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Molecular detection of staphylococcus aureus enterotoxin genes

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***Abstract*---**In this work, S. aureus isolates from meat and meat products were examined for frequency, profiles of antibiotic susceptibility, and virulence genes (seb, sea, sec, sed, see, and fme A). Staphylococcal food poisoning is one of the most economically important foodborne diseases in the world. S. aureus is a prominent food-borne pathogen and common food contamination across the world. Some S. aureus strains generate staphylococcal enterotoxins (SEs), which can cause staphylococcal food poisoning (SFP). Staphylococcus aureus (S. aureus) is one of the most frequently studied foodborne bacteria due to its high pathogenicity, production of heat-stable enterotoxins, and its continued development of resistance to multiple antibiotics. Despite enterotoxigenic staphylococci being thermally killed, cooked beef products may still contain staphylococcal enterotoxins SEs since these toxins are thermo persistent and cannot be removed by heat processing. Therefore, this investigation into the frequency of MRSA genes in certain fresh meat and canned meat was done to learn more about the disease. A total of 80 random samples, Twenty meat samples from butchers, and sixty samples each of Luncheon, Tune, Chicken pieces, Minced meat, Grilled meat, Hamburger Fresh meat and Sausage from some supermarkets in Hilla city in Iraq. The obtained results showed that among all the samples that were examined, 50% are from the family of Staphylococcus. Antimicrobial susceptibility testing of 40 staphylococcal isolates against different antibiotics clearly showed high susceptibility to Ciprofloxacin (CIP) (100%), Levofloxacin (LEV) (100%), Nitrofurantoin (F300) (100%), Gentamicin (95%), and Nitrofurantoin (F100) (82.5%). While high resistance was observed against Penicillin G (100%), Trimethoprim (5-10) (100%), followed by Erythromycin (90%), Rifampicin (RA) (90%), Chloramphenicol (C) (85 %) Clindamycin (AD)(82.5%), Tetracycline(TE) (77.5 %) ,Vancomycin (VA)(70%), and Oxacillin (65%).

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***Keywords*---**foodborne illness, staphylococcus aureus, enterotoxin gene antibiotic resistance.

Introduction

Foodborne illness caused by bacterial contamination is currently one of the most serious threats to human health and food safety(Wu et al.,2016). Bacterial contamination of meat is unavoidable owing to current processing circumstances, which expose meat to contaminated working surfaces, equipment, inadequate personal hygiene, and poor water quality(Endale and Hailey, 2013; Ruban,2018). The human body is home to a wide range of bacterial populations, both dangerous and beneficial. For this reason, in recent decades, the stresses associated with various parts of the body under various health conditions have been fully defined. Staphylococcus aureus, a normally innocuous commensal bacterium, is becoming more well acknowledged as an important opportunistic virus that causes a variety of illnesses across the world Momani, 2021;G Abril et al.,2020). Staphylococcus aureus is one of the most frequent causes of bacterial food poisoning in the United States, where there are yearly estimates of 241,148 cases and 6 deaths. (Wang et al 2018; Fetsch et al.,2014; Tiemersma et al., 2004). Staphylococcus aureus is a Gram-positive coccus with a cluster-shaped organization that has been described as the principal causative agent of a variety of clinical disorders across the world. This bacterium causes a lot of infections, both in the community and in hospitals. Unfortunately, because of the spread of microorganisms that are multi-drug resistant (MDR), therapy remains difficult. (Taylor and Unakal, 2020) S. aureus possesses virulent aggravating properties, produces enterotoxins, and is resistant to antibiotics, as well as having proteolytic and lipolytic activity at various temperature settings, causing food spoiling (Puah et al., 2016). Methicillin-resistant S. aureus is one of the most common nosocomial pathogens, causing food poisoning, post-operative wound infections, pneumonia, and nosocomial infections across the world (Turner et al., 2010). The virulence factors associated with drug resistance and affinity for staphylococcal enterotoxin production are referred to as S. aureus pathogenicity (Cheung et al., 2021). Staphylococcal food poisoning (SFP) occurs when S. aureus infects food, replicates, and produces extracellular heat-stable enterotoxins, rendering the food deadly despite its appearance (Zeaki et al., 2019). SFP symptoms include nausea, vomiting, and diarrhea, which are caused by Staphylococcal enterotoxins (SEs), most of which are type-A (CDC, 2018). PCR is a precise and effective method that can be used to classify and identify Staphylococcus aureus isolates. It has been used as a rapid detection method for Methicillin-Resistant Staphylococcus aureus (MRSA) can produce thousands to millions of copies of a certain DNA sequence by amplifying a single or a few copies of a fragment of DNA over multiple orders of magnitude (Ali et al., 2014). Therefore, this study was conducted to investigate the occurrence of pathogenic bacteria, especially drug-resistant (MDR) strains in fresh and canned meat, and detect the fem A gene for molecular identification of S. aureus.

