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OMPA GENE FOR AN OUTER MEMBRANE PROTEIN OF *RIEMERELLA ANATIPESTIFER* AND METHODS OF USE

BACKGROUND OF THE INVENTION

1. Technical Field

This invention relates to a gene present in *Riemerella anatipester*, purified protein encoded by this gene, and vaccine and diagnostic methods and products using the gene and protein. Specifically, this gene (*Omp*A) encodes a major outer membrane protein which is highly antigenic and therefore useful in the preparation of vaccines and serodetective diagnostic assays.

2. Description of the Background Art

Riemerella anatipestifer is a Gram-negative, non-motile, non-sporulating rod-shaped bacterium. It belongs to the family *Flavobacteriaceae* in rRNA superfamily V based on 16S rRNA gene sequence analyses. It is the etiological agent of septicemia anserum exsudativa, an enzootic, contagious, septicemic disease of domesticated ducklings and other birds. The disease causes a serious problem in the duck industry and has a worldwide distribution. Endemic infections usually are restricted to commercial duck and turkey flocks but

other poultry species such as chickens and geese are also susceptible to the infection, including wild fowl such as swans. In Singapore and other countries of South East Asia, *R. anatipestifer* infection has been a continued problem for the production of meat ducks using modern intensive production methods since 1982. Mortality and morbidity rates are usually between 10-30%, but mortality as high as 75% has been recorded in infected duck farms.

Slide and tube agglutination tests differentiate 21 separate serotypes of *R. anatipestifer*. Serotypes 1, 2, 3, 5 and 15 are most prevalent in outbreaks of septicemia anserum exsudativa. The occurrence of more than one *R. anatipestifer* serotype in infected ducks at any one time, and changes in serotypes from year to year within a single farm, make vaccination with antigen from a single serotype largely ineffective. Bacterial antigen vaccines have been shown to confer some protection against infection with homologous strains or serotypes but only very poor protection has been observed when heterologous strains were used for challenge. Strong variations of virulence as assessed by mortality and morbidity rates in outbreaks have been reported for the different serotypes of *R. anatipestifer*. In addition, differences in virulence were also observed within a given serotype. However, the molecular bases for these differences are unknown, because as yet, no virulence factors of *R. anatipestifer* have been found. Thus far, fibrinolytic enzymes, hemolysins and a lipopolysaccharide have been

postulated as virulence factors for *R. anatipestifer*, but the presence of these factors has not been established.

In addition, in view of the limited knowledge about the immunogenic factors of *R. anatipestifer*, production of efficient vaccines and diagnostics have been hampered. Knowledge of the predominant immunogenic components of an infectious agent is important for the analysis of the molecular mechanisms of virulence, the study of the route of infection, the serological diagnosis of the disease and the development of strategies for effective immune protection and eradication of the disease.

Outer membrane proteins of pathogenic bacteria are generally very immunogenic; they play an important role in virulence and immunity of bacterial diseases. The outer membranes of Gram-negative bacteria contain a limited number of major outer membrane proteins, usually present in very high copy numbers. Of these, outer membrane protein A (*Omp*A) is necessary for maintenance of structural integrity of cell envelopes. It is also involved in bacterial conjugation and attachment, colicin uptake, porin activity and as receptors for certain bacteriophages. *Omp*A is also known to stimulate a strong antibody response and may play an important role in virulence, since *Omp*A-deficient mutant *E. coli* K-1 demonstrate reduced virulence in an infant rat model of bacteremia. Antibodies against *Omp*A and several *Omp*A family

proteins generally are bactericidal, opsonic or protective.

The *Omp*A proteins of different bacterial genera show a high degree of homology, suggesting an important role in the cell, perhaps related to virulence. *Omp*A-like proteins are present in a wide variety of Gram-negative bacteria (Beher et al., J. Bacteriol. 143:906-913, 1980) and have been well characterized in *E. coli*. In *E. coli*, the N-terminus consists of a long, relatively hydrophobic sequence which spans the outer membrane eight times. The C-terminus, located in the periplasmic space, is very hydrophilic and appears to contain the immunodominant epitopes. Antibodies to *Omp*A are produced during enteric bacterial infection, and are reported to cross-react among some bacterial species. Although *Omp*A proteins have been described in members of the family *Enterobacteriaceae*, and some Gram-positive species (e.g. *Bacillus subtilis*), *Omp*A proteins have not been described in the scientific literature either for species of *R. anatipestifer* or in any other related species of *Flavobacteriaceae*.

SUMMARY OF THE INVENTION

The present invention relates to the cloning and isolation of the gene encoding *R.anatipestifer OmpA*. The invention further relates to the *OmpA* protein substantially free of other *R.anatipestifer* proteins. The invention further relates to a nucleic acid comprising nucleotide bases 82-1242 of SEQ ID NO:17 and a polypeptide having the sequence of SEQ ID NO:18.

In other embodiments, the invention relates to vectors comprising The *R.anatipestifer OmpA* gene, host cells transformed with such vectors, methods of producing the *R.anatipestifer OmpA* protein and immunogenic fragments thereof, antibodies to such peptides, vaccine compositions using such peptides or DNA and immunodiagnostic and vaccination methods using the peptides and antibodies directed against them.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides the structure of the 2.2kb insert in plasmid pJffRa*Omp*A15 produced in Example 1.

Figure 2 depicts an immunoblot of total cell lysate of *R. anatipestifer* strain CVL110/89, serotype 15 (lane 1) and purified recombinant 6xHis-*Omp*A-10xHis protein (lane 2) reacted with monospecific polyclonal anti-*Omp*A antiserum.

Figure 3 depicts an immunoblot as in Figure 2, but reacted with a serum from convalescent ducks that had been experimentally infected with *R. anatipestifer* serotype 15.

Figure 4 depicts an autoradiograph of the same blot as in Figures 2 and 3, reacted with $^{45}Ca^{++}$.

Figure 5 is an immunoblot of whole cell lysates of several different serotypes of *R. anatipestifer* reacted with anti-6xHis-*Omp*A polyclonal antiserum.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The gene *Omp*A, encoding a major antigenic outer membrane protein of *Riemerella anatipestifer*, has been discovered, cloned and analyzed. (See Table 3 for sequence information.) The Genbank DNA sequence accession number is AF104936. The protein encoded by *R. anatipestifer Omp*A is a predominant, specific antigen of the species. The *R. anatipestifer Omp*A gene and protein are useful in the preparation of vaccines for the prevention and amelioration of the septicemic disease of birds caused by *R. anatipestifer*, and *R. anatipestifer* serodiagnostics.

