
Characterization of Virulence Gene of *E. coli* and *Staphylococcus aureus* Isolated from Meat Products

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ABSTRACT

This study was conducted on beef burger, luncheon and sausage (35 of each) collected from different supermarkets at Menoufia Governorates for Detection of virulence genes in isolated *E. coli* and *S. aureus* using Multiplex PCR of *E. coli* and *S. aureus*. The data obtained from this study showed that the incidence of serologically identified *E. coli* was O₁, O₂₆, O₁₂₅ and O₁₄₂. Multiplex PCR showed Shiga toxin 2 gene (*stx2*) detected in O₂₆ and O₁₂₅, while shiga toxin 1 gene (*stx1*) detected in O₁, O₁₄₂ and O₂₆. While O₂₆ Positive *E. coli* strains for *stx1*, *stx2*, *eaeA* and *hlyA* genes, O₁, O₁₄₂ Positive *E. coli* strains for *stx1*, *eaeA* and *hlyA* and O₁₂₅ Positive *E. coli* strains for *stx2* and *hlyA*. Regarding to *S. aureus* was isolated from examined products showed that 7 strains were toxin-producing and the most detected enterotoxin gene in the examined strains was SEA (2 isolates), followed by SEC (3 isolates), then SEB and SEA (1 isolates) and SED and SEA (1 isolates) and 8 strains were negative for 4 enterotoxin-producing genes.

Keywords: *E. Coli*, *S. aureus*, Meat products, PCR, virulence Gene.

INTRODUCTION

Meat products are an excellent source of a wide variety of nutrients, high quality proteins, vitamins and certain minerals. In Egypt, beef meat products such as beef burger, luncheon and sausage are gaining popularity because they represent quick easily prepared meat meals and solve the problem of the shortage in fresh meat of high price that is not within the reach of large numbers of families with limited income. On the other hand, meat products are considered as an ideal culture media for growth of many organisms because of their high moisture content, their high percentage of nitrogenous compounds and good supply of minerals and fermentable carbohydrates (glycogen) as well as their favorable pH for growth of most microorganisms. (Al-Mutairi, 2011).

In general, *E. coli* is a major component of the normal intestinal flora of human and other mammals which are usually harmless to the host and only cause diseases in immunocompromised hosts or when the gastrointestinal barriers are breached. However, some specific *E. coli* strains represent primary pathogens with enhanced potential to cause disease after acquiring specific virulence attributes. These virulence attributes are normally encoded on genetic elements that can be exchanged between different strains or on those elements once having been mobile but later becoming fixed into the genome. (Li, et al., 2005).

S. aureus could cause food poisoning and if it grows in large numbers can leave toxins in the product, which may survive heating. It lives on the skins of humans and animals and can easily

be transferred to food products (Mousa, *et al.*, 2014). Staphylococcal food poisoning is due to the ingestion of foods containing one or more performed enterotoxins (SE) produced by *S. aureus*. Most staphylococcal intoxication outbreaks are related to recontamination of cooked products by food handlers, followed by improper holding temperature (Su and Wong, 1997). Multiplex PCR assay for detection of staphylococcal enterotoxins genes (SEA, SEB, SEC, SED and SEE) was developed and proved to be specific, sensitive, and rapid method (Omoe *et al.*, 2002 and Zschock *et al.*, 2005).

MATERIALS AND METHODS

Collection of samples:

A total of 105 samples of meat products including; luncheon, Beef burger and Sausage (35 of each) retailed for sale in different supermarkets at Menoufia Province were randomly collected. The samples were placed separately in clean sterile plastic bags and transferred in an insulated ice box to the laboratory of Food Control Department, Faculty of Veterinary Medicine, University of Sadat City under aseptic conditions without delay. All collected samples were subjected to microbiological examination.

Preparation of Samples according to (APHA, 1992)

25gm of samples were added to 225 ml of buffered pepton water and mixed well in stomacher for 2 minutes.

Isolation and identification of *E. coli* according to (ICMSF, 1996)

Detection of Toxin- producing genes in isolated *E. coli* using Multiplex PCR:

Materials used for PCR:

1. Reagents used for Agarose gel electrophoresis:

1.1. Agarose powder, Biotechnology grade (Bioshop^R, Canda inc. lot No: OE16323).

It prepared in concentration 2% in 1× TAE buffer.

1.2. Tris acetate EDTA (TAE) electrophoresis buffer (50×liquid concentration) (Bioshop^R, Canda inc. lot No: 9E11854).

The solution diluted to 1× by adding 1 ml stock solution to 49 ml double dist. Water to be used in the preparation of the gel or as a running buffer.

