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Sensitive detection of *Mycobacterium bovis* in spiked milk using a polymerase chain reaction assay

*Marvin A. Villanueva[1,2], Yasuhiko Suzuki[1], Chie Nakajima[1], Haruka Suzuki[1], Yukari Fukushima[1], Claro N. Mingala[2]**

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[1]*Division of Bioresources, Hokkaido University International Institute for Zoonosis Control, Kita 20, Nishi 10, Kita-ku, Sapporo 001-0020, Japan*
[2]*Philippine Carabao Center National Headquarters and Gene Pool, Science City of Muñoz 3120, Nueva Ecija, Philippines*

Correspondence email: cnmingala@gmail.com

Abstract: Bovine tuberculosis is a chronic zoonotic disease that affects both animal and human health and imposes serious public health concerns in the world. Intake of non-pasteurized milk is considered the most probable vehicle for the transmission of pathogenic bacteria. In this study, the detection of *Mycobacterium bovis* BCG in spiked milk using a polymerase chain reaction was performed. The performance of two DNA extraction methods, CTAB/phenol:chloroform:isoamyl alcohol and EXTRAGENMB were also evaluated. In addition, Mycobacterial concentration was tried to determine using the Standard/ Viable Plate Count Method and Spectrophotometric (Turbidimetric) Method. PCR successfully detected *M. bovis* BCG in spiked milk, detecting approximately up to two bacilli per reaction. The two DNA extraction methods were effective in the isolation of amplifiable DNA, having the advantage of EXTRAGENMB in terms of (1) shorter duration of DNA extraction, (2) less sample manipulation, and (3) ease of execution of the procedure. Quantitative determination of the Mycobacterial population, however, failed to quantify the bacterial concentration per dilution, suggesting that CFU concentration should be considered an approximation. It is expected that this method can be used for the detection of *M. bovis* in raw milk samples.
Keywords: Mycobacterium bovis; BCG; spiked milk; PCR

1. Introduction

Bovine tuberculosis is an important zoonotic disease worldwide. *Mycobacterium bovis (M. bovis)*, the causative agent of this chronic disease in cattle, is also a pathogen for livestock andhumans, generating financial losses to the industry and causing serious public health problems in the world. *M. bovis* is a member of the *M. tuberculosis* complex, a group that includes *M. tuberculosis* and *M. africanum* (Antognoli *et al.,* 2001; Di Pinto *et al.,* 2006; Rodriguez *et al.,* 1995; Zanini *et al.,* 1998).

Bovine TB is generally transmitted in three ways: inhalation of infectious droplet nuclei containing *M. bovis*; ingestion of contaminated material, usually milk; and direct contact, occurring among workers who are in contact with sick animals or vice versa. People at greatest risk are those in direct and prolonged contact with infected animals, such as farmers, farmworkers, and veterinarians (Di Pinto *et al.,* 2006).

The Philippines ranks ninth on the list of 22 high-burden tuberculosis (TB) countries in the world, according to the World Health Organization (WHO) Global TB Report in 2009, and had the second-highest number of cases in the WHO Western Pacific Region in 2007, making TB as the sixth-greatest cause of morbidity and mortality in the country (Vianzon *et al.,* 2013). Efforts have been made to detect *M. tuberculosis* and reduce the number of infected cases. However, very little data regarding *M. bovis* was available which can also contribute to the overall human TB cases in the country.

