Global Veterinaria 13 (3): 385-390, 2014
ISSN 1992-6197
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DOI: 10.5829/idosi.gv.2014.13.03.84269

Evaluation of In-House Solid Media for *Mycobacterium avium* Subspecies *paratuberculosis* Cultivation

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Abstract: Paratuberculosis is chronic granulomatous enteritis in ruminants having an economic impact. Cultivation of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from faecal and tissues samples is a definitive test for the detection of paratuberculosis in animals and also a gold standard method. Herrold's egg yolk medium (HEYM) and Lowenstein-Jensen (LJ) are solid media which are commonly used for cultivation of MAP. However, commercial HEYM is quite expensive in Indonesia. The aim of this study is to evaluate the ability of Ogawa medium which was modified with mycobactin J (Mmj) for cultivation of MAP and will be compared to commercial HEYM with mycobactin (Hmj). Suspension of MAP in PBS with concentration of 10^0-10^3 was inoculated each 0,1 ml in 4 Mmj and Hmj. After incubation period of 16 weeks, bacterial growth showed starting at the dilution of 10^1-10^3 in both media. While at dilution of 10^0 no bacteria were found. Hmj bility for isolation of MAP better than Mmj, but the sensitivity of the both media no difference. In general, for the purpose of MAP isolation, Hmj better than Mmj. However, Mmj was more economic than commercial media and can be used as an alternative medium for the cultivation of MAP.

Key words: *Mycobacteriuma vium* Subspecies paratuberculosis · Cultivation · Media

INTRODUCTION

Paratuberculosis or Johne's disease (JD) is chronic granulomatous enteritis in ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) [1, 2]. Besides infecting ruminants, MAP also affects non ruminants such as rabbit, raccoon, fox, badger and wild boar [3-5]. In clinical case, the symptoms detected are profuse diarrhea and emaciation with large amount of bacterial shedding in feces. Paratuberculosis causes huge economic losses in dairy farms due to decline in milk production, reproductive disorders, increased calving interval, reduce of carcass weight and early culling [6-9]. The disease is mostly transmitted horizontally through the fecal-oral route transmission from infected adult animals to young animals (under 6 months of age) [10, 11]. Also, the transmission was reported vertically through intra-uterine route [12, 13]. In Indonesia, paratuberculosis cases were reported in 2008, in which the MAP was isolated from dairy cows with an estimated prevalence of 2 % [14].

Isolation and culture of MAP from fecal or tissues samples are a definitive method for the detection of paratuberculosis in animals. To cultivate MAP in a solid media requires incubation time from 8 to 16 weeks and utilizes a special media. To date, feces culture is the gold standard test, because it can detect subclinical paratuberculosis [15-18]. Solid media which are widely used for the MAP culture are Herrold's egg yolk medium (HEYM) and Löwenstein medium (LJ) with mycobactin [19-21].

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Ogawa media is a media normally used to culture *Mycobacterium tuberculosis* [22-24]. However, its ability to be used for the purpose of MAP cultivation has not been reported. This media is more economic, very simple and easy to manufacture. HEYM is already commercially available. This media however is an imported product and quite expensive in Indonesia. This is an obstacle in conducting MAP cultivation. To overcome this problem, we conducted a study to make a simpler and more economic solid media for the cultivation of MAP. This media is the Ogawa media which have been modified. The primary objective of this study was to evaluate the ability of the solid medium to be used for MAP cultivation compared to a commercial HEYM with mycobactin (Becton Dickinson-BBL, USA).

MATERIALS AND METHODS

***Mycobacterium avium* Subspecies *paratuberculosis* Isolate:** MAP used in this study is an isolate collected from dairy cattle which had been confirmed by using Polymerase Chain Reaction (PCR) with IS900 and F57 primers, as well as the mycobactin dependency of MAP. This isolate was obtained from Balitvet Culture Collection (BCC) with identification number of B2788.

Modified Ogawa Medium with mycobatin J (Mmj): The formulation of culture media used for 1 L was as follows, 3.0 g monopotassium dihydrogen phosphat anhydrous (Merck, German), 3.0 g monosodium glutamate (Ajinomoto), 2.5 g L-asparagine (Merck, German), 4.0 g sodium pruvate (Merck, German), 20.0 ml glycerol (Merck, German), 2.0 mg mycobactin J (Allied Monitor, USA), 15.5 g agar noble (BD-BBL, USA). The materials were then dissolved in distilled water up to 790 ml, pH 7.0 and sterilized with autoclave at 121°C for 20 minutes. Media was cooled to a temperature of 50°C and after that 200.0 ml of egg yolks, 10.0 ml of 2% malachite green were added. Media solution then was put into a 10.0 ml sterile tube and placed slant to solidify. To check the sterility, the media was incubated at 37°C for 48 hours and stored at 4°C until being used.

HEYM with mycobactin J used in this study was a commercial product from Beckton Dickinson-BBL, USA (Hmj).

***Mycobacterium avium* Subspecies *paratuberculosis* Isolate Suspension:** MAP colonies were put into tubes containing sterile PBS and glass bead. Tubes then were vortex-mixed until homogeneous. The concentration was measured according to the method described by Ristow *et al.* [25], which was calculated using McFarland formula number 1. The estimated concentration of the suspension was approximately 10^7 CFU/ml. Bacterial suspension was further diluted into 10^3, 10^2, 10^1, 10^0 CFU/ml.

