ARTICLE / INVESTIGACIÓN

Molecular detection of some virulence genes, Shiga toxins and enterotoxin of *E. coli* and *S. aureus* isolated from dairy cows, workers and shared farm environments in Karbala Governorate-Iraq

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Abstract: *Staphylococcus aureus* and *E. coli* may contain one or more genes that encode toxins, including the staphylococcal enterotoxins, Shiga toxins and shock syndrome toxins. These can interact with several cellular targets to produce diseases, such as food poisoning and toxic shock syndrome. This study was designed to characterize the prevalence of Shiga toxins-producing E. coli (STEC) and enterotoxins-producing *S. aureus* among cow farms, worker farms and shared environments. Five dairy farms were recruited in the Karbala governorate. After gaining the owner's permission to visit the farms, A total of 400 samples were collected from cows (1 nose and 1 teat sample from each cow and 10 cows from each farm), (1 nose and 1 hand sample from each worker and ten workers from each farm), (one sample of milk from each cow and ten cows from each farm) and 30 environment samples per farm (10 swabs from milking tools, 10 swabs from the feeding place and 10 swabs from the cows' shelter). Sampling took place between June 2021 to October 2021. Farm workers were invited to volunteer to participate in the survey and sampling. The molecular profiling of *E. coli* isolates showed negative results for the presence of *eaeA, stx1* and *stx2* genes.10% (4 out of 40) of *S. aureus* isolates exhibited favorable PCR products for *femA* gene and the remaining 36 as *femA* negative. However, none of the *S. aureus* isolates were positive for SE production (sea and seb). In conclusion, the risk for severe human infections is low due to the loss of virulence, Shiga and enterotoxins-associated genes in *E. coli* and *S. aureus* adapted to livestock.

Key words: Shiga toxins, Enterotoxins, *eaeA*, *femA*, Cows, Human, Environment.

Introduction

Staphylococcus aureus and *E. coli* are major causative pathogens that threaten farmers; they are commonly found in environmental settings, such as bedding, clothes, farmers' hands, and water used on farms. It ubiquitous bacterial organisms that are found in a wide variety of places, including the human intestine, where they can lead to diarrheal disease and a range of extraintestinal infections[1]. Developing countries are affected mainly by foodborne infections, numerous epidemiological reports have implicated raw milk is usually colonized by a variety of zoonotic foodborne pathogens.These pathogens have originated from the environment on the farm, mixing clean milk with mastitis milk, manure, soil, and contaminated water. Therefore, food-producing animals are primary sources of most foodborne pathogens[2,3].

The significant virulence genes of STEC are the stx genes encoding the Shiga toxins and the eaeA gene encoding the intimin protein, which is responsible for adhesion. This protein is essential for the tight binding of bacteria to target cells and is encoded on a chromosomal pathogenicity island termed the locus for enterocyte effacement (LEE). The LEE has an attaching and effacing (A/E) lesion, which allows the binding of the toxins and, hence, results in an infection. The vast majority of virulence factors are encoded in mobile elements of the DNA: pathogenicity islands, transposons, plasmids and phages (Martin, 2017). The mechanism of disease cause, *E. coli*, is divided into six groups of pathotypes. Enteropathogenic *E. coli* (EPEC), Attaching and effacing *E. coli* (A/EEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), EHEC and Enteroaggregative *E. coli* (EAEC). *E. coli* strains that produce the Stx toxins have been referred to as Vero Toxin-producing *E. coli* (VTEC), Shiga-toxigenic *E. coli* (STEC) and enterohaemorrhagic *E. coli* (EHEC)[4]. The events of pathogenesis can be summarized as follows[5]: (i) the colonization of the gut,(ii) the effect of the virulence factors on the host and (iii) disease caused by the virulence factors. STEC contamination is not limited to cattle products, but there has been a higher frequency of cases involving beef or veal[5]. Ruminants, especially cattle and sheep, unspecified meat and sheep meat, young cattle and unpasteurized milk from cattle have constituted a vast range of STEC. It is not surprising that humans most frequently become infected with STEC by ingesting contaminated food or water or by direct contact with animals, resulting in sporadic disease or outbreaks involving up to several thousand individuals. The polymerase chain reaction assay should aid in quickly detecting this virulent serotype and help curb the severe epidemic of human diseases associated with STEC infections[6].

