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Original Research Article

Diagnosis of Brucella Infections using in-house PCR

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Abstract

Purpose: Brucellosis is a significant and increasing public health and veterinary problem in India, with 80% of the population lives in close contact with domestic/wild animal. Hence, human population stands at a greater risk of acquiring zoonotic diseases including brucellosis. This zoonotic infection is endemic to North Karnataka and has been reported from several districts of this region consistently. The aim of this study was to standardise a PCR protocol for early and specific diagnosis of Brucellosis using whole blood samples. Using PCR for detection would cut down on the time required for diagnosis and provide higher sensitivity of detection.

Method: Blood samples of 19 patients collected over a span of two years from 2017 to 2019 who were seropositive and/or culture positive were used and DNA was extracted using a commercial spin column kit. PCR amplification was done for the detection of the BSCP31 gene which is common in all *brucella* species. Primers B4 and B5 were used for PCR assay.

Result: A total of five (5/19; 26.3%) samples were positive for the BSCP31 gene of which, two were both culture and serology positive, two were only seropositive and one was only culture.

Conclusion: The study shows that in-house PCR is useful in early and specific diagnosis of brucellosis reducing the risk of exposure for laboratory staff handling the samples as results are available in less than 24 hours. Further studies must be conducted to standardize the assay, assess the utility of this test for more chronic cases of infection, and to follow up of patients under treatment.

Keywords: Brucella PCR, brucellosis, zoonotic, North Karnataka.

Introduction

Human brucellosis is a major bacterial zoonosis reported worldwide. The causative agents belong to genus *Brucella*, which are Gram-negative, non-spore-forming, and non-encapsulated coccobacilli. More than 500,000 new cases are reported globally every year and the annual incidence varies from < 2 to 500 per 1,000,000 population in different geographical regions.[1] Brucellosis remains a major debilitating illness, causing severe human disease and high economic losses. Brucellosis is a significant and increasing veterinary and public health problem in India. In India 80% of the population living in approximately 5,75,000 villages and thousands of small towns; has close contact with domestic animals.[1] Close animal contact predisposes this huge human population to zoonotic diseases, major being brucellosis. Brucellosis is endemic to North Karnataka.[2] The chief modes of transmission are consumption of unboiled/ unpasteurized milk, inhalation/inoculation of pathogens through cuts/abrasions & exposure of mucus membranes to infected material etc.[1] Undulant fever, weight loss, and night sweats are the major symptoms of brucellosis. It is an important differential diagnosis of fever of prolonged duration in endemic areas.[3,4] Brucellosis is often misdiagnosed or underdiagnosed due to overlapping clinical manifestations with many other infectious and non-infectious conditions. The disease, therefore, cannot be diagnosed on clinical grounds alone, and microbiological confirmation is a must for evidence-based antibiotic therapy. The organism being a potential laboratory hazard, needs meticulous handling.[5,6,7] Dharwad and neighbouring districts show presence of brucellosis.[2] In our laboratory, we frequently encounter blood culture-positive & seropositive cases of brucellosis. Serology is the mainstay of diagnosis of brucellosis; however, it has a lot of limitations. The development of PCR assays has the potential to overcome major disadvantages associated with conventional laboratory

methods.[8]. In the present study, we aim to design a genus-specific PCR assay using blood samples from suspected cases of brucellosis.

Materials and Methods

1) Clinical samples were collected from suspected cases of brucellosis which were either culture-positive or seropositive by standard agglutination test (SAT).
2) Extraction of DNA was done by commercially available spin column kit (QIAamp DNA Mini Kit).
3) The extracted DNA was subjected to PCR assay targeting a conserved 223-bp sequence of the gene *bcsp*31encoding an immunogenic outer membrane protein common to all *Brucella* species using the primers B4 and B5.
4) PCR protocol for a 50 µl reaction was as follows

Table 1: PCR protocol for 50 µl reaction

Reagents	Quantity (µl)
Buffer 10x	05.0
$MgCl_2$ 25 mM	02.0
dNTP (2mM each)	20.0
Primer B4	01.0
Primer B5	01.0
Taq	00.5
DNA template	05.0
Water	15.5
Total	50.0

The amplification protocol run was as follows -

1. Initial denaturation – 93°C for 5 minutes
2. Amplification - 40 cycles

a. denaturation at 90°C for 1min
b. annealing at 60°C for 1 min
c. extension at 72°C for 1 min

3. Final extension - 72°C for 10 min

Amplicons were detected by electrophoresis in 2% agarose gel in the presence of ethidium bromide (1 µg/ml) using Vilber Lourmat, France gel documentation system.[9]

Observation and Results

In this study, a total of 19 EDTA blood samples were collected over a period of two years (From July 2017 to July 2019) from equal number of suspected cases of brucellosis. These samples were either *brucella* culture positive and/or seropositive by SAT.

