Brazilian Journal of Microbiology (2005) 36:29-35
ISSN 1517-8382

APPLICATION OF BROAD-RANGE BACTERIAL PCR AMPLIFICATION AND DIRECT SEQUENCING ON THE DIAGNOSIS OF NEONATAL SEPSIS

Rubia Denise Ruppenthal[1]; Fabiana de Souza Pereira[3]; Vlademir Vicente Cantarelli[3]; Irene Silveira Schrank*[1,2]

[1]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil; [2]Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil; [3]Laboratório Weinnman Ltda., Porto Alegre, RS, Brasil

Submitted: March 31, 2004; Returned to authors for corrections: August 25, 2004; Approved: March 10, 2005

ABSTRACT

A broad-range bacterial PCR target to conserved regions of the 23S rDNA was applied to 306 blood culture samples from 295 infants (up to one year of age) admitted to a neonatal intensive care unit. Classic blood culture results were compared to DNA sequencing analysis of the PCR amplification products. Culture results were in agreement to DNA sequencing in 90.5% (277) of 306 samples tested, including 263 PCR and culture negative samples and 29 culture and PCR positive samples. The sensitivity of the PCR method combined with sequencing was 88%, and the specificity was 96.3%, with positive and negative predictive values of 74.3 e 98.5%, respectively. The PCR-based approach directly applied to blood culture samples, correlated well with blood culture results from neonates with presumptive diagnosis of bacterial sepsis. The PCR/sequencing approach is suggested to be a valuable complementary data for diagnosis of neonatal sepsis. This methodology is relatively easy and reliable giving accurate results that can be applied to samples colleted during antimicrobial treatment or by a hospital clinical procedure, especially when routine cultures are negative. It can also be useful for the identification of rare bacterial species and for those isolates not readily identified by microbiological tests.

Key words: sepsis, molecular diagnosis, neonatal, PCR

INTRODUCTION

Blood infections remain an important cause of mortality and morbidity in hospital neonatal units. The frequency of neonatal bacterial infections varies in different countries and in Brazil some factors as preterm with low birth weight increase mortality ranging from 10 up to 50% (6,12,24,29). Clinical diagnosis of sepsis is troublesome. The results of blood culture are not available before 48-72 h and usually the antimicrobial therapy has to start before the laboratory results become available. A common approach is to start microbial therapy to all infants with clinical or laboratory signs of blood infection as well as to infants with high risk of early onset sepsis. A large number of babies with presumptive diagnosis of sepsis do not have confirmation by laboratory tests. Therefore, faster and more specific tests are required to avoid the inappropriate use of antibiotics, which has been implicated in the development of multiresistant bacterial strains in hospitals (28).

Currently, blood culture is considered the gold standard for neonatal bacterial sepsis diagnosis (14). Molecular techniques, as PCR, have increasingly been used to detect and identify pathogenic organisms in clinical samples with higher degrees of sensibility and specificity (4,14,19,26). PCR primers targeted to conserve rDNA gene sequences allowed the DNA amplification of virtually any bacterial species (10,16,20). After DNA sequencing of the amplification product, the bacterium can be located in the phylogenetic tree. However, to date, the application of PCR to diagnose bacterial infections in blood

*Corresponding Author. Mailing address: Centro de Biotecnologia, Departamento de Biologia Molecular e Biotecnologia. Av. Bento Gonçalves, 9500, Prédio 43421, Caixa Postal 15005. 91591-970, Porto Alegre, RS, Brasil. Fax: (+5551) 3316-7309. E-mail: irene@dna.cbiot.ufrgs.br

samples is limited (15,21,27) and even blood culturing techniques can render unacceptable low sensitivity (30). It is well established that smaller volume of blood directly correlates with lower chances to detect the implicated organism (3,8,17). A common limitation of PCR methods is the lack of amplification due to the presence of inhibitory substances in the sample. Initial attempts to apply PCR detection to bacteria from blood cultures failed due to reaction inhibition. However, Fredricks and Relman (9) have identified a PCR inhibitor (sodium polyanetholesulfate) and described a method for removing it allowing the amplification of bacterial DNA from blood cultures without diluting the sample.

The rapid and accurate detection of bacteremia in newborn infants might have a significant impact in shortening hospital stays within the neonatal intensive care unit (NICU) as well as reducing the costs to the health care system. The aim of this study was to establish a new diagnostic method to detect neonatal bacteremia, using universal primers targeted to 23S rRNA and/or 16S followed by DNA sequencing, directly from blood culture bottles.

