



RESEARCH ARTICLE

Riemerella anatipestifer Infection in Chickens

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ABSTRACT

Riemerella anatipestifer (RA) is the causative agent of septicemic and exudative disease for a variety of bird species. Although RA had been isolated from chickens, whether can bring damages to them is not unrevealed yet. In this study, we report a flock of SanHuang chickens infected by RA with 15% morbidity and less than 8% mortality. The infection is further substantiated by case duplicate. The tested chickens demonstrate typical signs of pericarditis, air sacculitis and perihepatitis that are completely consistent with the field outbreak. The results suggest that RA is pathogenic to SanHuang chickens, which can then be theoretically and practicably incorporated into its infection spectrum.

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INTRODUCTION

Duck septicemia generally caused by *Riemerella anatipestifer* (RA) and previously known as *Pasteurella anatipestifer* or *Moraxella anatipestifer*, is a bacterial zoonosis of world-wide concern. Till now, more than 20 serovars have been identified (Sandhu and Harry, 1981; Ryll and Hinz, 2000). This serious and widespread disease causes major losses to duck industry through high mortality, reduced growth rate, poor feed conversion, increased condemnations, and high treatment costs. In east and south-east Asia, RA infection has been a critical problem in intensive production of meat ducks since 1982 (Loh *et al.*, 1992; Pathanasophon *et al.*, 2002). Currently, RA has been reported to cause disease in domestic geese, turkeys, swans and pheasants (Sandhu and Rimler, 1997; Rubbenstroth *et al.*, 2009). Outbreaks have also been reported in semi-domestic black ducks and in several species of captive wild waterfowl (Karstad *et al.*, 1970). But, the actual transmitting mechanism among different species is still unknown. The present study describes the disease of RA in ShanHuang chickens in southwest of China in December 2007 and the experimental infection of the isolated RA in chickens.

MATERIALS AND METHODS

On December 21, 2007, one owner possessing 3,500 17-day-old SanHuang chickens submitted 7 birds to our diagnostic laboratory, who reports that the morbidity is approximately 15% and mortality is less than 8%. The

birds exhibit abnormal clinical signs, such as dyspnea, droopiness, white fluid feces and stunting. The birds were then killed by cervical bloodletting with the help of butaylone anesthesia treatment. Necropsy of all birds revealed existence of typical pericarditis, air sacculitis and perihepatitis. Tissue samples including liver, brain and spleen were collected and immediately analyzed, or stored at -18°C.

RA isolation and identification

Appropriately treated samples were streaked on 10% rabbit blood agar plate and then placed at 37°C for 24-48h cultivation in 5% CO₂ incubator. After single colony formation, it was transferred into Brain Heart Infusion agar medium (Difco, BD, Franklin Lakes, NJ, USA) for further cultivation. The obtained pathogens were biochemically tested by VITEK32 (BiosMerieux, France). Growth test on MacConkey agar and Simmons citrate agar, and gelatin hydrolysis test were performed as described by Vandamme *et al.* (1998). Isolates were serotyped by rapid slide agglutination test using undiluted antiserum (Bisgaard, 1982). Cultures which showed cross-reactions were further confirmed by tube agglutination tests (Bisgaard, 1982) and gel-diffusion precipitin test (Pathanasophon *et al.*, 2002).

The 16SrRNA gene fragment was amplified using primer pairs 16S-L (AGAGTTTGATCCTGGCTCAG) and 16S-R (ACGGCTACCTTGTTACGACTT) (Stackebrandt *et al.*, 1994; Subramaniam *et al.*, 1997). Amplification conditions: 150ng genomic DNA, 5μl 10×Taq buffer, 2μl dNTP mix (2mM each), 1μl of each

primer (10pmol/ml) and 0.2µl Taq DNA polymerase (1U) in a volume of 50µl. The reaction parameters: a denaturation step (60s at 95°C) was followed by 35 cycles of denaturation (40s at 95°C), annealing (60s at 53°C) and elongation (100s at 72°C). An elongation step (300s at 72°C) terminated the PCR. The DNA was purified and sequencing was done (TaKaRa Biotech., Dalian, China).

