



PCR Detection of Nontuberculous Mycobacteria 16s Rrna in Cows and Sheep Subclinical Mastitis

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Abstract | The primary goal of the present study was to determine the association of NTM with subclinical mastitis in dairy cows and ewes through the following objectives: Estimation of Subclinical mastitis prevalence and Investigation of usefulness of 16SrDNA for rapid PCR detection of NTM in clinical samples. A total of 120 milk and faecal samples were collected from 70 cow and 50 ewes to identify subclinical mastitis cases using The Modified White Side Test (MWST) and 16S rDNA-based PCR. The results of MWST revealed that relatively higher prevalence (83%) of SCM was observed in cows followed by prevalence of SCM in ewes (76%). The difference between this two prevalence was not considered to be statistically significant ($p > 0.05$). The highest prevalence of slight (38%) and severe (31.4 %) SCM reactions are observed in ewes and cows respectively. PCR Detection of NTM 16S rRNA in milk samples indicated that the higher prevalence (22%) of SCM was observed in ewes followed by prevalence of SCM in cows (6%). The difference between this two prevalence was considered to be statistically significant ($p < 0.05$). The role of NTM as a causal agent of SCM was investigated by PCR amplification of NTM 16S rRNA gene in faecal samples of cows and ewes. The higher prevalence (21.7%) of SCM was observed in cows followed by prevalence of SCM in ewes (16%). The difference between this two prevalence was not considered to be statistically significant ($p > 0.05$).

Keywords | 16S rRNA, Mastitis, NTM

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INTRODUCTION

Non-tuberculous mycobacteria (NTM) are non-motile, acid-fast rods with varying sizes; they also do not produce spores. Members of this genus' cell walls are rich in lipids, making them resistant to host defenses and causing

chronic illness. These microorganisms can be identified by their ability to produce mycolic acid, which offers resistance against host cell defenses, virulence, pathogenicity, and acid-fast characteristics of the cell wall (Daffé *et al.*, 2017).

For most people living in low- to middle-income nations,

milk and dairy products are their main source of protein. The world's milk output is largely derived from the raising of cattle and buffaloes. Kumar *et al.* (2007). Nevertheless, production per head has stayed low despite genetic upgrading and contemporary techniques for raising animals (Ramesh and Divya 2014). Mastitis, particularly subclinical forms, may be one cause of this. Still ranks among the dairy industry's most prevalent global economic issues, which is primarily caused by bacteria, causes inflammation and pathophysiological changes in the udder tissue, which lowers milk production and compromises milk quality.

Clinical and subclinical forms of the disease can be distinguished based on the degree of inflammation (Viguiet *et al.*, 2009). Subclinical mastitis (SCM) is harder to diagnose than clinical mastitis due to asymptomatic symptoms. However, two thirds of the financial losses in the whole milk production industry are caused by SCM (FAO 2014). As a result, understanding routine diagnostic screening procedures for early diagnosis of mastitis is important for treating the problem and avoiding eventual economic losses (Galdhar and Roy, 2003).

There are a lot of indirect tests to detect subclinical mastitis like Modified White Side Test (MWST), California mastitis test (CMT), MDST (Mastrip) and bromthymol blue card test (BTB). The basis of Whiteside test and CMT is same which reflects the presence of excess leukocytes in milk as an indicator of inflammation. The Whiteside test is quick, simple and inexpensive in comparison to CMT (Kahir *et al.*, 2008)

Due to the limitations of traditional approaches, molecular techniques—which have the advantages of being quick, sensitive, and specific—have been developed for the identification of Mycobacteria. Over the last two decades, molecular approaches for identifying mycobacteria have considerably advanced (Yu *et al.*, 2014; Kim *et al.*, 2005). The most significant and specific method for detecting NTM in clinical specimens is 16S rRNA replication followed by gene sequencing. In some cases, Mycobacterium species can be identified to the species level by sequencing the whole 16S rRNA gene (Tortoli, 2014; Tortoli *et al.*, 2017). The sequencing of conserved genomic regions is widely accepted as the gold standard for identifying mycobacteria (Perez-Martinez *et al.*, 2005). A recent study found that the owner's saliva and camel milk both had nontuberculous mycobacteria. This implies that human and camelid infections can occur in the same manner (Asaava *et al.*, 2020). Because of the losses incurred from incorrect diagnoses of other diseases caused by notifiable mycobacteria, NTM in cattle are typically of production and economic significance (Biet and Boschioli, 2014). Furthermore, a number of NTM that were identified from animals may be zoonotic (Araya and Fikre, 2014; Thoen *et al.*, 2014). Numerous

