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MOLECULAR CHARACTERIZATION OF *STAPH. AUREUS* AND SOME ENTERIC BACTERIA PRODUCING TOXINS IN MINCED MEAT SOLED IN PORT-SAID CITY MARKETS

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ABSTRACT

A total of 100 minced meat samples were collected from different butchers in Port-Said city for isolation and identification of *Staph. aureus*, *Escherichia coli* and *Salmonella* spp.. The results revealed that *Staph. aureus*, *E. coli* and *Salmonella* spp. could be detected in a percentage of 14%, 12% and 1% respectively from minced meat samples. Molecular characterizations of all isolated *S. aureus*, *E. coli* and *Salmonella* spp. were confirmed using *16S rRNA*, *phoA* and *invA*, respectively by conventional PCR at 791 bp; 720 bp and 284 bp, respectively. Multiplex PCR was developed with specific primers for the detection of different enterotoxin genes (*Sea*, *Seb*, *Sec*, *Sed* and *See*) of *Staph. aureus*, (*Stx1*, *Stx2*, *STa* and *lt*) of *E. coli* and (*stn)* of *Salmonella* which may be considered a significant in food safety threat. The obtained results showed that the positive serotypes for enterotoxin genes were (*Seb* in 3 isolates and *Sed* in one isolate) of *Staph. aureus* at 164 bp and 278 bp respectively; (*Stx2* in 2 isolates and *STa* in 2 isolates) of *E. coli* at 779 bp and 229 bp, respectively. Conventional PCR is rapid methods for the confirmation of *Staph. aureus*, *E. coli* and *Salmonella* spp., while multiplex PCR is a useful technique for detection of enterotoxin genes. The public health hazards of this isolated organism, as well as recommended measures to improve quality status of minced meat were discussed.

Key words: *Staph. aureus*, *E. coli* and *Salmonella* spp., PCR, enterotoxin genes, minced meat.

INTRODUCTION

Minced meat that has been minced into fragments and contains less than 1% salt. Minced meat has an important role in human nutrition as they are desirable foodstuff (Biesalski, 2005). In a whole cut from an animal, the interior of the meat is essentially sterile, even before cooking; any bacterial contamination is on the outer surface of the meat. When meat is ground, bacterial contamination from the surface can be distributed throughout the meat. If ground beef is not well cooked all the way through, there is a significant chance that enough pathogenic bacteria will survive to cause illness. Food-borne illness is a major international health problem (Mensah *et al.*, 2002 and Ayten *et al.*, 2014).

Food-borne diseases coming from pathogenic bacteria have been of vital concern to public health. *Staph. aureus*; *E. coli* and *Salmonella* spp. are considered more frequent human pathogens. They are often simultaneously found in some contaminated food matrices, such as meat products (Leclerc *et al.*, 2002). *E. coli*, is generally used as an indicator of fecal pollution and some strains may cause severe diseases (Ahmed *et al.*, 2007). *Salmonella* spp. and *Staph. aureus* remains a major cause of morbidity and mortality worldwide (Threlfall 2008 and Schreiber *et al.*, 2011). Each year, millions of persons become ill from food-borne diseases, though many cases are not reported Centers for Disease Control and Prevention (1997). If you're getting mince from a butcher, it's likely to be made from cheaper cuts like chuck steak, from the front shoulders of the cow, and thin flank from the cow's belly. It will also probably include trimmings of meat from steaks, roasting joints and others. The butchers will also make sure that there's a percentage of fat in the mince because it needs a certain amount to give it moisture and flavor as it cooks. Food-borne pathogens such as bacteria or their toxins may lead to human disease when contaminated food is eaten. The source of contamination may vary but harmful bacteria are mostly responsible for causing gastrointestinal infections (Scallan *et al.*, 2011). Food-borne illnesses and intoxications can occur due to the presence of certain bacteria such as *Staph. aureus*, *E. coli* and *Salmonella* spp. (Elmalıand Yaman 2005 and Tachbele *et al.*, 2006). Microbial quality of minced meat as one of meat products plays an important role in increasing public health issue all over the world. During the last decades, there was a great improvement in hygienic technique for

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production of meat products with attention of a lot of consumers towards healthy nutrition Ahmedand Ismail, (2010).

