Indian Journal of Animal Sciences **82** (11): 1285–1289, November 2012

Development of a novel multiplex PCR for detection of *Brucella*, *Leptospira* and Bovine herpesvirus-1

S K BHURE[1], S CHANDAN[2], R G AMACHAWADI[3], S S PATIL[4], RAJESWARI SHOME[5], N L GANGADHAR[6], M R GAJENDRAGAD[7] and K PRABHUDAS[8]

Project Directorate on Animal Disease Monitoring and Surveillance, Hebbal, Bengaluru, Karnataka 560 024, India.

Received: 1 September 2011; Accepted: 9 April 2012

ABSTRACT

A novel multiplex PCR (mPCR) was developed for detection of *Brucella*, *Leptospira* and bovine herpesvirus-1 *(*BoHV-1*)* targeting conserved regions of *BCSP31, LipL32* and *gB* genes, respectively. No template controls were also run with each test. Electrophoresis of mPCR products showed bands specific to genus *Brucella, Leptospira* and BHV-1, respectively. The assay detected as low as 15 pg of each template. The mPCR showed specific bands with 4 *Brucella* species, 14 pathogenic *Leptospira* serovars, BoHV-1. In addition, 10 (out of 135 bull semen samples) and 5 (out of 17 bovine blood samples) showed a ~640bp amplicon specific to brucellosis. Five human urine samples were tested; 3 found positive for *Leptospira* and other 2 were positive for both *Leptospira* and *Brucella*. The assay is accurate, specific and cost effective tool for detection of *Brucella*, *Leptospira* and bovine herpesvirus-1 and will also be a valuable tool for the diagnoses of co-infection. The assay could be a preferable detection methodology for routine screening and molecular epidemiology of brucellosis, leptospirosis and infectious bovine rhenotracheitis.

Key words: Bovine Herpesvirus-1, *Brucella*, *Leptospira*, Infectious bovine rhenotracheitis, Multiplex PCR

Brucellosis, leptospirosis and infectious bovine rhinotracheitis (IBR) are widely distributed around the world. The *Brucella* affects range of different mammals including man, cattle, sheep, goats, swine, rodents and marine mammals. In most host species, disease primarily affects the reproductive system with loss in productivity of affected animals (Cutler *et al.* 2005). Leptospirosis, a communicable infectious disease, caused by more than 250 leptospiral serovars known to infect more than 160 species of mammals (Dutta and Christopher 2005). Leptospirosis causes reproductive losses due to abortions, stillbirths, infertility and non-reproductive losses due to septicemia, nephritis. In addition, leptospirosis is presumed to be the most widespread zoonotic disease in the world (Levett 2001, Gangadhar *et al.* 2008). The BoHV-1 is considered to be the most common viral pathogen found in bovine semen (Gibbs and Rweyemamu 1977). It is responsible for a variety of clinical conditions in cattle and buffaloes, including pustular vulvovaginitis, abortion, mastitis, and balano-posthitis of bulls, infertility, tracheitis, conjunctivitis-keratoconjunctivitis, encephalitis and fatal disease in newborn calves (Deka *et al.* 2005).

A disease- free germplasm is a prerequisite for getting healthy progeny. All 3 pathogens are transmitted through semen, either by natural service or artificial insemination. The intermittent excretion of *Brucella* (Amin *et al.* 2001), *Leptospira* (Ellis *et al.* 1986, Silva *et al.* 2007) and BoHV-1 (Rola *et al.* 2003, Deka *et al.* 2005) and their transmission through artificial insemination have been reported. Furthermore, leptospires can survive the freezing temperatures used to store the semen (Eaglesome and Garcia 1992).

