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Prevalence of aerobactin and adhesin genes in *Escherichia coli* isolates from blood of bacteremic severely ill neonatal calves

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ABSTRACT

Escherichia coli isolates are one of the most prevalent bacteria in the blood of bacteremic calves. Twenty two *E. coli* were isolated from the blood of severely diseased calves. The O-serogroup of *E. coli* isolates was determined. The presence and prevalence of virulence genes included *iucD* for (aerobactin), *afaE-VIII* (afimbrial adhesin), *clpG* (CS31A adhesin), *f5* (F5 fimbria), *f17A* (F17 fimbrial family), *f17a-A, f17b-A, f17c-A* and *f17d-A* genes (F17a-A. F17b-A, F17c-A and F17d-A fimbria), *f41* (F41 fimbria) and *papC* (P fimbrial adhesin) were examined by PCR assays. Nine different O serogroup O78 (3 isolates), O115 (2), O21 (2), O8 (1), O9 (1), O15 (1), O20 (1), O141 (1) and O153 (1) were determined and 9 isolates were nontypeable. Most of the isolates (20/22) were positive for at least one of the examined genes. All the isolates were negative for F5, F41, P, F17a-A and F17b-A encoding genes. The *iucD* was the most prevalent virulence gene with positive isolates belonging to 5 serogroups. Eight *f17A* positive isolates belonged to *f17b-A* and *f17c-A* variants equally. CS31A and AfaE-VIII encoding genes were detected in 6 and 4 isolates respectively. Six different combinations of virulence genes were detected: the two most prevalent combinations of *afaE-VIII- f17A* and *iucD-clpG* were found in 4 and 3 isolates respectively.

Key words: *Escherichia coli,* aerobactin, adhesin, bacteremia, calf

Introduction

Escherichia coli (*E. coli*) strains are commensal inhabitants of the gastrointestinal tract, although they are associated with diarrhea and a range of extra-intestinal diseases in human and animals (WEST et al., 2007). Gram negative bacteria such as *E. coli, Salmonella* spp. and *Pasturella* spp. and gram positive species (*Listeria monocytogenes*

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and *Streptococcus* spp.) are possible causes of bacteremia and/or septicemia in farm animals. However *E. coli* isolates are one of the most prevalent bacteria in the blood of bacteremic calves (RADOSTITS et al., 2007). In bacteremic cases, exposure of a colostrum deprived calf to an *E. coli* strain, which has the ability to invade and multiply in the blood and internal organs, is a determinant factor (FECTEAU et al., 2001). Bacteremia and subsequently septicemia occurs in a considerable percentage of severely ill calves with diarrhea, especially in calves with the complete or partial failure in transfer of passive immunity (MOXLEY, 2004; RENTER and SARGEANT, 2002). Enterotoxigenic *E. coli* (ETEC) strains as the important cause of sever diarrhea in newborn calves carry fimbriae (such as F5 and F41) mediating binding of the bacteria to microvilli of enterocytes of the small intestine (NAGY and FEKETE, 2005; VAN GERVEN et al., 2008). The septicemia associated *E. coli* isolates from calves often carry some of the adhesins such as P, F17, AfaE-III and CS31A. These adhesins may promote the mucosal adherence of *E. coli* and allow the bacteria to compete on the mucosal surface favoring host invasion and entry to the blood (GYLES et al., 2004). The F17 fimbrial family (encoded by the *f17A* operon) was detected on bovine and ovine *E. coli* isolates with diarrhea or septicemia. F17A family consists of four antigenic variants F17a-A, F17b-A, F17c-A and F111 (F17d-A) fimbriae (GHANBARPOUR and OSWALD, 2008). Pathogenic *E. coli* isolates expressing *afa* operon (afimbrial adhesins) are frequently associated with diarrhea and extraintestinal infections (LE BOUGUENEC, 2005). The *afaE* gene code, antigenically distinct adhesion subunits and an AfaE-VIII variant, has been found in pathogenic *E. coli* isolates from calves (GERARDIN et al., 2000). CS31A antigen encoded by the *clpG* operon, clearly differs from typical fimbriae and is associated with pathogenic *E. coli* isolates from septicemic and diarrheic calves (CROST et al., 2004). P fimbria encoded by the *pap* operon was detected in CS31A and F17A positive bovine isolates (BERTIN et al., 2000). Iron is an essential cofactor for bacterial metabolism, and septicemic *E. coli* isolates produce iron uptake systems, which bind to iron with high affinity (MOKADY et al., 2005). The possession of an aerobactin system allows *E. coli* isolates to be invasive, causing septicemia and bacteremia (VAN BOST et al., 2003).

