Comparison of five real-time PCR assays for detecting virulence genes in isolates of *Escherichia coli* from septicaemic neonatal foals

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Fifty-five isolates of *Escherichia coli* from septicaemic neonatal foals were used to validate five real-time PCR assays targeting different known virulence factor genes: curli fibre (*csgD*), ferric hydroxamate uptake (*fhuA*), type 1A pilin (*fimA*), aerobactin (*lutA*) and yersiniabactin (*fyuA*). A PCR assay targeting a universal sequence of the bacterial 16S rRNA gene served as quality control. The PCR assays showed good analytical specificity and sensitivity on the basis of sequencing the PCR products, their lack of cross-reactivity with non-*E coli* organisms, high amplification efficiency and a limit of detection as low as 25 *E coli* colony-forming units. There were differences between the detection rates and amplification efficiencies for the five virulence genes. The PCR assays targeting genes *csgD*, *fhuA* and *fyuA* were able to detect all 55 *E coli* isolates, with gene *csgD* having the best amplification efficiency. The lowest detection rate and amplification efficiency of the *E coli* isolates was found for the *lutA* gene.

SEPTICAEMIA is a common cause of illness and death in newborn foals (Sanchez 2005). The reported relative frequency of isolation of individual bacterial species from septicaemic foals varies, but Gram-negative microorganisms, especially *Escherichia coli*, are predominant everywhere. Other organisms, such as *Actinobacillus*, *Klebsiella*, *Enterobacter*, *Proteus*, *Salmonella*, *Pasteurella*, *Pseudomonas*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Clostridium*, *Acinetobacter* and *Citrobacter* species, have also been isolated from the blood of septicaemic foals (Koterba and others 1984, Wilson and Madigan 1989, Paradis 1994, Raisis and others 1996, Marsh and Palmer 2001, Stewart and others 2002). In the early stages it is difficult to recognise sepsis in neonatal foals because the clinical signs of disease are often vague and may be similar to those seen in other, non-infectious conditions. The culture of a pathogen from blood collected at the onset of clinical signs or from a local site of suspected infection remains the gold standard for definitive diagnosis of systemic bacterial infection (Sanchez 2005). However, the culture of blood and other samples has several inherent limitations. The results are typically not available for at least 48 hours, and false-negative results, which may be caused by a low, circulating bacterial population, a low blood volume collected for culture, the blood culture system used or the previous use of antimicrobial drugs, are common (Wilson and Madigan 1989, Lorenzo-Figueras and others 2006). Incorporating an additional, reliable and rapid assay to detect specific bacteria directly from blood would greatly facilitate timely diagnosis and appropriate care of neonatal foals suspected to be septicaemic. Several real-time PCR assays have been validated to augment culture-based methods for diagnosing neonatal sepsis in human beings (Jordan and Durso 2005, Shang and others 2005).

Virulence factors of intestinal and extraintestinal strains of *E coli* are being widely studied because they constitute an increasing problem for human health, due in particular to the high incidence of drug resistance (Mokady and others 2005a). The virulence factors in strains of *E coli* capable of causing septicaemia have not been fully identified, and neither have the differences between the virulence mechanisms of different pathogenic serotypes. A comparison of septicaemic strains of *E coli* isolated from human beings and animals has recently identified a few traits that are shared by all the strains in one form or another (Mokady and others 2005b). These common virulence factors are presumably important, or even essential, for the establishment of septicaemic infection. Virulence factors such as yersiniabactin, aerobactin and ferrichrome-iron receptor, which are associated with uptake of iron, are particularly important because iron is an essential cofactor for bacterial metabolism. The high affinity of these factors for iron allows bacteria to scavenge iron from the host (Gophna and others 2001). Adhesins located on fimbriae or pili are also important virulence factors because they promote bacterial attachment to and colonisation of the host's tissues. Septicaemic *E coli* strains have been shown to contain a variety of adherence factors, such as type I pili, AC/I pili, P pili, non-fimbrial adhesin, long polar fimbriae, and curli fibre (Ideses and others 2005).

The objective of this study was to validate real-time PCR assays targeting five virulence genes of *E coli* to assess their potential value as an adjunct to bacterial culture in the diagnostic evaluation of foals with suspected sepsis. The five target genes were selected on the basis of previous reports defining their importance as virulence genes for *E coli* strains capable of inducing neonatal sepsis and on the availability of sequence information for the design of the PCR assays (Gophna and others 2001, Mokady and others 2005a, b).

