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USE OF POLYMERASE CHAIN REACTION TO DETECT BACTEREMIA IN CRITICALLY ILL NEONATAL CALVES

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Summary

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The aim of the present study was to establish polymerase chain reaction (PCR) to detect bacterial 16S-rRNA genes in the blood of critically ill calves and to compare the results with those of the present "gold standard" of bacteraemia diagnostics, i.e. blood culture. For this purpose, a total of 126 blood samples were taken under sterile conditions from 53 severely ill and potentially bacteraemic calves and from 20 healthy control animals that were 1 to 23 days old and examined for bacterial growth and the presence of bacterial DNA in duplicate by means of blood culture and PCR. Blood sampling and laboratory diagnostic examinations were performed after establishment of the clinical diagnosis and repeated 8 to 12 h thereafter. No evidence of bacteria was found in 60 out of the total of 126 samples examined either in the blood culture or in the PCR. The positive result of blood culture and PCR agreed in 28 blood samples (22.2%). The positive blood culture result of 5 samples was not substantiated by the PCR investigation, which represents a percentage of 4.0%. However, bacterial DNA was detected in another 33 (26.2%) samples by means of PCR, which was not capable of inducing bacterial growth in the blood culture. The rate of contamination of the blood culture was 3% on the basis of the data obtained in this study and 4.9% in the case of the PCR detection.

Key words: 16S-rRNA, blood culture, bovine, septicaemia, universal primer

INTRODUCTION

In livestock housing, losses on account of neonatal diseases are substantial despite the constant further development of rearing management and veterinary care.

Besides intestinal and pulmonary diseases, sepsis, a general disease that is fatal in most cases, is one of the most frequent causes of losses in calf rearing (Virtala *et al.*, 1996; Donovan *et al.*, 1998). The methods of laboratory diagnostics available for verifying the presumptive clinical diagnosis of sepsis have up to now been limited. Blood culture, which is still regarded as the "gold standard", is only rarely used in practice to verify the presumptive clinical diagnosis. This is due to the fact that sepsis is acute in most cases and thus pathogens are not determined for reasons of time, and antibiotic treatment that has been initiated is changed if there

is no improvement. Sometimes it can be observed that patients that are clinically classified as septic reveal negative blood culture results. There are either no bacteria in the circulating blood at the time of sampling or their growth is suppressed by bacteriostatically acting components of the blood so that no bacterial growth can be induced in the culture. It is also assumed nowadays that the severe disturbance of the state of health of animals suffering from sepsis cannot be regarded as the sole consequence of bacteraemia. These effects are rather attributed to pathogens and their products that penetrate the circulatory pathway from a focus of infection and activate the inflammatory cascade through the formation of endogenous mediators (cytokines) and lead to a systemic inflammatory reaction that is no longer controlled (Glauser *et al.*, 1991; Anonymous, 1992; Bone *et al.*, 1997; Franz *et al.*, 1999; Faust *et al.*, 2001).

Polymerase chain reaction (PCR), which has been increasingly used in modern diagnostics since the middle of the eighties, has made it possible to solve some of the existing problems. Even very small amounts of bacteria are sufficient to provide positive evidence. The period until a reliable result is available is considerably shorter as compared to the blood culture one. Moreover, after antibiotic treatment has been initiated, a pathogen possibly present in the blood can be detected since the PCR is not dependent on the presence of pathogens capable of multiplication or metabolically active but only on the presence of parts of the genetic information (Kane *et al.*, 1998).

Bacterial rRNA is coded by several genes in the bacterial cell. DNA probes that are complementary to the 16S-rRNA genes, which are also referred to as 16S rDNA, are especially suitable for the direct detection of pathogens above all in the case of a low density of pathogens since they have a lot of identical sections (Amann *et al.*, 1995; Ley, 1998; Cursons *et al.*, 1999). Detection of these 16S-rRNA genes provides proof of the presence of bacterial DNA since eukaryotic organisms do not have a ribosomal subunit of this size (Woese *et al.*, 1990; Amann *et al.*, 1995).

Neonatology in human medicine provided the basis for this method of investigation. Laforgia *et al.* (1997) for the first time used the detection of 16S-rRNA genes to diagnose neonatal sepsis. Later, Cursons and Sleigh with coauthors (Cursons *et al.*, 1999; Sleigh & Cursons, 2000; Sleigh *et al.*, 2001) described this method of investigation in adult patients.

In the present study, a universal primer (Cursons *et al.*, 1999) was used to detect a highly conserved region of the 16S-rRNA gene of almost all pathogenic bacteria to provide evidence for the presence of bacterial DNA in the blood plasma and leukocytes of critically ill calves.

