Detection of bloodstream infection in neonatal foals with suspected sepsis using real-time PCR

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BACTERIAL infection is a major cause of morbidity and mortality in neonatal foals (Koterba and others 1984, Wilson and Madigan 1989, Paradis 1994, Raisis and others 1996, Marsh and Palmer 2001, Corley and others 2007). Early recognition of sepsis in neonatal foals is difficult, because the clinical signs are often vague and may be similar to those seen in other non-infectious conditions. 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 the definitive diagnosis of systemic bacterial infection (Sanchez 2005). The relative frequency of isolation of individual bacterial species from septicaemic foals varies between different reports, but Gram-negative microorganisms, especially *Escherichia coli*, are the organisms most isolated from neonatal foals with septicaemia in all geographical regions. Other microorganisms, 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). However, the culture of blood and other biological samples has several inherent limitations. Culture results are typically not available for at least 48 to 72 hours, and false-negative results may be caused by low numbers of circulating bacteria or the collection of a low volume of blood for culture; the results may also vary with the type of blood culture system used or previous use of antimicrobials (Wilson and Madigan 1989, Lorenzo-Figueras and others 2006). Incorporating an additional, reliable yet rapid assay to detect specific microorganisms directly from blood would greatly facilitate rapid diagnosis and appropriate care of neonatal foals with suspected sepsis. Recently, several real-time PCR assays have been validated in order to augment culture-based methods for diagnosing neonatal sepsis in human beings (Jordan and Durso 2005, Shang and others 2005). The aim of this study was to determine whether real-time PCR testing for a panel of seven preselected pathogens would be more sensitive than conventional culture using blood from neonatal foals evaluated for sepsis.

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Eighty-six client-owned foals, all less than 10 days of age, presented to the William R. Pritchard Veterinary Medical Teaching Hospital (VMTH) at the University of California, Davis, from January 2006 to June 2008, were included in the study. Using historical, physical and clinicopathological data, a sepsis score was calculated for each foal, according to Brewer and Koterba (1988). Based on the sepsis score and culture results, the 86 foals were assigned to one of two groups: sick, non-septic (sepsis score <11 and negative culture; 53 foals) or septic (sepsis score ≥11 with or without positive culture; 33 foals). The sick, non-septic group comprised foals diagnosed with meconium impaction, hypoxic encephalopathy, partial failure of passive transfer of maternal immunity, congenital diseases, trauma and bladder rupture. The sepsis score of the non-septic foals ranged from 1 to 10 (mean [sd] 5·5 [2·6]). The 33 septic foals had a sepsis score ranging from 11 to 21 (mean [sd] 13·2 [2·5]). The following pathogens were isolated or detected from the blood, faeces or tissues at postmortem examination from 22 of the 33 septic foals: *Escherichia coli* (5), *Actinobacillus equuli* (5), *Klebsiella pneumoniae* (2), *Enterococcus faecium* (2), *Clostridium perfringens* type C (2), *Pasteurella* species (2), *Pseudomonas aeruginosa* (1), *Streptococcus equi* subspecies *zooepidemicus* (1), *Corynebacterium* species (1) and equine herpesvirus type 1 (EHV-1) (1).

An additional 20 foals less than 10 days of age served as healthy controls. These foals were born at the Center for Equine Health, University of California, Davis, and were considered healthy based on normal physical and clinicopathological findings.

At the time of admission of each foal, 12 ml whole blood was collected in a sterile fashion via a newly placed jugular catheter or via direct venepuncture. The blood was transferred into a commercial resin-containing blood collection system (5 to 10 ml, BBL Septi-Chek TSB with resins; Becton Dickinson) as well as into a sodium citrate-containing blood collection tube (1 to 2 ml, Monoject; Kendall). The blood collection system bottles were incubated in a stationary position at 35°C without venting for 24 hours, without prior processing. Thereafter, a sample of each bottle was transferred by use of a sterile needle and syringe on to sheep blood agar and MacConkey's agar plates and incubated at 35°C with 5 per cent carbon dioxide. Microorganisms were identified by the use of Gram stain and standard microbiological procedures. An additional 1 ml aliquot was collected from each bottle into a sterile tube at 24 hours of incubation. In addition, 200 µl of whole blood collected into the sodium citrate tube on admission and the inoculated broth collected following 24 hours of incubation were processed for DNA purification using an automated nucleic acid extraction system (CAS-1820 X-tractor Gene; Corbett Life Science) according to the manufacturer's recommendations. Nucleic acid samples from the whole blood and incubated medium were assayed for the presence of a selected panel of microorganisms routinely associated with sepsis. The panel targeted the curli fibre (csgD) gene of *E coli* (Mapes and others 2007), the translation initiation factor (IF2A) gene of *A equuli*, the 16S rRNA gene of *S aureus*, *S equi* subspecies *zooepidemicus* and *K pneumoniae*, the 23S rRNA gene of *Enterococcus* species and the glycoprotein B gene of EHV-1 (Pusterla and others 2006). Furthermore, a real-time PCR assay targeting a universal sequence of the bacterial 16S rRNA gene was used to determine the molecular presence of bacterial organisms (Windsor and others 2006, Mapes and others 2007). For each assay 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). For pathogens, such as *S aureus*, *S equi* subspecies *zooepidemicus* and *K pneumoniae*, with high sequence variability between strains, several primers or primer pairs were used to maximise analytical specificity. Each real-time PCR reaction contained 400nM of each primer, 80nM of the TaqMan probe and commercially available TaqMan universal PCR mastermix (Applied Biosystems), containing 10mM Tris-HC1 (pH 8·3), 50mM potassium

