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Virulence Characterization of *Escherichia coli* Isolates Collected from Bovine Clinical and Subclinical Mastitic Milk Samples

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The Virulence characterization of *Escherichia coli* in clinical and subclinical mastitic milk samples was studied in this study. Bacteriological examination of 292 milk samples collected from Egypt revealed 44 *E. coli* organisms (15.068 %).The result showed that the *E. coli* incidence in clinical mastitic milk higher than subclinical mastitic milk. Serotyping *E. coli* isolates revealed 41 strains were typed (15.06%). Detection of verotoxin activity of *E. coli* strains showed that 5 strains (6.4%) belonged to O128: K67, O111: K58 and O126: K58 from clinical mastitic milk samples, while 15 strains (7.01 %) were from subclinical mastitic cases. For detection of shiga toxin type 2 (*stx*2) and intimin (*eaeA*) genes by multiplex PCR, the results revealed positive amplification of intimin gene in 4 strains O111 (9.76%), 3 strains of O128:K67 (7.32%), 2 strains of O26:K60, O55:K59 and O119:K69 (4.88%), one strain of O126:K7(2.44%). Simultaneous amplification of shiga toxin type 2 gene and intimin gene in all 4 *E. coli* serovar O157: H7, while 3 from serovars O111 (7.32%), from serovars O128 and O26:K60 were 2 (4.88%) and one strain of O55:K59 (2.44%) which confirm that multiplex PCR as-say are useful for identification of STEC possessing the *eaeA* and *stx*2 genes.

Key words: Mastitis, *E. coli*, Multiplex PCR, Shiga toxin type 2, Intimin gene.

Bovine mastitis is the most common infectious disease affecting cows and remains the most economically important disease of dairy industries around the world. Dairy producers' losses are primarily due to lower milk yield, reduced milk quality, higher production costs, or even destruction of mammary gland [1].

Moreover, mastitis is considered of vital importance to the public health due to association with many zoonotic disease in which milk acts as a vehicle for infectious agent.[2] The most common mastitis pathogens are *Staphylococcus aureus, Escherichia coli, Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis.*

The serious effects of mastitis are mostly due to its subclinical form, during which the causative micro-organism act as invisible potential source of spreading infection in the herd, therefore, it is important to identify quickly the new clinical cases to control infection in the herd.

Presence of *E. coli* in milk products indicates the presence of enteropathogenic microorganisms, which constitute a public health hazard. Enteropathogenic *E. coli* can cause severe diarrhoea and vomiting in infants and young children.[3]

Shiga toxin producing *Escherichia coli* (STEC) is the most important recently emerged group of foodborne pathogens.[4] It can cause severe gastrointestinal disease, including fatal infections, and is being detected with increasing frequency worldwide. Transmission occurs usually through consumption of undercooked meat, unpasteurized dairy products and vegetables or

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water contaminated by faeces of carriers.[5]

The present study was aimed at investigating the prevalence of *E. coli* strains in milk samples of clinical and subclinical mastitis in different of governorates in Egypt. The virulence characteristics of isolates were also studied.

Samples

During the period of 25th May to 25th October 2012, a total of 292 bulk-tank milk samples(78 clinical mastitic milk samples and 214 subclinical mastitic milk samples) were collected from 10 cow farms located in different Egyptian governorates. The samples were placed on ice and transported immediately to the laboratory.

Isolation and identification of *E. coli:*

Milk samples were centrifuged at 3000 rpm for 15 minutes and after centrifugation the supernatant as well as the sediment were cultured. Milk samples was primary cultured on MacConkey agar medium, incubated aerobically at 37°C. After overnight incubation, a part of single typical well isolated lactose fer-menting colonies were tested for sorbitol fermentation by culturing on sorbitol MacConkey agar and sorbitol phenol red agar media, incubated at 37°C overnight. Morphological, cultural and biochemical examination were carried out according to methods described by Quinn et al.[6] Samples were cultured in MacConkey (MAC) medium. Agar plates were incubated at 37°C and bacterial growth was evaluated after 24 and 48 h, respectively. Gram-negative microorganisms were isolated from MAC agar and determined at the species level using cytochrome oxidase, triple sugar iron agar, urea, and indole tests as putatively *E. coli*[7]. Reference strains used were *E. coli* strain ATCC 35150 (O157:H7, *stx1, stx2, eae, hly*) (positive control) and *Staphylococcus aureus* ATCC 29737 (negative control).[8]

Serotyping of *E. coli:*

Isolates that were primarily identified by biochemical tests as *E. coli* were subjected to serological identification using diagnostic polyvalent and monovalent *E. coli* antisera (Welcome *E. coli* diagnostic antisera). Diagnostic *E. coli*- O157 antisera (Difco code 2970-47-7) and H7 antisera (Difco code 2159-47-0) were used for serologi-cal identification of *E. coli* O157: H7.