MATERIALS AND METHODS

Test groups

A total of 53 ill calves (groups 1 and 2) and 20 healthy control animals (group 3) of different breeds and breed crosses that were 1–23 days old were included in the investigation from inpatient and outpatient cohorts of the clinic.

At least three of the following criteria had to be met for an inclusion in the study:

- Severely disturbed general condition;
- Presence of a local infection;
- Body temperature > 39.0°C or < 37.5°C;
- Absence of sucking reflex;

- Altered colour of the mucosa.

The allocation to groups was based on the findings of the clinical examination (Table 1).

The blood samples were taken by sterile puncturing of the jugular vein using a Safety Monovette (Sarstedt, Nümbrecht, Germany) after the clinical diagnosis had been established in the animal and 8 to 12 h later as secondary samples. The amount of blood taken was always divided and equal amounts were subjected to further microbiological and molecular biological processing.

Blood culture investigations

The SIGNAL blood culture system BC 100® of Oxoid (Wesel, Germany) was used as blood culture medium. The samples provided with the signal top were incubated in the incubator at 37°C. The liquid that ascended into the signal top was transferred to a blood agar plate at an interval of two days to detect possible pathogen growth. Further differentiation was carried out according to standardized microbiological methods. Findings were finally recorded on the 6th or 7th day after preparation of the samples.

Isolation of plasma and leukocytes

To be able to investigate blood plasma and leukocytes separately from each other, plasma was first extracted by centrifugation of 9 mL EDTA blood with 1500 rpm (400 RCF) for six minutes (Rotanta 96 R, Hettich, Tuttlingen, Germany). The plasma obtained in this way was stored in a 2-mL tube at −20°C until it was processed further.

Twenty mL red blood cell lysis solution (RBCLS) consisting of 8.29 g ammonium chloride; 1.0 g potassium hydrogen carbonate; 0.5 mL EDTA solution (0.2 M), *ad* 1000 mL bidistilled water), was added to the remaining blood column including the buffy coat. After mixing the blood column with the solution by shaking it briefly and 5-minute incubation at room temperature, the solution was centrifuged off at 1500 rpm (400 RCF) and the supernatant was decanted. RBCLS was added to the remaining pellet anew and the mixture was shaken. This procedure was repeated until the leukocyte pellet turned white.

Extraction of bacterial DNA from plasma and leukocytes

Plasma was centrifuged at 7500 rpm (5780 RCF) for 10 min (EBA 12 R, Hettich, Tuttlingen, Germany). After the supernatant had been discarded, the residue was resuspended with 180 µL lysozyme solution consisting of 1 mL Tris-HCl 0.5 M (USB, Cleveland, USA), 0.1 mL EDTA 0.5 M (Serva, Heidelberg, Germany), 0.3 mL triton 100% (Roth, Karlsruhe), 0.5 g lysozyme (Roth, Karlsruhe, Germany), *ad* 25.0 mL bidistilled water (Roth, Karlsruhe, Germany).

Table 1. Allocation of groups on the basis of clinical findings

Group	Clinical finding	Number (n)
1	Clinical sepsis	19
2	Disease of an individual organ	34
3	Healthy control animals	20

The leukocyte pellet was also mixed with 180 µL of this lysozyme solution.

The specific mixture of lysozyme solution and sample was incubated at 37°C for 12 h. This led to the hydrolytic cleavage of the leukocyte proteins, on the one hand, and to the breakup of the thick murein layer of the Gram-positive bacteria, on the other hand.

The QIAmp DNA extraction kit (Qiagen, Hombrechtikon, Switzerland) was used for DNA extraction. The DNA isolated in this way was stored at –20°C until it was processed further.

PCR

The total volume for amplification was 25 µL and consisted of 2.5 µL buffer (10x PCR Buffer II, Roche, New Jersey, USA), 1.1 µL $MgCl_2$ solution (25 mM, Roche, New Jersey, USA), 0.5 µL dNTP (10 mM, Roth, Karlsruhe, Germany), 13.6 µL bidistilled water (Roth, Karlsruhe, Germany), 0.3 µL AmpliTaq polymerase (5 U/µL, Roche, New Jersey, USA), 1.0 µL upstream primer (Roth, Karlsruhe, Germany), 1.0 µL downstream primer (Roth, Karlsruhe, Germany) and 5.0 µL DNA isolated from the sample (our own production).

The oligonucleotide primers for the detection of 16S-rRNA genes (rDNA) amplify products comprising 365 base pairs between nucleotides 1175 and 1540 (*Escherichia coli* for example) and have the sequences 5`GAG GAA GGI GIG GAI GAC GT 3` (upstream) and 5`AGG AGG TGA TCC AAC CGC 3` (downstream).