TABLE 1: Nucleotide sequences of the PCR primers and TaqMan probes used to amplify organisms commonly associated with sepsis in neonatal foals

Target gene	Primer/probe*	Sequences (5′→3′)	Reference/GenBank accession number
Escherichia coli curli fiber (*csgD*)	*csgD*-197f	AGCTTATCCATTATTGGCAGGAT	Mapes and others (2007)
	csgD-275r	TCTTCAGGCGTATTTAGCAACAA	
	csgD-probe	FAM-GAGCAGGA-MGB	
Actinobacillus equuli translation initiation factor IF2A†	*A equuli*-126f	CGGGACACGCAGCGTTTA	AY508859
	A equuli-222r	TGTGGCATTACACCATCGTCTG	
	A equuli-probe	FAM-GCGTGCACGTGGTGCGAAAGCA-TAMRA	
Staphylococcus aureus 16S rRNA†	*S aureus*-891f	TGGAGCATGTGGTTTAATTCGA	AF015929
	S aureus-1051r1	TGCGGGACTTAACCCAACA	
	S aureus-727r2	ACGACACGAGCTGACGACAAC	
	S aureus-probe	FAM-TCCTTTGACAACTCTAGAGATAGAGCCTTCCCCT-TAMRA	
Streptococcus equi subspecies *equi* 16S-23S rRNA intergenic spacer†	*S zoo*-71f	GAGAGCGCCTGCTTTGCA	AJ704861, AF489601, DQ204516
	S zoo-159r1	GGTAAACCGAACCGTCTGTTAGTATC	
	S zoo-159r2	GGTAACCCAAACATTTCTTTGTATCC	
	S zoo-159r3	GGTAACCCAAACTTCTCTTTGTATCC	
	S zoo-probe	FAM-CAGGAGGTCAGCGGTTCGATCCC-TAMRA	
Klebsiella pneumoniae 16S rRNA†	*Kleb*-41f1	GGTAGCACAGAGAGCTTGCTCTC	AY233333
	Kleb-14f2	GCAGGCCTAACACATGCAAGT	
	Kleb-33f3	AGTCGAGCGGTAGCACAGAGA	
	Kleb-172r1	CCACTTTGGTCTTGCGACGTTA	
	Kleb-130r2	CCAGTAGTTATCCCCCTCCATC	
	Kleb-179r3	AGGTCCCCCACTTTGGTCTT	
	Kleb-probe	FAM-CGGACGGGTGAGTAATGTCTGGGAAACT-TAMRA	
Enterococcus species 23S rRNA†	*Entero*-722f	CAGGTTGAAGGTGCGGTAAAAC	EU360259
	Entero-790r	CCTCATCCCCGCACTTTTC	
	Entero-probe	FAM-CTGGAGGA-MGB	
Equine herpesvirus type 1 glycoprotein B	EHV1-387f	TATACTCGCTGAGGATGGAGACTTT	Pusterla and others (2006)
	EHV1-476r	TTGGGGCAAGTTCTAGGTGGTT	
	EHV1-probe	FAM-ACACCTGCCCACCGCCTACCG-TAMRA	
Universal bacterial 16S rRNA (*panB*)	panB-283f	GGATGATCAGCCACACTGGA	Windsor and others (2006)
	panB-352r	CCAATATTCCTCACTGCTGCC	
	panB-probe	FAM-CCCGTAGGAGTCTGGACCGTGTCTCA-TAMRA	