Culturing of pathogens to diagnose infections is difficult, time consuming and often associated with a risk of laboratory acquired infection. Nucleic acid based techniques, particularly PCR, have been used for the diagnosis of slow growing or fastidious microorganisms. Monomeric PCR methods for identification of *Brucella* species (Romero *et al.* 1995, Rijpens *et al.* 1996, Navarro *et al.* 2002) in different clinical samples and pathogenic *Leptospira* (Bomfim *et al.* 2008, Bhure *et al.* 2009) have been reported. Researchers have developed PCR assays for BoHV-1 targeting the envelope glycoprotein genes, viz. *gB*, *gC* and *gD* (Kataria *et al.* 1997, Cândido 2000, Clause *et al.* 2005, Deka *et al.* 2005, Grom *et al.* 2006) from infected bull semen. There are no reports on the use of PCR in multiplex format for

Present address:[1] Senior Scientist (sdbhure@rediffmail.com), Division of Biochemistry, Indian Veterinary Research Institute, Izatnagar.[2]Department of Biotechnology, University of Mysore, Manasagangotri, Mysore–06, India.[3,4,5,6,7,8] Project Directorate on Animal Disease Monitoring and Surveillance.

simultaneous detection of *Brucella*, *Leptospira* and BoHV-1. We here reported the standardised mPCR as a rapid, low cost, sensitive tool for simultenious detection of *Brucella*, *Leptospira* and BoHV-1 pathogens.

MATERIALS AND METHODS

Reference strains

Brucella abortus S_{99} strain, *B. melitensis, B. canis, B. suis* and 15 *Leptospira* serovars (Icterohaemorrhagiae, Habdomadis, Canicola, Gryppotyphosa, Autumnalis, Javonica, Pyrogenes, Australis, Copenhageni, Hardzoprajictni, Hardjobovis, Tarassovi, Ballum, Pamona and Patoc) and BoHV-1 isolate (PD_ADMAS–01) were used as positive controls.

Isolation of DNA from reference strains and from blood of different species of animals

The genomic DNA of *Brucella abortus* stain S_{99}, *B. melitensis, B. suis, B. canis,* and 15 *Leptospira* serovars were extracted using genomic DNA extraction kit following the bacterial DNA extraction protocol. The DNA was extracted from plaque purified BoHV-1 using viral DNA extraction kit as per the manufacturer's protocol. The whole blood genomic DNA was isolated from cattle, buffalo,camel, sheep, goat, pig, dog and human using DNeasy blood and tissue kit.

Oligonucleotide primers

Three sets of primers for each of the pathogen that specifically amplified partial *LipL32* gene of *leptospira*, *BCSP31* gene of *Brucella* and *gB* gene of BoHV-1 were designed (Table 1) from the conserved region using GeneTool Biolite soft ware. Oligonucleotides were synthesised commercially.

Detection and characterization of PCR products

Initially the reactions were set up for detection of *Brucella*, *Leptospira* and BoHV-1 in individual PCR format under the following conditions using gradient PCR: 10 mM Tris.HCl pH 8.8, 50 mM KCl, 0.08% Non idet P40 and 1.5 mM $MgCl_2$, 200 µmoles of each dNTP, 25 pmoles of each primer and 2.0 U of Taq DNA polymerase in a final volume of 25 µl. The individual PCR reactions standardised for the 3 templates were: initial denaturation–95°C for 5 min followed by cycle denaturation at 95°C for 30 s; annealing at 65°C for 30 s; extension at 72°C for 30 s for 35 cycles and final extension at 95°C for 5 min using DNA extracted from *Brucella*, *Leptospira* and BoHV-1 as templates. The amplified PCR products were analysed by electrophoresis on a 2.0% agarose gel, stained with ethidium bromide (1 µg/ml) in TBE buffer (89 mM Tris; 89 mM Boric acid; 2 mM EDTA) and visualised under UV light. PCR products were characterised by restriction enzyme digestion with *Bgl*I, *Hinf*I for *Brucella*, *Bam*HI, *Hinf*I for *Leptospira* and *Sac*II, *Dpn*I for BoHV-I amplicons under the recommended conditions.