The purposes of this study were to characterize *E. coli* isolates from blood of ill neonatal calves and determine the virulence associated genes of the isolates.

Materials and methods

Sampling and E. coli isolation. Twenty two *E. coli* isolates were recovered from the same number of severely ill calves (with or without diarrhea) submitted to the Clinical Sciences Department of the Veterinary Medicine Faculty of Kerman, Iran. The general clinical symptoms of the severely ill calves were congestion of mucosa, anorexia, high fever, increased thirst, depression, dullness, weakness and recumbency. The sampled calves were aged between 1 to 9 days. For isolation of the bacteria, 3-5 milliliters of whole

blood samples from each calf were obtained over a period of 4 years (September 2002 - October 2005 and November 2006 - December 2007). The criteria used for isolation of *E. coli* were as previously described by FECTEAU et al. (1997). The biochemical identification of isolates was carried out according to QUINN et al. (1994). The API 20E identification system (BioMérieux, Marcy l'Etoile, France) were used for confirmation of *E. coli* isolates. The isolates were stored in Luria-Bertani broth (LBB) with 30% sterile glycerol at -80 °C. The O serogroup determination of *E. coli* isolates was performed by using antisera provided by the LREC, University of Santiago de Compostela, Lugo, Spain. Isolates examined by O antisera included: O1, O2, O8, O9, O15, O20, O21, O26, O45, O49, O64, O78, O101, O103, O109, O115, O128, O138, O139, O141, O147, O149, O153 and O157. Preparation of O antigen suspension was carried out according to the manufacture procedure; Briefly the isolates were inoculated on tryptone soya agar (TSA) and incubated overnight at 37 ºC. In two tubes a suspension of the growth from the TSA was prepared in concentration adjusted to the tube number 6 of the Mac Farland Barium Sulfate Scale. One of the tubes was heated in boiling water (1 h at 100 ºC) and the other in an autoclave (2.5 h at 121 ºC) to inactivate K antigens. After cooling the tubes, the O antigen suspension was used for serogroup determination by O antisera.

DNA extraction and PCR assays. *E. coli* isolates were incubated in LBB at 37 °C overnight. 300 μL of the bacterial suspension was centrifuged at 13000 rpm for 30 second. The pellet was suspended in 50 μL of sterile water, incubated at 100 °C for 10 minutes and centrifuged. The supernatant was used in the PCR reaction.

The presence and prevalence of virulence genes was examined by PCR assays according to the previously described methods. The examined genes included, *iucD* for (aerobactin), *afaE-VIII* (afimbrial adhesin), *clpG* (CS31A adhesin), *f*5 (F5 fimbria), *f17A* (F17 fimbrial family), *f17a-A, f17b-A, f17c-A* and *f17d-A* genes (F17a-A. F17b-A, F17c-A and F17d-A fimbria), *f41* (F41 fimbria) and *papC* (P fimbrial adhesin).

The isolates were examined for the presence of the genes encoding F5 and F41 fimbriae as described earlier (FRANK et al., 1998). The F17 family and AfaE-VIII encoding genes were identified using PCR described by VAN BOST et al. (2003). The *F17A* variants (*f17a-A, f17b-A, f17c-A*, and *f17d-A* genes) were detected by the method described previously (BERTIN et al., 1996). The CS31A encoding gene (*clpG*) was determined by a protocol established by BERTIN et al. (1998). The *papC* and *iucD* encoding operons were tested according to the primers and PCR amplification condition, as described by YAMAMAT0 et al. (1995).

