Applied Biological Research 16 (1): 119-121; (2014)
DOI: 10.5958/0974-4517.2014.00057.3

Short Note

DIAGNOSIS OF BRUCELLOSIS IN BUFFALO SERUM BY POLYMERASE CHAIN REACTION

Malik Raies Ul Islam[1]*, Mohinder Pratap Gupta, Gursimran Filia, Pritam Kaur Sidhu, Tauseef Ahmed Malik[2], Ghulam Rasool Bhat[3], Suhail Ahmed Bhat[4] and Sayed Ahaq Hussain Shah[2]

Animal Disease Research Centre, [2]Department Veterinary Medicine, [3]Animal Reproduction Gynecology and Obstetrics, [4]Veterinary Parasitology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana – 141 004, Punjab (India)
[1]*Krishi Vigyan Kendra, S.K. University of Agricultural Sciences and Technology of Kashmir, Malangpora, Pulwama 192 301, Jammu & Kashmir (India)*
***e-**mail: *malikrayees@gmail.com, malik_rayees@yahoo.com*

(Received 29 September, 2013; accepted 28 December, 2013)

Brucellosis, a chronic infectious disease, is worldwide in distribution. Though it has been eradicated in many developed countries, it still remains an uncontrolled serious public health problem in most of the developing countries (Refai, 2002). Brucellosis is caused by the members of genus *Brucella*. Bovine brucellosis is mostly caused by *B. abortus*, and less frequently by *B. melitensis* (OIE, 2009). Oral route is the common portal of entry of this agent (Crawford *et al.*, 1990). The main clinical signs of brucellosis are abortion, retained placenta, still births and orchitis (Enright *et al.*, 1984). Laboratory diagnosis of brucellosis can be made by various primary and secondary binding immuno-assays. However, unequivocal diagnosis requires bacteriological demonstration of the agent. As isolation of this agent is laborious and poses potential health hazard to the laboratory workers, an alternative to it is polymerase chain reaction (PCR). PCR has been applied for the diagnosis of *Brucella* species (Rijpens *et al.*, 1996). Previous studies have documented the use of different clinical specimen *viz.*, peripheral blood of human (Queipo-Ortuno *et al.*, 1999), buffaloes (Guarino *et al.*, 2000), dog (Keid *et al.*, 2009), goat milk (Leal-Klevezas *et al.*, 1995), human serum (Zerva *et al.*, 2001), camel (Alshaikh *et al.*, 2007) and sheep (Singh *et al.*, 2010) for the diagnosis of *Brucella* species by PCR. The present study describes the application of PCR for diagnosis of brucellosis in serum samples from buffalo.

A total of 178 animals were selected from an organized buffalo herd in Ludhiana (Punjab). Animals less than 1 year old were excluded. None of the animals were vaccinated against brucellosis**.** The rose Bengal plate test (RBPT) was performed as described by Alton *et al.* (1988). Thirty microliters of serum was mixed with an equal volume of rose Bengal antigen on a clean grease-free slide to produce a zone approximately 2 cm in dia. Both the drops were then mixed by a disposable stirring stick, spread over the full surface of circle. The slide was rotated manually for 4 min. and analyzed for the presence or absence of any degree of agglutination. Controls were run using known positive and known negative sera. For enzyme immune assay, a commercially available indirect enzyme linked immune-sorbent assay (I-ELISA) kit was obtained from BioNote (Korea). I-ELISA was performed as per the manufacturer's instructions. The optical density (OD) was used to calculate the percent positivity. The test sera were categorized as positive or negative on the basis of percent positivity value with the samples having percent positivity of $\geq$25 as positive and <25 as negative.