1.3. Ethidium bromide solution (stock solution) biotechnology grade (Bioshop @ Canda Inc., Lot No: 0A14667):

The stock solution was diluted by 25µl /200ml double distilled water and stored covered at 4°C. It was used for staining of PCR products that electrophoreses on Agarose gel to be visualized by UV light.

1.4. Gel loading buffer (6×stock solution) (Fermentas, lot No: 00056239).

The components were dissolved in sterile double distilled water and stored covered with aluminum foil at room temperature.

1.5. DNA ladder (molecular marker): 100 bp (Fermentas, lot No: 00052518).

1.6. 5X Taq master (Fermentas):

Containing polymerase enzyme, Magnesium chloride (Mg Cl₂), Deoxy nucleotide triphosphate (dNTP) and PCR grade water.

1.7. Primer sequences of *E. coli* used for PCR identification system:

Application of PCR for identification of shiga toxins (*Stx1* & *stx2*) and Intimin (*eaeA*) as virulence genes of *E. coli* was performed essentially by using primers (Pharmacia Biotech) as shown in table1.

2. DNA Extraction using QIA amp kit (Shah *et al.*, 2009):

3. DNA amplification:

3.1. Amplification reaction of *E. coli* (Fagan *et al.*, 1999):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). PCR assays were carried out in 1 ml of nucleic acid template prepared by using reference EHEC isolates (approximately 30 ng of DNA), 10 mM Tris-HCl (pH 8.4), 10 mM KCl, 3 mM MgCl₂; 2 mM concentrations of each primer, 0.2 mM concentrations of each 29-deoxynucleoside 59-triphosphate, and 4 U of AmpliTaq DNA polymerase (Perkin-Elmer).

Isolation and identification of *S. aureus* according to (ICMSF, 1996)

Table (1): Primer for detection of virulence genes in *E. coli*

Primer	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
<i>Stx1</i> (F)	5' AACTGGATGATCTCAGTGG '3	614	Dhanashree and Mallya (2008)
<i>Stx1</i> (R)	5' CTGAATCCCCCTCCATTATG '3		
<i>Stx2</i> (F)	5' CCATGACAACGGACAGCAGTT '3	779	Dhanashree and Mallya (2008)
<i>Stx2</i> (R)	5' CCTGTCAACTGAGCAGCACTTTG '3		
<i>eaeA</i> (F)	5' GTGGCGAATACTGGCGAGACT '3	890	Mazaheri et al., (2014)
<i>eaeA</i> (R)	5' CCCCATTTCTTTTTCACCGTCG '3		
<i>hlyA</i> (F)	5' ACGATGTGGTTTATTCTGGA '3	165	Fratamico et al., (1995)
<i>hlyA</i> (R)	5' CTTACGTGACCATACATAT '3		

Detection of Toxin- producing genes in isolated *S. aureus* using Multiplex PCR:

The demonstration of virulence factors including enterotoxin genes (A, B, C & D) of *S. aureus* was adopted by using the primers shown in table 2.

1. Primer sequences of *S. aureus* used for PCR identification system:

Table (2): Primer for detection of enterotoxin genes *S. aureus*

Target gene	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
<i>Sea</i> (F)	5' TTGGAAACGGTTAAAACGAA'3	120	Rall et al., (2008)
<i>Sea</i> (R)	5' GAACCTTCCCATCAAAAACA '3		
<i>Seb</i> (F)	5' TCGCATCAAACGACAAACG '3	478	
<i>Seb</i> (R)	5' GCGGTACTCTATAAGTGCC '3		
<i>Sec</i> (F)	5' GACATAAAAGCTAGGAATTT '3	257	
<i>Sec</i> (R)	5' AAATCGGATTAACATTATCC '3		
<i>Sed</i> (F)	5' CTAGTTTGGTAATATCTCCT '3	317	
<i>Sed</i> (R)	5' TAATGCTATATCTTATAGGG '3		

2. Amplification of enterotoxin genes of *S. aureus* (Mehrotra et al., 2000):

Ten µl of DNA sample was diluted in 990 µl of nuclease free water for PCR. The genomic DNA samples were amplified by PCR in a reaction mixture (25µl) containing 13.25 sterile dH₂O, 2.5ml 10 x buffer, 0.63ml 10mMNTPs, 1ml 25Mm Mgcl₂, 1.25 µl primer F(20pmol/ml), 1.25 µl primer R (20pmol/ml) and fill up to 25 µl PCR grade water. Concerning the primers used for demonstration of *S. aureus* enterotoxins (*sea*, *seb*, *sec*, *sed* & *see*), the amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany).

