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Comparison of isolation and histopathology methods in diagnosis of pigeon tuberculosis

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A B S T R A C T

Tuberculosis is the most important infectious disease between humans and animals. Avian tuberculosis often affects birds' gastrointestinal tract and causes the bird's death. It affects most species of birds, such as laying hens, pigeons, turkeys, parrots, pheasants, waterfowl and wild birds. The culture method is the most definitive method in diagnosing mycobacterial infection in birds, and other methods, including the histopathology method, are helpful in confirming the diagnosis. In the present study, two methods of isolation and histopathology were compared to diagnose tuberculosis in pigeons. In this research, more than 700 pigeons were examined, and 101 pigeons suspected with clinical signs were selected. After euthanization, tissue samples were collected for culture and histopathology tests. Among 101 pigeons suspected of avian tuberculosis, 38.61 % were positive in the culture method, and 37.62 % were positive in the histopathology method. Statistical results showed that 97.43 % of positive cases in the culture method were also diagnosed as positive by the histopathology method. A nodule was observed in lung tissue (pulmonary tuberculosis) in one case. It was concluded that avian tuberculosis among pigeon flocks in Ahvaz city is high, and the culture method is still the golden and definitive method, but it takes a long time, and diagnosis of the disease in the pathology method is faster than the culture method (correlation 97.43%).

Keywords: *Pigeon, Tuberculosis, Diagnosis, Culture Method, Pathology Method*

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1 Introduction

Tuberculosis is present in almost all countries of the world, but the disease's prevalence in humans and animals is different (1). According to the available statistical data, tuberculosis is the most important infectious disease between humans and animals. Nearly one-third of the world's people are infected with tuberculosis, and a significant percentage of animals are infected with tuberculosis bacillus (2). According to the available reports in 2007, there were about 13.7 million active cases of chronic tuberculosis in human societies (3). In 2010, 8.8 million new cases of tuberculosis and 1.45 million deaths were identified, most of which occurred in developing countries (4). All birds, under certain conditions, are susceptible to tuberculosis. So far, tuberculosis disease has been reported in parrots, long-tailed parrots, terns, sparrows, starlings, Australian ostriches, partridges, quails, pigeons, buzzards, owls, pheasants, cockatoos, crows, turkeys, vultures, scavengers and songbirds (5, 6). The highest prevalence of tuberculosis is related to areas such as zoos, bird collections, and areas with a large bird population (7). Nowadays, the role of parrots in the transmission of tuberculosis disease to humans is considered important (8). This disease affects pigeons and waterfowl more than chickens. The disease mainly occurs in adult and old birds due to extended exposure to bacteria and longer incubation time. Unlike mammals, most avian species with tuberculosis show signs of disease in the gastrointestinal tract. Avian tuberculosis is one of the diseases that are difficult to diagnose and control. Several species of mycobacteria are involved in avian tuberculosis, but the disease is most often caused by *Mycobacterium avium* (MA) serotypes 1, 2 and 3 ($IS901^+$ and $IS1245^+$) and *Mycobacterium genavens* (9-13). There are four subspecies of *Mycobacterium avium* based on phenotypic, genotypic, pathogenicity, and host type. The subspecies are *Mycobacterium avium* subsp. *avium*, *Mycobacterium avium* subsp. *Paratuberculosis*, *Mycobacterium avium* subsp. *sylvaticum*, and *Mycobacterium avium* subsp. *hominissuis* (14, 15). *Mycobacterium avium* complex has 28 serotypes (16). Members of the *Mycobacterium avium* complex can infect a wide range of mammals, such as pigs, rabbits, minks, cows, deer, sheep, goats, horses, cats and dogs and cause local lesions in them. Among mammals, pigs are more affected. In humans, all members of the *Mycobacterium avium* complex and *Mycobacterium genavens* can cause a progressive and treatment-resistant disease in immunocompromised patients with symptoms such as pneumonia, lymphadenitis, meningitis, and millet tuberculosis (17). The disease agent mainly enters the body of susceptible birds through the digestive system. *Mycobacterium avium* can be isolated from eggs in natural infection; however, hatchlings failed to develop avian tuberculosis (18). The primary diagnosis of the disease is based on the history of the disease, clinical Signs, and necropsy. In most cases, observing acid-fast bacilli in the slides prepared for the liver, spleen, or other organs helps to confirm the diagnosis (17-19). In suspected birds, fecal tests, staining, and PCR are also helpful in diagnosis (17). The culture method is still considered the "gold standard" method in disease diagnosis, but in this method, much time is needed for the growth of bacteria (17). In microscopic examination of affected organs using Ziehl-Neelsen (ZN) staining, a large number of acid-fast bacteria can be observed, which is helpful in the diagnosis (20). In the histopathological examination, epithelial macrophages, lymphocytes, multinucleated giant cells, and a fibrin capsule can be seen in the mesenteric nucleus of tubercles caused by tuberculosis (21). Avian tuberculosis is generally diagnosed based on clinical signs, lesions after death and by showing acid-fast bacilli in the lesions using a microscope. If acid-fast bacteria are not observed and despite clinical signs in the bird, the organism should be cultured (22). The present research was conducted to compare two methods of isolation (culture) and Histopathological in diagnosing pigeon tuberculosis.