Ruminants, especially water buffalo (*Bubalus bubalis*) have been considered a family member in rural areas in the Philippines. Water buffalo are used mainly for draft purposes, as well as a source of milk. Pasteurization of milk is seldom practiced in rural areas. Non-pasteurized milk is the most probable vehicle for the transmission of pathogenic mycobacteria, especially in developing countries where the prevalence of bovine TB is higher (Collins and Grange, 2003; Lermo *et al.,* 2010). Isolation of *M. bovis* from storage tanks, inadequately pasteurized milk, and milk samples from tuberculin non-reactive cattle has been reported (Lermo *et al.,* 2010; Pardo *et al.,* 2001).A typically infected udder may excrete tubercle bacteria to the extent of 5 x 102 -5 x 105 per ml of milk (Zanini *et al.,* 1998). As such, the detection of *M. bovis* in milk samples serves as an indirect diagnostic method distinguishing infected from non-infected animals and controlling airborne contamination that prevents the further spread of the disease (Pardo *et al.,* 2001). Diagnosis of bovine tuberculosis is done by intradermal tuberculin test/ bovine skin test, mycobacterial isolation through traditional culture methods, and biochemical tests. Although mycobacterial isolation is considered the gold standard, it is acknowledged that it is of limited use in identifying individual infected animals due to the slow process, and requires a long time for a reaction to show (Di Pinto *et al.,* 2006; Hancox, 2002).

Mycobacterial isolation is highly specific, unfortunately, it has poor sensitivity and is time-consuming and labor-intensive (Antognoli *et al.,* 2001). Moreover, the low sensitivity and specificity of the bovine skin test (Francis *et al.,* 1978; Gardner and Hird, 1989; Neill *et al.,* 1994; Seiler, 1979) is the cause of decreased efficacy in eradication campaigns and leads to a greater risk in public health programs and cause economic losses in the cattle industry. Therefore, the need for a rapid diagnostic method with 100% sensitivity and specificity is deemed necessary.

The objectives of this study were to use a PCR-based method for the detection of *M. bovis* in spiked milk, evaluate the performance of two extraction methods in yielding high *M. bovis* DNA, and identify the lowest mycobacterial number that can be detected in milk. This study also tried to determine the bacterial population of *Mycobacterium* spp.

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2. Materials e Methods

A bacterial strain Seed culture was obtained from *M. bovis* BCG Tokyo 172 culture in Ogawa medium and incubated at 37ºC for six weeks.

2.1. Sample preparation

The mycobacterial colony was harvested and inoculated in Difco Middlebrook 7H9 broth with BBL Middlebrook OADC Enrichment (Becton Dickinson, USA) and 0.05% Tween 80. Two sets of cultures were prepared. The first set had five pieces (5 mm) of beads and the second set had no beads. These mycobacterial cultures were vortexed for 10 sec every day for one week. After one week, the two sets of cultures were vortexed again for 10 sec and allowed the large clumps of bacteria to settle down for 5 min and then the supernatant was collected. The first set of cultures was filtered using a 5 µm syringe filter (Acrodisc 32 mm syringe filter with 5 µm Supor membrane) to separate large clumps of bacteria while the second set was not filtered. Both sets were subjected to Spectrophotometric (Turbidimetric) Analysis (WPA Colour Wave, CO7500 Colorimeter) at 590 nm. Results were referred to MacFarland Standard (Biomerieux, France). A 10-fold six serial dilution (X10-1 - X10-6) was performed followed by the Standard/ Viable Plate Count Method (PCM) using DifcoMiddlebrook 7H10 agar with BBL Middlebrook OADC Enrichment (Becton Dickinson, USA). Cultures were prepared in duplicate each of the six dilutions and incubated at 37ºC for three weeks. After three weeks, the bacterial colony was counted and bacterial concentration was calculated. Results from the two methods were compared to verify the accuracy of bacterial concentration for each dilution. Commercially available pasteurized milk was used where nine milliliters of milk were spiked with 1 ml of each of six dilutions having the final concentration of 1000 5 cells/ml, 100 cells/ml, 10 cells/ml, and 1 cell/ml.