Materials and Methods

Samples Collection

Eight meat samples were randomly collected from different markets and butcher shops in Hilla city in Iraq, including canned luncheon meat 15 (25%), sausage 10 (16.6), tuna 9(15%), Chicken nuggets pieces 9(15%), Minced meat 7(11.6), roasted meat 5 (8.3), and hamburger 5 (8.3%) and 20 (25%)sample from butcher Figure 1. the samples were collected during the period from November 2021 to January 2022 by using sterile, clean and dry.

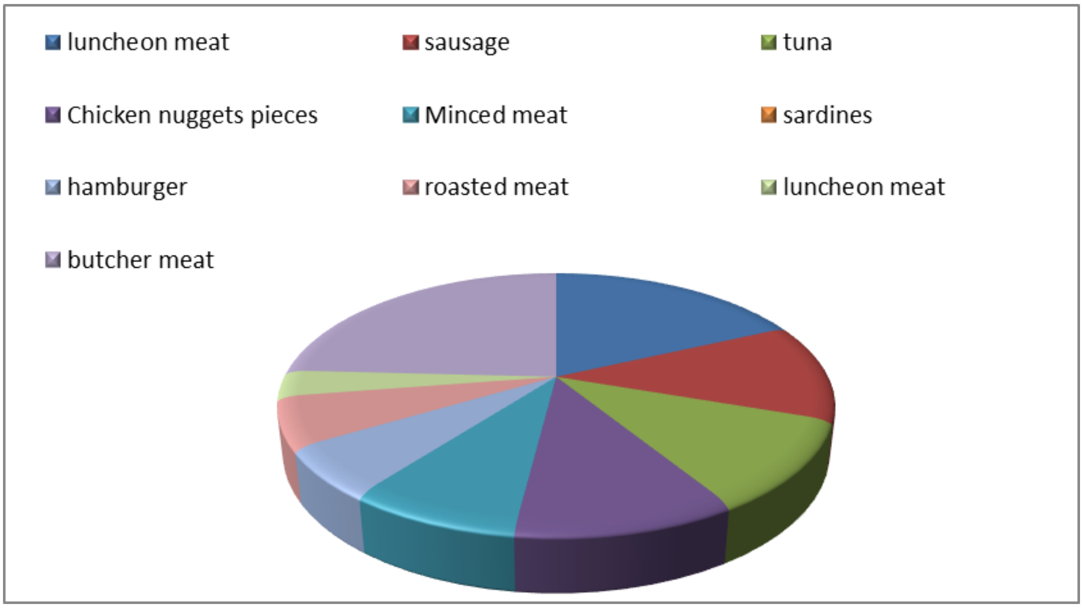

Figure 1. Types Distribution of food samples from meat and meat products Discussion

Preparation of samples and Isolation of bacteria

One gram of the material was weighed and homogenized before being dissolved in nine milliliters of sterile distilled water. The sample was thereafter serially diluted. With the use of Pasteur pipettes, an aliquot of 100µL of the 105, and 106 dilution was transferred into a sterile Petri dish, and the sterilized molten medium Nutrient agar and Chapman culture media (Mannitol salt agar) was put into each labeled Petri dish at 37°C. The plates were inverted and incubated for 24-48 hours under aerobic conditions to promote bacterial growth. (Samaha et al., 2012; Khalafallah et al., 2020).

Identification of bacteria isolates

Then Gram stain was done for all samples, as well as biochemically characterized for catalase (using 3% hydrogen peroxide), oxidase testing and maltose fermentation before stock preparation (Nair and Surendran, 2005).