MATERIALS AND METHODS

Blood culture and patient selection

All infants up to 1-month old admitted to the NICU for sepsis evaluation who had blood samples drawn for concomitant culture were eligible for inclusion in the study. The samples were obtained during a three-month period in NICUs of two hospitals located in Porto Alegre, Rio Grande do Sul, Brasil.. No additional blood samples were collected from the infants for the purposes of PCR analysis.

The automated continuous-monitoring blood culture system BacT/Alert (Organon Teknika Corporation, Durham, North Carolina, USA) was used for blood culture processing. Between 0.5 and 1.0 ml of whole blood was added to each blood culture bottle. The pediatric-sample-sized blood culture bottles were prefilled with blood collected from infants at their bedsides. The bottles were taken immediately to the microbiology laboratory, and incubated for at least 5 days, following the recommendation of the manufacturers. In the case of bacterial growth, aliquots were subcultured on agar plates for identification and susceptibility tests by conventional microbiology methods (13). The time of positivity was registered at this moment.

Inoculated blood culture broth was used to test the sensitivity of the PCR amplification. A blood culture bottle containing 500 µL of human blood, previously tested for the absence of bacteria, was inoculated with 10^1 CFU/mL of *Escherichia coli* grown on Luria-Bertani (LB) broth in the logarithmic phase and incubated at 37ºC overnight. The numbers of CFU in the inoculated blood sample, were determined by plating serial dilutions of *E. coli* on LB agar plates and counting the colonies after overnight incubation.

DNA extraction and PCR amplification

Bacterial DNA was extracted from 306 different blood culture bottles using a previously described wash/ alkali/ heat lysis method (23). Briefly, blood culture fluid (0.5 mL) was added to the alkali wash solution (0.5M NaOH and 0.05M sodium citrate), mixed by inversion and subsequently centrifuged. The cell pellet containing any microbial DNA was washed twice in 0.5M Tris-HCl, ressuspended in Tris-EDTA (10 mM Tris-HCl and 1 mM EDTA) and heated at 100ºC for 1 h. The sample was freeze/ thawed twice and subsequently centrifuged. The supernatant containing the extracted DNA was transferred to a clean tube and stored at -20ºC prior to PCR. A first sample was taken after a 24h incubation period and a second aliquot was removed when bacteria was detected by the equipment system. The DNA assay was performed without knowledge of the patient clinical details or the initial Gram staining result.

PCR amplification was performed with broadly conserved bacterial 23S rRNA genes primers MS37 (5´-AGGATGTTG GCTTAGAAGCAGCCA-3´) and MS38 (5´-CCCGACAAGG AATTTCGCTACCTTA-3´) and the conditions used for PCR amplifications were as described in the original study (20). For experiments in which a bacterial 16S rRNA gene was amplified, the universal primer pairs 285 and 261 were used in the PCR protocol as previously described (18). The Taq DNA Polimerase (CenBiot Enzyme, Centro de Biotecnologia, UFRGS, Porto Alegre, Brazil) enzyme was treated under UV light as previously described (7).

DNA sequencing and sequence analysis

Amplification products were directly sequenced with ABI PRISM 377 DNA sequencer (Applied Biosystem). Preliminary sequence associations of directly sequenced 23S or 16S rRNA genes were determined by using CHROMAS program (Technelysium, Austrália) and the BLAST search algorithm (1) of GenBank database (2) (National Center for Biotechnology Information). The best matches and sequence homologies were reported according to the original results. Some sequence analysis results were reported only at the genus level. The 16S rDNA PCR/sequencing was performed when the 23S sequencing needed confirmation.

RESULTS AND DISCUSSION

General data

We have investigated a novel diagnostic method for the identification of organisms causing neonatal sepsis. The results of the bacterial culture, PCR analysis, and DNA sequencing of the samples from blood culture are presented in Table 1. A total of 306 samples from infants admitted to the NICU suspected of sepsis were analyzed by PCR for the 23S rDNA and sequencing, and the results were compared to the concomitant blood culture tests. Thirty-three samples (10.7%) were positive for blood culture

and/ or 23S rDNA PCR analysis. Twenty-nine out of the 33 samples were positive for both methods. The 29 (87.9%) blood culture samples gave positive results on the automated blood culture system at 36 hours and only four samples were positive between 36 and 72 hours. The remaining 263 samples were negative after an incubation period of 5 days. The median time for positivity of the organisms considered bacterial pathogens (Tables 2 and 3) with a real clinical importance was 20 hours.