2.2. DNA extraction

Treatment 1. DNA for PCR was extracted from spiked milk as described previously with minor modifications (Serrano-Moreno *et al.,* 2007). Spikedmilk (10ml) was centrifuged at 13,000 g at 10ºC for 30 min. About 500 µl of the precipitate was washed with 1.5 ml of PBS. The pellet was homogenized with 400 µl of TE (100 mM Tris–HCl, pH 8.0, 10 mM EDTA), and 50 µl of lysozyme (100 mg/ml) was added and incubated for 1 h at 37ºC. Then, 70 µl of 10% SDS was added, followed by 3 µl of proteinase K solution (20 mg/ml), and incubated for 10 min at 65ºC. After incubation, 100 µl of 5 M NaCl was added to each sample followed by 80µl of 5 M NaCl solution with 5% N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB). The mixture was incubated for 10 min at 65ºC. DNA was extracted with one volume of phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with 0.6 volumes of absolute ethanol, then washed with 1 ml of 70% ethanol. DNA was resuspended in 25 µl of DDW.

Treatment 2. (EXTRAGEN MB, Japan). About 500 µl of the precipitate from the centrifuged-spiked milk was washed with 1.5 ml of PBS. The pellet was added with 500µl washing reagent and incubated at 70ºC for 3 min, centrifuged at 16,000 g for 5 min at 4ºC, and the supernatant was removed. 25 µl of bacteriolysis reagent was added and placed in an ultrasonic washer for 5 min, then centrifuged at 16,000 g for 5 min at 4ºC. The supernatant was then transferred to a new tube.

2.3. Primers

A pair of primers, forward 5'-CCTGCGAGCGTAGGCGTCGG-3' and reverse 5'-CTCGTCCAGCGCCGCTTCGG-3' were used to amplify a 123 bp (base pairs) fragment of the 6 insertion sequence IS6110 of *M. tuberculosis* complex (Hermans *et al.,* 1990; Kolk *et al.,* 1992; Leite *et al.,* 2003; Thierry *et al.,* 1990; Vitale *et al.,* 1998).

2.4. DNA amplification by PCR

The PCR reaction was performed in a final volume of 20 µl. The PCR mix contained a final concentration of 5X green GoTaq reaction buffer (Promega, USA), 10 mM of each deoxynucleotide solution mix, 10 µM of each primer, 5 µ/µl GoTaq DNA polymerase (Promega, USA), 1 µl of the template and an appropriate volume of double distilled water. The mixture was processed in iCyclerThermal Cycler (BIO-RAD, USA) with an initial denaturation step of 96ºC for 1 min, followed by 35 cycles of denaturation at 96ºC for 10 sec, annealing at 60ºC for 10 sec, extension at 72ºC for 15 sec and final extension at 72ºC for 5 min.

2.5. Amplified PCR product detection

PCR amplified products were analyzed by electrophoresis on 2% (w/v) agarose gel (Wako, Japan) in 1X TAE buffer and visualized by ethidium bromide staining and UV trans-illuminator (ATTO, Japan). The sizes of PCR products were determined using a 50 bp DNA ladder (New England BioLabs, UK) as markers.

3. Results

3.1. Quantitative determination of the Mycobacterial population

The mycobacterial concentration/ population of the first set of culture, i.e., with beads and was filtered has an average count of 3.475 x 105 cells while the other set with no beads has a count of 7.02 x 107 cells (Table 1). Results obtained from two methods of culture preparation showed the difference in bacterial count. Vortexing of mycobacterial suspension using 5 mm beads and filtration of large clumps using a 5 µm filter syringe did not contribute to separating the clumps of *M. bovis* BCG to individual cells,

as confirmed by the large colonies grown in the 7H10 agar (Fig. 1-A). Vortexing alone resulted in variations of colony sizes, composed mostly of small colonies, which representsingle cells (Fig. 1-B).

Treatments	Bacterial counting method	
	Standard/ Viable Plate Count Method	Spectrophotometric (Turbidimetric) Method
Vortexed with 5 mm glass beads and filtered using 5 µm filter syringe (1st batch)	2.3 x 10^5	7.5 x 10^7
Vortexed with 5 mm glass beads and filtered using 5 µm filter syringe (2nd batch)	4.65 x 10^5	7.5 x 10^7
Vortexed only	7.02 x 10^7	7 x 10^8

Table 1 – Quantitative determination of the Mycobacterial population.