Optimization of multiplex PCR conditions

The multiplex assay conditions were standerdized by taking different concentrations of $MgCl_2$ (1 to 3mM). The standardised mPCR protocol contained 10 mM Tris-HCl pH8.8, 50 mM KCl, 0.08% Non-idet P40, 200 µmoles of each dNTPs, 2.0 U of Taq DNA polymerase, 10 pmoles of each of 6 primers, 1.5 mM $MgCl_2$ and extracted DNA from *Brucella*, *Leptospira* and BoHV-1 in a final volume of 25 µl. Thermal cycling comprised an initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec and extension at 72°C for 30 sec. The sample was then heated at 72°C for 5 min for a final extension. Negative controls with no template DNA were also run with each assay. mPCR products were run on agarose gel electrophoresis.

Accuracy of multiplex PCR assay

The accuracy of the assay under the standardised conditions for detections of *Brucella*, *Leptospira* and BoHV-I template was evaluated by taking serial 2-fold dilutions of template mix (200 pg/µl) spiked to a 100 ng of bovine control DNA isolated from whole blood.

The mPCR reactions were set up following the above standardised conditions using bovine total genomic DNA spiked with a known amount DNA of BoHV-1, *Brucella* organisms (*B. abortus* stain S99, *B. melitensis, B. suis and B. canis*) and 14 pathogenic *Leptospira* serovars, viz. *L. interrogans serovars* (Icterohaemorrhagiae, Habdomadis, Canicola, Gryppotyphosa, Autumnalis, Javonica, Pyrogenes, Australis, Copenhageni, Hardzoprajictni), *L. borgpetersenii*

Table 1. Primers used in the study

Organisms (gene)	Primers (5'–3')	Length (bp)	Accession number	Location of the sequence
Brucella abortus S99 (*bcsp31*)	TCCGTCGCTTGGCTGTTGC TACCGTTCGAGATGGCCAGTTC	636	M20504	584–1219
Leptospira (LipL32)	GAACCAGGCGACGGAGACTTAGTA TGGATCAACGGGCTCACACCT	422	CP000350	1939251–1939672
Bovine herpes virus 1 *(gB)*	CGGACGAAATGCTGCGAGAC CGCCAGGTACGTCTCCAAGCT	218	AJ004801.1	56541–56758

serovars (Hardjobovis, Tarassovi, Ballum), *L. noguchii serovar* Pamona *and L. biflexa serovar* Patoc. Further, the assay was also performed to check for non-specific amplification with control genomic DNA of cattle, buffalo, camel, sheep, goat, pig, dog and human. Reactions with no template DNA served as negative controls.

Detection of leptospira, Brucella *and BoHV-1 in semen/blood/ urine of clinical cases*

Bull semen samples (127) were collected from 2 organised semen collection centers of Bengaluru, India. The DNA was extracted from 200 µl of semen using minikit as per the manufacturer's protocol. The DNA extractions from 17 bovine blood samples with a history of abortion or repeat breeding and 3 human urine samples were screened by mPCR.

RESULTS AND DISCUSSION

The PCR has become an invaluable diagnostic tool because of its speed, simplicity and applicablity to different clinical samples. Monomeric PCR for identification of *Brucella* in different clinical samples was reported (Romero *et al.* 1995, Rijpens *et al.* 1996, Navarro *et al.* 2002). No reports of a triplex assay for detection of *Brucella*, *Leptospira* and BoHV-1 are available. A number of nucleic acid sequences have been targeted for the development of *Brucella* genus-specific PCR assays, including 16S rRNA, the 16–23S intergenic spacer region, *omp*2 and *bcsp*31 (Romero *et al.* 1995, Rijpens *et al.* 1996, Navarro *et al.* 2002). The most frequently described PCR target for diagnosis of human brucellosis is *BCSP31* gene encoding a 31 kDa protein conserved among the *Brucella* species (Navarro *et al.* 2002, Morata *et al.* 2003, Sohn *et al.* 2003, Bounaadja *et al.* 2009). The primers were designed from this conserved region for the multiplex assay. For detection of leptospires by PCR, the 16S rRNA gene (Merien *et al*. 1992) could not differentiate pathogenic and non-pathogenic leptospires. The major outer-membrane lipoprotein, encoded by *LipL32* gene, is an important virulence factor (Haake *et al.* 2000) and is confined to strains of pathigenic *Leptospira* species. Hence, the primers were designed targeting the partial sequence of *LipL32* in the multiplex assay. Researchers have developed PCR assays for BoHV-1 targeting the envelope glycoprotein genes, viz. *gB*, *gC* and *gD* (Kataria *et al.* 1997, Cândido 2000, Clause *et al.* 2005, Deka *et al.* 2005, Grom *et al.* 2006) from infected bull semen. An mPCR has been developed to detect and diagnose the BoHV-1 and BoHV-5 infection targeting the amino terminal sequence of *gB* gene (Clause *et al.* 2005). Hence, the partial *gB* gene was selected for primer designing in mPCR.

