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Mutation of *oppF* gene in the *Mycoplasma synoviae* MS-H vaccine strain and its implication for differential serological responses to vaccination versus field challenge

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ABSTRACT

Mycoplasma synoviae (MS) is a major pathogen of poultry globally, causing chronic respiratory disease and arthritis. Vaccination is an effective means for the control of the disease. The MS-H vaccine is an attenuated strain developed through chemical mutagenesis of an Australian field strain, 86079/7NS. Analysis of whole genome of MS-H and its comparison with that of 86079/7NS has revealed a frameshift mutation early in a gene (*oppF*) that codes for an oligopeptide transporter permease, OppF.

Monospecific antibodies raised against peptides upstream and downstream of the mutation in OppF revealed that only N-terminus of the OppF was expressed in MS-H while the full version was expressed in 86079/7NS. Also, examination of the recombinant N- (OppF-N) and C termini (OppF-C) of OppF, upstream and downstream of the mutation site respectively, as well as the full length OppF in Western immunoblotting experiments showed that serum from MS-H vaccinated chicken strongly bound OppF-N while serum from 86079/7NS challenged chicken detected OppF, OppF-N and OppF-C.

The potential of the recombinant OppF, OppF-N and OppF-C to discriminate antibody responses to MS-H reisolates with wild or vaccine type OppF was assessed against 88 chicken sera in indirect ELISA and ratios were calculated between optical densities (OD) over those obtained in MS major membrane protein MSPB ELISA. Comparison of the OD ratios revealed that the MSPB/OppF and MSPB/OppF-C OD ratios of the sera against isolates with vaccine type OppF were significantly higher than those against isolates with wild type OppF. These results are in accordance with *oppF* gene mutation in MS-H and confirms that MS-H does not express OppF beyond the frame shift mutation found in its *oppF* gene.

Also, the indirect ELISA based on OppF-C in combination with the MSPB has the potential to differentiate between MS-H and field strain antibody responses.

1. Introduction

Mycoplasma synoviae (MS) is a worldwide poultry pathogen, causing airsacculitis and infectious synovitis in poultry (Noormohammadi et al., 1997). It causes subclinical respiratory diseases, eggshell apex abnormality and synovitis in chickens and turkeys, leading to considerable economic losses to the poultry industry breeding due to deterioration in eggshell quality, carcass condemnation and culling of lame birds (Landman, 2014). The clinical signs differ from sub-clinical to severe forms, depending on the pathogenicity of the strain and the level of immunity (Landman and Feberwee, 2004). Diagnosis of MS infection is based on clinical signs of the disease, epidemiological data, analysis of gross and microscopic lesions, serology and/or isolation and identification of the organism (Luciano et al., 2011). Several tests such as serological tests are available for monitoring of MS in flocks including rapid serum agglutination (RSA), enzyme-linked immunosorbent assays (ELISA) and hemagglutination inhibition (HI) (Luciano et al., 2011). The most effective strategy to control MS infection is by maintaining breeding flocks free of this pathogen although this has been proven impractical in many countries. Instead vaccination has become an effective means for control in many countries. The MS-H vaccine (Vaxsafe MS®; Bioproperties Ltd., Ringwood, Victoria, Australia) is a temperature-sensitive (ts^{+}) attenuated strain which was developed by chemical mutagenesis of the Australian field strain 86079/7NS and widely used

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around the globe (Markham et al., 1998).

Differentiation of MS-H vaccine from wild strains is important for monitoring the efficacy of vaccination programs in poultry industry and to determine whether the vaccine has colonised the upper respiratory tract of the birds (Shahid et al., 2014a, 2014b). A wide range of molecular techniques are used for the discrimination of the MS-H from field strains. These include PCR-based techniques targeting *vlhA* gene routinely employed for MS genotyping (Bencina et al., 2001; Hammond et al., 2009; Hong et al., 2004; Wetzel et al., 2010). Given that the *vlhA* typing results are not always consistent with genotyping results defined by conventional typing methods such as restriction fragment length polymorphism (RFLP), this gene may not be totally reliable for the discrimination of MS-H from some of the Australian (Jeffery et al., 2007) and European strains (Catania et al., 2016; Dijkman et al., 2014; Felde et al., 2018). Detection of specific point mutations in *obg* gene through high resolution melting-curve (HRM) analysis or mismatch amplification mutation assays (MAMAs) has also been described for the discrimination of MS-H strain and field strains (Kreizinger et al., 2017; Shahid et al., 2013). Recently whole genomic sequencing of MS-H and its comparison with that of its parent strain 86079/7NS (Zhu et al., 2018) has revealed a frameshift mutation at position 468 in one of the two copies of the *oppF* genes, and this is presumed to result in expression of a truncated form of OppF. The *oppF* gene codes for an oligopeptide transporter permease. This mutation is consistent in MS-H reisolates obtained after 5 *consecutive in vivo* passages in experimentally MS-H inoculated SPF birds (Zhu et al., 2017).

