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Serum Proteomics Identifies Candidate Proteins Respond to Avian Tuberculosis in Thai Domestic Duck (*Anas platyrhynchos* Domestica)

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Abstract: Avian tuberculosis caused by the infection of *Mycobacterium avium*. The incident of *M. avium* infection in poultry, especially, domestic duck was reported in the lower Northern Region of Thailand. This bacterial specie can cross-infecting to other animals and more likely to occur in persons with pre-existent diseases and in immune systems impaired-person. Laboratory routine methods for detection of *M. avium* are acid fast bacilli staining and subsequently culture in specific medium which time-consuming and false negative results were often reported. This study aims to explore the expression profiles and identification of serum proteins that responding to avian tuberculosis in domestic duck. Serum samples of *M. avium* infected and healthy ducks were analysed by two-dimensional electrophoresis. Hyper-intensity protein spots as estimated as approximately 22, 35 and 40 kDa in *M. avium*-infected serum were identified by LC-MS/MS. They were identified as Apolipoprotein A-1, transthyretin and immunoglobulin Y heavy chain. These proteins maybe hypothesized-upregulated for responding to *M. avium* infection and might be serving as alternative biomarkers for further development of screening test kit in poultry serum or secretion especially, for preventing the epidemiology of these pathogens.

Key words: Avian tuberculosis, duck, proteomics, serum protein, Thailand, pathogens

INTRODUCTION

Mycobacterium avium was recognised as a cause of tuberculosis in most avian species, primarily in ducks, chickens, bird species and other fowls (Dhama *et al*., 2011). It can also infect to an extensive variety of different animals by generally transmits via direct contact with infected poultry (Thoen, 1997). This pathogen can susceptible to the human or most livestock species such as bovine, after ingestion of contaminated feed and water (Prasanna and Mehra, 2013). The susceptibility is high in domestic fowl, pheasants, wild birds, swine, rabbit and human with immunocompromised (Thorel *et al*, 2001). Even human is considered as the highly resistant but there are a number of infected cases in persons with pre-existent diseases especially, involving the lungs or with an immune system impaired (Ramirez *et al*., 2008).

Routine microbiological techniques for examination of *Mycobacteria* species are microscopic examination of Acid Fast Bacilli (AFB) staining or subsequently specific culture in lowenstein-jensen medium. They presented a low sensitivity especially, in early or lightly infected cases, need of technician's proficiency and time-consuming.

In the field of veterinary diagnosis or epidemiologically survey, the protein-based biomarkers are ideal applicants for the easier accessibility. The 2-Dimensional polyacrylamide gel Electrophoresis (2-DE) coupled with Mass Spectrometry (MS) is a powerful tool for discovering of the biomarkers (Anderson and Anderson, 1998). Applications of proteomics to new biomarker investigation in domestic animal diseases were demonstrated in cow, pig small ruminants, horse, dog and cat using their fluidic-specimens such as serum, whey, milk, urine, tear or synovial, cerebrospinal and bronchoalveolar-fluid (Ceciliani *et al*., 2014).

In poultry, there are some applications of proteomic approaches. For examples, to study the metabolic differences (metabolism, stress response and antioxidant systems) in the livers of fat duck for identification of

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potential proteins for functional analysis to explore the changes in cellular protein profiles in duck heptatitis B virus infected primary duck hepatocytes for providing a valuable *in vitro* model of viral infection (Zhao *et al*, 2010; zheng *et al*., 2014), to analysis of serum proteome of hens at different developmental stages for exploring the physiology of growth (Dhama *et al*., 2011), to characterized serum proteins possibly involved in resistant to avian pathogenic *Escherichia coli* (Tyler *et al*., 2008) or to evaluation of the serum proteome response to coccidiosis by Eimeria infection into broilers for identify pharmacological targets (Gilbert *et al*., 2011). In this study we attempt to demonstrate the different serum proteomes of *M. avium*-infected ducks and healthy subjects. The upregulated proteins in *M. avium*-infected serum can be suggested for serving as an alternative biomarker for differential diagnosis of *M. avium* infection in Thai domestic ducks.

