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Amyloid A amyloidosis secondary to avian tuberculosis in naturally infected domestic pekin ducks (*Anas platyrhynchos* domestica)

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ABSTRACT

To investigate the correlation between avian tuberculosis and duck amyloidosis, the liver, lung, spleen, kidney, duodenum and pectoralis muscle of ducks naturally infected with *Mycobacterium avium* subsp. *avium* were used to detect amyloidosis by Congo red staining and potassium permanganate-Congo red staining. The expression level of IL-1β, IL-6, IL-10, TNF-α and SAA2 were detected by quantitative real-time RT-PCR (qRT-PCR). The results showed that the liver, lung, spleen, kidney, duodenum and pectoralis muscle of the infected ducks exhibited amyloid proteins under ordinary light microscopy and the polarization light under polarized light microscopy. However, no amyloid deposition in potassium permanganate-Congo red staining sections indicated that the amyloidosis was AA amyloidosis. In addition, the expression level of IL-1β, IL-6, IL-10, TNF-α and SAA2 increased from 4 to 43. This study showed that avian tuberculosis could induce secondary amyloidosis in naturally infected ducks.

1. Introduction

Amyloidosis is a disease caused by the abnormal deposition of insoluble amyloid proteins extracellular or blood vessels [1]. Amyloid A amyloidosis (AA amyloidosis) is systematic amyloidosis, which can occur in humans, mammals and poultry [2]. The precursor substance of AA amyloidosis is serum amyloid A (SAA), which is mainly synthesized by the liver. Drastically increased levels of IL-1, IL-6 and TNF-α stimulate the liver to synthesize SAA when the body is exposed to trauma, chronic infections, inflammatory stimuli and sustained immune stimulation [3,4]. The remaining N-terminal amino acids are converted to amyloid protein A after the partial C-terminal amino acid degradation of SAA [5,6]. The pro-inflammatory cytokines IL-1β and TNF-α are primary cytokines that stimulating the release of SAA [7]. Although, IL-6 primarily stimulates the secretion of haptoglobin. It can modulate the production of IL-1 and TNF-α, and synergistically induces the synthesis of SAA [7]. IL-10 acts as anti-inflammatory cytokines, inhibits the release of pro-inflammatory mediators from monocytes/macrophages and therefore inhibits the secretion of TNF-α, IL-1β, IL-6 [8].

Avian amyloidosis, belongs to AA amyloidosis [1], occurs in numerous bird species [9]. The occurrence of avian amyloidosis is related to the age, species, feeding management and inflammatory disease. Amyloidosis occurs in older birds [9]. Exotic, poorly adapted or excessively aggressive birds are more susceptible to amyloidosis [10]. Chicken AA amyloidosis is referred to as amyloid arthropathy and is associated with *Enterococcus faecalis* infection [11]. In waterfowl, AA amyloidosis is frequently secondary to bumblefoot caused by *Staphylococcus* infection [2]. Investigations have claimed that birds with tuberculosis could be accompanied by amyloidosis [12]. At present, there are reports about amyloidosis occurrence in wild boars that have been naturally infected with tuberculosis [3]. This study attempted to explore the correlation between avian amyloidosis and avian tuberculosis, in order to supplement the research amyloidosis occurs in domestic Pekin duck (*Anas platyrhynchos* domestica).

Abbreviations: MAA, Mycobacterium. avium subsp. avium; AA amyloidosis, Amyloid A amyloidosis; qRT-PCR, quantitative real-time RT-PCR

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2. Materials and methods

2.1. Animals selection

Sixteen *Mycobacterium. avium* subsp. *avium* (MAA) infected breeder Pekin ducks from a duck farm in Sichuan Province, China and two non-infected ducks from another flock of the same age were used for this study. Infection and non-infection were confirmed by postmortems, histology, and PCR amplification of the IS*901* and the 16S rRNA genes, as we reported previously [13]. Ziehl-Neelsen staining was positive for the tissues with gross lesions. And the single colony incubated by the tissues homogenate on the Middlebrook 7H10 agar was identified as MAA by PCR amplification. All eighteen ducks were anesthetized (pentobarbital sodium, 30 mg/kg of body weight, intravenous injection) and humanely killed by cervical dislocation. The animal procedures were approved by the Animal Ethics Committee of the Sichuan Agricultural University (approval 2015-018). Liver, spleen, lung, kidney, duodenum and pectoralis muscles were collected from the same regions from all ducks. These organs were fixed in 4% paraformaldehyde within 24 h followed by standard paraffin embedding.