Antibiotic Susceptibility Testing

The Kirby-Bauer disc diffusion procedure was used to assess the antibiotic susceptibility of S. aureus isolated from canned foods. antibiotic discs were used in this study were 51 discs as shown in Table 1 of antibiotics, their classification, abbreviation, and. Potency. results were reported according to Clinical and Laboratory Standards Institute guidelines (CLSI,2021). Colonies (2-4) of the bacterial isolate were transferred to a test tube containing 5 ml of Nutrient Broth and incubated at 37 °C for 2 hours then growth was reduced with physiological saline, and the growth in the tube was compared with the Macfarlane tube (0.5) standards. The bacteria were spread on the Muller Hinton Agar by a cotton swab dipped in the broth. Then the discs were placed around the plate at the rate of four tablets and one in the center. The dishes were kept at 37°C for 16-18 hours for all types of antibiotics. Then the damping diameters were measured using a measuring ruler and compared with the standard values. Results were reported based on the CLSI guidelines.

Table 1
Antibiotics, abbreviations, and potency

Antibiotic	Abbreviation	Potency (Disc load (µg))
Cefalexin	FOX	30
Erythromycin	E	15
Chloramphenicol	C	30
Ciprofloxacin	CIP	10
Trimethoprim	TMP	5 - 10
nitrofurantoin	F	100 - 300
Penicillin G	P	10
Clindamycin	DA	15
Vancomycin	VA	30
gentamicin	CN	10
Rifampicin	RA	5
Tetracycline	TE	10
Levofloxacin	LEV	5

Genomic DNA Extraction

Staphylococci were incubated in Luria broth and incubated at 37 °C for 24 hours, then 1 milliliter of cell suspension was transferred to a 1.5 mL Eppendorf tube for bacterial DNA extraction by silica-based membrane technology DNA extraction method Extraction of genomic DNA was performed according to the protocol provided with the Genome Kit.

Detection of sea, sec, see, sed, seb, 16SrRNA and fem A in S. aureus isolates by PCR

Using the primers given in Table 2, the presence of the staphylococcal enterotoxin genes sed, see, sec, sea, and seb was determined Table 3. A single PCR was carried out. There were 25 µl of the reaction mixture in each PCR tube Table 2.

Table 2
Contents of the reaction mixture of PCR

NO.	Contents of the reaction mixture		Volume
1	master mix		12.5µl
2	Primer	forward	2 µl
3		Reverse	2 µl
4	DNA template		4µl
5	Nuclease free water		4.5µl
Total volume			25µl

Table 3
Primer pairs, sequences, and Amplicon Size of Phylogenetic typing.

Target	Primer sequence (5′→3′)	Amplicon size (bp)	References
femA	AAAAAAGCACATAACAAGCG GATAAAGAAGAAACCAGCAG	132	Da Silva et al.,2005
sea	CCTTTGGAAACGGTTAAAACG TCTGAACCTTCCCATCAAAAAC	127	Omoe et al., 2005
seb	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	477	Omoe et al., 2005
sec	CTCAAGAACTAGACATAAAAGCTAGGT CAAAATCGGATTAACATTATCC	271	Omoe et al., 2005
sed	CTAGTTTGGTAATATCTCCTTTAAACGT TAATGCTATATCTTATAGGGTAAACATC	319	Omoe et al., 2005
see	CAGTACCTATAGATAAAGTTAAAACAA GCTAACTTACCGTGGACCCTTC	178	Omoe et al., 2005
16sRNA	TATGGAGGAACACCAGTGGCGAAG TCATCGTTTACGGCGTGGACTACC	791	Abdel-Tawab et al., 2016

PCR Thermal cycling conditions for the detection of different gene in Staphylococcus aureus in Table 4.