Molecular detection methods based on PCR are increasingly accepted as alternatives to conventional cultural/ biochemical methods for the detection of bacterial contamination in food (De Boer and Beumer, 1999). PCR technique is considered as a sensitive detection method for specific pathogens. Multiplex PCR assay seems to be a useful technique for rapid and specific detection of pathogens in food and has been used for the control and prevention of food-borne epidemics (Kawasaki *et al.*, 2009).

Due to the progressive increase in the incidence of food borne infections, there is an urgent need for control and/or prophylaxis for food poisoning outbreaks associated with meat products. It depends greatly on investigating the causative agents in mincedmeat, eliminating them to ensure food safety and to protect public health from microbial contamination. The aim of the current study was to determine the bacteriolological quality of minced meat obtained from different butchers in Port-Said city through determine the incidence of *Staph. aureus*, *E. coli* and *Salmonella* spp. with regarding to the public health as well as confirmed and determined some virulence genes by using PCR technique.

MATERIALS AND METHODS

1- Samples collection: One hundred samples of minced meat were randomly collected from different butchers in Port- Said city. Each sample was aseptically transported in ice-box to laboratory quicklyas soon as possible for detection of *Staph. aureus*, *E. coli* and *Salmonella* spp.

2. Bacteriological examination:

2.1- Isolation and Identification of *Staph. aureus*: Isolation of *Staph. aureus* was attempted according to ISO (1999). 10 gram of sample was homogenized with 90 ml sterile enrichment broth peptone water and enriched for 24 hrs at 37 °C. A loopful of inoculum from enrichment broth was streaked on Baird Parker Agar (BPA) and incubated for 48 hours at 37°C. Characteristic appearance of jet black colonies surrounded by a white halo was considered to be presumptive *Staph. aureus*. The pure cultures were streaked on Nutrient agar, incubated for 24 hours at 37°C for further characterized.

2.1.1- Morphological characteristics of *Staph. aureus*: The smear was prepared from the isolated culture and stained with Gram's stain. The stained smear revealed Gram positive, spherical cells arranged in irregular clusters resembling to bunch of grapes according to Cruickshank *et al.* (1975).

2.1.2- Biochemical examination: The biochemical tests were performed to confirm *Staph. aureus* using Catalase test, Coagulase test, DNase test, Acetoin production, Oxidase test and D-mannitol fermentation according to Thaker *et al.* (2013).

2.2- Isolation and Identification of *E. coli*: Isolation of *E. coli* was attempted according to Qunin *et al.* (2002). 10 gram of sample was homogenized with 90 ml sterile enrichment broth peptone water and incubated for 24 hrs at 37°C. A loopful from inoculated broth was streaked on the surface of Eosin methylene blue agar plate. Inoculated plate was incubated at 37°C for 24- 48hr. The pure cultures were streaked on Nutrient agar and were incubated for 24 hours at 37°C for further characterized.

2.2.1- Morphological characteristics of *E. coli*: according to (Qunin *et al.*, 2002).

2.2.2- Biochemical examination: The biochemical tests of ***E. coli*** using Oxidase test, Indole production, Methyl red, Voges Proskauer test, Utilization of citrate, hydrogen sulfide production on Triple Sugar Iron agar (TSI), Hydrolysis of urea and Sugar fermentation test according to (Qunin *et al.*, 2002).

2.3-Isolation and Identification of *Salmonella* spp.: Isolation of ***Salmonella* spp.** was attempted according to ISO (2002).

2.3.1- Morphological characteristics of *Salmonella* spp.: according to (Qunin *et al.*, 2002).

2.3.2-Biochemical examination: The biochemical tests were performed to confirm ***E. coli*** using Oxidase test, hydrogen sulfide production on Triple Sugar Iron agar (TSI), Hydrolysis of urea and Lysine iron agar according to (Qunin *et al.*, 2002).

