

Detection of *Streptococcus dysgalactiae* subsp. *equisimilis* in equine nasopharyngeal swabs by PCR

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Streptococcus (*S.*) *dysgalactiae* subsp. *equisimilis* is responsible for severe diseases in humans, including primary bacteraemia, pneumonia, endocarditis, and toxic shock syndrome. Infection in some animal species can also occur, although a few studies have looked into cross-species infectivity. In horses, *S. equisimilis* is generally considered infrequent or opportunistic, but has recently been isolated from cases of strangles-like disease. Rapid and sensitive diagnostic techniques could enable epidemiological studies and effective investigation of outbreaks involving these bacteria. In this study, PCR protocols previously described in cattle and in humans to detect the species *S. dysgalactiae* and the subspecies *equisimilis* were evaluated to detect specific sequences in equine samples. For this purpose, 99 monolateral nasal swabs were collected from horses from stud farms with a history of *S. equisimilis* infection and were tested blindly by bacteriological isolation and by single and duplex PCR. DNA for PCR was extracted both from the colonies grown on agar media and from enrichment broth aliquots after incubation with nasal swab samples. *S. equisimilis* was identified by bacteriological isolation in 23 out of 99 swab samples, and PCR assays on these colonies were fully concordant with bacteriological identification (kappa statistic = 1.00). In addition, PCR of the enrichment broth aliquots confirmed the bacteriological results and detected *S. equisimilis* in 6 samples more than the bacteriological examination (kappa statistic = 0.84). The PCR protocols appeared to be reliable for the rapid identification of *S. equisimilis* in equine nasal swab samples, and could be useful for microbiological diagnosis.

Keywords: diagnosis, horse, PCR, *Streptococcus equisimilis*

Introduction

Streptococcus (*S.*) *dysgalactiae* subsp. *equisimilis* are beta haemolytic streptococci belonging to the Lancefield group C. Their normal habitat in the horse appears to be the skin and mucous membrane, though their isolation from aborted placentas and from abscessed lymph nodes suggest a possible pathogenic role [16]. Recently, the interest in these bacteria has increased because of their isolation from cases of strangles-like disease in absence of *Streptococcus equi* infection [10,16]. Furthermore, *S. equisimilis* infections in humans are increasing and are responsible for different clinical syndromes, including primary bacteraemia, pneumonia, endocarditis, arthritis, and streptococcal toxic shock syndrome [2,5,9,14].

In veterinary medicine, retrospective examination of bibliographic data about *S. equisimilis* infection is complicated by the frequent nomenclature changes due to the animal origin of the strains. At the beginning, the name *S. equisimilis* was proposed for beta-haemolytic C-streptococci frequently isolated from humans and thought to be uncommon in domestic animals. Further studies resulted in including these bacteria in the species *S. dysgalactiae*, while the name *S. equisimilis* lost its official value because it was not included in the new classification [17]. The two most recent taxonomic studies [17,18] were concordant in distinguishing two different subspecies of *S. dysgalactiae*, called *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis*, although they were discordant about the strains to be included in the two subspecies. Consequently, on the basis of these studies, within a few years the name of the animal isolates changed from the original *S. dysgalactiae*, first into *S. dysgalactiae* subsp. *dysgalactiae* subsp. *nov.*, and then into *S. dysgalactiae* subsp. *equisimilis*. Nevertheless, invalid or obsolete names continued to be used in the meantime.