2 Materials and Methods

2.1 Sampling

Sampling was done for one year among ten flocks of pigeons suspected of tuberculosis in Ahvaz city. Based on clinical signs and inappropriate physical conditions, 101 pigeons were selected from more than 700 suspicious pigeons. Among the 101 samples collected, 75 were male and 26 were female pigeons. After clinical examination and recording of the signs, the pigeons were euthanized. In the necropsy, the observed necropsy injuries were recorded. Tissue samples were collected from the liver and spleen, and if tissue injuries were observed, samples were collected from the affected organ. The samples were collected in special sampling containers, and some were placed in containers containing 10% formalin for pathological tests. Then, samples were sent to the tuberculosis reference laboratory and the Department of Animal Pathology of Razi Vaccine

and Serum Institute for culture and pathological methods of diagnosis.

2.2 Culture of samples

This study used the Lowenstein-Johnson medium containing glycerin, Lowenstein-Johnson medium containing pyruvate, and Heroldegg medium to grow tuberculosis bacteria. Tissue samples were placed in the tray under the hood. Small pieces 5 cm in size were separated from different organs and their injuries and transferred into sterile mortars. Then, the same volume of the removed samples, the solution containing sodium citrate, sodium hydroxide, and N-acetyl-N-cysteine was added to each container. After 20 minutes, about 15 to 20 cc of the solution was taken from the top of the solution inside the mortar with a sterile syringe and poured into the Falcon tube containing five cc of hydrochloric acid, one drop of methylene blue reagent, and a green solution was prepared. The Falcon tubes were centrifuged at 4500 rpm for 15 minutes. The supernatant was discarded, and 2.5 cc of phenol-free phosphate buffer with pH = 6.8 was added to the sediments at the bottom of the tubes. After stirring the sediments, it was removed from the solutions with a sterile syringe, and about one cc was inoculated inside the culture media. In order to compare the growth, the cultured media were incubated at 37°C and 41°C, and the growth of bacteria was studied for three months.

2.3 Histopathology

Sections of the tissues and organs, including the liver, spleen, and other suspected organs, were collected from the euthanized pigeons after clinical examination and recording of the signs. Then, they were placed in 10% neutral buffered formalin for histopathology. The formalin-fixed tissues were embedded in paraffin cassette blocks, sectioned at 5µm, and finally stained with hematoxylin and eosin. The observation was evaluated qualitatively and quantitatively using a binocular light microscope with a magnification 10×. Experimental procedures were undertaken following the Animal Ethics Monitoring Committee and Animal Welfare Committee of Razi Vaccine & Serum Research Institute.

2.4 Statistical analysis

The statistical analysis of the data was performed using the chi-square test with a significance level of 5% and Pearson's correlation test (23).

3 Results

According to the results, the minimum distance between flocks infected with avian tuberculosis in the present study was 5 km, and the maximum distance was 13 km.

3.1 Clinical observation

According to Table 1. Antibiotic resistance profiles for blaCTX-M-1 and non-blaCTX-M-1 Salmonella isolates, the most common clinical signs in the examined pigeons were related to excessive thinness, the presence of nodules in the leg area and inability to move (Figure 1).

3.2 Culture results

Among the 101 samples collected, 39 samples showed positive results in the culture test. The positive samples grew well in all three culture media, but the bacterial growth was faster in the mycobactin medium, and the size of the colonies was larger. Based on the observed results, bacterial growth was better at 41°C, and the number and size of colonies were larger (Figure 2).

