Haryana Vet. (Dec., 2015) **54 (2)**, 99-102

Research Article

DEVELOPMENT OF REAL TIME PCR ASSAY FOR DIAGNOSIS OF *BRUCELLA* SPP.

VINAY KUMAR, AMAN KUMAR, ASHIS DEBNATH, KANISHT BATRA, NITISH BANSAL, AKHIL K. GUPTA, ANUJ TIWARI and SUSHILA MAAN*

Department of Animal Biotechnology, College of Veterinary Sciences
Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125 004, India

Received: 04.07.2015; Accepted: 13.11.2015

ABSTRACT

Brucellosis is one of the zoonotic diseases of major concern and can cause huge economic losses to livestock industry. We evaluated the performance of a newly designed TaqMan real-time PCR assay targeting the insertion sequence IS711 for the detection of *Brucella* genome at genus level. The IS711 gene-based real-time PCR assay was found to be highly sensitive and detected as low as single copy of DNA (0.2 fg) in the sample. The assay was highly specific, efficient and had good reproducibility and repeatability and therefore can be used for rapid and safe detection of *Brucella* spp.

Key words: *Brucella* spp., IS711 real-time PCR, sensitivity, specificity

Brucellosis is a widespread highly infectious disease which has a great impact on public health and animal production (Corbel, 1997). The genus *Brucella* is composed of nine recognized species, six of which are the "classical" members *(B. abortus, B. suis, B. melitensis, B. canis, B. ovis and B. neotomae)* (Cutler *et al.,* 2005). *B. abortus* affects primarily cattle and other bividae as well as cervidae; *B. suis* is isolated from swine and several forms of wildlife; *B. melitensis* infects mainly sheep and goats. Even though there has been considerable improvement in managing the spread of brucellosis in many countries, there are still areas where the disease is present in wild and domestic animal populations. These pockets of infection and potential transmission can represent significant health hazards to human populations (Treanor *et al*., 2011). Abortion due to *Brucella* infection causes huge economic loss to animal husbandry sector which is approximately US $ 3.4 billion/annum (Singh *et al*., 2015). Hence, it is paramount to detect infection caused by *Brucella* species with high sensitivity to impose control measures at the earliest.

"The gold standard" for laboratory detection of *Brucella* and species identification is based largely on serological tests and/or bacterial isolation and phenotypic characterization. These procedures are lengthy and labour intensive and also it has reduced sensitivity in chronic infections. Isolation has been associated with a high risk of laboratory acquired infection as *Brucella* organisms are class 3 pathogens (Alton *et al*., 1988; OIE, 2009). Serological assays are easy to perform, but lack specificity due to cross-reactions with other bacteria, particularly with *Yersinia enterocolitica* O:9 (Garin-Bastuji *et al*., 1999; Nielsen *et al*., 2004).

Nucleic acid based techniques (PCR-based) methods are simpler, faster, less hazardous and usually more sensitive (Bricker, 2002), especially those based on 16S rRNA (O'Leary *et al*., 2006) and the *Brucella* cell surface 31 kDa protein genes (bcsp31) (Baily *et al*., 1992; Da Costa *et al*., 1996), which are highly conserved in the genus *Brucella*. Real-time PCR results can either be qualitative or quantitative. Additionally, real-time PCR data can be evaluated without gel electrophoresis, resulting in reduced experiment time and increased throughput. Although there are many published real time assays available for *Brucella* but very few of them are based on dual labelled hydrolysis probe targeting IS711. Therefore, this study was aimed to develop and evaluate TaqMan probe based real time PCR assay targeting IS711 gene of *Brucella* spp.

MATERIALS AND METHODS

Commercially Synthesized Gene as Positive Control: The *Brucella* genus specific IS711 gene construct was commercially synthesized from Gene Oracle Company and was cloned in the pGOv6 synthetic construct cloning vector in the forward orientation. These *E. coli* transformants were grown on LB (Lauria Bertani) agar and further expanded in L B broth and were used for plasmid isolation.