S. equisimilis has been isolated infrequently from placentas from aborted, stillborn, and premature foals [6].

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Recently, it has been isolated from horses with a history of respiratory disease or strangles-like disease [10]. However, infection in horses continues to be considered infrequent and opportunistic [16], though exhaustive epidemiological studies are not available and routine diagnostic tests are usually directed to identify other streptococci rather than *S. equisimilis*. Endocarditis and arthritis due to *S. equisimilis* infection have been recently described in swine [8], while the data published before refer to isolation of *S. dysgalactiae* without subspecies distinction [7]. Similarly, subspecies identification has not been carried out in cases of *S. dysgalactiae* infection associated with high mortality in amberjack (*Seriola dumerili*) and yellowtail (*Seriola quinqueradiata*) [13], and with polyarthritis in dairy goats [3]. In summary, exhaustive epidemiological data about *S. equisimilis* infection in animals are not available and, despite the fact that it is considered by some authors as an opportunistic pathogen, specific and definitive studies have been not carried out. Furthermore, considering the lack of information, it is also difficult to evaluate if the animals play a role in the maintenance and transmission of this potential zoonotic agent.

A PCR protocol to detect a common sequence of the species *S. dysgalactiae* in bovine milk has been published [15], and an *in situ*-PCR protocol for the detection of the subspecies *equisimilis* has been previously described in human formalin-fixed pulmonary samples from medico-legal autopsy cases [12]. In this latter case, the primers were specific for the streptokinase precursor gene and designed off the sequence of the equine isolate *S. equisimilis* 87-542-W. Considering the differences in the sequence of this gene among strains isolated from human, pig and horse [4], these primers are probably better suitable on equine samples because their high homology with the sequence of the equine strain 87-542-W. However, no data on PCR protocols were available to directly detect *S. equisimilis* in equine samples.

The aim of this study was to verify the reliability of a PCR protocol for rapid detection and identification of *S. equisimilis* both in streptococcal colonies and in equine nasopharyngeal swab samples in order to develop a diagnostic protocol for future epidemiological and pathogenetic studies. The evaluation of the test was based on a blind comparison among PCR and bacteriological results.

Materials and Methods

Samples and experimental design

A total of 99 monolateral nasal swab samples were collected from horses with a history of respiratory diseases for bacteriological and PCR investigations. Since *S. equisimilis* infections are considered to be infrequent and since the aim of this study was not to carry out an epidemiological survey, the samples were collected from horses from stud

farms with a history of previous *S. equisimilis* infection in order to detect as many *S. equisimilis* positive swabs as possible. The nasal swab samples were briefly incubated in enrichment broth, as described below. After incubation, a loop of the enrichment broth was spread on blood agar for bacterial culture, and 1 mL of the remaining broth was used for DNA extraction and PCR. By this method it has been possible to use the same swab sample to test by both bacteriology and PCR instead of collecting two separate swabs.

Beta-hemolytic and catalase negative streptococci grown on sheep blood agar were used for the determination of the Lancefield group for biochemical identification and for PCR. Bacteriological and bio-molecular assays were carried out blindly by different operators in different laboratories, and comparison among the results was done only after both assays were completed.

Bacterial culture and biochemical identification

Swab samples were incubated in 4 mL T14 enrichment broth [phosphate buffered saline pH 7.2, 2% fetal calf serum (Celbio, Italy), 0.0005% amphotericin B (Bristol-Myers Squibb, Italy)] at 37°C for 6 h and then spread on agar plates containing 5% sheep blood and *Streptococcus* selective supplement (Oxoid, Italy). Beta-hemolytic and catalase negative colonies were subcultured for purity, processed for Lancefield group classification by latex agglutination test (Slidex Strepto-Kit; BioMerieux, Italy), and identified by their biochemical profile using API 20 Strep (BioMerieux, Italy) following the manufacturer instructions. A suspension of *S. equisimilis* ATCC 10009 was used as positive control for each batch of API 20 Strep tests.

Template preparation for PCR assays

The reference strain of *S. equisimilis* ATCC 10009 and other 18 *S. equisimilis* strains previously isolated from horses in Central Italy and conserved in the laboratory collection (Laboratory of Medical Microbiology and Infectious Diseases - University of Camerino, Italy) were used to optimize DNA extraction and PCR protocols from colonies. Furthermore, a loop of a pure culture of the reference strain was dissolved in 4 mL of T14 enrichment broth, incubated at 37°C for 6 h and used to optimize the DNA extraction protocol from broth samples.