RESULTS

This study revealed that presence of Different serotypes of *E. coli* which include O₂₆ which harbor the four virulence gene *stx1*, *stx2*, *eae* and *hlyA* also we isolated *E. coli* O₁₂₅ which carry *stx2* and *hlyA* genes and O₁ which contain *stx1*, *eae* and *hlyA* virulence genes.(table 1 and Fig 1).

In relation to *S. aureus* we detected different enterotoxin genes include A, B, C and D enterotoxins (table 2 fig 2)

Table (3): Occurrence of virulence genes of Shiga toxin-producing *E. coli* isolated from the examined meat products:

<i>E. coli</i> Serovars	<i>stx1</i>	<i>stx2</i>	<i>eae</i>	<i>hlyA</i>
O ₂₆	+	+	+	+
O ₁₂₅	-	+	-	+
O ₁	+	-	+	+
O ₁₄₂	+	-	+	+



Fig (1): Agarose gel electrophoresis of multiplex PCR of *Stx1* (614 bp), *stx2* (779 bp), *eaeA* (890 bp) and *hlyA* (165 bp) genes for characterization of *E. coli*.

Lane M: 100 bp ladder as molecular size DNA marker.

Lane 1: Control positive *E. coli* for *Stx1*, *stx2*, *eaeA* and *hlyA* genes.

Lane 2: Control negative.

Lane 3 (*E. coli* O₂₆): Positive strains for *Stx1*, *stx2*, *eaeA* and *hlyA* genes.

Lane 4 (*E. coli* O₁₂₅): Positive strain for *stx2* and *hlyA* genes.

Lane 5 (*E. coli* O₁): Positive strain for *Stx1*, *eaeA* and *hlyA* genes.

Lanes 6 (*E. coli* O₁₄₂): Positive strains for *Stx1* & *eaeA* genes.

Table (4): Occurrence of enterotoxin genes of some *S.aureus* strains isolated from the examined samples :

<i>Staph. aureus</i> enterotoxins	No.	%
A	2	13.33
C	3	20.00
A+B	1	6.67
A+D	1	6.67
-ve	8	53.33
Total	15	100

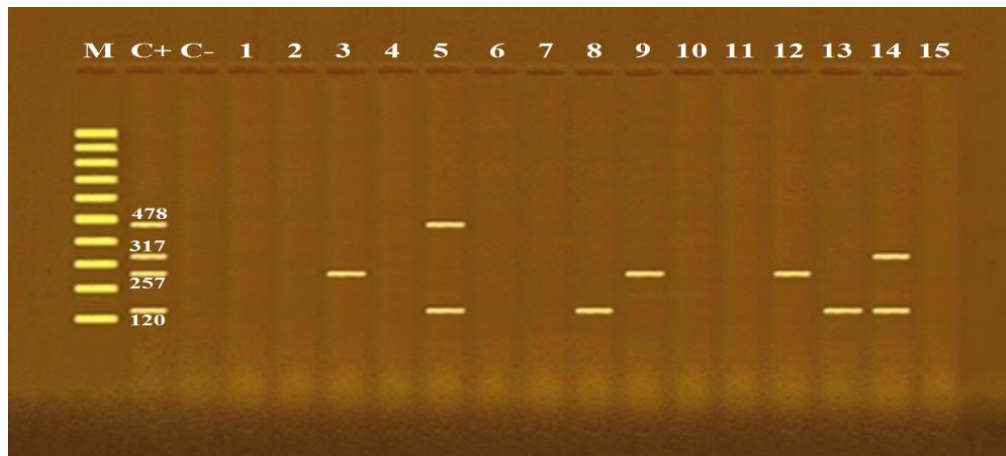


Fig (2): Agarose gel electrophoresis of multiplex PCR of *sea* (120 bp), *seb* (478 bp), *sec* (257 bp) and *sed* (317 bp) enterotoxin genes for characterization of *S. aureus*.

Lane M: 100 bp ladder as molecular size DNA marker.

Lane C+: Control positive for *sea*, *seb*, *sec* and *sed* genes.

Lane C-: Control negative.

Lanes 3, 9 & 12: Positive *S. aureus* strains for *sec* gene.

Lanes 8 & 13: Positive *S. aureus* strains for *sea* gene.