The types strains, serotype reference strains and field isolates of *R. anatipestifer* used to isolate and clone the *Omp*A gene are listed in Table 1, below. Any suitable strain of *R. anatipestifer* may be used to isolate the *Omp*A gene. All of the strains were grown on Columbia agar plates at 37°C in air enriched to 5% CO_2 for 24 hours. For gene cloning and expression, a variety of bacterial, mammalian, plant and insect host cells may be used. The following *E. coli* strains were used in the cloning and expression described herein: XL1-Blue (*E. coli* K-12, *recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 lac[F' *pro AB lacI*^{q}ZΔM15 Tn*10* (Tet^r)]) (Stratagene, La Jolla, CA), XL1-Blue MRF' (*E. coli* K-12,Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *supE*44 *thi*-1, *recA*1, *gyrA*96, *relA*1, lac [F' *proAB*, *lacI*^{q}ZΔM15, Tn*10* (Tet^r)]) (Stratagene) and XLOLR (*E. coli* K-12, Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *thi*-1, *recA*1, *gyrA*96, *relA*1, *lac* [F' *proAB*, *lacI*^{q}ZΔM15, Tn*10*

(Tet^r)]λ^R, Su$^-$) (Stratagene), BL21 (DE3) (*E. coli* B F$^-$ *dcm omp T hsdS*(r_B^- m_B^-) *gal* λ(DE3 T7*pol*)) (Stratagene). *E. coli* strains were grown in Luria Broth. Those of skill in the art are well acquainted with other appropriate and useful methods for growing the cells, cloning and expression.

Table 1. *R. anatipestifer* strains

Strain[a]	Serotype
ATCC11845[T]	ND[b]
HPRS1795[R]	1
HPRS2527[R]	2
HPRS2554[R]	3
HPRS2550[R]	5
CVL389/89[R]	6
DRL27179[R]	7
HPRS1785[R]	9
CCUG25055-890822[R]	11
DRL28020[R]	11
CCUG25012-890804[R]	13
CVL664/83[R]	14
CVL743/85[R]	15
DRLS-4801[R]	16
CVL977/83[R]	17
CVL540/86[R]	18
CVL30/90[R]	19
CVL110/89[C]	15

[a]ATCC, American Type Culture Collection; HPRS, Houghton Poultry Research Station, England; CVL, Central Veterinary Laboratory, Singapore; DRL, Duch Research Laboratory, New York, USA; CCUG, Culture Collection, University of Göteborg, Sweden.
[T]Type Strain
[C]Field Strain
[R]Serotype Reference Strain
[b]Not Determined

Numerous expression and cloning vectors are known to those skilled int he art and may be used for claiming and expressing the *R.anatipestifer OmpA* gene of this invention. Plasmid vectors used for cloning and expression described herein were pBluescriptIISK$^-$ (Stratagene) and pBK-CMV (Stratagene). For expression of poly-histidine tailed proteins, plasmid pETHIS-1 (a

ColE1 derived high-copy number expression vector containing the *bla* ampicillin resistance gene) was used for selection. A specific promoter sequence was used for the T7-polymerase dependent expression of cloned genes (see Table 2). This promoter allows the expression of fusion proteins with an N-terminal histidine hexamer, a C-terminal histidine decamer or both (R. Segers and J. Frey, GenBank/EMBL accession number AF012911).

Cloning and expression vectors containing the *A.anatipestifer OmpA* gene advantageously contain selectable markers, as is well known in the art. In the work described herein, selection of transformants and maintenance of plasmids, was accomplished by applicants the medium with 100 μg/ml ampicillin for pBluescriptIISK$^-$ and pETHIS-1 or with 50 μg/ml kanamycin for cloning vector pBK-CMV. Induction of cloned genes on expression vector pETHIS-1 in strain BL21 (DE3) was made by the addition of 0.3 mM (final concentration) isopropyl-ß-D-thiogalactopyranoside (IPTG) at mid-exponential growth phase and incubation of the cultures for a further 3 hours.

The oligonucleotide primers and their annealing temperatures used in this study are listed in Table 2 and in the accompanying sequence listing. Primers pETHIS1-3' and T7 match the segments flanking the codons of the multiple cloning sited in vector pETHIS-1 and were used for verifying the correct fusions of the genes cloned in pETHIS-1. The PCR were carried out in

a DNA thermal cycler (GeneAmp 9600; Perkin Elmer Cetus) in 50 µl reaction mix (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 170 µM of each deoxynucleotide triphosphate, 20 pmol of each primer, 5 ng plasmid DNA or 200 ng genomic DNA and 1.5 U *Taq* polymerase (Boehringer Mannheim)). The PCR thermal parameters used were 35 cycles of amplification with 30 seconds at 94°C, 30 seconds at the corresponding annealing temperature (Table 2) and 1 minute at 72°C.

When DNA fragments were produced by PCR for subsequent cloning and expression, or for DNA sequence analysis, the elongation steps were increased to 2 min at 72°C, and 2.5 U of *Taq/Pfu* polymerase mix (Boehringer Mannheim) was used instead of *Taq* polymerase. In addition, an extension step of 7 min at 72°C was added at the end of the last cycle in order to ensure full length synthesis of the different fragments. Digoxigenin(Dig)-labeled DNA probes were made by supplementing the PCR reaction mixture with a final concentration of 50 µM Digoxigenin-11-dUTP (Boehringer Mannheim).

Table 2. Oligonucleotides Used in Polymerase Chain Reactions.

Primer	Sequence	SEQ ID NO	T_m
RA6OMPA-L	GCAACAGGAGCATTACAAGGTA	1	50°C
RA6OMPA-R	CTGTCTTTCATTCTCTCTTTC	2	50°C
RAOMPAH1-	CGCTATGGATCCTTATTTTCTTTTCTT	3	48°C
RAOMPAH1	TACGGATCCTTTTCTTTTCTTTTTTACTA	4	48°C
RAOMPAH1-	CGCAGCCATATGGGTTAAAGAATTT	5	48°C
RAOMPAF1	GACTGGCAAACTTCAGTAGG	6	55°C
RAOMPAF2	TGGTCTTGGTATCCAAGGGG	7	55°C
RAOMPAF3	AATAACGGTTGCCCTTGGCC	8	55°C
RAOMPAF4	GCTGCTTTAGAAGCTAGAGG	9	55°C
RAOMPAR1	CAATGAAGCTGACGCTTGCC	10	55°C
RAOMPAR2	GCCCAGGAACTGTAGGACAC	11	55°C
RAOMPAR3	GTAGCTTCAGCAGAACCAAC	12	55°C
RAOMPAR4	CAACGAGCCATGCTTAGAGGC	13	55°C
PETHIS1-	CGTCTTCAAGCTCATGTTTG	14	55°C
T7	TAATACGACTCACTATAGGG	15	55°C
T3B-S	GCGCGCAATTAACCCTCACTAAAG	16	73°C

Underlined letters specify nucleotides that were added to the ompA sequence to create restriction enzyme recognition sites for cloning. T_m = annealing temperature.

DNA sequence analysis was done using an AmpliTaq FS dye terminator kit (Perkin-Elmer Cetus) in reactions containing approximately 500 ng plasmid DNA and 5 pmol of oligonucleotide primer. The ends of cloned DNA fragments in vectors pBKCMV pETHIS-1 and pBluescriptIISK$^-$ were sequenced with primers T3B-S, T7, PETHIS1-3' (Table 2) matching the sequences flanking the vectors' multiple cloning sites (MCS). The complete nucleotide sequences of cloned fragments were determined by primer walking. Sequences were assembled and edited by using the Sequencher 3.0 program

(GeneCodes, Ann Arbor, Ml) to obtain contiguous sequences. Comparison of the nucleotide sequences in searches for related sequences was performed using the NCBI BLASTN and BLASTX programs. The DNA and amino acid sequences were analyzed using the PCGENE programs PROSITE and PSORT and the GCG programs. The *Omp*A gene was cloned and expressed in *E. coli*. See Example 1.

