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Research Article

Compare Culture and Designed ELISA Methods to Diagnose Tuberculosis in Suspected Pigeons

Mahsa Lari Baghal[1], Mansour Mayahi[2]*, Nader Mosavary[3] and Zahra Boroomand[2]

[1]Ph.D. Candidate in Avian Health and Diseases, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

[2]Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

[3]Bovine Reference Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

***Corresponding Author:** Mansour Mayahi, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.

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Abstract

Backgrounds: Avian Tuberculosis (TB) is a chronic disease and caused by Mycobacterium avium subsp. avium and *Mycobacterium genavense*.

Aims: To diagnose the avian tuberculosis disease by culture and designed Elisa methods and compare results of two diagnostic methods.

Methods: One hundred one suspected pigeons were selected based on clinical signs and poor physical condition and transferred to the Avian Diseases Laboratory of Veterinary Medicine Faculty of Ahvaz. Blood sample was collected via pigeon wing vein and serum was collected. Then pigeons were euthanized and post mortem was performed, any gross lesions were recorded and sample from liver and spleen and each organ with gross lesions was collected and stored in freezer at -40 ˚C. All samples were placed on dry ice and transferred to reference tuberculosis laboratory of Razi Vaccine and Serum Research Institute, Karaj. Each sample after preparation inoculated into glycerinated Lowenstein-Jensen medium, pyruvate-enriched Lowenstein-Jensen medium and Herrold-egg yolk medium and bacterial growth was monitored for 3 months. Antibody against mycobacterium was were measured by designed Elisa test.

Results: Out of 101 pigeons, 39 (38.6%) and 13 pigeons (12.9%) were tuberculosis positive respectively by culture and designed Elisa methods. Correlation between results of culture and designed Elisa methods was significant (0.485) ($p < 0.01$).

Conclusions: It was concluded that mycobacterium affected pigeons lungs and there is significant correlation (0.485) ($p < 0.01$) between culture and designed ELISA system tests. The ELISA test due to easy sampling, speed, low price, non-invasive, prefer test for screening affected pigeons flocks.

Keywords: Avian Tuberculosis; Culture; Designed Elisa Test; Mycobacterium

Introduction

Tuberculosis is infectious diseases common between humans and animals and causes deaths every year and still one of the health problems [36]. In poor areas of the world people are the most frequent victims of TB [17]. Due to the commonality between bird and human, increasing risk of this disease in human colonies [23]. Avian tuberculosis is a very important disease that is seen in free-living and predatory birds and in domestic and wild mammals

[30]. Eradication of bovine tuberculosis in Czechoslovakia in 1968, Mycobacterium avium became the most important pathogenic mycobacterium in animal farms and also in birds and mammals [27]. The disease is a chronic and generally affects the bird's gastrointestinal tract and often causes bird death [25]. Due to the damage caused by tuberculosis in zoos, the risk of extinction of some of the rare birds, the zoonosis disease and the difficulty in controlling, it is very important to diagnose the disease right time [11]. The disease is most often caused by *Mycobacterium avium* subsp. avium and *Mycobacterium genavense* [29]. There are more than ten species of Mycobacterium (*Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycobacterium intercellular*, *Mycobacterium fortuitum*, *Mycobacterium scrpfulaceum*) reported in infected birds [7,16,28,29]. *Mycobacterium avium* subsp. avium is the most important cause of poultry disease [11]. Seed-eating birds, main source of *Mycobacterium avium* have been considered for a long period. Pigeons and aquatic birds infected more than poultry [27]. The disease diagnosed by culture, serologic tests [10] acid fast staining and radiography [20,26,30]. The clinical signs are not pathogenic and there are so many differences in clinical signs [2001]. Birds without clinical signs could be susceptible to tuberculosis if acid-fast bacteria are present and prove bacterial culture [28]. Although culture is the most definitive method to confirm the infections, but this method has problems such as invasion, slow growth of mycobacteria, and need appropriate temperature, nutrient and oxygen requirements [29]. Serological diagnosis tests of avian tuberculosis include hemagglutination (HA), complement fixation (CF) and Elisa [18,33]. These tests are species-specific and are only available for a limited number of species [3]. The sensitivity of the ELISA test is high, but is difficult and requires some specific antigens and false positive results can be high [11,30]. Available information is mainly limited to the detection of avian tuberculosis by culture and post-mortem lesions. Published study regarding to diagnosis of pigeon tuberculosis by serological method is rare. Therefore, the study was designed to diagnose the avian tuberculosis disease by culture and ELISA methods.

