Journal of Animal and Veterinary Advances 11 (21): 4073-4078, 2012
ISSN: 1680-5593

Biochemical Characterization of the Outer Membrane Enzyme OMPLA from *Riemerella anatipestifer*

Shenquan Liao, Jianfeng Yuan, Zonghua Qin, Caiyan Wu, Minna Lv,
Nanshan Qi, Jianping Cai and Mingfei Sun
Guangdong Academy of Agricultural Sciences, Institute of Veterinary Medicine,
510642 Guangzhou, People's Republic of China

Abstract: A gene (*pldA*) encoding a 33.0 kDa protein with significant homology to the *Escherichia coli* outer membrane phospholipase A was identified from *Riemerella anatipestifer* genome database. The results of this study revealed that this gene encoded an Outer Membrane Phospholipase A (OMPLA) in *Riemerella anatipestifer*. The recombinant OMPLA protein was expressed and its general biochemical features were also, determined. Methyl Arachidonyl Fluorophosphonate (MAFP) could specifically inhibit the OMPLA activity. These observations suggest that OMPLA could be exploited as a drug target in the bacteria and MAFP may serve as a lead compound for drug discovery against *R. anatipestifer*.

Key words: *Riemerella anatipestifer*, outer membrane phospholipase A, methyl arachidonyl fluorophosphonate, protein, database

INTRODUCTION

The Gram-negative, *Riemerella anatipestifer* is a major cause of disease in farm ducks. It was known as infectious serositis, new duck disease, duck septicemia or anatipestifer septicemia (Sandhu and Rimler, 1997). It caused the characterized syndrome in ducks by fibrinous serositis caseous salpingitis and arthritis which can lead to high mortality and consequently to great economic losses (Glunder and Hinz, 1989). The occurrence of different serotypes of *R. anatipestifer* has been reported (Sandhu and Harry, 1981; Loh *et al*., 1992). Currently, at least twenty-one serotypes of *R. anatipestifer* have been identified. However, there is little or no significant cross-protection between the serotypes (Sandhu, 1979; Pathanasophon *et al*., 1996; Higgins *et al*., 2000). It limited the protective efficacy of vaccines.

There are little known about the pathogenesis and virulence factors of *Riemerella anatipestifer* and so far, no virulence factors have been identified except for VapD (Chang *et al*., 1998), CAMP cohemolysin (Crasta *et al*., 2002) and OmpA (Hu *et al*., 2011). OMPLA is one of the few enzymes present in the outer membrane of Gram-negative bacteria and is likely to be involved in bacterial invasion and pathogenesis. OMPLA is strictly calcium dependent and have a much broader range of activities, i.e., PLA_2, lyso-PLA_1 and lyso-PLA_2 Dekker (2000). This enzyme is a dimer and has an a typical Ser-His-Asn catalytic triad (Snijder *et al*., 1999). OMPLA is widespread among Gram-negative bacteria which has been elucidated as virulence determinant in some species such as *Helicobacter pylori* (Dorrell *et al*., 1999; Zhang *et al*., 2002), *Yersinia* sp. (Karlyshev *et al*., 2001), *Legionella pneumophila* (Banerji *et al*., 2008; Schunder *et al*., 2010) and *Campylobacter* sp. (Grant *et al*., 1997; Ziprin *et al*., 2001), the key enzyme has been served as an attractive target for chemotherapeutic intervention (Ubarretxena-Belandia *et al*., 1999). However, to date, nothing about the encoding gene or biochemical function of *R. anatipestifer* OMPLA have been reported.

In the present study, researchers identified the genetic characterization of a gene (*pldA*) encoding a outer membrane phospholipase A from *R. anatipestifer* and thoroughly characterized the biochemical features. In addition, the inhibitor MAFP was discovered to specifically inhibit the OMPLA from *R. anatipestifer*.

MATERIALS AND METHODS

Reagents: 1 and 2-Dimyristoyl-sn-glycero-3-Phosphocholine (DMPC), myristoyl acid and lysophospholipid were purchased from Sigma (USA). The inhibitor used in this study, Methyl Arachidonyl Fluorophosphonate (MAFP) was purchased from Cayman (USA). The enzyme used in the assay was expressed and purified as described. Water used in this study was double-distilled (dd-H_2O) and all other reagents were of analytical grade.