MATERIALS AND METHODS

Ethical statement and sample collection: The animal experiments and related-protocols in this study were approved, the researchers were certified by the Institutional Animal Care and Use Committee (Thai-IACUC) cooperated with the Institute of Animal for Scientific Purposes Development (IAD), permit number: U1-05294-2559. Based on the Ethics of Animal Experimentation of the National Research Council of Thailand. Blood samples of Thai domestic duck (*Anas platyrhynchos* domestica) were routinely collected by the veterinarians from Veterinary Research and Development Centre (VRDC)-Lower Northern Region, Phitsanulok, Thailand. In fact, the samples in this study were the portions of the leftover specimens received from microbiological laboratory. Serum samples of *M. avium* infected (n = 5) and healthy control (n = 5, those underwent annual farm screening by VRDC) were subjected to this study.

Preparation of serum and isoelectro focusing on Immobilized pH Gradient (IPG) strips: Sera were separated after centrifugation at 3000×g for 15 min at 4°C and subsequently stored at -80°C until use. The concentration of protein in serum samples was determined according to the Bradford Assay using bovine serum albumin as a standard (Bradford, 1976). They were diluted in rehydration buffer (7 M Urea, 2 M Thiourea, 0.5% IPG buffer, 0.002% Bromophenol blue and 2.8 mg/mL Dithiothreitol (DTT)) for a total volume of 125 μL. After that each serum sample was applied to a 7 cm Ready Strip™ IPG linear strips (Bio-Rad Lab., Hercules, California, USA), pH 3-10 (150 μg of serum protein loading) or pH 4-7 (100 μg of serum protein loading). For the first-dimension electrophoresis, the Iso Electro Focusing (IEF) was performed according to the standard protocol of the manufacturer, briefly, 50 V for 15 min in rehydration, 300 V for 30 min in step and hold, 1000 V for 30 min in Gradient-1 step, 5000 V for 90 min in Gradient-2 step and 5000 V for 15 min in step and hold.

Polyacrylamide Gel Electrophoresis (PAGE) of IPG strips: The strips were equilibrated in Sodium Dodecyl Sulfate (SDS) equilibration buffer (6M Urea, 75 mM Tris-HCl pH 8.8, 29.3% Glycerol, 2% SDS, 0.002% Bromophenol blue and 10 mg/mL DTT). Then, the second-dimension electrophoresis was performed on a 10% SDS-PAGE until the tracking dye reached the bottom of the gel. The gels were stained with colloidal Coomassie Brilliant Blue G-250 (Sigma-Aldrich, St Louis, MO, USA). Gel images were captured using an ImageQuant LAS 500 (GE Healthcare Life Sciences, Little Chalfont, UK). Selection of the protein spots which presented the hyper-intensity in *M. avium*-infected sera from healthy sera were considered.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis: Candidate protein spots were isolated and in-gel digested with trypsin (Promega, Madison, WI, USA) according to the standard protocol. The digested peptide mixtures were analysed by LC-MS/MS using the Waters SYNAPT™ HDMS™ system (Synapt HDMS, Waters Corp., Manchester, England). The 1D-nano LC was carried out with an incorporated-waters nano ACQUITY UPLC system. Samples were electro-sprayed into a Mass Spectrometer for MS/MS analysis of peptides. Spectral data were generated in a micromass file support for MS/MS Ion search using the MASCOT engine program (http://matrixscience.com). Protein hits with the highest score were used for spot identifications.