2.2. Histopathology of target tissues

All liver, lung, spleen, kidney, duodenum and pectoralis muscle sections were routinely dewaxed and rehydrated before being stained with H&E, Congo red and potassium permanganate-Congo red staining. The specific Congo red staining steps followed the instructions described by the Bennhold Congo Red dyeing kit (Solarbio, Beijing). Briefly, sections were stained in hematoxylin for 5 min, then were stained in Congo red dye for 15 min and were differentiated in 70% alcohol for 30 s. The steps of potassium permanganate-Congo red staining are as follows: sections were oxidized in potassium permanganate for 5 min and were neutralized with 5% oxalic acid solution, then sections were stained in hematoxylin for 5 min, stained in Congo red dye for 15 min and differentiated in 70% alcohol for 30 s. All sections were dehydrated in graded alcohol and xylene and then mounted. All sections were observed under ordinary light microscopy (Nikon Eclipse 80i, Japan) ×400 magnification and Congo red stained sections also were observed under DMLP polarized light microscopy (Leica, Germany) at ×400 magnification. All images were taken using a Spot Flex camera (Diagnostic, USA). The results of Congo red staining were classified as follow: (-), no amyloid deposition; (1 +), mild amyloid deposition; (2 +), moderate amyloid deposition; (3 +), severe amyloid deposition.

2.3. Quantitative real-time RT-PCR for immunological factor monitoring

The total RNA of six infected ducks and two no-infected ducks were extracted from whole blood following the instructions described by the RNAiso Plus (Total RNA extraction reagents) (Takara, Japan). cDNA was inversely transcribed from RNA, following the instructions of the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). The expression of IL-1β, IL-6, IL-10, TNF-α and SAA2 were measured using SYBR Green method with SYBR® Premix Ex Taq™II (Tli RnaseH Plus) (Takara, Japan). The GAPDH gene was used as the internal reference gene. The nucleotide coding region of IL-1β, IL-6, IL-10, TNF-α, SAA2 and GAPDH were found through the National Center of Biotechnology Information (NCBI) database. These specific primers were designed with Primer Premier 5.0 software. The primer information for each gene is shown in the Table 1. The qRT-PCR reaction system was as follows: 2 μL cDNA template, 10 μL SYBR® Premix Ex Taq II, 0.8 μL forward primer, 0.8 μL reverse primer, 6.4 μL RNase Free H_2O. The reaction procedure was as follows: predegeneration 95 °C, 30 s; degeneration 95 °C, 3 s; annealing and extension 60 °C, 30 s, 39 cycles; 65 °C, 5 s.

The results were analyzed using the $2^{-\Delta\Delta Ct}$ method by Bio-Rad CFX manager version 3.0 (Bio-Rad). Each gene was tested independently three times and repeated three times. The statistical significance of the data was analyzed using unpaired Student's t-tests performed in GraphPad Prism 7 software. The statistical significance of the difference is indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Table 1
Primers utilized in the qRT-PCR.

primer	Sequence (5˜3′)	product size (bp)	annealing temperature(°C)
IL-1β F	GCTACACCCGCTCACAGTCCTT	123	61.4
IL-1β R	GCCTCACTTTCTGGCTGGATG		
IL-6 F	TACCCAGAAATCCCTCCTCACA	125	61.4
IL-6 R	AATAGCGAACAGCCCTCACG		
IL-10 F	GCCTCCACTTGTCTGACCTCCTA	181	64.5
IL-10 R	AACCGCATCATCTCCAGCAC		
TNF-α F	TTTTATGACCGCCCAGTT	114	59
TNF-α R	TAGGCAGAGGCCACCA		
SAA2 F	GCTGTTATTGTGTGTGCAAGTGC	179	61.4
SAA2 R	CCTCTTCGGGCAGCATCAT		
GAPDH F	TGCTTGCTGCTCCTCCTCAT	110	59
GAPDH R	TGGCTACCACTTGGACTTTGC		