For PCR analysis, DNA was extracted from serum samples collected from of 104 adult buffaloes that tested positive to either RBPT or I-ELISA. DNA was extracted from 100 µL serum using the DNeasy blood kit (Qiagen) according to manufacturer's instructions and was eluted in 100 µL elution buffer supplied with kit. DNA amplification using primers (JPF – GCGCTCAGGC

TGCCGACGCAA and JPR - ACCAGCCATTGCGGTCGGTA) originally described by Leal-Klevezas *et al.* (1995) was performed on 8 µL DNA sample in a 25 µL reaction mixture containing per litre 10 mmol Tris-HCl (pH 9.0), 50 mmol KCl, 3 mmol $MgCl_2$ and 200 µmol of each dNTP, 50 pmol of each primer and 2.5 units *Taq* polymerase. After initial denaturation at 94ºC for 4 min., 35 amplification cycles were performed. Each amplification cycle consisted of 1 min. at 94ºC, 1 min. at 60ºC, and 1 min. at 72ºC and followed by a final extension step at 72ºC for 3 min. All the PCR reactions were performed with the appropriate inclusion of positive and negative controls. Eight microlitres of amplification reaction was taken and resolved on 1.5% agarose gel containing 1 × TBE, stained with an ethidium bromide solution and visualized under ultraviolet light.

Out of 104 samples, amplicons of 193bp (fig 1) were detected in 47 samples by PCR and the rest samples were found negative. Positive control gave positive amplification of expected size. Serological methods are traditionally used for diagnosis of brucellosis. However, none of the currently available serological assays are 100% sensitive and specific when compared to gold standard. The most incontrovertible diagnosis is isolation of the etiological agent (Alton *et al.*, 1988) but it is less sensitive, time consuming and poses potential health hazard to the laboratory workers. As a result many serological tests are in routine use, like RBPT, complement fixation test, buffered plate antigen test, besides many others. These tests have high sensitivity but low specificity owing to cross reacting antigens found in other Gram negative bacteria.

Several PCR methods have been developed and used for the detection of *Brucella* strains (Gupta *et al.*, 2006; Fretin *et al.*, 2008; Huber *et al.*, 2009; Singh *et al.*, 2010). In this study a genus specific PCR method previously described by Leal- Klevezas *et al.* (1995) was applied to detect the presence of *Brucella* DNA in the serum from buffaloes. Use of serum instead of whole blood offers several advantages for nucleic acid amplification methods, as inhibition by anticoagulants, haemoglobin and host DNA is circumvented (Zerva *et al.*, 2001). Regarding the origin of pathogen nucleic acid in serum, Zerva *et al.* (2001) suggested that they are released in circulation as breakdown products during bacteremic phase. Further, at the equilibrium of host-parasite interaction the brucellae may persist for quite some time in the circulation before getting localized in their preferred sites.

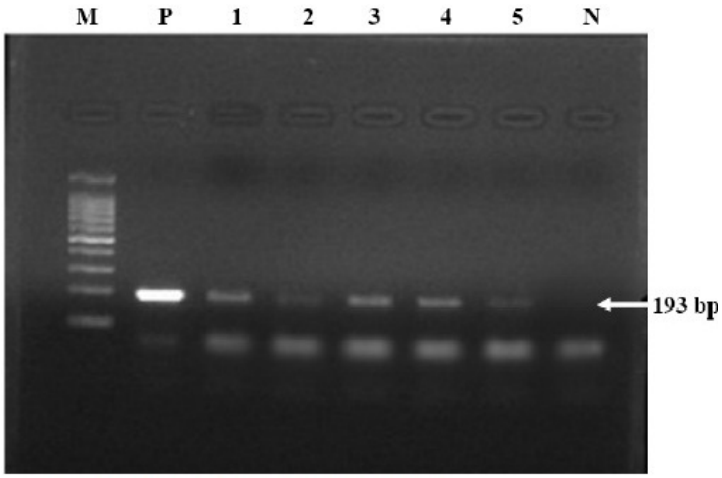

Fig. 1: Results of Brucella genus specific PCR from serum
Lane M: Molecular Weight Marker (100bp)
Lane P: Positive Control
Lane 1-5: Positve Samples
Lane N: Negative Control

The host-parasite interaction in case of brucellosis is complex. It is difficult to determine at which time the brucellae may be present in the circulation. Further, the chronic nature of infection and symptomless course in heifers and young ones makes it difficult to diagnose the infection before disease manifestation. PCR in this regard may prove as an indispensable tool for early detection of infection, before the organisms hide in their preferred locations. It may be concluded that PCR alone cannot be used for routine diagnosis of brucellosis using serum as starting material. However, it can be used to complement the serological tests for detection of brucellosis in buffaloes using serum, especially in those animals which may probably be in the early phase of infection when the immune response is undetectable, which will further augment in the control of this infection.