Materials and Methods

Sampling

The study was conducted according to license EE/98.24.3.45458/scu.ac.ir, ethics committee of Veterinary Medicine, Shahid Chamran University of Ahvaz during 2018 to 2019. From 10 suspected flocks with more than 700 pigeons, 101 pigeons were selected based on clinical signs and poor physical condition and transferred to the Avian Diseases lab of Veterinary Medicine, Shahid Chamran University of Ahvaz. Information of each pigeon was recorded in information sheet and blood sample was collected via pigeon wing vein and after centrifuging, serum was collected and stored in small vials in freezer at -40 ˚C up to test. Then pigeons were euthanized and post mortem was performed and any gross lesions were recorded in information sheet and sample from liver and spleen of each pigeon was collected and if lesions were observed in each organ, biopsies were taken and stored in freezer at -40 ˚C. Then all samples were placed on dried ice and transferred to reference tuberculosis laboratory of Razi Vaccine and Serum Research Institute, Karaj for diagnosis by culture and serology methods.

Culture

The samples within the trays were placed under the biological hood (Model SDS-156). Then, using forceps and a sterile scalpel, 5 cm pieces were cut off from the organs and placed in sterile mortars. Sterile sand was poured onto the samples to be ground. For decontamination, 50 ml of sodium citrate, sodium hydroxide and N-acetyl N-cysteine solutions were added to each sample and a homogeneous and uniform mixture was obtained. After 20 min, about 15 to 20 cc of the solution from the top of the solution in mortar is removed by sterile Poir and poured into a Falcon tube containing 5 cc of hydrochloric acid and a drop of methylene blue reagent. Then the Falcon tubes were centrifuged at 4500 for 15 min. The supernatant liquid was discarded. To the bottom sediments was added 2.5 cc of phenol-free phosphate buffer with pH=6.8. After, about 1 cc was inoculated into glycerinated Lowenstein-Jensen medium, pyruvate-enriched Lowenstein-Jensen medium and Herrold-egg yolk medium. Inoculated media were incubated at 37 ˚C and bacterial growth was monitored for 3 months.

Designed elisa system

Mycobacterium avium subsp. avium D4 strain antigens at Microbial Bank of Karaj Razi Vaccine and Serum Research Institute were used for serological tests. The concentration of 40 µg to lower concentrations designed to determine the best concentration of antigen and antibody to Checkerboard. In order to achieve positive control, two to three pigeons were injected with standard D4 strain after blood sampling (as negative control- after no antibody to *Mycobacterium avium* subsp. avium was identified). After one month, blood was collected again and their serum was used as a positive control on the Checkerboard.

Checkerboard process

Antigen dilution was performed from 100 µg/ml in the well without antigen. Used coating buffer was 0.1 molar bicarbonate carbonate buffer and antigen dilution was performed with this buffer and added to each well of 100 µl. The plate was covered with foil and it was stored at refrigerator temperature for 16 to 18 h. After this process, it was washed three times with buffer PBS 0.01 molar.

Blocking step

150 µl of 2% BSA blocking solution was poured into wells and then the plate was covered with foil and incubated for 1 h at room temperature. After 1 h, the blocking solution was empty and 30 min were taken until the wells were completely dry and the plate was ready for use.

Add antibody (serum sample)

Charged cow serum; using the antigen of *Mycobacterium avium* subsp. avium (standard strain D4) as a positive control and uncharged cow serum was considered as negative control. And 1/100 dilution was used for the check-in process. Sera were prepared by diluting buffer (1% PBST with 2% BSA) and incubated at room temperature for 30 min. The plate was washed 5 times and dilution of 1/10000 peroxidase conjugated antibody was used. After 30 min of incubation at room temperature the plate was washed again.