In this work, an mPCR is described and applied for detection of the 3 important pathogens in a single reaction. Optimization of a multiplex reaction requires optimization of concentration of reagents, annealing temperature, and/or cycling conditions to obtain the best overall strategy for amplification of more than 1 target sequence. Choice of primer pairs for a multiplex assay that would exhibit equal sensitivity for their target sequences would require extensive testing.

The mPCR specifically amplified a 636 bp, 422 bp and 218 bp target sequence of 4 *Brucella* species, 14 pathogenic *Leptospira* serovars and BoHV-1, respectively (Figs. 1, 2a,

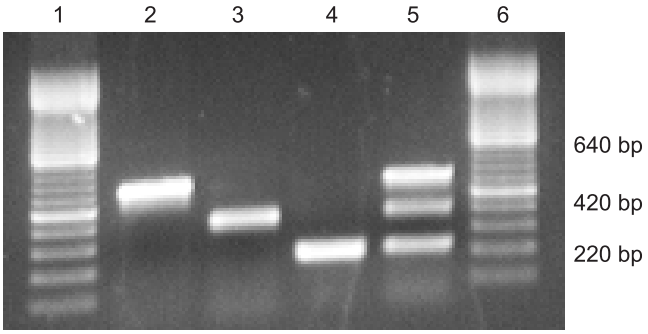

Fig. 1. 2% Agarose gel electrophoresis showing amplified PCR products in monomeric and multiplexPCR assay. Lanes 1 and 6: 100 bp DNA ladder, Lanes 2 to 4: monomeric PCR products of *brucella*, *leptospira* and BoHV-1, respectively, Lane 4: MultiplexPCR products of *brucella* (640 bp), *leptospira* (420 bp) and Bovine Herpesvirus-1(220 bp).

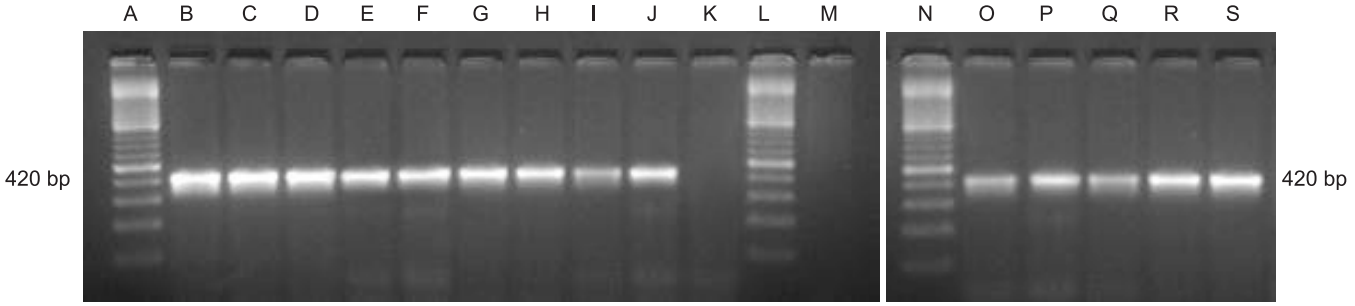

Fig. 2a. 2.0% agarose gel electrophoresis showing the amplification of pathogenic *leptospira* serovars and no amplification with non-pathogenic *Leptospira biflexa* serovar Patoc.