In this study the full length OppF and two truncated versions of it spanning upstream and downstream of the mutation point in MS-H were used for their potential in distinguishing antibody responses to MS strains/isolates with wild type or vaccine type OppF.

2. Materials and methods

2.1. Expression of recombinant OppF, OppF-N and OppF-C in Escherichia coli

The complete *oppF* coding DNA sequence of 86079/7NS was obtained from Zhu et al (Zhu et al., 2017).

In order to express full-length and truncated versions of *oppF* gene, the *oppF* sequence of 86079/7NS was optimised and synthesised for expression in *Escherichia coli* (*E. coli*) (Thermo Fisher Scientific, Australia). A His-tag sequence and two restriction sites *Bam*HI and *Xho*I at 5′ and 3′ ends, respectively, were also included and the construct was cloned in the pET151D/TOPO expression vector (Thermo Fisher Scientific, Australia).

Using Primer3 (version 2.3.4) (Rozen and Skaletsky, 2000) and AmplifX 1.7.0 (http://crn2m.univ-mrs.fr/pub/amplifx-dist) three pairs of oligonucleotide primers used in PCR (S1 Table) were designed (Geneworks, Thebarton, SA, Australia).

Two primer pairs oppF-N-F/R and oppF-C-F/R with *Bam*HI and *Xho*I restriction sites were used to amplify the 5′ and 3′ ends of the *oppF* by PCR, respectively.

For both oppF-N and oppF-C PCRs, the 25 µl PCR reaction mixture contained 2 µl of (~ 100 pg/µl) pET151D/TOPO harbouring *oppF* gene as template, 0.4 µl of each of 25 µM solutions of the forward and reverse primers, 1 µl of 50 mM $MgSO_4$, 4 µl of a 1.25 mM solution of each dNTP (Promega, Alexandria, NSW, Australia), 0.1 µl (1 U) of High Fidelity Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 2.5 µl of 10 × Platinum Taq DNA polymerase buffer (Invitrogen), and 14.6 µl of nuclease free water. The reaction mixture was incubated at 94 °C for 2 min, and then subjected to 30 cycles of 94 °C for 15 s, 66 °C for 30 s, followed by extension of 35 s and 2 min for oppF-N and oppF-C, respectively at 68 °C and final extension of 10 min at 68 °C. In each set of reactions, nuclease free water was included as negative control. All PCR reactions were carried out using an iCycler thermocycler (Biorad, Gladesville, Australia). Three microliters of all amplified DNA were analysed using electrophoresis through 1.1% agarose gels containing GelRed™ (Biotium, Fremont, CA, USA) at 80 V for 70 min. DNA bands were visualised under UV transillumination using the ChemiDoc™ Imaging System (Bio-Rad, Gladesville, NSW, Australia). Amplicons of expected size were gel-purified using the AxyPrep DNA gel extraction kit (Axygen, Australia) and subsequently digested using *Bam*HI and *Xho*I restriction endonucleases. The expression vector pET151D/TOPO was also digested with *Bam*HI and *Xho*I. Digested amplicons and vector were gel-purified as described above. The digested plasmid and PCR products were ligated using T4 DNA ligase (Promega, Australia) resulting in the pET151D/-oppF-N and pET151D-oppF-C constructs. The plasmid was then transformed into the chemically competent *E. coli* Rosetta (DE3) cells using the manufacturer instructions (Novagen, Merck, Australia). The transformation mixture was spread onto Luria-Bertani (LB) agar plates containing 100 µg/ml of ampicillin and incubated at 37 °C overnight. Individual ampicillin resistant colonies were selected and subcultured in LB broth containing 100 µg/ml of ampicillin (LB/Amp) at 37 °C overnight. Recombinant plasmids were isolated using Monarch® Plasmid Miniprep Kit (Genesearch, e-Freezer, Australia) and subjected to a double digestion using *Bam*HI and *Xho*I to confirm the identity of pET151D/-oppF-N and pET151D/-oppF-C.