3. Results

3.1. Qualitative assessment of amyloid deposits in tissue samples

H&E and Congo red staining showed that amyloid proteins could be detected in the liver, lung, spleen, kidney, duodenum and pectoralis muscle. For the same tissue, the areas of amyloid protein were detected by the two methods were consistent (Fig. 1). In the liver, amyloid proteins were mainly deposited in hepatic sinusoids, and a large amount of amyloid proteins extruded hepatocytes. In addition, there was a certain amount of amyloid deposition around the blood vessel wall of the liver. In the lung, only a small amount of amyloid deposits was detected around the walls of the blood vessels. In the spleen, diffuse amyloid proteins were deposited between the splenic pulp. In the kidney, amyloid proteins were deposited in the glomerular capillary interstitium, the renal tubule interstitial tissue and around the walls of the blood vessels. In the duodenum, diffuse amyloid deposits were deposited in the lamina propria of the intestinal villi. There was a small amount of amyloid deposition around the walls of the blood vessels of the duodenum serosa. In the pectoralis muscle, no granulomatous formed, only a small amount of amyloid deposits was detected around the walls of the blood vessels. The polarization light under polarized light microscopy could be observed in all the sections with Congo red positive staining (Fig. 1).

Potassium permanganate-Congo red staining sections were observed under ordinary light microscopy at ×400 magnification. There was no amyloid protein deposition in 16 infected duck liver, lung, spleen, kidney, duodenum and pectoralis muscle samples (Fig. 2). Amyloid protein can no longer be stained by Congo red dye after oxidation of potassium permanganate.

3.2. Quantitative assessment of amyloid deposits in tissue samples

Although amyloid protein deposition could be observed in the liver, lung, spleen, kidney, duodenum and pectoralis muscle, the degree of amyloidosis was different (Table 2). The most severe occurrence of amyloid protein was observed in the duodenum. 14 (87.50%) duodenum samples showed amyloidosis, of which 12 (75.00%) had serious amyloidosis. The liver and spleen showed moderately severe amyloidosis, both of which had amyloidosis detected in 11 samples, and the sample sizes of severe amyloidosis were 5 (31.25%) and 7 (43.75%), respectively. Mild amyloidosis occurred in the kidneys; although