Table 1. Antibiotic resistance profiles for blaCTX-M-1 and non-blaCTX-M-1 Salmonella isolates

Clinical signs/Numbers	excessive thinness	leg nodules	Inability to move	diarrhea	loss of appetite	conjunctival nodule	wing nodule	facial nodule
101 pigeons	101	15	23	3	2	15	3	6
positive results	39	11	11	1	1	4	1	0

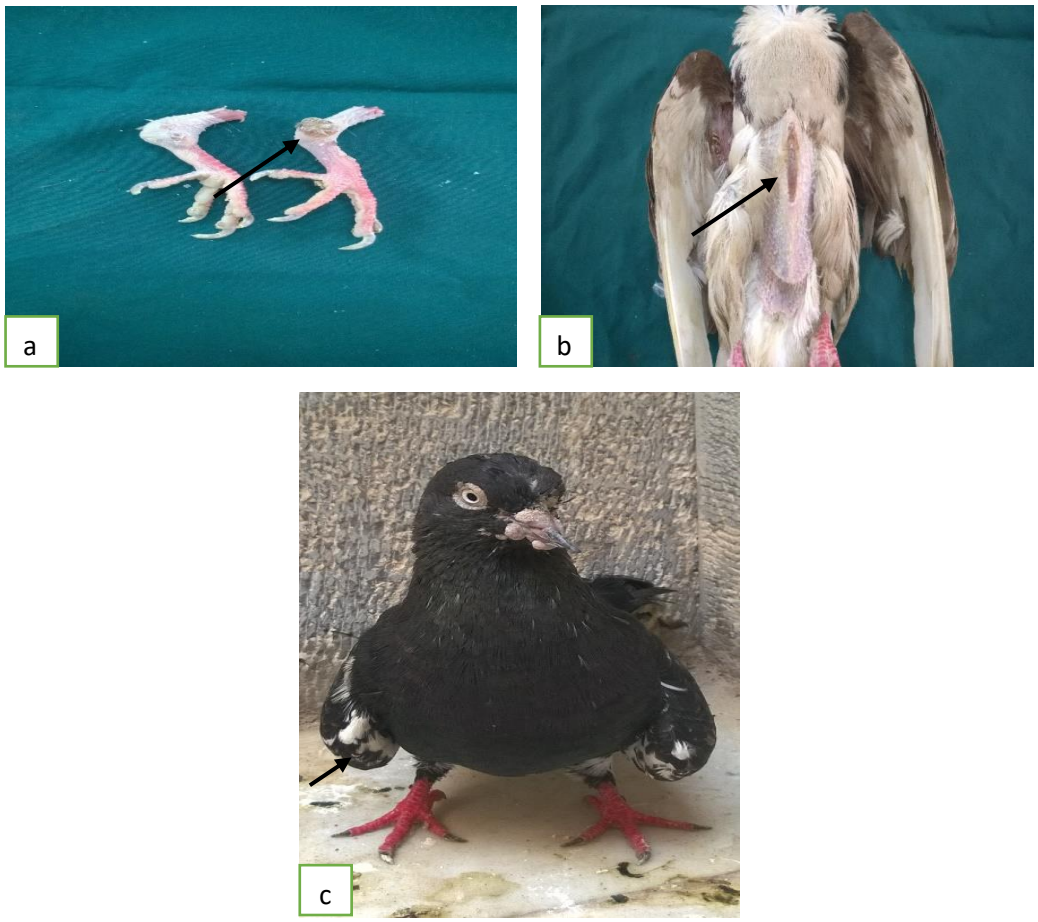

Figure 1. a- Tuberculous nodule in the stifle joint; b- Destruction of chest muscles and excessive thinness (knife-edged); c- Wing drooping due to tubercle nodule in the wing area.

Figure 2. Positive culture sample. Colonies of Mycobacterium avium grown in specific media.

3.3 Necropsy findings

In the sections prepared from different tissues of the examined pigeons, the liver was the most affected organ and bones and skins had the most minor damage to the organs, and no macroscopic lesions were found in the gizzard, proventriculus, gonads, kidneys and central nervous system, abdominal cavity and heart. According to the observations, the most common form of tuberculosis disease in the pigeons of this research was the hepatic form. Also, gastrointestinal parasite infection was the only chronic disease observed in all pigeons with avian avian tuberculosis.