Add substrate (3, 3', 5, 5' - Tetra methyl benzidine (TMB))

In each well, 100 landa TMB substrate solution was added and incubated for 10 min at room temperature and darkness.

Stop Solution

100 landa stop solution was poured into well.

Reading OD

The plate was read at 450 nm.

Designed Elisa system test procedure

- To perform the Elisa test, the standard *Mycobacterium avium* subsp. avium strain D4 antigen was used to coat the bottom of the Elisa plate.
- Molar bicarbonate carbonate buffer was prepared and 11 ml of the resulting solution was mixed with 100 landa D4 strain standard antigen.
- 100 landa of obtained solution were coated in each well of the Elisa plate.
- The Elisa plate was incubated in 2-8 ˚C for 24 h.
- After 24 h, the Elisa plate was removed from the refridgerator and the solution was discharged into the wells.
- Elisa plates were washed 3 times with 300 landa PBS without Tween.
- 150 landa Blocking solution was poured into the wells and left for 1 h.
- After 1 h, the blocking solution was discharged and the time was 20 min.
- Serum samples were diluted 1: 150.
- Then 100 landa of diluted sample was removed and poured into Elisa plate.
- 30 min were given.
- Elisa plates were washed 5 times with 300 landa washing solution.
- 100 landau Gout Anti chicken 1x was added to all wells of Elisa plate and given 30 min.
- Elisa plates were washed 5 times with 300 landa washing solution.
- 100 landa TMB was added to all wells and the plate was incubated in the dark for 10 min.
- 100 landa Stop solution was added to all wells.
- At the end, the Elisa plate was read with the Elisa Reader.

Results

The pigeon's clinical signs are presented in table 1. The most clinical signs were related to progressive loss of weight and then hook joints nodules and inability to move.

Gross pathology

Lesions were seen most frequently in liver, spleen, cloaca and lungs respectively. Gross lesions were not observed in gonads, kidneys, abdominal cavity and heart (Table 2).

The largest tuberculosis nodule was seen in lung with 15 mm and in liver and spleen, 9 and 8 mm respectively. Other tuberculous lesions in various organs were between 1 and 8 mm. All nodules were yellowish brown and were firm but incised easily.

Clinical signs / Number of pigeons	Progressive loss of weight	Wing drooping	inability to move	Diarrhea	The conjunctival nodule	Wing nodule	Beak nodule
101 pigeons	39	15	23	3	15	3	6
Number of positive results in culture method.	39	11	11	1	4	1	0

Table 1: Clinical signs in 101 suspected pigeons.

Gross pathology / Number of pigeons	Liver	Spleen	Lung	Cloaca	Hook joints
101 pigeons	19	13	2	2	15
Number of positive results in culture method	15	10	1	1	11

Table 2: Gross pathology in 101 suspected pigeons.

Culture results

Out of 101 pigeons, 39 (38.6%) had tuberculosis and from 75 male pigeons 27 (36%) and from 26 female pigeons 12 (46.2%) were positive. Among thirty-nine pigeons' positive tuberculosis cultures, 23 (58.9%), 5 (12.8%), 9 (23%) and 1 (2.5%) and 1 (2.5%) showed, 1, 2, 3, 4 and 5 clinical signs subsequently. In 18 pigeons (46.1%) gross lesion was not seen and in 14 (35.9%) and 7 (17.9%) pigeons gross lesions were seen in one and in 2 organs subsequently.

Designed Elisa system test results

Designed Elisa system test showed that 13 pigeons (12.9%) were positive. Among 75 male and 26 female pigeons, 8 (10.7%) and 5 (19.2%) were positive subsequently in designed Elisa system test. Among 13 pigeons positive tuberculosis cultures, 7 (53.8%), 1 (7.6%), 3 (23%) and 1 (7.6%) and 1 (7.6%) showed, 1, 2, 3, 4 and 5 clinical signs respectively. In 3 pigeons (23%) gross lesion was not seen and in 5 (38.4%) and 5 (38.4%) pigeons gross lesions were seen in one and in 2 organs subsequently.