Various virulence factors work together in the pathoge-

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nic process of *S. aureus*. The broad range of infections by *S. aureus* is related to several virulence factors that allow it to adhere to the surface, invade or avoid the immune system, and cause harmful toxic effects to the host[7]. The *femA* and *femB* genes encode proteins that influence the methicillin resistance level. Both are involved in the formation of the cell wall. The *femA* gene is involved in the glycine content of peptidoglycan and peptidoglycan biosynthesis of *S. aureus*. It mediates the effect on drug sensitivity, and thus, it is involved in methicillin resistance[8]. There are nine major antigenic types of staphylococcal enterotoxins (SEs) have been reported (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and SEJ), while only one serotype of TSST (comprised of TSST-1 and TSSTovine) has been characterized[9]. This study aimed to detect the Shigathe toxin of *E. coli* and enterotoxin of *S. aureus* on cows, humans and shared environments using PCR.

Materials and methods

Study area

Five dairy farms were recruited in the Karbala governorate. After gaining the owner's permission to visit the farms, samples were collected from cows (1 nose and 1 teat sample from each cow and ten cows from each farm), (1 nose and 1 hand sample from each worker and ten workers from each farm), (one sample of milk from each cow and ten cows from each farm) and 30 environment samples per farm (10 swabs from milking tools, 10 swabs from the feeding place and 10 swabs from the cows' shelter). Sampling took place between June 2021 to October 2021. Farm workers were invited to volunteer to participate in the survey and sampling.

Bacterial diagnosis

In other research, we isolated and identified E. coli and S. aureus isolates based on cultural and biochemical criteria and confirmed by Vitek-2-system and molecular detection of the 16SrRNA gene for each bacteria. The obtained results were 33 E. coli and 40 S. aureus from 400 samples collected.

Molecular detection

Polymerase chain reaction (PCR) was performed to amplify target genes using primers for the following genes: *eaeA*, *Stx1* and *Stx2* for *E. coli*, and *femA*, Sea and Seb for *S. aureus*. The sequences of these primers are listed in Table 1.

DNA extraction of bacterial genome

Chromosomal DNA of the overnight broth culture of *E. coli* and *S. aureus* isolates was obtained according to the protocol of ABIOpure Total DNA Kit (ABIOpure, USA); the procedure was explained in detail in the user's manual. The extracted DNA was validated by Nanodrop and stored at −20°C in the refrigerator until further usage. After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was utterly dependable on the extracted DNA criteria[10].

Molecular detection of Shiga toxin and *eaeA* gene in *E. coli* isolates

The PCR technique was used for molecular profiling of *E. coli* isolates through amplifying Shiga toxin-encoding genes *stx1, stx2*, and the intimin-encoding gene (even). The PCR reaction was performed using primers listed in (Table 1) in a Thermal Cycler (Thermo Fisher Scientific, USA). Approximately 2 ng of bacterial DNA was added to 10 µl Master Mix (10X), 1 µl of each primer and the final volume was adjusted to 20 µl by 6 µl adding Nuclease Free Water. The amplification conditions started with initial denaturation for 3 min at 95°C followed by 30 cycles of 95°C for 3 min, 63.4, 62.5 and 58°C for 40 s, and 72°C for 2 min. The final cycle was followed by a 72°C final extension for 5 min. The amplified DNA fragments were separated by 1.5% agarose gel electrophoresis (Optima, Japan) in 1x TBE buffer and

Organisms	Primer Name	Sequence 5`- 3`	Annealing Temp (ºC)	Product Size (bp)	Fagan et a
	STX1-F	F:5′- ACACTGGATGATCTCAGTGG -3′	62.5	890	Fagan et al.,1999
	STX1-R	R:5′- CTGAATCCCCCTCCATTATG - '3			
	STX2-F	F- 5′ ACCATGACAACGGACAGCAGTT - '3	58	614	Fagan et al.,1999
	STX2-R	R- 5′ CCTGTCAACTGAGCAGCACTTTG - '3			
	EaeA-F	F- 5′ GTGGCGAATACTGGCGAGACT- '3	63.4	799	Gannon et al., 1993
	EaeA-R	R- 5′ CCCCATTCTTTTTCACCGTCG - '3			
S. aureus	Sea-F	F:5′- TTGGAAACGGTTAAAACGAA -3′	58	120	Rall et al., 2008
	Sea-R	R:5′- - GAACCTTCCCATCAAAAACA 3′			
	Seb-F	F- 5′ TCGCATCAAACTGACAAACG- '3	58	478	Rall et al., 2008
	Seb-R	R- 5′ GCGGTACTCTATAAGTGCC- '3			
	FemA-F	F- 5′ TCGCATCAAACTGACAAACG- '3	55	98	Brahma et al., 2019
	FemA-R	R: 5′ TCCTAAGTTACTCATTTTATCAAA-GAAC -3′			

Table 1. Sequences of primers used in the conventional PCR.

captured and visualized on a UV transilluminator. A 100 bp plus DNA Ladder was used to determine each amplicon size.