Out of these 19 cases, 13 were males and 6 were females. The age group ranged from one year to 74 years. All *brucella* culture-positive cases gave a history of contact with live stocks and their products.

Five out of nineteen amplicons gave the expected band (223 bp) on the gel run. Known positive *brucella* DNA was used as positive control for the PCR. The amplicon was used in addition to DNA ladder. Out of five PCR-positive samples, two were culture and serology positive (1:320 and 1:1280), one was culture positive and two were serology positive with titres of 1:640 and 1:2560. Figure No 1 shows the gel electrophoresis picture with two samples giving a faint band. Figure No 2 shows expected band at 223 bp along with the known positive *brucella* DNA band.

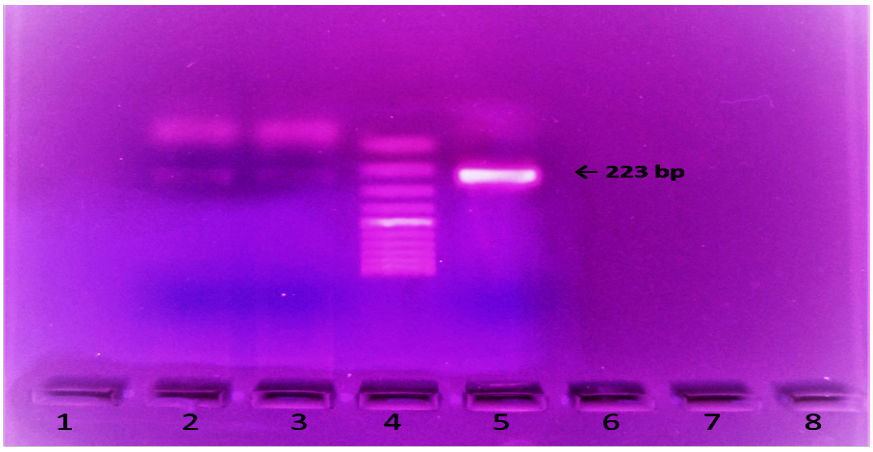

Figure No 1. Gel Electrophoresis picture. Lane number 2 shows faint positive band of Brucella culture sample at 223bp. Lane number 3 shows faint positive band of Brucella culture and serology positive (1:1280) sample. Lane number 4 shows DNA ladder. Lane number 5 shows known Brucella DNA at 223bp.