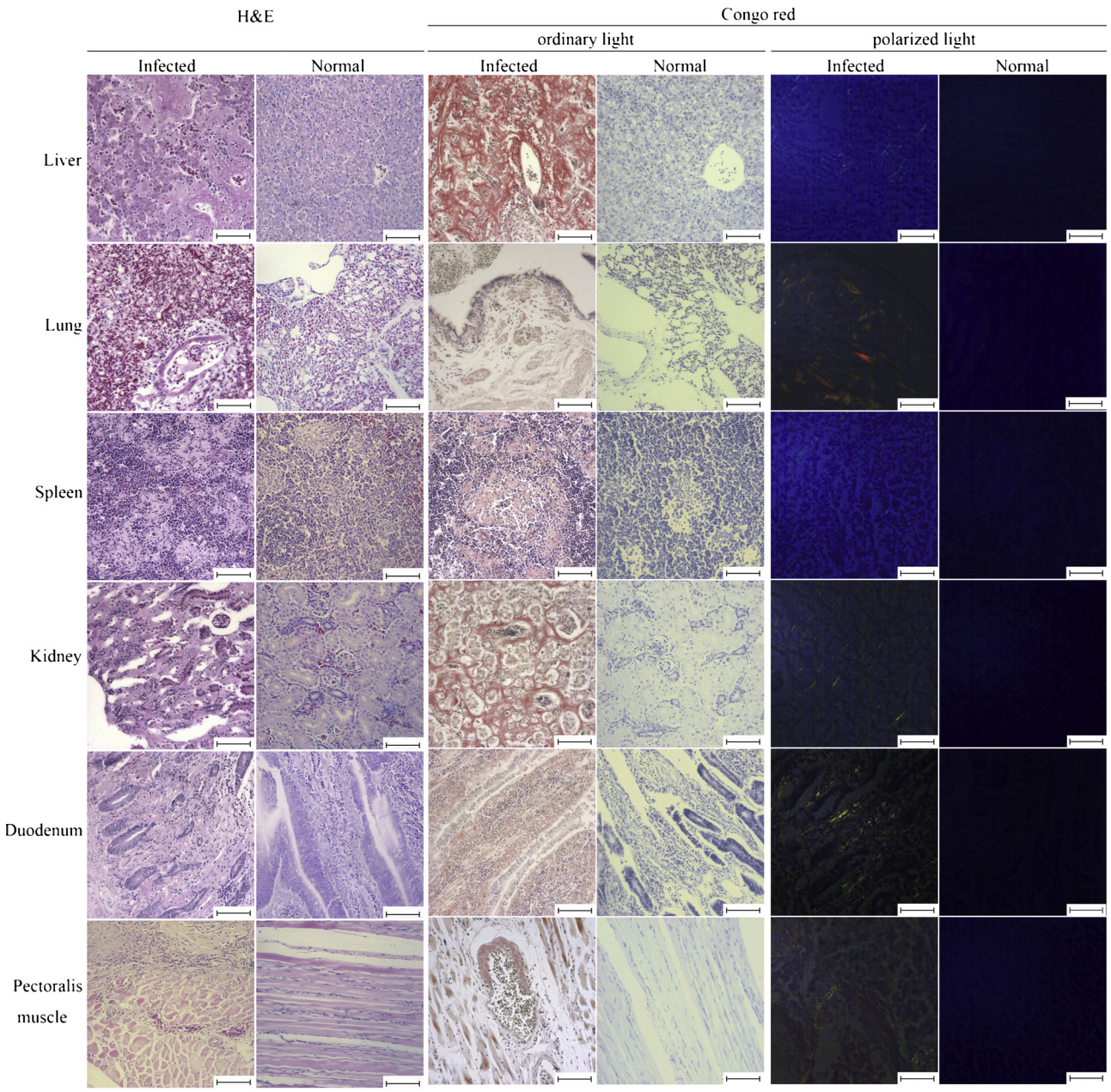

Fig. 1. H&E and Congo red staining staining of liver, lung, spleen, kidney, duodenum and pectoralis muscle sections in domestic Pekin ducks infected with avian tuberculosis and normal Pekin ducks observed under ordinary light microscopy. And congo red staining sections observed under polarized light microscopy. Bar = 75 μm.

amyloidosis was detected in 8 (50.00%) kidney samples, but only 3 (18.75%) instances of severe amyloidosis occurred. In the pectoralis muscle, only 6 (37.50%) of the samples showed mild amyloidosis. In the lung, amyloidosis was only detected in 5 (31.25%) of the samples, of which 4 (25.00%) samples were mild cases of amyloidosis.

3.3. Quantitative real-time RT-PCR for immunological factor monitoring

The results showed that the levels of IL-1, IL-6 and IL-10 increased to different degrees in six blood samples, and the levels of TNF-α and SAA2 were significantly increase in some blood samples (Fig. 3). The expression of IL-1 increased by 4–43 fold in six blood samples. The expression of IL-6 increased by 4–17 fold in six blood samples. The expression of IL-10 approximately increased by 4 fold in the six blood samples. There was no significant change in the expression of TNF-α in two blood samples, but the levels of TNF-α increased by 4–9 fold in other four samples. The levels of SAA2 increased by 6–28 fold in four blood sample, while no significant changes was observed in other two blood samples. The comparison results of IL-1, IL-6 and SAA2 expression were shown in Fig. 4. The expression of IL-6 was higher than that of corresponding IL-10 in four blood samples, the expression level of IL-6 was similar to that of corresponding IL-10 in other two blood samples, and the increase of the expression level of IL-6 was lower.

