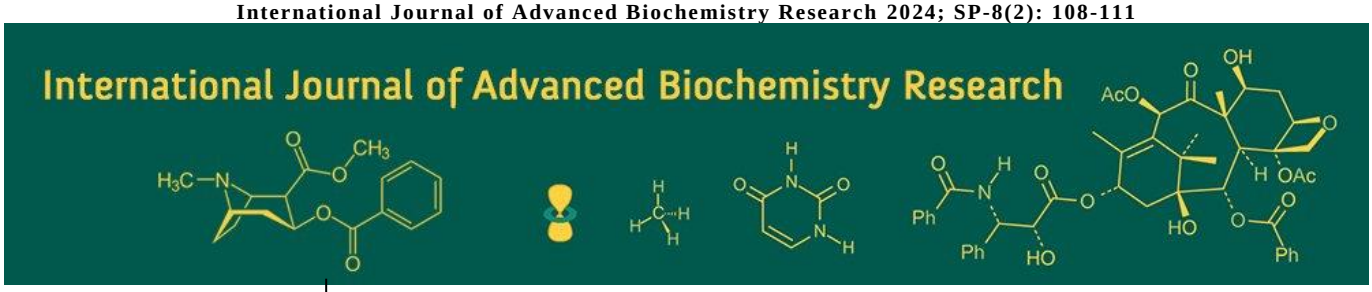

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Evaluation of conventional and molecular technique in detection of nontuberculous mycobacterium from nasal swab in cattle

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Abstract
Screening and identification of mycobacterium species from nasal swabs collected from cattle by culture isolation and PCR based test was performed for the detection of pathogen. Nasal swab was collected randomly from 112 cattle for the definite identification of Mycobacterial infection. Polymerase chain reaction was performed on the DNA extracted from nasal swabs targeting a 644 bp hsp65 gene which revealed 24 positive samples by amplifying specific region. Further culture, isolation from the nasal swab in Middlebrooks 7H10 medium shown growth from two samples and confirmed by acid fast staining. The two isolates were identified as *Mycolicibacterium smegmatis* and uncultured mycobacteria by partial sequencing of hsp65 gene. The prevalence of Nontuberculous mycobacteria by PCR from nasal swab was found to be 21.4% and PCR was found to have high sensitivity and specificity. It is suggested that PCR from nasal swab combined with other screening tests in live animals could be useful for early diagnosis and prevention of human infection from animal contact.

Keywords: Nontuberculous mycobacteria, NTM, *Mycolicibacterium smegmatis*, nasal swab, PCR

Introduction
Mycobacterium is the single genus in the family *Mycobacteriaceae* within the Order *Actinomycetales* (Magee and Ward, 2012; Nouioui *et al*., 2018) [12, 17]. Mycobacteria are aerobic to microaerophilic, non-motile straight or slightly curved, acid-fast bacilli with complex cell wall and the genome with high G + C content (57-73%) (Magee and Ward, 2012) [12]. The genus *Mycobacterium* contains nearly 200 species including strict and opportunistic pathogens affecting humans as well as animals. Among the strict pathogens, the principal pathogens of humans include *Mycobacterium tuberculosis* causing tuberculosis and *M. leprae* causing leprosy (Turenne, 2019) [25]. The term nontuberculous mycobacteria (NTM) generally refer to mycobacteria other than the *Mycobacterium* tuberculosis complex and *M. leprae*. Non-tuberculous mycobacteria (NTM) are known by several names including environmental mycobacteria, atypical mycobacteria or anonymous mycobacteria and MOTT-mycobacteria other than *Mycobacterium* tuberculosis (Sharma & Upadhyay, 2020) [21]. These organisms are ubiquitous in the environment *viz*., air, soil, dust, plants, natural and drinking water sources and are considered as saprophytic earlier. Now NTM are potentially pathogenic and emerging as serious public health impact particularly in immunocompromised humans and animals (Koh, 2017; Zulu *et al*., 2021) [11, 29].
Diagnosis of mycobacterial species by isolation and identification was definitive and gold standard but it is time consuming, unreliable and cumbersome with complex procedures. Rapid alternative methods had been attempted but PCR proves to be most promising (de la Rua-Domenech *et al*., 2006) [3]. The hsp65 gene is present in all mycobacteria, a highly conserved and more variable than 16S rRNA gene sequence (Telenti *et al*., 1993) [23]. The hsp65 gene can be used effectively for species identification of cultured clinical isolates of the genus *Mycobacterium* (Kim *et al*., 2005) [10].
In the present study, our aim is to evaluate the appliance of PCR amplification of DNA extracted from nasal swab and compare with conventional isolation methods in the early diagnosis of mycobacterial infection in cattle.