Single transformant colonies harbouring the plasmid constructs for *oppF*, *oppF-N* and *oppF-C* were grown in 5 mL of LB/Amp at 37 °C overnight with shaking at 250 rpm. Four and a half ml of prewarmed LB/Amp broth was inoculated with 500 µl of overnight culture and incubated at 37 °C until OD at 600 nm reached approximately 0.5. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the final concentration of 0.3 M and the culture was incubated at 37 °C for further 4 h (OD ˜ 1.5 at 600 nm). Small volume of *E. coli* cells was removed and harvested before and after IPTG induction by centrifugation at 12,000 × *g* for 5 min at 4 °C. The pellets were resuspended in 50 µl of 1 × sodium dodecyl sulfate (SDS) gel loading buffer (Laemmli, 1970), heated at 100 °C for 5 min and 10 µl of the lysate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and/or transferred onto PVDF membrane (Bio-Rad). The blot was probed with His-tag monoclonal antibody (GenScript, China), sera from MS-H vaccinated and 86079/7NS challenged chickens, as well as two mono-specific rabbit sera raised against N terminus and C terminus of OppF (S2 Table).

2.2. Evaluation of OppF expression in MS-H and 86079/7NS

One ml volumes of mycoplasma broth (MB) containing nicotinamide adenine dinucleotide (NAD) (Whithear, 1993) were inoculated with 1/10 dilution of strains MS-H and 86079/7NS and grown to late exponential phase (˜ pH 6.8) at 37 °C. The cells were harvested by centrifugation at 16,000 × *g* for 5 min at 4 °C and the cell pellets were washed with sterile Phosphate Buffered Saline (PBS), resuspended in 1 × SDS gel loading buffer and subjected to SDS-PAGE and Western blotting as described above. *E. coli* whole-cell lysates containing recombinant OppF, OppF-N and OppF-C were used as controls.

2.3. Protein purification

Purification of the His-tagged OppF, OppF-N and OppF-C fusion proteins from *E. coli* cultures was performed using Profinity Immobilized Metal Ion Affinity Chromatography (IMAC) Resin (Bio-Rad, Australia) as instructed by the manufacturer.

Briefly, *E. coli* cells were harvested by centrifugation at 8000 × g for 10 min, resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, 8 M urea and lysonase 20 µl/g of cell paste) and the cell lysate was sonicated (Sonics, USA) 4 times at 80% amplitude for 20 s each with 1 min intervals on ice. The homogenised slurry was centrifuged at 12,000 × *g* for 20 min at 4 °C, and the pellet (the inclusion bodies) resuspended in 1:10 ratio (w/v) in 1 × PBS/8 M urea (pH 7.5).

An IMAC column was equilibrated with 5 column volumes of binding buffer (20 mM sodium phosphate, 300 mM NaCl and 20 mM imidazole) and the lysate was loaded at a flow rate 0.5 ml/min. This step was followed by washing with 10 column volumes of wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole and 8 M urea) to elute unbound proteins. A linear Imidazole gradient from10 to 500 mM in elution buffer was used to determine the optimal purification of His-tagged proteins. The presence and purity of His-tagged OppF, OppF-N and OppF-C in eluted fractions were assessed using SDS-PAGE and Western blot analysis with His-tag antibody and sera from MS-H vaccinated and 86079/7NS challenged chickens (S2 Table).

2.4. Antisera

Two groups of chicken sera (S2 Table) were used in ELISA (Noormohammadi et al., 2003, 1999). Group W (55 serum samples) raised against MS strains/isolates with wild type (full length) OppF consisting of 86079/7NS, an Australian field strain 94011/18d, and non-temperature sensitive (ts-) MS-H reisolates MS-H[1], MS-H[3]and MS-H[4]. Group V (33 serum samples) was from chickens vaccinated/inoculated with MS-H or its ts- reisolate MS-H[5].