Legends: A, 100 bp ladder; B. *L. Icterohaemorrhagiae*; C, *L. Habdomadis*; D, *L. Canicola*; E, *L. Gryppotyphosa*; F, *L. Autumnalis*; G, *L. Javonica*; H, *L. Pyrogenes*; I, *L. Australis*; J, *L. Copenhageni*; K, *L. Patoc*; L, *100 bp ladder*; M, Negative control; N, *L. Pyrogenes*; O, *L. Australis*; P, *L. Copenhageni*; Q, *L. Patoc*; R, 100 bp ladder; S, Negative control.

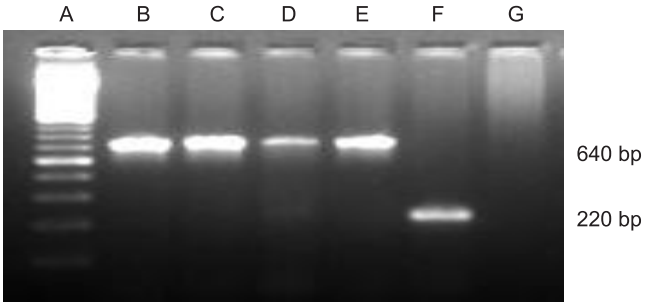

Fig. 2b. 2.0% agarose gel electrophoresis showing the selective amplification of different species with *brucella* and BoHV-1.

Legends: A, 100 bp ladder; B, *B. Abortus* S9; C, *B. mellitensis*; D, *B. suis*; E, *B. canis*; F, BoHV-1; G, Buffalo DNA (control).

and Fig. 2b). Additionally, as the sequence of primers designed from the conserved region of *BCSP31* gene are highly homologous to that of *Brucella ovis*. This mPCR would detect B. *ovis* as well (NCBI GenBank unpublished observation*).* All monomeric PCR amplicons showed the expected pattern after restriction endonuclease analysis. The assay performed to check for non-specific amplification with control genomic DNA of cattle, buffalo, camel, sheep, goat, pig, dog and human did not show amplification.

Serial 2-fold dilutions of template mix of the 3 DNA in a multiplex reaction could detect as low as 15 to 17 pg of each template in a 25 µl reaction volume consistently and was better than the other conventional PCR assays targeting the same set of genes described for detecting *Brucella* (Navarro *et al.* 2002), pathogenic *Leptospira* (Bomfim *et al.* 2008). A real-time triplex assay has been used in a multiplex format for rapid confirmation of *Brucella* species capable of detecting 150 fg of DNA (Probert *et al.* 2004) and a rapid conventional PCR for detection of pathogenic leptospires targeting *LipL32* capable of detecting as low as 2.5 pg of DNA in biological samples has been described (Bhure *et al.* 2009). The target of *IS1533* could determine leptospiral serovars, but the study did not show the sensitivity and specificity of the tested primers (Zuerner *et al.* 1995). Variants of conventional PCR with slightly higher accuracy of detection are reported but mPCR assay described here has the advantage of detecting 3 major reproductive diseases in single tube. Higher accuracy is desirable but any diagnostic method which can detect a pathogen in a clinical sample at a concentration normally present is acceptable. Although the sensitivity of some targets could be reduced, the improvement of both turnaround time and cost effectiveness would compensate for this minor reduction suggesting its potential application as a useful method for routine molecular diagnostic purposes.

This is the first report describing an mPCR for detection of genus *Brucella*, *Leptospira* and BoHV- 1 in semen and clinical samples. Among 127 bovine semen samples screened, 3 semen samples of Jersey bulls showed a ~640 bp amplicon specific to *Brucella*. Five out of 17 bovine blood samples from cattle with a history of abortion or repeat breeding showed the same specific amplicon of ~640 bp. None of the semen and blood samples were positive for BoHV-1 genomic DNA as the infected animals or carriers will shed the virus only in stressed conditions. Among 3 human urine samples, 1 sample was positive for *Leptospira* and other 2 were positive for both *Leptospira* and *Brucella*.