Vero cell cytotoxicity

E. coli isolates were grown in brain heart infusion broth for 8 h at 41°C. Then, 5ml of each isolate was subcultured into 50ml Casamino acid–yeast extract–salts medium without glucose. The cells were allowed to aerobically grow at 37°C.

After 18–20 h, the cells were removed by centrifugation at 12,000 g for 15 min at 4°C. The cell pellet was resuspended in 0.5ml sterile phosphate-buffered saline (0.1 M, pH 7.2). The cells were ultrasonically disrupted continuously for 2 min in an ice bath using a sonicator. The cytotoxicity assay was performed with Vero cells as previously described.[9]

Extraction of DNA:

The DNA of the standards strains and of the other bacterial isolates yielded from bacteriological examination was extracted by hexadecyl trimethyl ammonium bromide (CTAB), according to Sambrook et al.[10] Meanwhile, the extractions of DNA from milk samples were carried out according to Riffon et al.,[11] and Meiri-Bendek et al.[12] One ml of each culture was centrifuged at 5000 rpm / 5 minutes, and then the sedi-ment was washed five times with sterilized water and finally suspended in 1.0 ml of sterilized water. The suspension was kept at 95°C for 15 minutes, and after centrifu-gation at 15,000 rpm for 5 minutes, 10 ml of the supernatant was directly used for PCR.

PCR design and **amplification conditions according to Paton and Paton[13]**

PCR primer pairs were designed with reference to published sequence data for shiga toxin type 2 (*stx2*)[14], intimin gene (*eaeA*).[15] Details of the nucleotide sequence, the specific gene region amplified, and the size of the PCR product for each primer pair are listed in Table 1. The extracted DNA of the standard strains and of the bacterial isolates yielded from bacteriological examination was tested with multiplex-PCR using the oligo-nucleotide primers specific for (*stx2)* and *(eae A)* genes. Concurrently the crude DNA extracted from milk samples were tested by the same primer pair. All reactions were carried out in a final volume of 50ml in micro--amplification tube (PCR tubes). The reaction mixture consisted of 10ml (200mg) of the extracted DNA template from the bacterial cultures or 5ml of the extracted DNA tem-plate from the milk samples, 5ml of 10X PCR buffer (BIO-TOOLS) (75mM Tris-HCI, pH 9.0, 2mM $MgCI_2$, 50mM KCI,20 mM $(NH_4)_2$ SO_4, 1ml dNTPs *(40mM)* (BIOTOOLS), 1ml (1 U Amplitaq DNA polymerase) (Qiagen), 1ml (50 pmol) from the forward and reverse

primers. The volume of the reaction mixture was completed to 50ml using deionized distilled water (DDW). 40ml paraffin oil was added and the samples were subjected to PCR cycles, each consisting of 1 min of denaturation at 95°C; 2 min of an-nealing at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15; and 1.5 min of elongation at 72°C, incrementing to 2.5 min from cycles 25 to 35. Final extension was carried out at 72°C for 10 min, and the PCR products were visualized by agarose gel electrophoresis according to Sambrook et al.[10]

RESULTS AND DISCUSSION

E. coli, a gram-negative environmental pathogen associated with bovine mastitis, was isolated from 292 mastitic milk samples (78 clinical mastitic milk samples and 214 subclinical mastitic milk samples) collected from different Governorates revealed the presence of 44 (15.068 %) *E. coli* isolates, 32 (14.95%) among subclinical mastitic milk samples and 12 (15.38%) clinical mastitic milk samples. In France, *E. coli* (16%) was identified as one of the major causative agents of mastitis[16], a prevalence that is very close to our findings in Egypt (15.38 %). The results obtained in the present study, indicated that the incidence of *E. coli* in clinical mastitis is higher than subclinical mastitis. The presence of this pathogen in milk proved to be variable in different regions may be contributed to many factors such as geographical location, season, farm size, number of animals on the farm, hygiene status, farm management practices, variation in sampling, variation in types of samples evaluated, and differences in detection methods.

Serotyping of the *E. coli* isolates yielded from bacteriological examination of milk samples revealed 41 strains out of 44 *E. coli* isolates were typed (15.06%) as follows; 7 strains as O119: K69 (2.39%), 6 strains as O128: K67 (2.05%), 6 strains as O111: K58 (2.05%), 5 strains as O126: K58 (1.71%), , 4 strains as O55: K59 (1.37%), 4 strains as O26: K60 (1.37%), 4 strains as O157: H7 (1.37 %), 3 strains as O86: K61 (1.027%)and 5 strains (1.71%) were untypable.

