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Virulence Factors of *Escherichia coli* Isolated from Recurrent Cases of Clinical and Subclinical Mastitis in Buffaloes

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Abstract: Eight hundreds and forty milk samples from 220 lactating buffaloes with previous history of recurrent mastitis were examined for detection of clinical and subclinical mastitis. The clinical mastitis prevalence rates were 25.55% in Sharkia and 29.23% in Dakahlia Governorates. The incidence of subclinical mastitis was 40 and 33.84% in Sharkia and Dakahlia Governorates, respectively. *E. coli* was isolated from 63 (18.47%) mastitis milk samples out of 341 examined ones. 63 *E. coli* isolates were serotyped to O157, O153, O55, O18, O114, O126, O26, O1 and 21 untyped strains. Virulence tests were performed on *E. coli* serogroups isolated from mastitis cases. It was found that 50 strains (79.37%) were serum resistant, 24 (38.1%) had Congo red binding activity, 20 (31.75%) were invasive and 8 (12.7%) had hemolytic activity. PCR was applied to detect the presence of *stx2* and *eaeA* genes, where *stx2* was found in all strains tested, while *eaeA* gene was found in 3 *E. coli* strains only.

Key words: *E. coli* · Virulence Genes · Mastitis · Buffaloes · PCR

INTRODUCTION

Mastitis is known as a disease that causes heavy financial losses to milk producers and to the dairy industry [1-6]. This is attributed to severe drop in milk production [7], decrease in milk quality, increased veterinary expenses due to excessive use of medications, increased labor costs and increased culling rate and decreased reproductive efficiency in high producing animals [8-10].

Gram-negative bacteria are the etiological agents most often isolated from severe clinical cases of mastitis. Many studies have implicated coliforms as most common pathogens isolated from cases of bovine mastitis [11-16]. Although some cases are severe and occasionally fatal, most cases are self-limiting and would be resolved without therapy. Chronic coliform infections also occur, which may be subclinical but typically elicit recurrent clinical episodes [17]. *E. coli* is the most important coliform that has received more attention due to its high incidence relatively to other mastitis pathogens [7, 18,19]. *E. coli* is a risky microorganism due to the great zoonotic importance of some of its strains as O157:H7 [20]. Numerous studies were directed to identify virulence factors of *E. coli* isolated from animals with clinical mastitis [21-23]. These studies stated that *E. coli* isolated from acute mastitis cases lack virulence factors commonly observed in other *E. coli* groups associated with disease except serum resistance and this made some studies suppose that serum resistance is the only characteristic factor that could be related to virulence in *E. coli* causing intramammary infections.

The present work was planned to detect virulence factors of *E. coli* recovered from mastitis in buffaloes, mainly serum resistance, Congo red binding activity, invasiveness and hemolytic activities, in addition to the detection of shiga toxin type 2 and intimin genes in *E. coli* isolated from recurrent clinical mastitis and subclinical mastitis in buffaloes.

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MATERIALS AND METHODS

Diagnostic Antisera: Polyvalent and monovalent diagnostic *E. coli* antisera "Denka Seiken Co. LTD" were used for serotyping of *E. coli* isolates. They included 8 vials of polyvalent in addition to the 43 vials of monovalent antisera.

Laboratory Animals: Rabbits 2-2.5 kg body weights were used for invasiveness assay.

Oligonucleotides Primers Used for Amplification of *Stx*2 and *Eae*a Genes of *E. Coli*: The following specific oligonucleotides primers for amplification of *stx2* and *eae*A genes of *E. coli* were selected [24]:

Stx2	F: 5'GGCACTGTCTGAAACTGCTCC3'
	R: 5'TCGCCAGTTATCTGACATTCTG3'
*eae*A	F: 5'CCACCTGCAGCAACAAGAGG3'
	R: 5'GACCCGGCACAAGCATAAGC3'

Collection of Milk Samples: A total of 840 quarters of 220 lactating buffaloes with a previous history of mastitis were examined for recurrent clinical and subclinical mastitis. The buffaloes were from Sharkia and Dakahlia Governorates; 61 buffaloes with 161 quarters showed clinical signs of mastitis and the rest of the quarters were examined for the presence of subclinical mastitis by California mastitis test [25, 26].

