Comparison of immunohistochemistry and Ziehl-Neelsen staining for detecting the distribution of *Mycobacterium avium* subsp *avium* in naturally infected domestic Pekin ducks (*Anas platyrhynchos domestica*)

Dekang Zhu1,2 | Hongxi Chen1,2 | Xumin Ou1,2 | Mafeng Liu1,3 | Mingshu Wang1,3 | Xinlin Zhao1,3 | Renyong Jia1,2,3 | Shun Chen1,3 | Kunfeng Sun1,3 | Qiao Yang1,3 | Ying Wu1,3 | Xiaoyue Chen1,2 | Anchun Cheng1,2,3

1Research Center of Avian Diseases, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, China
2Key Laboratory of Animal Disease and Human Health of Sichuan Province, Chengdu, Sichuan, China
3Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, China

Correspondence Anchun Cheng, Research Center of Avian Diseases, College of Veterinary Medicine, Sichuan Agricultural University, No. 211, Huimin Road, Chengdu 611130, Sichuan, China. Email: chenganchun@vip.163.com

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Abstract

In order to detect the distribution of *Mycobacterium avium* subsp *avium* (MAA) in naturally infected domestic Pekin ducks, immunohistochemistry (IHC) and Ziehl-Neelsen (ZN) staining were used and compared. Six organs, the liver, spleen, lung, kidney, duodenum and pectoralis muscle, were collected from naturally infected Pekin ducks. Paraffin embedded tissues were examined, and the results were compared. Statistical analysis was performed using Chi-Square test. The results showed that the detection rates by IHC were similar with ZN staining in liver, lung, spleen and pectoralis muscle, but the detection rates by IHC were much higher than ZN staining in kidney and duodenum (p = .013, p = .0044). The liver (87.5%) and lung (81.3%) had the highest detection rates. Acid-fast bacilli (AFB) were primarily found intracellularly in six organs using ZN staining. Similarly, the MAA antigens in those selected organs were also detected in the cytoplasm with different cell types. Specifically, MAA antigen was distributed in epithelioid macrophages and necrotic centres within the liver, lung, spleen and kidney, while they were observed in macrophages of the lamina propria and duodenal glands and degenerative myocytes in the pectoralis muscle. This comparative study provides an important insight into the distribution of MAA in infected domestic ducks and indicates that the detection rate by IHC was higher than that of ZN staining.

KEYWORDS
distribution, domestic ducks, haematoxylin and eosin, immunohistochemistry, *Mycobacterium avium* subsp *avium*, Ziehl-Neelsen staining
Avian tuberculosis is a contagious disease caused by *Mycobacterium avium* subsp. *avium* (MAA). The primary modes of transmission are via oral and airborne routes. Avian tuberculosis can result in progressive weight loss, reduction in egg production, and ultimately, mortality of birds. It has been reported that avian tuberculosis can infect domestic poultry (Tell, Woods, & Cromie, 2001), companion psittacine birds (Lennox, 2007) and captive exotic birds (MONTALI, Bush, Thoen, & Smith, 1976). However, disease susceptibility varies from species to species. While it has been reported that domestic geese and ducks are moderately resistant to *M. avium* (Hejliveck & Treml, 1995), an outbreak of avian tuberculosis in a commercial domestic duck flock was recently reported (Song et al., 2016; ZHU et.al., 2016). A strain has been isolated from organs of infected ducks and sequenced using PacBio single-molecule real-time sequencing technology. Based on the complete genome of isolates (GenBank accession no. CP016396), it has been determined that the subspecies identified is MAA (Song et al., 2016).

To the authors’ knowledge, detection of MAA in ducks using immunohistochemistry (IHC) has not been reported. However, exploring MAA distribution in the organs of naturally infected domestic ducks is necessary to understand avian tuberculosis. Therefore, Ziehl-Neelsen (ZN) staining and IHC staining of a variety of organs of infected ducks is necessary to understand avian tuberculosis. Therefore, Ziehl-Neelsen (ZN) staining and IHC staining of the infected organs was conducted as reported previously (Chen et al., 2009). Briefly, 5 μm paraffin sections were deparaffinized in xylene and rehydrated in graded alcohol.