A phage library was established in *E. coli* from genomic DNA *Hin*dIII fragments extracted from *R. Anatipestifer* serotype 15 strain CVL110/89. The library was screened with a Digoxigenin-labeled probe derived from the sequence of *Omp*A and anti-Digoxigenin antibodies, producing a strongly reactive clone containing the *Omp*A gene. The gene was amplified by PCR and cloned into an expression vector which produced the coding frame of *Omp*A fused to 6 histidine codons at the 5' terminus and a second vector having 6 histidine codons at the 5' terminus and 10 histidine codons at the 3' terminus. The plasmids were transformed into *E.*

Table 3. Sequences of a 2.2kb Fragment of Plasmid pJFFRa*Omp*A15 Containing the *Omp*A Gene of *Riemerella anatipestifer* and its Gene Product.

acagttgcta gaaacttgaa caaggcgtta gttcttgact ggcaaacttc agtaggtaat
attgataata agagaattgg aatgggtaaa gaatttatgt tgatgactgg acttggtctt
cagcttaaat ttgcaggtct tctttttggc aacgaagatg catggtttga cccttatgta
agagttggag ccaactattt gagacacgac tatacaggtc ttacgttccc tgtgactgat
agctacaatg atgtaactta cgcggggtat agcgaaaata aaccatacac tcaaggaaga
gcggatcatt ttgctttatc aacaggttta ggtacaaaca tttggttaac taagaacttt
ggtcttggta tccaagggga ttatgtttct actccagtag ataaatctag attggctaac
ttttggcaag cgtcagcttc attgaacttt agatttggta acagagataa ggataaggat
ggagtgttag ataaagacga tttatgttca gaaacaccag gtttacctga attccaaggt
tgtccagata cagatggtga cggtgttcca gataaagatg ataactgtcc agaagtagca
ggaccagtag aaaacaatgg ttgcccttgg ccagatacag acaaagatgg tgtattggat
aaagacgatg cttgtgttga tgtagcagga ccagctgaaa ataacggttg cccttggcca
gatacggata atgatggtgt gttagataaa gatgataagt gtcctacagt tcctgggctt
ccacagtacg atggatgtcc taagccacag tctgcatttg cagctgaagc aacaggagca
ttacaaggta tattcttcaa ctttaataag gcgtctatca gatctgaatc taatactaag
ttagatcaag ctgctgaggt aattaagtct tctaacggag gtactttctt agtggtaggt
catacggatg ttaagggtaa tgctaactac aacttgaaac tttctagaga aagagctgca
tctgtagtag ctgctttaga agctagagga gttaatccat ctcagttaaa atctaaaggg
gttggttctg ctgaagctac agtaccagcg tctgcttcta acgaagagag aatgaaagac
agaaaagtgg ttgtagaagc aatcagcgga tctgcttggg aagctcttca aaagtctgac
cttccagtag tgaagaaaaa agtagtaaaa aagaaaagaa aataattagt attttctaat
cttaaaaata aacgccctct tttgaaaagg gcgtttttt attgtattaa aattagtatt
tttgcacatc taaatcatat tataattatg ggacgtgcgt ttgaatatag aaaagcctct
aagatggctc gttgggataa aatggcaaaa acttttttcta aaataggaaa agatattgcg
ttagcagtaa aagctggcgg tccagatcca gactctaatc cagcgttgag aagatgtata
caaaatgcta aaggggctaa tatgcctaaa gataatgtag aaagagccat taaaaaggca
agtggtgcag atgctgagaa ctatgaggag attacttacg aaggatatgg acaaggaggt
gttgcatttt ttgtagaatg tactactaat aactcaacta gaactgtggc taatgtaaga
gctatcttta ataaatttga cggtaacctt gggaagaatg gagagctttc tttcttattc
gatagaaaag ggatatttac tttagaaaaa tctttgataa acatggattg ggaagagttt
gagatggaaa tgatagacgg aggtgcggaa gatatagact ctgatgaaac agaagttatg
gtaactacgg cgtttgagga ttttgggtct ttatcacata agttagacga gctggggata
gaggttaaga atgcagaact gcaaaggata cctaatatta gtaaatctgt atcagaagag
caatttattg cgaatatgaa aatgttacaa aggtttgagg aagatgatga tgtacagaat
gtatatcata acatggaaat tacagacgag ctaatgaaga aactataaaa tagaaaaaag
gctacttaga ataggtagcc ttttttattt tttgtttacg aaaggagtaa gccattgaga
taaacttgat aatcaatgcc gacattgggt tctaaagttt tggataccga acaatatttt
tcaaaagaaa gttgagcagc cttcaaagct t (SEQ ID NO:17)

MGKEFMLMTGLGLQLKFAGLLFGNEDAWFDPYVRVGANYLRHDYTGLTFPVTDSYNDVTYAGYSEN
KPYTQGRADHFALSTGLGTNIWLTKNFGLGIQGDYVSTPVDKSRLANFWQASASLNFRFGNRDKDK
DGVLDKDDLCSETPGLPEFQGCPDTDGDGVPDKDDNCPEVAGPVENNGCPWPDTDKDGVLDKDDAC
VDVAGPAENNGCPWPDTDNDGVLDKDDKCPTVPGLPQYDGCPKPQSAFAAEATGALQGIFFNFNKA
SIRSESNTKLDQAAEVIKSSNGGTFLVVGHTDVKGNANYNLKLSRERAASVVAALEARGVNPSQLK
SKGVGSAEATVPASASNEERMKDRKVVVEAISGSAWEALQKSDLPVVKKKVVKKKRK (SEQ ID
NO:18)

coli cells, and the *Omp*A gene constructs were expressed by IPTG induction. The sequence is provided in Table 3.

Recombinant *Omp*A displayed a molecular mass similar to that predicted from the nucleotide sequence of the *Omp*A gene, but smaller than that observed in total cell lysates of *R. anatipestifer*. The sequence data obtained from the insert of plasmid pJFFRa*Omp*A15 revealed an open reading frame (ORF) of 1163bp encoding the 387 amino acid *Omp*A protein with a deduced molecular mass of 41,696 Daltons and a calculated pI of 4.91. It is preceded by a 6 nucleotide consensus sequence for a ribosome binding site (RBS) upstream from the ATG methionine start codon. Upstream of the RBS, there is a canonical promoter sequence with a -10 box (TAATAT, SEQ ID NO:19) and a -35 box (TTGACT, SEQ ID NO:20) optimally spaced by 16 nucleotides. This spacing is characteristic of promoters recognized by *E. coli* C532 RNA polymerases. The short segment further upstream did not reveal any homology to known nucleotide or amino acid sequences.

An inverted repeat structure is located just downstream of the open reading frame of *Omp*A. This structure represents a potential transcription stop signal. See Figure 1. The promoter sequence upstream of *OmpA* is indicated by a filled triangle, and the inverted repeat structure is indicated by a hairpin. Immediately downstream to *OmpA*, there is a partial open reading frame, ORFX, showing similarity to the 17 kDa

Bacillus subtilis spore coat protein. This region has the following sequence:

MAKTFSKIGKDIALAVKAGGPDPDSNPALRRCIQNAKGANMPKDN
VERAIKKASGADAENYEEITYEGYGQGGVAFFVECTTNNSTRTVA
NVRAIFNKFDGNLGKNGELSFLFDRKGIFTLEKSLINMDWEEFEM
EMIDGGAEDIDSDETEVMVTTAFEDFGSLSHKLDELGIEVKNAEL
QRIPNISKSVSEEQFIANMKMLQRFEEDDDVQNVYHNMEITDELM
KKL (SEQ ID NO:21).

