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Research Article

Direct DNA Modified CTAB Preparation from Nasal Exudate in Live M. *bovis* Infected Cattle in Mexico Provide with a Valuable Assay Extrapolated to Humans TB Diagnostic Test

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ABSTRACT

Detection, identification, and differentiation of members of the MTB complex rely on specificity, sensitivity, and accuracy of the methods that have been developed since the decades of the '90s. Despite this, still, in endemic areas of developing countries tuberculin field test as well as conventional techniques (histopathology and bacteriology) are performed due primarily to the costs and availability. Therefore, it is an urgent need to have a routine assay to boost field test (false positive and negative tests) in live cows while avoiding the unnecessary sacrifice of animals. To this end, in the present work, we designed a dual experimental strategy that can be used as a routine assay for the M. *bovis* or M. *tuberculosis* detection through PCR mediated amplification of RD's. DNA can be prepared from fast-growing colonies (7 to 8 days) or from homogenized tissue, nasal exudate and purification mediated cetyl-trimethyl-ammonium bromide (CTAB) cationic buffer. The method was extraplated to positive TB positive nasal/oral human exudate.with similar results. Collectively these findings indicate that this strategy represent a valuable tool for TBb epidemiological survey and research.

Keywords: M. *bovis*; M. *tuberculosis*; Cetyl-trimethyl-ammonium (CTAB); PCR; DNA DNA RD's

INTRODUCTION

Bovine tuberculosis (TBb) is a zoonotic, chronic disease of cattle caused by *Mycobacterium bovis*, a member of the M. *tuberculosis* complex that represents a serious health problem [1,2]. Accordingly with a recent report [3], it has been estimated that more than 50 million cows are infected worldwide with M. *bovis* leading to $3 billion annually losses in Agriculture [3]. The problem is aggravated by the number of asymptomatic infected animals that have not been notified, diagnosed leading to an underestimation of TBb in endemic regions [4,5]. Despite extensive efforts to control and eradicate the disease in endemic regions, still there an urgent need to make available faster test from live cows, since most of the diagnostic test rely; either in the field tests (caudal fold, comparative cervical and simple cervical) and/or confirmatory assays (negative or positive) (bacteriological and histopathological) in slaughtered cattle [6-8].

From the annals of the literature toward the development of molecular detection methods in TBb, a test that relies in the rRNA determination was found but not further studies were made [9]. Instead, in the last decades, M. *bovis* molecular detection based on different types of PCR (nested, multiplex, real-time) have been developed with promising results [10,11-16] For example, Liébana et al. [10] performed a simple detection of M. *bovis*, directly from samples of bovine tissue by means of a PCR technique, but the test was not able to distinguish between the different members of the M. *tuberculosis* complex [10] In addition, PCR test was not as sensitive as the culture and it did not always detect those samples that contained a small number

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of organisms, which could be due to the difficulty of extracting DNA from the isolates (tissue) of infected animals [17]. Moreover, the sensitivity of PCR can be affected by inhibitors in clinical samples, inhibitors reagents derived from the extraction method or the yield of DNA [18] or procedures for bacteria lysis and DNA extraction involve time-consuming steps and costly which is not always available in developing countries [19], or a lack of specificity and sensitivity because the region to be amplified is not the optimal [18-20]. In contrast, Lázaro et al. [21] and Cepeda and Vázquez. [22], respectively, showed that RD4 amplification could lead to higher specificity and sensitivity to distinguish between *M. bovis* from the rest of the *Mycobacteria* that belong to the complex of *M. tuberculosis* (from 20/20, 100% sensible and specific) [20] and *M. bovis* in the nasal exudate of TST+ cows. To note is that DNA preparation for PCR amplification was undertaken with a cationic buffer, the cetyl trimethyl ammonium bromide (CTAB) [22].