Corresponding Author: Mingfei Sun, Guangdong Academy of Agricultural Sciences, Institute of Veterinary Medicine, 510642 Guangzhou, People's Republic of China

Table 1: The bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source
DH5α	SupE4; ΔLacU09 (Φ80LacZΔM15); hsdR17; recA1; endA1; gyrA96; thi-l; relA1	Lab collection
BL21 (DE3) pLysS	F-, ompT, hsdS_B (r_B-,m_B-), dcm, gal, λ(DE3), pLysS, Cm^r	Promega
R. anatipestifer	-	Lab collection
pMD18-T vector	Amp^r	Takara
pCold TF	Amp^r	Takara

Table 2: The primers used in this study

Primers	Sequences	Restriction sites (underlined)
pldAF1	5' CCTCCCCGTTTCGTGAAACNAAYTAYGA 3'	-
pldAR1	5' TTCGCCATAGCCGGTATAAAMYTGNGTRTANA 3'	-
pldAF2	5' CAATTAACATGCCGGTTTGTGCTTC 3'	-
pldAR2	5' TTCACCGTTGCCTCTTGTGCATATA 3'	-
pldAF3	5' CTTTTTCGTAACGCTCCAGTTCCAT 3'	-
pldAR3	5' TCGCCAAAATAGTTCAGCAATACCA 3'	-
pldAF4	5' CGCGGATCCATGCGGACTCTGCAGGGCT 3'	BamHI
pldAR4	5' CGCAAGCTTTCAAAACAAATCGTTTAGCATAACCC 3'	HindIII

Substrate preparation: The substrate preparation was refered to Huang *et al*. (2006). The stock substrate solution prepared by sonication. About 50 μL of DMPC solution (40 mM, methanol) and 15 μL of deoxycholic acid solution (40 mM, methanol) were mixed and quickly added into dd-H_2O (1 mL). The solution was stirred for 1 min, sonicated in a water bath for 5 min.

Bacterial strains and media and plasmids: Plasmids and bacterial strains used in the present study are listed in Table 1. *E. coli* strain was grown in Luria-Bertani (LB) broth. *R. anatipestifer* strain was grown in Tryptic Soy Broth (TSB) at 37°C. Antibiotics were used at the following concentrations: 100 μg mL^{-1} of ampicillin for *E. coli* only when necessary.

Recombinant DNA techniques and nucleotide sequencing: Standard techniques including plasmid minipreparations, restriction endonuclease digestions, ligations and transformation into *E. coli* were performed according to standard procedures (Maniatis *et al*., 1982). Various primers were used to amplify RapldA fragments by PCR from genomic DNA isolated from *R. anatipestifer* (Guangdong strain). The primers used in this study are listed in Table 2. Partial *R. anatipestifer pldA* gene was cloned by PCR amplification using Consensus-Degenerate Hybrid Oligonucleotide Primers (CODEHOP). The degenerated primers pldAF1 and pldAR1 were designed from consensus regions of OMPLA protein using the CODEHOP strategy (Rose *et al*., 2003). Refer to the sequence of *pldA* gene in *E. coli* O157:H7 EDL933, the primers were designed to amplified the upstream and downstream fragments of the partial *pldA* gene in *R. anatipestifer*. The products were purified and ligated into pGEM-T-vector and clones were sequenced to confirm its identity. An error-free clone was then used as a template for subsequent gene manipulation and reengineering into expression vectors.

