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DETECTION OF *MYCOBACTERIUM AVIUM* SUB SP. *PARATUBERCULOSIS* (MAP) BY PCR IN THE FAECES OF DAIRY CATTLE OF CHITWAN, NEPAL

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ABSTRACT

Johne's disease or *Mycobacterium avium* subspecies *paratuberculosis* (MAP) causes chronic granulomatous enteritis with decrease in production resulting huge economic losses with high negative impact on the livestock industry. Diagnosis of MAP is difficult due to lack of characteristics clinical signs, prolong incubation period in cultivation of MAP, and non-specific results in diagnostic tests. To the best of our knowledge there is no report on faecal culture and molecular detection of MAP in dairy cattle of Nepal. The main objective of this research was to access the herd level prevalence of Johne's disease in the representative dairy farms of Chitwan district with the use of modern techniques as faecal polymerase chain reaction (fPCR) to know the MAP distribution in dairy cattle. A total of 265 individual dairy cattle faeces sample were collected during February 2017 to January 2018 from dairy farms of three different geographical location of Chitwan district, Nepal. Faeces were decontaminated and subjected for faecal culture as well as fPCR to have molecular detection of MAP. Findings revealed that bio-load of MAP in dairy cattle were 13.57% by faecal culture, and 16.59% by fPCR detection method. The overall prevalence of MAP in dairy cattle was detected as 16.59 % by fPCR. Likewise, IS*900* PCR assay proved to be a more sensitive and reliable test than faecal culture for the detection of MAP in faecal sample of clinically suspected dairy cattle as the PCR assay was able to detect significantly ($p < 0.01$) more positive cases than faecal culture. Findings of this study suggests that IS*900*-PCR-based detection of MAP could be used as a potential diagnostic tool for rapid and effective Johne's disease (JD) surveillance as compared with faecal culture detection method due to its advantage for JD control programs by reducing the time of definitive diagnosis from several months to a few days. This is the first molecular level of diagnostic research performed and reporting of MAP in dairy cattle of Nepal. These results will be useful in designing suitable disease control strategy for livestock industry.

Key words: Johne's disease, Diagnosis, *Mycobacterium avium* subsp. *paratuberculosis,* Faecal culture, fPCR, Nepal.

INTRODUCTION

Paratuberculosis, also known as Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is an important, endemic in many parts of the world, and considered as highly prevalent disease of domestic and wild ruminants manifest as a chronic granulomatous enteritis with decreased milk production, and in more serious cases leading to progressive emaciation and death (Yue et al., 2016). Clinical symptoms of paratuberculosis include slowly progressive wasting and chronic or intermittent, therapy-resistant diarrhoea (Stabel, 1997), which are intermittent at first, but become progressively more severe until they are constantly present. Affected animals become increasingly emaciated and usually die as a result of dehydration and severe cachexia (Cheng et al., 2020).

Johne's disease cause huge economic losses, and has high impact on livestock industry, due to premature culling of animals, reduced weight gain, reduced feed efficiency, high morbidity (Kaur et al., 2011); reduced carcass value, reduced milk production, increased susceptibility to mastitis and reproductive disorders resulting in increased calving intervals, reduced fertility and extra veterinary costs (Hasonova & Pavlik, 2006). Paratuberculosis is responsible for considerable economic losses causing disease within the dairy industry in the United States, estimated about more than $200 million per year (Groenendaal et al., 2015), and thus draws significant in developing as well as well developed countries. No country claims that it is free on MAP (Yue et al., 2016). The disease is considered one of the most important diseases of ruminant population (Sivakumar et al., 2005), however, MAP has also been isolated from a wide range of non-ruminant including horses, pigs, rabbits, foxes, stoats, weasels (Beard et al., 2001), humans (Chiodini et al., 1984) and non-human primates (McClure et al., 1987). Paratuberculosis shows similarities to Crohn's disease in humans (McAloon et al., 2019), suggesting that MAP is potentially a causative agent of Crohn's disease (Sechi & Dow, 2015), and has increased the possible zoonotic threat.

