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Original Article

Comparison of PCR and designed ELISA methods to detect avian tuberculosis in suspected pigeons

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Abstract

Avian tuberculosis is a chronic disease that generally affects the bird's gastrointestinal tract and it often results in bird death. *Mycobacterium avium* subsp. avium is the most important cause of disease in birds. From October 2018 to October 2019, one hundred one suspected pigeons were selected based on clinical signs and poor physical condition. For ELISA system design, a blood sample was collected via pigeon wing vein and serum was collected. For the PCR method, pigeons were euthanized and post mortem were performed, and samples from liver and spleen and each organ with gross lesions were collected and stored in a freezer at -40 °C. *Mycobacterium avium* subsp. avium strain D4 antigen in Microbial Bank of Vaccine Research Institute was used in the design of the ELISA system. 16S rRNA, IS901 and IS1245 primers were used for molecular testing. The results showed that 39 out of 101 suspected pigeons were positive for the IS901 and IS1245 genomic sequences in PCR. But, only 13 cases out of 101 pigeons, were positive by the designed ELISA system. These 13 cases also were positive in the PCR test. Correlation between PCR and designed ELISA methods results was significant (0.485). Clinical sensitivity and specificity of the PCR method were 100% and the sensitivity and specificity of the ELISA method were 33.33% and 100%, respectively. It was concluded that the rate of avian tuberculosis among pigeon flocks in Ahvaz is relatively high and prevention and control plans should be applied by pigeon keeper and veterinary organization. The sensitivity of PCR to detect avian tuberculosis is higher than the designed ELISA system and ELISA test could be used for primary screening pigeon flocks in the early stages of avian tuberculosis.

Keywords: Avian Tuberculosis, Mycobacterium, PCR, Designed, ELISA

Introduction

Tuberculosis is one of the oldest infectious diseases common between humans and animals that despite various treatments every year, many people die and is still one of the health problems and challenges of human society (Taroudi zadeh and Imani fouladi 2013, Velayati 1988). According to research, about 10.4 million people are infected with tuberculosis each year and almost one-third of the world's population carries the tuberculosis bacille and are at risk of developing the active disease (Barberis et al. 2017) and every year, 3 million people die from tuberculosis

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in the world. Avian tuberculosis is a chronic disease that generally affects the bird's gastrointestinal tract and it often results in bird death. Due to the economic importance and the damage caused by tuberculosis in zoos, the risk of extinction of some of the rare birds, the zoonosis of the disease and the difficulty of controlling it, it is very important to diagnose the disease (Fulton and Sanchez 2008). The disease is most often caused by *Mycobacterium avium* subsp. avium and *Mycobacterium genavense* (Tell et al. 2001). Although more than ten species of Mycobacterium have been reported in infected birds, *Mycobacterium avium* subsp. avium is the most important cause of poultry disease (Fulton and Sanchez 2008). *Mycobacterium avium* affects pigeons and aquatic birds more than poultry (Pavlas et al. 1992). Nowadays, molecular PCR tests based on IS6110, IS901 and IS1245 genes, as well as techniques such as enzymatic digestion (RFLP) using specific probes arevery useful for detection of mycobacteria. These methods can easily detect and confirm human or animal tuberculosis within one day (Jabbar zadeh and Saifi 2011). Molecular methods for detection and identification of specific antigens or genes of pathogenic mycobacteria have created a great revolution. The advantages of these methods include rapid results, the ability to detect low levels of contamination in the Samples, and accurate identification of Mycobacterium species (Tell et al. 2001). Despite the high rate of detection in this method, intermittent excretion of the organism and the difficulty in finding the target tissue are problems with this method. (Jabbar zadeh and Saifi 2011). Functional serological tests for the diagnosis of avian tuberculosis include hemagglutination (HA), complement fixation (CF) and ELISA (Jorgensen 1978, Thoen et al. 1979). These experiments are species-specific and are only available for a limited number of species (Christal 2013). HA test can be done using blood or serum. For the first time in 1943, an agglutination test by using serum samples was used to diagnose tuberculosis in poultry (Pavlas et al. 1992). Whole blood agglutination is a rapid diagnostic method for finding infected birds in the flock. However, false-positives are considered a weak point (Fulton and Sanchez 2008). The use of fresh whole blood is more sensitive than whole blood including EDTA or serum and it shows real positive results. Specific antigens are required to obtain reliable results. Although it can be used as a screening and evaluation test for flocks (Christal 2013, Cromie et al. 1993, Fulton and Sanchez 2008, Tell et al. 2001). This test can be done to aquatic birds, domestic birds and Hunting birds (Christal 2013, Cromie et al. 1993, Hawkey et al. 1990). The sensitivity of the ELISA test is high, however this test is difficult and requires some specific antigens and the false positive results in this test can be high (Fulton and Sanchez 2008, Tell et al. 2001). A literature review indicated that there is not an available published article regarding the serological diagnosis of avian tuberculosis. This study aimed to diagnose avian tuberculosis disease by PCR methods and designed the ELISA test and compare the results of two methods.

