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Short Communication: Molecular identification and assessment of bacterial contamination of frozen local and imported meat and chicken in Basrah, Iraq using 16S rDNA gene

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Abstract. *Mohammad AJ, Alyousif NA. 2022. Short Communication: Molecular identification and assessment of bacterial contamination of frozen local and imported meat and chicken in Basrah, Iraq using 16S rDNA gene. Biodiversitas 23: 1598-1604.* Meat is the main food source of daily meals with highly beneficial effects on human health. It is susceptible to the growth and reproduction of various bacteria due to its composition, which leads to its spoilage, and being an important carrier of pathogenic bacteria. The study aimed to isolate and identify the bacteria contamination of frozen local and imported meat and chicken samples using 16S rDNA gene. Twenty meat samples were collected randomly from local and imported meat sold in markets of Basrah province, Iraq. The results showed that the total aerobic bacteria count was within acceptable limits in all samples. Fifty-seven bacterial isolates were isolated and identified by amplification and sequencing of 16S rDNA gene, where the gram-positive bacteria were the most abundant 34(59.6%), and *S. pasteuri* is the most common species in the samples with 14 (24%) bacterial isolates out of the total isolates. Fifteen isolates were identified as new strains and their sequences were deposited at GenBank (NCBI) under different accession numbers (MZ964582-MZ964596). The meat and chicken have many bacteria on their surface, therefore the products must be frozen, and sanitary precautions should be taken into consideration.

Keywords: Bacterial contamination, chicken, food spoilage, meat, 16S rDNA gene

INTRODUCTION

Meat is the main food source of daily meals with highly beneficial effects on human health (Kheyri et al. 2014). Meat of all kinds is considered decomposable food due to its chemical composition that is rich in protein, water, fats, minerals, and vitamins. This exposes it to internal enzymatic activity and makes it more sensitive to oxidation and invasion of bacteria (Doulgerakia et al. 2012). Meat constitutes a suitable medium for bacterial growth and reproduction due to the availability of the necessary growth factors such as mineral elements, neutral pH and high moisture (Mboto et al. 2012). Meat and meat products could be contaminated with bacteria during slaughter and cutting as well as in general from knives, air, workers, carts, boxes, and equipment (Gautam et al. 2019).

The two most important groups of bacteria are spoilage and pathogenic bacteria. Although there are no known health risks associated with spoilage bacteria, their presence can cause food to lose freshness and quality (Pennacchia et al. 2011). The most common foodborne pathogenic bacteria cause of bacterial infections in humans are *Escherichia*, *Salmonella*, *Shigella*, *Enterobacter*, *Klebsiella*, *Proteus*, *Serratia*, and *Yersinia* (Gautam et al. 2019). Haleem et al. (2013) isolated bacteria from frozen and fresh local and imported poultry meat in Iraqi markets including *Pseudomonas*, *E. coli*, fecal *Streptococcus*, *Staphylococcus*, *Salmonella*, and fecal coliform. According to Qader and AlKhafaji (2019), bacterial contamination, such as *K. pneumoniae*, *E. coli*, *Citrobacter freundii*, *Salmonella*, and *Shigella* were reported in imported chicken meat in Baghdad city. Ghazi (2020) demonstrated total count in red meat for *E. coli*, *K. pneumonia* and *Proteus* spp. was above permitted limit in Basrah city. The necessary procedure must be taken in storage, handling, and consumption of meat due to the rapid decomposition, spoilage, and contamination of meat with pathogenic bacteria and bacterial toxins that are transmitted to humans through their consumption under certain conditions (Abd-Elghany et al. 2015).

The polymerase chain reaction (PCR) and 16S rDNA gene sequencing technique, are currently being successfully utilized to identify the bacteria isolated from various sources. It is considered the best tool to identify bacterial isolates because they are sensitive, reliable and fast (Foysal and Lisa 2018; Alyousif 2022). Husain and Aziz (2021) used a universal 16S rDNA primer to identify the bacterial isolates that were isolated from meat and chicken frozen in Misan province, their results showed the PCR method which is better than traditional methods.

The bacterial contamination of frozen local and imported meat and chicken can constitute a problem for people health. The present study aims to determine the occurrence, prevalence of bacterial contamination from frozen local and imported meat and chicken sold in markets of Basrah by isolation and molecular identification of bacteria.