DNA samples were prepared from colonies grown on blood agar and from 1 mL T14 enrichment broth after incubation at 37°C for 6 h with nasal swab samples. Briefly, DNA extraction from colonies was carried out similarly as previously described with few modifications [1]. One loop of colonies from the pure culture was suspended in 50 µL digestion buffer (Tris-HCl 10 mmol/L, EDTA 1 mmol/L, pH 8.0 containing 5 U/µL lysozyme; Sigma, Italy) and incubated at 37°C for 30 min in a water bath. 0.75 µL proteinase K 20

$\mu\text{g}/\mu\text{L}$ (Eurobio, France) were subsequently added, and the solution was incubated at 56°C for 30 min. After boiling for 10 min, the samples were centrifuged at $10,844 \times g$ for 5 min and the supernatant was transferred in a new tube and cooled before use.

Furthermore, 1 mL of T14 enrichment broth, after incubation with nasal swab, was centrifuged at $21,255 \times g$ for 10 min, the supernatant was discarded, and the pellet was suspended in 25 μL digestion buffer at 37°C for 60 min. After adding 0.38 μL proteinase K 20 $\mu\text{g}/\mu\text{L}$ (Eurobio, France), the samples were boiled, centrifuged and transferred in new tubes as described above.

PCR protocol

Three separate PCR tests, based on published primers, were used to detect *S. dysgalactiae* and the subspecies *equisimilis*. For *S. dysgalactiae* detection, the primers *Sdy519-F* 5'-GGC TCA ACC ACT NTA CGC TT-3' and *Sdy920-R* 5'-ATC TCT AGA CCG GTC AGG A -3' were used for the amplification of a 401 bp sequence of the 16SrRNA region [15]. PCR was carried out in a Hybaid PCR Express Thermal Cycler (Hybaid, UK), and the PCR reaction mixture (25 μL) contained 12.5 μL of Taq PCR mastermix (Qiagen GmbH, Germany), 10 pmol of each primer and 2 μL of DNA template prepared from colonies or 5 μL of DNA template prepared from T14 enrichment broth. In order to verify the absence of inhibition or cross-contamination, *S. equisimilis* ATCC 10009 DNA or water were used in each PCR run instead of the template DNA respectively as positive and negative control samples. PCR was conducted with the following program: 94°C for 3 min, 35 cycles at 94°C for 30 sec, at 57°C for 30 sec, and at 72°C for 40 sec, followed by a final extension at 72°C for 7 min.

For the detection of *S. equisimilis*, the primers *eqsim-F* 5'-TCA AAT CGG TTG GCA CAG AC-3' and *eqsim-R* 5'-CGT CCT TAG CAT AGA AGG ATT GG-3' flanking a 279 bp fragment of the streptokinase precursor gene were used [12]. The PCR was conducted as described above, but the annealing temperature was 55°C instead of 57°C .

The presence of PCR products was determined by the electrophoresis of 10 μL reaction products in 1.5% agarose gel containing 0.5 $\mu\text{g}/\text{mL}$ of ethidium bromide with Tris-borate-EDTA buffer (TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA pH 8.3). A sample was considered positive for *S. equisimilis* when both *Sdy* and *eqsim* PCR were positive.

In parallel experiments, the *Sdy* and *eqsim* primers were used in a duplex PCR using the PCR mixture and the protocol described above. On the basis of preliminary experiments using DNA extracted from the reference strain of *S. equisimilis*, the annealing temperature for the multiplex PCR experiments was fixed in 57°C and no additional MgCl_2 was included in the mixture.