Table 1. Comparison of results of 23S rDNA PCR and BacTec/ for detection of bacteria in neonatal blood samples.

PCR test result	Number of samples with culture result:		Total number of samples
	Positive	Negative	
Positive	29	10	39
Negative	4	263	267
Total	33	273	306

PCR and sequencing

The bacterial 23S rRNA gene was amplified from blood cultures samples after DNA extraction for removing inhibitory substances found on blood culture systems (9,23). We have used universal primers to amplify an 850 bp-conserved region of 23S the rDNA followed by sequencing. The broad-range bacterial primers for 23S rDNA cover several bacterial subdivisions, including gram-positive and gram-negative bacteria with low and high G+C contents, the *Cytophaga*, *Flexibacter and* Bacteriodes group, spirochetes and the purple bacteria (25).

The sensitivity of the 23S rDNA PCR assay was determined using blood culture samples inoculated with known amounts of viable bacteria. The inoculated blood culture samples were prepared and amplified exactly under the same conditions as

Table 2. Microorganism identification of samples positive by both PCR/sequencing and culture

Samples	23S rDNA PCR result	Identification			
		Sequencing result[a]	% Homology[b]	Culture result	Culture x sequening[c]
17W	+	*Streptococcus* sp[d]	96.9	*S. mitis*	C (g)
61W	+	*E. faecium*	100	*E. hirae*	C (g)
91W	+	*Staphylococcus* sp	96.6	*S. epidermidis*	C (g)
125W	+	*Staphylococcus* sp	99.1	*S. warneri*	C (g)
19P	+	*Staphylococcus* sp	97.1	CoNS	C (g)
40P	+	*Staphylococcus* sp	100	CoNS	C (g)
58P	+	*Staphylococcus* sp	99.3	CoNS	C (g)
72P	+	*Staphylococcus* sp	96.4	CoNS	C (g)
153P	+	*Staphylococcus* sp	99.1	CoNS	C (g)
108P	+	*Staphylococcus* sp	90.4	CoNS	C (g)
128P	+	*Staphylococcus* sp[d]	99.0	CoNS	C (g)
149P	+	*Staphylococcus* sp	95.7	CoNS	C (g)
124W	+	*E. faecalis*	98.0	*E. faecalis*	C (s)
38W	+	*P. aeruginosa*	100	*P. aeruginosa*	C (s)
141P	+	*S. aureus*	100	DCM	PC
82W	+	*X. campestris*	90.3	Non-fermenter GNB	PC
30P	+	*Burkolderia* sp	98.8	Non-fermenter GNB	PC
31P	+	*Burkolderia* sp	98.5	Non-fermenter GNB	PC
137W	+	*X. campestris*	100	*Candida sp*	D
49P	+	*Streptomyces* sp[d]	96.5	*Streptococcus* sp	D
73P	+	*R. solenacearum*	100	*Enterobacter* sp	D
143P	+	*G. adiacens*[d]	99.0	*Streptococcus* sp	D

[a]Identification was based on the 23S rDNA sequence; [b]The percent homology of the overlapping sequence for the best match are presented; [c]Comparison from blood culture and sequencing results: C, concordant result [in parentheses, the concordance level: (g) genus and (s) specie]; PC, partly concordant; D, discordant result; [d]Identification was based on 16S rDNA sequence. CoNS- coagulase-negative staphylococci; GNB- Gram-negative bacilli; DCM- double colony morphology.

those described for the clinical blood specimens. Using this approach, 10^3 UFC/mL of bacteria could be detected after gel electrophoresis and ethidium bromide staining (data not shown).

After PCR amplification and DNA sequencing the organism could be determined in 39 samples (Tables 2 and 3), 16 (41%) samples were characterized at the genus level and 11 (28.2%) samples to the species level. Among these 39 samples the identification was based on the 23S rDNA sequences for 18 samples, on the 16S rDNA sequences for 9 samples (Table 2 and 3) and 7 samples yielded inconclusive results. Overall Gram-positive bacteria accounted for 64% of the cases whereas Gram-negative accounted for 36%.