* f Forward primer, r Reverse primer
† Real-time PCR assays established in this study

chloride, 5mM magnesium chloride, 2·5mM deoxynucleotide triphosphates, 0·625 U AmpliTaq Gold DNA polymerase per reaction, 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) for 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 (C_t) values extracted with a threshold of 0·1 and baseline values of 3 to 15. The specificity of the assays was verified by analysing three field isolates of *E coli*, *A equuli*, *K pneumonia*, *Enterobacter* species, *Bordetella bronchiseptica*, *Salmonella* species, *Pasteurella* species, *Pseudomonas* species, *Enterococcus* species, *S equi* subspecies *zooepidemicus* and *S aureus* for cross-reactivity. The banked isolates originated from the blood of septicaemic foals. The specificity of the assays was also verified by sequencing real-time PCR products from each of the isolates. Amplification efficiency (E) was calculated using the slope of the standard curve (S) with the formula $E = 10^{[1/-s]}-1$. The slope of the standard curve was generated using 10-fold DNA dilutions from each specific target organism. High amplification efficiency for the target gene of 90 per cent or above indicated a high analytical sensitivity. The detection limit of each assay was determined using DNA samples from known numbers of colony-forming units for each of the six bacterial agents targeted by the assays.

The analytical specificity was 100 per cent for the six bacterial target genes, based on sequencing of real-time PCR products, and there was no cross-reactivity with non-related microorganisms (data not shown). The six bacterial assays were able to detect as few as 15 colony-forming units. High amplification efficiency (>90 per cent) was achieved for all bacterial target genes, indicating high analytical sensitivity.

None of the samples from healthy or sick, non-septic foals (blood and broth) had detectable pathogens by real-time PCR. All of the samples from the healthy and sick, non-septic foals tested PCR-negative for the universal bacterial 16S rRNA gene, which indicated the absence of bacterial organisms in the blood. In contrast, various pathogens were detected in the samples from septic foals (Table 2). Five foals had positive culture results for *E coli* from blood (four foals) or lung tissue (one foal). While all of these five foals tested PCR-negative for *E coli* in the direct blood samples, two foals tested PCR-positive for *E coli* in the 24-hour broth culture medium. The bacterial 16S rRNA gene was detected with universal primers in the blood and/or culture broth of three of the five foals with positive *E coli* culture results. *A equuli* was cultured from the blood (four foals) or lung tissue (one foal) of five foals. Three of the foals with positive blood culture results also tested PCR-positive for *A equuli* in blood and culture broth. The other foal with a positive *A equuli* blood culture tested negative by PCR in blood and culture broth. The foal with a positive *A equuli* lung culture and negative blood culture tested PCR-positive for *A equuli* in blood and culture broth. The bacterial 16S rRNA gene was detected by PCR in blood and culture broth of all the foals with positive *A equuli* PCR results. *K pneumoniae* was cultured in the blood of two septic foals; blood was PCR-positive for *K pneumoniae* in one foal and culture broth was positive in two foals and all the samples tested PCR-positive for the universal bacterial 16S rRNA gene. *E faecium* was cultured from the blood of one foal and the resected umbilicus of another foal. The foal with positive blood results for *E faecium* was positive by PCR for both the specific target gene and the bacterial 16S rRNA gene. The foal with an infected umbilicus and culture-negative blood was also PCR-negative in blood and culture broth for *E faecium* and the universal bacterial 16S rRNA gene. *C perfringens* type C was cultured from the faeces of two foals with sepsis. Both of these foals had negative blood results and tested PCR-negative for all the selected pathogens and for the bacterial 16S rRNA gene. Four foals with blood positive culture results (two for *Pasteurella* species, one for *P aeruginosa* and one for *Corynebacterium* species) tested PCR-positive in blood and culture broth for the bacterial 16S rRNA gene. One foal in the septic group with negative blood culture results and a positive abdominal fluid culture for *S equi* subspecies *zooepidemicus* tested PCR-positive for *S equi* subspecies *zooepidemicus* in blood and culture broth; these samples also

TABLE 2: Results of blood culture and real-time PCR for 22 septic foals with documented detection or isolation of microorganisms from blood, faeces or tissues

Organism	Sample of initial culture (number)	Culture broth	PCR	
			Blood	Broth
Escherichia coli	Blood (4)	4	0	2
	Lung (1)	0	0	0
Actinobacillus equuli	Blood (4)	4	3	3
	Lung (1)	0	1	1
Klebsiella pneumoniae	Blood (2)	2	1	2
Enterococcus faecium	Blood (1)	1	1	1
	Umbilicus (1)	0	0	0
Clostridium perfringens	Faeces (2)	0	0	0
Pasteurella species	Blood (2)	2	0	0
Pseudomonas aeruginosa	Blood (1)	1	0	0
Streptococcus equi subspecies *zooepidemicus*	Abdominal fluid (1)	0	1	1
Corynebacterium species	Blood (1)	1	0	0
Equine herpesvirus type 1	NA	NA	1	1