Table 4
Primer for characterization

Gene type	Conditions	Reference
Fem A	Step 1: 95.0°C, 2 min. Step 2: 95.0°C, 30 sec. Step 3: 63.9°C, 30 sec. Step 4: 72.0°C, 20.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72.0°C, 5 min. Step 7: 4.0°C, forever	Mori *et al.*,2014
Sea ***Seb*** ***Sec***	Step 1: 95°C, 2 min. Step 2: 95°C, 30 sec. Step 3: 52.1°C, 30 sec. Step 4: 72°C, 20.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72.0°C, 5 min. Step 7: 4.0°C, forever	**Haghi *et al.*,2021**
Sed	Step 1: 95°C, 2 min. Step 2: 95°C, 30 sec. Step 3: 59.1°C decrease 0.5°C per cycle, 30 sec. Step 4: 72°C, 30.0 sec. Step 5: Repeat steps 2-4 14 more times Step 6: 95°C, 30 sec. Step 7: 52.1°C, 30 sec. Step 8: 72°C, 30.0 sec. Step 9: Repeat steps 6-8 19 more times Step 10: 72°C, 5 min. Step 11: 4°C, forever	Haghi *et al.*,2021
see	Step 1: 95.0°C, 2 min. Step 2: 95.0°C, 30 sec. Step 3: 57.8°C, 30 sec. Step 4: 72.0°C, 40.0 sec. Step 5: Repeat steps 2-4 29 more times Step 6: 72.0°C, 5 min. Step 7: 4.0°C, forever	Haghi *et al.*,2021

Results and Discussion

Counting of bacteria

After incubation period the bacterial colonies were counted according the following equation:
CFU / mL= No. of colonies * dilution factor/inoculum volume (Hafez et al., 2020).
The highest bacterial count was recorded with 4.08 × 108 CFU\g from Tazaj beef luncheon samples, the lowest number was in fresh meat 1×108.

The highest levels of bacterial contamination were in Tazaj beef luncheon, Esalat chelavra, Chicken luncheon altazaj althabi, Sardines, Silou tuna and Chicken pieces Nasma, where the number of colonies exceeded the permissible numbers. Where the majority of canned foods were on the shelves at room temperature, as the temperature is very high in the summer in Iraq, which allows to grow the bacteria at the appropriate temperature. As for frozen meat products, in which the number of bacteria increased, this is due to several reasons, the most important of which is freezing and thawing the products and re-freezing them again due to the fluctuation of electric current.

Frequency of S. aureus in meat and meat products

All samples were cultivated on mannitol salt agar, the colonies of primary cultures were purified, and out of 80 samples, 22 isolates showed pinpoint colonies with yellow discoloration of the medium Figure 2. All of the isolates had gram-positive cocci with cluster organization on a microscopic level figure. The identification of the 16SrRNA housekeeping gene product, which corresponds to a 791 bp band size, on all of the isolated S. aureus was used to establish their identity Table 5.

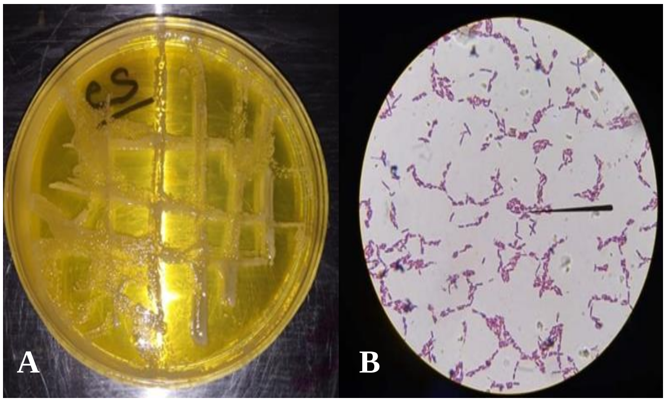

Figure 2. (a) typical culture growth on mannitol salt agar and (b) microscopic appearance upon grams staining.

Table 5
Number and percentage of Staphylococcus aureus isolated from different meat samples

No	Sample	Number of isolates	Percentage
1	Luncheon	4	50%
2	Sausage	2	50%
3	Tuna	3	75%
4	chicken pieces	2	50%
5	Minced meat	3	75%
6	grilled meat	2	50%
7	Hamburger	1	50%
8	fresh meat	4	35%

Staphylococcus aureus isolated from the Luncheon Sausage, Tuna, chicken pieces, Minced meat, Grilled meat, Hamburger, and Fresh meat respectively 4, 2, 3, 2, 3, 2, 1, and 4. The prevalence of S. aureus was closer to the present study which has been reported by Sahin et al. (2020) in Turkey (32%), Wang et al. (2013) in China (24.2%), Naas et al. (2019) in Libya (32%), another study that reported by (mathenge et al .,2015) in which an overall of 36% in meat and milk production was found in Nairobi county its surroundings, and higher than (Omwenga, 2022) who found the prevalence of Staphylococcus aureus was (20.3%).