3. Molecular characterization and detection of *Staph. aureus, E. coli* and *Salmonella* spp. enterotoxins genes:

The *Staph. aureus*, *E. coli* and *Salmonella* spp. isolated from minced meat samples were confirmed by PCR using (*16S rDNA* of *Staph. aureus*), (*phoA* of *E. coli*) and (*invA* of *Salmonella* spp.), also determining some enterotoxins genes using specific primers (*Sea*, *Seb*, *Sec*, *See* and *Sed*) for *Staph. aureus*, (*Stx1*, *Stx2*, *STa* and *lt*) for *E. coli* and (*stn)* for *Salmonella* spp.

3.1-DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 150 µl of elution buffer provided in the kit.

3.2-Oligonucleotide Primer: Primers are listed in **table (1)**.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondar denaturation	Annealing	Extension		
Staphylococcus Sea	GGTTATCAATGTG CGGGTGG	102	94˚C 5 min.	94˚C 30 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	***Mehrotra et al.,* (2000)**
	CGGCACTTTTTTC TCTTCGG							
Seb	GTATGGTGGTGTA ACTGAGC	164						
	CCAAATAGTGACG AGTTAGG							
Sec	AGATGAAGTAGTT GATGTGTATGG	451						
	CACACTTTTAGAA TCAACCG							
Sed	CCAATAATAGGA GAAAATAAAAG	278						
	ATTGGTATTTTTTT TCGTTC							
See	AGGTTTTTTCACA GGTCATCC	209						
	CTTTTTTTTCTTCG GTCAATC							
Staphylococcus 16S rRNA	CCTATAAGACTGG GATAACTTCGGG	791	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45sec.	72˚C 10 min.	**Mason *et al.,* (2001)**
	CTTTGAGTTTCAA CCTTGCGGTCG							
E. coli Stx1	ACACTGGATGATC TCAGTGG	614	94˚C 5 min.	94˚C 30 sec.	58˚C 45 sec.	72˚C 45sec.	72˚C 10 min.	**Dipineto*et al.,* (2006)**
	CTGAATCCCCCTC CATTATG							
Stx2	CCATGACAACGG ACAGCAGTT	779						
	CCTGTCAACTGAG CAGCACTTTG							
STa	GAAACAACATGA CGGGAGGT	229	94˚C 5 min.	94˚C 30 sec.	57˚C 45 sec.	72˚C 45sec.	72˚C 10 min.	**Lee*et al.,* (2008)**
	GCACAGGCAGGA TTACAACA							
LT	GGTTTCTGCGTTA GGTGGAA	605						
	GGGACTTCGACCT GAAATGT							
E. coli phoA	CGATTCTGGAAAT GGCAAAAG	720	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Hu *et al.* (2011)**
	CGTGATCAGCGGT GACTATGAC							
Salmonella Stn	TTG TGT CGC TAT CAC TGG CAA CC	617	94˚C 5 min.	94˚C 30 sec.	59˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Murugkar *et al.* (2003)**
	ATT CGT AAC CCG CTC TCG TCC							
Salmonella invA	GTGAAATTATCGC CACGTTCGGGCAA	284	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec	72˚C 30 sec	72˚C 7 min.	**Oliveira *et al.* (2003)**
	TCATCGCACCGTC AAAGGAACC							

3.3-PCR amplification: Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler. For multiplex PCR of enterotoxins, Primers were utilized in a 50- µl reaction containing 25 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 5 µl of water, and 10 µl of DNA template. For dultiplex PCR of *E. coli* virulence genes *(stx1* and *stx2*) or (*STa* and *LT*), Primers were utilized in a 50- µl reaction containing 25 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmolconcentration, 11 µl of water, and 10 µl of DNA template.

3.4-Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex PCR products and 40 µl of the multiplex PCR products were loaded in each gel slot. Gelpilot 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) and generuler 100 bpladder were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