The disease is transmitted via milk and colostrum to calves and by the faecal-oral route to animals of all ages (Yue et al., 2016). According to Stevenson (2015), Intra-uterine transmission can also occur, as well as MAP can also be detected in the saliva of cows, indicating this as a potential further mode of transmission. Incubation period of the disease is long and variable before manifestation of clinical signs (Salgado et al., 2005). Prevalence of the infection vary world-wide (Kennedy & Benedictus, 2001), but most notably the apparent prevalence varies as

per the test method and strategies used (Nielsen &Toft, 2008). A range of diagnostic techniques are used to detect infection with MAP in cattle, but their performance can vary widely depending on the stage of MAP infection (Nielsen &Toft, 2006; Whitlock et al., 2000). For an effective control, early and confirmatory diagnosis is highly important (Singh et al., 2018). The test and cull strategy of JD control programs require sensitive and specific diagnostic techniques. Diagnosis of MAP is difficult due to lack of characteristics clinical signs, prolong incubation period in cultivation of MAP and non-specific results in diagnostic tests (Singh et al., 2007). Faecal culture is considered as the gold standard for the diagnosis of MAP infected animals, but requires 12 - 16 weeks (Chiodini et al., 1984), therefore, alternative serological and molecular techniques have been employed for diagnosis. The IS*900* element is an insertion sequence considered to be a MAP-specific gene with 15 - 20 copies per genome and is a target for rapid detection of MAP by PCR (Green et al., 1989). Direct PCR contributes not only in reducing the time of diagnosis but also in detecting potentially uncultivable MAP strains.

Chitwan district has a thriving commercial cattle farming, including many largest dairy farms in Nepal. Under this context, this research was done with the objective to isolate MAP field strains in dairy cattle using solid media culture coupled with PCR for confirming the presence of MAP in Nepal. Since, till date no such research has been done in Nepal to diagnose and detect herd-level prevalence of MAP infection, this research was done also to compare the efficacy of faecal culture and IS*900* PCR assay of faecal samples for the detection of bio-load of MAP in dairy herds of Nepal.

MATERIAL AND METHODS

Sample and Sampling

A total of 265 individual bovine faeces samples were collected during February 2017 to January 2018, from 116 household dairy farms (organized as well as unorganized) located in three major dairy pocket areas (Eastern Chitwan: Ratnanagar; Central Chitwan: Gitanagar, and Western Chitwan: Mangalpur) of Chitwan district of Bagmati province (Figure 1). The collected samples were transported to Veterinary Medicine Laboratory of FAVF, AFU, Rampur. The cattle herds were selected on random base and sampling were done from individual lactating cows of age ≥ 2 years showing diarrhea and poor body score (purposive sampling) as well as some samples were collected from animals showing no clinical signs.

Approximately, five grams of faeces were collected from each animal with a gloved hand directly from the rectum and placed in a sterile, leak proof container. The faecal samples were stored at 4°C until they were transported to the laboratory and up to 48 hours prior to processing for culture and DNA extraction.

