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A Simple Cultural Method for Detection of *Mycoplasma bovis*

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Abstract: Simple method for the detection of *Mycoplasma bovis* in both liquid and solid cultures was established. Fresh media were easily prepared and dispatched into an eppendorf tube or a Petri dish, a new cultural procedure was followed with the eppendorf tube closed or the Petri dish sealed. Compared to the conventional methods, appropriate inoculating and subculturing operations got easier and a CO_2 incubator was not required; moreover, probable contamination by airborne fungi or bacteria could be reduced or eliminated during long-term incubation. Similarly, *M. bovis* grew normally and could be investigated by Color-Change Units (CCU) enumeration, colony morphology or PCR assay.

Key words: Culture, detect, method, *Mycoplasma bovis*, media, CO_2

INTRODUCTION

Mycoplasma bovis was first isolated in the USA from the milk of a mastitic cow (Hale *et al*., 1962). During the past decades, it has spread to numerous countries with the global transport of animals and sperm. *M. bovis* is considered one of the most frequent pathogens causing pneumonia, mastitis and arthritis in cattle. Its pathogenic role is often ignored although, several experimental infections have proved it (Gourlay and Houghtan, 1985; Thomas *et al*., 1986; Rodriguez *et al*., 1996; Kumar *et al*., 2012).

Methods used for definitive diagnosis of *M. bovis* infection include culture, fluorescent antibody test, serology and PCR assay. The most commonly used method is detection by culture. This method is time consuming and often problematic because of nonmycoplasmal bacterial overgrowth (Sachse *et al*., 1993). Moreover, selective media are necessary and inoculated plates must be incubated in 5-10% CO_2 for at least 2 days and should not be considered negative until 7 days have passed (Jasper, 1981). As a result, significant efforts have been made to develop new detection techniques including PCR assays (Ghadersohi *et al*., 1997; Hotzel *et al*., 1996; Pinnow *et al*., 2001; Bahador *et al*., 2006).

Researchers reported here a simple method for the detection of mycoplasma. Fresh medium was easily prepared and dispatched into eppendorf tubes or Petri dishes; appropriate inoculating and subculturing operations got easier and a CO_2 incubator was not required; moreover, contamination by airborne fungi or bacteria could be reduced or eliminated during long incubation following a simple cultural procedure with the eppendorf tube closed or the Petri dish sealed. Similar to the conventional methods, *M. bovis* grew normally and could be investigated by Color-Change Units (CCU) enumeration (Ueno *et al*., 2008), colony morphology or PCR assay.

MATERIALS AND METHODS

Culture media and growth conditions: Mycoplasma strain was cultured in modified Hayflick (1965)'s broth containing PPLO broth (Difco) 2.1 g, horse serum (Solarbio) 20 mL, 25% (w/v) fresh yeast extract 10 mL, glucose 0.2 g, sodium pyruvate 0.4 g, penicillin 20000 IU mL L^{-1}, 1% Phenol Red 0.2 mL and deionized water 70 mL. Medium was passed through 0.2 μm filters (Acrodisc® Syringe Filter, PALL Life Science) for sterilization and can be stored at 4°C for no >1 week.

For preparation of fresh yeast extract, 5 g dry bakers' yeast (Angel Yeast Co., Ltd. China)was crumbled and mixed with 20 mL deionized water in a 50 mL baker; the suspension was treated by microwave (Galanz WD900SL23-2II, mild strength, 10 sec for three times with intervals of 30 sec). After incubation for 3-5 min, the mixture was transferred to 2.0 mL eppendorf tubes and centrifuged for 10 min at 12000 rpm (Sigma 2 k15) and the supernatant was used as the extract for the preparation of the media.

Mycoplasmas cells were divided into 100 μL aliquots and each aliquot was inoculated into 900 μL fresh medium in 10 mL tubes (10×5) and incubated at 37°C with 5% CO_2 (Forma Scientific, Water-Jacketed Incubator 3250). In this research, 100 μL aliquot of *M. bovis* was tried to inoculate into 200-800 μL fresh media in 1.5 mL eppendorf tubes and incubated at 37°C without 5% CO_2.

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Table 1: Determination of growth of *M bovis* in 900 μL fresh medium in 10 mL tube (10×5) using Color-Change Units (CCU)

	Time (h)									
CCU	24	48	72	96	120	144	168	192	216	240
10^5	-	+								
10^4	-	-	+							
10^3	-	-	-	+						
10^2	-	-	-		+					
10^1	-	-	-	-	-	-	+			
10^0	-	-	-	-	-	-	-	-	-	-

For solid culture, agar plates were prepared by addition of purified agar (Oxoid) in modified Hayflick's broth to a final concentration of 1.0% (w/v). Serial decimal dilutions of *M. bovis* sample from 10^{-2} to 10^{-5} were prepared and a 200 μL aliquot was plated in duplicate on the culture media. The Petri plates were sealed with Parafilm® M (Li *et al*., 2005) and incubated at 37°C (Table 1).