The reference *E. coli* strains used in PCR assays were as follow: 510 (*F5+, F41+*); 1404 (*f17A+*); 239KH89 (*afaE-VIII+*); 25KH9 (*f17a-A+*), S5 (*f17b-A+*); 31A (*f17c-A+*, *clpG+*); 111KH89 (*F111+*); J96 (*iucD+*, *papC+*), A30 (*afaIB-C+*) and MG1655 (negative control). The reference strains were kindly provided by Dr. Eric Oswald (Microbiology

Department of Ecole Nationale Vétérinaire Toulouse, France). The primers used in this study are listed in Table 1.

Table 1. Primers used in this study

Gene	Primer Sequence (5′-3′)	Product size (bp)	Reference
f5	TATTATCTTAGG TGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314	FRANCK et al. (1998)
f41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCACCT	380	FRANCK et al. (1998)
f17A	GCAGAAAATTCAATTTATCCTTGG CTGATAAGCGATGGTGTAATTAAC	537	VAN BOST et al. (2003)
f17a-A	GCTGGAAGGGTGCAATACGCCTG CTGATAAGCGATGGTGTAATTAAC	321	BERTIN et al. (1996)
f17b-A	CAACTAACGGGATGTACAGTTTC CTGATAAGCGATGGTGTAATTAAC	323	BERTIN et al. (1996)
f17c-A	GCAGGAACCGCTCCCTTGGC CTGATAAGCGATGGTGTAATTAAC	416	BERTIN et al. (1996)
f111	GATAGTCATAACCTTAATATTGCA CTGATAAGCGATGGTGTAATTAAC	239	BERTIN et al. (1996)
afa E-VIII	CTAACTTGCCATGCTGTGACAGTA TTATCCCCTGCGTAGTTGTGAATC	302	VAN BOST et al. (2003)
clpG	GGGCGCTCTCTCCTTCAAC CGCCCTAATTGCTGGCGAC	402	BERTIN et al. (1998)
papC	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328	YAMAMATO et al. (1995)
iucD	TACCGGATTGTCATATGCAGACCG AATATCTTCCTCCAGTCCGGAGAAG	602	YAMAMATO et al. (1995)

Results

According to the serogroup determination results, 13(59.09%) isolates were typeable and belonged to 9 different O serogroups: O78 (3 isolates), O115 (2), O21 (2), O8 (1), O9 (1), O15 (1), O20 (1), O141 (1) and O153 (1), and 9 isolates (40.90%) were nontypeable.

The PCR analysis of *E. coli* isolates for the presence of virulence genes revealed that 90.90% of isolates (20/22) were positive for at least one of the examined genes. All the isolates were negative for F5, F41, P, F17a-A and F17b-A encoding genes (Table 2).

Out of 22 *E. coli* isolates, 12 (54.54%) were positive for *iucD* which was the most prevalent virulence associated gene. The 12 *iucD* positive isolates belong to 5 serogroups O8 (1), O9 (1), O21 (1), O78 (2) and O115 (1), whereas 6 isolates were O-nontypeable.

Table 2. Characteristics of 22 *E. coli* isolates from the blood of bacteremic calves

		Virulence genes									
		iucD	*afaE-VIII*	*clpG*	*f17A*				*papC*	*f5*	*f41*
Code N°	Serogroup				*f17a*	*f17b*	*f17c*	*f17d*			
2.7	O9	+	-	-	-	-	-	-	-	-	-
2.10	ON *	+	+	-	-	-	+	-	-	-	-
3.2	O78	+	-	-	-	-	-	-	-	-	-
3.5	ON	+	-	+	-	-	-	-	-	-	-
3.5	O21	-	+	-	-	-	+	-	-	-	-
3.9	ON	-	+	-	-	+	-	-	-	-	-
3.10	O153	-	-	-	-	+	-	-	-	-	-
4.3	O141	-	-	+	-	-	-	-	-	-	-
4.5	O78	+	-	-	-	-	-	-	-	-	-
4.11	ON	-	+	-	-	+	-	-	-	-	-
4.12	O8	+	-	-	-	-	-	-	-	-	-
4.12	ON	+	-	-	-	-	-	-	-	-	-
5.2	O115	-	-	+	-	-	-	-	-	-	-
5.4	ON	+	-	-	-	+	-	-	-	-	-
5.5	O20	-	-	-	-	-	+	-	-	-	-
5.8	ON	-	-	+	-	-	+	-	-	-	-
5.9	O78	-	-	-	-	-	-	-	-	-	-
6.12	ON	+	-	+	-	-	-	-	-	-	-
7.2	O115	+	-	-	-	-	-	-	-	-	-
7.6	O21	+	-	+	-	-	-	-	-	-	-
7.8	ON	+	-	-	-	-	-	-	-	-	-
7.12	O15	-	-	-	-	-	-	-	-	-	-
N° of positive isolates		12	4	6	-	4	4	-	-	-	-