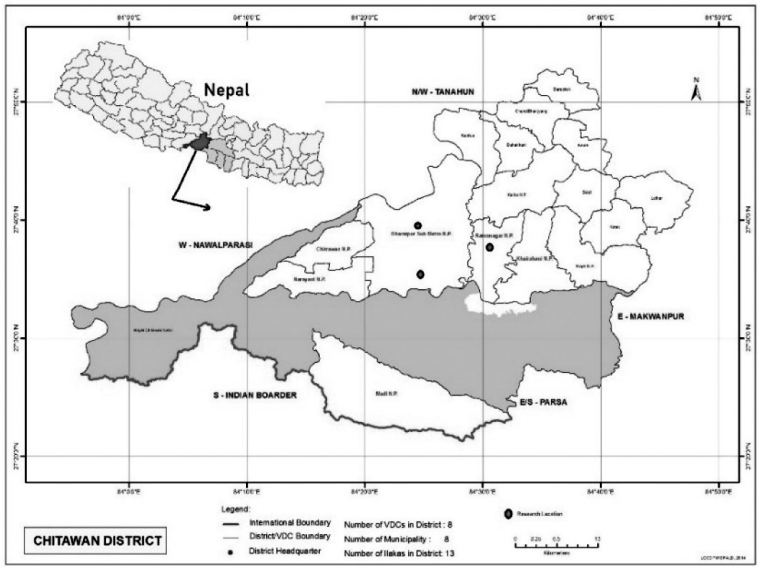

Figure 1. Map showing research location/sites

Decontamination of faecal samples

The faecal samples were decontaminated according to the protocol as described in OIE manual (2004), with some modification as described by Merkal (1970). One g of faecal sample was taken in 50 mL tube, added with 20 mL of sterile saline (to make 20 × solution). The solution was vigorously shake on vortex for 30 - 60 seconds and then stand for 30 minutes at room temperature. At this stage, the 5 mL supernatant as stock, 1 mL for PCR, and 5mL of supernatant for bacterial culture were used.

Then, transfer of 5 mL of the surface fluid was transferred to a fresh tube containing 20 mL of 0.75% hexadecylpyridinium chloride (HPC) in half strength brain heart infusion (BHI) broth was done and incubated overnight at 37°C. On the next day, centrifugation at 900×g for 30 minutes at room temperature was done and the supernatant was discarded by decantation. The pellet was re-suspended in 1 mL of a cocktail solution of antibiotics (100 µg/mL Vancomycin, 100 µg/mL Nalidixic acid and 50 µg/mL Amphotericin B) and proper mixing was done. Again, the solution was incubated at 37°C for 48 hours .

HEYM preparation and faecal culture

Herrold's Egg Yolk Medium (HEYM) supplemented with Mycobactin J was prepared as per manufacturer's guidelines and protocol. Mainly, the preparation protocol recommended by Ristow et al. (2006) and Stabel et al. (2004) with some modification followed to prepare the culture media.

The prepared 0.1 mL of sediment of faecal samples from the decontamination tubes were inoculated on slants tube of HEYM dispensed in Mc Cartney tubes using sterilized cotton swabs. The inoculated tubes were kept in slanted (horizontal) position for 4 - 5 days with caps loose and incubate at 37°C. Afterward, the tubes were returned to vertical position after the free moisture release from the slants. The caps of the tubes were tightened, and incubation was continued for 120 days period at 37°C. A control tube without mycobactin J was kept for each sample to demonstrate strict mycobactin dependency of MAP.

The incubated slants were observed at 15 days interval for colonies of MAP. Isolates of MAP were identified by their slow growth, acid fast staining, colonial morphology and mycobactin dependence. Isolated organisms were subjected to Ziehl-Neelsen staining and PCR analysis using IS*900* primers (Collins et al. 1993b) to confirm it as MAP.

Isolation of DNA from decontaminated faecal pellets and PCR

Faecal Pellets from decontaminated faecal sediment sample were processed for DNA isolation (van Soolingen et al., 1993) with some modifications. DNA was amplified from faecal sediment by faecal PCR (fPCR) using specific IS*900* Commercial PCR test primers (Vary et al., 1990).

Briefly, in a volume of 58 µL of master mix (forward primer: 150 C 24 mer, 1 µL, reverse primer: 921, 25 mer, 1 µL, Taq PCR master mix, (Qiagen), 30 µL and de-ionized water, 26 µL) and 2 µL of template DNA was added (total volume 60 µl). Total of 35 cycles were performed in a thermocycler (M J research) for complete amplification reaction. Reaction conditions were initial denaturation at 94 °C for 3 min. (one cycle), denaturation at 94 °C for 10 seconds, annealing at 61 °C for 10 seconds, extension at 72 °C for 10 seconds (35 cycles) and final extension at 72 °C for 3 minutes. Presence and yield of specific PCR product (229 bp) was analyzed by 1.8% agarose ethidium bromide gel electrophoresis. Positive (MAP 'Bison type') and negative (Sterile liquipure water) control were also run to check contamination.