In seven samples, the presence of several species was suggested on the basis of the typical electropherogram produced by the sequences and by bacterial culture. The bacterial cultures revealed the presence of *S. epidermidis, S. maltophilia,* coagulase-negative staphylococci (CoNS), yeast or *Candida* sp. (Table 3). Sequencing reaction in the other four samples gave an inconclusive result because the product of the reaction did not produce an interpretable sequence, and those four samples were negative for culture.

In our work, 25% (10/39) of PCR-positive samples were classified in the *Staphylococcus* sp. group, and species identification based on 23S rDNA sequence was not possible (Table 2). Bacterial identification based only on ribosomal sequences has its limitation. Some examples show that information on rDNA sequences are not in agreement with DNA-DNA relatedness or the phenotypic properties of the bacterial species (5).

Several studies have used DNA-based methods to identify a wide range of clinically significant bacterial species from clinical samples (14,22). A broad-range PCR assay for the 16S rRNA gene and sequencing was used to diagnose bacterial endocarditis in cardiac tissue specimens (10). Rantakollo-Jalava *et al.* (26) used the primers utilized in this work to amplify bacterial rRNA genes from biopsy tissue or body fluids.

Comparison of PCR and culture results

The comparison between the PCR results and sequencing and those obtained using BacT/Alert (considered the gold standard) are shown on Table 1. Results reveal a high level of agreement between the two methodologies, with sensitivity,

Table 3. Microorganism identification by only one method (PCR/sequencing or culture).

Samples	23S rDNA PCR result	Identification		
		Sequencing result[a]	% Homology[b]	Culture
106W	+	*Burkolderia* sp	89.8	-
75P	+	*Streptococcus* sp	96.9	-
83W	+	*Xantomonas campestris*	98.0	-
130W	+	*Xantomonas campestris*	100	-
134W	+	*Xantomonas campestris*	99.2	-
157P	+	non-conclusive[c]		-
89W	+	unsuccessful		-
96W	+	unsuccessful		-
142P	+	unsuccessful		-
146P	+	unsuccessful		-
18P	+	non-conclusive		CoNS
111P	+	non-conclusive		*Candida sp*
151P	+	non-conclusive		CoNS
154P	+	non-conclusive		Yeast (not identified)
19W	+	non-conclusive		*S. epidermidis*
44W	+	non-conclusive		*S. maltophilia*
152P	+	not attempted		CoNS
52P	-	not attempted		*Streptococcus pneumoniae*
95P	-	not attempted		*Streptococcus* sp
11P	-	not attempted		CoNS
150P	-	not attempted		CoNS

[a]Identifications based on 23S rDNA and on 16S rDNA sequence; [b]The percent homology of the overlapping sequence for the best match are presented. CoNS- coagulase-negative staphylococci. [c]Presence of several species was suggested on the basis of the typical electropherogram produced by the sequences.

specificity, and positive and negative predictive values of 87.9, 96.3, 74.3 and 98.5 %, respectively.

Of the 29 PCR and culture positive samples, sequencing was not successful for seven samples. The positivity percentage for blood specimens analyzed by culture and PCR/sequencing were 33 (10.7%) and 39 (12.7%) positive specimens from a total of 306 tested, respectively. A detailed description of the 22 samples, which were positive by both methods, is presented in Table 2. Concordant identification results with the two methods were found in 14 samples (12/14 at the genus level and 2/14 at species level). Classification of the results for four samples as partly concordant was based on incomplete bacterial identification by the culture method. In four other samples the results were discordant between the two methods. Among the concordant results 10 samples were identified, by sequencing, as *Staphylococcus* sp., and two of them could be identified by culture as *S. epidermidis* and *S. warneri*. Genus/species identification on samples classified as partly concordant was achieved only by sequencing (Table 2). In these cases, sequencing identification was critical to suggest the most adequate antimicrobial therapy to overcome the bacterial infection in neonates.

Among the 10 PCR-positive and culture-negative samples, sequencing gave an interpretable result for 5 samples, suggested the presence of several species in one sample, and was not successful for 4 samples. A description of these samples is given in Table 3. The five bacterial species sequenced have normal growth requirements. To confirm the 23S rDNA PCR the universal primer to amplify the 16S rDNA region was used, and the sequencing results yielded the same bacterial classification as that identified by the 23S rDNA sequencing. Samples 83W and 134W (Table 3) were blood specimens collected at different days from a neonate with septic episodes. PCR combined with sequencing was the only method that identified *Xanthomonas campestris* as the bacteria present in the sample, as blood culture remained negative.