NA not applicable

TABLE 3: Agreements (%) between the overall culture results (from blood, faeces and organs at postmortem examination) and blood culture and PCR results for selected pathogens associated with sepsis in neonatal foals

Organisms detected/cultured from blood, faeces or organs (number of foals)	Culture (blood)	PCR (blood)		PCR (broth)	
		Pathogen specific*	Universal 16S rRNA†	Pathogen specific*	Universal 16S rRNA†
Escherichia coli (5)	80	0	40	40	60
Actinobacillus equuli (5)	80	80	80	80	80
Klebsiella pneumoniae (2)	100	50	100	100	100
Enterococcus faecium (2)	50	0	0	50	50
Clostridium perfringens (2)	0	0	0	0	0
Pasteurella species (2)	100	NA	100	NA	100
Pseudomonas aeruginosa (1)	100	NA	100	NA	100
Streptococcus equi subspecies *zooepidemicus* (1)	0	100	100	100	100
Corynebacterium species (1)	100	NA	100	NA	100
Equine herpesvirus type 1 (1)	NA	100	NA	100	NA

* Pathogen-specific assay
† Universal bacterial 16S rRNA gene assay
NA not applicable

tested PCR-positive for the bacterial 16S rRNA gene. Only one foal with sepsis tested PCR-positive in blood and culture broth for EHV-1; and EHV-1 was confirmed at postmortem infection by the detection of the virus by immunohistochemistry in lung, liver, spleen and thymus. The remaining 11 septic foals with negative cultures in blood, faeces and tissues also tested PCR-negative in blood and enrichment broth.

Despite the small number of samples that were positive for a specific pathogen, agreements between the overall culture results (from blood, faeces or organs) and blood cultures varied with the pathogen, and ranged from 0 to 100 per cent (Table 3). The detection accuracy between the overall culture results and real-time PCR varied with the pathogen and also with the sample type (blood v broth). Overall, the real-time PCR results from broth for each specific pathogen were in stronger agreement with the culture results than the real-time PCR results from blood. The best agreement between culture and real-time PCR results was found for all pathogens using broth samples and targeting the universal bacterial 16S rRNA gene.

To the authors' knowledge, attempts to detect microorganisms in the blood of foals with suspected sepsis by real-time PCR has not been reported. Based on the hypothesis that real-time PCR may be faster and more sensitive than conventional blood culture to diagnose bacteraemia or viraemia in septic foals, pathogen-specific real-time PCR assays using blood from healthy and sick foals were established and validated, and the results were compared with conventional culture. The comparison of 106 neonatal blood samples from healthy, sick but non-septic, and septic foals revealed high levels of agreement between conventional blood culture and real-time PCR, with sensitivity of 82 per cent, specificity of 99 per cent, positive predictive value of 90 per cent and negative predictive value of 97 per cent for real-time PCR (results for the universal bacterial 16S rRNA gene in broth).

The results of conventional culture are often not available quickly enough to influence the initial antimicrobial treatment of sick neonatal foals. Generally, blood cultures with no growth are finalised after five days of culture, whereas, on average, culture-positive blood samples require 48 to 72 hours to detect and identify bacterial organisms. Recently, rapid real-time PCR has been used successfully to detect a variety of bacterial agents in the blood and human neonates (Van Haeften and others 2003, Golden and others 2004). However, most real-time PCR systems target specific bacterial species, with requires advance knowledge of which bacterial organism is suspected. Alternatively, PCR assays targeting a highly conserved region within the bacterial 16S rRNA gene have been successfully used to detect pathogens relevant to neonatal sepsis (Greisen and others 1994, Jordan and Durso 2000, 2005). In the present study, the authors choose to combine testing for selected pathogens with an assay targeting the universal bacterial 16S rRNA gene. The assays were targeted against the most common microorganisms (*E coli*, *A equuli*, *S aureus*, *S equi* subspecies *zooepidemicus*, *K pneumoniae* and *Enterococcus* species) associated with sepsis in neonatal foals, as previously reported (Wilson and Madigan 1989, Marsh and Palmer 2001). All six specific real-time PCR assays were determined to have good analytical specificity and sensitivity, based on sequencing of the PCR products, and lack of cross-reactivity with non-target organisms, high amplification efficiency, and a detection limit of as few as 15 organisms.