PCR detection limit

The detection limit of the PCR protocol for *S. equisimilis* amplification was determined using a series of 10-fold serial dilutions of DNA purified from *S. equisimilis* ATCC 10009 strain. The preparation of the template, PCR assays and visualization of the amplification products on agarose gel were carried out as above described for the tested samples. The starting DNA concentration was determined by spectrophotometer reading (Genova; Jenway, UK), and 10-fold dilutions were carried out in TE buffer pH 7.0 (1 M Tris-Cl, 0.5 M EDTA, pH 8.0).

PCR specificity

Although the primer sets to detect *S. dysgalactiae* spp. and the subspecies *equisimilis* have been previously published [12,15], their specificity was verified again in this study on different streptococci and equine respiratory bacteria obtained from the laboratory collection (Laboratory of Medical Microbiology and Infectious Diseases - University of Camerino, Italy). The cultures included *Streptococcus equi* subsp. *equi* (n = 5) (two of them kindly provided by Prof. J. Alber and Prof. C. Lämmle, Justus-Liebig-Universität of Gießen, Germany), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (n = 3), *Streptococcus equi* subsp. *zooepidemicus* (n = 5), *Streptococcus mitis* (n = 2), *Streptococcus pneumoniae* (n = 1), *Streptococcus pyogenes* (n = 4), *Streptococcus canis* (n = 3), *Staphylococcus aureus* (n = 4), *Staphylococcus intermedius* (n = 4), *Rhodococcus equi* (n = 5), *Escherichia coli* (n = 3), *Klebsiella pneumoniae* (n = 1), and *Pseudomonas aeruginosa* (n = 3). Furthermore, 40 nasal swab samples and 20 guttural pouches wash samples collected from 60 healthy horses that tested negative for *S. equisimilis* by bacteriology were used to verify the absence of non-specific PCR results.

Statistical analysis

The results from bacteriology and PCR assays were analyzed to assess the statistical agreement between the two methods. The kappa statistic was calculated as the observed agreement beyond chance divided by the maximum agreement beyond chance [11]. For convenience, a freeware computer program was used to perform the calculations (Kappa statistics calculator, version 02/07/2001 by Jen-Hsiang Chuang; Columbia University, USA). Values of kappa between 0.6 and 0.8 are indicative of substantial agreement, and values of kappa between 0.8 and 1.0 are indicative of almost perfect agreement [11].

Results

Bacteriological examination and biochemical identification

Beta-hemolytic and catalase negative streptococci of the Lancefield group C demonstrating the biochemical profile of *S. equisimilis* were isolated from 23 (23.23%) out of the

99 tested samples. In one case, the API 20 Strep test had to be repeated because an unacceptable profile was obtained after 24 hours of incubation.

PCR

PCR using *Sdy* primers resulted in a 401 bp segment in the *S. equisimilis* ATCC 10009 strain, in the 18 *S. equisimilis* positive controls, and in 23 (23.23%) isolates and 29 (29.29%) broth samples out of the 99 equine nasal swabs collected. Products 279 bp in size were obtained from the same samples by PCR using *eqsim* primers (Fig. 1). No bands were obtained by testing the negative controls including the bacteria from the laboratory collection and the 40 nasal swabs and the 20 guttural pouches wash samples collected from the uninfected horses. Identical results were obtained by multiplex PCR using *Sdy* and *eqsim* primers (Fig. 2). The bands visualised on agarose gel were well defined and no aspecific products were observed. Running 10 μ L of amplification products from DNA extracted from colonies often resulted in an overcharging of the wells, but it appeared useful for the visualisation of the PCR products from DNA extracted from broth samples, where running of a smaller amount of amplification products occasionally resulted in thin and weak bands (data not shown).

The detection limit of the *eqsim* primer set determined using a series of 10-fold dilutions of template DNA from the reference strain of *S. equisimilis* was 10 fg/reaction (Fig. 3).