Characterization of *Mycoplasms bovis*: Samples were collected from dying young cattles after dissection. Lungs which showed major fibrinous necrotizing and suppurative lesions, were cut in a sterile manner, kept at around 4°C and transported to the lab within a few hours and stored at -20°C. When the present study was undertaken, lung tissue scraps were obtained using an animal tissue sampler (China patent application number: 201120108302.2). After treatment by a Bullet Blender® homogenizer (Next Advance inc., USA) with 1.5 mL polypropylene tubes, the supernatant was divided into labeled aliquots, serially diluted and inoculated in fresh liquid media and cultivated at 37°C until the cell density was approximate 10^5 cells mL^{-1}.

At each day interval, Color-Change Units (CCU) which are directly related to the titre of viable mycoplasmas were determined as described previously (Ueno *et al*., 2008).

The colony morphology was investigated by microscopy. A nest or duplex PCR was carried out using a GeneAmp® PCR System 9700 (Applied Biosystems, USA) for verification.

The nested PCR was followed according to Pinnow *et al*. (2001) using outside primers (PpMB920-1 and PpMB920-2) and inside primers (PpSM5-1 and PpSM5-2). After 35 cycles of 94°C for 30 sec, 48°C for 60 sec and 72°C for 120 sec, the outside reaction product was diluted 1:100. The inside reaction was carried out under the same conditions except the annealing temperature of 54°C, a 442 bp DNA fragment was specifically amplified.

A duplex-PCR assay (Liu *et al*., 2010) was adapted for the differential detection of *M. bovis* and *M. mycoides* ssp. *mycoides* SC (MmmSC) using primers specific to *M. bovis* and MmmSC, respectively. A 528 bp DNA fragment was specifically amplified from the *M. bovis* and a 270 bp fragment from the MmmSC type strain PG1. The cycling conditions were an initial denaturing step at 94°C for 5 min followed by 35 cycles of 94°C for 45 sec, 52°C for 40 sec and 72°C for 60 sec.

Total 10 μL aliquots of the PCR product were separated by electrophoresis in a 1% (w/v) agarose gel. The gel was then stained with ethidium bromide (0.5 μg mL^{-1}) for photography. Positive bands were cut and verified via sequencing by Invitrogen Corporation Shanghai Representative Office (Table 2).

Table 2: Determination of growth of *M bovis* in 400 μL fresh medium in 1.5 mL eppendorf tube using Color-Change Units (CCU)

	Time (h)									
CCU	24	48	72	96	120	144	168	192	216	240
10^5	-	+								
10^4	-	-	+							
10^3	-	-	-	+						
10^2	-	-	-		+					
10^1	-	-	-	-	-	-	+			
10^0	-	-	-	-	-	-	-	-	-	-

CCU was determined over a period of 240 h from cultures inoculated with the approximate number of bacteria shown (100 μL aliquot of *M. bovis* inoculum with a 10 fold serial dilution of initial concentration of 8.6×10^5 CFU mL^{-1}). +: Indicates a change in the color of the medium from red to yellow; -: Indicates no change in medium color

RESULTS AND DISCUSSION

A contagious respiratory disease of cattle occurred in a farm in Guizhou Province of China in 2010. The main symptoms of this disease were coughing and high fever which led to the death of 4 out of 35 infected cattle. The pathogenic samples were collected from young calf lungs with a patented animal tissue sampler. As shown in Fig. 1, a positive sample was confirmed by nest and duplex PCR amplification. Targeting lipoprotein P80 DNA 504 bp fragment using pMB81-1 as a sequencing primer demonstrated 95 and 99% homology with *M. bovis* type strain PG45 clone MU clone A2 and *M. bovis* Hubei-1. When the sera were tested for antibody against *M. bovis* using a commercial kit (Biovet), positive results were obtained from all 35 samples.

M. bovis suspension was divided into aliquots inoculated in fresh liquid medium in 10 mL tubes (10×5) or 1.5 mL eppendorf tubes and incubated at 37°C with or without 5% CO_2. As is shown in Fig. 2, a change in the color of the medium from red to yellow increases when the initial medium volume decreases (i.e., the upper air space increases). The 400 μL fresh medium plus 100 μL inoculum was chosen as the optimum with regard to a comparative test of the liquid cultural method for sufficient *M. bovis*

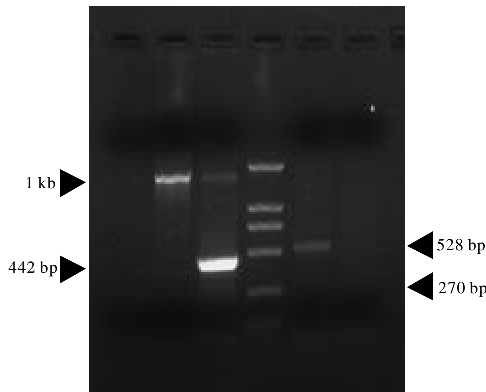

Fig. 1: A PCR-confirmed *M. bovis* positive sample. Lane 1: nested PCR 1000 bp product using outer primers; Lane 2: nested PCR 442 bp product using inner primers; Lane 3: ML2000 Marker (TaKaLa); Lane 4: 528 bp DNA fragment specific to *M. bovis*, 270 bp DNA fragment specific to MmmSC type strain PG1 was nagtive