Molecular detection of enterotoxin and *femA* gene in *S. aureus*

The presence of enterotoxins (sea and seb) and *femA* gene amplification were detected using PCR. The PCR mixture reactions were performed for *E. coli*. The amplification conditions started with initial denaturation for 5 min at 95°C followed by 30 cycles of 95°C for 3 min, 58, 58 and 55°C for 3 min, and 72°C for 3 min. The final cycle was followed by a 72°C final extension for 7 min. Sequences of the used primers are listed in (Table 1). The amplified DNA fragments were separated by 1.5% agarose gel electrophoresis (Optima, Japan) in 1x TBE buffer, captured, and visualized on a UV transilluminator. A 100 bp plus DNA Ladder was used to determine each amplicon size.

Results

Detection of *eaeA* and Shiga toxin (*stx1, stx2*) genes

The polymerase chain reaction assay should aid quick detection of the virulent serotype and help suppress the severe epidemic of human diseases associated with STEC infections. It is well known that STEC is a zoonotic food and water-borne pathogen associated with diarrhea and renal failure, particularly in children, and can cause attaching and effacing properties in diarrhea cases[11]. Also, previous reports revealed that virulence genes were detected in only a few *E. coli* strains, which may be due to the fact that there are occasional strains that have the genes but do not express the toxins[15]. On the other hand, many studies reveal that cattle have been recognized as the main reservoir of STEC strains[16].

Detection *femA* and enterotoxins (Sea, Seb) genes

The *femA* gene, which encodes a protein precursor, is involved in peptidoglycan biosynthesis and has been used as a molecular marker for identifying *S. aureus* (Jukes *et al.*, 2010). The amplified DNA (98-bp) region of *femA* gene could be identified through Agarose gel electrophoresis with a suitable 100bp ladder DNA marker (Figure 2). 10% (4 out of 40) of *S. aureus* isolates exhibited favorable PCR products for *femA* gene and the remaining 36 as *femA* negative (Table 3), which disagreed with the results of 19 who found that the *femA* (147) and *femB* (138) genes were the most frequent in 148 samples of *S. spp* samples.

Staphylococcus aureus isolates can produce enterotoxins, posing a public health threat. This means that the detection of Enterotoxins is very crucial[21]. In the current study, the molecular detection of *sea* and *seb* genes was beneficial for properly characterizing Enterotoxins-producing *S. aureus*. PCR was used to screen the existence of the enterotoxins (*sea* and *seb*) genes in 40 *S. aureus* isolates (Figure 3). None of the 40 *S. aureus* isolates were positive for SE production (*sea* and *seb*) (Table 3).

Discussion

Several primers that amplify these genes have previously been reported, including the ones used in this