Analysis of the *Ompa* amino acid sequence (SEQ ID NO:18) deduced from the *OmpA* gene sequence of both strain CVL110/89 and of strain ATCC11845 revealed characteristic features of *OmpA* proteins found in other Gram-negative bacteria. The C-terminus encompasses a characteristic 45 amino acid *Ompa*-like domain. The C-terminus of the protein, in particular amino acid residues 125-229, is predominantly hydrophilic. The N-terminal region (amino acids 4-22) is highly hydrophobic and shows no similarity to other outer membrane proteins. These features also are common to *Ompa* proteins, which generally are heterogeneous at their N-terminus.

Amino acid residues 5-22 form an inside to outside transmembrane helix locating the N-terminus of *Ompa* on the inside of the cell. The absence of alanine-proline or proline rich regions in *R. anatipestifer Ompa* is remarkable since such domains generally are found in the outer membrane proteins of other bacterial species at the junction of the periplasmic domains and the transmembrane domains.

The sequence also revealed the presence of six EF-hand calcium-binding domains between amino acids 129 and 141 and two PEST regions (amino acids 139-164 and amino acids 166-187). The presence of calcium-binding domains and PEST regions and the absence of proline-rich regions suggest that *Omp*A may have additional roles in *R. anatipestifer* which are different from those commonly associated to outer membrane proteins in other species. It is known that calcium-binding proteins play a central role in intracellular signal transduction pathways and are associated with a wide-range of effects on disease production. The finding of six EF-hand calcium-binding domains in *R. anatipestifer Omp*A is notable since other *Omp*A proteins do not contain such domains.

Adjacent to the calcium-binding domains are two PEST regions. These peptide sequences are enriched in proline, glutamic acid, serine and threonine residues and form motifs that target proteins for destruction through a yet unknown mechanism. PEST sequences are found in key metabolic enzymes, transcription factors, protein kinases, protein phosphatases and cyclins and are also abundant among proteins that give rise to immunogenic peptides presented on MHC I molecules. Because PEST sequences are hydrophilic, it is likely they are solvent-exposed. While PEST sequences are often present as carboxy-terminal extensions of proteins, they are located in the middle of the *R. anatipestifer Omp*A. The presence of two PEST regions adjacent to the EF-hand calcium-binding domains is an

indication that *Omp*A may be a preferred calpain substrate.

In the lower part of Figure 1, the different domains of *Omp*A are diagrammed. The inside to outside transmembrane helix spanning domain is represented by a checkered box. The six EF-hand calcium-binding domains are shown by a vertically hatched box. The two gray boxes represent the PEST regions (amino acids 139-164 and 166-187) and the black box indicates the *Omp*A-like domain in the C-terminal half of the protein.

The *Omp*A proteins of serotype 15 strain CVL110/89 and type strain ATCC11845 only differ from each other in 7 amino acids which lie outside of these characteristic structures and are clustered between amino acid positions 228 and 255. Comparison of the deduced amino acid sequence of the 42 kDa *R. anatipestifer Omp*A gene with sequences in the Swiss Prot Databank using the NCBI BLASTX computer program revealed a high percentage of amino acid identity with the known sequences of membrane proteins in certain other Gram-negative bacteria, including the porin protein *Opr*F of *Pseudomonas aeruginosa*. See Table 4. The *Omp*A-like domains had 33-37% identical amino acids and 47-59% identity including conservative substitutions. *R. anatipestifer Omp*A showed highest similarity to *Bordetella avium Omp*A, the etiological agent of turkey bordetellosis, a highly contagious upper respiratory disease of turkeys, characterized by signs and symptoms similar to those caused by *R.*

anatipestifer in ducks and other birds. The N-terminal half of *R. anatipestifer OmpA* showed no similarity to other proteins in the Swiss Prot and GenBank/EMBL1 databases.

PCR analysis using the primers RAOMPAH1-L and RAOMPAH1A-R (see Table 2) showed the presence of 1177 bp amplificates in all *R. anatipestifer* type- and

Table 4. Percent Amino Acid Homology (Identity) of *R. anatipestifer OmpA* with Other Known Bacterial Proteins.

Bacterial Protein	Percent Homology
Bordetella avium OmpA (accession no. Q05146)	38%
Serafia marcescens OmpA (accession no. P04845)	28%
Enterobacter aerogenes OmpA (accession no. P09146)	28%
Pseudomonas aeruginosa OprF (accession no. P13794)	29%

serotype reference strains analyzed. PCR analysis of genomic DNA of the *R. anatipestifer* type strain and serotype reference strains together with restriction analysis of the amplified DNA segments shows that the *ompA* gene is common to *R. anatipestifer*. However, restriction fragment length polymorphism (RFLP) analysis of the PCR products with the frequently cutting enzyme *AluI*, indicated some heterogeneities in the *OmpA* gene. Three different groups with similar enzyme profiles were noted (see Table 5). Although the gene shows a certain degree of variation among the different serotypes, these minor intra-species variations are predominantly silent mutations not affecting the phenotype. Variable regions are found at

the 3'-end of *ompA* corresponding to the domain where nucleotide differences were detected between the *OmpA* sequences of strain CVL110/89 and ATCC11845. Generally, the highest divergence among *OmpA* genes of different bacterial species is found in this region as well.

Table 5. *AluI* Restriction Enzyme Groups of *R. anatipestifer* Serotypes.

Group	*R. anatipestifer* serotype or strain
1	ATCC11845 serotypes 1, 2, 3, 5, 7, 17
2	serotypes 6, 13, 14, 16, 19
3	serotypes 9, 11, 15, 18

Immunoreactions of total cell lysates of *R. anatipestifer* serotype 15 strain CVL110/89 and of purified recombinant 6xHis-OmpA-10xHis were studied on immunoblots using monospecific polyclonal anti-6xHis-*OmpA* hyperimmune serum, and convalescent serum from ducks experimentally infected with a *R. anatipestifer* serotype 15 strain. See Figures 2-4.

Proteins were separated on 10% SDS-PAGE after first boiling 10 minutes in an equal volume of SDS sample buffer (62.2 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue), and then transferred to nitrocellulose membranes. The membranes were soaked in

calcium-binding buffer (60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole hydrochloride, pH 7.2) for 10 min. Subsequently the membranes were incubated in calcium-binding buffer supplemented with 1.0 μCi of $^{45}Ca^{++}$ per ml (0.02 mCi of $^{45}CaCl_2$ per μg; Amersham Corp.) for 20 min, then rinsed twice with deionized water for 5 min and dried at room temperature. Bound ^{45}Ca was visualized by autoradiography.

Monospecific polyclonal antisera to *Omp*A protein were produced in mice and ducklings. Mice were immunized with 330 μg purified recombinant polyhistidine-tailed fusion protein (6xHis-OmpA) protein mixed 1:1 with complete Freund's adjuvant (Difco Laboratories, Detroit, MI) in a total volume of 200 μl. A booster immunization was given two weeks later containing 330 μg purified recombinant protein in incomplete Freund's adjuvant. Serum was collected from the mice seven days after the second immunization.

