Short Paper

Comparison of multiplex and ordinary PCR for diagnosis of paratuberculosis and tuberculosis in blood samples (buffy coat) of cattle and buffaloes

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Abstract

Background: Paratuberculosis and tuberculosis (TB) caused by *Mycobacterium avium paratuberculosis* (MAP) and *Mycobacterium tuberculosis* complex (MTC), respectively are economically important, chronic debilitating diseases affecting the dairy herds and are also potential zoonotic threats. **Aims:** Differential diagnosis of paratuberculosis and TB in blood samples of cattle and buffaloes. **Methods:** In this study, an in-house developed multiplex polymerase chain reaction (PCR) targeting MAP, *Mycobacterium bovis* and *Mycobacterium smegmatis* was used in blood samples (buffy coat) parallel with IS*900* PCR and *esx*B PCR for diagnosis of paratuberculosis and TB, respectively; in a total of 202 cattle and buffaloes. **Results:** Out of 202 animals, 12 (5.9%) and 17 (8.4%) animals were positive for MAP by multiplex PCR and IS*900* PCR, respectively; from which only 8 (4%) animals were positive by both tests; whereas 4 and 9 animals were exclusively positive by multiplex PCR and IS*900* PCR, respectively. None of the animals were found to be positive for *M. bovis* and *M. smegmatis* by the multiplex PCR. However, the *esx*B PCR detected 13 (6.4%) animals positive for TB. In fact, 3 (1.5%) animals were found to be co-infected by both paratuberculosis and TB. **Conclusion:** The in-house multiplex PCR detected MAP in buffy coat and there was a fair degree of agreement between the multiplex PCR and IS*900* PCR in detection of MAP DNA though the latter detected more number of animals to be positive for MAP. Besides, *esx*B PCR showed a high diagnostic potential and can be used for diagnosis of TB from blood.

Key words: Blood samples, Multiplex PCR, Paratuberculosis, Tuberculosis

Introduction

Paratuberculosis or Johne's disease (JD), caused by *Mycobacterium avium paratuberculosis* (MAP), is a chronic and progressive enteritis of domestic and wild ruminants, and is endemic in cattle of developing countries (OIE, 2019a). *Mycobacterium avium paratuberculosis* has impact on food safety and is also associated with Crohn's disease, which is a chronic inflammatory bowel disease in humans (Hermon-Taylor, 2009; Singh *et al*., 2010; Carvalho *et al*., 2012). Polymerase chain reaction (PCR) assays using primers specific for *F57*, IS*Mav2*, IS*MAP02*, and IS*MAP04* elements have been used for specific detection of MAP DNA. However, the higher number of copies of IS*900* element in comparison to other IS elements makes IS*900*-based detection very sensitive (Singh *et al*., 2010; Carvalho *et al*., 2012).

Tuberculosis (TB) is also an infectious chronic debilitating disease of zoonotic importance, affecting a wide range of livestock, domestic and wild animals, besides humans (OIE, 2019b). It is caused by the pathogenic mycobacterial species, *Mycobacterium tuberculosis* complex (MTC) comprised of *Mycobacterium bovis*, *M. tuberculosis*, *Mycobacterium africanum*, *Mycobacterium canneti*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, and *Mycobacterium caprae* (Olsen *et al*., 2010). Tuberculosis in milch animal is mainly caused by *M. bovis* (OIE, 2009). Molecular diagnosis of TB using *esxB* (CFP-10) PCR targeting *esxB* gene present in the region of difference 1 (RD1) region of the pathogenic mycobacterial species, is a fast diagnostic tool having higher sensitivity and specificity (Dikshit *et al*., 2012; Brahma *et al*., 2017).

Opportunistic mycobacterial infections due to environmental mycobacteria may also occur in immunocompromised animals. One such example is the *Mycobacterium smegmatis*, which is a saprophytic, rapid growing, atypical, non-tuberculous mycobacteria (NTM) (Quinn *et al*., 2011) and has the potential of causing opportunistic infection. *Mycobacterium smegmatis* has been reported from relapsing polygranulomatous mastitis in cattle (Siqueira *et al*., 2016) and was also detected in milk samples among other atypical mycobacteria, which

may be a public health concern (Franco *et al*., 2013; Bolaños *et al*., 2017).

Therefore, compared to the traditional culture and microscopy method of diagnosis, the molecular diagnostic technique using Blood PCR is rapid and has a high potential for diagnosis of JD (Bhide *et al*., 2006; Singh *et al*., 2013), as well as TB (Zali *et al*., 2014), especially during the bacteremic stage in subclinical or clinical stage. Therefore, in this study an in-house developed multiplex-PCR which differentiates mycobacterial infections caused by MAP, *M. bovis* and *M. smegmatis* was used in blood samples (buffy coat) of cattle and buffaloes for differential diagnosis of JD, TB and *M. smegmatis* infection. Further, this multiplex PCR was similarly complemented by conventional IS*900* PCR and *esx*B (CFP-10) PCR for comparison of the diagnostic potential for MAP and MTC, respectively.

