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Immunoproteomics selection of cross-protective vaccine candidates from *Riemerella anatipestifer* serotypes 1 and 2

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ABSTRACT

Riemerella anatipestifer (RA) is one of the most important bacterial pathogens of ducks and other avian species worldwide. Current approaches for controlling RA are hindered by the absence of effective vaccines, particularly cross-protective vaccines. In this present study, an immunoproteomics approach was used to identify cross-protective vaccine candidates against RA serotype 1 strain RAf63 and serotype 2 strain RAf153. First, whole-cell and secreted proteins of RAf153 and RAf63 were separated by two-dimensional gel electrophoresis. Then, western blotting of the proteome was performed using duck convalescent serum against RAf153, followed by matrix-assisted laser desorption/ionization time of flight mass spectrometry and peptide mass fingerprinting. Finally, common immunoreactive proteins from RAf153 and RAf63 were identified as cross-protective vaccine candidates, and so these were cloned and expressed recombinantly. The cross-protection abilities of purified recombinant protein vaccines were tested against homologous and heterologous virulent strains in a challenge model that followed vaccination. Six proteins were identified as cross-protective vaccine candidates. Three of these proteins showed reactivity with convalescent sera after prokaryotic expression, and the recombinant outer membrane protein A (OmpA) showed high protective indices against challenges with RAf153 (60%) and RAf63 (50%). In summary, we have developed a high-throughout, accurate, rapid and efficient method for the successful selection of cross-protective vaccine candidates.

1. Introduction

Riemerella anatipestifer (RA) is one of the most important bacterial pathogens of avian species worldwide. RA predominantly infects ducks (Gerlach, 1970) and, less frequently, geese (Pierce and Vorhies, 1973) and turkeys (Helfer and Helmboldt, 1977). 16S rRNA gene sequences have identified RA to be a member of the family *Flavobacteriaceae* in rRNA superfamily V (Subramaniam et al., 1997). The majority of the *Flavobacteriaceae*, including *Flavobacterium columnare* (Decostere et al., 1998), *Flavobacterium psychrophilum* (Madetoj et al., 2002) and *Flavobacterium branchiophilum* (Speare et al., 1995), cause disease in freshwater fish but these species have not been isolated from waterfowl. However, RA is an important pathogen of ducks and infection is associated with a range of diseases, including septicemia and polyserositis, which cause severe symptoms such as depression, lack of coordination and tremors of the head and neck (Turbahn et al., 1997). Infection can result in high economic losses and ducklings of 2–5 week old are typically affected (Sandhu and Rimler, 1997), but susceptibility to infection decreases with age.

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Amongst the 21 RA serotypes described to date (Loh et al., 1992; Pathanasophon et al., 1994), 16 serotypes have been isolated and identified in China, and serotypes 1 and 2 are the most prevalent pathogens responsible for outbreaks across the country (Zhang et al., 2006). RA is usually treated with antibiotics, including ceftiofur, cephalothin, chloramphenicol, flumequine and kanamycin (Chang et al., 2003); however, the excessive and inappropriate use of antibiotics may result in the selection of resistant bacterial strains and undesirable antibiotic residues in duck-related products.

Vaccines based on inactivated and live bacteria provide some protection against infection with homologous strains or serotypes, but no significant cross-protection is conferred against heterologous strains (Pathanasophon et al., 1996; Sandhu, 1979, 1991). Thus, subunit vaccines, particularly those that provide cross-protection against different serotypes, may aid the control of this disease, though little progress has been made to this end. In this present study, a new method was used to identify cross-protective vaccine candidates against RA serotypes 1 and 2. We hypothesized that cross-protective antibodies should exist in the convalescent sera of ducks challenged with heterogenous strains. Briefly, whole-cell and secreted proteins from two virulent RA strains, serotype 1 RAf63 and serotype 2 RAf153, were identified using an immunoproteomics approach as described previously (Zhai et al., 2012). Then, common immunoreactive proteins from RAf153 and RAf63 were cloned, expressed and detected again in convalescent duck serum. Finally, purified recombinant proteins were used for to investigate cross-protection in an *in vivo* challenge model. Using this approach, it was possible to establish a library of cross-protective antigens.

2. Materials and methods

2.1. Bacterial strains and culture conditions

RA strains RAf63 and RAf153 were isolated from two septicemic domestic ducks in Fujian Province (China) (Table 1). All strains were cultured in tryptic soy broth (TSB; Merck & Co., Inc.), and cultures were incubated overnight at 37 °C in a rotary shaker until late exponential phase of growth.

2.2. Sera preparation

RAf63 and RAf153 whole cell enzyme-linked immunosorbent assays (ELISA) were used to detect RA infection.

Table 1
RA China isolates used in this research.

Strain	Serotype	Isolation area
RAf63	1	Fujian
RAf153	2	Fujian
RAf167	6	Fujian
RAf10	10	Fujian
RAf74	11	Fujian
RAf43	13	Fujian
RAf27	14	Fujian
RAf17	17	Fujian

Five ducks with no history of RA infections were challenged on day 10 by subcutaneous injection in the abdomen of 1 ml (5×10^7 CFU/ml) of a RAf153 suspension. Classic clinical signs, such as depression and lack of coordination, were observed 1–3 d after injection and these lasted usually for 4–5 d. Eating disorders were a significant obstacle during this period, and death or recovery was the two possible outcomes. Surviving ducks with classic symptoms of RA infection were challenged with a second dose of RAf153 (0.5 ml; 5×10^7 CFU/ml). Serum was collected from each surviving duck. This experiment lasted for approximately 20 d. Titer of each convalescent serum sample was evaluated using the RAf153 whole cell ELISA. Convalescent serum with the greatest titer was used in the following experimentation.

Rabbit hyperimmune sera were also prepared against RAf63 (serotype 1), RAf153 (serotype 2), RAf167 (serotype 6), RAf10 (serotype 10), RAf74 (serotype 11), RAf43 (serotype 13), RAf27 (serotype 14) and RAf17 (serotype 17). Two rabbits, determined to be negative for RA antibodies using whole cell ELISA, were immunized with each formaldehyde-inactivated RA strain, using Montanide ISA 206 (SEPPIC Co. Ltd.) as the adjuvant. Two doses of 1.0×10^9 cells/rabbit were administered by subcutaneous injection into the back 3 weeks apart. Sera from control and immunized rabbits were collected before the first and after the second immunizations. Serum titers were evaluated using whole cell ELISA and serum with the greatest titer was used for subsequent western blot analysis. All experimentation was conducted following permission from the Ministry of Science and Technology of Jiangsu Province (China).

2.3. Immunoproteomics of RAf153 and RAf63

The immunoproteomics of RAf153 and RAf63 was performed according to a previously described protocol (Zhai et al., 2012), which included protein preparation, isoelectric focusing (IEF), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, image processing, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), database searches and bioinformatic analyses.

Both the whole cell and secreted proteomes were examined. Whole cell proteins were extracted from cells harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C, and the supernatant was used for the extraction of secreted proteins.

2.4. Conservation analysis of recombinant proteins expressed in Escherichia coli BL21 (DE3)

Polymerase chain reaction (PCR) primers were designed to contain specific restriction sites for directional cloning (Table 4). The open reading frames (ORFs) of six cross-immunoreactive proteins were amplified using purified genomic DNA as the template. PCRs were carried out in 100 μl volumes containing 10 μl of 10× reaction buffer, 8 μl dNTPs (10 mM), 4 μl of each primer (1 pM/μl), 4 μl DNA template and 4 U *Taq* polymerase. Amplifications