Statistical methods

Data were tabulated using Microsoft Office Excel 2010 and analyzed using SPSS 21. Statistical comparisons between faecal culture and fPCR were done using the Chi square test (Snedecor & Cochran 1989) with $p < 0.05$ denoting statistical significance. SPSS software (Chicago, IL, USA) was used.

RESULTS AND DISCUSSION

This research presents a simple technique of detection of MAP by faecal culture and DNA extraction for direct identification of MAP by PCR.

Faecal Culture

A total of 36 out of 265 samples (13.57%) were positive in faecal culture. The isolates were only from the mycobactin J supplemented culture appeared at incubation period of 8 - 16 weeks. Primary bacterial colony characteristics were tiny (about one mm diameter), colorless, translucent and hemispherical (Figure 2).

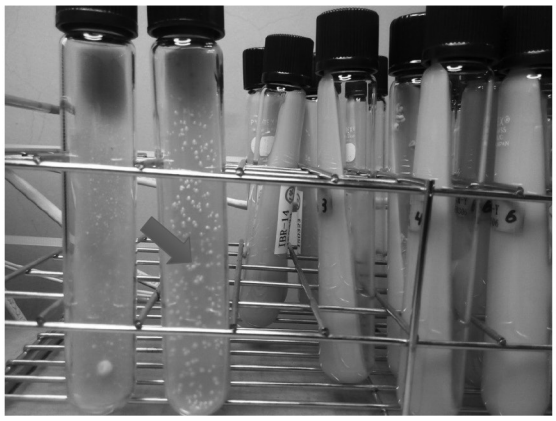

Figure 2. Growth of several bacterial colonies in 2 – 4 months of incubation in Mycobactin J supplemented HEYM media

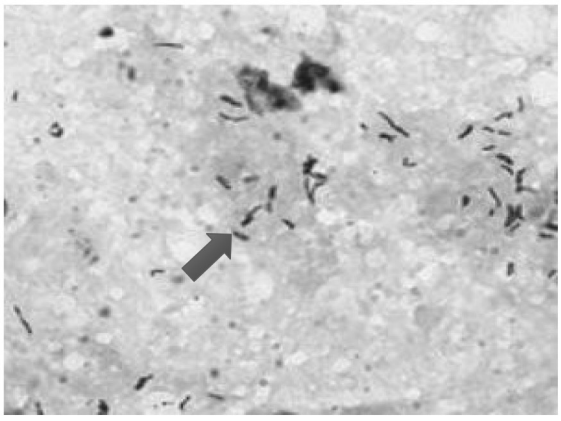

Figure 3. MAP in a ZN-stained smear of faecal sample demonstrating the numerous, slender, red-staining rods characteristic of MAP (×1000)

The colony's margins were round and even, and surface was smooth and glistening. Later, the colonies became rougher, opaque and increased in size on prolonged incubation.

After 4 months of incubation, tubes of HEYM from 3 samples were overgrown with contaminants. The smears prepared from all the isolates showed the presence of acid-fast bacilli such as the morphology of MAP. The DNA prepared from the 36 culture isolates of MAP was subjected to IS*900* primary PCR assay.

In this study, MAP isolates on culturing showed moist, smooth and glistening colonies in HEYM, which agreed with the reports of Merkal & Curran (1974), and OIE (2004). The growth of isolates was observed only in HEYM supplemented with mycobactin J whereas no growth was noticed in medium without mycobactin J supplementation. Mycobactin dependence of MAP strains for initial isolation was also reported by Tripathi et al. (2006), and Ronald (2007). Unlike most species of mycobacteria, MAP is a mycobactin auxotroph, unable to synthesize its own mycobactin and is therefore, required as a supplement in the culture media for growth. The smears prepared from all the isolates in this study showed the presence of acid-fast bacilli similar as the morphology of MAP (Figure 3), which is in accordance with the findings of Collins et al. (1993a) and Tripathi et al. (2006). Detection of MAP by culture is almost 100% specific but may yield false positive results due to the 'pass-through' effect in uninfected cattle that ingest MAP from the environment (Whittington & Sergeant, 2001). In our study, the percentage of positivity for faecal culture in dairy cattle was 13.57%. This result is quite low with that of Souyma et al. (2009) as the authors had reported 52.5% positivity in faecal culture in dairy cattle of India. This support the scientific evidence that the prevalence of MAP for faecal culture in dairy cattle of Nepal is comparatively lower, and thus the disease can be well managed on timely implementation of control measures.