The ability of mPCR to detect infecting agent or its genomic DNA in semen can make the test suitable for routine screening of semen samples for possible shedding of the organisms; thereby preventing the spread of infection by artificial insemination and natural service. Since it was possible to detect *Brucella*, *Leptospira* and BoHV-1 genomic DNAs in routine agarose gel electrophoresis in different clinical samples; it can be a low-cost alternative of real time PCR. Further, the validated mPCR assay described here would be ready for application for the detection of *Brucella*, *Leptospira* and BoHV-1 from other types of clinical samples, once a suitable DNA extraction method has been selected. All these advantages make this mPCR a preferable detection methodology for routine diagnosis, screening and molecular epidemiology of brucellosis, leptospirosis and IBR.

ACKNOWLEDGEMENTS

The authors wish to thank, Indian Council of Agricultural Research, New Delhi, India for providing the necessary facilities and financial support for the research work and also IAH and VB, Bengaluru, Karnataka; Institute of Veterinary Preventive Medicine, Ranipet, Tamil Nadu; Dr D. K. Singh and Dr Sabrinath of IVRI, Izatnagar, for providing the reference strains of *Brucella* and *Leptospira*.

REFERENCES

Amin A S, Hamdy M E and Ibrahim A K. 2001. Detection of *Brucella melitensis* in semen using the polymerase chain reaction assay. *Veterinary Microbiology* **83**: 37–44,

Bhure S K, Lakshmisha K V, Patil S S, Rajeswari S, Shome B R, Gangadhar N L, Gajendragad M R and Pabhudas K. 2009. Rapid polymerase chain reaction (PCR) for detection of pathogenic Leptospires in biological samples. *Indian Journal of Animal Sciences* **79**: 781–83.

Bomfim M R, Barbosa-Stancioli E F and Koury M C. 2008. Detection of pathogenic Leptospires in urine from naturally infected cattle by nested PCR. *Veterinary Journal* **178**:251–56.

Bounaadja L, Albert D, Chénais B, Hénault S, Zygmunt M S, Poliak S and Garin-Bastuji B. 2009. Real-time PCR for identification of *Brucella* spp.: a comparative study of IS711, *bcsp31* and per target genes. *Veterinary Microbiology* **137**: 156–64.

Cândido A, Bontempo E and Resende M. 2000. Polymerase chain reaction amplification of genomic fragments of bovine herpesvirus-1. *Memorias do InstitutoOswaldo Cruz* **95**: 225–26.

Clause M P, Alfieri A F, Folgueras-Flatschart A V, Wosiacki SR, Medici K C and Alfieri A. 2005. A rapid detection and differentiation of bovine herepesvirus 1 and 5 glycoprotein C gene in clinical specimens by multiplex-PCR. *Journal of*

Virological Methods **128**: 183–88.

Cutler S J, Whatmore A M and Commander N J. 2005. Brucellosis – new aspects of an old disease. *Journal of Applied Microbiology* **98**:1270–81.

Deka D, Ramneek M N K and Oberoi M S. 2005. Detection of bovine herpesvirus-1 infection in breeding bull semen by virus isolation and polymerase chain reaction. *Revue Scientifeque et Technique (International office of Epizootics)* **24**: 1085–94.

Dutta T K and Christopher M. 2005. Leptospirosis – An Overview. *The Journal of the association of Physicians of India* **53**:545–51.

Eaglesome M D and Garcia M M. 1992. Microbial agents associated with bovine genital tract infections and semen. Part 1. *Brucella abortus, leptospira, Campylobacter fetus* and *Tritrichomonas foetus*. *Veterinary Bulletin* **62**: 743–75.

Ellis W A, Cassels J A and Doyle J. 1986. Genital leptospirosis in bulls. *Veterinary Record* **118,** 333.

Gangadhar N L, Prabhudas K, Bhushan S, Sulthana M, Barbuddhe S B and Rehaman H. 2008. *Leptospira* infection in animals and humans: a potential public health risk in India. *Revue Scientifeque et Technique (International office of Epizootics)* **27**: 885–92.

Gibbs E P J and Rweyemamu M M. 1977. Bovine herpesviruses. Part I. Bovine herpesvirus 1. *Veterinary Bulletin* **47**: 317–43.