mycobacteria other than tuberculosis are real or competing pathogens for humans (Gcebe *et al.*, 2018; Nishiuchi *et al.*, 2017). These NTM species are commonly found as biofilms in the environment (Nishiuchi *et al.*, 2017).

NTM species are geographically diverse; the *M. avium* complex is the most frequently isolated, followed by mycobacteria that grow quickly, like *M. fortuitum* and *M. abscessus* (Honda *et al.*, 2018). Since most countries' public health agencies don't consider NTM to be communicable diseases, there are insufficient requirements for reporting, and there are few reliable NTM statistics worldwide (Winthrop *et al.*, 2017). With the exception of a few studies on Mycobacterium avium sub sp. paratuberculosis, the etiological agent of Johne's disease, conducted in Basrah, Iraq, reports of animal infections caused by NTM are rare (Alzaidi, 2018; Kawther *et al.*, 2018; Kawther *et al.*, 2019). NTM was found in clinical samples suspected of containing Mycobacterium tuberculosis complex (MTBC) in other studies were carried out in Basra, Iraq (Al-Mussawi, 2014; Al-Sulami *et al.*, 2016). Consequently, the present study was conducted to determine the association of NTM with subclinical mastitis in dairy cows and ewes through the following objectives: (1) Estimation of Subclinical mastitis prevalence (2) Investigation of usefulness of 16SrDNA for rapid PCR detection of NTM in clinical samples.

MATERIALS AND METHODS

In several regions of the southern Iraqi provinces of Basrah and Thi-Qar, the current study was conducted between March, 2022 and December, 2022. In collaboration with a veterinary hospital staff and animal owners, 70 cows and 50 ewes of different breeds and ages provided milk and dung for clinical testing. A total of 0.2-10 g of fresh faeces were collected and put into screw capped clean tubes. Following udder cleaning and discarding the first three jets, 10 ml of milk was manually milked from each of the four quarters into a sterile tube.

SAMPLES COLLECTION

MILK SAMPLES: After washing the udder with a piece of cloth, the samples were collected using 70% alcohol-moistened cotton and the first milk flow was withdrawn and 10 ml collected in a sterile tube, then transported to Laboratory with an ice box (Khaleel *et al.*, 2016). Mastitis quarter milk samples were obtained, and milk samples from healthy cows from one of the cow's hind quarters were collected at random. Upon arrival, the samples collected were immediately stored at 4 ° C for a period of 24 hours, until the next day they were separated into 2ml aliquot and placed for further processing at 20°C.

FAECAL SAMPLES: In sterile, disposable, lidded plastic

containers, fresh, moist feces samples ranging in weight from 0.2 to 10 grams were collected. All samples were transported under cold circumstances to the laboratory and stored at -20 degrees Celsius until use.

MASTITIS SCREENING USING (MWST)

The WST was performed as per the modified test described by Kahir *et al.*, (2008). The reagent used for modified Whitteside test is 4% sodium hydroxide solution. Five drops of well mixed milk are placed on a glass plate with a dark background and two drops of 4 % sodium hydroxide are added to the milk. The mixture of milk and reagent should be stirred rapidly with an applicator stick for 20 to 25 seconds milk from normal animals will have no change after the addition of sodium hydroxide. Milk from a cow suffering with acute or subacute mastitis will become thick and viscid, but that from an animal with a chronic case may have only a few white flakes. The basis of MWST and CMT is same which reflects the presence of excess leukocytes in milk as an indicator of inflammation. The scores for the MWST reactions are as follows:

- Negative (N): There is no precipitant present at all, and the combination is opaque and milky.
- 1+: The background is less opaque but still slightly milky, with larger particles of coagulated materials present and densely dispersed across the area. A minor amount of clumping is seen.
- 2+: The background appears more watery, with numerous clumps of coagulated material present. If the stirring was rapid, fine threads or strings could be present.
- 3+: The background is extremely watery and whey-like, with enormous amounts of coagulated material forming into strings and shreds.