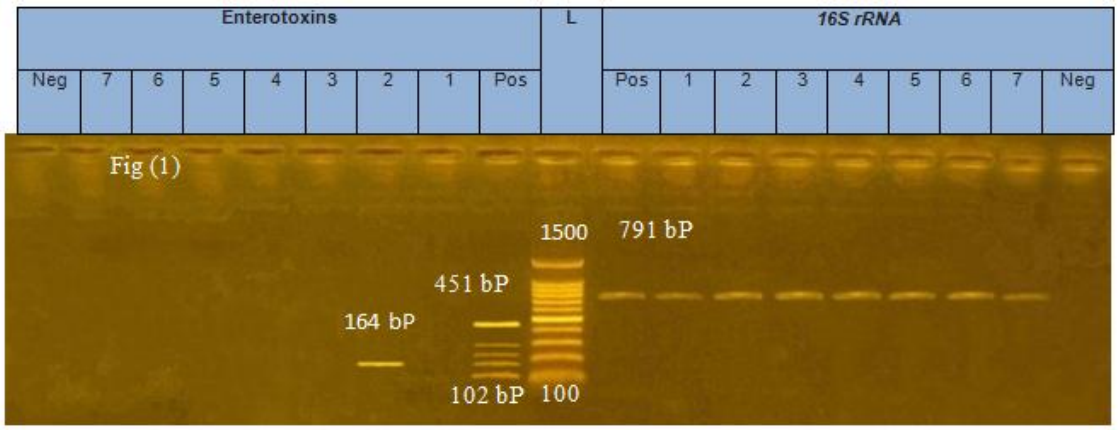
RESULTS

Table 2: Prevalence of *Staph. aureus*, *E. coli* and *Salmonella* spp. in mincedmeat samples (n=100).

isolated organisms	No.	%
Staph. aureus	**14**	**14%**
E. coli	**12**	**12%**
Salmonella	**1**	**1%**

Table 3: Molecular characterization and some enterotoxin genes of *Staph.aureus* isolated from minced meat samples.

No. of tested isolates	*16S rRNA*		enterotoxin genes									
			Sea		***Seb***		***Sec***		***Sed***		***See***	
	No.	***%***	***No.***	***%***	***No.***	***%***	***No.***	***%***	***No.***	***%***	***No.***	***%***
14	**14**	**100**	**-**	**0.0**	**3**	**21.4**	**-**	**0.0**	**1**	**7.1**	**-**	**0.0**