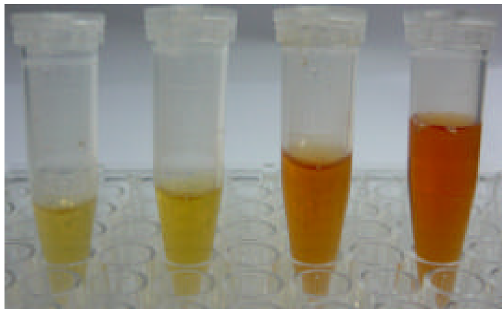

Fig. 2: Effect of the upper air space on the change in the color of the medium. The 200, 400, 600 or 800 μL fresh medium (from left to right) plus 100 μL inoculum (8.6×10^5 CFU mL^{-1}) in each eppendorf tube was incubated at 37°C for 4 days

reproduction. The most suggestive evidence in growth behavior by the two broth cultural methods was obtained when their growth dynamics were examined by CCU enumeration.

As expected, both methods showed evident growth between starting inocula of 10^5 and 10^1, for at least 10 starting organisms were required to establish growth in broth culture (Table 1 and 2).

When cultured in an open dish (Fig. 3a) at 37°C with 5% CO_2, *M. bovis* had a typical colony formation commonly described as fried egg due to the opaque granular central zone of growth penetrating the agar surrounded by a flat translucent peripheral zone on the surface while cultured in a sealed dish, colony formation changed a bit with a smaller opaque granular central zone

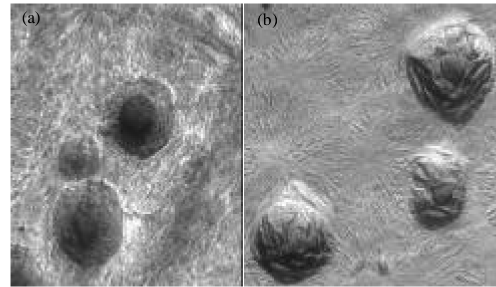

Fig. 3: Micrograph of *M. bovis* colonies (40x). a) Colonies in an open dish and b) a sealed dish were photographed by using an inverted microscope (eye piece of WH10x-H22 and object lens of UPlanF1 4x/0.13 PHL, Olympus IX70) at day 8th post-inoculation

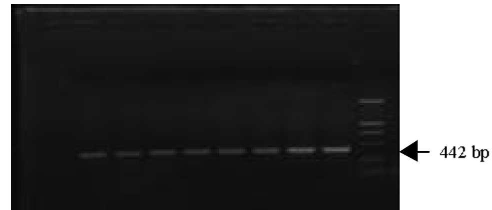

Fig. 4: Nested PCR-amplified *M. bovis* 442 bp products from 10 fold serially diluted inoculated cultures. Lane 1: Negative control (fresh liquid medium); Lane 2: 8.6×10^{-1}; Lane 3: 8.6×10^0; Lane 4: 8.6×10^1; Lane 5: 8.6×10^2; Lane 6: 8.6×10^3; Lane 7: 8.6×10^4; Lane 8: 4.3×10^4; Lane 9: 8.6×10^5; Lane 10: Positive control (frozen preserved *M. bovis* broth) and Lane 11: ML2000 Marker (TaKaLa)

and more irregular peripheral zone (Fig. 3b). Both methods of plating were very convenient for *M. bovis* culture because the colonies could be distinguished when viewed by a low-power microscope and could be easily applied for further culture and investigation. As shown in Fig. 4, a nested PCR was used to validate the starting inocula from 8.6×10^0 to 8.6×10^5 CFU mL^{-1}, the amplified 442 bp *M. bovis* DNA products showed that initial 100 μL inocula aliquot of 8.6×10^1 CFU mL^{-1} *M. bovis* suspension into 400 μL fresh medium in 1.5 mL eppendorf tubes should be detected after incubation at 37°C for 4 days, i.e., 3 days earlier when compared with CCU enumeration.

There are several circumstances under which the use of culture and microscopy detection, coupling with PCR identification seems to offer some advantages. One application is the direct examination of clinical material. The method requires some experience and also to some

extent, special optical equipment. This will certainly limit the number of laboratories in which cultural and microscopic examination can be used as a diagnostic tool for detection of *M. bovis*. However, with the fresh medium easily prepared and dispatched into eppendorf tubes, the initial liquid cultural procedure could perhaps provide possibility for rapid diagnosis of the disease by Color-Change Units (CCU) enumeration. Further solid culture procedure with the Petri dish sealed by Parafilm® M provides a simple way to characterize *M. bovis* colonies. It is easy to generate microaerobic conditions without a CO_2 incubator, to prevent further drying of the medium and to prevent contaminating from fungi or bacteria after long time incubation.

CONCLUSION

The results showed an improved method for the detection of *M. bovis* using an eppendorf tube and a sealed plate which can be easily handled and can generate microaerobic conditions suitable to growth of *M. bovis*. The combination of the two cultural methods and following Color-Change Units (CCU) enumeration, colony morphology or PCR assay shows an effective procedure to detect *M. bovis* which has the potential for easy diagnostic applications.

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