**RESULTS**

Histopathological results revealed granulomas without necrosis (Figure 1a) and granulomas with necrosis (Figure 1b–d and f) in the liver, lung, spleen, kidney and pectoralis muscle. Interestingly, no granulomas were observed in the duodenum on microscopic examination (Figure 1e). Granulomas with necrosis were characterized by caseous necrosis that contained nuclear debris, surrounded by epithelioid macrophages and lymphocytes. Many small necrotizing foci appeared to fuse, forming large necrotizing granulomas, as observed in the spleen (Figure 1c), kidneys (Figure 1d) and pectoralis muscle (Figure 1f). The pectoralis muscle, with tubercle nodules, showed caseous necrosis that was surrounded by degenerative myocytes, with some lymphocytic infiltration, rather than the presence of granulomas (Figure 1f). Specifically, multinucleated giant cells were rarely observed in granulomas of the liver, lung, spleen and kidney.

ZN staining showed that acid-fast bacilli (AFB) were present in all six organs examined. AFB were primarily found intracellularly, with...
few AFB found extracellularly. However, the number of AFB in each organ was different. Large concentrations of AFB were observed in the liver (Figure 2a), lung (Figure 2b) and pectoralis muscle, while few were found in the spleen (Figure 2c), kidneys (Figure 2d) and duodenum (Figure 2e). The detection rates were different among organs. For example, 87.5% (14/16) of livers demonstrated the

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**TABLE 1** Percentage of positively stained organs of the MAA infected domestic Pekin ducks by ZN staining and IHC

| Total | ZN Positive | Percentage | IHC Positive | Percentage | χ² | p  |
|-------|-------------|------------|-------------|------------|----|----|
| Liver | 16          | 14         | 87.5%       | NA         | NA | NA |
| Lung  | 16          | 12         | 75.0%       | 13         | 81.3% | 0.18 | .69 |
| Spleen| 16          | 8          | 50.0%       | 11         | 68.8% | 1.17 | .28 |
| Kidney| 16          | 5          | 31.3%       | 12         | 75.0% | 6.15 | .013|
| Duodenum| 16         | 3          | 18.8%       | 11         | 68.8% | 8.13 | .0044|
| Pectoralis muscle | 16 | 12 | 75.0% | 12 | 75.0% | NA | NA |

Note: Percentage: the ratio of the number of tissues had positive staining to the total number of tissues. The Chi-Square test: p < .05 is significance. NA, not applicable.
presence of AFB, while only 18.8% (3/16) of duodenum had detectable AFB (Table 1).

MAA infection of Pekin ducks was further examined by IHC. Similar to ZN staining, positive staining was observed in all six organs examined (Figure 3). All negative controls showed no brown staining. In liver sections with non-necrotizing granulomas, positive staining was primarily observed in the cytoplasm of epithelioid macrophages and denatured hepatocytes (Figure 3a). In lung (Figure 3b), spleen (Figure 3c) and kidney sections (Figure 3d) with necrotizing granulomas, positive staining was detected in the cytoplasm of epithelioid macrophages as well as in necrotic centres. Positive staining was also observed in macrophages of the lamina propria and duodenal glands (Figure 3e), as well as in degenerative myocytes in the pectoralis muscle (Figure 3f). Consistent with ZN staining, immunostaining showed that the less abundant stainings were observed in the liver, lung and pectoralis muscle, while relatively slight brown stainings were observed in the spleen, kidney and duodenum (Table 2). However, the MAA-positive detection rates among the six organs showed no difference (p > 0.05).