Grom J, Hostnik P, Toplak I and Barlic-Maganja D. 2006. Molecular detection of BHV-1 in artificially inoculated semen and in the semen of a latently infected bull treated with dexamethasone. *Veterinary Journal* **171**: 539–44.

Haake D A, Chao G, Zuerner R L, Barnett J K, Barnett D, Mazel M, Matsunaga J, Levett P N and Bolin C A. 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infection and Immununity* **68**: 2276–85.

Karsen H, Karahocagil M K, Irmak H and Demiröz A P. 2008. A meningitis case of *Brucella* and tuberculosis co-infection. *Mikrobiyoloji Bulteni* **42**: 689–94.

Kataria R S, Tiwari A K, Gupta P K, Mehrotra M L, Rai A and Bandyopadhyay S K. 1997. Detection of bovine herpesvirus 1 (BHV-1) genomic sequences in bovine semen inoculated with BHV-1 by polymerase chain reaction. *Acta Virologica* **41**: 311-15.

Kee S H, Kim I K, Choi M S and Chang W H. 1994. Detection of leptospiral DNA by PCR. *Journal of Clinical Microbiology* **32**: 1035–39.

Levett P N. 2001. Leptospirosis. *Clinical Microbiology Reveiw* **14**:296–26.

Merien F, Amouriauz P, Perolat P, Baranton G and Saint Girons I. 1992. Polymerase chain reaction for detection of *Leptospira* species in clinical samples. *Journal of Clinical Microbiology* **30**: 2219–24.

Morata P, Queipo-Ortuño M I, Reguera J M, García-Ordoñez M A, Cárdenas A, and Colmenero J D. 2003. Development and evaluation of a PCR–enzyme-linked immunosorbent assay for diagnosis of human brucellosis. *Journal of Clinical Microbiology* **41**: 144–48.

Navarro E, Escribano J, Fernandez J and Solera J. 2002. Comparison of three different PCR methods for detection of *Brucella* spp. in human blood samples. *FEMS Immunology and Medical Microbiology* **34**: 147–51.

Probert W S, Schrader K N, Khuong N Y, Bystrom S L and Graves M H. 2004. Real-Time multiplex PCR assay for detection of *Brucella* spp, *B. abortus* and *B. melitensis. Journal of Clinical Microbiology* **42**: 1290–93.

Rijpens N P, Jannes G, Van Asbroeck M, Rossau R and Herman L M. 1996. Direct detection of *Brucella* spp. in raw milk by PCR and reverse hybridization with 16S–23S rRNA spacer probe. *Applied and Environmental Microbiology* **62**: 1683–88.

Rola J, Miroslaw P and Polak Jan F Zmudzinski. 2003. Amplification of DNA of BHV1 isolated from semen of naturally infected bulls. *Bulletin of Veterinary Institute in Pulawy* **47**:71–75.

Romero C, Gamazo C, Pardo M, López-Goñi I. 1995. Specific detection of *Brucella* DNA by PCR. *Journal of Clinical Microbiology* **33**:615–17.

Silva E F, Brod C S, Cerqueira G M, Bourscheidt D, Seyffert N, Queiroz A, Santos C S, Ko A I and Dellagostin O A. 2007. Isolation of *Leptospira noguchii* from sheep. *Veterinary Microbiology* **121**: 144–49.

Sohn A H, Probert W S, Glaser C A, Gupta N, Bollen A W, Wong J D, Grace E M and McDonald W C. 2003. Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. *Emerging Infectious Diseases* **9**: 485–88.

van Eys G J J M, Gravekamp C, Gerritsen M J, Quint W, Cornelissen M T E, Schegget J and Terpstra W J. 1989. Detection of Leptospires in urine by polymerase chain reaction. *Journal of Clinical Microbiology* **2**:2258–62.

Zuerner R L, Alt D and Bolin C A. 1995. IS1533–based PCR assay for identification of *Leptospira* interrogans sensu lato serovars. *Journal of Clinical Microbiology* **33**: 3284–89.