Isolation and Quantification of Plasmid: Plasmid was isolated using commercial plasmid extraction

*Corresponding author: sushilamaan105@gmail.com

minikit (QIAGEN, Germany) as per manufacturer's protocol. The concentration and purity of the plasmid isolated from bacterial colonies was measured spectrophotometrically (BIO-RAD, India) by measuring the wavelength at A_{260} and A_{280} and their purity was assessed by taking the 260/280 ratio (Sambrook and Russel, 2001). The concentration of DNA was calculated using the following formula.

DNA concentration (µg DNA/ml) = OD 260 x 50 x dilution factor

Primer and Probe Designing for Real Time TaqMan Assay: Sequences of insertion element IS711, gene was used for designing primers and probe for genus and species specific real time TaqMan assay. The probe was dual labeled with VIC at 5' end and BHQ1 at 3' end (Table 1). The specificity of primers and probe was tested *in silico* in Bioedit ver. 7.0 software. The sequences of insertion element IS711 that were available online until February, 2015 were used for primer and probe design.

Optimization of TaqMan Based Assay: TaqMan based real-time PCR amplification was performed using Stratgene MX3500P Real-Time PCR System (Agilent technologies, USA) using Applied Biosystems master mix. The plasmid DNA of *Brucella* was used in triplicate for optimization of real time PCR. Thermal amplification was carried out as follows: preheating at 50^0C for 2 min, initial denturation at 95^0C for 10 min, 40 cycles of denaturation at 95^0C for 15 sec, annealing and extension at 61^0C for 1 min. The annealing temperature was optimized from 60^0C to 65^0C for finding maximum efficiency. The reaction was carried out in a 20 µl reaction volume using 10 µl Universal TaqMan based Master Mix (2X) (ABI). Different concentrations of primers starting from 5 pmol to 20 pmol of each of forward and reverse primer were used for standardization as per checkerboard method. For qPCR, various concentrations of probes 2.5 pmol to 10 pmol were used to test efficiency of amplification.

Analytical Sensitivity: Analytical sensitivity was determined using 10 fold serial dilution of plasmid DNA product from 10^{-1} to 10^{-12} dilutions. Serial dilutions of plasmid DNA was quantitated using Qubit® dsDNA HS (High Sensitivity) assay kit for use with Qubit® 2.0 Fluorometer (Invitrogen, USA). These dilutions were then used as template in real time PCR and were run in triplicate for generating standard deviation curve. Thermal amplification was carried as discussed under previous heading. All the reactions were run in triplicate. The copy number was calculated as follows:

Copy number = [A x No.]/[length (plasmid + insert size) $\times 1 \times 10^9 \times 660$].

A=amount of plasmid DNA (ng); No=Avogadro's number 6.022×10^{23}

Average molecular weight of a nucleotide base pair (bp) as 660 Dalton.

Amplification efficiency, E%, was calculated from the slope of the standard curve using the formula:

$$E\% = (10^{-1/slope} - 1) \times 100$$

Analytical Specificity: It was calculated by employing same procedure and conditions on related bacteria belonging to α–2 subdivision of Proteobacteria like *Yersinia enterocolitica.*

Evaluation of the Assay: One Hundred samples (blood, semen and cervical swabs) collected from the livestock farms in the neighbouring area and Teaching Veterinary Clinical Complex, LUVAS, Hisar were tested for *Brucella* spp infection. DNA was isolated by Purelink DNA isolation kit (Invitrogen, USA). The purified DNA was used as template in real time PCR.

Comparison of Sensitivity of conventional PCR and Real Time PCR Assays: Serial ten fold dilutions (from 10^{-1} to 10^{-11}) of plasmid DNA of *Brucella* genus specific construct, was used as template for comparison of conventional and real time PCR. The conventional PCR was performed in thermal cycler (Veriti, ABI) in 12.5 µl reaction containing 3 µl of template DNA with high fidelity Phusion Taq mastermix (5 X) and GC buffer (New England Biolabs, USA). Cyclic conditions used in PCR were: Initial denaturation at 98^0C for 45 sec, and 35 cycles of denaturation at 98^0C for 15 sec, annealing at 60^0C for 30 sec and elongation at 72^0C for 30 sec followed by final elongation at 72^0C for 5 min. The real time PCR was performed as discussed above.