Isolation and Identification of *E. coli*: Milk samples were inoculated onto MacConkey's agar plates and incubated aerobically at 37°C for 24 hours. Pure colonies of the *E. coli* isolates were confirmed based on their biochemical characters [27].

Serological Typing of *E. coli*: Isolates were serotyped by polyvalent and monovalent antisera [28].

Detection of Virulence Factors of *E. coli* Isolates Causing Mastitis: Tests used were; Serum resistance test [21], Congo red binding [29], invasiveness assay [30] and hemolysin production [31].

Detection of stx2 and intimin Genes Using PCR: 1. Extraction of DNA from *E. c*oli isolates [32], 2- PCR amplification and cycling protocol [33] 3-Screening PCR products by agarose gel electrophoresis [34].

RESULTS

Incidence of Clinical and Subclinical Mastitis among Lactating Buffaloes: As shown in Table 1, in this study a total of 220 lactating buffaloes with previous history of mastitis (90 from Sharkia Governorate and 130 from Dakahlia Governorate) were examined. Clinical examination revealed that the incidence of clinical recurrent mastitis among examined buffaloes was 25.5% in Sharkia Governorate and 29.2% in Dakahlia Governorate. On the other hand, the incidences on the quarter level were 17.6 and 20.2 % in Sharkia and Dakahlia Governorates, respectively.

Subclinical mastitis was detected by testing quarter milk samples, which recorded incidence of 25.21 and 18.78% in Sharkia and Dakahlia Governorates,

Respectively, while the incidence of subclinical mastitis among buffaloes were 40 and 33.8% in Sharkia and Dakahlia Governorates, respectively. Distribution of mastitis among quarters revealed that clinical mastitis was more common to affect 4 and 3 quarters with an incidence of 24.02 and 38.84%. On the other hand, subclinical mastitis in 1 and 2 quarters was more common with incidences of 32.1 and 28.7%, respectively (Table 2).

Isolation and Identification of *E. coli*: *E. coli* was isolated from 63 out of 341 examined mastitis milk samples with an incidence rate of 18.47%. It was recovered from 35 out of 141 mastitic buffaloes with an incidence of 24.82%. Incidence of *E. coli* isolated from clinically mastitic buffaloes was 26.08 and 36.8% in Sharkia and Dakahlia Governorates, respectively. The corresponding rates in subclinical cases were 16.66 and 20.45%, respectively.

As shown in Table 3, 42 out of 63 *E. coli* strains isolated from clinically and subclinically mastitis milk samples, were serotyped into O157 (4 isolates), O55 (6 isolates), O78 (6 isolates), O114 (7 isolates), O18 (5 isolates), O153 (4 isolates), O1 (2 isolates), 0126 (3 isolates) and O26 (5 isolates), in addition to 21 untypable strains.

Detection of Virulence Factors of *E. Coli* Isolated from Mastitis Milk Samples: The results of testing *E. coli* for 4 phenotypic virulence factors were depicted in Table 4. Concerning the virulence factors of *E. coli* strains; serum resistance ability was detected in 50 out of 63 *E. coli* strains isolated from mastitis milk samples in an incidence of 79.37%. Thirty two (86.48%) and 18 (69.23%) *E. coli*

Table 1: Incidence of mastitis among examined lactating buffaloes

	Sharkia Governorate				Dakahlia Goverorate				Total	
	No. ex.	C+	SC+	Total	No. Ex.	C+	SC+	Total	No ex.	No+
A	90	23 25.5%	36 40%	59 65.5%	130	38 29.2%	44 33.8%	82 63.1%	220	141 64.1%
Q	345	61 17.6%	87 25.2%	148 42.8%	495	100 20.2%	93 18.7%	193 38.9%	840	341 40.6%

No. ex.: No. examined, A: Animal, Q: Quarter, C: Clinical mastitis, SC: Subclinical mastitis

Table 2: Incidence of mastitis in different buffaloes quarters

	Governorate													
	Sharkia						Dakahlia							
No. of quarters affected	CM (23)		SCM (36)		Total (59)		CM (38)		SCM (42)		Total (82)		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No	%
4 quarters	5	21.73	9	25	14	23.72	10	26.31	6	13.63	16	19.51	30	21.27%
3 quarters	10	43.47	7	19.44	17	28.81	13	34.21	9	20.45	22	26.82	39	27.65%
2 quarters	3	13.04	10	27.77	13	22.03	6	15.78	13	29.54	19	23.17	32	22.69%
1 quarter	5	21.73%	10	27.77	15	25.42	9	23.68	16	36.36	25	30.48	40	28.36%