After the samples had been transferred into a thermal cycler (GeneAmp PCR system 2400, Perkin Elmer, Überdingen, Germany), they were amplified in 30 cycles (denaturation: 95°C, 15 s; annealing: 55°C, 45 s; elongation: 72°C, 45 s).

Optical presentation of the PCR products

For optical presentation, 2% agarose gel was used with the addition of ethydium bromide. The pockets of the finished gel were loaded with 10 µL of the specific PCR product in the electrophoresis chamber (Owl Separation Systems, Portsmouth, USA) and then gel electrophoresis was started at 90 V for about 45 min.

Optical presentation by means of UV light (FotoPrep I, Fotodyne, Biometra, Göttingen, Germany) and printout via a thermoprinter (Digital Graphic Printer UP-D860E, Sony, Cologne, Germany) were used for permanent documentation of the result.

PCR control samples

At least one positive and one negative control were concurrently used to check the validity of the PCR product. One µL pure bacterial DNA was used as positive control. The negative control consisted of 1 µL bidistilled water instead of the sample DNA in the PCR preparation.

The usual laboratory precautions were taken to avoid false positive detection due to contamination during sampling preparation.

Statistics

The data were evaluated statistically by the biomathematics and data processing working group of the Veterinary Medical Faculty of Justus-Liebig University of Giessen (Dr. K. Failing) using the statistics program package BMDP/Dynamic, Release 7.0 (DIXON, 1993) and the biometric analysis system (BiAS) for Windows. Chi^2 and McNemar tests were used for calculation. The level of significance of $P \leq 0.05$ was used as a basis for the assessment of statistical significances.

RESULTS

The result of the determination of sensitivity carried out beforehand showed that there were different detection limits between the two laboratory diagnostic methods of investigation with regard to the number of colony-forming units (CFU) for different bacterial species and also with regard to the number of incubation days in the blood culture (Table 2). Therefore, in order to be able to compare the validity of the PCR results directly with those of the blood cultures the quantitative statement of the "gold standard" blood culture was regarded as decisive. Every detection of bacteria without consideration of the type and number of bacteria detected in one of the two samples examined microbiologically was assessed as a positive result. With regard to the PCR evidence, a positive result in the blood plasma or leukocytes in one of the two samples examined molecular biologically was a successful detection.

No evidence of bacteria was found in 60 of the total of 126 samples examined either in the blood culture or in the PCR. The positive result of blood culture and PCR agreed in 28 blood samples (22.2%). The positive blood culture result of 5 samples was not substantiated by the PCR investigation, which represents a percentage of 4.0%. However, bacterial DNA by means of PCR was detected in 33 (26.2%) of the samples, this not being observed in the blood culture (Table 3).

Table 2. Sensitivity limit of the PCR and blood culture in CFU

Type of bacterium	Detection limit (CFU)	
	PCR	Blood culture
Staphylococcus aureus	100	10 (1)
Arcanobacterium pyogenes	1	10 (1)
Salmonella Typhimurium	1	1
Klebsiella pneumoniae	10	1
Escherichia coli	1	1

( ) = values after 2 days of incubation.

Table 3. Results of the PCR detection and examination of the total blood samples by blood culture

PCR	Blood culture		Total
	Negative	Positive	
Negative	60	5	65
Positive	33	28	61
Total	93	33	126*

* The data of 20 series are incomplete here since the animals were no longer available for further sampling.

Table 4. Assessment of the PCR negative and blood culture positive results (individual detection) taking into account diagnosis and fate

Result of the blood culture	Clinical diagnosis	PCR	Outcome	Assessment
Staphylococcus epidermidis	*Control animal*	–	Healthy control animal	True negative
Klebsiella pneumoniae	Diarrhoea	–	Cured after 6 days	Unclear
Aerobic bacilli	Diarrhoea	–	Cured after 8 days	Unclear
Corynebacterium spp.	Diarrhoea	–	Discharged chronically ill after 14 days	Unclear
Neisseria species	Omphalitis	–	Cured after 7 days	Unclear

The high proportion of pure cultures (n=27) was striking when the microbiologically detected bacteria were differentiated. Only six blood cultures showed mixtures of two or three different bacteria.

E.coli was the bacterium detected most frequently (24.2% in pure culture), followed by *Pseudomonas* spp. with five positive individual detections and a proportion of 15.2%. *Staphylococcus epidermidis*, *Klebsiella pneumoniae* and *Enterococcus*, *Corynebacterium* and *Neisseria* spp. were each detected in a fraction of 6% of the individual detections of the total of 33 positive cultures.