The data in Table (2) showed that O119: K69, O128: K67, O111: K58 and O126: K58 were

Table 1. PCR primers used for multiplex PCR

Primer	Sequence (5' – 3')	Specificity	Amplicon size
eaeA F	GAC CCG GCA CAA GCA TAA GC	Intimin gene	384
eaeA R	CCA CCT GCA GCA ACA AGA GG		
stx2 F	GGC ACT GTC TGA AAC TGC TCC	Shiga toxintype 2	255
stx2 R	TCG CCA GTT ATC TGA CAT TCT G		

Table 2. Serotyping of E. coli recovered from subclinical and clinical mastitic of milk samples

Serovars	Samples from subclinical mastitis (214)		Samples from clinical mastitis (78)		Total (292)	
	No.	%	No.	%	No.	%
O119: K69	3	1.4	4	5.13	7	2.39
O128: K67	3	1.4	3	3.85	6	2.05
O111: K58	3	1.4	3	3.85	6	2.05
O126: K71	3	1.4	2	2.56	5	1.71
O86: K61	3	1.4	-	0	3	1.027
O55: K59	4	1.86	-	0	4	1.37
O26: K60	4	1.86	-	0	4	1.37
O157: H7	4	1.86	-	0	4	1.37
Untyable	5	2.34	-	0	5	1.71
Total	32	14.95	12	15.38	44	15.06

%: was calculated according to the number of examined samples between brackets.

detected from the clinical mastitic samples with an incidence of 5.13%, 3.85%, 3.85%, 2.56% respectively. Meanwhile O119: K69, O128: K67, O111: K58, O126: K58, O55: K59 (1.37%), O26: K60, O157: H7, O86: K61 and untypable strains were recovered from subclinical mastitic cases.

Our results indicated that *E. coli* recovered from mastitic cases belonged to a very broad range of O serogroups which confirm the conclusion of Fuse et al.[17], Harmon et al., [18] and Garber et al..[19]

Detection of verotoxin activity of *E. coli* strains using vero cells was investigated. Table (3) showed that 5 strains (6.4%) belonged to O128: K67, O111: K58 and O126: K58 from clinical mastitic milk samples, meanwhile, 15 strains (7.01 %) were recovered from subclinical mastitic cases. The results coincided with that mentioned by Karmali et al [20] and Chapman et al.[21]

The *E. coli* serovars recovered by bacteriological examination were tested by multiplex PCR using stx2 F & stx2 R and eaeA F&

Table. 3 Cytotoxicity of E. coli serovars isolated from subclinical and clinical mastitic of milk samples

E. coli serovars	Subclinical mastitic samples (214)			Clinical mastitic samples (78)			Total (292)		
	No. of serovars	Cytotoxic activity +ve		No. of serovars	Cytotoxic activity +ve		No. of serovars	Cytotoxic activity +ve	
		No.	%		No.	%		No.	%
O119: K69	3	0	0	4	-	0	7	-	0
O128: K67	3	2	0.93	3	2	2.56	6	4	1.37
O111: K58	3	3	1.4	3	2	2.56	6	5	1.71
O126: K71	3	1	0.46	2	1	1.28	5	2	0.68
O86: K61	3	0	0	-	-	0	3	-	0
O55: K59	4	3	1.4	-	-	0	4	3	1.02
O26: K60	4	2	0.93	-	-	0	4	2	0
O157: H7	4	4	1.86	-	-	0	4	4	1.037
Untyable	5	0	0	-	-	0	5	-	0
Total	32	15	7.01	12	5	6.4	44	20	6.84

%: was calculated according to the number of examined samples between brackets

Table 4. Characterization of the recovered E. coli serovars by multiplex PCR using stx2 F, stx2 R and eaeA F, eaeA R primers:

E. coli serovars	Number of examined serovars	Multiplex PCR			
		Positive for eaeA* gene		Positive for stx2** gene	
		No.	%	No.	%
O119: K69	7	2	4.88	0	0.00
O111: K58	6	4	9.76	3	7.32
O128: K67	6	3	7.32	2	4.88
O86: K61	3	0	0.00	0	0.00
O126: K71	5	1	2.44	0	0.00
O26: K60	4	2	4.88	1	4.88
O55: K59	4	2	4.88	1	2.44
O157: H7	4	4	9.76	4	9.76
Total	39	18	43.90	12	29.27

%: was calculated according to the total number of typable strains.
* Intimin gene; ** Shiga toxin type-2 gene.

eaeA R primers. The results observed in Table (4) revealed positive amplification of intimin gene from the DNA extracted of 4 strains O111(9.76%), 3 strains of O128:K67 (7.32%), 2 strains of O26:K60, O55:K59 and O119:K69 (4.88%),one strain of O126:K7(2.44%). Simultaneous amplification of 255 bp fragment of shiga toxin type 2 gene and 384 bp fragment of intimin gene from all 4 *E. coli* serovar O157: H7, while 3 from serovars O111 (7.32%), from serovar O128 and O26:K60 were 2(4.88%) and one strain of O55:K59 (2.44%). No amplification could be observed with *E. coli* serovars O86:K61, O119:K69 and O126:K61.

CONCLUSION

Our results demonstrated that incidence of *E. coli* strains is higher in clinical than subclinical mastitis. Also, the results were of importance to the microbiologists seeking to use a simple, reliable and rapid procedure that can detect the major virulence factors of *E. coli.*

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