Materials and Methods

Sampling

The study was conducted according to license EE/98.24.3.45458/scu.ac.ir, the ethics committee of Veterinary Medicine, Shahid Chamran University of Ahvaz from October 2018 to October 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 the information sheet and a blood sample was collected via pigeon wing vein and after centrifuging, serum was collected and stored in small vials in a freezer at -40 ˚C up

to test. Then pigeons were euthanized and post mortem was performed and any gross lesions were recorded in the information sheet and sample from liver and spleen of each pigeon was collected and if lesions were observed in each organ, necropsies were taken and stored in a 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 PCR and serology methods.

Designed the 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. To achieve positive control, two to three pigeons were injected intravenously with 0.2 cc 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 minutes 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 a 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 minutes. The plate was washed 5 times and dilution of 1/10000 peroxidase conjugated-antibody (HRP Conjegate Abcam/UK goat anti bovine [1]) was used. After 30 minutes of incubation at room temperature, the plate was washed again.

Add substrate (3, 3', 5, 5' – Tetramethyl benzidine (TMB))

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

Stop Solution

100 µl stop solution was poured into well.

Reading OD

The plate was read at 450 nm.

Designed ELISA system test procedure

1. To perform the ELISA test, the standard *Mycobacterium avium* subsp. avium strain D4 antigen was used to coat the bottom of the ELISA plate.
2. 0.1 molar bicarbonate carbonate buffer was prepared and 11 ml of the resulting solution was mixed with 100 µl D4 strain standard antigen.
3. 100 µl of the obtained solution was coated in each well of the ELISA plate.
4. The ELISA plate was incubated in 2-8 ˚C for 24 h.

1 Horseradish peroxidase

5. After 24 h, the ELISA plate was removed from the refrigerator and the solution was discharged into the wells.
6. ELISA plates were washed 3 times with 300 µl PBS without Tween.
7. 150 µl Blocking solution was poured into the wells and left for 1 h.
8. After 1 h, the blocking solution was discharged and the time was 20 min.
9. Serum samples were diluted 1: 150.
10. Then 100 µl of diluted sample was removed and poured into ELISA plate.
11. 30 minutes was given.
12. ELISA plates were washed 5 times with 300 µl washing solution.
13. 100 µl goat Anti chicken 1x was added to all wells of ELISA plate and given 30 min.
14. ELISA plates were washed 5 times with 300 µl washing solution.
15. 100 µl TMB was added to all wells and the plate was incubated in the dark for 10 min.
16. 100 µl stop solution was added to all wells.
17. In the end, the ELISA plate was read with the ELISA Reader.