Materials and Methods

Collection of blood samples and DNA extraction

A total of 202 animals (42 cattle and 160 buffaloes) aged 2 years and above were selected randomly from an organized dairy farm in Ludhiana. Whole blood samples were collected and buffy coat was obtained from 1 ml each of whole blood samples by centrifugation at 12,000 g for 10 min and then washed with phosphate-buffered saline (PBS) for 2-3 times by re-centrifugation until white pellet is obtained. The buffy coat was subjected to DNA extraction using QIAamp DNA blood mini kit (Qiagen). The DNA sample was stored at -20°C until further use.

In-house developed multiplex PCR

Multiplex PCR primers (Brahma *et al*., 2017) designed with the help of *in-silico* PCR targeting three mycobacterial species: MAP, *M. bovis*, and *M. smegmatis* (Table 1) were used.

Brahma *et al*. (2017), assessed the sensitivity of the in-house multiplex primers by using ten-fold serial dilution of the standard DNA of *M. tuberculosis* (IMTECH, Chandigarh), MAP (GENEKAM, Germany) and *M. Smegmatis* (Microbiologics). Specificity of the primers were cross-tested individually against genomic DNA of *M. tuberculosis*, MAP, *M. smegmatis*, and NTM species (*Mycobacterium fortuitum* and *Mycobacterium kansasii*) and non-mycobacterial species (*Brucella abortus*, *Pasteurella multocida* and *Escherichia coli*).

The DNA extracted from the buffy coat was amplified by in-house multiplex PCR primers and PCR conditions were followed as per Brahma *et al*. (2017).

IS*900* PCR

The DNA extracted from the buffy coat was also amplified by MAP species specific PCR based on the insertion sequence IS*900*. The sequences are as follows: Forward (IS*900*/150C): 5´- CCG CTA ATT GAG AGA TGC GAT TGG - 3´ and Reverse (IS*900*/921): 5´- AAT CAA CTC CAG CAG CAG CGC GGC CTC G -3´ designed to amplify a 229 bp target sequence (Vary *et al*., 1990; Singh *et al*., 2008). Reaction volume and PCR cycling conditions were followed as per Brahma *et al*. (2017).

*esx*B (CFP-10) PCR

The primer sequences for CFP-10 were as follows: Forward: 5´-ATG GCA GAG ATG AAG ACC GAT GCC GCT-3´ and Reverse: 5´-TCA GAA GCC CAT TTG CGA GGA CAG CGC C-3´ (Dikshit *et al*., 2012) giving a product size of 302 bp. Sample DNA from buffy coat was amplified by *esx*B (CFP-10) PCR, for detection of MTC. Reaction volume and PCR cycling conditions were followed as per Brahma *et al*. (2017) with a little modification, i.e., out of total 25 µL reaction volume, the DNA template was increased from 6 µL to 8 µL and nuclease free water was reduced from 4.5 µL to 2.5 µL, so as to increase the chances of detection, whereas the other reagent compositions were unchanged.

Results

In-house developed multiplex PCR

The sensitivity of *M. bovis*, MAP, and *M. smegmatis* primers were as little as 170 fg/µL, 300 fg/µL, and 51 fg/µL of genomic DNA, respectively. None of the organisms other than the specific standard DNA had

Table 1: Primer sequences for the in-house designed multiplex PCR

Target organism and strain	Primer sequence		Location of primer gene sequence	Size of PCR product
M. avium subsp. *paratuberculosis* MAP4 CP005928.1	Forward	5´- CGCGCGTACC TGACAAAAC - 3´	562055 - 562037	187 bp
	Reverse	5´- TCACCCTGAC ACTGACAGACA - 3´	561869 - 561889	
M. bovis strain SP38 CP015773.1	Forward	5´- GATGGTGGAA CACGACCACT - 3´	4138314 - 4138333	571 bp
	Reverse	5´- TTGATCGACC GTTCCGGTTT - 3´	4138865 - 4138884	
M. smegmatis MC2 155 CP009494.1	Forward	5´- ACCATGTCTAT CTCAGTGTGCT - 3´	3877883 - 3877904	628 bp
	Reverse	5´- ACGCTCGAGGT CCACTACAA - 3´	3878510 - 3878491	

amplification at the standardised annealing temperature i.e. 65.5-68°C which clearly indicates the specificity of in-house multiplex PCR (Brahma *et al*., 2017).