DETECTION OF NTM BY PCR

DNA extraction: Following the manufacturer’s instructions, the genomic DNA was extracted from milk and samples using the SYNCTM DNA Extraction Kit (Genaid, Korea) and the PrestoTM Stool DNA Extraction Kit (Genaid, Korea), respectively. On 1% agarose gel stained with Green Star TM Nucleic Acid Staining BioNeer (Korea), the extracted DNA was verified. The Nanodrop spectrophotometer (Quawell, USA) was then used to measure the concentration and purity of the extracted DNA using a calibration of 260/280 nm.

PCR amplification: The AccuPower PCR premix (Cat

K-2012, Bioneer, Korea); as the PCR reaction premix; which contained 1 U of Top DNA, 250 μM of dNTPs, and 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, and 1.5 mM of MgCl₂, Taq PCR Premix (5 μl), forward primer (1 μl), reverse primer (1 μl), DNA template (3 μl), nuclease free water (10 μl) made up the reaction mixture (20μ l). A PCR thermocycler (Bioneer, Korea) was used to amplify the 16S rDNA gene (976 pb) using the forward (AGAGTTT-GATCMTGGCTCAG) and reverse (GTAAGGTTCT-KCGCGTTGC) primers (Joao *et al.*, 2014). The amplification conditions included three minutes at 95°C, thirty-five cycles of 45 sec at 95°C, 45 sec at 55°C, one min at 72°C, and a final extension for 5 min at 72°C. The PCR results were examined in 2% agarose gel using a UV-trans-illuminator (Vilber Lourmal CE; Taiwan) following electrophoresis with a safety dye (Green DNA DYE, Biotech, USA) at 110 volts for 60 min.

THE STATISTICAL ANALYSIS

In order to collect and clear up all the data, MS Excel version 2016 was used. To determine significantly different prevalence of SCM in cows and ewes; at a 5% significance level; z test was employed.

RESULTS

RESULTS OF MODIFIED WHITE SIDE TEST SCREENING

The overall prevalence of SCM and the distribution of SCM in cows and ewes were shown in Table 1. relatively higher prevalence (83%) of SCM was observed in cows followed by prevalence of SCM that found in ewes (76%). The difference between this two prevalence was not considered to be statistically significant (The value of z is 0.9466. The value of p is .34212. The result is not significant at p < .05).

Prevalence of SCM according to different reaction score were displayed in Table 2. The prevalence of SCM in cows and ewes. The highest prevalence of slight (38%) and severe (31.4 %) SCM reactions are observed in ewes and cows respectively.

Table 1. Prevalence and distribution of subclinical mastitis in cows and ewes in cows and ewes.

Samples	No.	+ve (%)	-ve (%)	p-value
Cows	70	58 (83)	12 (17)	> 0.05
Ewes	50	38 (76)	12 (24)	> 0.05

Table 2. Subclinical mastitis reaction scores (%) in cow and ewes.

Samples	Positive samples	Reaction WST			
		Slight (+)	Moderate (+ +)	Severe (+ + +)	Negative
Cows	70	25 (35.7 %)	11 (15.8 %)	22 (31.4 %)	12(17.1%)
Ewes	50	19 (38%)	8 (16%)	11 (22%)	12(24%)

DETECTING NTM 16S rRNA IN MILK SAMPLES VIA PCR

The amplified products of the NTM 16SrRNA gene sequence (976 bp) was detected in 3 cow and 11 sheep milk samples (Figure 1). In Table 3, higher prevalence (22%) of SCM was observed in ewes followed by prevalence of SCM in cows (6%). The difference between this two prevalence was considered to be statistically significant (The value of z is -2.4874. The value of p is .01278. The result is significant at $p < .05$).

