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Prevalence of Virulence Factor Genes in *Escherichia coli* Isolates from Dairy Cows with Mastitis

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To investigate into the prevalence of virulence factor genes in mastitis-causing bacteria and prevent bovine mastitis optimistically, one hundred and twenty three *Escherichia coli* isolates from bovine mastitis were examined to detect the virulence associated genes by using polymerase chain reaction (PCR) methods. The data showed that 11 virulence genes were detected, including ETT2, STb, CNF2, Tra, LTa, HPI, Hly, Aer, Pfa, Sfa and F17, and none of the isolates contained the genes for Stx2e, STa, CNF1, LEE, Afa and CS31A were found. All of the detected virulence genes were present alone or in combination with each other, there into, 87 (70.73%) isolates had at least one virulence gene, and ETT2 locus was the most common gene in the examined isolates (38.21%), followed by STb (25.20%), CNF2 (19.51%), Tra (17.07%), LTa (14.63%), HPI (14.63%), Hly (7.32%), Aer (4.07%), Pfa (2.44%), Sfa (2.44%) and F17 (1.63%).

Key words: Dairy cow, Mastitis; *Escherichia coli*, Virulence factor.

Mastitis is the most important disease in dairy milk production worldwide (Kossaibati *et al*., 1997), and the infectious pathogeny is the most important cause that frequently due to infection by one and/or the other pathogens, such as bacteria, viruses, mycoplasma, yeasts and algae (Chaneton *et al*., 2008; Malinowski *et al*., 2006; Osumi *et al*., 2008; Watts, 1988; Wellenberg *et al*., 2002). Thereinto, the vast majority of mastitis is of bacterial origin and just a few of species of bacteria account for most cases, such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae* (Chaneton *et al*., 2008; Cheng *et al*., 2010; Dogan *et al*., 2006; Kuang *et al*., 2009).

Bacteriological, epidemiological and clinical studies indicate that *E. coli* is one of the major agents of bovine mastitis worldwide (Blum *et al*., 2008; Cheng *et al*., 2010; Wenz *et al*., 2006), and several virulence factors were detected in the isolates associated with bovine diseases. These include F17 fimbriae, P fimbriae (Pfa), S fimbriae (Sfa), afimbrial adhesins(Afa), CS31A adhesin, Aerobactin (Aer), Cytotoxic necrotizing factors (CNF), hemolysin (Hly), transfer surface exclusion protein (Tra), high-pathogenicity island (HPI), locus of enterocyte effacement (LEE), etc. (Barrow and Hill, 1989; Burns *et al*., 1996; Hogan *et al*., 1990; Kaipainen *et al*., 2002; Lipman *et al*., 1995; Nemeth *et al*., 1994; Pohl *et al*., 1993; Sanchez-

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Carlo *et al*., 1984; Valente *et al*., 1988). However, there are few reports on the role of *E. coli* in bovine mastitis, and no detailed information on the virulence factors which presence in *E. coli* isolates has been reported in China dairy farms so far.

The biggest challenge facing the modern dairy industry is the pressure to reduce the incidence of mastitis, and the extensive investigation and research of mastitis etiology may be capable of helping to provide an important and optimistic approach to control this disease. In pursuit of these goals, a total of 123 bacterial isolates were submitted to PCR detection to investigate the prevalence of the putative virulence factors among the *E. coli* isolates from bovine mastitis in Jiangsu province of China, and determine the relationship between the detected virulence factors.

MATERIALS AND METHODS

E. coli isolates

E. coli isolates (n = 123) from raw milk samples from the dairy cows with bovine mastitis in 5 farms in the Jiangsu Province, China. Secretions from individual mammary glands with clinical mastitis were collected in sterile vials following teat end disinfection with 75% ethanol and removal of the first 3~4 streams of milk. A loopfull of each samlpe was streaked on Mackonkey agar plate (Shanghai China Academy of Sciences Shanghai Hexapod Technology Development Co., Ltd.) and then incubated at 37 °C for 24 h under aerobic conditions. Then single colonies were obtained for *E. coli* isolation. The isolates were identified as *E. coli* based on colony morphology and colour, Gram stain and standard biochemical tests. All of the bacteral isolates were stored in Luria-Bertani broth with 20% sterile glycerol at –70 °C.