Lane 5: Positive *S. aureus* strain for *sea* and *seb* genes.

Lane 14: Positive *S. aureus* strain for *sea* and *sed* genes.

Lanes 1, 2, 4, 6, 7, 10, 11 & 15: Negative *S. aureus* strains for enterotoxins.

DISCUSSION

Pathogenic *E. coli* have public health impact on humans by causing gastroenteritis including hemorrhagic colitis and hemolytic uremic syndrome (Pavithra and Ghosh, 2013). *E. coli* are responsible for three major types of clinical infections like enteric and diarrheal diseases, urinary tract infections, sepsis and meningitis (Maheux *et al.*, 2009).

There are several pathovars (enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), with certain sets of serogroups and virulence factors causing enteric and diarrheal disease (Bonyadian *et al.*, 2010). So, identification and Serotyping of *E. coli* strains in food and environmental samples is important to understand the epidemiology (Monaghan *et al.*, 2012).

There are two major Shiga toxin gene families – *stx 1* and *stx 2* – and multiple Shiga toxin genotypes within each family. The *stx* genotype is unrelated to the *in vitro* cytotoxicity or quantity of Shiga toxin produced but does appear to be associated with the severity of clinical illness (Orth *et al.*, 2007). *Stx2* is the most heterogeneous group statistically associated with severe symptoms, including bloody diarrhea and HUS (Persson *et al.*, 2007). Shiga toxin genes are found in more than 200 serotypes of different strains of *E. coli* strains which are well known for disease causing ability (Page and Liles, 2013).

Rapid and highly reliable methods of detecting and identifying STEC causing gastroenteric illnesses are needed to prevent food borne outbreaks such as PCR technique (Goji *et al.*, 2015). The multiplex polymerase chain reaction (MPCR) used for detection of the presence of shiga-toxin producing *E. coli* virulence markers genes encoding Shiga toxin 1 and 2 (*Stx1* and *Stx2*) and intimin (*eae A*) (Murinda *et al.*, 2004), because PCR-based techniques are more reliable, sensitive and rapid and offer a sensitive and specific detection of pathogens and can discriminate virulent bacteria from avirulent members of the same species as well (Olsen, 2000).

The results declared in Fig (1) showed Agarose gel electrophoresis of multiplex PCR of *stx1*, *stx2*, *eaeA* and *hlyA* virulence genes for

characterization of Enteropathogenic *E. coli* and & table (7) clarified that from the examined 9 *E. coli* strains O₁, O₂₆, O₁₂₅ and O₁₄₂, it was found that *E. coli* O₁ (ETEC) carry (3) genes of *Stx1*, *eaeA* and *hlyA*; *E. coli* O₂₆ (EHEC) carries (4) *Stx1*, *Stx2*, *eaeA* and *hlyA* genes; *E. coli* O₁₂₅ (EPEC) carries (2) *Stx2* and *hlyA* genes; *E. coli* O₁₄₂ (EPEC) carries (3) *Stx1*, *eaeA* and *hlyA* genes. Moreover, Shiga toxin producing *E. coli* poses one gene was *E. coli* O₁ (ETEC), O₂₆ (EHEC) and O₁₄₂ (EPEC) carry (1) *Stx1* gene and *E. coli* O₂₆ (EPEC) and O₁₂₅ (EPEC) carries (1) *Stx2*. The current results agree to some extent to those obtained by Feng and Monday (2000), Pelisser *et al.* (2009), Pereria *et al.* (2009), Awny *et al.* (2010), Hassan (2012) and Shawish (2015) who could detect *Stx* and *eaeA* in *E. coli* isolated from meat products. While, Awadallah-Maysa *et al.* (2014) and Nadim-Samaa (2016) found that O₁₂₄ serovars of *E. coli* that isolated from meat products.

Severe disease in the form of bloody diarrhea and the hemolytic uremic syndrome is attributable to Shiga toxin (*Stx*), which exists as 2 major types, *Stx1* and *Stx2*. The *stx* genes are encoded on temperate bacteriophages in the chromosome of the bacteria, and production and release of the toxin are highly dependent on induction of the phages (Gyles, 2014)

The majority of human diseases are associated with strains of STEC that produce either Shiga toxin 1 (*Stx1*) and/or Shiga toxin 2 (*Stx2*) (Brudzinski and Harrison, 1998). *Stx2* is the primary virulence factor in systemic host responses produced by clinical isolates (Imamovic *et al.* 2009). The predominance of *Stx2* either alone or in combination with *Stx1* has been highly associated with HUS (Wani *et al.*, 2004) and considered to be the most important virulence factor as compared to *Stx1* (Mainil *et al.*, 1993); but several studies revealed that *Stx1* might have a role in diarrhea (Sandhu *et al.*, 1996). However, STEC strains that didn't carry intimin have been reported to cause HUS and HC in human (Barrett *et al.*, 1992).