Interestingly, Duarte de Silva et al. [15] were able to detect *M. bovis* directly in bovine milk and blood samples using real-time PCR. However, the milk sample that tested positive for *M. bovis* did not belong to any of the animals that had positive blood samples whereas the presence of proteins and milk fats that may have altered the extraction of *M. bovis* DNA. In a more recent study, it was compared the analytical performance of a multiplex real-time PCR assay for the detection of *M. bovis*, *M. californicum* and *M. bovigenitalium* in culture versus samples of bovine milk, semen, and nasal exudate. Samples were also seeded on the appropriate solid growing medium and except for the nasal exudate; colonies were observed [23]. The authors concluded that the PCR technique has a better analytical performance than the traditional culture for the detection of mycobacteria in nasal exudates of bovines [24]. To note is the several advancements for the improvement of detection, differentiation of the members of the MTB complex [25-31], each other have provided with a key point to take in the account either for TBb or human tuberculosis [25-31].

In the light of all these data from the literature, we pursued to design an experimental strategy that can be used as routine assay for the *M. bovis* or *M. tuberculosis* detection through PCR mediated amplification of Regions of Difference (RD's) DNA can be prepared from fast-growing colonies (7 to 8 days) or from homogenized tissue, nasal exudate, and purification mediated cetyl-trimethyl-ammonium bromide (CTAB) cationic buffer. The protocol was applied on TB positive human nasal/oral exudate with similar results. Collectively these findings indicate that this strategy represent a valuable tool for TBb epidemiological survey and research.

MATERIALS AND METHODS

Animals

Cattle were selected based on the inclusion criteria and according to the NOM-031-ZOO-15. Negative and positive bovines to the Tuberculin Skin Test (TST) for live studies: TST (-) to the caudal anus and the comparative cervical test while TST (±) to either of the field tests but negative for bacteriological and histopathological exams, considered as a reactor. TST (+) was considered positive either to field tests as well as for bacteriological and histopathological exams was considered as positive.

Samples collection

Tissues: A piece of tissue samples of slaughtered cattle were collected in PBS or borate buffer, homogenized and processed as described below.

Exudate: The exudate is collected by introducing a sterile swab into the nostril of the immobilized animal. Thereafter, a nasal swab is placed and homogenized in 5 ml of phosphate buffer (PBS) and maintained on ice. The swab is removed and the suspension is centrifuged at 10,000 rpm/4°C. The supernatant is discarded and the pellet is recovered, which is resuspended in TE buffer and stored at -30°C until processing.

Culture middlebrook medium

Solid Middlebrook (7H10) medium dissolved in 900 ml of sterile MQ water, with vigorous stirring. Subsequently, 5 ml of glycerol is added and it is homogenized with agitation. The medium is sterilized and OADC, (dextrose, catalase, and albumin) (Gibco, Co) PANTA and THF (2-thiophenocarboxylizocidhydrazide) (2 mg/ml) (Sigma, Aldrich, Co were added and Petri dishes were prepared [32].

Direct DNA preparation using cationic cethyl-tri-methyl-ammonium bromide (CTAB) from tissue or exudate

The extraction of DNA from the tissue sample is carried out from tissue homogenate (milteny homogenizer) in TE buffer. Incubate for 2 hours at a temperature of 37°C with 50 µl of lysozyme (10 mg/ml). Time to which 75 µl of 10% SDS are added, and mixed by inversion. 50 µl Proteinase K (1 mg/ml) is added, with gentle shaking and incubated at 65°C for 20 minutes [22,33]. Subsequently, 100 µl of 5 M NaCl is added and mixed gently by inversion. 100 µl of pre-warmed CTAB/NaCl buffer is added, and shake in the vortex until the sample is white incubating at 65°C for 10 minutes. The separation of the organic and aqueous phases is carried out by the addition of 750 µl of chloroform/isoamyl alcohol (24:1) and centrifugation at 12,000 rpm/5 min/room temperature. DNA is precipitated from the aqueous phase with 750 µl of isopropanol, incubation for 30 min at -20°C. Centrifuge at 12,000 rpm/5min. The pellet is recovered, washed with 1 ml of 70% ethanol and finally resuspended in 50 µl of DEPEC water and stored at -30°C until use.