4. Discussion

Amyloid proteins were observed in each tissue under ordinary light microscopy and special light birefringence were observed under polarized light microscopy in Congo red staining sections. The results showed that amyloidosis occurred in the ducks infected with MAA, but the severity of amyloidosis was different. Amyloidosis was severe in the duodenum, liver and spleen, but amyloidosis was mild in the lung,

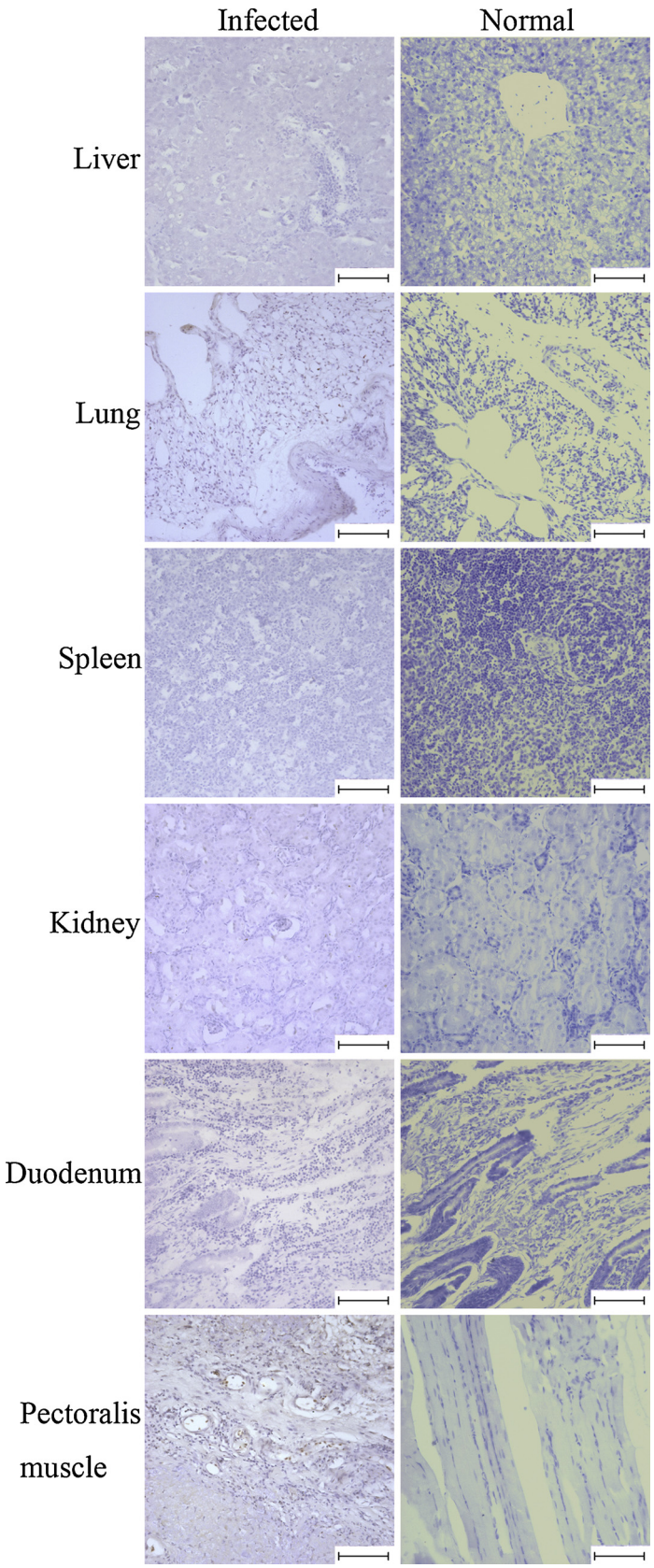

Fig. 2. Potassium permanganate-Congo red staining of liver, lung, spleen, kidney, duodenum and pectoralis muscle sections in domestic Pekin ducks infected with avian tuberculosis and normal Pekin ducks observed under ordinary light microscopy. Bar = 75 μm.

Table 2
Results from Congo red staining of the MAA infected duck tissues.

		Number of positive results (%)				
	Total	-	1+	2+	3+	Positive
Liver	16	5(31.25)	2(12.50)	4(25.00)	5(31.25)	11(68.75)
Lung	16	11(68.75)	4(25.00)	1(6.25)	0(0.00)	5(31.25)
Spleen	16	5(31.25)	2(12.50)	2(12.50)	7(43.75)	11(68.75)
Kidney	16	8(50.00)	4(25.00)	1(6.25)	3(18.75)	8(50.00)
Duodenum	16	2(12.50)	0(0.00)	2(12.50)	12(75.00)	14(87.50)
Pectoralis muscle	16	10(62.50)	6(37.50)	0(0.00)	0(0.00)	6(37.50)