Antibiotic susceptibility test (AST)

Antibiotic susceptibility. testing was performed using 15 types of antibiotics. The results showed that the highest resistance was against Trimethoprim (TMP5)100%, Trimethoprim (TMP10)100%. and a penicillin G (P)100%, followed by Erythromycin (E) (91.25%), Clindamycin (AD) (90%) ,chloramphenicol (C) (88.75%) , Vancomycin (VA) (85%), tetracycline (TE) (82.5%) , Oxacillin (OX) (80%), the lowest resistance against Nitrofurantoin (F-100) (13,75%), rifampicin (RA) (5%), and gentamicin (CN) 1.25%), and no isolates found resistant to Nitrofurantoin (F300), Ciprofloxacin (CIP), and Levofloxacin (LEV) which make them a good choices for treatment Table 6.

Table 6
Antibiotics susceptibility test of bacterial isolates.

No.	Antibiotic	Resistant	Sensitive	Intermediate
1	Trimethoprim (TMP)5	(100%)	-----	
2	Trimethoprim (TMP)10	(100%)	-----	
3	Penicillin G (P)	(100%)	-----	
4	Erythromycin (E)	(91.25%)	(8.75%)	
5	Clindamycin (AD)	(90%)	(10%)	
6	Chloramphenicol (C)	(88.75%)	(11.25%)	
7	Vancomycin (VA)	(85%)	(15 %)	
8	Tetracycline (TE)	(82.5%)	(17.5%)	
9	Oxacillin (OX)	(80%)	(20%)	
10	Nitrofurantoin (F-100)	(13,75%)	(86,25%)	
11	Rifampicin (RA)	(5%)	(88,75%)	(6,25%)
12	Gentamicin (CN)	(1.25%)	(98,75%)	
13	Nitrofurantoin (F300)	----	(100%)	
14	Levofloxacin (LEV)	----	(100%)	
15	Ciprofloxacin (CIP)	----	(100%)	

From table 4, It has been found that Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria, and this agrees with each of the following research (Zhou, 2021; Breijyeh et al.,2020), Because of the increased protection provided by the outer membrane, Gram-negative bacteria are more resistant to antimicrobial agents than Gram-positive bacteria. The Methicillin Resistant S. aureus is identified utilizing the Oxacillin disc antibiotic, according to (Palavecino, 2020).

Drug-resistant Staphylococcus spp. are one of the main dangers to public health because they cause nosocomial and other communicable diseases Sarrafzadeh et al., 2014. The greatest rate of penicillin resistance was found in the current investigation (100 %). animals and humans both use penicillin to cure illnesses. However, bacterial resistance has been caused through abuse of this antibiotic.

PCR Detection of 16s rRNA Gene

Using a particular housekeeping gene primer (16s), all samples were determined to be S. aureus as reported in Table 1. Unfavorable samples were not included.

The 25 µL combination of 2 µ L Maxime PCR Premix, 0.5 µ L of each primer, 2 µ L of template DNA, and 20 µ L of double-distilled water was used to accomplish the DNA amplifications. Three phases were included in the amplification process: a preliminary denaturation step at 95 °C for 2 min; 35 successful cycles of denaturation at 95 °C for 30 sec, annealing at 63.9 °C for 30 sec, and extension at 72 °C for 30 sec; and the concluding extension step at 72 °C for 5 min.After isolating 22 samples of Staphylococcus aureus identified by 16s RNA, the result was positive as shown in the figure. The results in Figure-1 show that 22 isolates of S. aureus gave positive resultsfor16S rRNA as a single DNA band of PCR product with amolecular base of 791 bp.