DNA preparation extraction using ethyl-tri-methyl-ammonium bromide (CTAB) and filter mediated purification

The direct DNA extraction method using the CTAB extraction reagent (materials and methods) [23] yielded a DNA of varying concentration. To optimize this parameter, the aqueous phase that is usually added isopropanol to precipitate the DNA, which was done was transferred to a column of the Qiagen kit (indirect method) and centrifuged at 10,000 rpm for 30 seconds. The DNA bound to the membrane of the column was washed with

the AW1 buffer and the AW2 buffer, respectively, and centrifuged each time at 10,000 rpm for 20 seconds. In a third wash, 50 µl of the elution buffer was added and incubated at room temperature/1 min, and again centrifugation at 10,000 rpm. This step was repeated until obtaining a final volume of 100 µl, which was stored until its use [32].

Exudate DNA extraction

the nasal swabs were removed from the tubes containing PBS and these were centrifuged to subsequently eliminate the liquid phase, while the pellet was homogenized in 400 µl of TE buffer and subsequently incubated at 37°C with 50 µl of lysozyme at a concentration of 10 mg/ml). Purification of DNA was made as above described [22,33].

PCR amplification of the RD4 (*M. bovis*) or RD5 (*M. tuberculosis*) DNA fragment purified by the cethyl-trimethyl-ammonium bromide (CTAB) modified method

The amplification reaction of a *M. bovis* DNA fragment was carried out, following the instructions of the manufacturer (Qiagen protocol). Briefly, to each reaction mixture, 0.25 µl of the forward, *M. bovis* RD4 region forward (F-AACGCGACGACCTCATATTC) and 0.25 µl of the reverse (R- AAGGCGAACAGATTCAGCAT). *M. tuberculosis* RD1-RD5 region: forward, F (GGTTTTGGGTCTGACGAC); reverse, R (CCGAGAGGGGACGGAAAC) [21,22,34-36] were added and incubated at temperature environment for 15 minutes, then add 25 µl of the master mix or master mix it contains, the enzyme Hot Star Taq polymerase, dNTPs (dATP, dCTP, dGTP, dTTP), $MgCl_2$ and KCl. Mix gently and add 16.5 µl of DEPEC wáter. Finally, 8 µl of the DNA sample (˜ 0.7-1 µg/ml) are added. They are homogenized gently. For the amplification of a DNA fragment, the one recommended by the manufacturer (QIAGEN, Co) was followed. The analysis of the PCR products was carried out on a 1% agarose gel in TAE buffer and syber safe staining. The gel is prepared by weighing 0.30 g of agarose and dissolved in 30 ml of the buffer with heating. Allow to warm, and add 1 µl of syber Safe (Invitrogen, Co). The DNA samples are prepared by mixing a given volume with 4 µl of loading buffer. The gel is run in TAE buffer in a 90 volt electrophoresis chamber. The DNA bands are visualized in a photodocument (Chemidoc) (Bio-Rad).

RESULTS

A protocol designed for the molecular detection of *Mycobacterium bovis* from live cows

Despite the huge amount of methodologies for the molecular detection of members of the MTB complex, still there are a percentage of variability and sensitivity [12] in the data depending of the experimental settings used for that purpose. Therefore, we aimed to design a simple protocol to be used as a routine assay for extensive epidemiological survey as well as for research. To this end, we collected nasal swabs from TST (+) cattle and TST (-). From this, we followed two mains steps for DNA preparation (Figure 1). Nasal swabs were also seeded as described in Figure 1 and the suspention in buffer was used for DNA extraction. As positive controls we used *M. bovis* BCG and *M. bovis* AN5 [35,36] and RD1 primers (present only in AN5). In either case it was obtained a band of around 7 kb (the amplicon) (Figure 2, lanes 1 and 6). Not product was detected without DNA (lane 2); with only primers (lane 3); with DNA from Escherichia coli (E. coli) (lane 5). RD4 amplification was obtained for M. bovis BCG (lane 4); for AN5 (lane 7). RD1 amplification for AN5 (lane 8) but not for M. bovis BCG (lane 9).