MATERIALS AND METHODS

Sample collection

The current study was conducted to detect the presence of bacteria in frozen local and imported meat and chicken in Basrah province, southern Iraq. Twenty samples of frozen products of different brands were collected randomly from the local markets from January to June 2021. The samples included six minced meat, ten chicken meat and four chicken liver (Table 1).

Bacterial isolation

Twenty-five grams of each sample was blended with 225 mL of sterile water and homogenized using a grinder under aseptic condition. One mL of each homogenized sample was added into a separate tube containing 9 mL of sterile water to perform tenfold serial dilutions (Shrestha et al. 2017).

Enumeration of total aerobic bacteria

The total aerobic bacteria were isolated by spreading 0.1 mL from appropriate dilutions on the surface of the nutrient agar plates (Himedia, India). The plates were incubated for 24-48 h at 37ºC. A colony counter was used to count the number of distinct colonies on each plate, the number of colonies ranging from 30 to 300 on each plate was accepted. Colony forming units (cfu) per g of sample were calculated using the formula as below: cfu per g: Number of colonies *Inverted dilution factor/ inoculum volume (APHA 2001).

Microscopic examination

The colonies with different morphologies were selected after incubation period, purified and stained with gram stain (Machfaddin 2000). Gram staining properties and the shapes of cells were observed, the isolates were grown on nutrient agar slants and stored at 4°C for further studies.

DNA extraction, PCR and electrophoresis

The 16S rDNA gene was used to identify the bacterial isolates up to species level. Chromosomal DNA was isolated using the PrestoTM Mini gDNA bacteria kit from the (Geneaid) company. The universal primers adopted from Miyoshi et al. (2005) 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3) were used to amplify DNA using the polymerase chain reaction (PCR) technique. PCR reactions were carried out in a total volume of 50 µL. An initial denaturation step of 96°C for 3 minutes was followed by 27 cycles of 96°C for 30 seconds, the annealing temperature of 56°C for 25 seconds, extension at 72°C for 15 seconds, and a final extension of 72°C for 10 minutes (Miyoshi et al. 2005).

The PCR products of the 16S rDNA gene were separated using a 1% (w/v) agarose gel-based on molecular weight, which was made with TBE buffer. DNA bands were visualized under UV light using ethidium bromide stain. The amplified 16S rDNA genes were purified and sequenced by the Macrogen Company (South Korea).

Data analysis

The obtained 16S rDNA gene sequences were proofread and compared with nucleotide sequences of NCBI using BLAST tools from http://www.ncbi.nlm.nih.gov to assess the sequence homology and identification of isolates.

RESULTS AND DISCUSSION

Isolation and enumeration of total aerobic bacteria

The detection of the total number of bacteria in meat products indicates the extent of contamination and the suitability of the products for human consumption. The high number of bacteria reflects the poor health conditions that the animal is exposed to before and after slaughter, while the low number reflects the good conditions. The total number of aerobic bacteria in samples of local and imported minced meat sold in the markets of Basrah province is shown in Table 2. The results showed that the total number of aerobic bacteria was within acceptable limits, which counted to $\geq 10^7$ according to APHA (2001). The imported minced meat had more bacteria than the local minced meat (Table 2). This result is similar to Abid Ali et al. (2013) in their study in Baghdad city. Bacterial spoilage of meat depends on the initial number of bacteria, time, temperature, storage conditions and chemical and physical properties of meat (Doulgeraki et al. 2012).

The imported chicken meat and chicken liver contain more bacteria than other imported and local chicken (Table 3). The result is similar to Azize et al. (2016) based on their study in Hilla city, that frozen Turkish chicken was the most contaminated with bacteria at a rate of 21.4%. Mohamed et al. (2017) is also found that Sadia chicken was less contaminated than the rest of the imported chicken. The contamination of meat and chicken with bacteria is attributed to several reasons, including transportation, storage, manual handling, and exposure for long periods away from sanitary conditions (Qader et al 2019).