One of the limitations of this study was the number of foals with confirmed sepsis that were studied. Although sepsis was suspected in 33 sick foals, positive culture results from blood, faeces or tissue were obtained for only 22 of them. False-negative blood culture results may have been caused by a low circulating bacterial population, a low volume of blood collected for culture, the type of blood culture system used, or the previous use of antimicrobials (Wilson and Madigan 1989, Lorenzo-Figueras and others 2006). The study also included two septic foals with *C perfringens* type *C enterocolitis*, for which positive blood results were not to be expected, as the pathology of the disease is mainly caused by exotoxins produced by the microorganisms. However, the agreement between the results of blood culture and real-time PCR was high. Negative blood culture and real-time PCR results were found in 88 foals (20 healthy, 53 sick but non-septic, and 15 septic foals). Positive blood results with negative real-time PCR results were found in two foals (one for *E coli* and one for *A equuli*), and negative blood culture results with positive real-time PCR results were found in two foals (one for *A equuli* and one for *S equi* subspecies *zooepidemicus*). Thirteen foals with positive blood culture results also had positive real-time PCR results for the specific target organism, or the universal bacterial 16S rRNA gene, or both. The only foal that tested PCR-positive for EHV-1 did not have viral culture performed on its blood, but was confirmed to be EHV-1-positive on postmortem examination by immunohistochemistry.

It is not clear why three foals with positive blood culture results (two for *E coli* and one for *A equuli*) tested PCR-negative for the respective specific target genes. The three isolates from culture were successfully amplified by real-time PCR using the specific target gene, ruling out lack of primer recognition (data not shown). The samples may have contained too few bacteria to allow molecular detection from whole blood or following 24 hours of pre-enrichment. Interestingly, the blood sample in which *A equuli* could not be detected by PCR had the highest white blood cell

(WBC) count (15·88 x 10^9 cells/l) of all the five foals with *A equuli* septicaemia (in the other foals the WBC count ranged from 1·09 x 10^9 to 1·89 x 10^9 cells/l). Previous studies have shown that the high level of genomic DNA that exists in samples with a high WBC count can interfere with the ability of the relatively lower level of bacterial DNA within the sample to bind to the extraction column (Jordan and Durso 2005). It remains to be determined whether blood samples with high WBC counts affect the assay sensitivity. The dilution of samples with high WBC counts before purification of the sample could possibly be useful in these cases.

Another aim of this study was to compare the results of real-time PCR on whole blood samples with the results from pre-enrichment samples, in order to potentially decrease the detection time. Positive real-time PCR results in whole blood were found in eight (24 per cent) septic foals for the specific bacterial target gene, and in 14 (42 per cent) for the universal bacterial 16S rRNA gene. PCR-positive blood samples were commonly found for foals infected with *A equuli* (four of five foals). Following 24 hours' incubation time, the pre-enrichment broth tested PCR-positive in 11 (33 per cent) septic foals for the specific target gene, and in 15 (45 per cent) for the universal bacterial 16S rRNA gene. In comparison, blood culture following pre-enrichment was positive in 15 septic foals. These data suggest that real-time PCR for specific microorganisms or the bacterial 16S rRNA gene would be useful for rapidly detecting bacterial DNA directly from whole blood or pre-enriched samples. Compared with conventional blood culture, the time saved by real-time PCR analysis ranged from 20 to 44 hours. The minimum of 20 hours of time saved applied to samples that tested PCR-positive (DNA extraction and PCR amplification takes an average of four hours) in enrichment broth, and culture-positive 24 hours after plating of the enriched blood. The minimum time saved was 44 hours for samples that tested PCR-positive in blood and culture-positive in enrichment broth. Molecular techniques incorporating very sensitive assays must take into account the risk of contamination from the environment, the sites of venepuncture and the reagents used for DNA purification and amplification. The use of a sterile blood collection technique and good laboratory practices are recommended to reduce the likelihood of generating false-positive PCR results.

In conclusion, this study represents a limited assessment of real-time PCR assays for the detection of selected microorganisms in foals with suspected sepsis. Nucleic acid amplification testing, such as real-time PCR, can be performed rapidly and has been used with success to diagnose a wide range of infectious diseases, including septicaemia in human infants. For the foreseeable future, PCR-based testing will not replace conventional culture due to the requirement for purified culture isolates in antimicrobial susceptibility testing. However, if an amplification assay could reliably rule out neonatal bacteraemia or viraemia more rapidly than culture, it would allow the identification and treatment of neonatal foals with true infections, thus reducing the use of broad-spectrum antibiotics and the potential for sick neonates that are not septic of acquiring drug-resistant bacteria. This approach would also shorten the time during which neonatal foals would have to stay in intensive care, and significantly reduce the overall medical costs.

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