CM: Clinical mastitis

SCM: Subclinical mastitis

Table 3: Serogroups of *E. coli* isolates recovered from mastitis milk samples

	Clinical mastitis		Subclinical mastitis		Total mastitis	
E. coli serogroup	No	%	No	%	No	%
O114	4	10.8%	3	11.54%	7	11.11%
O55	3	8.10%	3	11.54%	6	9.52%
O78	4	10.8%	2	7.69%	6	9.52%
O18	5	13.5%	-	-	5	7.93%
O26	3	8.10%	2	7.69%	5	7.93%
O153	-	-	4	15.38%	4	6.35%
O157	4	10.8%	-	-	4	6.35%
O126	3	8.10%	-	-	3	4.76%
O1	-	-	2	7.69%	2	3.17%
Untyped	11	29.72%	10	38.46%	21	33.33%
Total	37		26		63	

Table 4: Phenotypic virulence factors of *E. coli* isolated from mastitic buffaloe milk samples

	Clinical mastitis (37)		Subclinical mastitis (26)		Total mastitis (63)	
Virulence factor	No+	%	No+	%	No+	%
SR	32	86.5 %	18	69.23%	50	79.37%
CRB	16	43.24%	8	30.80%	24	38.10%
IA	15	40.54%	5	19.23%	20	31.75%
HA	5	13.50%	3	11.54%	8	12.70%

SR: Serum resistance, CRB: Congo red binding, IA: Invasvness activity, HA: Hemolytic activity

Table 5: Relationship between different serogroups and phenotypic virulence factors

		Serum resistance		Congo red binding		Invasiveness		Hemolytic activity	
Serogroups	No	No +	%	No+	%	No+	%	No+	%
O1	2	0	0.0%	1	50.0%	0	0.0%	0	0.0%
O18	5	4	80.0%	2	40.0%	2	40.0%	0	0.0%
O26	5	5	100.0%	3	60.0%	3	60.0%	0	0.0%
O55	6	6	100.0%	3	50.0%	0	0.0%	3	50.0%
O78	6	3	50.0%	4	66.6%	0	0.0%	0	0.0%
O114	7	7	100.0%	5	71.42%	5	71.42%	2	28.6%
O126	3	3	100.0%	1	33.3%	2	66.6%	0	0.0%
O153	4	3	75.0%	1	25.0%	0	0.0%	1	25.0%
O157	4	4	100.0%	3	75.0%	4	100.0%	2	50.0%
Untyped	21	15	71.42%	1	4.76%	4	19.0%	0	0.0%
Total	63	50	76.92%	24	38.1%	20	31.75%	8	12.7%

SR: Serum resistance, CRB: Congo red binding, IA: Invasiveness activity, HA: Hemolytic activity

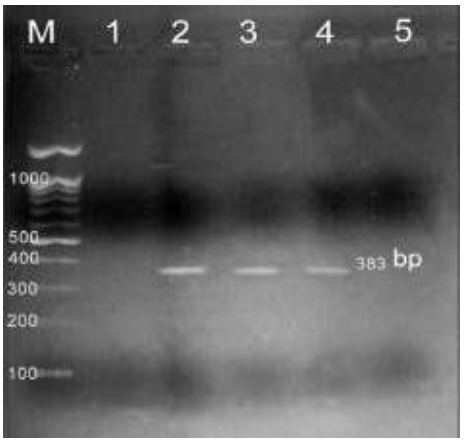

Photo 1: Agarose gel electrophoresis of eaeA gene from extracted DNA of E. coli isolated from mastitis milk samples. M: Marker, 100 bp DNA ladder. Lanes "1&5": negative amplification of eaeA gene representing E. coli serogroups O114 and O55, respectively. Lanes "2-4": positive amplification of eaeA gene representing E. coli serogroups O18,, O78 and O157, respectively.