Table 1. Frozen meat products in Basrah markets, Iraq used in the study

Samples	Local	Imported
Minced meat	5	1
Chicken	4	6
Chicken's livers	2	2

Table 2. The number of total aerobic bacteria in minced meat (cfu/g)

Source	Number of bacteria (cfu/ g)
Local	33×10^2
Local	41×10^2
Local	73×10^2
Local	67×10^2
Local	92×10^2
Imported	106×10^2

Table 3. The number of total aerobic bacteria in chicken meat and chicken liver (cfu/g)

Source	Number of bacteria (cfu/ g)
Local	43×10^2
Local	32×10^2
Local	86×10^2
Local	62×10^2
Imported	31×10^2
Imported	57×10^2
Imported	93×10^2
Imported	68×10^2
Imported	82×10^2
Imported	78×10^2
Local	55×10^2
Local	35×10^2
Imported	73×10^2
Imported	42×10^2

Morphological and molecular Identification bacterial isolates

Fifty-seven bacteria were isolated and purified from the collected samples. The result of Gram staining of bacterial isolates revealed that gram-positive bacteria were the most abundant, and their number was 34 (59.6%) and 23 (40.4%) of gram-negative bacterial isolates. The isolation of 28 bacterial isolates (49.1%) from local and imported chicken meat, 18 bacterial isolates (31.6%) from local and imported minced meat, and 11 bacterial isolates (19.3%) from local and imported chicken liver. The bacterial isolates were identified by amplification and sequencing of 16S rDNA gene of all bacterial isolates. The results of identification showed that the most abundant bacterial genus in the samples was *Staphylococcus* with a number of 22 of the total bacterial isolates (38.5%), where *S. pasteuri* is the most common species in the samples with 14 bacterial isolates (24%) out of the total isolates (Table 4). This result is similar to Mohamed et al. (2017) that the imported chicken had a high percentage of bacterial contamination of *Staphylococcus*, especially *S. aureus* in Salah Al-Din Governorate and *S. epidermidis* was also found in a few samples.

Table 4. Gram-positive bacteria isolated from minced meat, chicken meat, and chicken liver

Bacterial species	Number of isolates	% of bacterial species
Staphylococcus pasteuri	14	24.5
Staphylococcus warneri	4	7.0
Staphylococcus haemolyticus	3	5.2
Staphylococcus epidermidis	1	1.7
Enterococcus faecalis	2	3.5
Enterococcus faecium	1	1.7
Enterococcus durans	1	1.7
Enterococcus aguimarinus	1	1.7
Macrococcus caseolyticus	2	3.5
Lactococcus garvieae	1	1.7
Carnobacterium divergens	1	1.7
Kurthia gibsonii	1	1.7
Rothia endophytica	1	1.7
Kocuria rhizophila	1	1.7

Four species of *Enterococcus* could be isolated from the samples in present study (Table 4). *Enterococcus* is part of the normal flora bacteria in the digestive system of humans and animals, and it may also be found in the skin and reproductive system. It is one of the opportunistic pathogens that causes infection significantly when the host's immunity is compromised (Robbins et al. 2012). Tyson et al. (2018) demonstrated that more than 90% of meat samples were contaminated with *Enterococcus* especially *E. faecium* and *E. faecalis* in the United States in periods 2002 to 2014.

Two isolates of *Macrococcus caseolyticus* are isolated in meat samples of current study (Table 4). *M. caseolyticus* is a Gram-positive, closely related to *Staphylococcus* and increasingly recognized as veterinary pathogens (MacFadyen et al. 2018). Organji et al. (2018) isolated *M. caseolyticus* from meat and dairy products produced in Saudi Arabia. One isolate of both *Lactococcus garvieae* is isolated in minced meat sample of current study (Table 4). The species is found as natural microbiota in GIT of both humans and animals. It causes a septicemic called lactococcosis in humans (Abdelfatah and Mahboub 2018). *Lactococcus garvieae* and *Carnobacterium divergens* are lactic acid bacteria and both associated with meat spoilage (Andreevskaya et al. 2018). *Kurthia gibsonii* is recorded in one meat sample of the current study. This bacterium is isolated in previous studies from poultry meat and smoked fish (Maïworé et al. 2021; Lozica et al. 2022). One isolate of *Rothia endophytica* is recorded in meat sample. It is an aerobic coccoid to rod-shaped, non-motile and non-sporogenic, Gram-positive bacterium, it could be isolated from different sources such as food, plants, and soils (Xiong et al. 2013; Augustynska-Prejsnar et al. 2020).