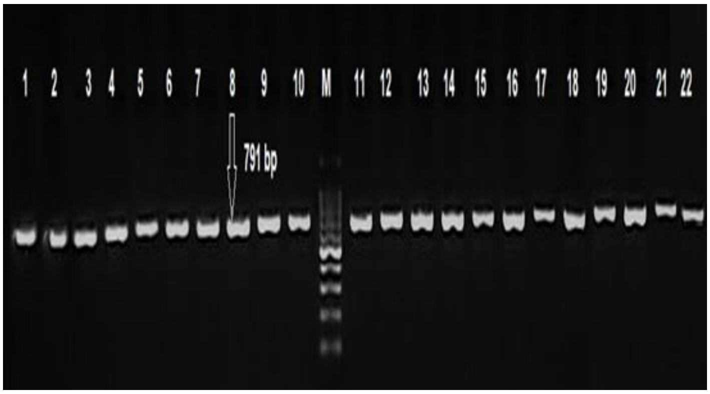

Figure 2. PCR amplification of 16SrRNA gene of S. aureus on 1.5% Agarose gel electrophoresis

Distribution of Enterotoxin genes in S. aureus isolates

Enterotoxins are one of the most significant factors that determine the pathogenicity of S. aureus (SE). The protein architectures of about 15 different forms of SE have been identified Pourmand et al., 2009. A standard PCR experiment was used to further investigate the isolates for the presence of the fem A and enterotoxin genes using a specified starting material. The fem A gene of Staphylococcus aureus was not found. However, an isolate of S. aureus showed the presence of an enterotoxin produced from the gene sea, seb, sec, sed, see, and femA Table7.

Table 7
Staphylococcus aureus isolates with enterotoxin-producing genes are frequently found in meat and meat products.

Toxin gene	Type of meat sample								Total
	luncheon	sausage	tuna	chicken pieces	Minced meat	grilled meat	hamburger	fresh meat	n=(22)
sea	2(13.3%)	1(10%)	1(11.1%)	------	1(14.3%)	1(20%)	1(20%)	----	7(31%)
seb	1(6.6%	1(10	2(22.2	1(11.1	2(28.6	1(20	1(20%)	----	9(40.9

	)	%)	%)	%)	%)	%)			%)
sec	1(6.6%)	2(20%)	-----	1(11.1%)	-----	1(20%)	1(20%)	----	5(22.7%)
sed	1(6.6%)	----	-----	------	------	------	1(20%)	-----	2(9.09%)
see	------	1(10%)	-----	1(11.1%)	------	------	-----	------	2(9.09%)
fem A	----	----	-----	-----	---	-----	-----	------	-----

The most frequent foodborne pathogen, Staphylococcus aureus, is a serious public health concern in underdeveloped nations (Haghi et al.,2021). S. aureus may infect both human and animal skin and mucous membranes (as the primary reservoir). As a result, during the preparation process, these bacteria can readily penetrate meals that require processing (meat, milk, and vegetables). The majority of these widely spread bacteria are saprophytes and nonpathogenic in nature, however some are pathogenic to people and animals.

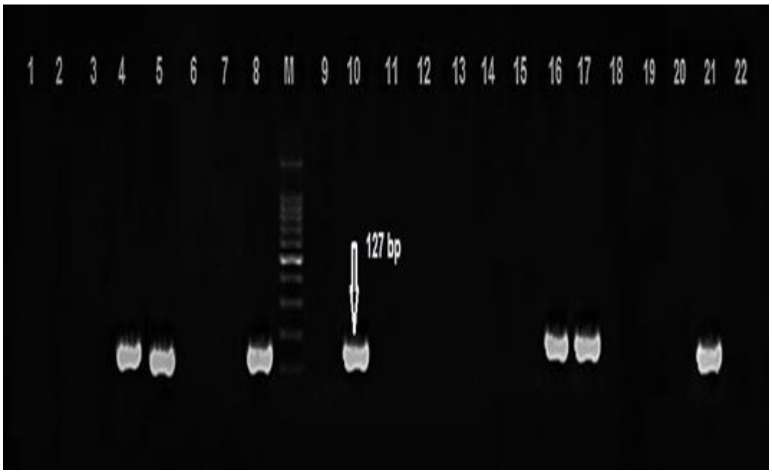

Figure 3. Agarose gel electrophoresis staining with red safe stains (1.5% Agarose, 70 volts for 60 min) for PCR product for Staphylococcus aureus. Enterotoxin A (sea) gene. Lane (M) DNA molecular size marker (100-bp ladder). Lanes (2-5-8-10-16-17-21) of Staphylococcus aureus isolates show positive results. other Lanes of Staphylococcus aureus isolates show negative results