STEC are food borne pathogens that have been associated worldwide with outbreaks (Mora *et al.*, 2011). As it transmitted to human mainly through contaminated food of animal origin as well as cross contamination due to

inappropriate food handling and preparation and/or insufficient heat treatment (Gyles, 2007) and cause different symptoms included diarrhea, abdominal pain, vomiting and fever. The onset of illness is typically 3-8 days after infection and most patients recover within 10 days. Acute STEC infections (HC) are characterized by severe abdominal cramps and bloody diarrhea. In some cases, patients develop HUS which can lead to kidney failure. HUS can also have neurological effects and cause seizure, stroke and coma. The fatality rate of HUS is 3-5% (FSANZ, 2013). Also, Shiga toxin induced the release of complement-coated red blood cell-derived microvesicles and the release of hemoglobin and lactate dehydrogenase in whole blood, may be thus Stx contributed to the induction of hemolysis (Arvidsson, 2015). It has been suggested that SEs stimulate the vagus nerve in the abdominal viscera, which transmits the signal to the vomiting center in the brain. In addition, SEs are able to penetrate the gut lining and activate local and systemic immune responses (Argudin *et al.*, 2010).

All traditional methods used for detection of food borne pathogens are often time consuming, therefore, for accurate identification of microbial contamination in meat product; it is strongly recommended to use PCR parallel with cultural identification (Tarabees *et al.*, 2015).

As, Polymerase Chain Reaction (PCR) involves detection of specific gene fragments by in vitro enzymatic amplification of the target DNA; followed by detection of the amplified DNA molecule electrophoresis or other techniques. The PCR method is a highly rapid, specific and sensitive method used for detection and characterization of pathogens and toxins. So, it is very useful in epidemiological investigations of food-borne illness as *S. aureus* enterotoxins (Vasavada, 2001).

The results declared in Fig (2) which showed Agarose gel electrophoresis of multiplex PCR of SEA, SEB, SEC and SED enterotoxin genes for characterization of *S. aureus*; indicated that from the examined selected 15 positive *S. aureus* strains it was found only 7 isolates were enterotoxin-producing strains, 4 strains of *S. aureus* carry SEA gene, 1 strain carry SEB gene, 3 strains carry SEC gene and 1 strain

carry SED gene. While, 8 isolates were negative for producing 4 enterotoxins (SEA, SEB, SEC and SED).

It is obvious from the results that there are *S. aureus* strains which carry more than one enterotoxin genes, such as strains in the lane 5 (SEA and SEB), and lane 14 (SEA and SED), and presence of these strains in meat products imposes potential hazards and induce food poisoning if the conditions are suitable for toxin production.

These results agree to some extent to those obtained by Pelisser *et al.* (2009), Pereira *et al.* (2009), Awany *et al.* (2010), and Hassan (2012) who used multiplex PCR for detection of *S. aureus* enterotoxin genes.

SEA is the most common toxin in staphylococcus-related food poisoning. SED is suggested to be the second most common staphylococcal toxin associated with food poisoning worldwide, and SEB, while it is associated with food poisoning, has been studied for potential use as an inhaled bioweapon (Ler *et al.*, 2006).

The amount of staphylococcal enterotoxins required for establishment of typical symptoms of food poisoning is very low, ranging from 20 ng to 1µg, which corresponds to approximately 10⁵ staphylococci cfu/g of food (Normanno *et al.*, 2007).

The *S. aureus* enterotoxins (SEs) are potent gastrointestinal exotoxins synthesized by *S. aureus* throughout the logarithmic phase of growth or during the transition from the exponential to the stationary phase (Derzelle *et al.*, 2009). SEs are superantigenic toxins, they are 5 major classical types, i.e., SEA, SEB, SEC and SED (Chiang *et al.*, 2008). SEA and SED are the most common enterotoxin recovered from food poisoning outbreaks (Pexara *et al.*, 2010).

SEs belong to the broad family of pyrogenic toxin super antigens that able to activate a large number of T-cells followed by proliferation and massive release of inflammatory cytokines that may led to potentially lethal toxic shock syndrome (Balaban and Rasooly, 2000).

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