Percentage (%): the ratio of the number of tissues had positive staining to the total number of tissues. (-): No amyloid deposition; (1+) : Mild amyloid deposition; (2+): Moderate amyloid deposition; (3+): Severe amyloid deposition.

kidney and pectoralis muscle. Some study have claimed that the liver, spleen, intestines and kidneys are the major organs affected by amyloidosis [14,15], similar to the study results. More serious amyloidosis was observed in the duodenum, liver and spleen, which might be caused by their normal structure containing abundant macrophages which one of the main cells that secrete the inflammatory cytokines [7]. Thus they could stimulate the synthesis of serum amyloid protein, leading to a more severe amyloid protein deposition. Muscle amyloidosis was rare, but it was observed occurring in the muscle of chickens that had received multiple vaccinations. The muscle that had received vaccinations could form granulomas, and amyloids could be deposited in the granulomas [16,17]. These previous observations are different from the results of this study in which the pectoralis muscle did not have typical granulomatous formations and the amyloids were only deposited only around the vessel wall.

Potassium permanganate-Congo red staining and immunohistochemistry can distinguish AA amyloidosis and AL amyloidosis. Because of the lack of specific antibodies, the use of immunohistochemistry is limited. In contrast, potassium permanganate-Congo red staining has the advantages of simple operation, low cost and high practicability [18,19]. Potassium permanganate-Congo red staining sections did not observed amyloidosis. The results showed that amyloid protein could not be stained with Congo red dye because the structure was changed after the potassium permanganate oxidation reaction, which is consistent with the characteristics of secondary amyloid proteins. In conclusion, the liver, lung, spleen, kidney, duodenum and pectoralis muscle of ducks naturally infected with MAA had AA amyloidosis.

The relative expression of IL-1 and IL-6 were increased to different degrees. These results were consistent with previous study. The levels of

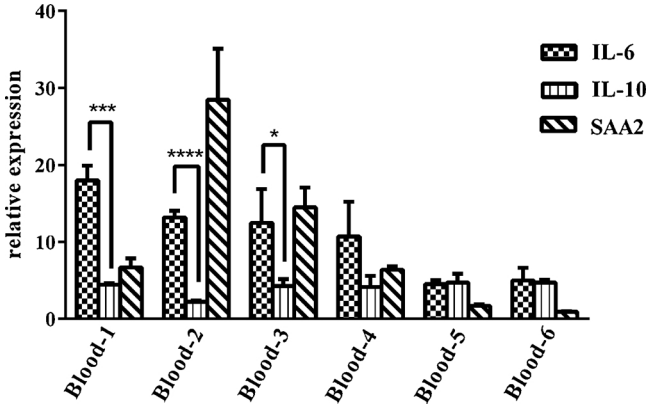

Fig. 4. The comparsion result of the relative expression of IL-6, IL-10 and SAA2. The differences between the expression of IL-6 and IL-10 for each group were calculated separately, and the statistical significance of the difference is indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Statistical significance between groups was assessed by unpaired Student's t-tests (n = 3).