MATERIALS AND METHODS

Escherichia coli isolates

A total of 55 *E coli* isolates, 46 from blood, four from abdominal fluid, three from the umbilicus and two from internal abscesses of septicaemic foals examined at the Veterinary Medical Teaching Hospital of the University of California, Davis, between 1990 and 2005 were used. The isolates, which had been stored on glass beads at –80°C, were retrieved, inoculated on to bovine blood agar plates, and incubated overnight at 37°C. For each DNA extraction, one colony of each isolate was collected under a biosafety cabinet directly into a deep-well plate (Masterblock; Greiner Bio-One) containing 150 µl NucPrep digestion buffer (Applied Biosystems), 50 µl proteinase K (Applied Biosystems), and 600 µl NucPrep solution (Applied Biosystems). Two stainless steel grinding beads 4 mm in diameter were added to the deep-well plate and the bacteria were homogenised in a grinder (Geno Grinder 2000; SpexCertiprep) for two minutes at 1000 strokes per minute. The samples were stored at –20°C for one hour to reduce foam, and the protein digestion was then completed at 56°C

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TABLE 1: Nucleotide sequences of the PCR primers and TaqMan probes used to amplify five virulence factor genes of *Escherichia coli* and the bacterial 16S rRNA gene

Target gene	Primer/probe	Sequences 5'→3'	Reference sequence
Curli fibre (*csgD*)	*csgD*-197f*	AGCTTATCCATTATTGGCAGGAT	AE005174
	csgD-275r†	TCTTCAGGCGTATTTAGCAACAA	
	csgD-probe	GAGCAGGA	
Ferric hydroxamate uptake (*fhuA*)	*fhuA*-4f	TCGCAGTTGTAGTAGCCACAGC	AE005174
	fhuA-157r	TTTCTTGCGGCGCAGGT	
	fhuA-probe	CAGGCAGC	
Type 1A pilin (*fimA*)	*fimA*-28f	GTTCTGTCGGCTCTGTCCCT	AE005174
	fimA-166r	GAACGGTTTGATCAACAGAGCC	
	fimA-probe	GGTGGGACCGTTCACTTTAAAGGGGAAGT	
Aerobactin (*lutA*)	*lutA*-223f	CAAACCACCTGGGTTATCGAAA	AE014075
	lutA-354r	CATACCGTAGTTGGTTCGGCTC	
	lutA-probe	CAGCAGCC	
Yersiniabactin (*fyuA*)	*fyuA*-488f	GCTATATTGAAGGCGGCGTC	AY233333
	fyuA-622r	GGTTAATCATGTCGCCGTCAT	
	fyuA-probe	GCCTGCTG	
Bacterial 16S rRNA gene (*panB*)	*panB*-283f	GGATGATCAGCCACACTGGA	Reference 17
	panB-352r	CCAATATTCCTCACTGCTGCC	
	panB-probe	CCCGTAGGAGTCTGGACCGTGTCTCA	

* f Forward primer
† r Reverse primer

for 30 minutes. The DNA was extracted from the bacterial lysates using an automated nucleic acid work station (6100 Nucleic Acid Prep Station; Applied Biosystems) according to the manufacturer's instructions.

Real-time PCR assays

For each of the five *E coli* virulence factor target genes (curli fibre [*csgD*], ferric hydroxamate uptake [*fhuA*], type 1A pilin [*fimA*], aerobactin [*lutA*], and yersiniabactin [*fyuA*]), two primers and one internal, fluorescent-labelled TaqMan probe (5' end reporter dye FAM [6-carboxyfluorescein], 3' end quencher dye TAMRA [6-carboxytetramethylrhodamine] were designed using Primer Express software (Applied Biosystems) (Table 1). In addition, a TaqMan assay targeting a universal sequence of the bacterial 16S rRNA gene was used as quality control for the DNA extraction as described by Windsor and others (2006). Each PCR reaction contained 400nM of each primer, 80nM of the TaqMan probe, and commercially available TaqMan universal PCR mastermix (Applied Biosystems), containing 10mM Tris-HCl (pH 8·3), 50mM potassium chloride, 5mM magnesium chloride, 2·5mM deoxynucleotide triphosphates, 0·625 U AmpliTaq Gold DNA polymerase and 0·25 U AmpErase UNG per reaction, and 1 µl of the DNA sample, in a final volume of 12 µl. The samples were placed in 384-well plates and amplified in an automated fluorometer (ABI PRISM 7900 HTA FAST; Applied Biosystems). The amplification protocol was two minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Fluorescent signals were collected during the annealing temperature and cycle threshold values were extracted with a threshold of 0·1 and baseline values of 3 to 15. Analytical specificity was verified by sequencing the PCR products for the target gene and by analysing several different microorganisms, such as *Actinobacillus equuli*, *Klebsiella pneumoniae*, *Bordetella bronchiseptica*, *Enterobacter*, *Salmonella*, *Pasteurella*, *Pseudomonas* and *Enterococcus* species, *Streptococcus equi* subspecies *zooepidemicus* and *Staphylococcus aureus*, for cross-reactivity.

Each assay's amplification efficiency (E) was calculated from the slope of the standard curve (S) by the formula $E = 10^{1/-s} - 1$. The standard curve was generated by using 10-fold DNA dilutions from *E coli*. An amplification efficiency for the target gene of 90 per cent or more indicated a high analytical sensitivity. The detection limit of each assay was determined by using a DNA sample from an isolate of *E coli* positive for all five target genes, with a known number of colony-forming units (cfu). The mean (sd) values of the cycle threshold were calculated for each target gene. The extraction efficiencies of the five *E coli* assays were compared by using the Mann-Whitney test (GraphPad Prism; GraphPad Software) and differences with $P<0·05$ were considered significant).