Convalescent duck serum was produced as follows. Four ducklings aged eight days were immunized subcutaneously with 1 ml of killed antigen preparation. Killed antigen was prepared by heating *R. anatipestifer* serotype 15 strain CVL110/89 bacteria (105 cfu/ml) at 100°C for 1 hour. A second immunization with an equal volume of the antigen preparation was given 11 days later. Serum was collected from the ducklings 10 days after the second immunization and pooled. These sera were used at a dilution of 1:2000 to detect antigen on SDS-polyacrylamide gels (SDS-PAGE)

and immunoblot assays according to known methods. Visualization of the bound antibodies was performed using phosphatase-labeled goat anti-mouse antibodies (IgG/IgM; KPL #0751806) or goat anti-duck antibodies (IgG/IgM; KPL #052506 obtained from Kirkegaard and Perry Inc., Gaithersburg, Md. USA).

Immunoblots of total cell lysate of *R. anatipestifer* serotype 15 strain CVL110/89 revealed three bands at 55 kDa, 53 kDa and 51 kDa which reacted with serum directed against 6xHis-*Omp*A (Figure 2, lane 1). Immunoblots of recombinant 6xHis-OmpA-10xHis protein revealed three bands of lower molecular mass (46 kDa, 44 kDa and 42 kDa) when reacted with the same serum (Figure 2, lane 2). St indicates molecular weight standards. Molecular masses are given in kDa.

Immunoblots of total *R. anatipestifer* cell lysate which was reacted with the convalescent duck serum revealed the same *Omp*A triplet at 55 kDa, 53 kDa and 51 kDa, plus additional immunoreactive proteins (Figure 3, lane 1). This pooled duck convalescent serum also reacted with recombinant 6xHis-OmpA-10xHis protein, showing the characteristic triplet at 46 kDa, 44 kDa and 42 kDa (Figure 3, lane 2), as did the polyclonal mouse anti-*Omp*A antibodies. Sera from ducks that had been immunized with total cell antigen from *R. anatipestifer* strain CVL110/89 gave the same results on immunoblots as convalescent serum (data not shown).

In immunoblots of total cell lysates of the type strain and different serotype reference strains of *R. anatipestifer*, all reacted with recombinant 6xHis-*Omp*A

polyclonal serum, showing the characteristic triplet bands at 55 kDa, 53 kDa and 51 kDa as was found for *R. anatipestifer* serotype 15 strain CVL110/89 (Figure 4). In Figure 4, T indicates *R. anatipestifer* type strain ATCC11845, C indicates purified recombinant 6xHis-*Omp*A010xHis protein used as a control. The numbers indicate the serotype used. For serotype 11, [a] indicates strain CCUG25055-890822 and [b] indicates strain DRL28020. St indicates a prestained molecular weight protein marker as in all Figures.

The presence of three distinct bands of 55 kDa, 53 kDa and 50 kDa may be due to the protein's being detected at different stages of processing as was reported for *Omp*A in *E. coli* and *B. avium* (16) where multiple bands were identified. They have been interpreted to represent *Omp*A precursor which contains the signal peptide and is located in the cytoplasm or is associated with the cytoplasmic membrane (pro-*Omp*A), immature processed *Omp*A without the signal peptide found in the periplasm or attached to the inner face of the outer membrane (imp-*Omp*A) and mature *Omp*A. The molecular masses of the protein bands of recombinant *Omp*A were approximately 10 kDa smaller and corresponded better to the calculated molecular mass of *Omp*A than those from endogenous *R. anatipestifer Omp*A. The difference in molecular masses could be due to further posttranslational modifications of *Omp*A in *R. anatipestifer* such as additions of glycosaminoglycan

chains which do not occur when recombinant *Omp*A is expressed in *E. coli*.

Ca^{2+} binding experiments with *R. anatipestifer* total antigen showed a major band of 51-55 kDa corresponding to the *Omp*A triplet at 55 kDa, 53 kDa and 51 kDa and three minor bands of 40 kDa, 32 kDa and 30 kDa which bound $^{45}Ca^{++}$ (Figure 5, lane 1). However, recombinant 6xHis-*Omp*A-10xHis protein bound $^{45}Ca^{++}$, showing a strong band in the range of 42-46 kDa corresponding to the 6xHis-OmpA-10xHis triplet seen in the immunoblot (compare Figure 5, lane 2 and Figure 3, lane 2). The individual bands of the triplet which were seen on immunoblots can not be differentiated in these $^{45}Ca^{++}$ blots due to the lower separation capability of autoradiography.

The *Omp*A protein in *E. coli* is known to contain a transmembrane segment spanning amino acids 1 to 177. In contrast, *R. anatipestifer Omp*A shows no analogous region at the N-terminus, but does contain a short inside to outside transmembrane helix comprising 17 amino acids. The long stretches of hydrophilic residues suggest that a large part of *R. anatipestifer Omp*A is exposed to the surface. This is consistent with and would explain its strong antigenicity.

The structural features of this protein suggest it to be an essential antigen in a formulation of a vaccine against *R. anatipestifer*. The presence of calcium-binding domains and PEST sequences on *R. anatipestifer Omp*A are particularly interesting in this

regard, since calcium-binding proteins are known to be associated with a wide range of effects on disease production, while PEST sequences are abundant among proteins that give rise to immunogenic peptides preserved in MHC I molecules. Moreover, hyperimmune serum directed against recombinant *Omp*A protein reacts with *Omp*A and pre-*Omp*A of all serotypes.

The high immunoreactivity and conservation across different serotypes of this protein make *Omp*A a candidate for the design of specific diagnostic methods and vaccines for *R. anatipestifer* with the advantage of cross-protection against all serotypes of the bacteria using a single antigen. This is in contradistinction to early reports in the prior art indicating that outer membrane protein vaccines are not useful in enteric bacteria since the potentially antigenic proteins are not accessible on the surface of these cells. Clearly the abundance and the surface accessibility to the antigen processing pathway of this new major outer membrane protein are advantageous to its immunogenicity.

Immunization of poultry against *R.anatipestifer* infection can be achieved by administering to birds susceptible to infection with the organism a protein according to the invention in an immunogenic composition as a so-called subunit vaccine. The subunit vaccine according to the invention may comprise the *Omp*A protein isolated from the native bacterial cells, optionally in the presence of a pharmaceutically acceptable carrier.

It will be recognized by those skilled in the art that immunogenic subunits or fragments of the *Omp*A protein may also be used in vaccines as well as diagnostic methods. Similarly, recombinant proteins in which one or more epitopes are combined may be employed.

In some cases the ability to raise protective immunity using these proteins per se may be low. Small fragments are preferably conjugated to carrier molecules in order to raise their immunogenicity. Suitable carriers for this purpose are macromolecules, such as natural polymers (proteins like keyhole limpet hemocyanin, albumin, toxins), synthetic polymers like polyamino acids (polylysine, polyalanine), or micelles of amphiphilic compounds like saponins. Alternatively these fragments may be provided as polymers thereof, preferably linear polymers.

