella presence from the fresh raw chicken and frozen chicken were similar; however, significantly more samples were contaminated with Salmonella compared to other categories. The prevalence of Salmonella in the UK poultry was found to be 25-29% using the cultural methods (Soumet et al., 1999). Using the multiplex-PCR method, Salmonella typhimurium and Salmonella enteritidis strains were detected in 30.6% of environmental swabs of poultry houses in Ploufragan, France (Zarei et al., 2012) Thus, the incidences of Salmonella in Baghdad, Iraq were nearly similar to those reported for the European poultry.

As shown in Table 2, the occurrence of L. monocytogenes was highest in giblets (14%) followed by leg and breast (10%). L. monocytogenes can be found in a wide variety of raw and processed foods. Various meats and meat products such as beef, lamb, pork, and chicken, milk and dairy products, seafood and fish products have all been associated with Listeria contaminations (Rocourt and Cossart, 1997). We have previously reported that 4% of white cheese samples contaminated with this pathogen (AL-Joobori and Aboodi, 2015).

| Species                     | Target gene | PCR primers’ sequences ( 5’ – 3’ )                                      | Product size | References       |
|-----------------------------|-------------|------------------------------------------------------------------------|--------------|------------------|
| Salmonella spp.             | invA        | invA F:GTGAAATTATCGCCACGTTCGG                                         | 284 bp       | Rahn et al., 1992|
|                             |             | invA R:TCATCGCACCCTGCAAGGG                                              |              |                  |
| Escherichia coli            | uidR        | UidR F:TGGTTACGTCCTGTAGAAAAGCC                                       | 153 bp       | Bej et al., 1991 |
|                             |             | R:AAAACTGCCGCGGACAGCAAT                                                  |              |                  |
| Listeria monocytogenes      | LIS         | LIS F:TCATCGACCGCACCCTTCGG                                              | 217 bp       | Germini et al., 2009|
|                             |             | LISR:TGAGCAACGTATCCTCCAGAGT                                             |              |                  |
Table 2 Incidence of *E. coli*, *Salmonella spp* and *L. monocytogenes* in examined samples.

| Samples   | Incidence of pathogens mPCR |   |
|-----------|------------------------------|---|
|           | *E. coli*                  | *S. spp* | *L. monocytogenes* |
| Leg       | 22(44%)                    | 9(18%)   | 5(10%)            |
| Breast    | 19(38%)                    | 9(18%)   | 5(10%)            |
| Giblets   | 26(52%)                    | 8(16%)   | 7(14%)            |
| Total     | 67(45%)                    | 26(17%)  | 17(11%)           |

Fig. 1 Multiplex PCR applied to multiple Pathogen detection. Lane M: 100 bp DNA ladder, Lane 1,2,3,4,5,6 Multiple pathogen, *E. coli* 152 bp, *Salmonella* 284 bp, and *L. monocytogenes* 217bp, Lane:7,8 Negative control.

Fig. 2 Distribution of *E. coli*, *Salmonella spp* and *L. monocytogenes* in five selected origin based on results of mPCR.
According to the previous reports from Iran, 2.6% of the beef and 6% of the lamb samples were contaminated with this pathogen (Jalali and Abedi, 2008). No contamination with L. monocytogenes was reported in 200 beef carcasses, in Northern Ireland (Madden et al., 2001). However, 0.42% of bison carcasses in USA were contaminated with this pathogen (Li et al., 2004). This is consistent with observations that L. monocytogenes can grow over a wide range of environmental conditions such as refrigeration temperatures (Naravaneni and Jami, 2005), which allows it to overcome the food preservation and safety barriers, and creates a potential risk to human health. The prevalence of L. monocytogenes in retail poultry in Leon, Spain, was 32% (Miettinen et al., 2001). In addition, the difference in prevalence of L. monocytogenes in different parts of chicken might be due to the variation in water content and nutrient level of chicken parts. Montville and Matthews (2008) stated that listerial growth is affected by humidity and nutrient contents of the food.

In the present study, E. coli was detected in 63 and 53% of origin Turkish and Brazilian followed by Iranian 40%. Samples of origin Turkish and Ukrainian were the most frequently contaminated by Salmonella 27 and 20% respectively, and L. monocytogenes 17 (Table 2). The differences in these contamination percentages are probably related to numerous factors, including the origin of the chicken lots, the hygiene-sanitary conditions in the abattoirs, and cross-contamination that occurred during plucking, washing, cooling and wrapping, and the way of displayed the product in the markets. When comparing our results to other authors, the discrepancies could be partly due to differences in sampling techniques and the detection methods. Besides, slaughter hygiene, cross contamination of the products at different stages throughout the food chain should be considered. Variations in the prevalence of foodborne pathogens from different food samples in different studies could be due in part to several factors including; differences in the reservoir, ecological origin of pathogenic strains, sensitivity of detection methods, detected genes, number of samples, type of sample time of sampling (Zhao et al., 2001; Adwan et al., 2005) and storage conditions. The finding of high level of bacterial contamination as well as the occurrence of virulence factors in food pathogens strongly indicates the need for the implementation of surveillance programs for food products in Iraq. In conclusion, the mPCR is rapid, effective and sensitive method in detection of foodborne pathogens. Contamination of chicken meat and giblets indicate bad microbiological quality of retail chicken which may due to contamination occur during processing or distribution. It is necessary to put more emphasis on meat hygiene. Therefore, the surveillance of potential contaminant bacteria in different kinds of meat is crucial to safeguard the public health.

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