RESULTS AND DISCUSSION

Identification of hyper-intensity protein spots by 2-DE: Using IEF-linear separating of pH range from 4-7 for SDS-PAGE, two hyper-intensity proteins spots which estimated Molecular Weight (MW) as approximately 25 and 35 kDa were obviously identified in *M. avium*-infected serum samples when compared with healthy controls (Fig. 1). Repeatedly, these 2 protein spots were obtainable in the gels from IEF-linear separating of pH range from 3-10 with an additional hyper-intensity protein spot at estimated MW as approximately 40 kDa is also remarked in this condition (Fig. 2).

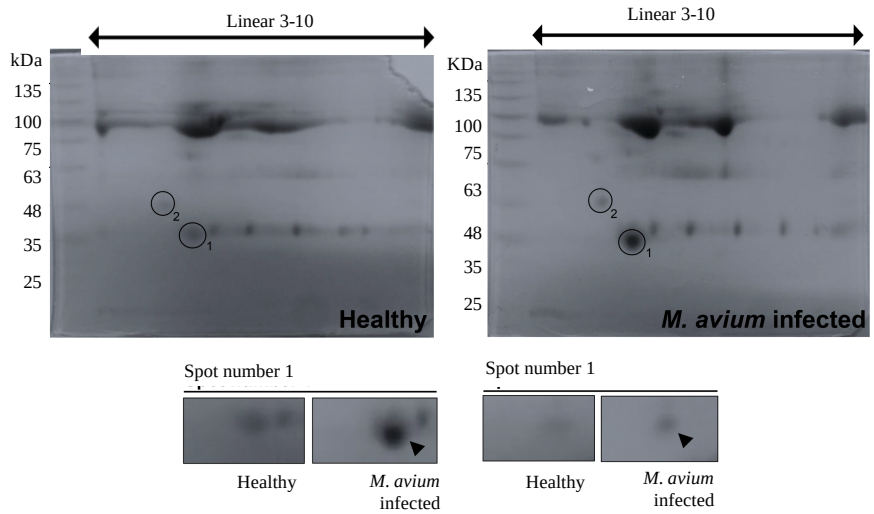

Fig. 1: Upper panel presented the representative 2DE profiles of serum samples from *M. avium*-infected ducks and healthy controls when IEF-linear separating of pH range from 3-10. Two spots with increasing intensity in *M. avium*-infected serum are circled and numbered. Lower panel emphasized the hyper-intensity of each spot by the arrow

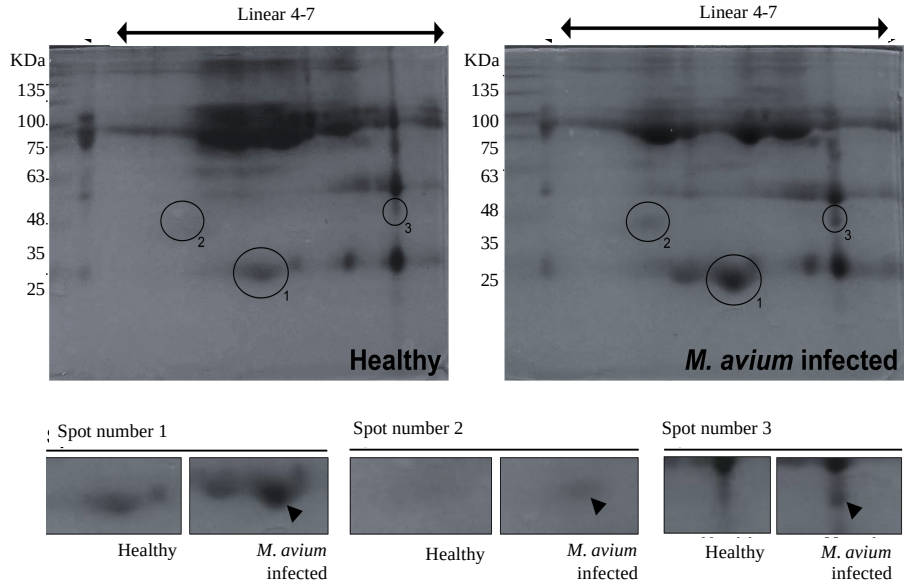

Fig. 2: Upper panel presented the representative 2DE profiles of serum samples from *M. avium*-infected ducks and healthy controls when IEF-linear separating of pH range from 4-7. Three spots with increasing intensity in *M. avium*-infected serum are circled and numbered. Lower panel emphasized the hyper-intensity of each spot by the arrow