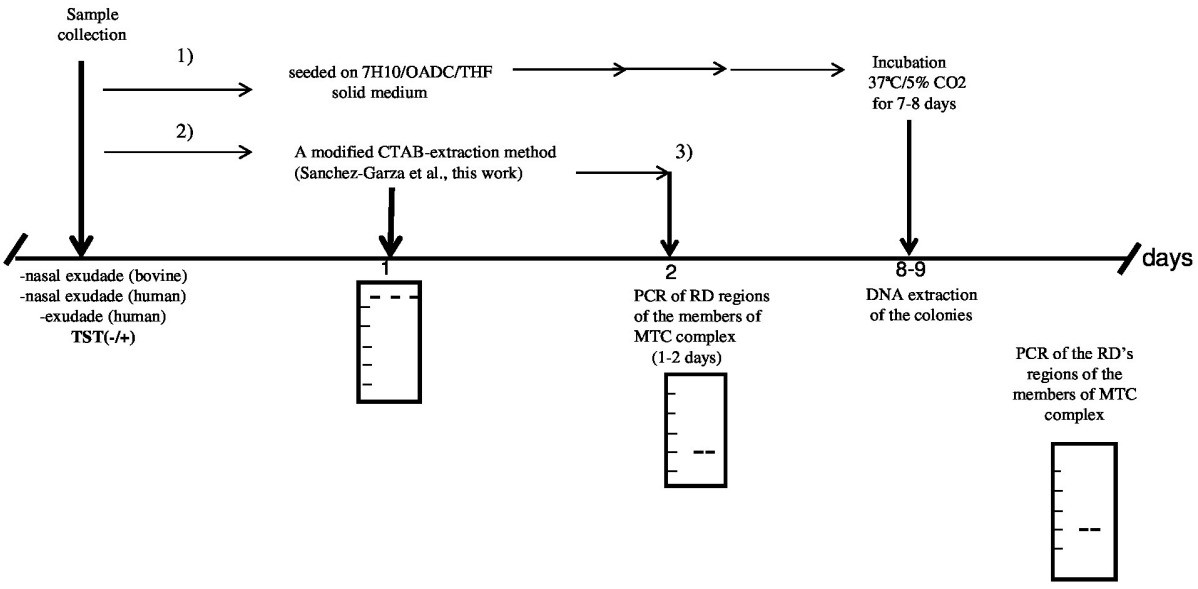

Figure 1: A experimental protocol designed for the study. A simple and rapid method was designed as a routine assay for *M. bovis* or *M. tubeculosis* detection either direct from the simple collected or from seeding in soild Middlebroth culture médium supplemented with 2-thiophenocarboxylizocidhydrazide (THF) THF as described in material and methods.

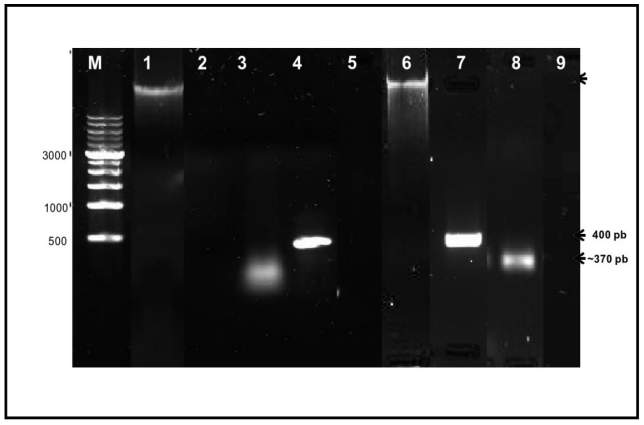

Figure 2: As positive controls, we used DNA (amplicon of aorund 7 kb) from a colony of *Mycobacteriym bovis* BCG grown on 7H10/OADC, M. bovis BCG (lane 1) or reference *M. bovis* strain AN5 grown on 7H10/OADC and supplemented with 2-thiophenocarboxylizocidhydrazide (THF) (lane 7). For PCR amplifcation, we used RD's (RD4 or RD1). In either case it was obtained a band of around 7 kb (the amplicon) (Figure 2, lanes 1 and 6). Not product was detected without DNA (lane 2); with only primers (lane 3); with DNA from Escherichia coli (E. coli) (lane 5). RD4 amplification was obtained for *M. bovis* BCG (lane 4); for AN5 (lane 7). RD1 amplification for AN5 (lane 8) but not for *M. bovis* BCG (lane 9).