Table 3. Prevalence of SCM according to 16S rRNA PCR results of the tested milk samples.

Samples	Examined	Positive (%)	Negative (%)	p-value
Cows	50	3 (6%)	47 (94%)	<0.05
Ewes	50	11 (22%)	39 (78%)	<0.05

PCR DETECTION OF NTM 16S rRNA

IN THE TESTED FAECAL SAMPLES

The role of NTM as a causal agent of SCM was investigated by PCR amplification of NTM 16S rRNA gene. The PCR results showed that amplified product (976 bp) was discovered in 13, and 8 faecal samples from cows and ewes, respectively. The higher prevalence (21.7%) of SCM was observed in cows followed by prevalence of SCM in ewes (16%) (Figure 1; Table 4). The difference between this two prevalence was not considered to be statistically significant (The value of z is 0.7944. The value of p is .42952. The result is not significant at $p < .05$).

Table 4. Prevalence of SCM according to 16S rRNA - PCR results of the examined faecal samples.

Samples	No.	Positive (%)	Negative (%)	p-value
Cows	60	13 (21.7)	47 (78.3)	>0.05
Ewes	50	8 (16)	42 (84)	>0.05

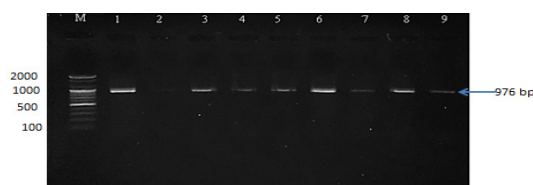


Figure 1. PCR result of NTM 16SrRNA gen (976bp), M: ladder, 1 and 3-9: positive results; and 2: negative result.

DISCUSSION

The occurrence of NTM is rarely studied in veterinary medicine, with the exception of *Mycobacterium avium* subsp. *Paratuberculosis* (Biet and Boschioli, 2014). Thus, iso-

lation of NTM is frequently a subsequent result in bovine tuberculosis (bTB) examinations. Consequently, the vast majority of articles report the isolation of multiple NTM species within the context of TB surveillance programs. Commercially available kits are expensive and ineffective at identifying uncommon or novel species of *Mycobacteria*, even though they are good at identifying the most prevalent clinical species (Tortoli *et al.*, 2010). In this case, other alternatives must be developed to provide an accurate diagnosis that is within the capabilities of most laboratories. This study used the highly conserved gene; 16S rRNA; to identify mycobacteria in order to accurately identify NTM. Many studies had been used 16S rRNA for identifying NTM. Joao *et al.* (2014) found that 16S rRNA should be examined first for accurate identification of NTM; because it's the gold standard and has demonstrated superior performance in identifying the most common NTM. Beside that other prior research has employed the 16S rRNA to identify this bacteria (Ghielmetti *et al.*, 2018; Abdullah and Abdullah, 2022).

According to milk samples PCR analysis, the overall percentage of subclinical mastitis of cows and ewes were 6% and 22%, respectively. The reported percentage of cow subclinical mastitis in the present study were lower than those of Abdullah and Abdullah (2022) (71.4%), but higher than those of ewes milk (20%). The results of this study were supported by other studies, Siqueira *et al.* (2016); and Escobar-Escamilla *et al.* (2014) who proved that the mastitis milk isolates were correctly identified as NTM in a single adult Holstein cow by performing phenotypic and molecular characterization. Moreover, *M. smegmatis* group species have been identified to cause clinical mastitis in dairy cows and ewes, as recorded by Franco *et al.* (2013); and Machado *et al.* (2015). Additionally; based on PCR data; Jayasumana *et al.* (2018) established the existence of NTM in 2 milk samples (0.8%), and Leão (2015) obtained 26% positive NTM out of 98 cattle milk samples. The discrepancy between these results could be attributed to a variety of factors, including animal age, lactation stage, number of lactations, milk supply, sample time, daily milking frequency, and number of lambs suckled (Raynal-Ljutovac *et al.*, 2007).