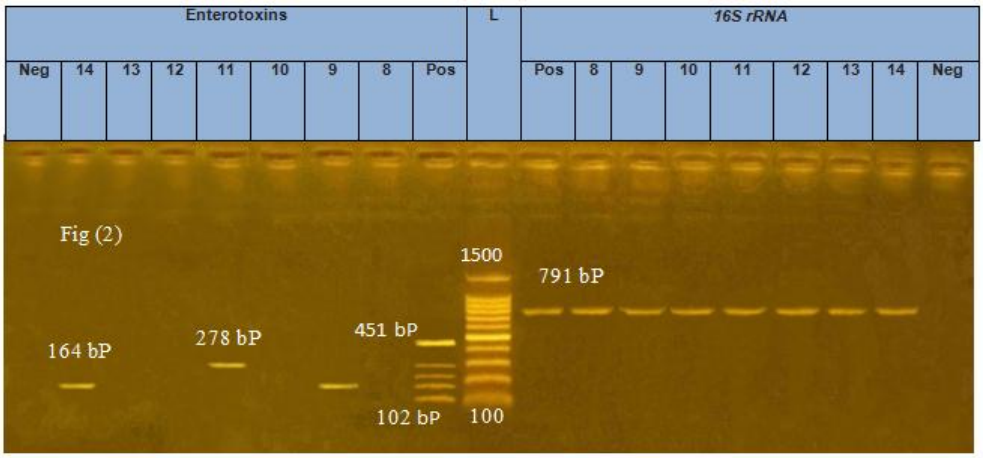
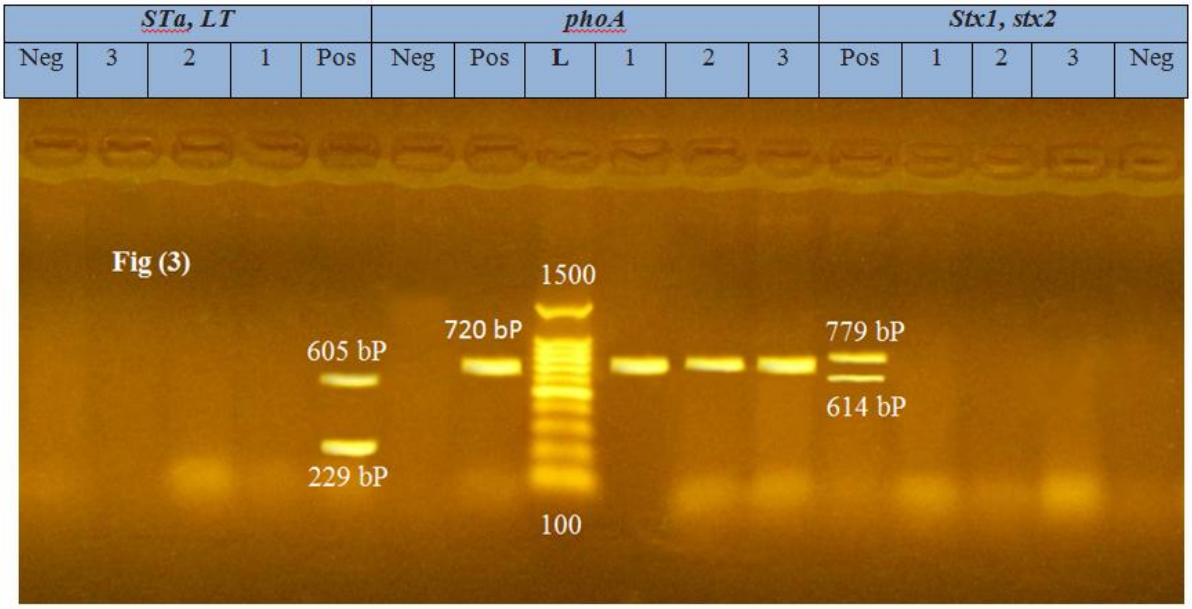
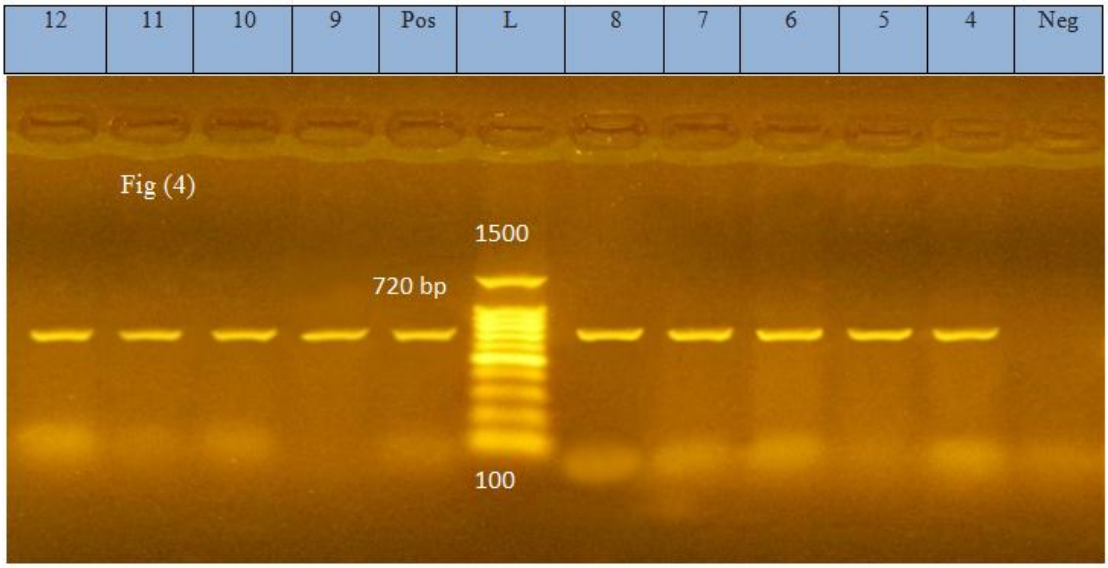


Fig. (1) & Fig. (2): Agarose gel electrophoresis of PCR products after amplification of:
1- ***Staph. aureus 16Sr DNA*** gene. MWM-molecular weight marker (100 – 1500 bp DNA ladder), + control (Positive, Negative) + different strains of ***Staph. aureus*** (***Staph. aureus16Sr DNA*** gene products at 791bp).
2- ***Sea***gene (***Sea*** gene products at 102 bp). **3-** ***Seb*** gene ***(Seb*** gene products at 164 bp).
4- ***Sec***gene (***Sec*** gene products at 451 bp). **5-** ***See*** gene (***Sec*** gene products at 209 bp).
6- ***Sed*** gene (***Sed*** gene products at 278 bp).