Acinetobacter spp., anaerobic Gram-negative rods and aerobic bacilli and *Micrococcus* spp. were found once in each pure culture.

Representatives of the *Staphylococcus* genus, α-haemolytic streptococci, *Enterococcus* and *Acinetobacter* spp.were detected as mixed cultures in the blood culture.

The statistical evaluation confirmed that both the relation and the difference between the result of the bacterial detection by means of PCR and the blood culture were significant ($P<0.001$).

On the basis of the comparison of the outcome and the diagnosis of the calves with a positive blood culture result, it can be more accurately assessed whether the result was true or false positive (Table 4).

It was obvious that only one blood culture can be regarded as false positive and that the PCR sample can thus be considered true negative since the calf affected was a healthy control animal. Since the *Staphylococcus epidermidis* was not recorded by the PCR, it is likely that there was a contamination during the transferral of the blood sample into the blood culture medium. Other cases, however, were classified as unclear since nonspecific bacteria were detected or the rapid recovery of the animals did not indicate a septic course of the disease. Since, however, transient bacteraemia could not be ruled out, the unclear findings were also regarded as "true positive" with regard to the blood culture result (Table 4).

All calves affected with mixed-culture detection suffered from severe diseases so that these mixed cultures were assessed as a consequence of the severity of the dis-

ease and thus as "true positive" rather than as contamination. Thus, on the basis of the data obtained in this study, the contamination rate of the blood culture was 3% (Table 5).

With regard to the molecular biological detection of bacteria, unclear results were assessed as "true positive" in parallel to the evaluation of questionable blood culture findings. The rate of false positive PCR detections thus was 4.9% and was in the range determined for the microbiological findings (Table 6).

DISCUSSION

On the basis of the present results, the observation made in trials in humans that 16S-rRNA universal primers are able to detect almost all pathogens and nonpathogens (Kane *et al.*, 1998; Cursons *et al.*, 1999; Reno *et al.*, 2001; Sleigh & Cursons, 2000; Sleigh *et al.*, 2001) was confirmed for veterinary medicine.

However, even in the pretests it was obvious that the detection was weak as regards Gram-positive bacteria in general and in the case of individual Gram-negative bacteria (*Klebsiella pneumoniae*). To differentiate the cause more precisely, it was therefore necessary to find out whether this was due to an insufficient amount of DNA in the starting material or to the amplification procedure. It was noted that, under standardized conditions, the DNA yield in Gram-positive bacteria was considerably slighter even during DNA isolation than in Gram-nega-

Table 5. Assessment of the PCR results of mixed culture positive calves taking into account diagnosis and fate

Result of the blood culture	Clinical diagnosis	PCR	Outcome	Assessment
Staphylococcus epidermidis *Acinetobacter* spp. α-haemolytic streptococci	*Sepsis*	+	Euthanized after 7 days	True positive
Enterococcus spp. *Acinetobacter* spp.	Sepsis	+	Died on the same day	True positive
Enterococcus spp. *Acinetobacter* spp. *Flavobacterium* spp.	Diarrhoea	+	Cured after 30 days	True positive
Pasteurella spp. *Micrococcus* spp. *Arcanobacterium pyogenes*	Sepsis	+	Died on the same day	True positive
Staphylococcus epidermidis α-haemolytic streptococci	Sepsis	+	Euthanized after 14 days	True positive
Staphylococcus epidermidis *Neisseria* spp.	Diarrhoea	+	Died on the same day	True positive

Table 6. Assessment of the PCR positive and blood culture negative results taking into account diagnosis and outcome

Diagnosis	Outcome	Assessment
Sepsis	Died on the same day	True positive
Sepsis	Euthanized on the same day	True positive
Omphalitis	Cured after 25 days	True positive
Omphalitis	Cured after 24 days	True positive
Omphalitis	Cured after 6 days	Unclear
Diarrhoea	Died on the same day	True positive
Omphalitis	Cured after 12 days	Unclear
Diarrhoea	Cured after 9 days	Unclear
Sepsis	Died on the same day	True positive
Omphalitis	Cured after 10 days	Unclear
Meningitis	Discharged chronically ill after 14 days	True positive
Sepsis	Died on the same day	True positive
Diarrhoea	Cured after 5 days	Unclear
Diarrhoea	Cured after 5 days	Unclear
Omphalitis	Discharged chronically ill after 18 days	True positive
Sepsis	Discharged chronically ill after 13 days	True positive
Sepsis	Discharged chronically ill after 12 days	True positive
Sepsis	Died after 15 days	True positive
Sepsis	Died on the same day	True positive
Diarrhoea	Cured after 30 days	True positive
Diarrhoea	Cured after 30 days	True positive
Bronchopneumonia	Discharged chronically ill after 14 days	Unclear
Bronchopneumonia	Discharged chronically ill after 11 days	Unclear
Diarrhoea	Died on the same day	True positive
Diarrhoea	Cured after 12 days	Unclear
Polyarthritis	Euthanized after 6 days	True positive