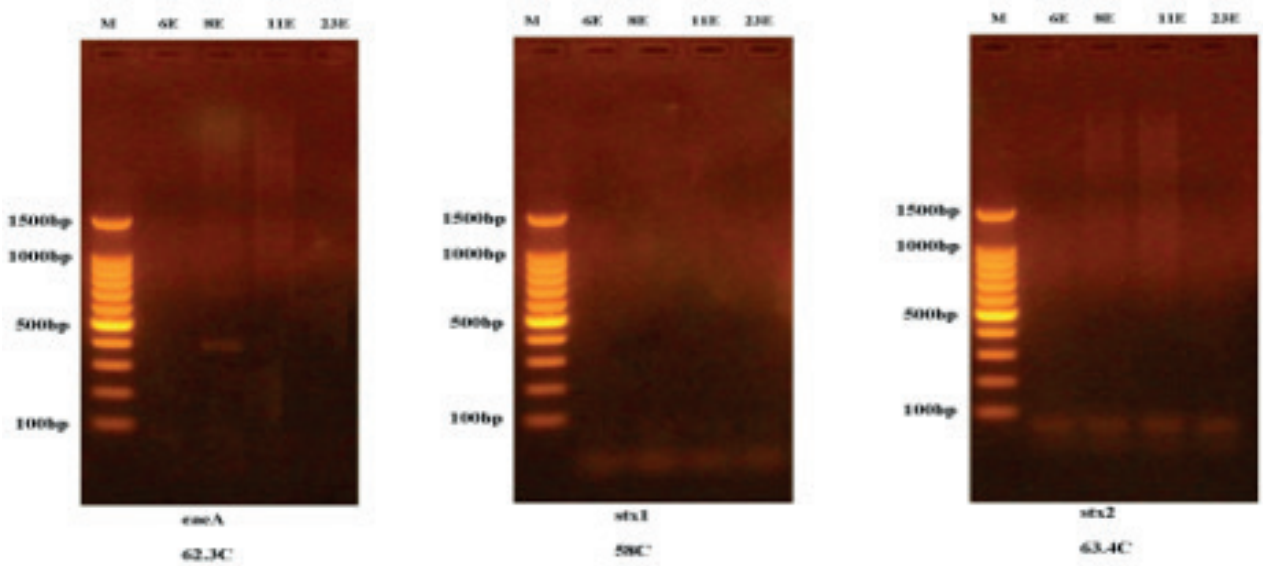

Figure 1. The amplification of *eaeA, stx1* and *stx2* genes of *E. coli* were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker.

Gene	Gene detection	Number	%
EaeA	Present	0	0
	Absent	33	100
Stx1	Present	0	0
	Absent	33	100
Stx2	Present	0	0
	Absent	33	100

Table 2. Detection of *16SrRNA* and some virulence genes in *E. coli* isolated from five areas in Karbala.

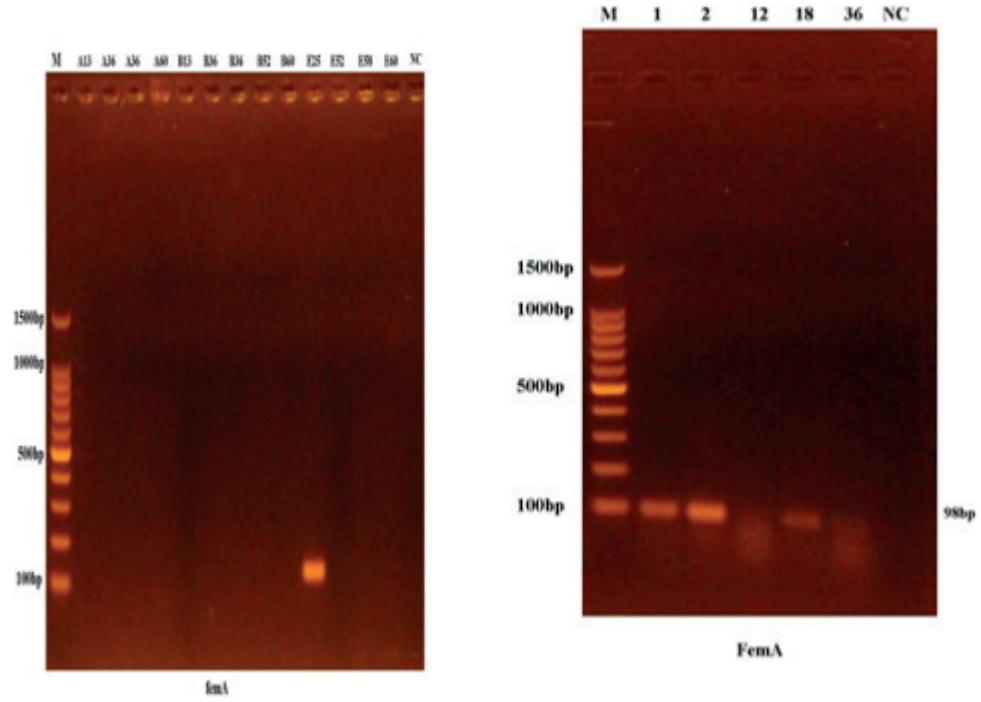

Figure 2. The amplification of *femA* gene were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker.

Gene	Gene detection	Number	%
femA	Present	4	10
	Absent	36	90
Sea	Present	0	0
	Absent	40	100
Seb	Present	0	0
	Absent	40	100

Table 3. Detection of 16SrRNA and some virulence genes in *S. aureus* isolated from five areas in Karbala.