Comparison between bacteriological and PCR results

Results of culture and PCR for *S. equisimilis* were concordant in 23 out of 23 samples ($\kappa = 1.00$ - "perfect agreement"). Furthermore, PCR was positive not only in the 23 T14 enrichment broth samples that were positive by bacteriology, but also in 6 out of the 76 T14 enrichment broth samples that were negative for *S. equisimilis* by culture ($\kappa = 0.84$ - "almost perfect agreement").

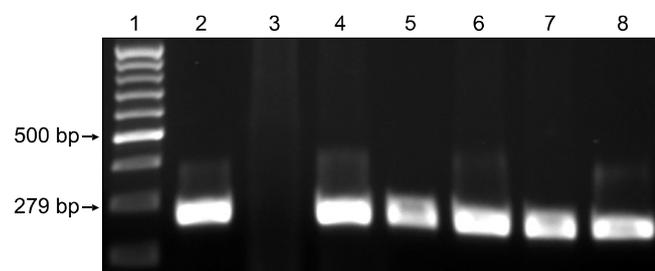


Fig. 1. Agarose gel electrophoresis results of the PCR assay for the detection of a 279 bp sequence of the streptokinase precursor gene of *Streptococcus* (*S.*) *equisimilis*. Lane 1: 100 bp ladder; Lane 2: positive control (*S. equisimilis* ATCC 10009); Lane 3: negative control; Lanes 4-8: *S. equisimilis* isolated from nasal swabs samples from five horses.

Discussion

S. equisimilis can infect humans and several animal species. Its pathogenic role has been well documented in humans, but no detailed data have been reported so far in horses. The availability of rapid and reliable diagnostic tools is fundamental for detecting *S. equisimilis* in clinical specimens and for its differentiation from other streptococci. Currently, bacterial culture and biochemical identification with commercial kits are widely used for *S. equisimilis* diagnosis. Nevertheless, overgrowing of other beta-haemolytic streptococci or mistakes in biochemical profile interpretation may be encountered during bacterial examination. PCR is widely used in many fields of microbiology, such as for direct bacterial detection in clinical specimens or for rapid identification of the cultured colonies. *S. equisimilis* secretes a plasminogen activator, known as streptokinase, which catalyses the

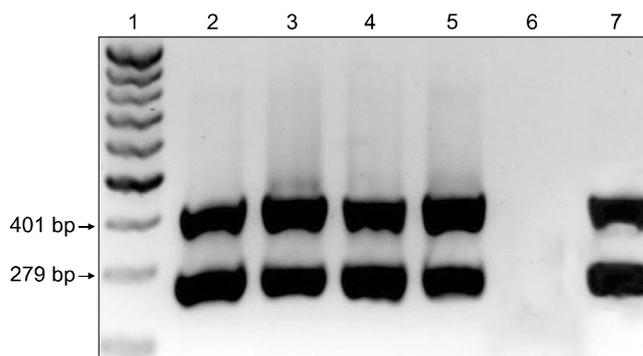


Fig. 2. Agarose gel electrophoresis results of the duplex PCR assay to detect a 401 bp sequence of the 16SrRNA region of the species *S. dysgalactiae* and a 279 bp sequence of the streptokinase precursor gene of *S. equisimilis*. Lane 1: 100 bp ladder; Lane 2-5: *S. equisimilis* detected in nasal swabs pre-incubated in enrichment broth; Lane 6: negative control; Lane 7: positive control (*S. equisimilis* ATCC 10009).