RESULTS

The analytical specificity for all five *E coli* target genes was 100 per cent, on the basis of the sequencing of the PCR products and no cross-reactivity with non-*E coli* microorganisms (data not shown). The five *E coli* assays were able to detect as few as 25 cfu. An amplification efficiency of more than 90 per cent was achieved for all five target genes, indicating high analytical sensitivity. DNA was successfully extracted from all 55 *E coli* isolates on the basis of their similar cycle threshold values, which had a mean (sd) of 17·8 (1·69), using the bacterial 16S rRNA gene TaqMan assay. The PCR assays targeting the

TABLE 2: Detection of 55 isolates of *Escherichia coli* from septicaemic neonatal foals using five different virulence factor target genes

	Target genes					
	csgD	*fhuA*	*fimA*	*lutA*	*fyuA*	*panB*
Number of isolates/total (%)	55/55 (100)	55/55 (100)	53/55 (96)	38/55 (69)	55/55 (100)	55/55 (100)
Mean (sd) cycle threshold*	20·4 (2·37)	21·8 (1·56)	NA	NA	27·4 (4·40)	17·8 (1·69)
Mean (sd) cycle threshold†	20·2 (2·61)	21·6 (1·29)	22·2 (2·15)	37·1 (5·38)	27·4 (4·51)	17·5 (1·69)

* For target genes with a signal for all 55 isolates
† For the 37 isolates detected by all five virulence factor target genes
NA Not applicable, *csgD* Curli fibre, *fhuA* Ferric hydroxamate uptake, *fimA* Type 1A pilin, *lutA* Aerobactin, *fyuA* Yersiniabactin, *panB* Bacterial 16S rRNA gene

csgD, *fhuA* and *fyuA* genes were able to detect all 55 *E coli* isolates (Table 2). There were significant differences (P=0·001) in amplification efficiency between the *csgD* gene and either the *fhuA* or *fyuA* gene, and between the *fhuA* and the *fyuA* gene. Thirty-seven of the *E coli* isolates were detected by targeting all five virulence genes, with variations in amplification efficiency. The lowest cycle threshold values, that is, the strongest PCR signals, for the 37 isolates were obtained for the *csgD* gene, and the highest values (the weakest PCR signals) were obtained for the *lutA* gene. There were significant differences (P=0·001) in amplification efficiency of the 37 *E coli* isolates between the *csgD* gene and the other four virulence genes, between the *fhuA* gene and either the *lutA* or the *fyuA* gene, and between the *fyuA* and the *lutA* gene, there was no significant difference in amplification efficiency between the *fhuA* and the *fimA* gene.

DISCUSSION

The five *E coli* PCR assays all had a good analytical specificity and sensitivity, on the basis of PCR product sequencing and their lack of cross-reactivity with non-*E coli* organisms, a high amplification efficiency, and a limit of detection of as few as 25 *E coli* cfu. Their detection and amplification efficiency varied between the five selected virulence genes. The virulence genes *csgD*, *fhuA* and *fyuA* were detected in all 55 isolates, but there were significant differences between the amplification efficiencies for the three genes. The *csgD* gene performed the best, having a mean cycle threshold value four times the value for *fhuA* and 24 times the value for *fyuA*. The lowest detection rate or amplification efficiency of the *E coli* isolates was for the *lutA* gene. The lower occurrence of this gene in isolates from neonatal foals may be due to different selective pressures, as has been shown for other *E coli* genes responsible for the uptake of iron (Perna and others 2001). Variations in DNA extraction were ruled out as the cause of the differences in detection rates because similar cycle threshold values were obtained for all the isolates when the bacterial 16S rRNA gene was targeted. Comparative studies have shown that there are differences in gene content between septicaemic and enterohaemorrhagic strains of *E coli* (Gophna and others 2001, Perna and others 2001, Jin and others 2002). The differences have been found between isolates recovered from human beings and animals. The results show that the majority of the target genes were present among the 55 *E coli* isolates, possibly in association with a homogeneous pool of strains associated with the same disease (septicaemia) and targeting the same type of host (neonatal foals) and the same tissue (blood). The gene variability of the 55 isolates did not appear to have changed over the period of 15 years during which they had been collected (data not shown). Further comparative genomic studies of *E coli* will be necessary to characterise isolates from septicaemic foals more fully. The clinical use of PCR for the detection of *E coli* in the blood of septicaemic neonatal foals will need to be compared with conventional culture to assess its accuracy. For the foreseeable future, culture will not be superseded by PCR-based testing, owing to the requirement for purified culture isolates in antimicrobial susceptibility testing. However, the results of this study suggest that the use of both conventional culture and PCR should facilitate more rapid confirmation of a diagnosis of septicaemia and thereby improve the therapeutic management of neonatal foals.

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