Out of 202 animals, 12 (5.9%) were found to be positive for MAP infection by in-house multiplex PCR (Fig. 1). However, none of the animals were found to be positive for *M. bovis* and *M. smegmatis.*

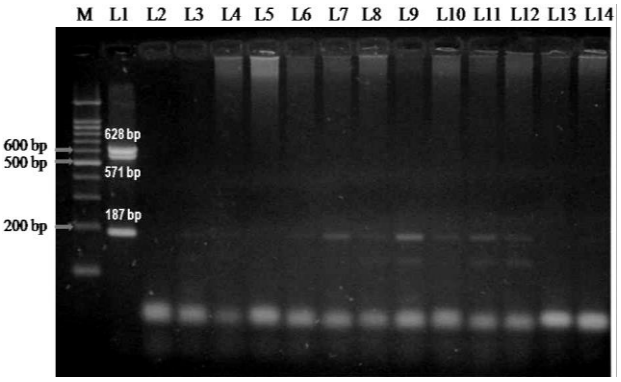

Fig. 1: Amplification of DNA from the buffy coat using in-house developed multiplex primers. Lane M: 100 bp plus ladder. L1: Positive control (*M. avium paratuberculosis*, *M. tuberculosis*, and *M. smegmatis*), L2: Negative control, and L3-L14: Blood samples

IS*900* PCR

The analytical sensitivity of the IS*900* PCR was upto 30 fg/µL of MAP DNA (GENEKAM, Germany) and no other bacteria other than the target species was detected indicating its specificity (Brahma *et al*., 2017).

Out of a total 202 animals screened for JD, a total of 17 (8.4%) animals were positive by IS*900* PCR (Fig. 2). However, only 8 (4%) out of 17 animals were positive by both the multiplex and IS*900* PCR; whereas 4 and 9 animals were exclusively positive by multiplex PCR and IS*900* PCR, respectively.

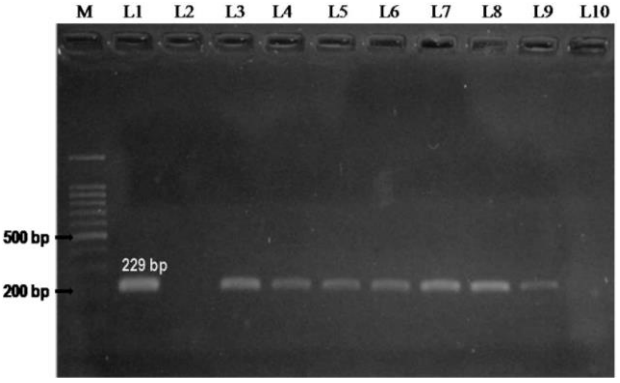

Fig. 2: Amplification of DNA from the buffy coat using IS*900* primers. Lane M: 100 bp plus ladder. L1: Positive control (*M. avium paratuberculosis*), L2: Negative control, and L3-L10: Blood samples

*esx*B (CFP-10) PCR

The analytical sensitivity of *esxB* (CFP-10) primers were upto 800 fg/µL of *M. tuberculosis* DNA (IMTECH, Chandigarh) and none of the NTM or other bacteria were detected (Brahma *et al*., 2017).

Out of the 202 animals screened for TB, only 13 (6.4%) animals were found to be positive for TB by *esx*B PCR (Fig. 3). In fact, the combined results of IS*900* and *esx*B PCR revealed incidences of co-infection with both JD and TB in 3 animals which had debilitated health condition.

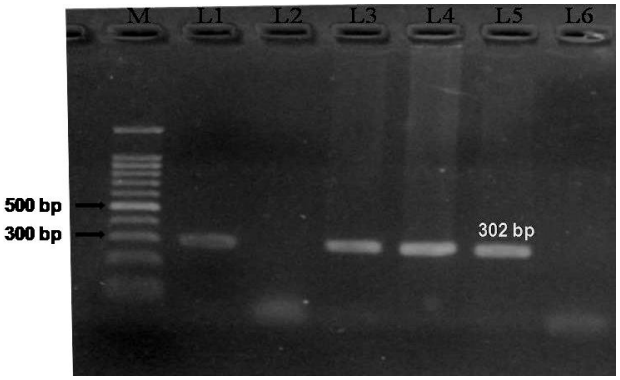

Fig. 3: Amplification of DNA from the buffy coat using *esx*B (CFP-10) primers. Lane M: 100 bp plus ladder. L1-L4: Blood samples, L5: Positive control (*M. tuberculosis*), and L6: Negative control

Discussion

In this study, the in-house developed multiplex PCR detected MAP in blood samples though it did not detect *M. bovis* and *M. smegmatis*. In contrast, Brahma *et al*. (2017), using the same multiplex PCR detected both MAP and *M. bovis* from tissue samples, though the sensitivity for detection of *M. bovis* was comparatively less than the *esx*B PCR. Amplification of *M. bovis* in multiplex PCR may be inhibited in blood samples due to PCR inhibitors or it could be presence of DNA in undetectably low concentration or due to presence of low copy number of the target sequence. In other studies by different workers, the accuracy of multiplex PCR for differential detection of *M. bovis* and *M. tuberculosis* was found to be 100% in terms of specificity and could detect as little as 20 pg of genomic DNA (Shah *et al*., 2002; Bakshi *et al*., 2005).