Materials and Methods

Nasal swab was collected from 112 cattle randomly by swabbing the nasal cavity 10-15 cm deep to adsorb the nasal secretion. The collected samples were prepared by immersing the swab in 2-3 ml PBS saline and decontamination were performed by modified Petroff's method with minor modification as described by Tripathi *et al*., 2014 [24]. Briefly, nasal swab was soaked in 2-3 ml of PBS for 30 min and swab was removed and then equal volume of 4% NaOH was added to the solution and incubated for 15 mins. Twenty millilitre of sterile distilled water was added to neutralize the solution and centrifuged at 3000 rpm for 15 mins. From the sediment, 100 µl was inoculated in to Middlebrook 7H10 medium, slants were incubated at 37^0C and observed for growth for 4 weeks. A part of nasal swab solution about 0.5 ml of phosphate-buffered saline solution was used for DNA extraction by HiPurA® Multi-Sample DNA Purification Kit (Himedia). A total of 200 µl of nasal swab solution was incubated with 20 µl Proteinase K and lysis buffer added at 55 °C for 10 min for lysis procedure. Further, 200 µl of ethanol was added to the mixture to prepare the mixture to bind in the spin column. The lysate was washed twice and the DNA was eluted with 100 µl of the elution buffer and stored at -80 °C for future use (Vitale *et al*., 1998) [26]. DNA was also extracted from the individual colonies of the isolate in the in the culture slants as described earlier by commercial kit method (Himedia). PCR reaction was performed as described by Kim *et al*. (2005) [10] with slight modification in a reaction mixture (25 µl) containing 12.5 µl of Taq master mix (Amplicon), 3 µl of DNA template and 0.2 µM of each primer [forward primer (5'-ATCGCCAAGGAGATCGAGCT-3'), reverse primer (5'-AAGGTGCCGCGGATCTTGTT-3')] targeting for hsp65 gene (644 bp PCR products). The cycling conditions were 30 cycles of amplification (denaturation at 95 ^{0}C for 60 s, annealing at 62 $^{\circ}$C for 45 s and extension at 72 ^{0}C90 s) followed by a 5 min final extension at 72 ^{0}C and PCR products were analysed by agarose gel electrophoresis using 1.5% agarose gel with ethidium bromide and visualized in gel documentation system. Species was identified by sequencing the amplified products by outsourcing. The obtained sequences of hsp65 gene were BLAST analyzed using Gene Bank online software of the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

PCR amplification of the DNA extracted from 112 nasal swabs revealed 24 samples as positive for hsp65 gene with a specific 644 bp amplification (Fig. 1) (Table 1). Isolation of mycobacterial species by inoculation of samples in Middlebrooks 7H10 medium shown growth in two samples within 5 days and identified as acid-fast bacilli. The prevalence of mycobacterial infection by PCR and Isolation from nasal swab was found to be 21.4% and 1.7% respectively. Samples which had mycobacterial growth were also given positive results in PCR conducted directly from nasal swab. Results of isolation was 100% in concordance with PCR results. The two isolates were identified as *Mycolicibacterium smegmatis* and uncultured mycobacteria by sequencing of hsp65 gene.

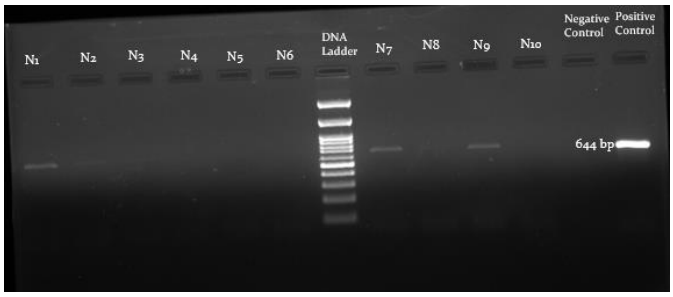

Fig 1: PCR amplification of 644bp hsp65 gene of Mycobacterial species. Samples N1, N2, N7, N9 are positive and N3-N6, N8 & N10 are negative by PCR