Experimental infection

Tested SanHuang chickens were obtained from a commercial hatchery at one day after birth, received drinking water and antibiotic-free commercial feed *ad libitum* and were used to explore infection profile by RA challenge at 10-day-old. Total ninety birds were randomly and averagely divided into three groups. Group I and II were injected routinely via foot pad with 1ml of saline containing 1×10^9 CFU of the isolate (Sarver *et al.*, 2005). Disease status was examined up to 6 days after post infection (p.i). Survival rate in group I was recorded from the beginning to the end. At each time point (1, 2, 3, 4, 5 and 6th d p.i), three infected birds of group II were sedated and killed for observing gross lesions and collecting tissue or organ samples. Group III, served as control, were injected synchronously inactivated RA.

Hematologic and serum biochemical assays

White blood cells (WBC) were counted using the hemocytometer method (Schalm *et al.*, 1975). Serum aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and α -amylase (AMY) levels were estimated by automatic biochemistry analyzer (Cobas Integra 400/400 plus, Germany). The diagnostic kits were obtained from Roche reagent (Germany, lot: 621978-01, 618874-01, 621102-01 and 621325-01).

Enumeration of bacteria and histopathology

Samples were aseptically removed and homogenized separately in 3ml of sterile saline in tissue homogenizer. Then 0.1ml volumes of serial 10-fold dilution were plated on blood agar plate. The culture conditions were in accordance with the protocol as before manipulated. RA colonies were identified by rapid slide agglutination test. The colonies were counted and calculated per ml or gram.

The prepared tissues were placed in 10% neutral formalin for 24h fixing. After thoroughly washing with PBS, tissues were embedded in paraffin using standard techniques. Thin sections (5µm) were cut and stained with haematoxylin and eosin for histopathological studies.

Statistical analysis

The results are expressed as the mean value \pm standard deviation (SD). A Student's t-test was used to evaluate the significance of differences between experiments and control. A P value <0.05 was considered statistically significant.

RESULTS

R. anatipestifer isolation and identification

We firstly examined the etiology status for suspected infection from most of the damaged tissues. RA-like bacteria were isolated from the samples. Microscopically all strains appeared as Gram-negative, non-sporulating,

pleomorphic rods. We secondly performed biochemical test for all isolates. Representative characteristics of isolates were listed in Table 1. The isolates were tentatively confirmed by positive gelatin liquefaction, catalase and urease, negative arginine dihydrolase, negative fermentation of glucose and galactose, the absence of growth on MacConkey agar and on litmus lactose agar. The results of the rapid slide agglutination tests demonstrated that the isolates had intense positive reaction with rabbit anti-RA serotype-2 serum, and did not cross-react with any of the other serotypes. In order to confirm their affiliation to the species RA, 16S rRNA fragment was amplified and sequenced for eleven isolates. The sequences were compared with those of type RA strain in GenBank. The eleven isolates had identical 16S rRNA sequences (HQ008052), which shared 98% similarity with the sequences of type strain RAf13(EU715005.1), C900(AY871842.2), 540/86 (AY871834.2) and 30/90 (AY871835.2).

Table 1: Biochemical properties of isolates compared to *Riemerella anatipestifer* ATCC11845

Parameters	Isolate strains tested	Isolate (strains)	<i>R.anatipestifer</i> ATCC 11845
Pigment	11	- (11)	-
MacConkeyagar	11	- (11)	-
Litmus lactose agar	11	- (11)	-
Catalase	11	+ (11)	+
Urease	11	+ (11)	+
Gelatine liquefaction	11	+ (11)	+
Nitrate reduction	11	- (11)	-
Indole	11	- (11)	-
Aesculin hydrolysis	11	- (11)	-
D (+)-Glucose	11	- (11)	-
D (-)-Fructose	11	- (11)	-
Lactose	11	- (11)	-
Maltose	11	- (11)	-
D (+)-Galactose	11	- (11)	-
D (+)-Mannose	11	- (11)	-
N-acetyl-D-glucosamine	11	- (11)	-
Dextrin	11	- (11)	-
Trehalose	11	- (11)	-
Lactulose	11	- (11)	-

Notes: "+" positive; "-" negative.

Case duplicate

We determined tentatively the pathogen of RA SWCQ071205 strain to ShanHuang chickens. During the 6-day observation, birds in group I developed typically dyspnea, droopiness, white fluid feces and stunting. Out of 30, 12 chicks displayed leg weakness and 7 died soon after the symptom emerged. After the challenge, the lesions in dead or tolerated birds in group I and II were typical exudative inflammation including pericarditis, air sacculitis and perihepatitis. From the first day to the sixth, pericarditis and air sacculitis also developed sporadically and gradually. Alternatively, perihepatitis was only observed since the third day. Fig. 1 illustrates the representative lesions on multiple organs. Histological examination results were displayed in Fig. 2. The heart carried with typical fibrinous picarditis, subepicardial edema, myocarditis and focal necrosis. The liver displayed fibrinous perihepatitis, vacuolar degeneration

and focal necrosis. The spleen emerged perisplenitis, lymphoid depletion, reticular cells hyperplasia, focal necrosis and vascular endothelial cell swelling. Focal pneumonia, fibrinous air sacculitis, oedema and cellular infiltration were also observed in lungs. Mucosa exuviation, folliculus lymphaticus analosis, lymphoid depletion, reticular cells hyperplasia were founded in bursa of fabricius.