Beside PCR testing of milk samples, MWST was used for subclinical mastitis primary screening in this study. The results of this test revealed that percentage of subclinical (82.9 and 76%) of cows and ewes respectively were far from the estimated results of PCR. The current MWST overall percentage (58%) of SCM in cows was supported by another researcher, Kahir *et al.*, (2008) who found that prevalence of SCM. Among different breeds of cattle was 54%. However, higher prevalence of SCM was reported by some earleir (Ramachandiraiah *et al.*, 1990) whereas some authors (Parai *et al.*, 1992) reported a lower prevalence than

this finding. The possible reasons for this variations may be small size, composition of samples (cross breeds only) and methods applied to detect SCM (Kahir *et al.*, 2008). Most of the authors reported a relatively lower sensitivity and specificity of WST than other tests (Dangore *et al.*, 2000). Some authors reported WST as a superior test than CMT (Prodhan *et al.*, 1996).

The association of fecal samples investigation, NTM 16SrRNA gene amplification and SCM distribution in cows and ewes. The current results of 16SrDNA based PCR revealed that 21.7% of cows and 16% of ewes were complained SCM which was caused by NTM. In contrast, other studies carried out in Basrah, Iraq by Abdullah and Abdullah (2022) who found NTM in the faeces of 48 cows (80%) and 11 sheep (22%). In addition, Alzaidi (2018) had been detecting NTM in 6% of cow's faecal samples. In other parts of world, Sumiyah *et al.* (2017) reported that 5.5% of 200 faecal samples were positive for NTM using a PCR. While Leão (2015) found that 83% of 155 faecal samples from sheep, cattle, and goats had positive NTM PCR results. On the other hand, Echeverría *et al.*, (2020) indicated that 20% of the faecal samples of cows yielded nontuberculous mycobacteria species. The reasons for the differences among the present results and the results of other studies might be due to age and breed of animals, in addition to type of genes and molecular methods of detection. Concerning the importance of NTMs presence in the digestive system of cows, Jenkins *et al.*, (2018); Echeverría *et al.*, (2020) reported that NTMs may also disrupt other diagnostic techniques including the interferon- γ and tuberculin skin tests. Beside that vanIngen *et al.*, (2018) found that NTM Presence in the digestive system or in milk can cause an animal to become sensitized to them and mount an immune response, which can lead to false-positive paratuberculosis tests. In conclusion, this study established NTM as the cause of ruminant subclinical mastitis in dairy cows and ewes. This link was discovered using MWST screening and 16SrDNA-based PCR confermentation of NTM in clinical samples. The present results, however, emphasize how critical it is to improve laboratory assessment and quality control procedures in order to raise the precision of microorganism identification in clinical specimens.

CONCLUSIONS

In conclusion, this study established NTM as the cause of ruminant subclinical mastitis in dairy cows and ewes. This link was discovered using MWST screening and 16SrDNA-based PCR confermentation of NTM in clinical samples. The present results, however, emphasize how critical it is to improve laboratory assessment and quality control procedures in order to raise the precision of microorganism identification in clinical specimens.

This study was conducted to determine the association of NTM with subclinical mastitis in dairy cows and ewes and Investigate the usefulness of 16SrDNA for rapid PCR detection of NTM in clinical samples. The research findings highlighted that established NTM as the cause of ruminant subclinical mastitis in dairy cows and ewes. This link was discovered using MWST screening and 16SrDNA-based PCR confermentation of NTM in clinical samples.

AUTHOR'S CONTRIBUTION

Study concept and design: N.R.A., F.A.A., N.R.A., A.A.K and H.S.G.

Acquisition of data: N.R.A., A.A.K and H.S.G.

Analysis and interpretation of data: N.R.A., F.A.A., N.R.A., A.A.K and H.S.G.

Drafting of the manuscript: N.R.A.

Critical revision of the manuscript for important intellectual content: F.A.A., N.R.A.

Statistical analysis: F.A.A.

Administrative, technical, and material support: N.R.A., F.A.A.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest, and all authors approve the manuscript for publication.

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