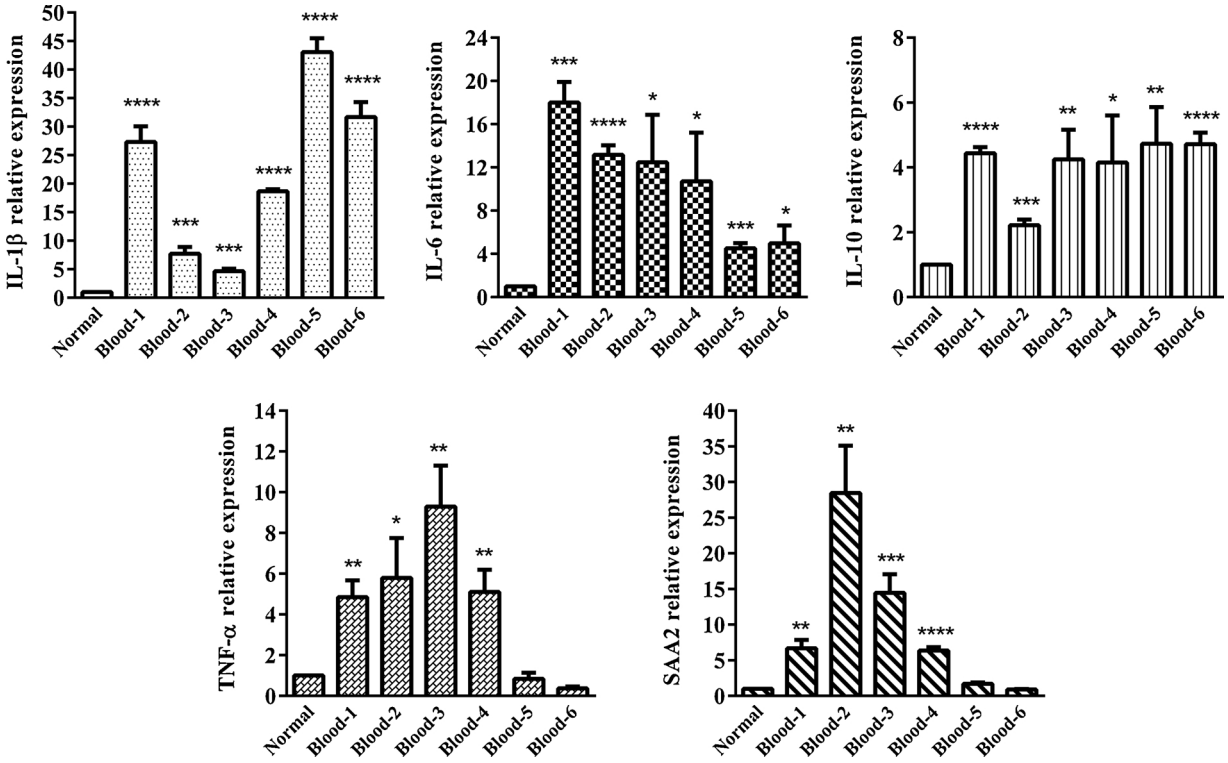

Fig. 3. The relative expression of IL-1β, IL-6, IL-10, TNF-α and SAA2 in blood of infected ducks. The differences between the infected blood and normal blood were calculated separately, and the statistical significance of the difference is indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Statistical significance between groups was assessed by unpaired Student's t-tests (n = 3).

IL-1 and IL-6 were significantly increased by experimental induced amyloidosis in chickens [20] and mice [21]. Muhammed et al research showed that the expression levels of IL-10 continuously drastically increased and the levels of IL-10 was higher than that of IL-6 when the mice were subjected to multiple acute stimuli [21,22]. The above results were contrary to the results obtained in this study, which the levels of IL-10 only slightly increased and the levels of IL-6 was higher than IL-10. This might be due to the animals were subjected different stimulated. Pekin ducks which were naturally infected with MAA, were subjected to a continuous inflammatory stimulus. Sevimli et al research showed that TNF-α was an important factor in inducing amyloidosis in chickens [23], which is basically consistent with this study that the levels of TNF-α increased to a certain extent. The expression of SAA2 did not change significantly in the other two blood samples. In these two blood samples, the levels of IL-6 and IL-10 were the same. It is possible that IL-6 and IL-10 are antagonistic to each other, resulting in decreased synthesis of SAA2 in the hepatic [8,24]. Some studies have shown that the increase of the blood acute phase protein in chronic inflammation was lower than in acute inflammation [7]. This finding could also support the observation of low expression level for SAA2 in this study.

Tuberculous granulomas in Pekin ducks that were confirmed only infected with MAA [13] include macrophages and fibroblasts, which are the main cells that secrete inflammatory cytokines [25]. Therefore, these Pekin ducks had long been stimulated by continuous inflammation, and synthesized excessive inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. These inflammatory cytokines stimulate the liver to synthesize excessive SAA, further forming AA amyloidosis. In short, secondary amyloidosis in these infected ducks was caused by avian tuberculosis.

However, the ducks in this study were naturally infected, so this study could not investigate the time at which the expression level of IL-1β, IL-6, IL-10, TNF-α and SAA2 started to increase and when amyloidosis formed after infection. Additionally, this study was unable to explore the correlation between the expression levels of IL-1β, IL-6, IL-10, TNF-α and SAA2 and the degree of amyloidosis.

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Declaration of interest

None.

Ethical approval

Not required.

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