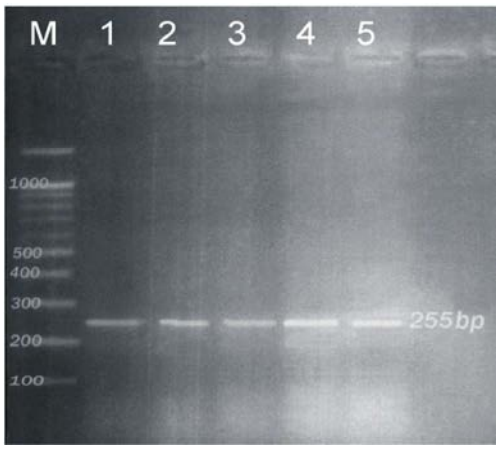

Photo 2: Agarose gel electrophoresis of stx2 gene from extracted DNA of E.coli isolated from mastitic milk samples. M: Marker: 100 bp DNA ladder, Lanes "1-5": positive amplification of stx2 gene representing E. coli serogroups O114, O18, O78, O157, and O55, respectively.

strains out of 37 and 26 strains isolated from clinical and subclinical mastitis milk samples, respectively, were able to survive in serum. The results of Congo red binding activity revealed that 24 (38.1%) were positive for this activity. The rate was higher in clinical isolates (3.24%) than in subclinical ones (30.80%).

The Sereny test used for detection of invasive *E. coli* strains indicated that 31.75% of the isolates are able to invade epithelial cells of cornea and induced keratoconjuctivitis in eyes of rabbits after 48 hours. The invasive activity was higher in clinical isolates (40.5%) than in subclinical ones (19.2%). Hemolytic activity was detected in only 8 out of sixty three examined *E. coli* strains recovered from mastitis milk samples with an incidence of 12.7%.

Relationship between Different Serogroups and Phenotypic Virulence Factors: Relationship between different serogroups and different virulence factors was illustrated in Table 5. It is evident that almost all serogroups tested possessed the serum resistance and Congo binding activities. Hemolytic activity and invasiveness was very poor. Only all isolates of *E. coli* O157 were invasive (100.0%), followed by 114 (71.42%), O126 (66.6%) and O26 (60%).

Detection of Shiga Toxin 2 and Intimin Virulence Genes: Five *E. coli* isolates representing five different serogroups (O114, O18, O78, O157 and O55) were subjected to conventional PCR to detect the presence of shiga toxin 2 and intimin genes. The results revealed that all 5 isolates have *stx2* gene, which gives a characteristic band at 255 bp, while 3 isolates (O18, O78 and O157) had *eaeA* gene, which gave a characteristic band at 383 bp.

DISCUSSION

E. coli is the most important coliform that has received more attention due to its high incidence relatively to other mastitis pathogens [21,35-39]. It is a risky microorganism of public health importance, as enteropathogenic *E. coli* serotypes (as O157:H7) have been implicated in many cases of gastroenteritis, food poisoning as well as sporadic cases of hemorrhagic colitis and hemolytic uremic syndrome [40, 41]. It is the dominant pathogen diagnosed more than once in the same lactation and usually elicits recurrent clinical episodes [7, 11], which may lead to culling of the cow. Moreover, *E. coli* is the pathogen associated with the most severe clinical cases of mastitis. In some cases, it ends with animal death and so it is considered as fatal mastitis pathogen [42].

The present study recorded that total isolation rate of *E. coli* from clinically mastitic buffaloes (recurrent mastitis cases) was 31.44%. This result is close to that detected by other authors [43-46], who reported incidences of 32, 31, 34.7 and 32%, respectively. Variable incidences of *E. coli* among clinically mastitic animals were also mentioned [14, 47-50].

In the present work, among the serotyped strains of *E. coli*, there was no predominant serogroup and this result agreed with [51-54] and this emphasized that serotyping of *E. coli* not of high significance in mastitis cases characterization. This observation had been suggested by many researchers [4, 55-57] and this was the cause that most researchers in this topic make further studies on virulence factors of these *E. coli* serogroups. Based on epidemiological studies, the wide range of *E. coli* serotypes supported the hypothesis that animals were infected with *E. coli* from their environment (feces or straw) and that was previously mentioned [52].

O157 was among the serotyped *E. coli* strains recovered from clinically mastitis cases and this serogroup is one of the most important STEC that causes severe disease in human and was mentioned by many other authors as a cause of mastitis [58, 59]. On the other hand, some authors stated that none of *E. coli* strains isolated from cases of bovine mastitis were identified as *E. coli* O157:H7.