Table 1: NTM identification by PCR and Isolation

Detection of NTM	Positive	Negative	Total
PCR from nasal swab	24(21.4%)	88	112
Culture, Isolation	2(1.7%)	110	112

Discussion

Nasal secretion and milk are reported to be the main route of excretion of mycobacteria and shedding of bacteria is higher during first four weeks of infection which can survive and sustain infectivity up to 18 days in humid conditions (Jalil *et al*., 2003, Kao *et al*., 2007, McCorry *et al*., 2005) [8, 9, 15]. Molecular screening of animals by PCR is a rapid, very sensitive and cost-effective method which could be employed as an rapid diagnostic tool and PCR from biological samples like nasal swab and other body fluids can be effective for early diagnosis of mycobacteria (Romero *et al*., 1999, Eustáquio de Souza Figueiredo *et al*., 2010; Vitale *et al*., 1998, Serrano-Moreno *et al*., 2008) [19, 4, 26, 20]. In the present study, 112 nasal swabs collected from cattle were used as samples, in which 24 samples carried *Mycobacterial* DNA as by PCR based method. Our study reported higher prevalence of 21.4% by PCR conducted directly from nasal swab and also much higher than isolation method (1.7%). The results indicates that PCR has higher sensitivity in identifying positive samples. Similarly, Eustáquio de Souza Figueiredo *et al.* (2009) [4], also reported higher prevalence of 5.9% by mPCR from nasal swab over isolation method (0%). Zanini *et al*. (1998) [27] and Zumarraga *et al*. (2005) [30] evaluated PCR from nasal swab and milk in routine diagnosis of mycobacteria and reported its high sensitivity and specificity. In contrast, Mayer *et al*. (2015) [14] reported that screening of nasal swab by PCR was not a suitable tool in the diagnosis of bovine tuberculosis by comparing with comparative cervical test. The prevalence of NTM by conventional isolation method was1.7%. Earlier worker reported higher prevalence than the present study which is 50% and 14.8% by Gcebe *et al*, (2013) [6] and Zulu *et al* (2021) [29] respectively. The less prevalence by isolation from nasal swab is due to sample design, isolation and decontamination procedure employed. Further, only two animals were found positive by conventional culture method this could be due to the fact that even though isolation is the gold standard test, it is a slow, cumbersome, unreliable due to decontamination procedure and it depends on time of shedding of the organism through nasal secretion.

The two isolates were identified as *Mycolicibacterium smegmatis* and uncultured mycobacteria by sequencing and annotation of hsp65 gene. *M. smegmatis* is a nonpathogenic

and fast-growing species and also reported to be ideal surrogate for mycobacterial research (Sparks *et al*., 2023) [22]. In animals *M. smegmatis* causes mastitis and has been isolated from milk samples by earlier researchers (Cvetnić *et al*., 2022; Ghielmetti *et al*., 2017; Masello *et al*., 2013) [2, 7, 13]. In humans, *M. smegmatis* was reported to cause skin or soft-tissue infections and disseminated infections related to immunosuppression (Newton *et al*., 1993, Pennekamp *et al*., 1997, Best & Best, 2009) [16, 18, 1]. Different NTM strains was also identified by previous researchers from nasal swab (Gcebe *et al*., 2013; Gcebe & Hlokwe, 2017; Zaragoza Bastida *et al*., 2017) [6, 5, 28]. Results of isolation was 100% in concordance with PCR results. Two samples were positive by both conventional and PCR which indicates PCR screening is highly sensitive as well as highly specific. Similar result was also reported by Vitale *et al*., (1998) [26] stating that PCR has high specificity and positive predictive value and nasal swab can be used as sample for general tuberculosis screening.

In this study, two isolates obtained by conventional method were identified as NTM, remaining 22 samples positive by PCR amplification needs further investigation and identification.

Conclusion

In conclusion, PCR from nasal swab amplified more number of positives, indicating that the PCR test is highly sensitive and can be combined with other screening tests being conducted in live animals for early diagnosis and adoption of preventive measures could be adopted accordingly. Since it is less time consuming, screening the samples by PCR prior to diagnostic tests can give presumptive idea on prevalence of mycobacteria in the study region.

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