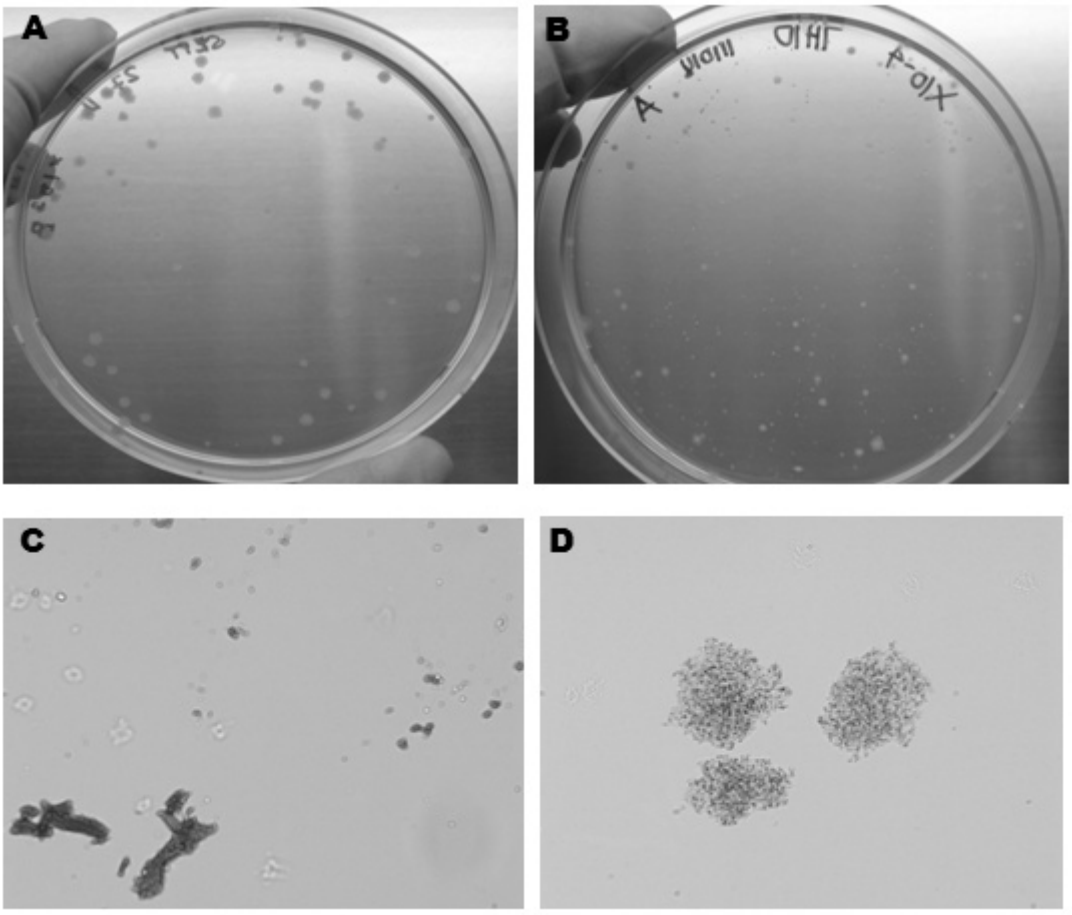

Figure 1 - (A) *M. bovis* BCG showing large colonies, vortexed with 5 mm glass beads and filtered using 5 µm syringe filter and (B) *M. bovis* BCG showing variations of colonies, vortexed alone before culture in Middlebrook 7H10 agar; (C) *M. bovis* BCG showing individual and small clumps and (D) *M. bovis* BCG showing large clumps, stained with crystal violet.

