

Short communication

INVESTIGATION OF VIRULENCE FACTORS IN *ESCHERICHIA COLI* ISOLATED FROM CLINICAL AND SUBCLINICAL BOVINE MASTITIS

H. MOMTAZ

Department of Microbiology, Faculty of Veterinary Medicine, Islamic Azad University – Shahrekord branch, Shahrekord, Iran

Summary

Momtaz, H., 2010. Investigation of virulence factors in *Escherichia coli* isolated from clinical and subclinical bovine mastitis. *Bulg. J. Vet. Med.*, 13, No 2, 122–126.

Escherichia coli, an opportunistic environmental pathogen, is one of the commonest causes of bovine clinical mastitis in the early lactation period. Shiga toxin-producing *E. coli* (STEC) whose pathogenesis is associated with enterotoxigenic genes (*stxI*, *stxII*, and *eaeA*) cause serious acute illness and long-term side effects in both animals and humans. The aim of this study was to investigate the rate of virulence factors of *E. coli* isolates from cows with mastitis in the Chaharmahal-Bakhtiari province, Iran. Milk samples from 400 cows with mastitis were obtained, cultured in MacConkey agar to select Gram-negative microorganisms and *E. coli* was further identified by microbiological tests. After DNA extraction, multiplex PCR amplification using specific primers was performed. Out of 400 samples, 42 specimens were found to be *E. coli*-positive according to the microbiological tests. The PCR results indicated that 14 out of 42 isolates carried the *eaeA* gene, 4 isolates were positive for the gene of *F41* fimbriae and 10 for *stxI* and *stxII* genes.

Key words: bovine mastitis, *E. coli*, multiplex PCR, virulence factors

Mastitis is a major problem in dairy farms (Kobori *et al.*, 2004). Gram-negative bacteria are environmental mastitis pathogens and are most commonly involved in acute clinical cases of mastitis. The term coliform mastitis is often used incorrectly to identify mammary disease caused by Gram-negative bacteria but genera classified as coliforms are *Escherichia*, *Klebsiella* and *Enterobacter* (Hogan & Smith, 2003). Acute coliform mastitis is primarily caused by *E. coli* (Wenz *et al.*, 2006). *E. coli*, which is considered to be an opportunistic pathogen and originate from a

contaminated environment (Lehtolainen *et al.*, 2003), is also one of the commonest causes of bovine clinical mastitis (Green *et al.*, 2005) and a major problem in lactating dairy cows (Kobori *et al.*, 2004). It is mostly observed in the early lactation period and in high-producing cows with low somatic cell counts (Guler & Gunduz, 2007). It has been reported that the incidence of *E. coli* mastitis has recently increased in some countries (Green *et al.*, 2005; Guler & Gunduz, 2007). Epidemiological studies show that the serotypes of *E. coli* which cause clinical bovine masti-

tis produce a group of virulence factors upon invading mammary tissue. It is believed that there is a relationship between mastitis and virulence factors produced by this pathogen (Franck *et al.*, 1998; Guler & Gunduz, 2007).

Shiga toxin-producing *E. coli* (STEC) are known to cause serious acute illness and long-term side effects in humans (Chern *et al.*, 2004). They are an important group of food-borne pathogens that can cause severe gastrointestinal diseases in humans and complications such as the haemolytic uremic syndrome (Chern *et al.*, 2004; Kobori *et al.*, 2004). Furthermore, enterotoxigenic *E. coli* (ETEC) are known to cause diarrhoeal diseases in both animals and humans. Their virulence is associated with ETEC shiga toxin I and II genes (*stxI*, *stxII*), and intimin gene (*eaeA*). The bacteria harbouring these toxin genes have the ability to colonize the small intestine of humans and animals (Chern *et al.*, 2004).

In a large-scale study, Fremaux *et al.* (2006) investigated the prevalence and presence of the gene for shiga toxinogenic *E. coli* in 13 dairy cattle farms in France. This survey showed the presence of *stx* genes in 35% (145 out of 415) of *E. coli* isolates in individual faeces and 20% (179 out of 894) of *E. coli* isolates in environmental niches. Of these, 46%, 86% and 29%, were carrying genes for virulence factors of *stxI*, *stxII* and *eaeA*, respectively (Fremaux *et al.*, 2006).