RD4 amplification from CTAB DNA prepared through direct sampling (nasal exudate) from live cattle in Mexico

Next, DNA was prepared from nasal swabs using cationic Cethyl-Tri-Methyl-Ammonium Bromide (CTAB) buffer and filter mediated purification (improved in this work) as described in material and methods. This buffer depending of the system, can eliminate genomic DNA or other undesirable components that affect the yield and integrity of DNA [10,11,23,33]. From figure 3I (lanes 1-6), an amplicon of around 7 kb was obtained and a PCR product of 400 pb with RD4 primers in all cases (Figure 3IB, lanes 1-6). This methodology was applied also to tissues (lymph nodes) with similar results (Figure 3II, lanes 1-4).in comparison nasal exudate (Figure 3II-C, lanes 5-6), yieldin in either case a fragment of 400 pb corresponding to RD4 of M. bovis genome (Figure 3II B-1; lanes 1-4 and lanes 5-6, respectively)[21;35;36]. As negative controls from TST(-) cows , no amplicon was obtained (FJOigure 3II-C, lanes 2-5), nor RD4 PCR product (Figure 3II-C-1, lnaes 2-5).

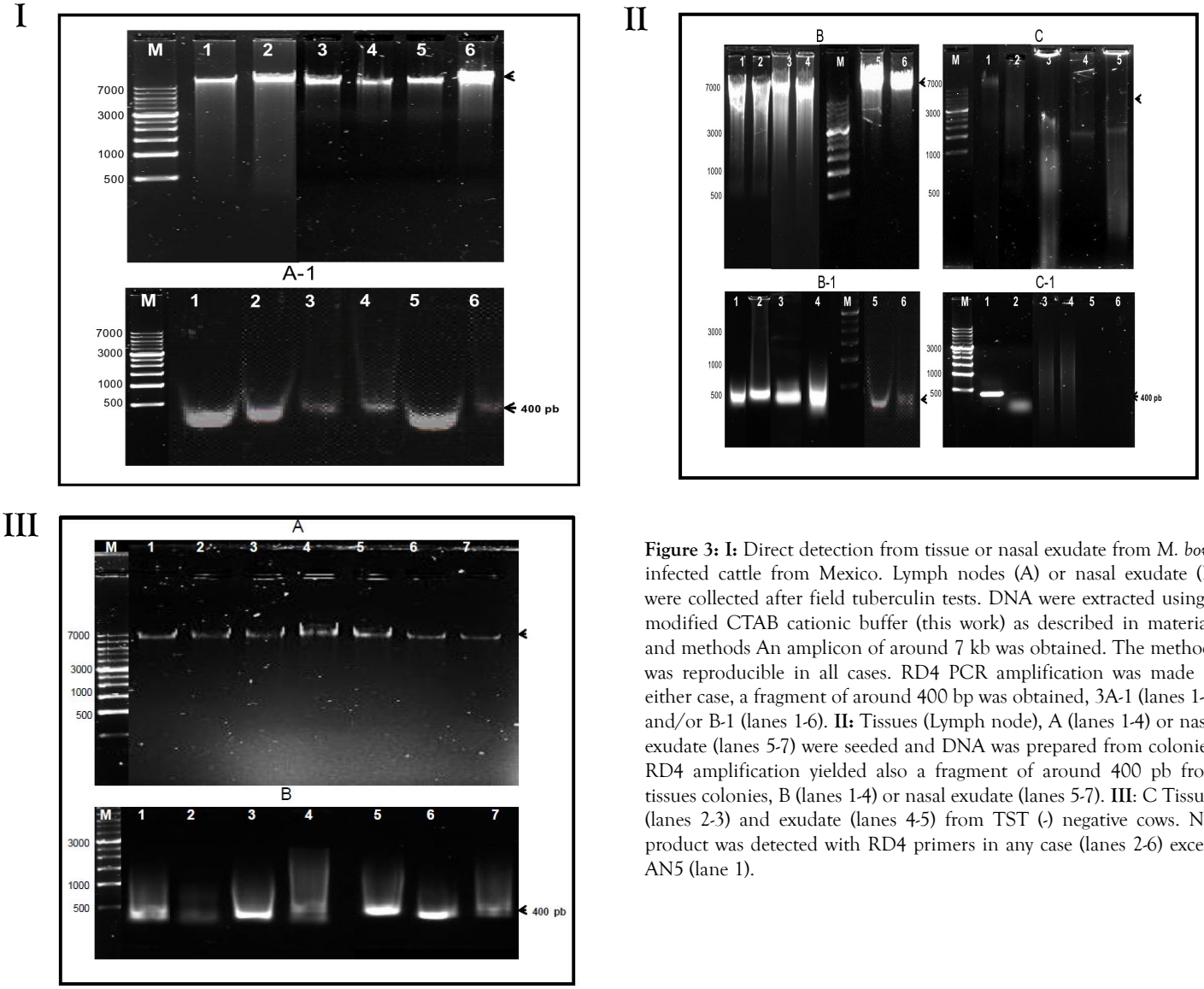

Figure 3: I: Direct detection from tissue or nasal exudate from M. *bovis* infected cattle from Mexico. Lymph nodes (A) or nasal exudate (B) were collected after field tuberculin tests. DNA were extracted using a modified CTAB cationic buffer (this work) as described in materials and methods An amplicon of around 7 kb was obtained. The methods was reproducible in all cases. RD4 PCR amplification was made in either case, a fragment of around 400 bp was obtained, 3A-1 (lanes 1-6) and/or B-1 (lanes 1-6). **II:** Tissues (Lymph node), A (lanes 1-4) or nasal exudate (lanes 5-7) were seeded and DNA was prepared from colonies. RD4 amplification yielded also a fragment of around 400 pb from tissues colonies, B (lanes 1-4) or nasal exudate (lanes 5-7). **III**: C Tissues (lanes 2-3) and exudate (lanes 4-5) from TST (-) negative cows. Not product was detected with RD4 primers in any case (lanes 2-6) except AN5 (lane 1).