Kocuria rhizophila isolate is recorded in meat sample of current study. This bacterium is isolated in previous study from foods and found to be show antimicrobial resistance, which increases the risk of gene transfer to pathogens in foods (Ramos et al. 2021). *Kocuria* species exist as coexisting bacteria, however many species of *Kocuria* have emerged as important pathogens responsible for many diseases such as endocarditis, meningitis, cystitis, urinary tract infection, and bacteremia (Purty et al. 2013; Dotis et al. 2015; Kandi et al. 2016).

The results of identification showed 5 isolates of *E. coli* (8.7%) in samples of minced meat, chicken meat, and chicken liver, in addition to that, 19 bacterial isolates belonging to 9 genera of the Enterobacteriaceae family were isolated and identified, including (*Escherichia*, *Enterobacter*, *Klebsiella*, *proteus*, *Pantoea*, *Citrobacter*, *Atlantibacter*, *Hafnia* and *Serratia*) and 4 other isolates belonging to 3 genera including *Atlantibacter*, *Acinetobacter* and *Pseudomonas* (Table 5).

Escherichia coli is gram-negative, rod-shaped bacteria that inhabit the intestine of warm blood animals. Its presence in meat sample refer to fecal contamination, and some of its strains can cause many diseases and diarrhea (Lubote et al. 2014; Wester et al. 2014). Enterobacteriaceae members are the most challenging and prevalent bacterial contaminants in raw and processed meat worldwide. *Salmonella*, *Klebsiella*, *Enterobacter*, and *E. coli* are the

most prevalent in food poisoning cases associated with meat and meat products (Al-mutairi 2011). Schwaiger et al. (2012) found that 80% of chicken meat samples were contaminated with Coliform bacteria, especially *E. coli*. This indicates that fecal contamination cannot be prevented completely through skinning processes. The species of genera *Klebsiella*, *Enterobacter* and *Citrobacter* are characterized as the dominant species of coliform bacteria in slaughterhouse samples according to their predominance in animal feces.

Five isolates of *Proteus* isolated from meat samples are belonging to *P. mirabilis*, *P. vulgaris*, and *P. penneri* (Table 5). *Proteus* is opportunistic pathogens for humans. The contamination of meat products with *Proteus* refers to fecal contamination and may cause poisoning when consuming *Proteus* contaminated water and food (DrZewiecka 2016). Study conducted by Jamaluddin et al. (2018) in Makassar city, Indonesia showed that 12.5% of chicken meat samples were contaminated with *P. mirabilis*. *Atlantibacter hermannii* is a rod-shaped Gram-negative bacteria. It is generally considered nonpathogenic bacteria but has been isolated from human wounds and eye infections (Girlich et al. 2021). *Acinobacter johnsonii* are ubiquitous Gram-negative coccobacilli. It is distributed in soil and water, as free-living saprophytes but it was occasionally recognized as agent of animal infections (Carvalheira et al. 2016).

Klebsiella pneumoniae is spread through food and is considered a foodborne pathogen. These bacteria can be found in seafood, frozen foods, and fresh chicken meat (Guo et al. 2016). Rafei et al. (2015) isolated different *Klebsiella* species from dairy products, such as milk, cheese, fish, and meat samples. *K. pneumoniae* is found in raw vegetables, powdered infant milk, meat, fish, and street foods, where it has been considered a foodborne pathogen (Davis and Price, 2016). These bacteria possess a variety of virulence factors, including capsule, endotoxin and iron removal systems. The capsule is an important virulence factor as it has two pathogenic mechanisms, i.e. the first is to protect bacteria from phagocytosis, and the second is to directly inhibit the host's immune response (Kang et al. 2015). Therefore, dietary intake is one of the ways to introduce antibiotic-resistant bacteria and their genes into the human digestive system (Milanovic et al. 2017). *Pantoea agglomerans* a plant-associated bacteria. It could be a cause of opportunistic human infections, usually by wound infection with plant material, or as a hospital-acquired illness, mostly in immunocompromised persons (Mardaneh and Dallal1 2013).