Checkerboard process include: coating and blocking, coating of the main plate, then, blocking of the plate, then, add antibody, then, add HRP antibody, then, add substrate (TMB), then, add stop solution and finally Reading OD

Molecular identification

DNA extraction

Genomic DNA was extracted by Van Soolingen method (Van Soolingen et al., 2001). All samples were tested by PCR assays targeting the 16S rRNA gene for identification of Mycobacterium members, (Amplicon size 543 bp), specifically IS1245 for MAC (Amplicon size 427 bp), and *IS901* for identification of MAA (Amplicon size 1108 bp), (Kunze et al., 1991, Huard et al., 2003) (Table 1). PCRs were conducted with the incorporation of positive controls (*Mycobacterium avium* subsp. *avium* D4 strain, ATCC number 35713), negative species control (*Mycobacterium bovis* AN5 strain, ATCC number 35726) and negative controls (distilled water) (Guerrero et al., 1995, Kul 2005). PCR products were analyzed on ethidium bromide-stained agarose gels (2%) in a submerged electrophoresis system (Fig. 1 and Fig. 2).

Table 1: Characteristics of the PCR assays used for the detection and identification of Mycobacterial isolates collected from pigeons (Guerrero et al. 1995, Huard et al. 2003, Kunze et al. 1991). F: forward, R: reverse

Primer type and the target locus	Nucleotide sequence
16S rRNA	F:5 ACG GTG GGT ACT AGG TGT GGG TTT C 3
	R:5 TCT GCG ATT ACT AGC GAC TCC GAC TTC A 3
IS901	F:5 GCA ACG GTT GTT GCT TGA AA 3
	R:5 TGA TAC GGC CGG AAT CGC GT 3
IS1245	F:5 AGG TGG CGT CGA GGA AGA 3
	R: 5´ GCC GCC GAA ACG ATC TAC 3

Results

Designed the ELISA system

Designed ELISA system test showed 13 pigeons (12.9%) were positive. Based on sex Among 75 male and 26 female pigeons, 8 (10.7 %) and 5 (19.2%) were positive respectively subsequently in designed ELISA system test. Among 13 pigeons' positive tuberculosis with designed ELISA system, 7 (53.8 %), 1 (7.6%), 3 (23%), 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%) pigeon's gross lesions were seen in one and in 2 organs subsequently.

Molecular identification test results

The molecular detection results of Mycobacterium in 101 suspected pigeons are presented in Table 2.

Table 2: Molecular identification of Mycobacterium based on sex variable in 101 suspected pigeons

Sex / PCR	Positive	Negative
Male	27 (36 %)	48 (64 %)
Female	12 (46/2 %)	14 (53/8 %)
Regardless of sex	39 (38/6 %)	62 (61/4 %)

In the PCR test, out of 101 pigeons, 39 (38.6%) had tuberculosis. base on sex, in this study there were 75 male and 26 female pigeons. Among male pigeons, 27 (36%) cases and among female pigeons, 12 (46.2%) cases had tuberculosis. Among thirty-nine pigeons' positive tuberculosis, 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%), pigeon's gross lesions were seen in one and 2 organs subsequently.

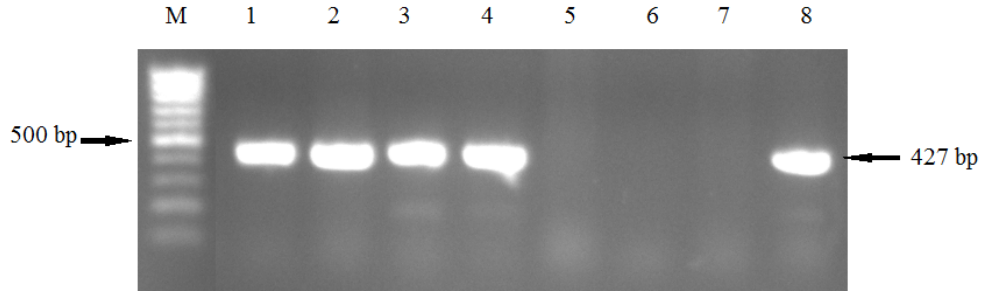

Fig. 1: PCR analysis. The 427 bp specific fragment from IS1245. Lane M, DNA size marker (100 base pair ladder). Lane 5 and 6, negative controls (distilled water). Lane 7, negative species control (*Mycobacterium bovis* AN5 strain, ATCC number 35726). Lane 8, positive control (*Mycobacterium avium* subsp. *avium* D4 strain, ATCC number 35713). Lane 1 to 4 samples tested for *Mycobacterium avium* subsp. *Avium*