Table 1

Primer and probe for real time PCR

Primer probe pair name	Label/position	Primer sequence (5'-3')	Amplicon size (bp)
Brucella IS711 F	Bru/IS711/706-728F	GACATATTCAAAGTCCGGCGTAT	
Brucella IS711 R	Bru/IS711/808-788R	CAAATGGACAGCGGTTCATGC	102
Brucella IS711 P	Bru/IS711/748-773P	VIC- CCTTTCCCATACACCGGCGTGCGACC-BHQ1	

RESULTS AND DISCUSSION

Standardization of Primer Probe Concentration: Evaluation of results showed that 10 pmol concentration of forward and reverse primers and 5 pmol concentration of probe gave lowest Ct value in real time PCR assay (Fig. 1). These lowest Ct values were used as final concentrations for measuring sensitivity and specificity of assay. Minimum cycle threshold value for annealing temperature optimization was observed at 61^0C.

Limit of Detection and Assay Linearity: The concentrations of plasmid DNA of *Brucella* spp. specific constructs was 9.54 ng/µl in undiluted sample and copy no. was found to be 4.5×10^9. Ten fold serial dilutions (from 10^{-1} to 10^{-10}) of plasmid DNA were made. Ct values were observed up to 10^{th} dilution and the assay could detect as low as single copy of plasmid DNA. Amplification plots were plotted between fluorescence and cycles as shown in Fig. 2. The standard deviation curve was plotted between fluorescence and initial quantity (copies) of DNA for calculation of copy number of bacteria (Fig. 3). For *Brucella* genus specific assay R^2 (coefficient of determination) was found to be 0.998, slope of the curve was found to be -3.28 and amplification factor was 2.01 (Fig. 3).

Evaluation of the Assay: Out of 100 samples 13 samples were found positive and were giving Ct value in between 28 to 34 for *Brucella* genus specific qPCR. Amplification plots were observed as shown in Fig. 4.

Specificity: For specificity of the assay *Y. enterocolitica* belonging was tested and found negative with the *Brucella* specific qPCR. No Ct value and no amplification plot were observed.

Comparison of Sensitivity: It was observed that 10^{-8} dilution of plasmid DNA (concentration 20 fg) for *Brucella* genus specific construct was detected by conventional PCR performed with high Phusion Taq (5X) with GC buffer. While 10^{-10} dilution of plasmid DNA (concentration 0.2 fg) for *Brucella* genus, specific

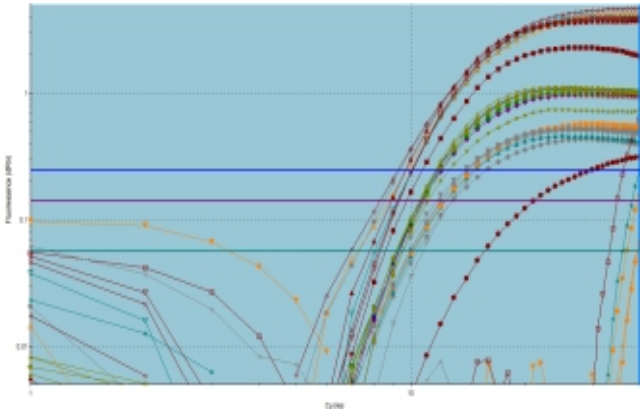

Fig 1. Amplification plots of *Brucella* genus specific qPCR for primer optimization

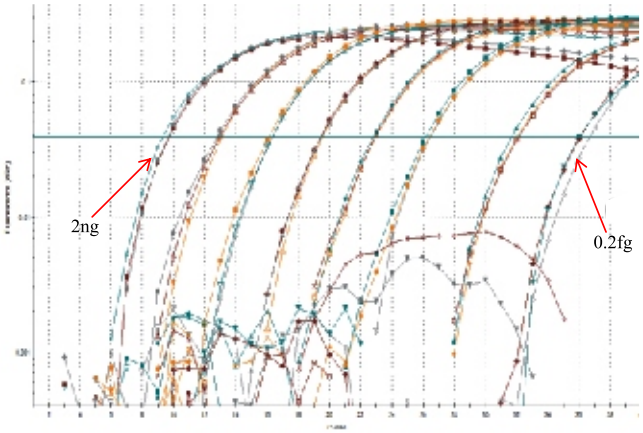

Fig 2. Amplification plots of serially 10 fold dilutions for *Brucella* genus specific qPCR