Of the four PCR-negative and culture-positive samples, the culture result was designated as contamination for one sample. This decision was made on the basis of the three independent blood culture that yielded negative results even after a prolonged culture period (52.4 hours). Among the three PCR-negative samples yielding significant growth (Table 3) the bacterium recovered was a Gram-positive organism. The possibility of PCR inhibition in these samples was excluded by successful amplification of added *S. aureus* DNA on these samples before the PCR amplification (data not shown).

Grunillicatella adiacens DNA identified by PCR from sample 143P (Table 2) is a nutritionally variant bacterium previously considered to belong to the genus *Streptococcus* but recently reclassified in a new genus, *Grunillicatella*, on the basis of phylogenetic and phenotypic 16S rDNA studies (10). This is the first report of this species detection from a blood culture in Brazil.

The only four PCR-negative and culture-positive samples revealed the presence of Gram-positive species (Table 3). On the basis of sequence analysis there was no discrimination of gram-positive species with the rDNA-specific primers used. Also, the 23S rDNA primers have successfully amplified several gram-positive bacteria (20), including *Streptococcus* and *Staphylococcus* (Table 2 and 3). Therefore, the problems faced are possibly due to the difficulties in breaking the cell walls of these organisms during DNA extraction, resulting in a failure in the PCR amplification. Previous work in diagnosis of bacterial infections also reported difficulties in detecting Gram-positive bacteria and mycobacteria by PCR (26).

The vast majority 85.9% (263 of 306 samples) of neonatal blood samples lacked detectable levels of bacteria by both culture and PCR analyses (Table 1).

Despite all efforts taken to avoid contamination, the risk is still higher for broad-range PCR than for more specific assays. Using a sensitive and rapid method combining broad-range PCR amplification of bacterial 16S rDNA fragments and pyrosequencing for detection, identification and typing, Grahn *et al*. (11) have found contaminating bacterial DNA in reagents used for PCR reactions, further identified as water-borne bacteria. Among the PCR-positive and culture-negative group (Table 3) we have identified *Xanthomonas campestris* on three samples and *Burkolderia* sp. in one sample. In comparison to culture results those PCR/ sequencing results are probably false-positive reactions due to the PCR contamination. Thus, conventional microbiology methods would still be required for confirmation of bacterial infections, identification of species and antibiotic susceptibility of any bacteria isolated from blood samples should not be replaced by the currently available molecular methods.

In conclusion, we report the comparison of broad-range PCR and sequencing and routine culture in bacteriological diagnoses of 306 blood culture samples. Although not competitive with conventional methods the PCR/sequencing approach appears to be a valuable complementary technique for diagnosis of neonatal sepsis. However, rDNA PCR and direct sequencing are relatively easy and reliable methods that give accurate results that can be recommended for samples obtained during antimicrobial treatment or by a demanding clinical procedure, especially when routine cultures remain negative.

ACKNOWLEDGEMENTS

Authors are grateful for technical assistance from Hospital Materno-Infantil Presidente Vargas and from Weinmann Laboratorio Ltda. This work was supported by Fundação de Amparo a Pesquisa do Rio Grande do Sul and Weinmann Laboratorio Ltda.

RESUMO

Aplicação de PCR associado ao seqüenciamento no diagnóstico da sepse neonatal

Primers universais, que amplificam regiões conservadas de rDNA 23S, foram utilizados para analisar 306 amostras de cultivo de sangue obtidas de 295 neonatos com um ano ou menos de idade admitidos em unidades hospitalares de tratamento intensivo. O diagnóstico molecular baseado em seqüenciamento dos produtos de PCR foi comparado com os resultados obtidos do cultivo das amostras de sangue. Os resultados foram concordantes para 277 (90.5%) das 306 amostras testadas, incluindo 263 amostras PCR-negativo e cultura-negativa e 29 amostras cultura-positiva e PCR-positivo. Comparado com o método de cultivo, a técnica de PCR combinada com seqüenciamento, apresentou maior especificidade, 88% e 96,3% respectivamente, com valores preditivos positivos e negativos de 74,3 e 98,5% respectivamente. Concluímos que a técnica baseada em PCR utilizando amostras de cultivo de sangue, obtidas de neonatos com suspeita de sepse bacteriana, apresenta boa correlação com os métodos de cultivo convencionais. A metodologia de PCR/seqüenciamento apresenta aplicabilidade como técnica complementar para o diagnóstico da sepse neonatal. Esta metodologia fácil de ser executada fornece resultados confiáveis podendo ser recomendada para utilização no diagnóstico de amostras obtidas durante o tratamento antimicrobiano especialmente quando o resultado do cultivo permanece negativo. Apresenta, também, potencial de utilização na identificação de espécies bacterianas com problemas de classificação pelos métodos convencionais microbiológicos.