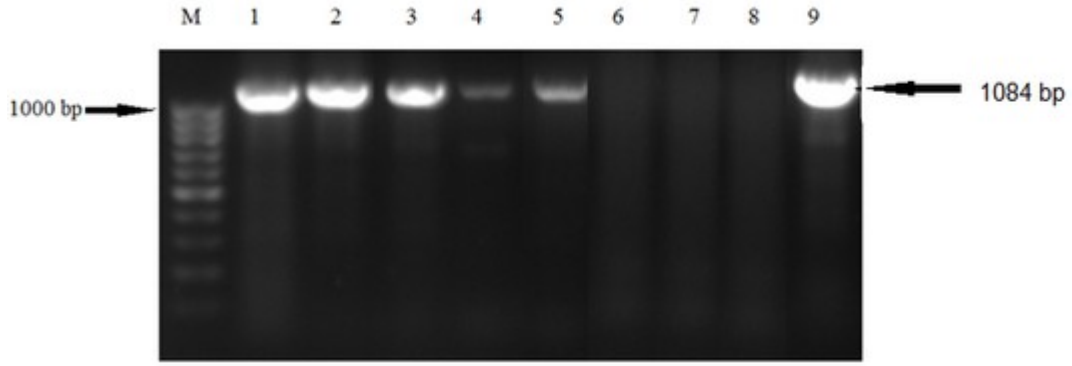

Fig. 2: PCR analysis. The 1084 bp specific fragment from *IS901*. Lane M, DNA size marker (100 base pair ladder). Lane 6 and 7, negative controls (distilled water). Lane 8, negative species control (*Mycobacterium bovis* AN5 strain, ATCC number 35726). Lane 9, positive control (*Mycobacterium avium* subsp. avium D4 strain, ATCC number 35713). Lane 1 to 5 samples tested for *Mycobacterium avium subsp. Avium*

Molecular detection and designed an ELISA system based on the age variable

In molecular detection, 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 infected with tuberculosis (The mature and immature criteria were based on a questionnaire that asked pigeon owners when inspecting pigeon flocks).

Comparison of molecular detection and designed ELISA system test

Thirty-nine (38.6%) pigeons in molecular detection and 13 (33.3%) pigeons in designed ELISA system test were positive. Correlation between these two tests was significant (0.485) ($p<0.01$). Sensitivity and specificity of the PCR method were 100% and the sensitivity and specificity of the ELISA method were 33.33% and 100%, respectively.

Discussion

It is difficult to diagnose tuberculosis in birds due to different and non-typical clinical symptoms, prolonged incubation period, presence of undiagnosed clinical cases, lack of appropriate serological tests and prolonged and difficult cultivation of the causative agent (Van der Heyden 1997). Historically keeping pigeons by human societies had many fans. Despite present suspected birds to tuberculosis among pigeons, owner not satisfied with necropsy of suspected pigeons for a definite diagnosis. Therefore, necessary to develop a serology method for screening infected pigeons. In this study, ELISA test was designed and test accuracy was evaluated by PCR method. Based on the results of molecular detection and designed ELISA system, 39 out of 101 pigeons were positive by PCR, and 13 cases were positive in the ELISA test. This means that the sensitivity of the PCR test in detecting disease is higher than the ELISA test. Since tuberculosis caused by an intracellular organism and its entry into the blood is related to the stage of the disease (the beginning of the disease) and because of the disease's agent stimulates cellular immunity more than humoral immunity (the production of non-protective antibodies) and also, according to the different stages of disease in different pigeons, less sensitivity of designed ELISA system than PCR can be justified (Cromie et al. 2000, Fulton and Thoen 2003). The sensitivity of designed ELISA system test in the present study was less than the sensitivity of other researchers reported in wild waterfowl (Cromie et al. 2000). Statistic work showed a significant correlation between molecular detection and designed ELISA system tests (0.485) ($p<0.01$) and due to easy sampling, speed and price, it seems an indirect designed ELISA test can be useful for detecting early stages of the disease and flocks screening. Due to scarce of published information regarding ELISA test for detecting tuberculosis in pigeon, we could not compare present results with another researcher but some research regarding other birds was available Shitaye et al., in 2008 worked on diagnostic testing of different stages of avian tuberculosis in naturally infected hens (Gallus domesticus) by the tuberculin skin and rapid agglutination tests, faecal and egg examinations. They did not consider the Rapid Agglutination Test (RAT) a reliable method for detecting infected birds and they pointed out that RAT is only sensitive to the diagnosis of highly infected birds (Shitaye et al. 2008). Cromie et al. in 1993 compared the results of necropsy with the results of ELISA and whole blood and serum agglutination tests in 178 wild goose and reported that the sensitivity of the ELISA test was such that it was able to detect the disease even at an early stage. However, whole blood and serum agglutination test have false positive and negative results despite speed and ease of work. However, ELISA and agglutination tests can be used to screen birds in these flocks (Cromie et al. 1993). The use of serological methods in the

