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Comparison of four different culture media for growth of *Mycobacterium avium* subsp. *avium* isolated from naturally infected lofts of domestic pigeons

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ABSTRACT

Background and Objectives: Diagnosis of avian tuberculosis by conventional culture method is still considered as the "gold standard" technique. The main objective of this study was to compare growth of *Mycobacterium avium* subsp. *avium* on four specific Mycobacterial cultures such as glycerinated Lowenstein-Jensen medium, pyruvate-enriched Lowenstein-Jensen medium, mycobactin J-supplemented Herrold-egg yolk medium and plain Herrold-egg yolk medium

Materials and Methods: Eighty out of more than 600 pigeons were selected based on their clinical signs and poor health conditions. The birds were numbered and their clinical signs were registered in the working sheets, and under standard condition, euthanized, subjecting to necropsy examinations, followed by bacterial culture on four specific media for *Mycobacterium avium* subsp. *avium*, including glycerinated Lowenstein-Jensen (LJG) medium, pyruvate-enriched Lowenstein-Jensen medium (LJP), mycobactin J-supplemented Herrold-egg yolk medium and plain Herrold-egg yolk medium.

Results: Fifty one *Mycobacterium avium* subsp. *avium* were isolated from pigeons. Mycobactin J-supplemented Herrold-egg yolk media yielded greater number of colonies in shorter incubation time in compare with other media.

Conclusion: It was concluded that most of the isolates need mycobactin as a growth factor.

Keywords: *Mycobacterium avium*, pigeon, culture, *Avium tuberculosis*

INTRODUCTION

Avian tuberculosis is one of the most important diseases that affect most orders of the birds (1, 2). Several Mycobacterial species can be involved in the etiology of avian tuberculosis, but the disease is most often caused by *Mycobacterium avium* (MA) belonging to serotypes 1, 2 and 3 (genotype IS901+ and IS1245+) and *Mycobacterium genavense* (3, 4). All species of birds can be infected with *M. avium*. Domesticated fowl or captive wild birds are affected more frequently than those living in a wild state (4). Symptoms of mycobacteriosis in birds include chronic illness characterized by weight loss, diarrhea, dyspnea, lameness and poor feathering, although a significant number of birds die acutely without recognized symptoms (2). The most common route of infection for susceptible birds is via the alimentary tract; however, pulmonary avian tuberculosis and egg transmission have also been described (3, 5). Lesions are seen most frequently in liver, spleen, intestines and bone marrow and less frequently in other organs (5). Isolation via culturing and/or molecular methods remains the most definitive means of diagnosing mycobacterial infection in birds (6). *Mycobacterium avium* grows best on media such as Lowenstein–Jensen, Herrold's medium, Middlebrook 7H10 and 7H11, or Coletsos medium, with 1% sodium pyruvate added (7). Cultures should be incubated for at least 8 weeks (7). Typically *M. avium* produces 'smooth'

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Fig. 1. The example of real PCR amplification product. The 427 bp specific fragment from IS1245. Lane M, DNA size marker (100 base pair ladder). Lane 1 and 2, negative controls (distilled water). Lane 3, negative species control (*Mycobacterium bovis* AN5 strain, ATCC number 35726). Lane 4, positive control (*Mycobacterium avium* subsp. *avium* D4 strain, ATCC number 35713). Lane 5 to 9 samples tested for *Mycobacterium avium* subsp. *avium*

colonies, within 2–4 weeks; rough variants do occur. Colonies are larger if the medium contains glycerin (8). Some strains of *M. avium* have been identified to have special requirement of mycobactin as a growth factor (7). The importance of avian tuberculosis and difficulty in culturing the organism motivated our interest to investigate the growth of some *Mycobacterium avium* subsp. *avium* (MAA) isolates from naturally infected lofts of domestic pigeons (*Columba livia* var. *domestica*), on four solid media.

MATERIALS AND METHODS

Eighty suspected pigeons (*Columba livia* var. *domestica*) to avian tuberculosis, out of a total of about 600 pigeons from more than 10 lofts, based on their clinical signs (swollen joints, lameness, emaciation, tubercle formation under the skin and granulomas in the conjunctival sac**)** and poor health conditions were selected and transferred to avian diseases section, Faculty of Veterinary Medicine of Ahvaz. The birds were numbered, euthanized and subjected to necropsy examinations. Gross lesions observed in the internal organs were noted on the working sheets and immediately tissue samples from the lesions from each bird which had lesions and liver samples from all the birds were aseptically collected in 50 ml screw cap containers and sent in dry ice chambers to the tuberculosis reference laboratory, Razi Vaccine and Serum Research Institute for definitive identification.

Mycobacterial isolation. After thawing the tissue samples in the tuberculosis reference laboratory, approximately 4 gram of tissues of each bird were pooled and grinded in a pestle and mortar containing sand using sterile materials and equipments. The homogenized mixtures were decontaminated according to the NALC-NAOH method (9). The inoculums were cultured on 4 culture slopes including glycerinated Lowenstein-Jensen (LJG) medium, pyruvate-enriched Lowenstein-Jensen medium (LJP), mycobactin J-supplemented Herrold-egg yolk medium (HM) and plain Herrold-egg yolk medium (H). The inoculated slopes were incubated at 41ºC for 8 to 16 weeks. All suspected colonies visualized with the naked eye. Genomic DNA of all isolates from each infected pigeons was extracted according to the van Soolingen method (10). All Acid- Fast Bacilli (AFB) were tested (11) by the PCR assays targeting the 16S rRNA gene for identification of mycobacterium members, IS1245 for mycobacterium avium complex and finally IS901 for identification of *Mycobacterium avium* subsp. *avium* (MAA) (12-14). Analyses of PCR products were conducted on ethidium bromide-stained 2% agarose gels in a submerged electrophoresis system.