Expression and purification: The pldA was cloned from *R. anatipestifer* genomic DNA by PCR and expressed in pCold TF (Takara, China). DNA sequencing verified the integrity of this and other constructs. rOMPLA expression was induced with 0.5 mM Isopropyl-1-Thio-β-D-Galactopyranoside (IPTG) at 16°C in *E. coli* BL21 (DE3) pLysS and bacteria were lysed by sonication in 300 mM NaCl, 50 mM phosphate buffer, pH 8.0, 10 mM imidazole and 1 mM phenylmethylsulfonyl fluoride. rOMPLA, present partly in the supernatant from the centrifuged lysate was purified by Ni-NTA His Bind Resins (Novagen, USA) and concentrated to 21 mg mL^{-1} (in 10 mM Tris, pH 8.0) and stored at -80°C. For Western blot analysis, the induced cells were collected and mixed 1:1 with SDS-PAGE sample buffer and boiled for 5 min before resolving on 10% SDS gel at 100 V constant voltage. The separated proteins and marker were transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosci-ences) using the Xcell Surelock System (Invitrogen, USA) in Tris Glycine transfer buffer (50 mM Tris, 192 mM glycine, 20% methanol) for 1 h at 100 V constant voltage. After blotting, the membrane was blocked with 1% milk powder in PBS. Membrane was incubated for 1 h at room temperature with mouse anti-His 6 antibody and washed three times with PBS for 5 min. Membranes were then incubated with alkaline phosphatase conjugated goat anti-mouse I gG (Jakson Immuno Research, USA) (1:264) for 1 h, washed three times with PBS for 5 min. Finally, the labelled proteins were developed using 5'-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP).

***In vitro* Phospholipase activity assays:** Phospholipase activity was measured by ANS, an interfacial probe which described by Huang *et al*. (2006). The reaction system contained 160 μL of reaction buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 5 μg mL^{-1} bovine serum albumin and 10 μM ANS), 20 μL of substrate stock solution and 10 μL

of 100 mM $CaCl_2$. Samples were vortexed and incubated at 25°C for 10 min. Reaction was started by adding OMPLA (0.75 μg) in 10 μL of buffer (300 mM NaCl, 50 mM phosphate buffer). The kinetic curves were recorded for 20 min. Using a Perkin Elmer Victor 3 multilabel counter plate reader 1420-524 with the excitation and emission wavelengths used were 377 and 470 nm, respectively.

Inhibition studies: The stock solution of MAFP is 10 mg mL^{-1} in methyl acetate. Serial dilutions of MAFP were prepared utilizing Dimethylsulfoxide (DMSO). Various amounts (2.5-40 μm, final concentration) of MAFP were added to the optimized fluorescence-based activity assay prior to the addition of rOMPLA which preincubated for 10 min at 25°C. The half-maximal inhibitory concentration (IC_{50}) was determined by fitting rOMPLA activity (Relative Fluorescence Units, RFU) at the various log concentrations of MAFP to a sigmoidal dose response Regression Model.

RESULTS AND DISCUSSION

Identification and cloning of the *R. anatipestifer pldA* gene: Partial *R. anatipestifer pldA* gene (400 bp named P1) was obtained using the CODEHOP strategy which permitted the design of specific primers to amplify the entire coding region by PCR. Refer to sequence of *pldA* gene in *E. coli* O157:H7 EDL933, the upstream (1300 bp) and downstream (2400 bp) upstream sequence were obtained, named as P2 and P3, respectively. P1, P2 and P3 were assembled, a 3400 bp sequence was obtained. The ORF algorithms predicted the assembled sequence contains an entire ORF which encoded 289 amino acids with a predicted molecular mass of 33.0 kDa.

The sequence comparison between *R. anatipestifer* OMPLA and 13 homologous enzymes from other gram-negative bacterias were alignmented which highlighted the conserved residues in the active site with the highly consensus sequence motif (YTQ-X_n-G-X_2-H-X-SNG) (Fig. 1).

The conserved residues were earlier shown as vital in the other gram-negative bacteria OMPLA (Istivan and Coloe, 2006).

Enzymatic activity of *R. anatipestifer* OMPLA: To characterize the biochemical features for the *R. anatipestifer* OMPLA, researchers have successfully expressed RaOMPLA as a Trigger Factor-tag fused protein in *E. coli*. The recombinant RaOMPLA was purified in the chromatographic step at 250 mM imidazole.

The purified RaOMPLA were analyzed by SDS-PAGE (Fig. 2a). The recombinant fused RaOMPLA protein was also detected a single band with a calculated molecular mass at ~85 kDa (Fig. 2b) by Western blot analysis which agrees well with the predicted size of Trigger Factor-tag fused protein RaOMPLA protein.

Enzymatic activity of the recombinant *R. anatipestifer* OMPLA was measured with DMPC to assay the phospholipase A activity. The catalytic activity exhibited kinetics, the data fitted to Michaelis-Menten kinetics (Fig. 3). The apparent Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) were determined using nonlinear curve-fitting regression analysis. The K_m and V_{max} values determined for DMPC were 9.3 μM and 315.5 nmol min^{-1}, respectively.