3.4 Histopathological findings

In the results of the histopathology examination of the samples, 38 cases were diagnosed, and 42 cases were

reported as suspicious. The culture results of these 42 cases were reported as negative. Necrosis of hepatic cells, portal hepatitis, multifocal infiltration of inflammatory cells, accumulation of inflammatory cells and the presence of foamy macrophage cells caused by contamination with tuberculosis bacteria in the liver tissue were seen. Microscopic lesions were created in the lung tissue, including Infiltration of multifocal inflammatory cells (primary foci of tuberculosis), lesions caused by *Mycobacterium avium* such as large foci with central necrosis, loss of alveolar spaces, oedema and hyperemia, and formation of hyaline membranes. Severe dermatitis with necrosis and the infiltration of heterophils and foamy macrophages (indicative of contamination with the mentioned bacteria) was observed in the prepared slides' skin tissue (Figures Figure 3, Figure 4 and Figure 5). Based on the obtained results, among 101 pigeons suspected of avian tuberculosis, 38.61 % were positive in the culture method, and 37.62 % were positive in the histopathology method. Based on the statistical results, 97.43 % of the positive cases in the culture method were also diagnosed as positive by the histopathology method.

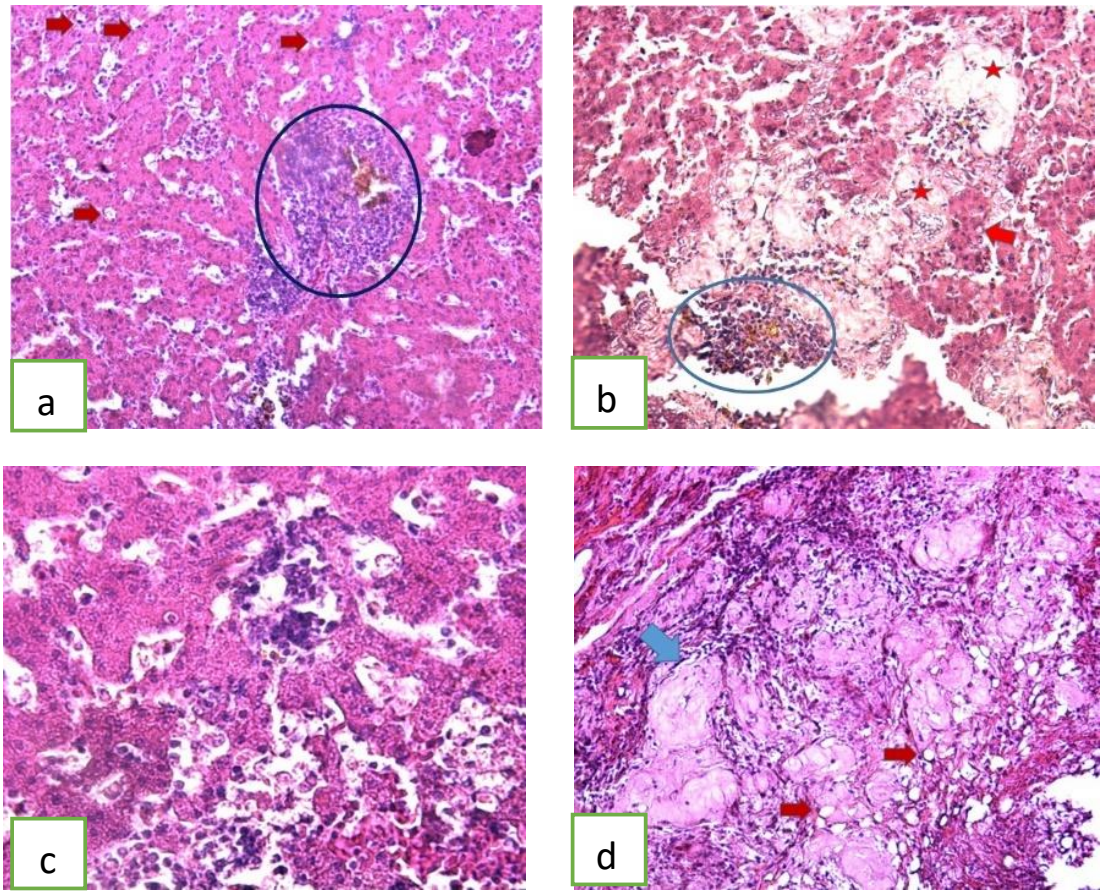

Figure 3. Liver. Hepatic cells necrosis (red star), accumulation of inflammatory cells (enclosed in blue circle) and foamy macrophages caused by Mycobacterium avium infection (red arrow)- Part of a tuberculosis foci. Foamy macrophages (red arrows) and signs of hepatic cell necrosis (blue arrow); H&E×200 (a&b). H&E×400 magnification (c&d).