*O-Nontypeable; Number of examined *E. coli* isolates = 22

The F17 fimbrial family encoding gene was one of the most frequent genes in the examined isolates with 8 (36.36%) isolates positive for the *f17A* gene. PCR assays showed that these isolates belonged equally to the two F17b-A and F17c-A variants. The 4 *f17b-A* positive isolates belonged to O153 (1) and O-nontypeable (3) serogroups whereas the *f17c-A* positive isolates were from the O20 (1), O21 (1) and O-nontypeable (2) serogroups.

Of the 22 *E. coli* isolates investigated, 6 (27.27%) possessed the *clpG* gene that belonged to the O21 (1), O115 (1), O141 (1) and O-nontypeable (3) groups.

The PCR assays for detection of *afaE-VIII* showed that 18.18% (4 isolates) were positive and belonged to O21 (1) and O-nontypeable (3) groups.

According to the PCR analysis, six different combinations of virulence genes were detected. The two most prevalent combinations of *afaE-VIII- f17A* and *iucD-clpG* were found in 4 and 3 isolates respectively (Table 2).

Discussion

Bacteremia occurs in a significant proportion of cows with a systemic disease or due to clinical coliform mastitis, where genotypically similar *E. coli* strains were isolated from the blood of affected cows (WENZ et al., 2006). In bacteremic calves, gram negative enteric bacteria are the most commonly isolated microorganisms, which supports the hypothesis that the early environment of the calf may be the source of infection. On the other hand, in 51% of blood culture from critically ill calves *E. coli* were isolated (FECTEAU et al., 1997).

In the present study 9 O-serogroups were identified; O78 and O115 were the two most prevalent groups. It is believed that O78 and O115 are non enterotoxigenic bovine isolates (CONTREPOIS et al., 1989). It seems that the O-antigens of these serogroups are important factors in the survival of bacteria in the blood and resistance to bacterial killing by the complement and phagocytosis activity of the host (BAHRANI-MOUGEOT et al., 2002). In adult and neonatal cattle endotoxemia and its pathophysiologic consequences are associated with systemic organ infections such as enteritis, mastitis and metritis, as well as gram-negative bacteremia (PEEK et al., 2008). In *E. coli* isolates, other than O-antigens, certain surface factors, such as K1 capsule have been recognized as responsible for serum resistance and anti phagocytic properties are instrumental for inducing a high degree of bacteremia (KIM, 2006).

In this study, aerobactin, F17A, CS31A and AfaE-VIII encoding genes were detected in 90.90% of the isolates. It was established that the pathogenicity of *E. coli* isolates from bacteremic calves was associated with the presence and production of virulence factors including siderophores, colicin V and CS31A surface antigen (MOHAMED OU SAID et al., 1988). To obtain iron, *E. coli* strains have evolved a number of transport systems (STUART et al., 1982). In the present study, the aerobactin encoding gene (*iucD)* was the most prevalent virulence gene that was detected alone (7 isolates) or in combination with *afaE-VIII- f17c-A* (1), *clpG* (3) and *f17b-A* (1). The *iucD* positive isolates were distributed in different O-serogroups and the O-nontypeable group. Similarly, FECTEAU et al. (2001) reported that aerobactin production is frequently observed in blood isolates with different serogroups such as O78 and O119; on the other hand, according to the results of the present study, *iucD* was observed alone and/or along with P, F17 and CS31A adhesin factors.