Polymerase chain reaction (PCR)

A total of 44 faecal samples out of 265 were positive by primary PCR assay for MAP yielding an expected PCR product of size 413 bp, as shown in Gel electrophoresis of IS*900* PCR product from faecal samples of dairy cattle (Figure 4). The percentage of positivity for fPCR was 16.59%. The DNA prepared from the 36 culture isolates of MAP was subjected to IS*900* primary PCR assay, which yielded a single amplicon of 413 bp.

IS*900* PCR assay was used in this study for the screening of faecal samples/ bacterial cultures. Several researchers (Giese & Ahrens, 2000; Semret et al., 2006; and Tripathi et al., 2006) have used IS*900* PCR in their studies and reported it to be highly specific as well as sensitive, which support its potential value in the rapid and effective diagnosis of paratuberculosis. The results of PCR in this study were 16.59% which was very low as compared with the findings of Soumya et al. (2009) who reported quite high as 70%. Similarly, higher prevalence of fPCR has also been reported (Collins et al., 1993b; Vinodh Kumar, 2003; and Ronald, 2007) using IS900 nPCR assay for the detection of MAP from bovine faecal samples. The PCRs for IS*900* are commonly used to screen samples for MAP because it is a sensitive target, but other mycobacteria can harbour IS*900*-like sequences and the assay may not be 100% specific (Cook & Britt, 2007). Cousins et al. (1999) have reported that a small number of isolates of mycobacteria other than MAP possess IS*900*-like sequences detectable by IS*900* PCR and have produced an amplified product of the same size as that expected from MAP.

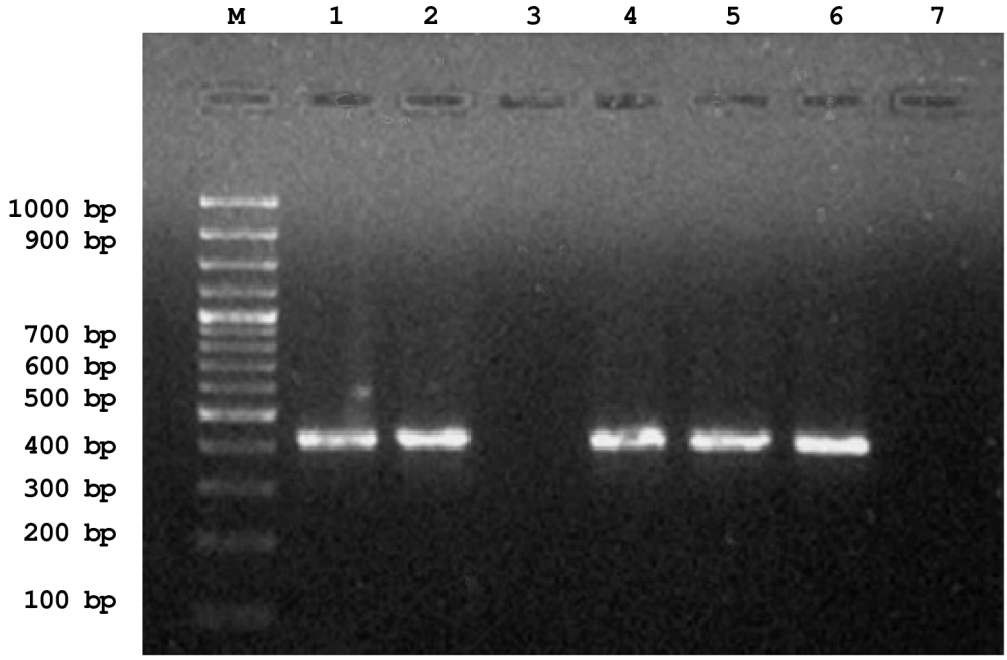

Figure 4. Gel electrophoresis of IS*900* PCR product from faecal samples of dairy cattle (Lane M as 100 bp DNA ladder, Lane 1, 2, 4 and 5 as PCR result of 413 bp from faecal samples whereas Lane 6 was PCR result from standard DNA