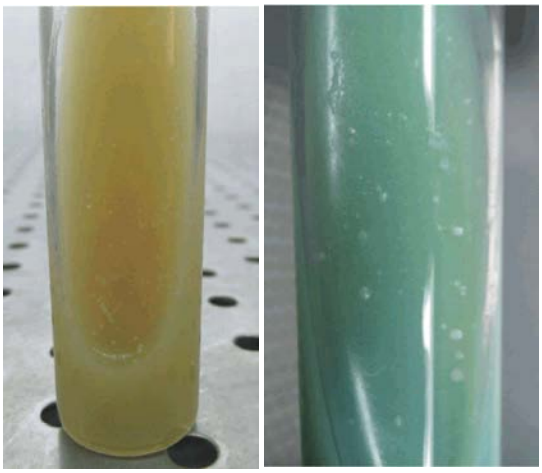
Evaluation of Culture Media: Around 0.1 ml of bacterial suspension with concentration of 10^0-10^3 CFU/ml was inoculated into 4 Hmj and Mmj. Tubes were incubated at 37° C for 16 weeks. In the first week, the tubes were placed slant and the lids were loosened. After that the tubes were placed upright with the lids were sealed and incubated again until the 16th week. Observation of MAP colonies growth was conducted every 2 weeks.

Statistical Analysis: To investigate the difference between two media evaluated, the average of MAP colonies growth in both media was analysed statistically using student t-test method with GraphPad Prism 6 software (GraphPad Software Inc., USA). The results was considered statistically significant if the p value was < 0.05.

RESULTS AND DISCUSSION

Several media used to cultivate MAP are Herrold's egg yolk medium (HEYM), modification of Dubos, modification of Middlebrook 7H10, Middlebrook 7H9 and Löwenstein Jensen (LJ). All those media are with mycobactin addition [21, 26, 27].

For MAP suspension in PBS with concentration of 10^3, MAP growth in the Hmj and Mmj media was observed in week 4. The average number of colonies growing, after 16 weeks incubation period, in Mmj and Hmj media were 52.0 and 89.25 colonies respectively (Figure 1). For MAP suspension with concentration of 10^2, the MAP growth in Mmj medium was seen in week 6 with the colony grew after incubation period was 11.25, while in Hmj medium the MAP growth was observed in week 4 with the number of colony of 28.5. The growth of colonies for MAP 10^1 suspension in Mmj and Hmj were detected in week 6, with the average number was 2.25 and 5.0 respectively. No bacteria grew at suspension of 10^0 on both media (Table 1). For dilution 10^2-10^3, Mmj and Hmj there re a significant differences ($P < 0,001$). The ability of Hmj for MAP cultivation better than Mmj, but the sensitivity of the both media was no difference.



A B

Fig 1: The growth of MAP in Hmj (A) nd Mmj(B)

Table 1: The growth of MAP in Mmj nd Hmj media

	The average number of MAP Colonies Growing	
MAP suspension	Mmj	Hmj
MAP in PBS/10^0	0	0
MAP in PBS/10^1	2.25	5.00
MAP in PBS/10^2	11.25	28.50
MAP in PBS/10^3	52.00	89.25

The composition of original Ogawa medium was unable to be used to cultivate MAP, possibly because it does not contain mycobactin. Mycobactin is siderophores compounds that help microorganisms absorb iron which is present in very limited amount in the environment. For in vitro growth on culture media, MAP was reported depend on and to require mycobactin from outside. This is because MAP could not produce mycobactin sufficiently [28, 29].

The modification of Ogawa is done, because the original Ogawa media already has a basic component that is adequate for the growth of mycobacteria.The addition of some other components is expected to provide better condition and nutrition for MAP to grow. On Mmj and Hmj, MAP was able to grow well. Glutamate and asparagine in Mmj medium gives an advantage because the bacteria have nitrogen resource from two amino acids. This in accordance with Leal *et al.* [30] showing that nitrogen resource for mycobacteria growth can be obtained from amino acid or other proteins which presence in the media.

Glycerol which is available in both media can be used as carbon resource for MAP growth [30, 31]. In addition, glycerol is also reported to reduce the resistance of MAP to the media acidity [32]. For the source of energy required by MAP, it may be obtained from sodium pyruvate which is presence in the media. Sodium pyruvate was reported as a source of energy and stimulating MAP growth by reducing the active glycolytic pathway [31, 33] and to prevent the effect of antibiotic [34].

In the modified Ogawa medium components whole egg is replaced with egg yolk. The use of egg yolk for the initial isolation of MAP is very important, although it is not for the purpose of subculture [35-37]. Harris *et al.* [38] reported that the growth of MAP in the ESP II media with addition of egg yolk was better than those without egg yolk, while Jorgensen [34], stated that the MAP colonies grew larger on the LJ egg-based media. The function of egg yolk for bacterial growth is not yet known, it is likely that egg yolk neutralizes the action of decontaminant, such as hexadecylpyridinium chloride (HPC) and benzalkonium chloride. Another role of the egg yolk is possibly as a source of iron [39,40], carbon and energy [37]. The content of egg yolk 12% (120 ml/L) will provide iron approximately 8-9 mg/L of medium [33]. The content of egg yolk in Mmj medium was 20%, so it is likely that the amount of iron in 1 L of medium was estimated 13-14 mg. Components in the medium Mmj re sufficient for the basic needs of growing MAP.

CONCLUSION

For the purpose of *Mycobacterium avium* subspecies *paratuberculosis* cultivation, commercial HEYM has better ability than the modified Ogawa medium, but regarding sensitivity of the both media there is no difference. In general, for the purpose of MAP isolation, commercial HEYM better than the modified Ogawa. However, Modified Ogawa can be used as solid media alternative which is more economic than commercial HEYM for the cultivation of MAP.

AKNOWLEDGEMENTS

I would thanks to Indonesian Research Center for Veterinary Science which has provided facilities and also isolate *Mycobacterium avium* subspecies *paratuberculosis* for this study. I would also thanks to all staff at Tuberculosis unit for their assistances during this project.

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