In the present study 8 isolates possessed *f17A* genes (*f17c-A* and *f17b-A* subtypes). The presence of these two subtypes was previously reported in septicemic and diarrheic bovine isolates of *E. coli* (MAINIL et al., 2000; VAN BOST et al., 2001). The operon coding F17A adhesins are normally chromosome- located, whereas in some strains the *Vir* plasmid can carry the operon coding for afimbrial adhesins (GERARDIN et al., 2000). According to the results of this study all the 4 *afaE-VIII* positive isolates were also positive for *f17c-A* and *f17b-A* genes equally. The strong association of F17A and CS31A adhesins in *E. coli* isolates from pathogenic conditions of calves has been established (BERTIN et al., 2000; BERTIN et al., 1998). In the current study six CS31A positive isolates from O21, O141, O115 and O-nontypeable groups were detected and 4 of them were in combination with *iucD* (3 isolates) and *f17c*-A (one isolate). In agreement with these results, FECTEAU et al. (2001) found that among six CS31A positive isolates, 3 isolates were F17A positive and some isolates were positive for aerobactin. GIRARDEAU et al. (1988) indicated that CS31A producing *E. coli* from septicemic calves belonged to O8, O20, O78, O86, O117, and O153 serogroups.

In this study *E. coli* isolates from the blood of sick calves, regardless of the presence of diarrhea, were examined for aerobactin and adhesin encoding genes. Although in this study some of the adhesin genes, P, F5 and F41 were not detected, 13 isolates were positive for at least one of the other examined adhesin genes. It is believed that in diarrheic calves there is an increased *E. coli* strain count in the intestine: colonization and damage of the bacteria in the small intestine increase susceptibility to bacteremia (RADOSTITS et al., 2007).

In conclusion, *E. coli* isolates from the blood of severely ill neonatal calves varied in their virulence genes and constitute a heterogeneous group. The presence of these virulence factors may not be a sufficient reason for translocation of the bacteria from the intestine and survival in the blood. The ability of these isolates to cause bacteremia and subsequently septicemia and death may be related to other virulence factors, which are unidentified and/or under certain conditions related to host dependent factors.

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SAŽETAK

Escherichia coli jedna je od najčešće prisutnih bakterija u krvi bakteremične teladi. U radu su 22 izolata *E. coli* bila izdvojena iz krvi teško bolesne teladi. Izolati su pripadali serološkoj skupini O. Lančanom reakcijom polimerazom istražena je prisutnost i prevalencija sljedećih gena odgovornih za virulenciju: iucD za aerobaktin, *afaE-VIII* za afimbrijski adhezin, *clpG* za adhezin CS31A, *f5* za fimbrije F5, *f17A* za fimbrijsku porodicu F17 gene *f17a-A, f17b-A, f17c-A* i *f17d-A* (fimbrije F17a-A. F17b-A, F17c-A i F17d-A), *f41* (fimbrije F41) i *papC* (fimbrijski adhezin P). Dokazano je bilo devet različitih seroloških skupina O: O78 (3 izolata), O115 (2), O21 (2), O8 (1), O9 (1), O15 (1), O20 (1), O141 (1) i O153 (1) dok se devet izolata nije moglo tipizirati. Većina izolata (20/22) bila je pozitivna najmanje za jedan od pretraživanih gena. Svi izolati bili su negativni za gene koji kodiraju za F5, F41, P, F17a-A i F17b-A. Gen iucD bio je najčešće ustanovljen gen za virulenciju, a izolati s tim genom pripadali su u pet seroloških skupina. Osam izolata pozitivnih za *f17A* pripadalo je varijantama *f17b-A* i *f17c-A*. Gen koji kodira za CS31A bio je dokazan u šest izolata, a gen koji kodira za AfaE-VIII u četiri izolata. Dokazano je šest različitih kombinacija gena za virulenciju. Dvije najčešće kombinacije bile su *afaE-VIII-f17A* koja je ustanovljena u četiri izolata i *iucD-clpG* ustanovljena u tri izolata.

Ključne riječi: *Escherichia coli*, aerobaktin, adhezin, bakteremija, telad