The current study recorded the isolation of *Citrobacter gillenii* (Table 5). Food can be an essential source for species of *Citrobacter* that is transmissible to humans (Liu et al. 2017). Hashim and Al khafaji (2018) isolated another species of *Citrobacter* bacterium namely *C. freundii* from chicken meat after collecting 25 samples of chicken meat randomly from Local markets in Baghdad city.

Table 5. Gram-negative bacteria isolated from minced meat, chicken meat, and chicken liver

Bacterial species	Number of isolates	% of bacterial species
Escherichia coli	5	8.7
Proteus mirabilis	3	5.2
Proteus valgaris	1	1.7
Proteus penneri	1	1.7
Atlantibacter hermanii	2	3.5
Acinobacter johnsonii	2	3.5
Hafnia paralvei	2	3.5
Klebsiella pneumoniae	1	1.7
Pantoea agglomerans	1	1.7
Pseudomonas fragi	1	1.7
Pseudomonas tolaasii	1	1.7
Citrobacter gillenii	1	1.7
Serratia marcescens	1	1.7
Enterobacter hormaechei	1	1.7
Hafnia alvei	1	1.7

The presence of *Serratia marcescens* in food is a cause for concern because these bacteria have been identified as potential pathogens capable of causing cytotoxic and inflammatory effects similar to those produced by classical pathogens (Ochieng et al. 2014). As for *Hafnia alvei* bacteria, the current study agreed to Lindterg et al. (1998), that *H. alvei* isolated from minced meat, meat products, and packaged beef are responsible for spoiling meat packed under low oxygen. *Pseudomonas fragi* is Gram-negative bacterium that is able to grow at low temperature. It can produce several enzymes that are responsible for the spoilage of meat and fish (Wang et al. 2017). While *Pseudomonas tolaasii* is the main pathogen causing brown bacterial blotch disease in several varieties of cultivated mushrooms (Yun et al. 2013).

The imported chicken product is the most contaminated with different species of bacteria (Table 6). This result is similar to Azize et al. (2020) based on their study in Hilla city, that frozen Turkish chicken was the most contaminated with bacteria. Mohamed et al. (2020) recorded the contamination of Turkish chicken with *S. aureus*, as it contained more significant numbers of these bacteria than other species in Tikrit city, Iraq. Mohamed et al. (2017) was also reported that the Brazilian Sadia chicken has the lowest contamination rate, which agree to the current study.

The presence of bacteria in local and imported samples due to the absence or poor hygiene practices in slaughter, processing, and evisceration as minced meat. These are the main reasons for the high surface contamination of beef carcasses by pathogenic and non-pathogenic bacteria (Niyonzima et al. 2013). As well as other reasons associated with storage conditions of meat products, such as inappropriate temperature and adopting incorrect storage conditions during import or circulation until it reaches the consumer (Qader et al 2019).

The GenBank accession numbers of new isolates

The current study showed the isolation and identification of 15 new isolates by amplification and sequencing of 16S rDNA gene and they were deposited in the NCBI GenBank database under the Genbank accession numbers. Six isolates were isolated from chicken livers, five from chicken meat, and four from minced meat (Table 7).

The DNA sequence may be changed as result of exposure to chemical mutagens, leading to inherited damage in DNA (Najafi and Pezeshki 2013). It occurs when bacteria are exposed to environmental variables and chemical mutagens such as radiation, heat, and HNO_2, which causes them to lose their ability to repair DNA damage and become inherited (Ilmjärv et al. 2017). This result enrich the 16S rDNA gene sequence data from frozen meat and chicken in Iraq after Husain and Aziz (2022) reported sixteen bacterial isolates as new isolates in GeneBank based on 16S rDNA gene from the markets in Misan province, Iraq.