If required, the proteins and fragments according to the invention, which are to be used in a vaccine can be modified *in vitro* or *in vivo*, for example by glycosylation, amidation, carboxylation or phosphorylation.

Additionally the vaccine may also contain an aqueous medium or a water containing suspension, often mixed with other constituents in order to increase the activity and/or the shelf life. These constituents may be salts, pH buffers, stabilizers (such as skimmed milk or casein hydrolysate), emulsifiers, adjuvants to improve the immune response (e.g. oils, muramyl

dipeptide, aluminium hydroxide, saponin, polyanions and amphipatic substances) and preservatives.

An alternative to subunit vaccines is live vaccines. A nucleic acid sequence encoding the *R.anatipestifer Omp*A protein or immunogenic fragment may be introduced by recombinant DNA techniques into a microorganism (e.g. a bacterium or virus) in such a way that the recombinant microorganism is still able to replicate, thereby expressing a polypeptide coded by the inserted nucleic acid sequence and eliciting an immune response in the infected host.

One embodiment of the present invention is a recombinant vector virus comprising a nucleic acid encoding the *R.anatipestifer Omp*A protein or an immunogenic fragment thereof, as described above, capable of expressing the nucleic acid in (a) host cell(s) or host bird infected with the recombinant vector virus. The invention comprises (a) host cell(s) or cell cultures infected with the recombinant vector virus, capable of producing the *R.anatipestifer Omp*A protein by expression of the nucleic acid sequence.

As described in U.S. patent 5,843,722, incorporated herein by reference, the well-known technique of *in vivo* homologous recombination can be used to introduce a nucleic acid according to the invention into the genome of a vector virus.

First, a DNA fragment corresponding with an insertion region of the vector genome, i.e. a region which can be used for the incorporation of a heterologous sequence without disrupting essential

functions of the vector, such as those necessary for infection or replication, is inserted into a cloning vector according to known recombinant DNA techniques. Insertion-regions have been reported for a large number of microorganisms (e.g. EP 80,806, EP 110,385, EP 83,286, EP 314,569, WO 88/02022, WO 88/07088, U.S. Pat. No. 4,769,330 and U.S. Pat. No. 4,722,848).

If desired, a deletion can be introduced into the insertion region present in the recombinant vector molecule obtained from the first step. This can be achieved for example by appropriate exonuclease III digestion or restriction enzyme treatment of the recombinant vector molecule from the first step.

The nucleic acid encoding the *R.anatipestifer OmpA* gene or immunogenic fragment is then inserted into the insertion-region in the recombinant vector of the first step or in place of the DNA deleted from said recombinant vector. The insertion region DNA sequence should be of appropriate length as to allow homologous recombination with the vector genome to occur. Thereafter, suitable cells can be infected with wild-type vector virus or transformed with vector genomic DNA in the presence of the recombinant vector containing the insertion flanked by appropriate vector DNA sequences whereby recombination occurs between the corresponding regions in the recombinant vector and the vector genome. Recombinant vector progeny can now be produced in cell culture and can be selected for example genotypically or phenotypically, e.g. by hybridization, or by detection of expression of a

co-integrated marker gene, or by immunological detection of the antigenic *R.anatipestifer OmpA* polypeptide expressed by the recombinant vector.

Next, this recombinant vector can be administered to poultry for immunization, whereafter it maintains itself for some time, or even replicates in the body of the inoculated animal, expressing *in vivo* the *R.anatipestifer OmpA* protein or immunogenic fragment, resulting in the stimulation of the immune system of the inoculated animal. Suitable vectors for the incorporation of a nucleic acid sequence according to the invention can be derived from viruses such as pox viruses, e.g. vaccinia virus (EP 110,385, EP 83,286, U.S. Pat. No. 4,769,330 and U.S. Pat. No. 4,722 848) or fowl pox virus (WO 88/02022), herpes viruses such as HVT (WO 88/07088) or Marek's Disease virus, adenovirus or influenza virus, or in bacteria such as *E. coli* or specific *Salmonella* species. With recombinant microorganisms of this type, in order to expose the *R.anatipestifer OmpA* protein or immunogenic fragments as a surface antigen, the protein or fragments may be expressed as a fusion with host OMP proteins, or pilus proteins of for example *E. coli* or synthetic provision of signal and anchor sequences which are recognized by the organism.

A vector vaccine according to the invention can be prepared by culturing a recombinant bacterium or a host cell infected with a recombinant vector comprising a nucleic acid sequence according to the invention, whereafter recombinant bacteria or vector containing

cells and/or recombinant vector viruses grown in the cells can be collected, optionally in substantially pure form, and formed into a vaccine optionally in a lyophilised form.

Host cells transformed with a recombinant vector according to the invention can also be cultured under conditions which are favorable for the expression of a nucleic acid sequence encoding the *R.anatipestifer OmpA* protein or immunogenic fragment. Vaccines may be prepared using samples of the crude culture, host cell lysates or host cell extracts, although in another embodiment more purified polypeptides according to the invention are formed into a vaccine, depending on its intended use. In order to purify the polypeptides produced, host cells transformed with a recombinant vector according to the invention are cultured in an adequate volume and the polypeptides produced are isolated from such cells, or from the medium if the protein is excreted. Polypeptides excreted into the medium can be isolated and purified by standard techniques, e.g. salt fractionation, centrifugation, ultrafiltration, chromatography, gel filtration or immunoaffinity chromatography, whereas intracellular polypeptides can be isolated by first collecting said cells, disrupting the cells, for example by sonication or by other mechanically disruptive means such as French press, followed by separation of the polypeptides from the other intracellular components and forming the polypeptides into a vaccine. Cell disruption could also be achieved by chemical (e.g.

EDTA or detergents such as Triton X114) or enzymatic means, such as lysozyme digestion.

Antibodies or antiserum directed against a polypeptide according to the invention may be used in passive immunotherapy, diagnostic immunoassays and generation of anti-idiotypic antibodies.

The *R.anatipestifer OmpA* protein and immunogenic fragments, as described above can be used to produce polyclonal, monospecific and monoclonal antibodies. If polyclonal antibodies are desired, techniques for producing and processing polyclonal sera are known in the art (e.g. Mayer and Walter. eds, Immunochemical Methods in Cell and Molecular Biology, Academic Press, London, 1987). Monospecific antibodies to an immunogen can be affinity purified from polyspecific antisera by a modification of the method of Hall et al. (Nature, 311, 379-387, 1984). Monospecific antibody, as used herein, is defined as a single antibody species or multiple antibody species with homogeneous binding characteristics for the relevant antigen. Homogeneous binding, as used herein, refers to the ability of the antibody species to bind to a specific antigen or epitope.

Monoclonal antibodies, reactive against the *R.anatipestifer OmpA* protein or an immunogenic fragment thereof, can be prepared by immunizing inbred mice by techniques known in the art (Kohler and Milstein, Nature, 256, 495-497, 1975).

Anti-idiotypic antibodies are immunoglobulins which carry an "internal image" of the antigen of the

pathogen against which protection is desired and can be used as an immunogen in a vaccine (Dreesman et al., J. Infect. Disease, 151, 761, 1985). Techniques for raising anti-idiotypic antibodies are known in the art (MacNamara et al., Science, 226, 1325, 1984).