Many of *E. coli* isolates detected in this study belonged to classical E.P.E.C serogroups as O114, O55, O18, O26, O78 and O126 that were also detected by others [50, 60-63] among serotyped *E. coli* strains from cases of mastitis.

E. coli mastitis as any disease results from interaction between host, pathogen and environment. Many studies were directed to study this interaction and detected that severity of *E. coli* mastitis is mainly determined by animal factors rather than by the pathogenicity of the invading pathogen [23, 64]. Virulence factors of the bacterial strain can give it a chance for colonization, multiplication and survival in udder in the face of host defense mechanism [22]. Virulence varies not only among different species, but also among strains of the same species. So numerous studies have been conducted to identify virulence factors of *E. coli* isolated from cases of bovine mastitis [21-23, 53, 56, 59].

Serum resistance was the most common virulence marker detected in this study and that agrees with other researchers and that made some of them to suggest that serum resistance is the only characteristic that could be related to virulence in *E. coli* strains isolated from bovine intramammary infections [53, 56].

Many studies were conducted to determine the correlation between Congo red binding ability and the virulence of *E. coli* strains and many of these studies emphasized that there is a strong correlation between expression of Congo red binding phenotype and virulence of *E. coli* [65, 66]. On the other side, some authors found that Congo red binding activity did not correlate well with pathogenicity [56, 67, 68]. The present study supports that point of view.

The term invasive *E. coli* is referred to those strains of *E. coli,* which are able to induce keratoconjuctivitis in eyes of Guinea pigs [69, 70]. In this study *E.coli* strains were found to be invasive with an incidence of 40.5% and 19.23% among those strains recovered from clinical and subclinical mastitis cases, respectively. This result is in contrast to that found by many researchers [50, 53] who found no invasive *E. coli* strains isolated from cases of mastitis or detected only one invasive strain among *E. coli* isolates. This incidence is lower than that detected by others [56], who found that 70.4% of *E. coli* isolated from

mastitis milk samples were invasive. Many researches were concerned with the ability of *E. coli* isolated from mastitis cases to invade mammary tissue. Old studies mentioned that *E. coli* are non invasive to mammary tissue but remain in the lumen of teat canal and lactiferous sinus [53, 54], but some recent studies indicated that *E. coli* strains isolated from clinical mastitis were able to invade bovine mammary epithelial cells under *in vitro* conditions [72, 73].

PCR analysis of some *E. coli* serogroups using eaeAF, eaeAR primers demonstrated the presence of *eae*A gene in three out of five examined *E. coli* strains, which represented serogroups O18, O78 and O157 respectively. These results are in line with those of other authors [26,74], who showed positive amplification of *eae*A gene by using same primers used in this study. Different primers, other than that used in this study, were also used in PCR assay for detection of *eaeA* [75].

O157: H7 gave positive amplification for the two examined genes and this is consistent with results reported by others [62,74,76], who detected the presence of these two genes in all *E. coli* serotype O157:H7, that have been examined. O55 showed positive amplification for *stx2* gene and negative amplification for *eaeA* gene [62]. All examined strains were found to carry *stx2* gene and 3 strains carried *eaeA* gene and this result is consistent with other authors, who detected the presence of these two genes in some *E. coli* strains that has been isolated from cases of mastitis [19, 61-63]. On the contrary, others had mentioned that *E. coli* isolated from cows with mastitis are negative for these two genes by PCR [77-80]. In this study, the detection of intimin gene in some *E. coli* strains examined suggested that adhesion of *E. coli* to mammary epithelial cells may have a role in pathogenesis of this disease. It was suggested that strains of *E. coli* recovered from mastitis cases in dairy cows adhere selectively to part of the cells [72,79]. On the contrary, many researches mentioned that there is no role for adherence in establishment of infection in cases of *E. coli* intramammary infection [81].

CONCLUSIONS

Special attention should be given to subclinical mastitis cases, which act as invisible potential source for infection spread. There was no predominant serogroup associated with mastitic cases, so serotyping of *E. coli* is not of high significance in mastitis cases characterization. Additionally, *E. coli* isolated from mastitis cases had *eaeA* and *Stx2* genes are of great importance in progression of the disease.

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