Kaipainen *et al.* (2002) performed a study upon virulence properties of *E. coli* isolated from clinical bovine mastitis in Israel and Finland by PCR. In this study, a total of 160 Finnish *E. coli* isolates from bovine mastitic milk were examined of which 37% carried F factor transfer (*traT*) genes, 14%: cytotoxic necrotizing factor 2 (*CNF2*) gene, 8%: cytotoxic necrotizing

factor 1 (*CNF1*) gene, 11 %: aerotaxis (*aer*) gene, 9%: *F17* gene, 8%: S fimbriae (*Sfa*), 7%: P fimbriae (*Pap*) gene, 1%: each of afimbrial adhesion genes *afa8D* and *afa8E*. Of 113 *E. coli* isolates from bovine mastitic milk in Israel 41% carried *CNF2* genes, 1% – *Sfa* gene, and 1% – fimbriae *F17* gene (Kaipainen *et al.*, 2002).

In 2006, having studied *E. coli* mastitis strains, Dogan *et al.* (2006) showed that virulence genes of *K99*, *F1845* and *CS31A* fimbriae; heat labile toxin (*LT*); heat stable toxins (*StA*, *StB*) (from ETEC), *StxI* and *StxII* (from enterohaemorrhagic *E. coli*), *CNF1*, *CNF2*, *Pap*, afimbrial adhesion I (*afaI*), haemolysin (*hly*), *Sfa* (from uropathogenic *E. coli*) and *eaeA* (from enteroinvasive *E. coli*), usually can cause gastroenteritis and genitourinary tract infections. In rarer cases, virulent *E. coli* strains are also responsible for mastitis (Dogan *et al.*, 2006). A survey made by Bean *et al.* (2004), similarly to studies by Dogan *et al.*, showed that there were no virulence genes *stxI*, *stxII*, *CNF1*, *CNF2* and *eaeA* in most *E. coli* mastitis isolates.

As seen from the reviewed literature, most *E. coli* strains isolated from bovine mastitis are carrying virulence genes, though in the different studies various virulence genes have been investigated and assessed. The outcome of bovine mastitis caused by *E. coli* ranges from severe clinical and even lethal signs, to possibly mild mastitis with mammary localized signs and depends on cow's response to bacteria and the type of bacterial virulence factors (Shpigel *et al.*, 1998).

The aim of this study was to detect the presence of the genes for some virulence factors Shiga toxins (*stxI* and *stxII*), intimin (*eaeA*), heat-stable enterotoxin a (*StA*), and fimbriae of *K99* and *F41* in *Escherichia coli*, isolated from clinical

and subclinical bovine mastitis in Chaharmahal-Bakhtiari province, Iran.

Milk samples from 400 mastitic cows were obtained aseptically in different dairy farms of Chaharmahal-Bakhtiari province in Iran between September 2006 and July 2008. All cows were milked 3 times daily and were housed in dry-lot pens or free stalls. The average monthly bulk tank SCC of cooperating dairies, reported by the processor, was less than 300,000 cells/mL during the sampling period. Clinical and subclinical mastitis were identified by the California mastitis test (CMT) and clinical examination, and samples were collected in both cases. Approximately 5 mL of milk were collected in sterile glass bottles, stored in a cool box and transported for culturing.

Samples were cultured in MacConkey's (MAC) agar. Agar plates were incubated at 37 °C and bacterial growth was evaluated after 24 and 48 h. 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* (Franck *et al.*, 1998).

To obtain O157:H7 *E. coli* the strains

were also subcultured on Sorbitol MacConkey Agar and incubated at 37 °C overnight. Sorbitol-negative colonies were tested with an O157 latex agglutination kit (Oxoid) for the detection of O157:H7 *E. coli* (Guler & Gunduz, 2007).

A multiplex PCR that detects the genes encoding Shiga toxins (*stxI* and *stxII*), intimin (*eaeA*), heat-stable enterotoxin a (*Sta*), and fimbriae of *F41* and *K99* was performed using the primers described by Franck *et al.* (1998) (Table 1). The PCR assay was carried out in a total volume of 50 µL of mixture containing PCR buffer 10×, 1.5 mM of MgCl₂, 250 µM of each of deoxynucleoside triphosphates, 0.5 µM of each of the virulence gene-specific primers, 1.5U of *Taq* polymerase (Sigma), and 5 µL of template DNA. The amplification conditions included 35 cycles of a denaturation step at 94 °C for 30 s, primer annealing at 55 °C for 45 s, and extension at 70 °C for 90 s. The extension time was ramped for an additional 3 s per cycle and a final extension step of 10 min at 70 °C was performed (Guler & Gunduz, 2007). The PCR products were analyzed by 1.5% agarose gel electrophoresis, after which