Inhibition of *R. anatipestifer* OMPLA activity: MAFP was discoved to inhibit both $iPLA_2$ and $cPLA_2$ by bound their active-site residue (Huang *et al*., 1994). Benson determined MAFP could inhibit ExoU from *Pseudomonas aeruginosa* (IC_{50} of 13.8 nmol L^{-1}) (Benson *et al*., 2010). The experiments also showed that this inhibitor could inhibit the recombinant RaOMPLA activity in a dose-dependent manner (Fig. 4). The IC_{50} value for this inhibition was determined as 1.73±0.06 μM, suggesting that the inhibitory effect was moderate but slightly better than that observed for *P. aeruginosa* ExoU.

OMPLA is one of the few enzymes presented in the outer membrane of gram-negative bacteria, the enzyme is strictly calcium dependent and displays broad substrate specificity. It hydrolyses the acyl ester bonds in phospholipids with PLA_1 and A_2 activities and also has 1-acyl and 2-acyl lysophospholipase activity as well as mono and diacylglyceride lipase activities (Horrevoets *et al*., 1989). Phospholipase activity has been linked to pathogenesis in bacterial species (Banerji *et al*., 2008; Istivan and Coloe, 2006). OMPLA was thought as a virulence in several gram-negative bacteria such as *Helicobacter pylori*, *Neisseria*, *Yersinia*, Legionella, *Campylobacter* sp. and so on. The key enzyme also has been persued as a novel drug target against gram-negative bacteria (Barh and Kumar, 2009).

OMPLA has been more thoroughly studied in several Gram-negative bacteria. However, although *R. anatipestifer* represents one of the major branches and a group of most significant avain pathogens within the gram-negative bacteria. Until recently, nothing about OMPLA from *R. anatipestifer* have been reported. In the present study, researchers identified the full length sequence of *R. anatipestifer pldA* gene from and