Table 6. The species of bacteria isolated from minced meat, chicken meat, and chicken liver of different local and imported products

Product type	Source	Number of isolates	Bacterial species
Minced meat	Local	2	*Staphylococcus pasteuri*
		1	*Atlantibacter hermanii*
Minced meat	Local	1	*Staphylococcus pasteuri*
Minced meat	Local	1	*Pseudomonas fragi*
		1	*Enterococcus faecium*
		2	*Staphylococcus pasteuri*
Minced meat	Local	2	*Proteus mirabilis*
Minced meat	Local	1	*Macrococcus caseolyticus*
		1	*Carnobacterium divergens*
		1	*Pseudomonas tolaasii*
		1	*Hafnia alvei*
Minced meat	Imported	1	*Enterococcus faecalis*
		1	*Lactococcus garvieae*
		1	*Kurthia gibsonii*
		1	*Staphylococcus warneri*
Boneless chicken pieces	Local	1	*Staphylococcus pasteuri*
		1	*Citrobacter gillenii*
Whole chicken	Local	1	*Kocuria rhizophila*
		1	*Staphylococcus epidermidis*
		1	*Proteus mirabilis*
Chicken wings	Local	1	*Staphylococcus pasteuri*
Whole chicken	Local	1	*Staphylococcus pasteuri*
		1	*Escherichia coli*
Chicken breasts	Imported	1	*Proteus valgaris*
Chicken breasts	Imported	1	*Macrococcus caseolyticus*
		2	*Staphylococcus pasteuri*
Chicken breasts	Imported	1	*Pantoea agglomerans*
		1	*Rothia endophytica*
		1	*Staphylococcus haemolyticus*
		1	*Acinobacter johnsonii*
		1	*Hafnia paralvei*
Chicken breasts	Imported	1	*Enterococcus aguimarinus*
		1	*Staphylococcus haemolyticus*
		1	*Staphylococcus warneri*
		1	*Escherichia coli*
Chicken breasts	Imported	1	*Staphylococcus pasteuri*
		1	*Staphylococcus warneri*
		1	*Proteus penneri*
Chicken breasts	Imported	1	*Acinobacter johnsonii*
		2	*Staphylococcus pasteuri*
		1	*Staphylococcus haemolyticus*
Chicken's liver	Local	1	*Enterococcus durans*
Chicken's liver	Local	3	*Escherichia coli*
Chicken's liver	Imported	1	*Staphylococcus warneri*
		1	*Enterococcus faecalis*
Chicken's liver	Imported	1	*Serratia marcescens*
		1	*Staphylococcus pasteuri*

Table 7. Fifteen new 16S rDNA sequences of bacterial isolates were deposited in the NCBI GenBank

Product type	Source	Accession no.	Bacteria name
Minced meat	Local	MZ964582	*Enterococcus faecium* strain ANSNAS1
Chicken	Local	MZ964583	*Citrobacter gillenii* strain ANSNAS2
Chicken's liver	Local	MZ964584	*Escherichia coli* strain ANSNAS3
Chicken's liver	Imported	MZ964585	*Serratia marcescens* strain ANSNAS4
Chicken's liver	Imported	MZ964586	*Enterobacter hormaechei* strain ANSNAS5
Chicken	Local	MZ964587	*Escherichia coli* strain ANSNAS6
Chicken's liver	Imported	MZ964588	*Atlantibacter hermanii* strain ANSNAS7
Minced meat	Local	MZ964589	*Atlantibacter hermanii* strain ANSNAS8
Chicken's liver	Imported	MZ964590	*Staphylococcus warneri* strain ANSNAS9
Chicken's liver	Imported	MZ964591	*Enterococcus faecalis* strain ANSNAS10
Minced meat	Imported	MZ964592	*Staphylococcus warneri* strain ANSNAS11
Chicken	Imported	MZ964593	*Acinobacter johnsonii* strain ANSNAS12
Chicken	Imported	MZ964594	*Acinobacter johnsonii* strain ANSNAS13
Chicken	Imported	MZ964595	*Staphylococcus warneri* strain ANSNAS14
Minced meat	Local	MZ964596	*Hafnia alvei* strain ANSNAS15

In conclusion meat and chicken in local markets in Basrah province, Iraq contain bacteria and imported samples were contaminated higher than local samples. Several pathogenic and spoilage bacteria were isolated and identified. Some of bacteria cause great public health concern and spoilage of food by changing its taste and smell, that lead to the economic losses. Fifteen isolates were identified as new strains and their sequences were deposited at GenBank (NCBI). Sanitary precautions should be used in meat control and preservation. It must be taken in consideration that all frozen red meat and chicken meat have many bacteria on their surfaces, therefore they must be refrigerated and frozen.

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