diagnosis of tuberculosis in other animals has always been of interest to researchers. Singhla et al. in 2019, Comparing three diagnostic methods (skin test, interferon-gamma assay and ELISA) in 128 dairy cows from 25 infected flocks with TB concluded that interferon-gamma levels and ELISA could be used as complementary diagnostic tests (Singhla et al. 2019). O'Brien et al. in 2017 examined the serum and milk of 500 adult goats vaccinated against *Mycobacterium avium* subsp. paratuberculosis. They reported elevated IgG antibodies in serum and milk of 100% of animals whose skin test was positive, 77.8% in serum and 95.4% of milk of animals whose skin test was negative. According to the researchers, a serological test of goat serum and milk can be useful in the diagnosis and management of tuberculosis in this animal (O'Brien et al. 2017). Wood et al., in 1992 reported that the sensitivity and specificity of the ELISA test for the diagnosis of bovine tuberculosis were 18/1 % and 96/4 %, respectively (Wood et al. 1992). Human medicine also uses serological methods to diagnose tuberculosis. Qadiri et al. in 2007 evaluated 176 serum samples of TB patients (124 pulmonary tuberculosis patients and 52 extra-pulmonary tuberculosis) by using ELISA. They reported that the ELISA method could be helpful in the diagnosis of TB. Although, the sensitivity of this method is not high (Qadiri et al. 2007). Izadi et al., in 2017, by examining the serum samples of 55 patients with tuberculosis and 28 healthy samples, by indirect ELISA method, concluded that the level of antibodies in patients against infection was significantly higher than in healthy subjects. The results of this study showed that the use of ELISA for determination of antibody titers can replace the old tests as a rapid and accurate diagnostic method (Izadi et al., 2017). Nowadays, molecular identification and culture methods are the most definitive methods for the diagnosis of tuberculosis in birds (Shitaye et al. 2008). In this study, all 39 PCR-positive samples were positive for IS901 and IS1245 genomic sequences which indicate that these isolates belong to serotypes 1, 2 and 3 of *Mycobacterium avium* and they are considered as acute *Mycobacterium avium* strains (Asadollahi et al. 2015, Dvorska et al. 2003, Tell et al. 2001). Asadollahi et al., in 2015 investigated 80 pigeons suspected of TB by PCR. They reported 51 positive samples infected with *Mycobacterium avium* subsp. avium (Asadollahi et al. 2015).

It was concluded that the rate of avian tuberculosis among pigeon flocks in Ahwaz is relatively high and prevention and control plan should be applied by pigeon keeper and veterinary organization. Based on the results obtained a sensitivity of PCR to detect avian tuberculosis is higher than designed ELISA system and ELISA test could be used for primary screening pigeon flocks in the early stages of avian tuberculosis.

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Conflict of interest

The authors declared no potential conflicts of interest to the research, authorship, and/or publication of this article.

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ELISA test could be used for primary screening pigeon flocks in the early stages of avian tuberculosis.

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