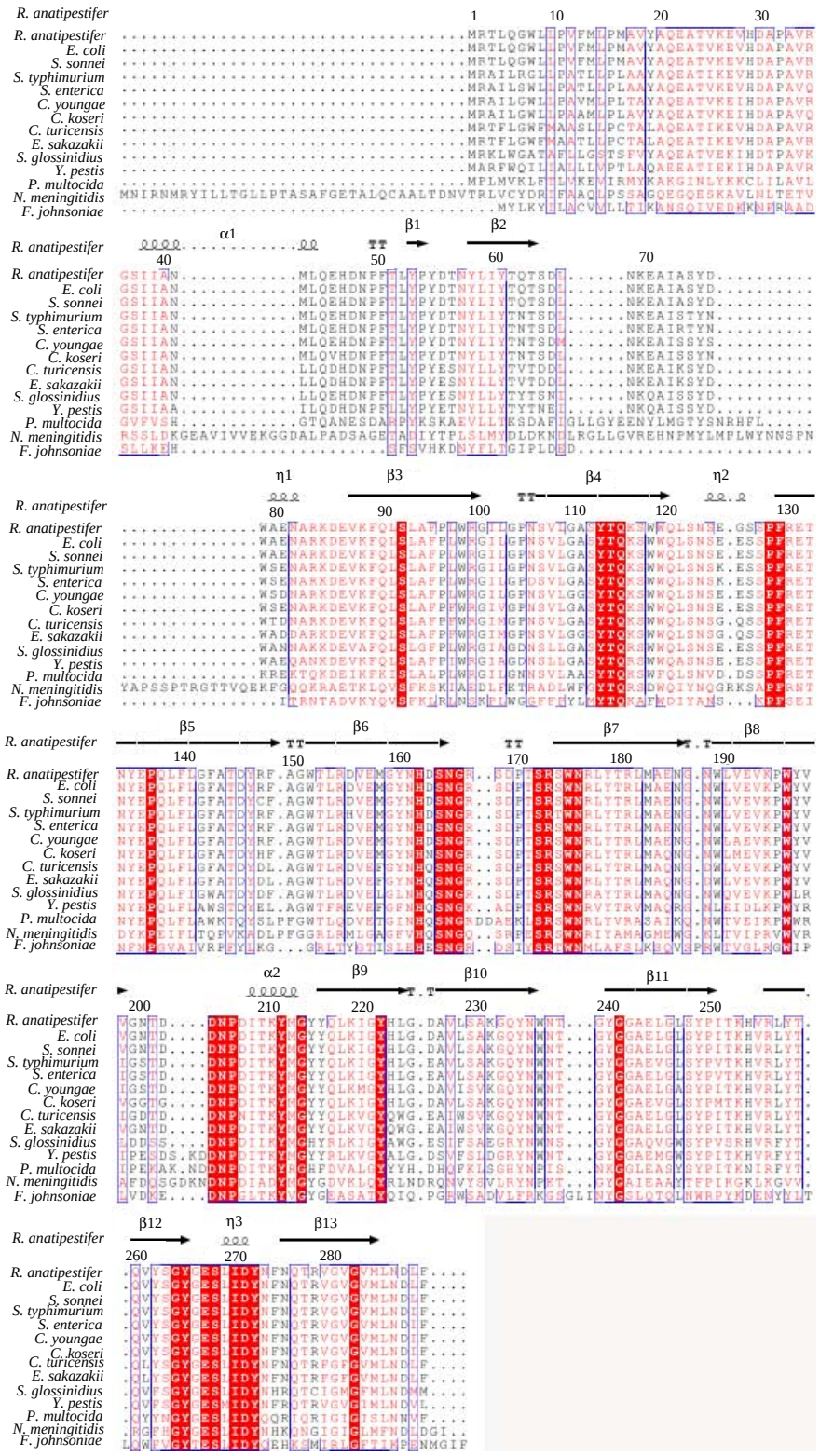

Fig. 1: Sequence alignment of *R. anatipestifer* OMPLA and 13 homologous enzymes from other Gram-negative bacterias. The alignment highlighted the conserved residues in the active site with the highly consensus sequence motif (YTQ-X_n-G-X_2-H-X-SNG). Strictly conserved residues are shown in red boxes

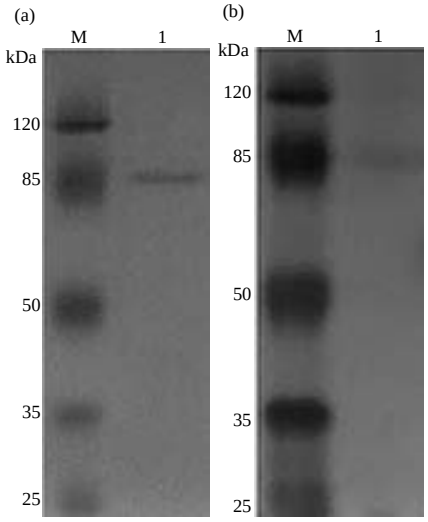

Fig. 2: SDS-PAGE and Western blot analysis of recombinant RaOMPLA. a) Lane 1: Molecular weight markers; Lane 2: Purified RaOMPLA. b) Lane 1: Molecular weight markers; Lane 2: Western blot analysis for the recombinant RaOMPLA

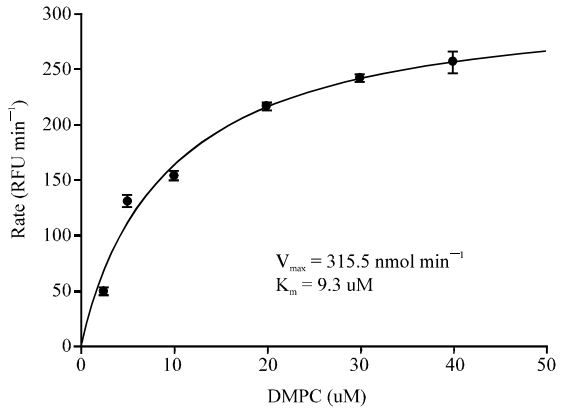

Fig. 3: Enzyme activity of *R. anatipestifer* OMPLA. Phospholipase A activity was determined as described for standard reactions and varying the substrate concentration. Each data point represents standard errors of the means derived from at least three replicates

thoroughly characterized the molecular and biochemical features. The result showed the *R. anatipestifer* OMPLA has phospholipase A activity.