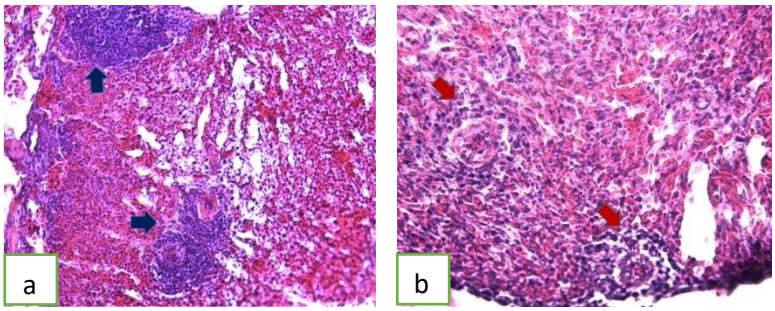

Figure 4. Lung. Granulomas caused by Mycobacterium avium bacteria (dark blue arrows) (a). H&E×200; Infiltration of inflammatory cells in the form of primary foci of granulomatous tuberculosis (red arrows), fibrinous edema and formation of hyaline membranes and loss of alveolar spaces (b). H&E×400.

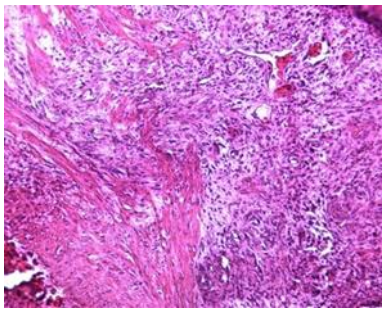

Figure 5. Skin. Severe dermatitis with necrosis and infiltration of heterophils and foamy macrophages caused by contamination with Mycobacterium avium and observing the formation of fleshy bud tissue. H&E×200.

The results of the present study, using both diagnostic methods, showed that among the 39 positive samples with avian tuberculosis, 27 cases were male, 12 were female, 35 were adults, and four were immature.

4 Discussion

For a long time, keeping pigeons has been popular among human societies. The frequency of tuberculosis disease among pigeons, the great interest of pigeon owners in this bird, and their lack of satisfaction in removing diseased pigeons in most cases have forced researchers to find the best and most accurate method for screening the existing flocks by examining different methods of disease diagnosis and thus prevent the spread of disease between bird species and their owners. Based on the available articles, limited information is available about avian tuberculosis in domestic and free-range pigeons (20). In many cases, this disease is associated with a low prevalence in nature, which increases if birds are placed together (13, 24). Although many years have passed since the existence of avian tuberculosis in Iran, there are few studies about this disease in Iran (25). In this study, the most clinical signs were related to excessive thinness, nodules in the leg area and inability to move. Although these signs are not pathognomonic of the disease, the existence of stress caused by the conditions of keeping and the high population of birds in cages can be considered as the cause of these signs and one of the factors of disease development in flocks (9, 11).

In the present study, the liver and spleen were the most involved internal organs, and tuberculous nodules were observed in the lung in only one pigeon (20, 26). However, in the histopathology studies of this research, the presence of tuberculosis bacillus in the lung tissue was confirmed in 25 cases out of 39 pigeons with tuberculosis. So far, only one case of tuberculous nodules in the lung of a pigeon has been reported, and the present research is the second report in this field (22). The existence of only one macroscopic case of tuberculosis nodule in the lung tissue in this research shows that the primary transmission of tuberculosis disease in birds is through the gastrointestinal tract. If the liver, spleen, and other organs are involved, the spread of infection through the blood to the lung tissue can create the pulmonary form of tuberculosis in birds. Also, exposure to the infectious agent through breathing can be considered adequate for