Extraction of DNA templates

DNA was extracted from the *E. coli* isolates by boiling (Cheng *et al*., 2006). One bacterial colony from an overnight Luria-Bertani agar plate culture was suspended in 50 ìl of sterile deionized water, boiled for 10 min, and centrifuged at 10,000 × g for 5 min. The supernatant was then used as the DNA template for PCR.

PCR to detect virulence factor genes

All the 123 *E. coli* isolates from bovine mastitis were submitted to PCR detection. The genes for shigatoxin 2e (Stx2e), heat-stable toxin type A (STa), heat-stable toxin type B (STb), heat labile toxin type A (LTa), HPI as well as LEE were analyzed by multiplex-PCR or duplex-PCR as described by Cheng *et al*. (2006). PCR identifications of genes for CNF1, Aer and Pfa were performed as recommended by Yamamoto *et al*. (1995). Simplex PCR reactions were carried out individually to determine F17 (Bertin *et al*., 1996), CS31A (Bertin et al., 1998), Sfa (Le Bouguenec *et al*., 1999), CNF2 (Kaipainen *et al*., 2002), Tra (Kaipainen *et al*., 2002), Hly (Schmidt *et al*., 1995) and *E. coli* type three secretion system 2 (ETT2) island. The PCR products were analyzed in appropriated agarose gel containing ethidium bromide. The oligonucleotide primers used for amplification of the genes and expected size of products are presented in Table 1.

RESULTS

Following PCR amplifications were performed, 11 virulence genes were detected, including ETT2, STb, CNF2, Tra, LTa, HPI, Hly, Aer, Pfa, Sfa and F17, and none of the isolates contained the genes for Stx2e, STa, CNF1, LEE, Afa and CS31A. Among the 123 China *E. coli* isolates from bovine mastitis (Table 2), 87 (70.73%) isolates had at least one virulence gene, whereas the remainder (39.27%) did not contain any virulence genes detected in this study.

All of the detected virulence genes were present alone or in combination with each other, there into, only ETT2 and CNF2 genes of partial isolates were present alone. Analyses of the results revealed 30 types of virulence factors pattern among the 87 isolates investigated. The combinations of virulence genes varied markedly and some combinations typically in only one to three isolates respectively.

The most common gene in the examined isolates was ETT2 locus which was positive in 47 isolates (38.21%) and alone or in combination with the genes for STb, CNF2, Tra, LTa, HPI, Hly, Aer, Pfa, Sfa or F17. STb was the second most prevalent (25.20%) virulence factor and alone or in combination with the genes for ETT2, STb, CNF2, Tra, LTa, HPI, Hly, Aer or F17, except Pfa and Sfa. Twenty-four (19.51%) isolates were positive