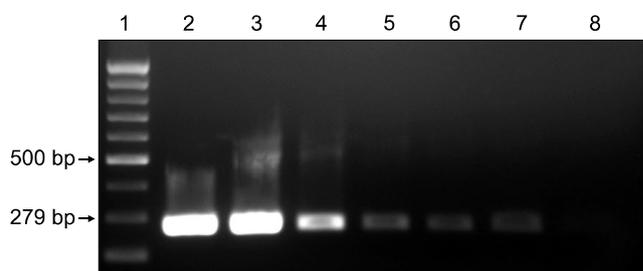


Fig. 3. Agarose gel electrophoresis of the amplification products, demonstrating the detection limit of the *eqsim-F/eqsim-R* PCR protocol on 10-fold dilutions of *S. equisimilis* ATCC 10009 DNA. Lane 1: 100 bp molecular size marker; Lane 2: 1 ng; lane 3: 100 pg; Lane 4: 10 pg; Lane 5: 1 pg; Lane 6: 100 fg; Lane 7: 10 fg; Lane 8: 1 fg.

conversion of plasminogen to plasmin, and may facilitate tissue invasion [4]. The nucleotide sequence of the streptokinase precursor gene of an equine strain of *S. equisimilis* has been sequenced and published [4] and may represent a target sequence for the binding of specific primers. Furthermore, a PCR protocol to detect a common sequence of the species *S. dysgalactiae* has been previously described [15] and may be used as a control for species identification.

The multiplex PCR protocol described in this study has been able to specifically detect both the streptokinase precursor gene of *S. equisimilis* and the 16SrRNA region of the species *S. dysgalactiae* in equine nasal swabs and in colonies grown on blood agar plates. There was “perfect agreement” ($\kappa = 1.00$) between bacteriological examinations and PCR in identifying *S. equisimilis* colonies. The agreement between the two methods in detecting *S. equisimilis* in enrichment broth samples inoculated with equine nasal swabs was “almost perfect” ($\kappa = 0.84$), and PCR protocols showed a higher sensitivity than bacteriology. All the samples where *S. equisimilis* was isolated by bacteriology were also positive by PCR. Furthermore, PCR was able to detect *S. equisimilis* DNA in 6 samples more than bacterial culture. This could be due to not only to the different characteristics of the two techniques, but also to the amounts of the starting samples examined by the two methods. The preliminary culture of the swabs in enrichment broth first causes multiplication, and consequently, a higher concentration of the bacteria. Nevertheless, only 100 μ L of this solution are tested by standard bacteriological examination, while 10-fold of this volume (one-fourth of the total volume) was analysed by PCR in this study without difficulty, resulting in its higher sensitivity.

Bacterial culture is a fundamental tool for *S. equisimilis* diagnosis, but the PCR assay can identify *S. equisimilis* isolates or detect *S. equisimilis* DNA directly in clinical specimens. In this latter case, a preliminary incubation in enrichment broth is recommended to increase the DNA yield. The PCR technique may be particularly useful when sample conservation does not guarantee the viability of the bacteria or when the samples have been treated with bacterial killing substances, such as ethanol or formalin, for other experiments (i.e. histology or cytology). In any case, the contemporary evaluation of diagnostic samples by bacterial culture and PCR could be combined for higher diagnostic sensitivity and specificity with the availability of live bacteria.

In conclusion, this study describes a rapid duplex PCR protocol for the detection and identification of *S. equisimilis* in equine nasal swabs by amplification of the streptokinase precursor gene and by contemporaneous amplification of a 16SrRNA region specific for the species *S. dysgalactiae*. This method could be useful for rapid

diagnosis and epidemiological purposes by the direct detection of *S. equisimilis* in equine nasal swabs.