The vaccine according to the invention can be administered in a conventional active immunization scheme: single or repeated administration in a manner compatible with the dosage formulation, and in such amount as will be prophylactically effective, i.e. the amount of immunizing antigen or recombinant microorganism capable of expressing said antigen that will induce immunity in poultry against challenge by virulent *R.anatipestifer*. As used herein, "immunity" means a level of protection against infection that is higher than that of an unvaccinated population of birds.

For live viral vector vaccines the dose rate per bird may range from 10^5 to 10^9 CFU (colony forming units). A typical subunit vaccine according to the invention comprises 0.01 to 1 mg of the immunogenic protein. Such vaccines can be administered intradermally, subcutaneously, intramuscularly, intraperitoneally, intravenously, orally or intranasally.

The novel *R.anatipestifer OmpA* protein and immunogenic fragments, antibodies thereto and nucleic acids encoding such proteins and fragments may be employed in diagnostic methods and kits for detecting the presence of *R.anatipestifer* in biological fluids.

Such diagnostic assays and kits may be of conventional format as immunoassays for antigens and antibodies and hybridization assays, optionally following amplification, for nucleic acids.

Immunoassay techniques are based upon formation of a complex between antigenic substances and an antibody or antibodies. One of the components of the complex may be labeled, permitting complex detection and/or quantitative analysis after separation of the complexed labeled antigen or antibody from an uncomplexed labeled antigen or antibody.

In a competitive immunoassay format, the antigenic substance, e.g., the *R.anatipestifer* protein or immunogenic fragment thereof, in a sample of fluid being tested for its presence competes with a known amount of labeled antigen for a limited quantity of antibody-antigen binding sites. The amount of labeled antigen bound to the antibody is inversely proportional to the amount of antigen in the sample.

In an immunometric or non-competitive assay format, a labeled antibody is employed in place of labeled antigen and the amount of labeled antibody associated with an insoluble ternary complex is directly proportional to the quantity of antigenic substance in the fluid sample.

Both competitive and immunometric immunoassays can be configured in one of two basic formats: heterogenous and homogenous assays. In a competitive immunoassay, both configurations involve the formation of a reaction mixture comprising a minimum of three reaction

components: a known amount of analyte or analyte conjugate, an analyte binding agent, and a sample fluid medium suspected of containing the analyte.

A heterogeneous or two phase assay comprising solid and liquid phases involves immobilization of one member of the analyte/analyte binding agent pair on a solid phase and conjugation of the other to a label or tracer such as an enzyme or radionuclide. The labeled analyte or conjugate competes with analyte suspected to be present in the sample fluid medium for a restricted number of analyte binding sites.

In a homogeneous assay, the separation and preincubation steps are eliminated by measuring the amount of enzyme activity of an analyte-enzyme conjugate rather than the amount of analyte conjugate attached to a support. The presence of an analyte in the sample fluid is established by an increase in activity of the enzyme conjugate (U.S. Pat. Nos. 4,067,774 and 3,817,837).

When either the added analyte conjugate or the sample analyte is bound by the analyte binding agent during an incubation step, the analyte becomes insoluble. The liquid and insoluble phases are then separated and the quantity of analyte in each phase quantitated. The amount of analyte in the sample fluid medium is determined from the quantity of insoluble analyte conjugate following both the incubation and separation steps. Since the amount of bound analyte conjugate is inversely proportional to the quantity of sample analyte, the greater the amount of sample

analyte in the sample fluid, the less the amount of analyte conjugate will be present in the insoluble phase.

Any of the various types of quantitative or qualitative immunoassay formats may be employed for diagnosing birds for *R.anatipestifer* infection.

A kit for the immunoassay of a biological fluid for *R.anatipestifer* advantageously comprises, (i) an antibody to the *R.antatipestifer OmpA* protein or an immunogenic fragment thereof and (ii) labeled *R.anatipestifer OmpA* protein or antigenic fragment (i.e., a fragment that forms an immunologic complex with the antibody) thereof. In such a kit, the antibody may be monoclonal, monospecific or polyclonal, as described above. If polyclonal, the antibody advantageously is selected such that interferring cross-reactions, and consequent false positives, are minimized. The moiety used for labeling of the *R.anatipestifer OmpA* protein or antigenic fragment may be any label conventionally used in heterogenous or simultaneous assay formats. For example, it may be a radioactive atom, an enzyme, a fluorescent moiety, a ligand, a luminescent moity or the like. The kit optionally may comprise unlabeled *R.anatipestifer OmpA* protein or antigenic fragment thereof for use as a control.

In an alternative embodiment, an immunoassay kit for detecting *R.anatipestifer* in biological fluids may comprise two or more antibodies to *R.anatipestifer OmpA* protein or an antigenic fragment thereof. At least one

of such antibodies is labeled in the manner described above, or alternatively, the kit may contain or be used in conjunction with a labeled reporter antibody that is capable of reacting immunologically with one of the antibodies to the *R.anatipestifer OmpA* protein or antigenic fragment. As described above, the kit may optionally contain *R.anatipestifer OmpA* protein or antigenic fragment as a control.

A kit for the detection of the *R.anatipestifer OmpA* gene (or RNA) comprises a detection nucleic acid that is capable of hybridizing to a sequence within the *R.anatipestifer OmpA* gene under stringent hybridization conditions. "Stringent hybridization conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0,015M NaCl/0.0015M sodium tatrate/0.1% sodium dodecylsulfate at 50°C., or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SC (0.75M NaCl, 0.075M sodium citrate), 50mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 mu g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C., with washes at 42°C. in 0.2 x SSC and 0.1% SDS. Alternatively, "stringent hybridization conditions" are those conditions in which a nucleic acid having greater

than about 80% homology, preferably greater than about 90% homology, most preferably, greater than about 95% homology to the *R.anatipestifer OmpA* gene will hybridize to that gene.

The nucleic acid may be labeled, e.g. using any of the type of labels described above, or alternatively, a labeled reporter nucleic acid that binds to the detection nucleic acid, may be employed.

Preferably, a kit for detection of the *R.anatipestifer* gene or RNA contains reagents for amplification of a target sequence within that gene prior to detection. Such reagents include primers or probes that flank the target sequence for carrying out PCR, LCR or other well-known nucleic acid amplification procedures.

In any of the foregoing kits, one or more of the reagents may be immobilized on a solid support, as is well known in the art. Such supports include for example, walls or bottoms of test tubes or microtiter plate wells, latex particles, test strips, bibulous materials, magnetic particles, slides, plates and the like.

The present invention comprises both diagnostic methods and kits for detecting *R.anatipestifer OmpA* nucleic acids, proteins and antibodies.

The invention is further illustrated by the following examples, which are not intended to be limiting.

EXAMPLES

Example 1. Creation and Screening of an *R. anatipestifer* gene library.

Genomic DNA was extracted from *R. anatipestifer* serotype 15 strain CVL110/89 by the rapid guanidium thiocyanate method. This strain was responsible for a severe outbreak with 25% mortality in duck farms in Singapore. Partially digested (*Sau*3A) genomic DNA was cloned into BamHI digested and dephosphorylated bacteriophage λ ZAP Express™ vector (Stratagene) and packaged with Gigapack II Gold Packaging Extract (Stratagene). The gene library was plated according to standard protocols using the *E. coli* strain XL1-Blue MRF'. Ligation products were transformed into XL1-Blue cells by the calcium chloride procedure. Selected kanamycin resistant clones were excised *in vivo* on plasmid vector pBK-CMV from the phage plaques after infection with the helper phage M13 according to the supplier's protocol. The library of *Hin*dIII fragments of genomic DNA from *R. anatipestifer* serotype 15 strain 110/89 was established using plasmid pBluescriptIISK⁻.