The result of the present study found the percentage of sea gene was 7(31%) in S. aureus isolated from canned meat including luncheon2(13.3%), sausage1(10%), tuna1(11.1%), Minced meat1(14.3%), grilled meat1(20%), hamburger1(20%). This result agrees with (Yoon et al.,2022) found that the percentage of sea was (32.6%). This result is identical from the percentage of sea gene found by another study in the city of Karbala, Muhammad and Alwan (2017) found that the percentage of sea was 20 (35%), 12 (37.5%) of frozen, and 8 (32%) of meat samples fresh . and agree with (Al-Ashmawy et al.,2016) who found that percentage of sea was 26.5%. in anthor study Zouharova and Rysanek (2008) detected sea in 27.1% of isolates s while the seb and sec genes were observed in 10% and 1.4% of the isolates.

The result of the present study found the percentage of seb gene was 9(40.9%), The highest percentage of enterotoxigenic genes was detected in this study. including: luncheon1 (6.6%), sausage1 (10%),tuna2 (22.2%),chicken pieces1 (11.1%), Minced meat2 (28.6%) grilled meat1 (20%),and hamburger 1(20%). These poisons are extremely resistant to high temperatures and are not broken down by intestinal proteases like pepsin. For instance, after 28 minutes at 121°C, the sea maintains its biological characteristics. (Nazari et al., 2014). The use of sea in food must be prevented due to its thermo-resistance property. SEA does not alter the flavor or color of food; therefore, conventional cooking techniques do not get rid of this toxin. In most parts of the world, nearly 50% of foodborne diseases are caused by the sea.

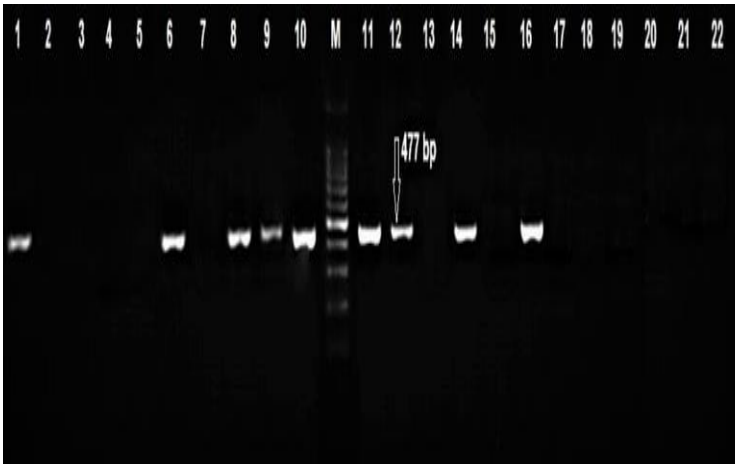

Figure 4. Agarose gel electrophoresis staining with red safe stains (1.5% Agarose, 70 volts for 60 min) to PCR product for Staphylococcus aureus Enterotoxin B (seb) gene. Lane (M) DNA molecular size marker (100-bp ladder). Lanes (1-6-8-9-10-11-12-14-16) of Staphylococcus aureus isolates show positive results. other Lanes of Staphylococcus aureus isolates show negative results

The result of the present study found the percentage of sec gene was 5 (22.7%), including luncheon1(6.6%), sausage2(20%), chicken pieces1(11.1%), grilled meat1(20%), and hamburger 1(20%) agree with (Al-Ashmawy et al.,2016) who found that percentage of sec was 33.9%.

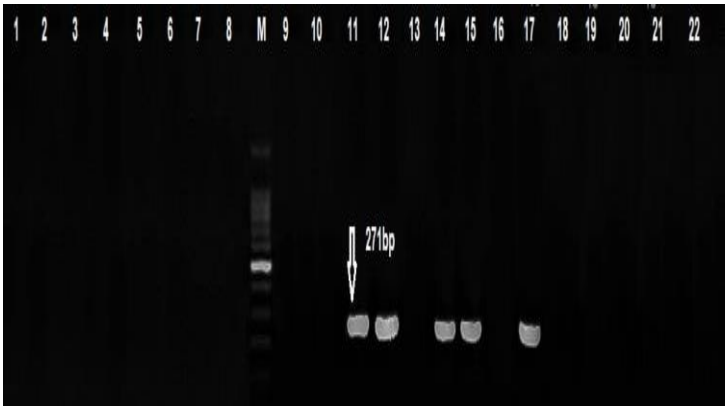

Figure 5. Agarose gel electrophoresis staining with red safe stains (1.5% Agarose, 70 volts for 60 min) to PCR product for Staphylococcus aureus Enterotoxin C (sec) gene. Lane (M) DNA molecular size marker (100-bp ladder). Lanes (11-12-14-15-17) of Staphylococcus aureus isolates show positive results. Other Lanes of Staphylococcus aureus isolates show negative results

The result of the present study found the percentage of sed gene was 2(9.09%) in S. aureus isolated from canned meat including: luncheon1(6.6%) %), hamburger1(20%).