Table 1. The PCR primers used in this study

virulence factor	Primer	Base sequence 5→3	Size of product (bp)	Reference
Stx2e	Stx2e-F	GAATGAAGAAGATGTTTATAGCGG	454	Cheng *et al*. (2006)
	Stx2e-R	TTTTATGGAACGTAGGTATTACC		
STa	STa-F	GGGTTGGCAATTTTTATTTCTGTA	183	Cheng *et al*. (2006)
	STa-R	ATTACAACAAAGTTCACAGCAGTA		
STb	STb-F	ATGTAAATACCTACAACGGGTGAT	360	Cheng *et al*. (2006)
	STb-R	TATTTGGGCGCCAAAGCATGCTCC		
LTa	LTa-F	TAGAGACCGGTATTACAGAAATCTGA	282	Cheng *et al*. (2006)
	LTa-R	TCATCCCGAATTCTGTTATATATGTC		
HPI	Irp2-F	AAGGATTCGCTGTTACCGGAC	280	Cheng *et al*. (2006)
	Irp2-R	TCGTCGGGCAGCGTTTCTTCT		
ETT2	Ecs3703-F	CATGCAATAGTTGCTCAATGC	552	This study
	Ecs3703-R	CCCATTCTCTTTTCGATTCG		
LEE	eae-F	ATATCCGTTTTAATGGCTATCT	425	Franck,*et al*. (1998)
	eae-R	AATCTTCTGCGTACTGTGTTCA		
Aer	aer-F	TACCGGATTGTAATATGCAGACCGT	602	Yamamoto *et al*. (1995)
	aer-R	AATATCTTCCTCCAGTCCGGAGAAG		
Pfa	pap-F	GCAACAGCAACGCTGGTTGCATCAT	336	Yamamoto *et al*. (1995).
	pap-R	AGAGAGAGCCACTCTTATACGGACA		
CNF1	cnf1-F	AAGATGGAGTTTCCTATGCAGGAG	498	Yamamoto *et al*. (1995)
	cnf1-R	CATTCAGAGTCCTGCCCTCATTATT		
CNF2	cnf2-F	ACTGAAGAAGAAGCGTGGAATA	654	Kaipainen *et al*. (2002
	cnf2-R	ATAAGTTGAGCCGAGCGAGG		
Afa	afaD8-F	GTTGAACTGAGTCTTAATACCAGTG	354	Lalioui *et al*. (1999)
	afaD8-R	TGAGCATTCTCCGCTAACTGATAAT		
	afaE8-F	CTAACTTGCCATGCTGTGACAGTA	302	Lalioui *et al*. (1999)
	afaE8-R	TTATCCCCTGCGTAGTTGTGAATC		
F17	F17-F	GCAGAAAATTCAATTTATCCTTGG	537	Bertin *et al*. (1996)
	F17-R	CTGATAAGCGATGGTGTAATTAAC		
CS31A	clpG-F	GGGCGCTCTCTCCTTCAAC	402	Bertin *et al*. (1998)
	clpG-R	CGCCCTAATTGCTGGCGAC		
Sfa	sfa-F	CTCCGGAGAACTGGGTGCATCTTAC	410	Le Bouguenec *et al*. (1999)
	sfa-R	CGGAGGAGTAATTACAAACCTGGCA		
Tra	tra-F	GATGGCTGAACCGTGGTTATG	307	Kaipainen *et al*. (2002)
	tra-R	CACACGGGTCTGGTATTTATGC		
Hly	hlyA1	GGTGCAGCAGAAAAAGTTGTAG	1550	Schmidt *et al*. (1995)
	hlyA4	TCTCGCCTGATAGTGTTTGGTA		

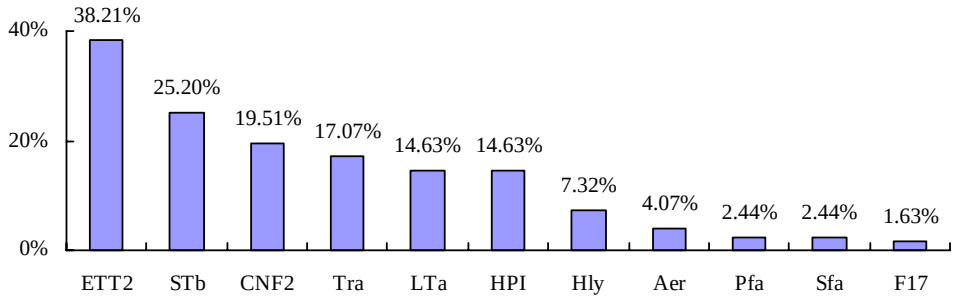

Fig. 1. Prevalence of virulence factor genes among the *E. coli* isolates from cows with mastitis in China

Table 2. Combinations of detected virulence genes among the *E. coli* isolates from cows with mastitis in China

Virulence type factor No.	ETT2	STb	CNF2	Tra	LTa	HPI	Hly	Aer	Pfa	Sfa	F17	Total
1.	+											11
2.	+						+		+			1
3.	+					+						6
4.	+			+		+				+		1
5.	+			+								9
6.	+		+			+						6
7.	+		+									2
8.	+	+	+									2
9.	+	+	+		+		+					2
10.	+	+	+		+							1
11.	+	+	+				+					1
12.	+	+										3
13.	+	+						+			+	1
14.	+	+			+							1
15.		+	+									1
16.		+	+		+		+					1
17.		+	+		+							1
18.		+	+				+					1
19.		+			+		+					1
20.		+			+							8
21.		+		+	+						+	1
22.		+		+				+				2
23.		+										3
24.		+				+						1
25.			+									6
26.				+		+				+		2
27.				+								6
28.						+						2
29.					+			+				2
30.							+		+			2
31.												36

for CNF2 coding gene, alone or in combination with the genes for ETT2, STb, CNF2, LTa, HPI or Hly, except Tra, Aer, Pfa, Sfa and F17. Other virulence genes coding for Tra, LTa, HPI, Hly, Aer, Pap, Sfa or F17 were also found in the examined isolates with 17.07%, 14.63%, 14.63%, 7.32%, 4.07%, 2.44%, 2.44% and 1.63% percentage rate respectively (Fig 1).