A Dig-labeled probe for the *R. anatipestifer Omp*A gene segment was produced by PCR using plasmid pJFFRA6 DNA as template and the primers RA60MPA-L and RA60MPA-R (see Table 2) which were derived from the sequence of the cloned fragment of *Omp*A. This Dig-labeled probe was used to screen the plasmid vector based gene library of *Hin*dIII digested genomic DNA. The gene library was screened with pooled convalescent sera from

ducks experimentally infected with a *R. anatipestifer* serotype 15 field isolate as described above.

To screen the recombinant *E. coli* clones, colonies were transferred from the plates with solid medium to nitrocellulose membrane filters (Schleicher & Schuell, Dassel, Germany). The recombinants were lysed *in situ* and DNA was cross-linked to the membrane by baking the membrane filters at 80°C for 2 h. The filters were then pre-incubated with hybridization buffer (750 mM NaCl, 75 mM trisodium citrate, 0.1% N-laurylsarcosine, 0.02% sodium dodecylsulfate (SDS) and 1% blocking reagent (Boehringer Mannheim, product #10961789), pH 7.7 at 68°C for 2 hours. Hybridization was then performed in hybridization buffer (750 mM NaCl, 75 mM trisodium citrate, 0.1% N-laurylsarcosine, 0.02% SDS) containing 1 µg Dig-labeled *Omp*A gene probe for 18 hours at 68°C. The membrane was washed twice for 15 minutes with buffer containing 30 mM NaCl, 3 mM trisodium citrate and 0.1% SDS, pH 7.7, at 68°C. Dig-labeled DNA probes were detected using phosphatase-labeled anti-Digoxigenin antibodies (Boehringer Mannheim) according to the producer's instructions.

A strongly immunoreactive clone was retained and converted to plasmid designated pJFFRA6. This clone contained a partial open reading frame of 859 bp showing significant similarity to the *Bordetella avium* outer membrane protein A. The corresponding gene on plasmid pJFFRA6 therefore was designated *Omp*A. Plasmid pJFFRa*Omp*A15 containing a 2.2kb *Hin*dIII fragment was

retained for further analysis according to methods known in the art, including sequencing (see Table 3).

Example 2. Expression of *Omp*A Fusion Proteins and Purification of Antigen.

To obtain purified recombinant *R. anatipestifer* *Omp*A antigen, the *Omp*A gene was amplified *in vitro* with *Taq/Pwo* polymerase mix and oligonucleotide primers RAOMPAH1-L and RAOMPAH1-R (see Table 2) using *R. anatipestifer* serotype 15 strain CVL110/89 genomic DNA as template. The purified PCR product was digested with *Nde*I and *Bam*HI and cloned into the expression vector pETHIS-1 to obtain plasmid pJFFOMPA which resulted in an in-frame fusion of six histidine codons at the 5' end of *Omp*A. A second plasmid, pJFFOMP13, was constructed analogously using primers RAOMPAH1-L and RAOMPAH1A-R. The resulting sequence produces the coding frame of *Omp*A fused 5' terminally to six histidine codons and 3' terminally to ten histidine codons. The cloned gene constructs in plasmid pJFFOMPA and pJFFOMP13 were verified by DNA sequence analysis.

The plasmid pJFFOMPA and pJFFOMP13 were transformed into *E. coli* host strain BL21 (DE3). *Omp*A fusion proteins (6xHis-*Omp*A and 6xHis-*Omp*A-10xHis) were expressed by IPTG induction of *E. coli* harboring the corresponding plasmid with the *Omp*A-fusion constructs. Following induction, the cells were harvested, washed in TES buffer (10 mM Tris, 1 mM EDTA, 0.8% NaCl, pH 8.0) and extracted in 50 mM phosphate buffer, pH 8.0

containing 6 M guanidine hydrochloride. The fusion proteins were purified from these cell extracts using Ni^{++}-chelate affinity chromatography (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. The bound polyhistidine tailed fusion proteins were eluted by slowly decreasing the pH from 8.0 to 4.5 with 50 mM phosphate-buffer containing 300 mM NaCl and 6 M guanidine hydrochloride. The fusion proteins eluted at pH 5.0. The fusion proteins were subsequently dialyzed against 50 mM phosphate buffer, pH 8.0, containing 300 mM NaCl. A second plasmid, pJFFOMP17, which is identical to pJFFOMP13, was constructed independently. Its gene product, 6xHis-*Omp*A-10xHis, showed the same characteristics to that obtained from pJFFOMP13 throughout the study.

CLAIMS

1. A nucleic acid encoding the *R.anatipestifer* *Omp*A protein or a fragment thereof.

2. A nucleic acid that will hybridize to a nucleic acid having the sequence of nucleotides 82-1242 of SEQ ID NO:17.

3. A nucleic acid comprising nucleotide bases 82-1242 of SEQ ID NO:17.

4. A vector which comprises the nucleic acid according to claim 1, 2 or 3 and a replicon operative in a host cell.

5. An expression vector comprising the nucleic acid of claim 1, 2 or 3, wherein the coding sequence of said nucleic acid is operably linked to control sequences capable of directing expression of said coding sequence in host cells.

6. Host cells transformed with a vector according to claim 4.

7. Host cells transformed with a vector according to claim 5.

8. An isolated polypeptide comprising SEQ ID NO:18.

9. An inmunogenic peptide fragment of the peptide according to claim 5.

10. A polypeptide according to claim 8, wherein the polypeptide is a fusion protein.

11. A method of producing the peptide according to claims 8, 9 or 10 comprising:

a) providing host cells transformed with an expression vector encoding said peptide operably linked to a control sequence capable of directing expression of said peptide;

b) culturing said host cells under conditions suitable for the production of said peptide;

c) recovering said peptide; and optionally

d) purifying said peptide.

12. An antibody capable of specifically binding the peptide according to claims 8, 9 or 10.

13. A vaccine composition for stimulating immune responses against *Riemerella anatipestifer* in a member of an avian species comprising a compound selected from the group consisting of a peptide having the sequence defined by SEQ ID NO:18, a nucleic acid comprising bases 82-1242 of SEQ ID NO:17, and an antibody capable of specifically binding a peptide having the sequence defined by SEQ ID NO:18.

14. A vaccine composition according to claim 13 comprising a viral vector capable of replication in an immunized host and expression of a nucleic acid encoding the *R.anatipestifer OmpA* protein or an immunogenic fragment itself in said host.

15. A vaccine composition according to claim 13 wherein the peptide is synthetic.

16. An immunological method of diagnosis of infection by *Riemerella anatipestifer* in a member of an avian species, comprising:

a) providing a sample consisting of blood, plasma, serum, tissue or a body fluid from a member of

an avian species suspected of being infected with *Riemerella anatipestifer*.

b) incubating said sample with an antibody according to claim 10; and

c) determining whether said antibody has specifically bound antigen.