Comparison of diagnostic tests

Various studies have reported that PCR has increased sensitivity than faecal culture. The herd prevalence of MAP in faecal culture and PCR assay in this study was 13.57% (36/265) and 16.59% (44/265), respectively. Of the 265 samples tested, 36 samples were found positive for both faecal culture and PCR whereas PCR could detect additionally 8 samples as positive which were not detected by faecal culture. The chi-square analysis showed a significant difference between the tests ($p<0.01$). The findings showed that the proportion of positives observed by PCR was calculated to be significantly higher ($p<0.01$) than that observed by faecal culture, proving IS*900* PCR assay to be a more sensitive test than faecal culture for the detection of MAP in faecal samples of clinically suspected dairy cattle. The low prevaence in faecal culture in this study could be due to intermittent shedding of the bacterium in the faeces (Stabel, 1997), or due to substantial decrease in the bacterial load during specimen decontamination protocol (Whittington & Sergeant 2001; Reddacliff et al., 2003). Another possible explanation for the low prevalence in faecal culture in this study is that faecal culture may not necessarily give positive results in cows which shed low numbers of MAP in the faeces, as reported by Visser (1999). Faecal culture detected the infected animal only at the time the organisms were released into the gastrointestinal tract (Pavlik et al., 2000). Halldorsdottir et al. (2002) reported that the faecal culture had the detection limit of 102 CFU/g of faeces whereas PCR was able to reliably detect 50 organisms/g of faeces with improved sensitivity than the single round amplification PCR (Collins et al., 1993b). The PCR may be more sensitive than faecal culture, for the detection of MAP but this is difficult to ascertain because culture is the current gold standard test (Clark et al., 2008).

Overall prevalence of MAP in dairy cattle

The overall prevalence of MAP in dairy cattle of Chitwan district was 16.59%. This results agrees with that of the prevalence of the MAP in Egypt as 19.6% (Selima et al., 2019), higher than China (11.7%) as reported by Yue et al., (2016) and lower than India (39.3%) as reported by Singh et al., (2014) as well as Japan where Kobayashi et al., (2007) have reported the prevalence as 27%. The prevalence of Paratuberculosis in cattle in Australia, New Zealand, and Europe ranges from 10 to 60% (Harris & Barletta, 2001).

CONCLUSION

Faecal culture and fPCR were adopted for the first time in Nepal to detect MAP in the dairy cattle. In this study, comparative efficacy of faecal culture and IS*900* PCR of faecal samples was investigated for the detection of MAP infection in suspected cases of Johne's disease in dairy cattle. Findings revealed that bio-load of MAP in

dairy cattle were 13.57% by faecal culture, and 16.59% by fPCR detection method. Thus, 16.59 % prevalence rate of MAP in dairy cattle of Chitwan district, Nepal, as confirmed by fPCR. In this study, IS*900* PCR assay proved to be a more sensitive and reliable than faecal culture for the detection of MAP in faecal samples of clinically suspected dairy cattle since the PCR assay was able to detect significantly ($p<0.01$) more positive cases than faecal culture. The findings of this study suggest that IS*900*-PCR-based detection of MAP could be used as a potential diagnostic tool for rapid and effective Johne's disease surveillance. This is the first molecular level of diagnostic research performed, and first reporting of MAP in Nepal. These results will be useful in designing suitable disease control strategy for livestock in this country. Employment of the direct PCR test for detection of MAP would be an advantage for JD control programs by reducing the time of definitive diagnosis from several months to a few days.

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