MAFP designed by Huang *et al.* (1994) was a selective inhibitor of $iPLA_2$ and $cPLA_2$. Earlier research revealed that OMPLA hydrolyses the acyl ester bonds in phospholipids with PLA_1 and PLA_2 activity (Horrevoets *et al.*, 1989).

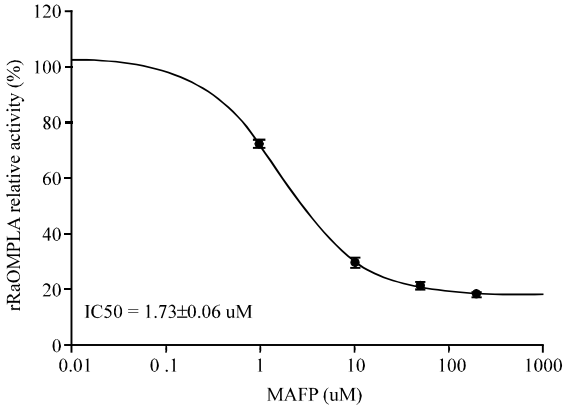

Fig. 4: Inhibition of *R. anatipestifer* recombinant OMPLA by MAFP. OMPLA activity was determined in the presence of various concentrations of MAFP. The IC50 value was 1.73 μM. Each data point represents standard errors of the means derived from at least three replicates

CONCLUSION

In this study, MAFP displayed an inhibitory effect on *R. anatipestifer* OMPLA activity (IC_{50} = 1.73 M). To the knowledge this is the first time that the efficacy of this compound on *R. anatipestifer* OMPLA has been tested. The specific inhibition of MAFP on the recombinant enzyme indicate that the compound may serve as a lead chemical for further modifications to increase selective inhibition against *R. anatipestifer*.

ACKNOWLEDGEMENTS

This research was supported by a NSFC grants (No: 30471300 and U0831004 to JC), international cooperating project from the Guangdong Science and Technology Department (No: 2008A050200015), NSF grant of Guangdong province (No: 104510600100612 6) and the President's Funds of Guangdong Academy of Agricultural Sciences (No: 201014 and 201115).

REFERENCES

Banerji, S., P. Aurass and A. Flieger, 2008. The manifold phospholipases a of *Legionella pneumophila*-identification, export, regulation and their link to bacterial virulence. Int. J. Med. Microbiol., 298: 169-181.

Barh, D. and A. Kumar, 2009. *In silico* identification of candidate drug and vaccine targets from various pathways in *Neisseria gonorrhoeae*. In Silico Biol., 9: 225-231.

Benson, M.A., K.M. Schmalzer and D.W. Frank, 2010. A sensitive fluorescence-based assay for the detection of ExoU-mediated PLA(2) activity. Clin. Chem. Acta, 411: 190-197.

Chang, C.F., P.E. Hung and Y.F. Chang, 1998. Molecular characterization of a plasmid isolated from *Riemerella anatipestifer*. Avian Pathol., 27: 339-345.

Crasta, K.C., K.L. Chua, S. Subramaniam, J. Frey, H. Loh and H.M. Tan, 2002. Identi?cation and characterization of CAMP cohemolysin as a potential virulence factor of *Riemerella anatipestifer*. J. Bacteriol., 184: 1932-1939.

Dekker, N., 2000. Outer-membrane phospholipase A: known structure, unknown biological function. Mol. Microbiol., 35: 711-717.

Dorrell, N., M.C. Martino, R.A. Stabler, S.J. Ward and Z.W. Zhang *et al*., 1999. Characterization of *Helicobacter pylori* PldA, a phospholipase with a role in colonization of the gastric mucosa. Gastroenterology, 117: 1098-1104.

Glunder, G. and K.H. Hinz, 1989. Isolation of *Moraxella anatipestifer* from embryonated goose eggs. Avian Pathol., 18: 351-355.

Grant, K.A., I.U. Belandia, N. Dekker, P.T. Richrdson and S.F. Park, 1997. Molecular characterization of pldA, the structural gene for a phospholipase: A from *Campylobacter coli* and its contribution to cell-associated hemolysis. Infect. Immun., 65: 1172-1180.

Higgins, D.A., R.R. Henry and Z.V. Kounev, 2000. Duck immune responses to *Riemerella anatipestifer* vaccines. Dev. Comput. Immunol., 24: 153-167.