DISCUSSIONS

As yet, bovine mastitis is still a multifactorial disease responsible for economic losses (Anaya-López *et al*., 2006). Bacteriological, epidemiological and clinical studies indicate that *E. coli* is one of the major agents of bovine mastitis worldwide (Blum *et al*., 2008; Cheng *et al*., 2010; Wenz *et al*., 2006). Different virulence factors have been reported in some previous studies. Such as, Nemeth *et al*. (1994) revealed that 20% of isolates from mastitis were Aer positive, which was significantly higher as in isolates from faeces. Pohl *et al*. (1993) and Burns *et al*. (1996) reported that CNF1 and CNF2 were found in only a few mastic strains, whereas CNF2 was very commonly found in the faecal strains. Nemeth *et al*. (1991) found 43% of *E. coli* strains isolated from mastitic milk contain *traT* gene, and Lipman *et al*. (1995) found

55% of isolates from mastitic milk in the Netherlands expressed F17-related fimbriae; but no genes for LT, STa, CNF2 or shigatoxin were detected. Kaipainen *et al*. (2002) found that the genes for Tra, CNF2, CNF1, Aer, F17, Sfa, Pfa and Afa were present in 160 Finnish isolates and 113 Israeli isolates. Colicin production, shigatoxicity, haemagglutination and enterotoxin (LT and STa) were not shown to be associated in mastic isolates (Sanchez-Carlo *et al*., 1984; Hogan *et al*., 1990).

Since there are few reports on the mastic *E. coli* in China dairy farms, it is necessary to determine the prevalence of the virulence genes of *E. coli* isolates from bovine mastitis in China. In this study, 17 genes coding for Stx2e, STa, STb, LTa, HPI, ETT2, LEE, Aer, CNF1, CNF2, Afa, Pfa, Sfa, F17, CS31A, Tra and Hly selected and detected by PCR. The data showed that 11 virulence genes were detected, including ETT2, STb, CNF2, Tra, LTa, HPI, Hly, Aer, Pfa, Sfa and F17, and none of the isolates contained the genes for Stx2e, STa, CNF1, LEE, Afa and CS31A were found. Analyses of the results revealed 30 types of combinations of virulence genes among the mastic isolates investigated.

Enterotoxin-, HPI- and CNF-harboring *E. coli* strains were isolated mainly from pig cattle, lambs and goats with intestinal infections (Cheng *et al*., 2006; De Rycke *et al*., 1999; Horne *et al*., 2004; Van Bost *et al*., 2001). Among the 123 mastic *E. coli* isolates, 25.20% were STb-positive, 19.51% were CNF2-positive and 14.63% were HPI-harboring. These bacteria isolate may well be diarrheagenic strains, for environment contamination with faeces is the main source of mastitis-causing *E. coli* strains (Houser *et al*., 2008). This finding just well suggested that the environmental pathogens, typically faecal strains, are the important opportunistic invaders of the mammary gland. Therefore, a matter of special importance is avoiding the contamination (directly or indirectly) with these invaders in dairy management, especially at or soon after milking or after teat damage.

It is worth notice that ETT2 locus was found among 38.21 *E. coli* from dairy cows with mastitis. ETT2 island, approximately 29.9 kb in size and localised adjacent to the tRNA locus *glyU*, was discovered through the analysis of genome sequences of enterohemorrhagic *E. coli* (Hayashi *et al*., 2001). There are at least 35 genes coding by ETT2 locus, including *yqe*, *yge*, *etr*, *epr*, *epa* and *eiv*, etc., which were similar to the sequence of several Salmonella pathogenicity islands (Hansen-Wester *et al*., 2001). The mutants deleted of ETT2 or *eivA* gene exhibited defects in invasion and intracellular survival compared with the parental *E. coli*, and this suggest that ETT2 plays a role in the pathogenesis of *E. coli* infection (Yao *et al*., 2009). Whether ETT2 could contribute to the virulence of mastic *E. coli* isolate remains a mystery, which needs to be revealed by comparing the virulence of the parental strain and that of the isogenic mutants in a suitable infection mode.

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