The blank/control tube (data not shown) in the first set of cultures was observed to have microparticles appearing after vortexing for one week. The media was thought to be contaminated, so it was cultured in 7H10 agar, but there was no growth of other bacteria.

3.2. Comparison of two DNA extraction methods and detection of M. bovis BCG DNA in spiked milk

Both methods used in the study succeeded in extracting *M. bovis* BCG DNA from spiked milk. PCR detected up to 2000 bacilli per reaction using CTAB/ Phenol:Chloroform:Isoamyl OH DNA extraction method, while up to two bacilli per reaction was detected by PCR using the EXTRAGEN MB DNA extraction method. EXTRAGENMB was easier and faster to perform than CTAB/ Phenol:Chloroform:Isoamyl OH DNA extraction method. (Fig. 2)

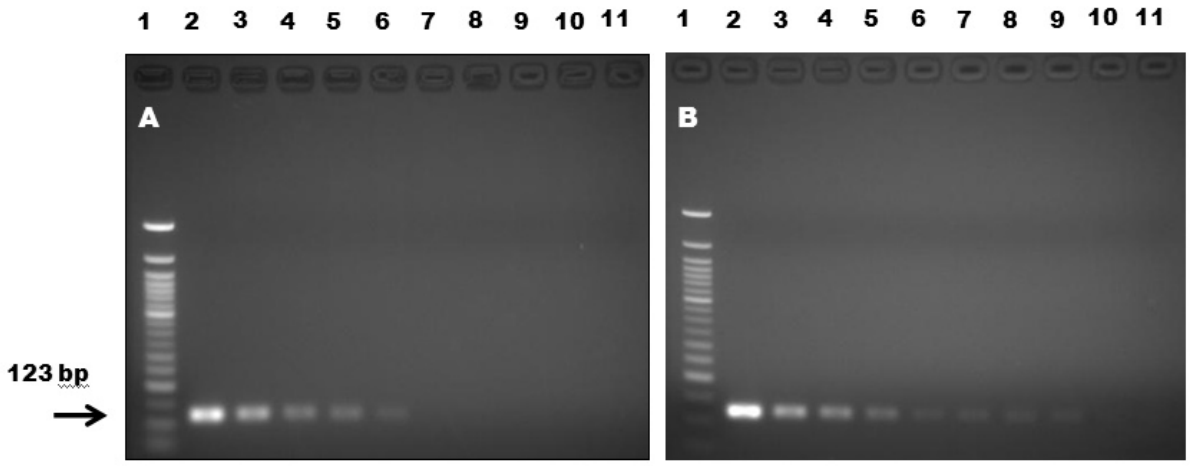

Figure 2 – Sensitivity of PCR assay for the detection of *M. bovis* BCG in spiked milk samples. Lane 1: 50bp DNA marker; Lane 2: positive control (BCG Tokyo); Lanes 3-10; milk contaminated with 5 x 107 - 5 x 100 after 10-fold serial dilution; Lane 11: negative control. (A) Result of PCR assay with all precipitate using CTAB/ Phenol:chloroform:isoamyl OH DNA extraction and (B) EXTRAGEN MB DNA extraction kit.