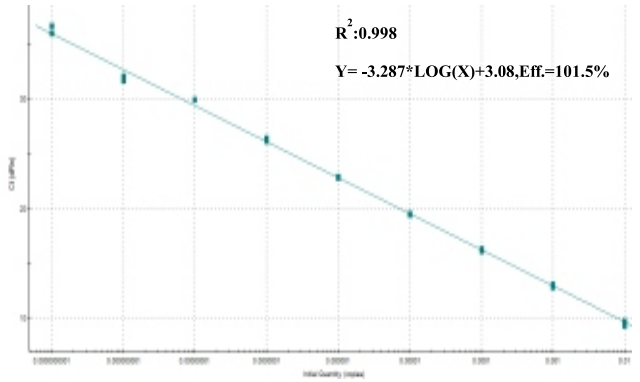

Fig 3. Standard curve along with linear equation using 10 fold serial dilution of *Brucella* genus specific qPCR

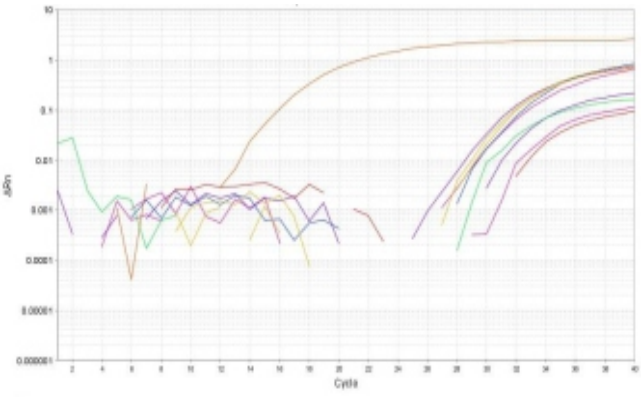

Fig 4. Amplification plots of field samples for *Brucella* genus specific qPCR

construct was detected by real time PCR (Fig. 2). These results indicated that real time PCR was 100 times more sensitive than conventional PCR.

Many diagnostic assays have been developed, tested and employed to support eradication of brucellosis (Probert *et al*., 2004; Bounaadja *et al*., 2009). New methods are continuously devised with an aim to reduce the turnaround time with high specificity and sensitivity. Real time PCR is one of the methods employed in the diagnosis of various diseases and is relatively recent in the field of diagnosis of brucellosis from clinical samples. Newby *et al*. (2003) described the IS711 based real time PCR assay with similar principle and the detection limit of the assay was found to be 7.5 fg for *B. abortus* DNA. In the present study, detection limit was found to be 0.2 fg for genus specific PCR. These results are in accordance with real time PCR assays described earlier (Redkar *et al*., 2001; Probert *et al*., 2004; Bounaadja *et al*., 2009) but our assay showed high sensitivity.

To check the perfection of developed assay the R^2 and efficiency (E) value of the generated standard curve was estimated. In the present study R^2 value and Amplification factor for *Brucella* genus specific real time PCR were 0.998 and 2.01, respectively. Similar kinds of results were observed by Newby *et al*. (2003) where the R^2 and Amplification factor were 0.9985 and 2.00, respectively. In this study, we also compared the sensitivity of TaqMan based qPCR assays with conventional gel based PCR. TaqMan based qPCR assays were found 100 times higher sensitive than conventional PCR assay. These results were in accordance with the study earlier described by Al Ajlan *et al*. (2011). Real time PCR based on dual labeled probe described in this study was found to be highly specific to *Brucella* spp. and didn't show any amplification with related bacteria such as *Y. enterocolitica*.