Horrevoets, A.J., T.M. Hackeng, H.M. Verheij, R. Dijkman and G.H. de Haas, 1989. Kinetic characterization of *Escherichia coli* outer membrane phospholipase: A using mixed detergent-lipid micelles. Biochemistry, 28: 1139-1147.

Hu, Q., X. Han, X. Zhou, C. Ding, Y. Zhu and S. Yu, 2011. OmpA is a virulence factor of *Riemerella anatipestifer*. Vet. Microbiol., 150: 278-283.

Huang, C., L. Zhou, Y. Liu and L. Lai, 2006. A continuous fluorescence assay for phospholipase A2 with nontagged lipid. Anal. Biochem., 351: 11-17.

Huang, Z., S. Liu, F. Laliberte, M. Ouellets and S. Desmaka *et al*., 1994. Methyl arachidonyl fluorophosphonate, a potent irreversible cPLA, inhibitor, blocks the mobilization of arachidonic acid in human platelets and neutrophils. Carl. J. Physiol. Phanruzcol., 72: 711-715.

Istivan, T.S. and P.J. Coloe, 2006. Phospholipase: A in Gram-negative bacteria and its role in pathogenesis. Microbiology, 152: 1263-1274.

Karlyshev, A.V., P.C. Oyston, K. Williams, G.C. Clark, R.W. Titball, E.A. Winzeler and B.W. Wren, 2001. Application of high-density array-based signature-tagged mutagenesis to discover novel *Yersinia virulence-associated* genes. Infect. Immun., 69: 7810-7819.

Loh, H., T.P. Teo and H.C. Tan, 1992. Serotypes of *Pasteurella anatipestifer* isolates from ducks in Singapore: A proposal of new serotypes. Avian Pathol., 21: 453-459.

Maniatis, T., E.F. Fritsch and J. Sambrock, 1982. Molecular Cloning: A Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Pathanasophon, P., T. Sawada, T. Pramoolsinsap and T. Tanticharoenyos, 1996. Immunogenicity of *Riemerella anatipestifer* broth culture bacterin and cell-free culture filtrate in ducks. Avian Pathol., 25: 705-719.

Rose, T.M., J.G. Henikoff and S. Henikoff, 2003. CODEHOP (Consensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. Nucleic Acids Res., 31: 3763-3766.

Sandhu, T. and E.G. Harry, 1981. Serotypes of *Pasteurella anatipestife* from commercial White Pekin ducks in the United States. Avian Dis., 25: 497-502.

Sandhu, T.S. and R.B. Rimler, 1997. *Riemerella anatipestifer* infection. In: Diseases of Poultry, Calnek, B.W. (Ed.). Iowa State University Press, Ames, IA., pp: 161-166.

Sandhu, T.S., 1979. Immunization of White Pekin ducklings against *Pasteurella anatipestifer* infection. Avian Dis., 23: 662-669.

Schunder, E., P. Adam, F. Higa, K.A. Remer and U. Lorenz *et al*., 2010. Phospholipase PlaB is a new virulence factor of *Legionella pneumophila*. Int. J. Med. Microbiol., 300: 313-323.

Snijder, H.J., I. Ubarretxena-Belandia, M. Blaauw, K.H. Kalk and H.M. Verheij *et al*., 1999. Structural evidence for dimerization-regulated activation of an integral membrane phospholipase. Nature, 401: 717-721.

Ubarretxena-Belandia, I., R.C. Cox, R. Dijkman, M.R. Egmond, H.M. Verheij and N. Dekker, 1999. Half-of-the-sites reactivity of outer-membrane phospholipase: A against an active-site-directed inhibitor. Eur. J. Biochem., 260: 794-800.

Zhang, Z.W., N. Dorrell, B.W. Wren and M.J.G. Farthing, 2002. *Helicobacter pylori* adherence to gastric epithelial cells: A role for non-adhesin virulence genes. J. Med. Microbiol., 51: 495-502.

Ziprin, R.L., C.R. Young, J.A. Byrd, L.H. Stanker and M.E. Hume *et al*., 2001. Role of *Campylobacter jejuni* potential virulence genes in cecal colonization. Avian Dis., 45: 549-557.