In order to get significant insight into the knowledge of germ-carrying time and organ-addiction, status of RA deposition in different organs was individually investigated and results were represented in Fig. 3. Peripheral blood contained approximately 2×10^4 CFU/ml at 1d p.i. and decreased to half at 2d p.i. During 3d to 5d p.i., an abrupt reduction in exponential format to approximately 10^2 CFU/ml was observed. At 6d p.i., approximately 2×10^3 CFU/ml were presented. The tendency in heart is very similar to the status with peripheral blood at every period. Between 1d to 6d p.i., RA was all recovered from the liver, spleen, kidney and brain. In liver and spleen, highest bacteria amount increased up to approximately 1.4×10^4 CFU/g and 4.0×10^4 CFU/g, respectively. In kidney, highest RA content emerged at 4d p.i.. In brain, the highest content is at 2d p.i. with 2.5×10^3 CFU/g. Then, it declined to approximately 40 CFU/g at 5d p.i.. Interestingly, it again elevated to 3×10^2 CFU/g at 6d p.i..

WBC count and biochemical parameters in chickens experimentally infected with RA train SWCQ071205 were listed in Table 2. There were no significant differences for white blood cells counting on 1d, 2d and 3d p.i.. The corresponding RA contents were $27.2 \pm 0.8 \times 10^9/L$, $27.1 \pm 4.0 \times 10^9/L$ and $32.7 \pm 4.5 \times 10^9/L$, respectively (T test, $P < 0.05$) compared with control which

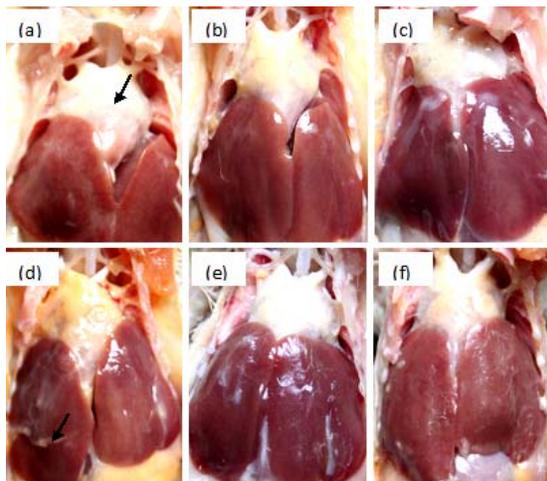


Fig. 1: Lesions observed in following foot pad infection of *Riemerella anatipestifer* strain SWCQ071205 in chickens. (a) 1d post-infection, (b) 2d post-infection, (c) 3d post-infection, (d) 4d post-infection, (e) 5d post-infection and (f) 6d post-infection. Arrows in (a) and (d) are typical pericarditis and perihepatitis, respectively.