Table 4: Molecular characterization and some virulence genes of *E. coli* isolated from minced meat samples.

No. of tested isolates	phoA		Enterotoxin genes							
			Stx1		*Stx2*		*STa*		*lt*	
	No.	%	No.	%	No.	%	No.	%	No.	%
12	12	100	-	0.0	2	-	2	-	-	0.0





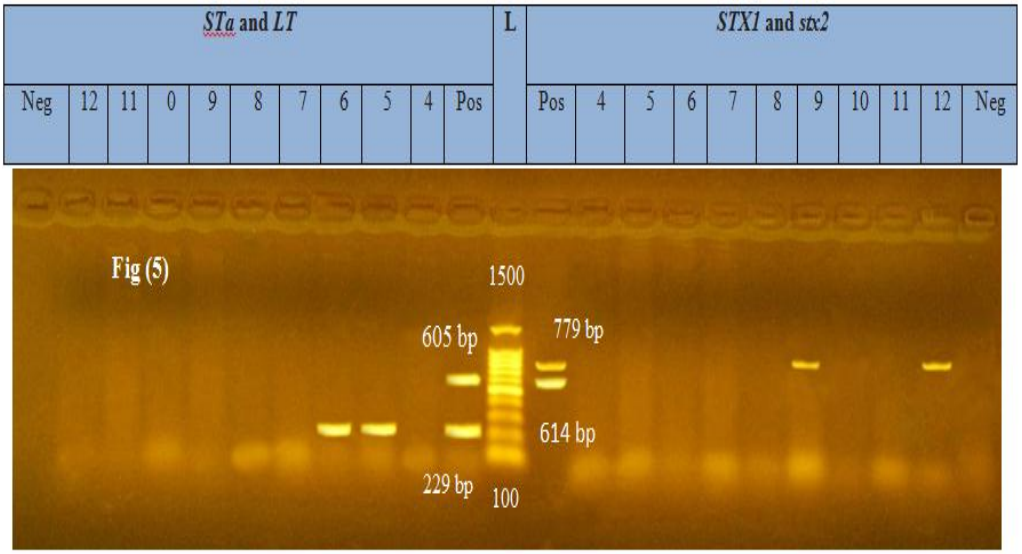


Fig. (3) & (4) & (5): Agarose gel electrophoresis of PCR products after amplification of:
1- ***E.coli PhoA*** gene. MWM-molecular weight marker (100 – 1500 bp DNA ladder), + control (Positive, Negative) + different strains of ***E.coli*** (***E.coli PhoA*** gene products at 720bp).
2- ***Sta*** gene (***Sta*** gene products at 229 bp). **3-*LT*** gene (***LT*** gene products at 605 bp).
4- ***Stx1*** gene (***Stx1***gene products at 614 bp). **5-** ***Stx2*** gene (***Stx2*** gene products at 779 bp).

Table 5: Molecular characterization and some virulence genes of *Salmonella* Spp. Isolated from minced meat samples.

No. of tested isolates	*invA*		Enterotoxin genes	
			stn	
	No.	%	No.	%
1	1	100	-	0.0