contracting the pulmonary form of tuberculosis in birds. In the present research, the heart, proventriculus, gizzard, kidneys, gonads, and central nervous system were the organs in which no macroscopic granulomatous lesions were observed. The present research result was compatible with other researchers' reports (22, 25, 27). In the present finding, the largest tuberculosis tubercles, except for one case, were observed in the liver and spleen (abdominal cavity); the reason for this can be the existence of sufficient space for the proliferation of the disease agent and the enlargement of the lesions, as well as the abundance of oxygen supply to these tissues. This was compatible with the reports of other researchers (25, 28). Although all the isolates were grown in mycobactin and pyruvate media, the number of colonies obtained in mycobactin media was more and more significant, which indicates that mycobactin is effective in bacterial growth. The result was compatible with the results of other studies (9, 25, 28). By comparing the growth of the isolates at 37°C and 41°C, their growth was faster at 41°C, which was compatible with the results of other studies (17). Kul et al. (2005) examined 66 birds from 6 different species and reported that the disease occurrence in the researched birds was 6%. They reported the presence of non-mineralized caseogranulomas in organs such as the liver, spleen, intestine, lung, and cloaca. In the histopathological examination, they observed acid-fast and osteonecrosis bacilli in the centre surrounded by epitheloid macrophages, lymphocytes and multinucleated giant cells. Multiple granulomas were seen in the liver, spleen, lung, cloaca, and intestinal serosa with non-mineralized caseous necrosis in the center surrounded by a layer of epithelioid cells, multinucleated giant cells, and macrophages, lymphocytes, and macrophages at the peripheral margin. In tissue section staining by the Ziel-Nielson method, they observed acid-fast bacilli in multinucleated giant cells in necrotic areas, consistent with the present research (18). In 2018, Rahman et al. studied a four-week-old flock of 20 turkeys with clinical signs such as fatigue, anorexia, panting, and, finally, death. They reported that non-mineralized nodules were found in the abdominal cavity's lung, rib membranes, and internal surface on post-mortem examination. The histopathological findings of this study showed that epithelioid macrophages, lymphocytes and multinucleated giant cells surrounded the lesions. In the microscopic examination of the lung parenchyma, the accumulation of macrophages inside the bronchi was observed at the same time as the lesions of bronchiolitis. Also, microscopic examination showed foamy macrophages surrounded by lymphocytes in tuberculous granulomas, which is compatible with the present research (29). Rahman et al. reported the presence of granulomas in infected turkeys, indicating exposure to the infectious agent through inhalation and not through the gastrointestinal tract.

Schmidt et al. 2022 examined 50 suspected pet and wild birds and successfully isolated mycobacterium from 34 birds (psittaciformes, psittaciformes, columbiformes and other orders) belonging to 26 species. *Mycobacterium genavens* was isolated from 15 birds (orders psittaciformes, passeriformes and falconiformes), and *Mycobacterium avium* was isolated from 22 birds (orders psittaciformes, passeriformes, columbiformes, musophagiformes, galliformes, falconiformes and pelicaniformes). In necropsy examination, they reported enlarged liver and spleen in 24 out of 34 birds, usually accompanied by white proliferative foci as the most common finding. They reported thinness in 22 of 34 birds and white to yellow capsular foci with cheesy content in various visceral organs from 1 mm to over 25 mm in 19 of 34 birds, which were compatible with the present research. Histopathology of the foci confirmed mycobacterial tubercles in the form of fibrinous granulomas with necrosis and fibrin in the center. It is surrounded by multinucleated giant cells, histiocytes, lymphocytes, heterophils, and varying degrees of fibroplasia. They also reported that acid-fast rod-shaped bacteria were detected in the center of granulomas and the cytoplasmic space of multinucleated giant cells. In the histopathology reports of birds that did not have tubercular foci, the accumulation of epithelioid-like cell macrophages with rod-shaped acid-fast bacteria was observed in various organs (30). These results were compatible with the results obtained in the present study. The present research revealed that the organs most affected by avian tuberculosis are the liver, spleen, lung, and cloaca. It was concluded that avian tuberculosis among pigeon flocks in Ahvaz city is high, and the culture method is still the golden and definitive method but needs prolonged time, but the pathology method is faster than the culture method with a high correlation (97.43%) with culture method.

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Conflict of Interest

All authors declare that they have no conflicts of interest.

Author Contributions

All authors equally contributed.

Data Availability Statement

Data are available from the corresponding author upon reasonable request.

Ethical Considerations

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.

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