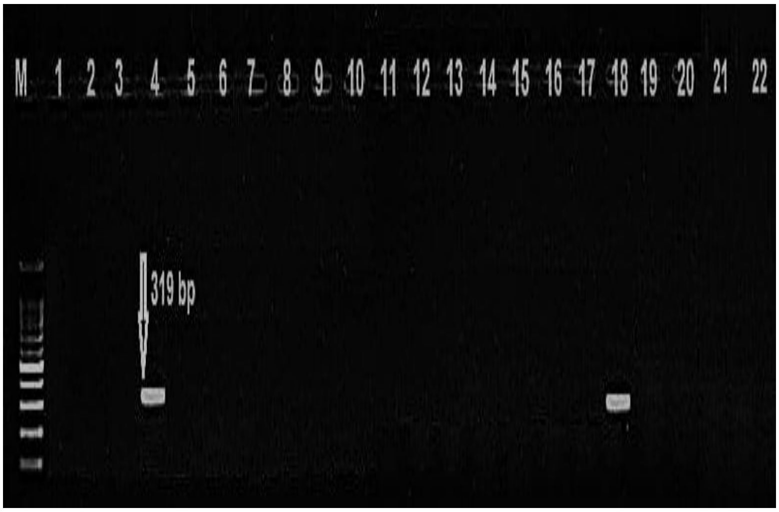

Figure 6: Agarose gel electrophoresis staining with red safe stains (1.5% Agarose, 70 volts for 60 min) to PCR product for Staphylococcus aureus Enterotoxin D (sed) gene product (amplified size 319 bp) using DNA template of Staphylococcus aureus isolates. Lane (M) DNA molecular size marker (100-bp ladder). Lanes (4-18) of Staphylococcus aureus isolates show positive results. other Lanes of Staphylococcus aureus isolates show negative results.

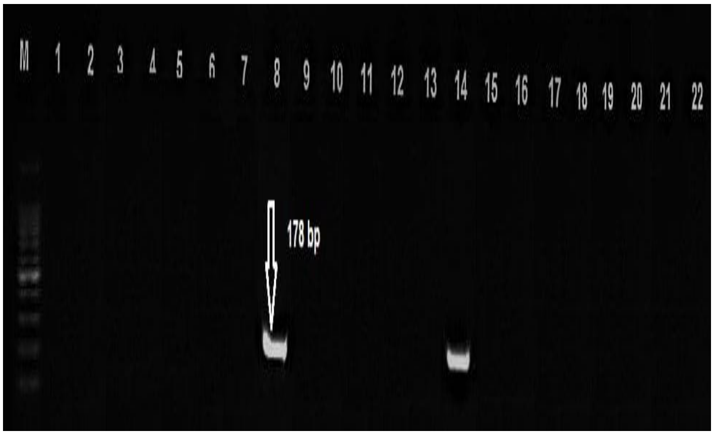

Figure 7: Agarose gel electrophoresis staining with red safe stains (1.5% Agarose, 70 volts for 60 min) to PCR product for Staphylococcus aureus Enterotoxin E (see) gene. Lane (M) DNA molecular size marker (100-bp ladder). Lanes (8-14) of Staphylococcus aureus isolates show positive results. other Lanes of Staphylococcus aureus isolates show negative results

This result does not agree with (Şanlıbaba,2022) where see genes were never detected. And also does not agree with (Omwenga et al., 2019)who found that the most frequently encountered gene was see

Conclusions

The study concluded that Staphylococcus aureus taken from different sample of meat and canned meat showed bacteria resistance to many antibiotics. Furthermore, the presence of enteric staphylococcal genes among these isolates indicates their potential to cause food poisoning.

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