Seeding of nasal exudate yielded fast growing colonies of *M. bovis* isolated from live cattle in Mexico

To confirm the above results (Figure 2 and 3I), nasal exudate and lymph nodes (retropharyngeal and mesenteric) from live cattle (TST+) and slaughtered cattle respectively were seeded on solid growing medium as described in Materials and Methods In either case, 7-8 days post seeding colonies were obtained. DNA preparation from these colonies were made using filter column as indicated by the manufacturer. A thiny band of the amplicon (around 7 kb) was obtained from tissue (Figure 3III-A, (lanes 1-4) or nasal exudate (Figure 3III-A, lanes 5-7) [25,35-36]. RD4 amplification of *M. bovis* DNA obtained from nasal exudate or from tissue was made and one fragment of around 400 pb was obtained in either case, tissue (Figure 3III-B, lanes 1-4) or exudate (Figure 3III-B, lanes 5-7).

Amplification of the RD4 region of *M. bovis* DNA obtained from human nasal/oral exudate of infected individuals

To extrapolate these findings from infected live or slaughtered cattle (in the case of the tissues), we applied the same methodology. DNA preparation from nasal/oral exudate respectively using the above described methodologies (cationic CTAB buffer) or filter colums yielded a band of an amplicon of around 7 kb (Figure 4A, lanesas 1-4, 4B, lanes 1-3). A fragment of around 400 bp was obtained for *M. bovis* (Figure 4B, lanes 1-4, 4D, lanes 1-3). Furthermore, DNA either from bovine or human were tested with *M. tuberculosis* primers yielding two bands (in preparation).