RESULTS

Fifty one AFB were isolated from pigeons. All of the AFB isolates were positive for IS1245 and IS901 in PCR (Figs. 1 and 2). Clinical signs, ecropsy findings, ZN staining and molecular identification confirmed that the pigeons were infected with

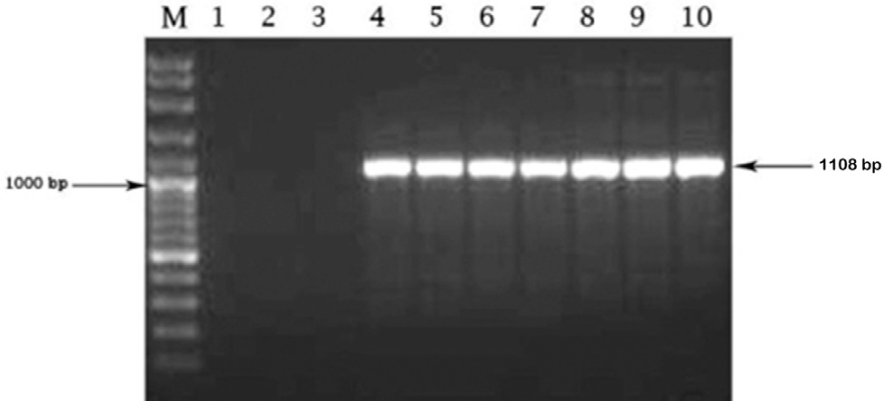

Fig. 2. The example of PCR amplification product. The 1108 bp specific fragment from *IS*901. Lane M, DNA size marker (100 base pair ladder). Lane 1 and 2, negative controls (distilled water). Lane 3, negative species control (*Mycobacterium bovis* AN5 strain, ATCC number 35726). Lane 4, positive control (*Mycobacterium avium* subsp. *avium* D4 strain, ATCC number 35713). Lane 5 to 10 samples tested for *Mycobacterium avium* subsp. *avium.*

MAA. In necropsy examinations firm grayish-yellow or grayish-white and raised nodules were found especially on liver and intestines. Liver and intestines were the most frequently affected organs, and lungs were the least affected organs while no macroscopic lesion was found in the gonads, kidneys and CNS. Forty six isolates out of 51 MAA isolates, first appeared on the HM media and then on the other media but with lesser colony significantly. Five out of 51 MAA isolates first appeared on the LJP media, then on the other media. The type of growth was also distinctive for each solid medium. The colonies in HM media were very small, usually in a high number distributed throughout the entire surface of the medium, and difficult to be observed macroscopically compared with the colonies in other culture media. In Lowenstein-Jensen medium, especially pyruvate-enriched, the colonies are rougher, larger, and more easily observed macroscopically. With the exception of 5 isolates, all MAA isolates grew on the HM media in less than 12 weeks incubation, but most of them grew on the other media after 12 weeks.

DISCUSSION

Even though diagnosis of avian tuberculosis by conventional culture is tedious and requires a long incubation period, it is still considered as the "golden standard" technique. It is important to use the proper media for detecting all the infected birds (15). In this study the growth of 51 MAA isolates which were isolated from naturally infected lofts of domestic pigeons were evaluated on 4 solid media. Several media are used for the primary isolation of *M. avium* and these include: 1) Egg-based media like the Lowenstein-Jensen medium and Herrold's egg-yolk medium, 2) synthetic media, such as, Middlebrook medium. Growth of certain strains of *M. avium* is dependent on mycobactin as a growth factor (8). Matthew *et al*. (1976) described the importance of mycobactin enriched medium for primary isolation of *M. avium* especially when a small number of bacteria exist (16). In another study he demonstrated a significant increase in isolations of *M. avium* complex on Herrold's egg-yolk medium with mycobactin in comparison with other media particularly Lowenstein-Jensen medium (17). To determine the best media for culturing tissue and fecal samples from infected Japanese quail with *M. avium*, Modified Herrold egg yolk with mycobactin, Lowenstein-Jensen, and Lowenstein Jensen with cycloheximide, nalidixic acid and lincomycin were evaluated. Lowenstein-Jensen media (without additives) provided more positive cultures, had greater numbers of colonies on positive tubes and had shorter incubation times than the other media (18). However in this study mycobactin J-supplemented Herrold-egg yolk media provided greater number of colonies on positive cultures and shorter incubation time than the other media. This indicates that these isolates need mycobactin as growth factor. According to our study, the best medium for isolation of MAA from domestic pigeons is HM medium. In this study approximately all of the MAA isolates, except 5 isolates which first appeared on the LJP media, grown on the HM media before 12 weeks, but most of them grown on the other

media in more than 12 weeks; therefore, increasing the incubation period up to 16 weeks to avoid false negative results due to relatively short incubation time is recommended. The use of two solid media, HM plus LJP, is highly recommended to prevent false-negative results or poor growth. In some molecular techniques such as restriction fragment length polymorphism (RFLP), large amount of extracted DNA is needed. For this reason, in some cases obtaining large number of colony is crucial. In conclusion, use of the proper media as well as extending the incubation period up to 16 weeks for detection of MAA in domestic pigeons is indispensable. Further research about other isolates of MAA from animals especially birds is needed to understand the growth requirements of the different MAA isolates from different sources.

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