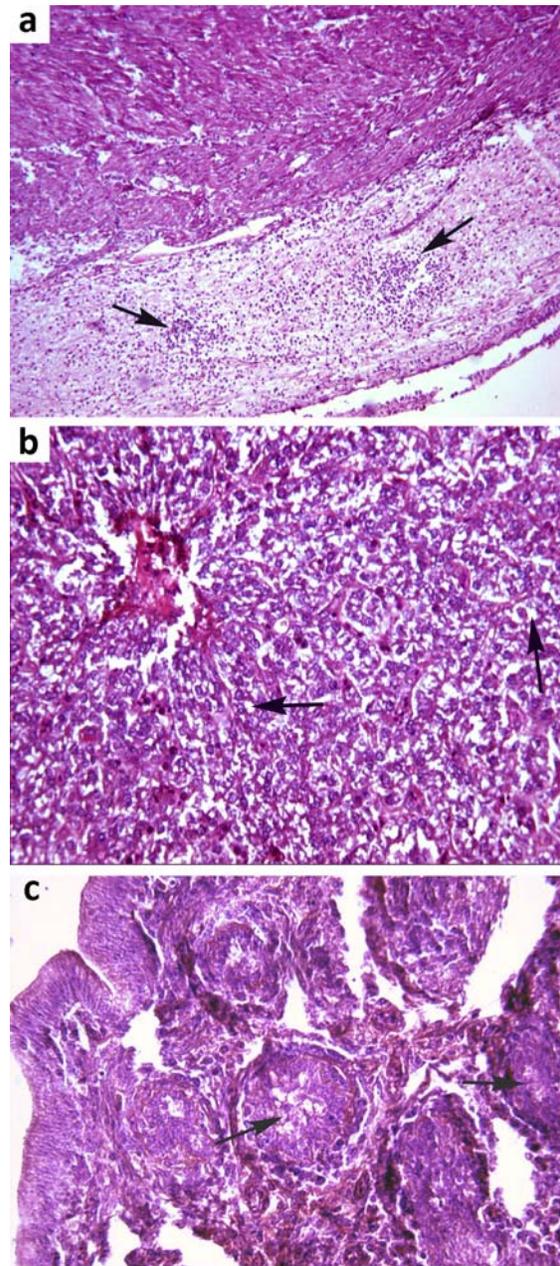


Fig. 2: Histopathological lesions in experimental infection of *Riemerella anatipestifer* strain SWCQ071205 in chickens at 3d p.i.. H & E staining. Section a (100 \times), b (400 \times) and c (400 \times) of heart, liver and bursa of Fabricius shows typical hemaleucin effusion, vacuolar degeneration of hepatocyte, and necrosis in germinal center (arrows), respectively.

is $26.5 \pm 1.8 \times 10^9/L$. But, it rose quickly to highest level with $60.2 \pm 5.6 \times 10^9/L$ on 4d p.i. After that, it declined to $43.0 \pm 2.6 \times 10^9/L$ on 5d p.i.. AST activity was increased at 2 d p.i. and 3 d p.i. LDH activity significantly increased from 1d p.i. to the end. ALP activity had the tendency of gradual depressing during the experimental process, and AMY activity significantly declined from 2d p.i. to 6d p.i.