Analysis of hyper-intensity protein spots by LC-MS/MS: Three identified protein spots were subjected to LC-MS/MS and the analysis of the MASCOT web-based analysis and Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST) are resulted. Apolipoprotein A-1 (PSI-BLAST: gi|546158), transthyretin (PSI-BLAST: gi|483524938) and immunoglobulin Y heavy chain (PSI-BLAST: gi|345642) were specific identified with the highest protein hit score for spot number 1 (~25 kDa), spot number 2 (~35 kDa) and spot number 3 (~40 kDa), respectively, (the spot number corresponding to the Fig. 2.

Apolipoprotein A-1 (ApoA1) that is identified from protein spot number 1. In the UniProt database, *Anas platyrhynchos* ApoA1 is available as UniProtKB - O42296 (APOA1_ANAPL). A previous study in duck

characterized the primary structure of 240 amino acid residues of ApoA1 by sequencing peptide fragments and alignment with chicken ApoA1 (Gu *et al*., 1993). There are no cross-reactivity between duck and human ApoA1 with a goat antiserum against human ApoA1 was found. Generally, ApoA1 is the major protein component of High-Density Lipoprotein (HDL) particles in plasma which has a specific role in lipid metabolism. ApoA1 enables efflux of lipids by accepting from within cells for transportation or excretion.

The protein that identified from spot number 2 is Transthyretin (TTR). Regarding to its structure, TTR was originally called as pre-albumin because it faster than albumin on experimentally serum electrophoresis. TTR expression was generally observed in the choroid plexus and liver of adult ducks, quails and pigeons (Southwell *et al*., 1991). Its function as a transporter in the serum for carrying the thyroid hormone, thyroxine and it also bound to retinol as retinol-binding protein.

In addition our study identifies IgY heavy chain from spot number 3. In avian species, the type of immunoglobulin is Ig Y which its functional similarity to mammalian IgG. In this case, Ig Y is hypothesized that regularly produced by the immune system for reacting to certain foreign substances or bacterial infection. Previous report established that ducks produce a truncated form of IgY which is missing part of the Fragment crystallizable (Fc) region (Lundqvist *et al*., 2001).

Comparison of these upregulated serum proteins under the condition of *M. avium* infection in our study. TTR is suggested to be the candidate responded-protein for further purpose. There are a several studies represented the correlation of infection with TTR especially using proteomics approach. A previous study in cattle, serum levels of TTR were significantly increased in *M. paratuberculosis*-infected animals compared with controls (Seth *et al*., 2009). They summarized that TTR were identified exclusively in *M. paratuberculosis* infection which corresponding to the hyper-intensity of TTR protein in our study of *M. avium* infection in duck.

CONCLUSION

This study highlights the serum protein which is an obvious target for the development of non-invasive biomarkers. We identified three overexpressed-proteins in a subgroup of well-characterized serum samples from November 23, 2018 *M. avium*-infected cases and healthy controls. The further exploration of statistically validation in a number of specimens and standardization of the sensitivity and specificity will reveal Transthyretin or TTR as an alternative potentially biomarker for epidemiological surveys of *M. avium* in domestic poultry, especially in Thailand where *M. avium* infection in ducks are usual informed.

Reading to the low sensitivity of bacterial staining or long-time bacterial culture in routine detection method. The proteomic approach in our study suggests that TTR may be suitable to complement the diagnostic decisions. The discovery of these biomarkers has significant impact on the elucidation of pathogenesis of mycobacterial diseases at the molecular level and can be applied in the development of mycobacterium-specific diagnostic tools for the epidemiologically survey.

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