4. Discussion

The cell wall of Genus Mycobacterium is rich in long-chain fatty acid esters known as "mycolic acids". It is chemically related to wax and gives the colonies their characteristic waxy appearance, and the cell has a tendency to clump and resist dispersion (Saviola and Bishai, 2006). Different culture preparation methods have been tried to prevent clumping such as repeated tuberculin needle passage (Garbaccio and Cataldi, 2010), vigorous vortexing with 2x TBT buffer (1), and many others to come up with a more reliable bacterial count. In this study, two culture preparation methods, i.e., vortexing with glass beads followed by filtration using a 5 µm syringe filter and vortexing alone were used, aiming to separate clumps of bacteria into individual cells. Both culture preparations were then subjected toSpectrophotometry analysis and Standard/Viable PCM for bacterial count determination. Results concluded that both culture preparation methods failed. The hypothesis that large clumps of Mycobacteria can be separated into individual cells using beads and filtration did not materialize. Instead, it contributes further clumping, as shown by the large colonies (Fig. 1-A). Moreover, bead-beating activity may cause the death of Mycobacteria, resulting in low bacterial count in the first culture preparation. Furthermore, the use of glass beads while vortexing produced microparticles that contaminate the culture. Vortexing alone, on the other hand, presented variations in colony sizes, which composed mostly of small colonies, indicative that these colonies came from a single bacterium. Results from two bacterial counting methods were comparable using this culture preparation, since variations of colony sizes are present. This indicates that the bacterial count from PCM could be higher than the calculated bacterial number based on counted colonies. Furthermore, the Mycobacterial count is said to be about 10% of the *E. coli* count. However, Mycobacterial count determination is still difficult because of its clumping characteristics.

EXTRAGEN MB, a Mycobacterial nucleic acid extraction reagent, is designed for sputum samples, as well as in pleural fluid, bronchial washings, urine, gastric juice, ascites, cerebrospinal fluid, lung lavage fluid, and pus. Through this study, milk can be used as a sample where this DNA extraction kit can be applied. It enables the removal of inhibitors present in the milk to successfully extract DNA. Moreover, it only requires a few samples and runs for approximately 30 min.

The result obtained from the PCR assay shows that it can amplify as low as two bacilli per reaction, which is very sensitive for animals that shed a minimal amount of bacterial number. However, it should be considered that the clumping pattern could have been different between cell dilutions in this study, and therefore the presence of amplified product would be different from the expected decreasing manner of bacterial count.

The use of DNA-based techniques like polymerase chain reaction is indeed a powerful tool for the diagnosis of zoonotic diseases like bovine tuberculosis, which is considered one of the difficult-to-diagnose diseases because of its chronic nature of infection. This method can shorten several months of culturing to confirm results over intradermal tuberculin testing, which is considered the gold standard method.

In developing countries like the Philippines, where cases of tuberculosis have alarming rates, focusing only on *M. tuberculosis* as a culprit of this devastating disease will not solve the problem. Tuberculosis causing *M. bovis* has an economic impact on livestock and of public health concerns that can contribute to the persistence of the disease. To date, existing data regarding the prevalence of bovine tuberculosis in the country is still limited. Considering that most Filipinos in rural areas rear water buffaloes, which can be used for draft as well as a source of milk and meat, the presence of this disease poses a great health threat.

Aside from milk samples, studies are currently being conducted searching for better samples that can be taken for the detection of Mycobacterium. Nasal swabs (Vitale *et al.,* 1998; Figueiredo *et al.,* 2010; Meikle *et al.,* 2007), semen (Niyaz *et al.,* 1999), urine (Napolitano *et al.,* 2008), blood (Condos *et al.,* 1996) and feces (Jha *et al.,* 2007) producedencouragingresults. Since

milk can be taken only from lactating animals, the non-invasive use of other samples from live animals is crucial to cover all sex and ages within the herd. However, because of the slow-growing nature and being an intermittent shedder of infected animals, so far, rapid diagnosis of infected animals is still difficult.

Several reports have also been published that besides *M. bovis*, transmission of *M. tuberculosis* from infected humans to animals and vice versa has been reported (Erwin *et* al., 2004; Fritsche *et al.,* 2004; Sjogren and Hillerdal, 1978). Hence, *M. bovis* and *M. tuberculosis* pose a potential health hazard to both animals and humans (Goodchild and Clifton-Hadely, 2001; Thoen *et al.,* 2006).

5. Conclusion

This PCR assay can be used as a confirmatory test for reactor animals tested by intradermal tuberculin testing. A more sensitive diagnostic technique is a reliable tool for gathering epidemiological data to evaluate the extent of *M. bovis* infection in the country.

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