Alshaikh, M.A.A., Al-Haidary, A.I., Aljumaah, R.S., Mohammed, O.B., Al-Korashi, M.M., Omer, S.A., Gar El Nabi, A.R. and Hussain, M.F. 2007. First detection of *Brucella abortus* in camel serum in Saudi Arabia using the polymerase chain reaction. *Journal of Applied Animal Research*, **31**: 149-152.

Alton, G., Jones, L.M., Angus, R.D. and Verger, J.M. 1988. *Techniques for the Brucellosis Laboratory* (2nd edn.). INRA, Paris, France.

Crawford, R.P., Huber J.D. and Adams, B.S. 1990. Epidemiology and surveillance. pp. 131-151. **In**: *Animal Brucellosis* (eds. K. Nielsen and J.R. Duncan). CRC Press, Boca Raton, USA.

Enright, F.M., Walker, J.V., Jeffers, G. and Deyoe B.L. 1984. Cellular and humoral responses of *Brucella abortus*-infected bovine fetuses. *American Journal of Veterinary Research*, **45**: 424-443.

Fretin, D., Whatmore, A.M., Al-Dahouk, S., Neubauer. H., Garin-bastuji, B. and Albert, D. 2008. *Brucella suis* identification and biovar typing by real-time PCR. *Veterinary Microbiology*, **131**: 376-385.

Guarino, A., Serpe, L., Fusco, G., Scaramuzzo, A. and Gallo, P. 2000. Detection of *Brucella* species in buffalo whole blood by gene-specific PCR. *Veterinary Records*, **147**: 634-636.

Gupta, V.K., Verma, D.K., Singh, K., Kumari, R., Singh, S.V. and Vihan, V.S. 2006. Single-step PCR for detection of *Brucella melitensis* from tissue and blood of goats. *Small Ruminant Research*, **66:** 169-174.

Huber, B., Scholz, H.C., Lucero, N. and Busse, H.J. 2009. Development of a PCR assay for typing and subtyping of *Brucella* species. *International Journal of Medical Microbiology*, **299**: 563-573.

Keid, L.B., Soares, R.M., Vasconcellos, S.A., Megid, J., Salgado, V.R. and Richtzenhain, L.J. 2009. Comparison of agar gel immunodiffusion test, rapid slide agglutination test, microbiological culture and PCR for the diagnosis of canine brucellosis. *Research in Veterinary Science*, **86**: 22-26.

Leal-Klevezas, D.S., Martinez, V.I.O., Lopez, M.A. and Martinez, S.J.P. 1995. Single step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *Journal of Clinical Microbiology*, **3**: 3087-3090.

OIE. 2009. *Bovine Brucellosis: Manual of the Diagnostic Tests and Vaccines for Terrestrial Animals*, Vol. 1 (5th edn.). OIE World Organization for Animal Health, 2.4.3. Office International Des Epizooties, Paris, France.

Queipo-Ortuno, M.I., Garcia-Ordenez, M.A., Colmenero, D.J. and Morata, P. 1999. Hydrogen peroxide improves the efficiency of peripheral blood PCR assay for diagnosis of human brucellosis. *Biotechniques*, **27**: 248-252.

Refai, M. 2002. Incidence and control of brucellosis in the Near East region. *Veterinary Microbiology*, **90**: 81-110.

Rijpens, N.P., Jannes G., Van A.M., Rossau, R. and Herman, L.M.F. 1996. Direct detection of *Brucella* spp. in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probe. *Applied Environmental Microbiology*, **62**: 1683-1688.

Singh, M., Singh, D.K., Shivaramu, K.V., Biswas, R., Rawat, S., Boral, R., Singh, S. and Cheema, P.S. 2010. Serum as clinical specimen in PCR for diagnosis of ovine brucellosis. *Indian Journal of Animal Sciences*, **80**:17-18.

Zerva, K., Bourantas, S., Mitka, Kansouzidou, A. and Legakis, N.J. 2001. Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. *Journal of Clinical Microbiology*, **39**: 1661-1664.