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References

1. Alber J, El-Sayed A, Lämmler C, Hassan AA, Weiss R, Zschöck M. Multiplex Polymerase Chain Reaction for identification and differentiation of *Streptococcus equi* subsp. zooepidemicus and *Streptococcus equi* subsp. equi. J Vet Med B Infect Dis Vet Public Health 2004, **51**, 455-458.
2. Berenguer J, Sampedro I, Cercenado E, Baraia J, Rodriguez-Cr eixems M, Bouza E. Group-C beta-hemolytic streptococcal bacteremia. Diagn Microbiol Infect Dis 1992, **15**, 151-155.
3. Blanchard PC, Fiser KM. *Streptococcus dysgalactiae* polyarthritis in dairy goats. J Am Vet Med Assoc 1994, **205**, 739-741.
4. Caballero AR, Lottenberg R, Johnston KH. Cloning, expression, sequence analysis, and characterization of streptokinases secreted by porcine and equine isolates of *Streptococcus equisimilis*. Infect Immun 1999, **67**, 6478-6486.
5. Hashikawa S, Iinuma Y, Furushita M, Ohkura T, Nada T, Torii K, Hasegawa T, Ohta M. Characterization of group C and G streptococcal strains that cause streptococcal toxic shock syndrome. J Clin Microbiol 2004, **42**, 186-192.
6. Hong CB, Donahue JM, Giles RC Jr, Petrites-Murphy MB, Poonacha KB, Roberts AW, Smith BJ, Tramontin RR, Tuttle PA, Swerczek TW. Etiology and pathology of equine placentitis. J Vet Diagn Invest 1993, **5**, 56-63.
7. Katsumi M, Kataoka Y, Takahashi T, Kikuchi N, Hiramune T. Bacterial isolation from slaughtered pigs associated with endocarditis, especially the isolation of *Streptococcus suis*. J Vet Med Sci 1997, **59**, 75-78.
8. Kawata K, Minakami T, Mori Y, Katsumi M, Kataoka Y, Ezawa A, Kikuchi N, Takahashi T. rDNA sequence analyses of *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from pigs. Int J Syst Evol Microbiol 2003, **53**, 1941-1946.
9. Kumar A, Sandoe J, Kumar N. Three cases of vertebral osteomyelitis caused by *Streptococcus dysgalactiae* subsp. *equisimilis*. J Med Microbiol 2005, **54**, 1103-1105.
10. Laus F, Preziuso S, Spaterna A, Berib e F, Tesei B, Cuteri V. Clinical and epidemiological investigation of chronic upper respiratory diseases caused by Beta-haemolytic Streptococci in horses. Comp Immunol Microbiol Infect Dis 2007, **30**, 247-260.
11. McGinn T, Wyer PC, Newman TB, Keitz S, Leipzig R, Guyatt G. Tips for learners of evidence-based medicine: 3. Measures of observer variability (κ statistic). CMAJ 2004, **171**, 1369-1373.

12. Nakamura M, Honda K, Tun Z, Ogura Y, Matoba R. Application of *in situ* PCR to diagnose pneumonia in medico-legal autopsy cases. *Leg Med* 2001, **3**, 127-133.
13. Nomoto R, Munasinghe LI, Jin DH, Shimahara Y, Yasuda H, Nakamura A, Misawa N, Itami T, Yoshida T. Lancefield group C *Streptococcus dysgalactiae* infection responsible for fish mortalities in Japan. *J Fish Dis* 2004, **27**, 679-686.
14. Ortel TL, Kallianos J, Gallis HA. Group C streptococcal arthritis: case report and review. *Rev Infect Dis* 1990, **12**, 829-837.
15. Riffon R, Sayasith K, Khalil H, Dubreuil P, Drolet M, Lagacé J. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *J Clin Microbiol* 2001, **39**, 2584-2589.
16. Timoney JF. The pathogenic equine streptococci. *Vet Res* 2004, **35**, 397-409.
17. Vandamme P, Pot B, Falsen E, Kersters K, Devriese LA. Taxonomic study of Lancefield streptococcal groups C, G, and L (*Streptococcus dysgalactiae*) and proposal of *S. dysgalactiae* subsp. *equisimilis* subsp. nov. *Int J Syst Bacteriol* 1996, **46**, 774-781.
18. Vieira VV, Teixeira LM, Zahner V, Momen H, Facklam RR, Steigerwalt AG, Brenner DJ, Castro ACD. Genetic relationships among the different phenotypes of *Streptococcus dysgalactiae* strains. *Int J Syst Bacteriol* 1998, **48**, 1231-1243.