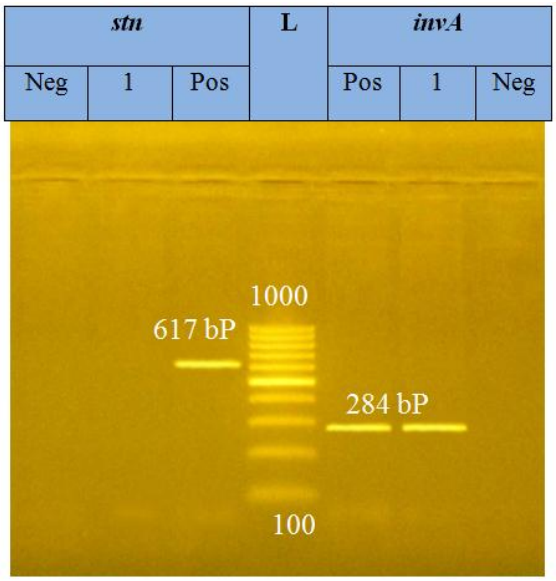


Fig (6): Agarose gel electrophoresis of PCR products after amplification of:
1- ***Salmonella*** spp. ***InvA*** gene. MWM-molecular weight marker (100 – 1000 bp DNA ladder), + control (Positive, Negative) + different strains of *Salmonella* (*Salmonella* spp. *invA* gene products at 284bp).
2- ***Stn***gene (*stn* gene products at 617 bp).

DISCUSSION

The present work was made in order to evaluate the prevalence of *Staph. aureus*, *E. coli* and *Salmonella* spp. among minced meat, also to confirm the isolated microbes and determined some enterotoxins genes characteristics of *Staph. aureus*, *E. coli* and *Salmonella* spp. using conventional PCR and multiplex PCR. So, a total of 100 minced meat samples showed bacterial contamination with species of the genera, *Staph. aureus*, *E. coli* and *Salmonella* spp. in percentages of 14 %, 12% and 1%, respectively in (Table 2). The presence of these isolates in the higher percentage in minced meat samples collected from different butchers in Port-Said city is an indication of unsatisfactory handling of minced meat and inadequate hygiene. Higher results were recorded by Sarah, (2014); Ezzat *et al.* (2014) and Raafat *et al.* (2011). The later isolated *Staph. aureus*, *E. coli* and *Salmonella* spp. in a percentages of 80 %, 28% and 20%, respectively from minced meat. The prevalence of *S. aureus* in different food products ranged from 5% to 100% (Adwan *et al.*, 2005; Vázquez-Sánchez *et al.*, 2012). The difference in the results may be attributed to difference in sampling procedure, locality, number of samples and difference in method used. Butthe prevalence of *E. coli* in different food ranged from 11% to 100% (Zhao *et al*., 2001; Ukut *et al.*, 2010; Abdellah *et al*., 2013; Iyer*et al*., 2013 and Adeyanju and Ishola, 2014). While the prevalence of *Salmonella* in different food products ranged from 2% to 100% (Cohen *et al.*, 2007; Aftab *et al.*, 2012; Iyer *et al.*, 2013; Anihouvi *et al.*, 2013; Adeyanju and Ishola, 2014).

Biochemical identified *Staph. aureus*, *E. coli* and *Salmonella* spp. (14, 12 and 1isolates, respectively) were submitted for molecular characterizations and confirmed by using (*16S rRNA* of *Staph. aureus*), (*phoA* of *E. coli*) and (*invA* of *Salmonella* spp.) by conventional PCR. The results proved that the isolates were Positive *Staph. aureus* (14 isolates), *E. coli* (12 isolates) and (one isolates) *Salmonella* spp. as recorded in tables (3, 4 and 5) and figures (1, 2, 3, 4 and 6). These results were agreement with those obtained by the conventional PCR assay with respective primers *16S rRNA* of *Staph. aureus*), (*phoA* of *E. coli*) and (*invA* of *Salmonella* spp.), suggesting PCR was able to confirm the *Staph. aureus*, *E. coli* and *Salmonella* infection (Manson *et al.*, 2001; Hu *et al.*, 2011 and Oliveira *et al.*, 2003).

Determination of some enterotoxin genes (*Sea*, *Seb*, *Sec*, *See*and *Sed*) of *Staph. aureus*, (*Stx1*, *Stx2*, *STa* and *lt*) of *E. coli* and (*stn)* of *Salmonella* spp. isolated from minced meat samples by multiplex PCR. Tables (3, 4 and 5) Figures (2 and 5) showed the positive serotypes for enterotoxin genes (*Seb* in 3 isolates and *Sed* in one isolate) of *S. aureus*; (*Stx2* in 2 isolates and *STa* in 2 isolates) of *E. coli*. None of the samples were positive for (*Sea*, *Sec* and *See*) of *S. aureus*, (*Stx1* and *lt*) of *E. coli* and (*stn)* of *Salmonella* spp. These results were in agreement with those obtained by the multiplex PCR assay with respective primers (*Sea*, *Seb*, *Sec*, *See* and *Sed*) of *S. aureus*, (*Stx1*, *Stx2*, *STa* and *lt*) of *E. coli* and (*stn)* of *Salmonella* spp.