Results of culture and designed Elisa system based on age variable

In culture, 35 (44.9%) adult pigeons and 4 (17.4%) immature pigeons and in designed Elisa system test, 11 adult pigeons (14.1%) and 2 (8.7%) immature pigeons were positive.

Comparison of culture and designed Elisa system test

39 (38.6%) and 13 (33.3%) pigeons were positive in culture and designed Elisa system test subsequently. Correlation between these two tests was significant (0.485) ($p < 0.01$).

Discussion and Conclusion

There is limited information about avian tuberculosis in pigeons [1,20]. The disease is low prevalence in nature, which increases when birds are placed in colony [22,32]. There is few study regarding pigeon tuberculosis in Iran [1,2]. The present study was designed to compare diagnose pigeon tuberculosis based on culture and designed ELISA methods. The most clinical signs seen in suspected pigeons were weight loss, nodules in hook and inability to move but these signs are not pathogonomic [1,8] and may be due to stress caused by keeping the birds in unusual conditions and a high number of birds in the cages [9]. We find prevalence of the disease in females than males, but this difference was not significant ($p < 0.05$), and was consistent with earlier reports [1,6]. The prevalence of the disease in young pigeons was lower than in older pigeons. This can be attributed to the higher exposure to the disease agent in older pigeons, the longer incubation period and the lack of development of the humoral immune response and the production of detectable antibody [1,12,13,15]. We find liver and spleen were the most involved internal organs [20,24]. This finding showed that the disease is transmitted through the intestine. It was interesting that in one case we detected Mycobacterium avium subsp. avium from nodule in the lung (Figure 5) by culture method. although tuberculosis in birds rarely affects the lungs, this can be considered as a interesting new finding. The involvement of the lung and liver, spleen and joint hook in the pigeon is probably due to the spread of infection through the bloodstream and secondary pulmonary infection. Macroscopic granulomatous lesions in heart, stomach, gizzard, kidney, testicles and central nervous system were not observed which correlated with another pub-

lished research [1,14,34]. In tubercular young pigeons only, sign was progressive loss of weight and no other clinical and autopsy symptoms were observed. This finding was consistent with some studies [1,31]. Largest tubular nodules were observed in the liver and spleen (abdominal cavity) except in one case, it seems enough space in liver and spleen besides oxygen abundance in these organs provides ideal condition for proliferation and enlargement of the lesions. This was consistent with the results of other studies [1,19]. According to different individual responses to the disease among birds of the same species, the differences in severity of autopsy lesions and clinical signs in the present study seem reasonable [1,21]. In culture method 39 cases were positive but 13 cases were positive in designed ELISA system test. This means that the sensitivity of the culture to disease diagnosis was higher than designed ELISA system test. Since the cause of disease is intracellular organism and time it takes to enter into blood stream is related to stage of the disease (the beginning of the disease) and cellular immunity plays a main role to protect the birds [4,6,35]. Also, according to the different stages of disease in pigeons, the sensitivity of the designed Elisa system test was lower than culture [10,30]. Although the sensitivity of designed ELISA system test in this study was less than sensitivity of other researchers in wild waterfowl [5]. It was concluded that mycobacterium may affected pigeons lungs and there is significant correlation (0.485) ($p < 0.01$) between culture and designed ELISA system tests. The ELISA test due to easy sampling, speed, low price, non-invasive, prefer test for screening affected pigeons flocks.

Figure 1: Loss of pectoral muscles and progressive loss of weight (knife-edged).

Figure 2: Wing drooping.

Figure 3: Tuberculosis nodules in liver.

Figure 4: Tuberculosis nodules in spleen.

Figure 5: Nodules in lungs.

Figure 6: Nodules in hook joint.

Figure 7: Positive sample culture of Mycobacterium avium in specific media (notice arrow).

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Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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