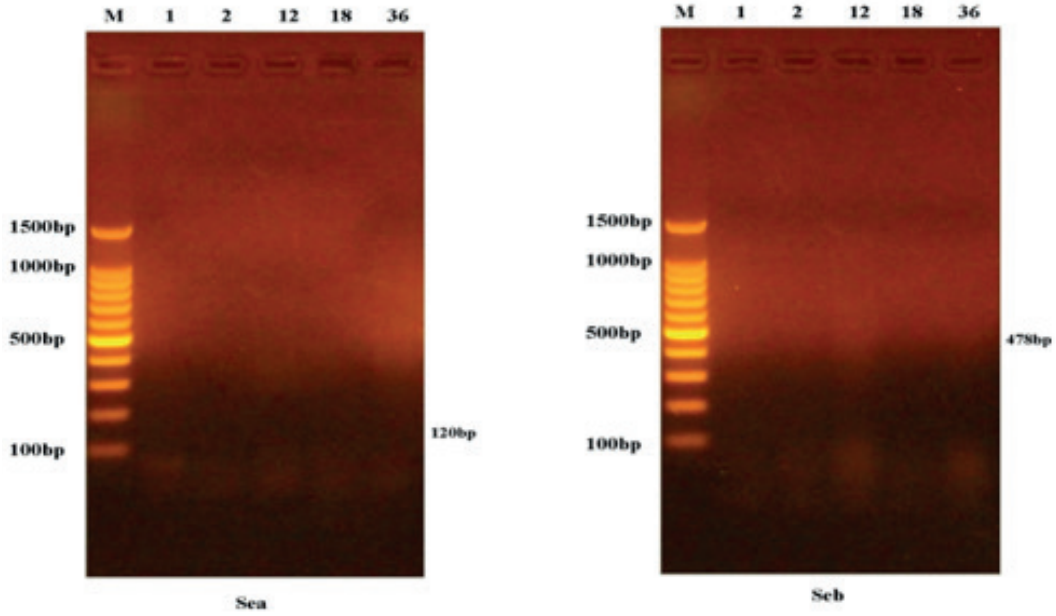

Figure 3. The amplification of sea and seb genes was fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker.

study[11,12]. The molecular profiling of *E. coli* isolates showed negative results for the presence of *eaeA*, *stx1* and *stx2* genes (Figure 1). These results were correlated with previous studies, which showed that eaeA-positive *E. coli* was not found in clinical mastitis cases[13], and with the results obtained by (14) none of the isolates had *stx2* shown by PCR. Numerous investigators have underlined the strong association between the carriage of *eaeA* gene and the capacity of STEC strains to cause severe human illnesses.

This study demonstrated the absence of pathogenic *E. coli* in cows, workers and environmental sources by detecting the virulence genes associated with the pathogenic (Table 2). These results imply that the presence of drug-resistant strains of non-pathogenic *E. coli* isolates from the

environment is possible. This can threaten management programs for farm[17] since one study also reported that non-pathogenic *E. coli* can serve as a reservoir of antibiotic resistance genes and could transfer the genes to other pathogenic *E. coli* if conditions are suitable[18].

The absence of *femA* and *femB* is related to glycine reduction in the peptidoglycan, thus making the cell wall more susceptible to beta-lactams[20].

The risk for acute human infections is low due to the loss of virulence-associated genes and adaptation of *S. aureus* to livestock[21]. These results agree with the previous result of other authors[22], who showed that sea and seb genes were not detected in all isolates. However, disagree with the results of (23) who reported that Of all 77 *staphylococcus* enterotoxin (SE)-positive isolates, more than 90% could produce enterotoxins, and (24) when used commercial test kits (SET-RPLA) showed that 21.4% (3 out of 14 isolates) of *S. aureus* isolates produced classic enterotoxins (sea, sec, sed). The findings of this study indicate that all isolates isolated from the five study areas do not produce enterotoxins.

Conclusions

The present study uses molecular tools to provide new information on the genotypic traits of *E. coli* and *S. aureus* isolates from cow farms, workers and shared environments. The risk for severe human infections is low due to the loss of virulence, Shiga and enterotoxins-associated genes in *E. coli* and *S. aureus* adapted to livestock. In the future, surveillance studies using large sample size should be conducted to make the findings robust.

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