IS*900* PCR-based detection of MAP from white blood cells (WBCs) has also been reported by some workers (Naser *et al*., 2004; Stott *et al*., 2005). The reason for detection of MAP in more number of samples by IS*900* PCR compared to the in-house developed multiplex PCR could be due to the higher copy number of IS*900* element as reported by Vary *et al*. (1990) and Singh *et al*. (2010). However, using tissue samples, Brahma *et al*. (2017) detected MAP DNA in 2 out of 2 histopathologically JD positive cases by the in-house multiplex PCR.

In other studies, it has been found that MAP could be readily detected in blood from cattle with disseminated infections, and possibly in the blood of infected young and asymptomatic animals (Juste *et al*., 2005). Blood PCR targeting IS*900* gene was rapid, highly sensitive, and specific for detecting MAP infection at any stage of infection in any age of goats (Singh *et al*., 2010).

Mycobacterium avium paratuberculosis DNA was also detected in WBCs from about 11% of 262 Indian cattle of unspecified age or disease status (Bhide *et al*., 2006). Singh *et al*. (2013) found substantial agreement between "microscopy" and blood PCR detecting 39.3 and 13.1% positive by faecal microscopy and blood PCR, respectively; though Blood PCR detected more cases from heavy MAP shedders in microscopy. It may be assumed that low positivity in blood PCR may be due to presence of MAP in blood stream for a limited period (Singh *et al*., 2013). Gwozdz *et al*. (2000) contrarily showed poor performance of "blood PCR" to detect subclinically infected sheep. Barrington *et al*. (2003) had also recorded lower sensitivity of "blood-PCR" in comparison to PCR applied on milk, liver and faecal samples of advanced subclinically infected cows. The overall sensitivity of blood PCR for JD diagnosis from different studies was 11-66%. However, Youssef *et al*. (2014), reported that, out of 150 faecal samples, 31.33%, 29.33%, and 42.66% were found positive whereas MAP DNA was detected in only 4.67%, 3.33%, and 5.33% of milk samples by conventional IS*900* PCR, conventional F57 PCR and IS*900* real-time (RT) PCR, respectively. Gümüşsoy *et al*. (2015), in PCR of milk and stool samples, detected MAP DNA in 20 (13.61%) and 42 (28.57%) samples, respectively. However, MAP is only shed via milk in a small proportion of cows with subclinical JD for a limited period of time (Khol *et al*., 2013) and the shedding of MAP in faeces and milk is not synchronized (Gao *et al*., 2009).

Several PCR systems have also been developed for the diagnosis of TB, e.g., PCR amplification of *esx*A and *esx*B genes targeting ESAT-6 and CFP-10 proteins, respectively present in pathogenic mycobacterial species can be used for detection of *M. tuberculosis* as well as *M. bovis* (Pinxteren *et al*., 2000; Rogerson *et al*., 2006; Dikhsit *et al*., 2012). Using *esx*B PCR on tissue samples Brahma *et al*. (2017) detected all 7 out of 7 histopathologically TB positive cases, indicating its efficiency in TB diagnosis. Zali *et al.* (2014), using JB21 and JB22 primers specific for *M. bovis*, detected *M. bovis* positive in 8% of blood and lymph node samples of cattle. In this study, PCR targeting *esx*B (CFP-10) gene in blood samples detected 13 (6.4%) TB positive animals which were not detected by the in-house multiplex PCR, thus, indicating the early diagnostic potential of *esx*B (CFP-10) PCR. After all, 3 of the TB infected animals found to be positive for JD indicates presence of co-infection with both the diseases within the herd. Infection of either JD or TB may make the animals more susceptible to co-infection due to the immuno-compromised condition (Brahma *et al*., 2017).

In conclusion, "Blood PCR" using buffy coat was rapid, highly sensitive and specific, and the combination of various PCR techniques increased the chances of successful diagnosis of both JD and TB infection in cattle and buffaloes. The use of multiplex-PCR was able to detect MAP and differentiate from other mycobacterial infections in blood samples, thus providing a rapid diagnosis and its confirmation for the specific disease of concern, JD. However, the probable limitation is that, a large-scale study is required to determine whether this PCR assay is adequate for JD and TB control program.

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