Table 1. Primers used in the multiplex PCR for *stxI*, *stxII*, intimin, *F41*, *F5* (*K99*) and *Sta* genes

Virulence factor	Primer sequence (5'-3')	Size of product (bp)
<i>stxI</i> (F)	TTC GCT CTG CAA TAG GTA	555
<i>stxI</i> (R)	TTC CCC AGT TCA ATG TAA GAT	
<i>stxII</i> (F)	GTG CCT GTT ACT GGG TTT TTC TTC	118
<i>stxII</i> (R)	AGG GGT CGA TAT CTC TGT CC	
Intimin (F)	ATA TCC GTT TTA ATG GCT ATC T	425
Intimin (R)	AAT CTT CTG CGT ACT GTG TTC A	
<i>F41</i> (F)	GCA TCA GCG GCA GTA TCT	380
<i>F41</i> (R)	GTC CCT AGC TCA GTA TTA TCA CCT	
<i>K99</i> (F)	TAT TAT CTT AGG TGG TAT GG	314
<i>K99</i> (R)	GGT ATC CTT TAG CAG CAG TAT TTC	
<i>Sta</i> (F)	GCT AAT GTT GGC AAT TTT TAT TTC TGT A	190
<i>Sta</i> (R)	AGG ATT ACA ACA AAG TTC ACA GCA GTA A	

the gel was stained with ethidium bromide and photographed. *E. coli* O157:H7 strain (Department of Microbiology, Faculty of Veterinary Medicine, Tehran University, Tehran-Iran) was used as positive control in the multiplex PCR.

Out of 400 samples that were suspected for mastitis, 42 specimens were found *E. coli*-positive according to the microbiological culture and negative for O157:H7 by both the latex agglutination test and PCR. The PCR results established that 4 (9.52%) isolates out of the 42 isolated bacterial samples, were positive for the gene of *F41* fimbriae, 14 (33.3%) – positive for the *eaeA* gene and 10 (23.8%) – positive for Shiga toxin genes (*stxI*, *stxII*) (Table 2).

In our study, 23.8% of *E. coli* isolated from bovine mastitis carried genes for some of the most important virulence factors – *stxI*, *stxII* or both. A similar prevalence of *stx* genes: 35% (145 out of 415) of in individual faeces and 20% (179 out of 894) in environmental niches is reported by Fremaux *et al.* (2006). In other studies, 30.8% of udders (54 out of 175) and teats 37.8% (49 out of 161) were *E. coli*-positive (Rahn *et al.*, 1997; Cobbold & Desmarchelier, 2000). Our findings however differed from those obtained by Dogan *et al.* (2006) and Bean *et al.* (2004) who have not established *stx* genes in their studies.

Table 2. Results of detection of some virulence genes in 42 *Escherichia coli* isolates from bovine mastitis

Virulence genes	Number (%) of samples
<i>stxI,II</i>	10 (23.8 %)
<i>eaeA</i>	14 (33.33 %)
F41	4 (9.52 %)

The incidence of 33.3% of the *eaeA* gene in *E. coli* isolated from bovine mastitis in this investigation was similar to results obtained by Blanco *et al.* (2004) in Spain where the *eaeA* gene was observed in 29% of *E. coli* isolates. It was however highly different from rates reported by Cobbold & Desmarchelier in Australia (2000) and by Irino *et al.* (2005) in Brazil – 0.7 % and 1.5 % respectively.

In general, different results reported from various studies indicate that factors such as geographical location can influence the type and the incidence of virulence genes presence in *E. coli* strains. For instance, Kaipainen *et al.* (2002) showed significant differences in the presence of some virulence genes of *E. coli* isolates in mastitic milk samples in different geographical regions (Finland and Israel).

The results of this study may therefore serve as a background for more complete studies on the distribution of virulence genes in *E. coli*, isolated from coliform mastitis in different regions of Iran.

ACKNOWLEDGEMENTS

We thank Dr. A. Sharifzadeh, Dr. S. Nekoie, Dr. S. Nejat for their cooperation. This work was supported by Grant No. 30289 from the Islamic Azad University of Shahr-e-kord Branch in Iran.