Palavras chave: sepse, diagnóstico molecular, neonatal, PCR

REFERENCES

1. Altschul S.F.; Gish W.; Miller W.; Myers E.W.; Lipman D.J. Basic local alignment search tool. *J. Mol. Biol.*, 215, 403-410, 1990.
2. Benson, D.A.; Karsch-Mizrachi, I.; Lipman, D.J.; Ostell, J.; Rapp, B.A.; Wheller, D.L.; Genbank. *Nucleic Acids Res.*, 30, 17-20, 2002.
3. Brown, D.R.; Kutler, D.; Rai, B.; Chan, T.; Cohen, M. Perinatal neonatal clinical presentation:bacterial concentration and blood volume required for a positive blood culture. *J. Perinatol.*, 15, 157-159, 1995.
4. Carroll, N.M.; Jaeger, E.M.; Choudhury, S.; Dunlop, A.; Matheson, M.M.; Adamson, P.; Okhravi, N.; Ligthman, S. Detection of and discrimination between gram-positive and gram-negative bacteria in intraocular samples by using nested PCR. *J. Clin. Microbiol.*, 38, 1753-1757, 2000.
5. Clyton, R.A.; Sutton, G.; Hinkle, P.S. Intraspecies variation in small-subunit rRNA sequences in Genbank: why single sequences may not adequately represent prokaryotic taxa. *Int. J. Syst. Bacteriol.*, 45, 5951-5993, 1995.
6. Cerase, P.A. Neonatal sepsis. *J. Perinat. Neonatal. Nurs.*, 3, 48-57, 1989.
7. Corless, C.E.; Guiver, M.; Borrow, R.; Edwards-Jones, V.; Kaczmarsky, E.B.; Fox, A.J. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. *J. Clin. Microbiol.*, 38, 1747-1752, 2000.
8. Dietzman, D.; Fischer, G.; Schoenknecht, F. Neonatal *Escherichia coli* septicemia: bacterial counts in blood. *J. Pediatr.*, 85, 128-130, 1974.
9. Fredericks, D.; Relman, D.A. Improved amplification of microbial DNA from blood culture by removal of the PCR inhibitor sodium polyanetholsulfonate. *J. Clin. Microbiol.*, 36, 2810-2816, 1998.
10. Goldenberg, D.; Kunzli, A.; Vogt, P.; Zbinden, R.; Altwengg, M. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. *J. Clin. Microbiol.*, 35, 2733-2739, 1997.
11. Grahn, N.; Olofsson, M.; Ellnebo-Svedlund, K.; Monstein, H.J.; Jonasson, J. Identification of mixed bacterial DNA contamination in broad-range PCR amplification of 16S rDNA V1 and V3 variable regions by pyrosequencing of cloned amplicons. *FEMS Microbiol. Lett.*, 219, 87-91, 2003.
12. Hervas, J.A.; Ballesteros, F.; Aalomar, A.; Gil, J.; Bendei, V.; Alberti, S. Increase of *Enterobacter* in neonatal sepsis: a twenty-two-year study. *Pediatr. Infect. Dis. J.*, 20, 134-140, 2001.
13. Isenberg, H.D. *Clinical microbiology procedures handbook*. Washington: American Society for Microbiology, 1992.
14. Jordan, J.A.; Durso, M.B. Comparison of 16S RNA gene PCR and BACTEC 9240 for Detection of Neonatal Bacteremia. *J. Clin. Microbiol.*, 38, 2574-2578, 2000.
15. Kane, T.D.; Alexander, J.W.; Johanninhman, J.A. The detection of microbial DNA in the blood: a sensitive method for diagnosing bacteremia and/or bacterial translocation in surgical patients. *Ann. Surg.*, 227, 1-9, 1998.
16. Kattar, M.M.; Chavez, J.F.; Limaye, A.P.; Rassoulian-Barret, L.; Yarfitz, L.; Carlson, L.C.; Houze, Y.; Swanzy, S.; Wood, B.L.; Cookson, B.T. Aplication of the 16S rRNA gene sequencing to identify *Bordetella hinzii* as the causative agent of fatal septicemia. *J. Clin. Microbiol*., 38, 789-794, 2000.
17. Kellog, J.A.; Ferrentino, F.L.; Liss, K.; Goodstein, M.H.; Shapiro, S.L.; Bankert, D.A. Frequency of the low bacteremia in infants from birth to two months of age. *Pediatr. Infect. Dis. J.*, 16, 381-385, 1997.
18. Kirschner, P.; Meier, A.; Bötgtger, E.C. Genotypic identification and detection of Mycobacteria-facing novel and ulcultured pathogens. *In:* Persing, D.H.; Smith, T.F.; Tenover, F.C.; White, T.J. (eds). *Diagnostic Molecular Microbiology - principles and applications*. D.C: American Society for Microbiology, Washington, 1993, pp. 173-190.
19. Klausegger, A.; Hell, M.; Berger, A.; Zinober, K.; BAaier, S.; Jones, N.; Sperl, W.; Kofler, B. Gram type-specific broad range amplification for rapid detection of 62 pathogenic bacteria. *J. Clin. Microbiol.*, 37, 464-466, 1999.
20. Kotilainen, P.; Jalava, J.; Meurman, O.; Lehtonen, O.; Rintala, E.; Seppalla, O.; Eerola, E.; Nikkary, S. Diagnosis of meningococcal meninitis by broad-range bactereial PCR with cerobrospinal fluid. *J. Clin. Microbiol.*, 36, 2205-2209, 1998.
21. Laforgia, N.; Coppola, B.; Carbone, R.; Grassi, A.; Mautone, A.; Iolascon, A. Rapid detection of neonatal sepsis using polymerase chain reaction. *Acta Paediatr.*, 86, 1097-1099, 1997.
22. McCabe, K.M.; Khan, G.; Zhang, Y.; Mason, E.O.; McCabe, E.R.B. Amplification of bacterial DNA using highly conserved sequences: automated analisys and potencial for molecular triage of sepsis. *Pediatr.*, 95, 165-169, 1995.
23. Millar, B.C.; Jiru, X.U.; Moore, J.E.; Earle, J.A.P. A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material. *J. Microbiol. Methods.*, 42, 139-147, 2000.
24. Miura, E. Sepse Neonatal. *In:* Pitrez, J.L.B.; Pitrez, P.M.C. *Pediatria Consulta Rápida*. (eds). Artmed, Porto Alegre, 1998, p. 428-429.