(Mehrotra *et al.,* 2000; Dipineto *et al.,* 2006 and Lee *et al.,* 2011 and Murugkar *et al.,* 2003).

The negative results in PCR may be attributed to PCR based detection mainly depends on the purity and amount of the template DNA used (Estrada *et al.,* 2007). The presence of PCR inhibitors in food samples and incomplete bacterial cell isolation lead to the production of false negative results in PCR based detection and the removal of PCR inhibitors, efficient bacterial cell isolation and efficient DNA extraction is important (Jeníkova *et al.,* 2000). The variation in the presence of enterotoxin genes among different serotypes isolated from different sources of minced meat samples revealed that the mechanisms of pathogenesis depends mainly on the presence of different virulence factors not to the different serotypes. *S. aureus*, *E. coli* and *Salmonella* spp. 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 and storage conditions (Zhao *et al*., 2001 and Adwan *et al*., 2005).

CONCLUSION AND RECOMMENDATION

From the obtained results, it can concluded that contamination by *Staph. aureus*, *E. coli* and *Salmonella* spp. were found in minced meat samples collected from different butchers in Port-Said city. The following suggestive measures should be considered to keep the examined products free from pathogens as possible:

- Routine microbiological examination should be adopted in minced meat factories, butchers shops, groceries and other food rendering outlet with a consequent certificate of nil presence food born bacteria.

- Hygienic awareness should be applied for personnel whom involved in handling and preparing of food at factories, home or restaurants.

- Careful handling and thorough cooking of minced meat, regardless of market source by the consumers is required to prevent food borne illness.

- Conventional and multiplex PCR is required as rapid, accurate and specific tool for isolated confirmation of isolated *Staph. aureus*, *E. coli* and *Salmonella* spp. and of detection of their enterotoxin genes.

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التوصيف الجزيئي لميكروب العنقودي الذهبي وبعض الميكروبات المعوية المنتجة للسموم في اللحوم المفرومة بأسواق مدينة بورسعيد المباعة

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في هذه الدراسة تم تجميع ١٠٠ عينة من اللحم المفروم المباعة في محلات الجزارة المختلفة بمدينة بورسعيد عشوائيا للكشف عن مدى تواجد الميكروب العنقودي الذهبي والايشيريشيا القولونية والسالمونيلا. وأظهرت نتائج الفحص البكتريولوجي والتعريف البيوكيميائي أن الميكروبات تم عزلها بنسبة ١٤%، ١٢% و ١% من العينات موضع الفحص علي التوالي. وباستخدام اختبار البلمرة المتسلسل (PCR) للتأكد من العترات المعزولة وذلك باستخدام (*16S rRNA*) لميكروب العنقودي الذهبي و (*phoA*) لميكروب الايشيريشيا القولونية و(*invA*) لميكروب السالمونيلا والذي اعطى نتائج ايجابية بنسبة١٠٠ %. وأيضا باستخدام (multiplex PCR) لتحديد جينات السموم (*Sea, Seb, Sec, Sed* and *See*) لميكروب العنقودي الذهبي و (*Stx1, Stx2, STa , lt*) لميكروب الايشيريشيا القولونية و (*stn*) لميكروب السالمونيلا، كشفت النتائج عن وجود كل من (*Sed* في عترة و *Seb* في 3 عترات لميكروب العنقودي الذهبي ، بينما تم تحديد (*Stx2* في عترتين و*STa* في عترتين لميكروب الايشيريشيا القولونية). مما يدل ان تقنية PCR طريقة سريعة تشخيصيه للتحري عن تواجد الميكروب العنقودي الذهبي والايشيريشيا القولونية والسالمونيلا. وقد تم مناقشة النتائج وبيان أهمية الميكروبات المعزولة وخطورتها علي الصحة العامة للمستهلك وعمل التوصيات للتقليل من مخاطرها.