REFERENCES

- Bean, A., J. Williamson & R. T. Cursons, 2004. Virulence genes of *Escherichia coli* strain isolated from mastitic milk. *Journal of Veterinary Medicine Series B*, **57**, 285–287.
- Blanco, M., J. E. Blanco, A. Mora, G. Dahbi, M. P. Alonso, E. A. Gonzalez, M. I. Bernardez & J. Blanco, 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia*

- coli* isolates from cattle in Spain and identification of a new intimin variant gene (*eae-xi*). *Journal of Clinical Microbiology*, **42**, 645–651.
- Chern, E. C., Y. L. Tsai & B. H. Olson, 2004. Occurrence of genes associated with enterotoxigenic and enterohemorrhagic *Escherichia coli* in agricultural waste lagoons. *Applied Environmental Microbiology*, **70**, 356–362.
- Cobbold, R. & P. Desmarchelier, 2000. A longitudinal study of Shiga-toxigenic *Escherichia coli* (STEC) prevalence in three Australian dairy herds. *Veterinary Microbiology*, **71**, 125–137.
- Dogan, B., S. Klaessig, M. Rishniw, R. A. Almeida, S. P. Oliver, K. Simpson & Y. H. Schukken, 2006. Adherent and invasive *Escherichia coli* are associated with persistent bovine mastitis. *Veterinary Microbiology*, **116**, 270–282.
- Franck, S. M., B. T. Bosworth & H. W. Moon, 1998. Multiplex PCR for enterotoxigenic, attaching and effacing, and Shiga toxin producing *Escherichia coli* strains from calves. *Journal of Clinical Microbiology*, **36**, 1795–1797.
- Fremaux, B., S. Raynaud, L. Beutin & C. V. Rozand, 2006. Dissemination and persistence of Shiga toxin-producing *Escherichia coli* (STEC) strains on French dairy farms. *Veterinary Microbiology*, **117**, 180–191.
- Green, M. J., L. E. Green, A. J. Bradley, P. R. Burton, Y. H. Schukken & G. F. Medley, 2005. Prevalence and associations between bacterial isolates from dry mammary glands of dairy cows. *The Veterinary Record*, **156**, 71–77.
- Guler, L. & K. Gunduz, 2007. Virulence properties of *Escherichia coli* isolated from clinical bovine mastitis. *Turkish Journal of Veterinary and Animal Science*, **31**, 361–365.
- Hogan, J. & K. L. Smith, 2003. Coliform mastitis. *Veterinary Research*, **34**, 507–519.
- Irino, K., M. A. Kato, T. M. Vaz, I. I. Ramos, M. A. Souza, A. S. Cruz, T. A. Gomes, M. A. Vieira & B. E. Guth, 2005. Serotypes and virulence markers of Shiga toxin-producing *Escherichia coli* (STEC) isolated from dairy cattle in São Paulo State, Brazil. *Veterinary Microbiology*, **105**, 29–36.
- Kaipainen, T., T. Pohjanvirta, N. Y. Shpigel, A. Shwimmer, S. Pyörälä & S. Pelkonen, 2002. Virulence factors of *Escherichia coli* isolated from bovine clinical mastitis. *Veterinary Microbiology*, **85**, 37–46.
- Kobori, D., E. C. Rigobelo, C. Macedo, J. M. Marin & F. A. Avila, 2004. Virulence properties of shiga toxin-producing *Escherichia coli* isolated from cases of bovine mastitis in Brazil. *Révue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux*, **57**, 15–20.
- Lehtolainen, T., T. Pohjanvirta, S. Pyörälä & S. Pelkonen, 2003. Association between virulence factors and clinical course of *Escherichia coli* mastitis. *Acta Veterinaria Scandinavica*, **44**, 203–205.
- Rahn, K., S. A. Renwick, R. P. Johnson, J. B. Wilson, R. C. Clarke, D. Alves, S. McEwen, H. Lior & J. Spika, 1997. Persistence of *Escherichia coli* O157:H7 in dairy cattle and the dairy farm environment. *Epidemiology and Infection*, **119**, 251–259.
- Shpigel, N. Y., M. Winkler, G. Ziv & A. Saran, 1998. Clinical, bacteriological and epidemiological aspects of clinical mastitis in Israeli dairy herds. *Preventive Veterinary Medicine*, **35**, 1–9.
- Wenz, J. R., G. M. Barrington, F. B. Garry, R. P. Ellis & R. J. Magnuson, 2006. *Escherichia coli* isolates, serotypes, genotypes, and virulence genes and clinical coliform mastitis severity. *Journal of Dairy Science*, **89**, 3408–3412.

Paper received 07.02.2009; accepted for publication 11.09.2009

Correspondence:

Dr. Hassan Momtaz
Department of Microbiology,
Faculty of Veterinary Medicine,
Islamic Azad University, Shahrekord branch,
P.O.Box 166, Shahrekord-Iran
e-mail: hamomtaz@iaushk.ac.ir