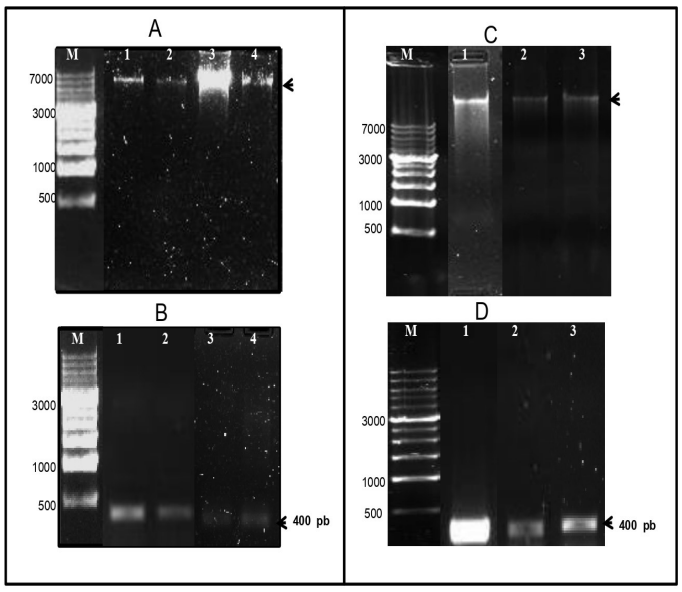

Figure 4: The validation of the designed methodology for molecular detection of *M. bovis* was extrapolated to human nasal/oral exudate.(2 independent individuals). The DNA preparation (amplicon of around 7 kb) was made using the the cationic CTAB modified protocol (this work) (A, lanes 1-4) as well as the filter column kit (4C, lanes 1-3). RD4 amplification (B, lanes 1-4 nasal/oral, respectively for 2 individual) and D, amplification of the AN5 RD4 DNA from colonies (lane 1) and from nasal/oral of one individual is shown (lanes 1-2).

DISCUSSION

In the present work, we are reporting a dual experimental strategy to be used as a routine assay from live cows for detection of *Mycobacterium bovis* or *M. tuberculosis* RD's. The assay validation was made on human nasal/oral exudate TB detection.

Detection, identification and differentiation of members of the MTBC complex rely in specificity, sensivity and accuracy of the methods that have been developed since the decades of the 90's. Despite this, still in endemic areas developing countries tuberculin field test as well as conventional techniques (histopathology and bacteriology) are performed due primarily to the costs and availability [25-31]. Therefore, it is a urgent need for endemic regions to have a routine assay to boost field test (false positive and negative tests) in live cows [37]. Moreover, the development of an optimal PCR methods that can boost field tuberculin tests (false positive/false negative) of live cows while avoiding unnecessary sacrifice of animals a molecular method should fulfill some basic requirements such as the high quality DNA, the regions to be amplified and primers design [8,16,22,24-31,37-39]. *M. bovis* detection either from cattle or humans samples have moderate sensitivity, mainly attributed to the difficult for DNA extraction (bacterial lysis, long manipulation steps) or quality of the sample collection [17,25-28]. Nowadays, there are several molecular methods for the detection of *M. bovis* that goes from simple PCR to multiplex real time PCR using either milk, semen, urine or nasal exudate [16,23,24-31], some of them fill one or two of the basic requirements mentioned above, but not all. In some of them, the results should be corroborated throughout histopathology and bacteriology, which would imply, time, and costs; and therefore, it can not be used extensively in epidemiological surveys [37,38,39-42]. Thus, in the present work, we aimed to design a dual experimental protocol that can be used as routine assay for the *M. bovis* or *M. tuberculosis* detection that involves DNA extraction prepared from fast growing colonies (7 to 8 days) (Figures 2 and 3III) or from direct from nasal exudate using cetyl-trimethyl-ammonium bromide (CTAB) cationic buffer which improved the quality of DNA for several reasons: a) denatures proteins, b) solubilize cell wall and lipid proteins [26] and c) PCR mediated molecular detection [25-31,38,39,43-44]. Furthermore, we observed complementation between the direct method for DNA preparation from exudate nasal/oral and PCR RD's amplification either from *M. bovis* or *M. tuberculosis* (in human MTb in preparation) with exudate seeding, colonies growth and RD's PCR amplification method [15-17,25] (Figures 2, 3III and 4B), To note is that tissues samples can be obtained only from slaughtered cattle, dampening more extensive screening studies. Thus, herein we are showing that through this improved and shortened *M. bovis* DNA preparation from nasal exudate, it is possible to avoid unnecessary sacrifice of animals, strenghtening tuberculin test, offering thus an advantage and a promising alternative that can be extrapolated on human molecular diagnostic and nasal/oral exudate sampling for TB epidemiological surveys and research.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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