To measure the difference in detection rates, a comparative study between IHC and ZN was conducted. As shown in Table 1, only in the liver and pectoralis muscle, where large concentrations of AFB were identified, were the detection rates of IHC and ZN staining identical. While in lung and spleen where found moderate concentrations of AFB, the detection rates of IHC (81.3% and 68.8%) were slightly higher than those of ZN staining (75.0%; p = 0.69 and 50.0%; p = 0.28, respectively). In kidney and duodenum where found few AFB, the detection rates of IHC (75.0% and 68.8%) were observably higher than those of ZN staining (31.3%; p = 0.013 and 18.8%; p = 0.0044, respectively).

4 | DISCUSSION

In general, multinucleated giant cells are commonly found in birds with avian tuberculosis. Multinucleated giant cells play an important role in resisting tuberculosis because they may limit the growth and spread of Mycobacterium tuberculosis (Dannenberg, 2006). Indeed, the presence of multinucleated giant cells has been reported in wild ducks infected with Mycobacterium avium (Roffe, 1989). However, multinucleated giant cells were rarely observed
in tissues of naturally infected Pekin ducks in this study, which is similar to a previous study (Saggese et al., 2007). We proposed a possible explanation that the stimulus that induces multinucleated giant cell formation is reduced. It is therefore possible that a defect in multinucleated giant cell formation in Pekin ducks increased their susceptibility to MAA infection, although it has been reported that geese and ducks are moderately resistant to MAA (Hejlícek & Treml, 1995). Some reports also claimed that decreased genetic diversity of commercial ducks leads to an increased susceptibility to MAA infection (Acevedo-Whitehouse, Gulland, Greig, & Amos, 2003; Bonneaud, Pérez-Tris, Federici, Chastel, & Sorci, 2006; Miller & Lambert, 2004). In addition, we suspected that this isolated strain is very virulent, but further studies are needed to confirm this suspicion.

The detection rates of MAA in spleen and duodenum were lower in both methods. The reason may be that there are a large number of lymphocytes and macrophages in the tissue structure of the spleen and duodenum, which is more effective in inhibiting the colonization of bacteria in tissues.

In this study IHC detected more MAA than ZN staining in the kidney and duodenum. It may suggest that immunostaining may be more sensitive than ZN staining in detecting MAA infection. As ZN staining requires bacteria with intact cell walls, which depend on the formation of mycolic acid-carbol fuchsin complex, this may lead to a comparatively lower detection rate (Martinson et al., 2008). Indeed, mycobacteria tend to lose acid-fast ability under stressful environments (Deb et al., 2009). In contrast, IHC can detect all types of bacterial proteins without needing intact cell walls and therefore provides a higher sensitivity, which is also in accordance with previous reports (Thoresen, Falk, & Evensen, 1994). Considering the technical advances in detecting MAA and relative higher detection rates, IHC will provide the potential to detect low concentrations of MAA, especially at early stage of infection.

The ducks in this study were naturally infected and as such this study did not investigate the dynamic distribution of MAA in ducks. An artificial infection model for avian tuberculosis will be established in the future. In addition, more bioinformatics analysis and identification of virulence of MAA are of significance to the understanding of the bacteria.

5 | CONCLUSION

The IHC and ZN staining methods can be used to detect the distribution of MAA in tissues of the naturally infected domestic Pekin ducks and the MAA antigens were primarily detected in the epithelioid macrophages in selected organs. There was no difference in MAA-positive detection rates between the six organs (liver, spleen, lung, kidney, duodenum and pectoralis muscle). Additionally, this study confirms that the detection rate of MAA in naturally infected domestic ducks by IHC was higher than that of ZN staining. These findings lay the foundation for further research on the pathogenesis of Avian tuberculosis.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL STATEMENT

The animal-use procedures were approved by the Animal Ethics Committee of the Sichuan Agricultural University.

ORCID

Dekang Zhu https://orcid.org/0000-0002-7314-1088
Anchun Cheng https://orcid.org/0000-0001-6093-353X

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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