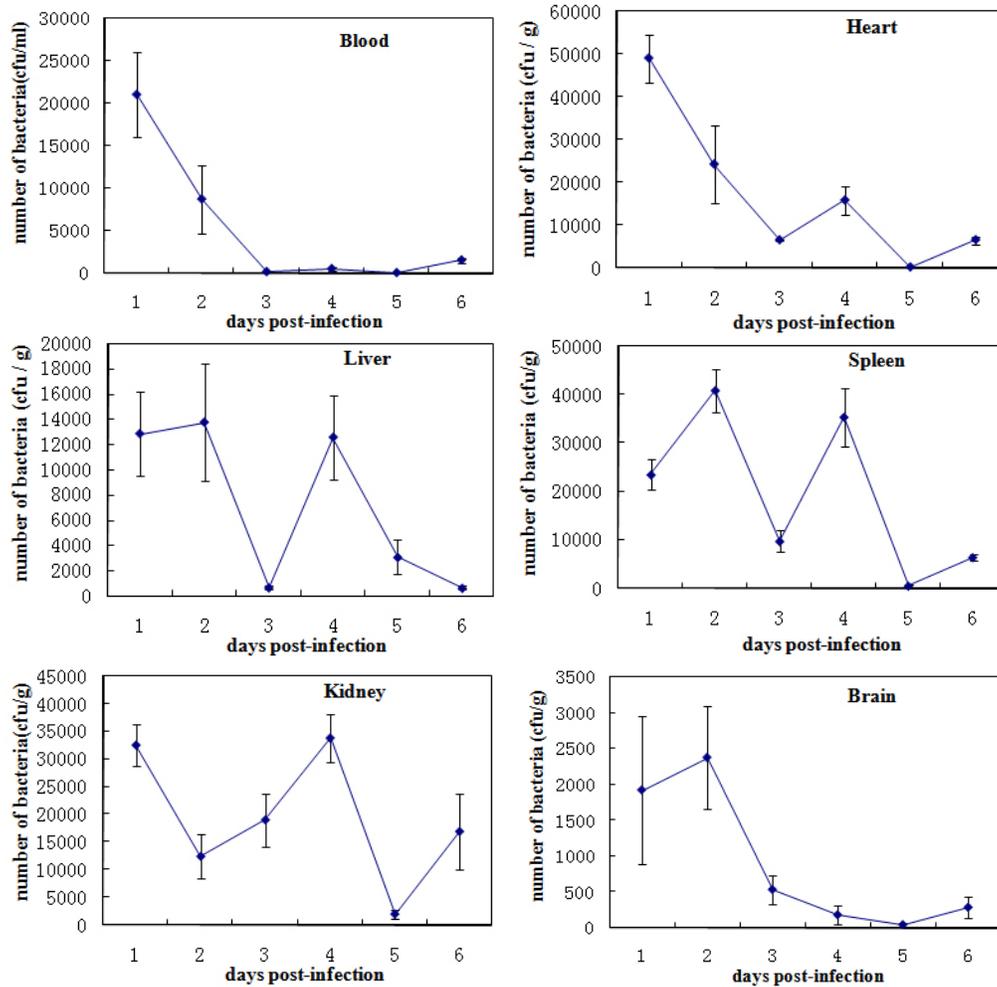


Fig. 3: Bacterial counts (CFU/g or CFU/ml) after SWCQ071205 strain challenge via foot pad injection from various organs in chickens. Data points represent mean value from three survives. Error bars are \pm standard deviation.

Table 2: WBC counts and biochemical parameters in chickens experimentally infected with *Riemerella anatipestifer* strain SWCQ071205

Parameters	Days after challenge						control
	1	2	3	4	5	6	
WBC ($\times 10^9/L$)	27.2 \pm 0.8	27.1 \pm 4.0	32.7 \pm 4.5	60.2 \pm 5.68*	43.0 \pm 2.6*	40.3 \pm 3.5*	26.5 \pm 1.8
AST (U/L)	225 \pm 4	237 \pm 5*	262 \pm 8*	227 \pm 6	224 \pm 7	219 \pm 5	213 \pm 7
ALP (U/L)	3402 \pm 239*	2938 \pm 410*	1827 \pm 34*	1597 \pm 7*	1551 \pm 14*	807 \pm 44*	4711 \pm 628
AMY (U/L)	715 \pm 18	605 \pm 23*	606 \pm 15*	588 \pm 18*	421 \pm 16*	556 \pm 13*	731 \pm 19
LDH (U/L)	698 \pm 20*	782 \pm 22*	854 \pm 13*	1334 \pm 65*	983 \pm 32*	707 \pm 12*	612 \pm 9

*Significantly different from the control ($P < 0.05$).

DISCUSSION

Riemerella anatipestifer (RA) infection is an economically important bacterial infectious disease of domestic ducks, historically referred to as infectious serositis, duck septicemia, and so on. A variety of work was conducted to extrapolate its mechanism of transmission and the infection spectrum is increasingly extended since its first report by Rimer (Glünder and Hinz, 1989). Previous reports of RA in turkeys in United States either have involved close association of the turkey flock with commercial ducks or dealt with isolated case in which no source of the infection was determined (Smith *et al.*, 1987), but experimental turkey did not duplicate the

severity of RA-related disease as observed under field conditions. The organism has been isolated from nasal swabs of clinically normal wild Canada geese (Harry, 1969) and migratory birds (Hubálek, 2004). It was reported that avian metapneumovirus (aMPV) may exacerbate RA pathogenesis (Rubbenstroth *et al.*, 2009). An isolate was pathogenic to 14-day-old duckling, but did not induce disease in 18-day-old chicken of White Leyhorn Hy-line strain (Baba *et al.*, 1987). Although Rosenfeld (1973) reported that RA had been isolated and identified to chickens, typical signs were not demonstrated. There were no related RA infections to chicken elsewhere. We reported here a field outbreak of RA in ShanHuang chickens and systemically

demonstrated by pathogen isolation and identification, as well as experimental infection by artificial inoculation.

As we all known, scatter-dispersed, blending-breed and low-intensification are the major pattern for bird husbandry in developing country. This situation is particular popular in China. Such mode is unfavorable for disease control due to the chances of microorganism transmitting increased by frequently intercourse among different flocks. The example in this study can be used to make a speculation. The owner narrate that the farmer is closely adjacent to a river which constantly habited large number of ducklings and geese. The ShanHuang chickens were permitted to stock along the river now and then. RA had been burst in both ducklings and geese. Therefore, it is not clear whether the chickens were infected by ducklings or geese. Some endeavors in detailed comparison such as serotype and gene variation from all of the species should be carried out. But, a useful suggest can be presented here that some attentions should be paid to the RA infection in chicken for avoiding unnecessary economic damage.

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