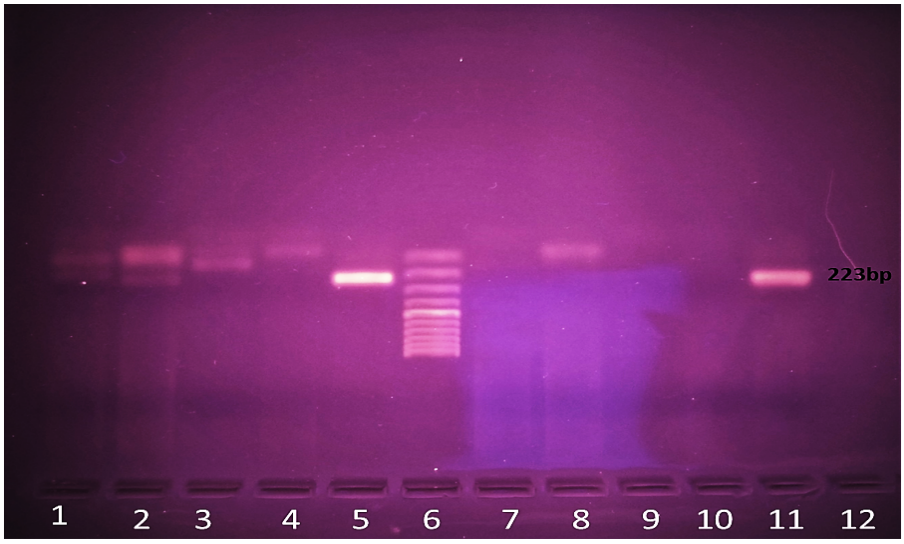

Figure No 2. Gel Electrophoresis picture. Lane number 2 shows positive band of brucella culture and serology positive (1:640) sample at 223bp. Lane number 5 shows known positive DNA band. Lane number 6 shows DNA ladder. Lane number 11 shows positive band of culture and serology positive (1:320) sample at 223bp.

Discussion

Culture is the gold standard for diagnosis of brucellosis. It commonly takes 5 days or longer and increases the hazards of handling the organism in the laboratory. Conventional serological methods have some basic limitations. Such methods display poor sensitivity in the early stages of the disease. Serological methods are also of limited value in assessing individuals who have been treated for brucellosis and are suspected of having a relapse. [5, 6, 7]

In this study, we tried to standardize the PCR assay on direct blood samples from suspected cases of brucellosis. Five of the 19 samples collected from suspected cases gave appropriate bands to confirm brucellosis. The assay proved to be sensitive because it detected *Brucella* DNA sequences directly in blood specimens from patients with blood culture-positive and serology-positive cases. The positive control *brucella* DNA used (223-bp fragment) in the assay gave the expected band in the gel run. The assay was rapid as it made available the results to the clinicians in less than 24 hours. Hazards of handling the organism in the laboratory were minimalized.[10]

The development of PCR assays is for there for some time; however, standardization of the methods for specific samples and organisms is lacking. A better assessment of clinical utility of PCR vis-à-vis conventional diagnostic methods is still needed. In our laboratory, we have established successfully *brucella* genus-specific PCR on isolates using B4 and B5 primers. The same PCR protocol was used on direct blood samples from suspected cases of brucellosis. The assay is more sensitive on blood culture-positive samples collected before initiating anti-brucella treatment as the bacterial DNA load reduces once treatment is started.[11] The assay is sensitive enough to give positive PCR results in low titre seropositive samples.[12]

In some reports this PCR method using the same primers showed different sensitivities and did not reproduce the same result, the reason being unknown. It is not surprising as the assay is complex and laboratory to laboratory variations are common.[8] It is, therefore, essential for the laboratories to standardize the protocols for their own setting. Matar and Morata showed excellent sensitivity using B4/B5 primers in diagnosing human brucellosis,[10, 13] but the same result was not reproduced by other groups.[14,15]

Serological methods have poor prognostic value in individuals who have been treated and when relapse is suspected as the antibodies remain in the blood for a very long period even after complete treatment.[7, 16]

Further work is required to assess the utility of this test for chronic cases and to follow the patients under treatment. The assay is prone to produce false-positive results in cases of relapse, which is not uncommon in *brucellosis*.

The application of PCR assay in diagnosis presents several advantages over the current methods used for *brucella* diagnosis and serotyping. The most important advantage is the speed to arrive at diagnosis in a single day compared to several weeks to culture the *brucella* organism. It is clear that PCR will be an important tool in the diagnosis of *Brucella* infections because of its sensitivity and specificity. More work on optimization and validation of this PCR assay especially to augment the sensitivity is essential. Modifications like dry reagent PCR [17]

will make it handy and hazzle free for diagnostic laboratories.

Conclusion

PCR assay for identification of *Brucella* from clinical samples is not available in any of the premier institutes of North Karnataka. It is cumbersome and potentially hazardous to handle the *Brucella* isolates for confirmation. This in-house genus-specific PCR assay will be an excellent addition to phenotypic methods of identification; it will reduce laboratory risk to the technical staff, will save time. It could be a specific and dependable assay for the diagnosis of brucellosis both in acute and chronic infections. More work on standardization of this PCR is needed in chronic and relapse cases.

Authors Contribution: All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Data Availability: All datasets generated or analyzed during this study are included in the manuscript.