To conclude, real-time PCR developed in this study for detection of *Brucella* nucleic acid at genus level in the clinical blood samples showed excellent sensitivity and good specificity. Hence, the above attributes makes this assay a potent diagnostic tool for rapid detection of *Brucella* organism.

REFERENCES

Al Ajlan, H.H., Ibrahim, A.S., Al Salamah, A.A. (2011). Comparison of different PCR methods for detection of *Brucella* spp. in human blood samples. *Polish J. Microbiol.* **60(1)**: 27-33.

Alton, G.G., Jones, L.M., Angus, R.D. and Verger, J.M. (1988). Techniques for the brucellosis laboratory. INRA Paris.

Baily, G.G., Krahn, J.B., Drasar, B.S. and Stoker, N.G. (1992). Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *J. Trop. Med. Hyg*. **95**: 271-275.

Bounaadja, L., Albert, D., Chenais, B., Henault, S., Zygmunt, M.S. and Poliak, S. (2009). Real-time PCR for identification of *Brucella* spp.: a comparative study of IS711, bcsp31 and per target genes. *Vet. Microbiol.* **137**: 156-64.

Bricker, B.J. (2002). PCR as a diagnostic tool for brucellosis. *Vet. Microbiol*. **90**: 435-446.

Corbel, M.J. (1997). Brucellosis: an overview. *Emerg. Infect. Dis.* **3**: 213-221.

Cutler, S.J, Whatmore, A.M. and Commander, N.J. (2005). Brucellosis-New aspect of an old disease. *J. Appl. Microbiol.* **98(6)**: 1270-81.

Da Costa, M., Guillou, J.P., Garin-Bastuji, B., Thiebau, M. and Dubrai, G. (1996). Specificity of six gene sequences for the detection of the genus *Brucella* by DNA amplification. *J. Appl. Bacteriol.* **81**: 267-275.

Garin-Bastuji, B., Hummel, N., Gerbier, G., Cau, C., Pouillot, R., Da Costa, M. and Fontaine, J.J. (1999). Non specific serological reactions in the diagnosis of bovine brucellosis: experimental oral infection of cattle with repeated doses of *Yersinia enterocolitica* O:9. *Vet. Microbiol*. **66**: 223-233.

Newby, D.T., Hadfield, T.L. and Roberto, F.F. (2003). Real-time PCR detection of *Brucella abortus*: a comparative study of SYBR green I, 5'-exonuclease and hybridization probe assays. *Appl. Environ. Microbiol.* **69**: 4753-4759.

Nielsen, K., Smith, P., Widdison, J., Gall, D., Kelly, L., Kelly, W. and Nicoletti, P. (2004). Serological relationship between cattle exposed to *Brucella abortus, Yersinia enterocolitica* O:9 *and Escherichia coli* O157:H7. *Vet. Microbiol.* **100**: 25-30.

OIE (2009). Terrestrial Animal Health Code Brucellosis. http://www.oie.int/.

O'Leary, S., Sheahan, M. and Sweeney, T. (2006). *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. *Res. Vet. Sci*. **81**: 170-176.

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*. *J. Clin. Microbiol.* **42**: 1290-1293.

Redkar R., Rose, S., Bricker, B. and Del Vecchio, V. (2001). Real-time detection of *Brucella abortus*, *Brucella melitensis* and *Brucella suis*. *Mol. Cell. Probes.* **15**: 43-52.

Sambrook, J., Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual. (3rd edn.), New York, Cold Spring Harbor Laboratory Press.

Singh, B.B., Dhand, N.K. and Gill, J.P.S. (2015). Economic losses occurring due to brucellosis in Indian livestock populations. *Prev. Vet. Med.* **119(3–4)**: 211-215.

Treanor, J.J., Geremia, C., Crowley, P.H., Cox, J.J., White, P.J., Wallen, R.L. and Blanton D.W. (2011). Estimating probabilities of active brucellosis infection in Yellow stone bison through quantitative serology and tissue culture. *J. Appl. Ecol.* **48**: 1324-1332.