Table 6. Assessment of the PCR positive and blood culture negative results taking into account diagnosis and outcome (continued)

Diagnosis	Outcome	Assessment
Polyarthritis	Euthanized after 6 days	True positive
Sepsis	Cured after 16 days	True positive
Sepsis	Died on the same day	True positive
Diarrhoea	Cured after 9 days	Unclear
Control animal	Control animal	False positive
Control animal	Control animal	False positive
Control animal	Control animal	False positive

tive bacteria. This observation may be explained by the different structure of the bacterial walls: The cell wall thickness of Gram-positive bacteria is 15–80 nm, whereas in Gram-negative pathogens – only 10–15 nm. This difference is based on the thick murein layer (peptidoglycan) of the Gram-positive bacteria. The problem could be solved in the present study by extending the incubation period of the lysozyme solutions, thus developing the method further and securing an adequate DNA yield in the case of Gram-positive bacteria as well. An extension of the period of action from 30 minutes to 20 hours led to a 3.78-fold increase in the concentration of the DNA of *Staphylococcus aureus* and a 7.75-fold increase of the DNA concentration of *Arcanobacterium pyogenes* in the specific sample. The Gram-negative bacteria *(Salmonella Typhimurium; Escherichia coli)* did not show any substantial changes in concentration as a result of the extended lysis periods.

Klebsiella pneumoniae turned out to be a further problematic bacterium. Both in the pretests and in the clinical study, blood culture was superior to PCR. Thus, the sensitivity limit was 1 CFU in the blood culture whereas it was 10 CFU in the PCR. One of the total of two detections of *Klebsiella pneumoniae* in the blood culture could not be substantiated by the PCR. The thick mucous capsule of this bacterium may be the cause of this failure in detection. *Neisseria* and *Corynebacterium* spp. and aerobic bacilli are other bacteria not recorded by the PCR or not recorded to the same extent.

Besides improving the reliability of detection, the adaptation of the method to problems of veterinary medicine on the basis of the example of critically ill, bovine neonates was a primary aim of the present study. The blood culture that has up to now been regarded as the "gold standard" was used as the reference method (Aronson & Bor, 1987). Although blood culture provides many important results that at present cannot be completely replaced by the PCR, such as an exact qualitative and quantitative detection of bacteria together with the specific test for resistance, the interval between sampling and the availability of a

useful result is so long that a reliable and specific treatment of an ill calf can be carried out only after a considerable delay.

It was demonstrated that the method of investigation used in the present study based on Cursons *et al.* (1999) and modified for the investigation of blood samples from calves to determine bacterial DNA in the blood of septic calves or calves with severe diseases of an individual organ by means of PCR may provide reliable and reproducible results with regard to the content of bacteria of the blood sample examined. The interval required to detect bacteremia is thus reduced by up to three days as compared with blood culture. The comparison of the results of the two methods illustrates the statistically verified superiority of the PCR. Almost half of the blood samples containing bacteria (28 of 61) would have remained undetected if blood culture had been the sole method of investigation.

Taking into account two blood examinations, 100% of the calves classified as septic showed positive PCR results, but only 61.5% were positive by means of blood culture. In the group of calves with severe diseases of an individual organ, it was also observed that the proportion of animals (58.8%) which demonstrated positive results even after one PCR investigation was higher than after repeated tests by means of blood culture (54.5%). This corresponds to a statistically significant increase of the predictive value even after one blood test ($P<0.001$).

When considering the total results of the comparison of the methods, the question arises whether the numerous positive PCR samples are true or false positive results. Bacteraemic episodes may also occur in healthy individuals, on the one hand, and it must be considered, on the other hand, that there may be a higher contamination rate overall although all measures to prevent contamination were observed. This question cannot be clarified ultimately on the basis of the present state of knowledge.

In conclusion, it can be stated that the application of molecular biological methods using universal primers is a suitable approach for detecting bacteria in the blood of critically ill calves and also a progress with regard to the sensitivity of detection and the saving of time until a result is available as compared with the detection of bacteria by blood culture. It might additionally be possible to assess the PCR result qualitatively with regard to a differentiation and specification of the bacterium by means of the subsequent investigation of positive blood samples using specific primer mixtures or also sequencing.

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