Ethics Statement: This study was approved by Institutional Ethics Committee, SDM College of Medical Sciences and Hospital, SDM University, Dharwad, Karnataka, India, with reference number IEC/NMCRC/46/2022.

References

1. Pathak AD, Zunjar B. Dubal, Doijad S, Raorane A. Human brucellosis among pyrexia of unknown origin cases and occupationally exposed individuals in Goa Region, India. Emerg Health Threats J. 2014; 7: 23846.
2. Patil DP, Ajantha GS, Shubhada C, Jain PA, Kalabhavi A, Shetty PC, et al. Trend of human brucellosis over a decade at tertiary care centre in North Karnataka. Indian J Med Microbiol 2016; 34:427-32.
3. Ciftc¸i E, Ince E, Dogru U. Pyrexia of unknown origin in children: a review of 102 patients from Turkey. Ann Trop Paediatr. 2003; 23:259 263.
4. Baba MM, Sarkindared SE, Brisibe F. Serological evidence of brucellosis among predisposed patients with pyrexia of unknown origin in the northeastern Nigeria. Cent Eur J Public Health. 2001; 9:158-161.
5. Avhoven, P., E. Jansson, and K. Aho. Marked cross-agglutination between brucellae and a subtype of Yersinia enterocolitica. Acta Pathol. Microbiol. Scand.1969; 75:291–295.
6. Corbel, M. J. The relationship between the protective and cross reacting antigenes of Brucella spp., Yersinia enterocoliticaO:9, and Salmonella serotypes of Kauffmann-White group. N. Contr. Microbiol. Immunol.1979; 5:50–63.
7. Pellicer, T., J. Ariza, A. Foz, R. Pallares, and F. Gudiol. Specific antibodies detected during relapses of human brucellosis. J. Infect. Dis.1988; 157:918–924.
8. Navarro E. M. A. Cassao, J. Solera. Diagnosis of human brucellosis using PCR. Expert Rev. Mol. Diagn.2004; 4:115–123.
9. Mitka et al: J Clin Microbiology, 2007; 45: 1211-1218.Bruce D. Note on the discovery of a micro-organism in Malta fever Practitioner. 1887, 39:161-170.
10. Matar G.M, Khneisser I.A, Abdelnoor A.M. Rapid laboratory confirmation of human brucellosis by PCR analysis of a target sequence on the 31-kilodalton Brucella antigen DNA. J. Clin. Microbiol. 1996; 34: 477-478Hench PS, Bauer W, Dawson H, et al. Sixth Rheumatism Review. Ann Intern Med. 1940; 13:1655-1739, 1837-1990. See p.1706.
11. Che, L., Qi, C., Bao, W., Ji, X., Liu, J., Du, N., Gao, L., Zhang, K., & Li, Y. Monitoring the course of Brucella infection with qPCR-based detection. International Journal of Infectious Diseases, 2019;89: 66–71.
12. Queipo Ortuño, Mª Isabel & Morata, P & Ocon, Pilar & Manchado, PP & Colmenero, J. Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. Journal of clinical microbiology. 1997;35: 2927-30.
13. Morata P, Queipo-Ortuno M.I, Colmenero J.D. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. J. Clin. Microbiol. 1998; 36: 2443-2446.
14. Zerva L, Bourantas K, Mitka S, Kansouzidou A, Legakis N.J. Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. J. Clin. Microbiol. 2001; 39:1661-1664Lulu, A. R., G. F. Araj, M. I. Khateeb, and M. Y. Mustafa. Human brucellosis in Kuwait: a prospective study of 400 cases. Q. J. Med. 1988; 66(249):39-54.
15. Navarro E, Fernandez J.A, Escribano J, Solera J. PCR assay for diagnosis of human brucellosis. J. Clin. Microbiol.1999; 37: 1654.
16. Khalil, I A S Phkrykian, A D Farr. Brucella antibodies in Sudanese blood donors. Med. Lab. Sci. 1988; 45:312.
17. Kulkarni RD, Mishra MN, Mohanraj J, Chandrasekhar A, Ajantha GS, Kulkarni S, et al. Development of a dry-reagent mix-based polymerase chain reaction as a novel tool for the identification of Acinetobacter species and its comparison with conventional polymerase chain reaction. J Lab Physicians. 2018; 10:68-72.