25. Olsen, G.J.; Woese, C.R.; Overbeek, R. The winds of (evolutionary) change: breathing new life into microbiology. *J. Bacteriol.*, 176, 1-6, 1994.
26. Rantakokko-Jalava, K.; Nikkary, S.; Jalava, J.; Eerola, E.; Skurnik, M.; Meurman, O.; Ruuskanen, O.; Alanen, A.; Kotilainen, E.; Toivanen, P.; Kotilainen, P. Direct amplification of rRNA genes in a diagnosis of bacterial infections. *J. Clin. Microbiol.*, 38, 32-39, 2000.
27. Shang, S.; Chen, Z.; Yu, X. Detection of bacterial DNA by PCR and reverse hybridization in the 16 rRNA gene with particular reference to neonatal septicemia. *Acta Paediatr*., 90, 179-183, 2001.
28. Terrone, D.A.; Rinehart, B.K.; Einstein, M.H.; Britt, L.B.; Martin, J.; Perry, K.G. Neonatal sepsis and death caused by resistant *Escherichia coli*: possible consequences of extended maternal ampicillin administration. *Am. J. Obstet. Gynecol.*, 180, 1345-1348, 1999.
29. Tessin, I.; Trollfors, B.; Thiringer, K. Incidence and etiology of neonatal septicemia and meningitis in Western Sweden 1975-1986. *Acta Paediatr. Scand.*, 79, 1023-1030, 1990.
30. Turrene, C.Y.; Witwicky, E.; Hoban, D.J.; Karlowsky, J.A.; Kabani A.M. Rapid identification of bacteria from positive blood cultures by fluorescence-based PCR-single-strand conformation polimorphism analysis of the 16S rRNA gene. *J. Clin. Microbiol.*, 38, 513-520, 2000.