Before use in ELISA, all serum samples were centrifuged at 10,000 × *g* for 10 min at 4 °C and diluted appropriately in ELISA buffer (0.1 M Tris, pH 7.4 (HCl); 0.5 M NaCl; 1 mM Na2EDTA; 2% (w/v) BSA; 3% (v/v) Triton X-100; and 3% (v/v) Tween 20).

Two mono-specific rabbit sera raised against N terminus and C terminus of OppF were also synthesized commercially (GenScript, China) (S2 Table).

2.5. Determination of the partial oppF gene sequence of MS strains/isolates

A region of the 86079/7NS *oppF* gene spanning nucleotides 266–892 (covering the mutation point nucleotide 468 in the MS-H) was amplified using oligonucleotide primers oppF-F and oppF-R (S1 Table) for sequencing. To extract genomic DNA, cultures of MS-H, 86079/7NS and MS-H reisolates MS-H[4], MS-H[5] and the Australian field strain 94011/18d were inoculated into mycoplasma culture medium containing 10% swine serum and 0.01% (w/v) NAD (Whithear, 1993) and grown until late logarithmic phase (pH of approximately 6.8) at 37 °C in a 50-mL final volume. Cells were collected by centrifugation at 10,000 × g for 30 min. Genomic DNA was prepared by proteinase K digestion of the pellet, phenol-chloroform extraction and ethanol precipitation (Gaillard and Strauss, 1990; Longmire et al., 1987). Genomic DNA of MS-H reisolates MS-H[1] and MS-H[3] were available in our laboratory. For PCR, a 25 µl PCR reaction mixture contained 2 µl of genomic DNA, 1.6 µl of a 1.25 mM solution of each dNTP (Promega, Alexandria, Australia), 1.25 ml of 25 mM $MgCl_2$, 2 µl of each 25 mM forward and reverse primers, 0.25 µl of Taq DNA polymerase (Promega), 5 µl of 5 × GoTaq flexi green buffer (Promega) and 12.9 µl of nuclease free water. The PCR reaction was subjected to one cycle of denaturation at 95 °C for 2 min followed by 34 cycles of 30 s at 95 °C, 20 s at 53 °C and 80 s at 72 °C, followed by a final extension of 5 min at 72 °C. All PCR reactions were carried out using an iCycler thermocycler (Biorad, Gladesville, Australia) and amplicons subsequently analysed by electrophoresis through 1.2% agarose gels stained with GelRed™ (Biotium, Fremont, CA, USA) and visualisation under UV transillumination using the ChemiDoc™ Imaging System (Bio-Rad, Gladesville, NSW, Australia). Amplicons of *oppF* PCR products were purified using the AxyPrep DNA gel extraction kit (Axygen), eluted in 40 µl nuclease-free water and then subjected to Sanger sequencing in both directions using primers oppF-F and oppF-R at the Micromon DNA Sequencing Facility (Monash University, Australia). The nucleotide sequences were compared with each other and 86079/7NS *oppF* sequence was used as reference (Geneious®, version 11.1.3).

2.6. ELISA

The recombinant purified OppF and MSPB proteins were used as coating antigens in indirect ELISAs to examine their capacity to discriminate antibody responses to MS-H and field strains. Antibody dilution and coating protein concentration were optimised as described before (Noormohammadi et al., 1999). Briefly, 100 µl of serial 1/5-fold dilutions (from 1/10 to 1/577) of the purified proteins in carbonate/bicarbonate buffer (0.032 M Na_2CO_3, 0.068 M $NaHCO_3$, pH 9.6) were used to coat duplicate wells of a 96-well flat-bottom plate (Maxisorp, Nunc-Immuno plate, Thermo Scientific, Denmark) and the plate was incubated overnight at 4 °C. The wells were washed three times with 200 µl 0.05% Tween 20 in PBS and the plate was incubated for 2 h with 200 µl per well of 1% (w/v) BSA in PBS at room temperature. The wells were washed as before and incubated for 1 h at room temperature with 100 µl per well of serial two-fold dilutions (from1/10 to 1/2560) of each MS-H and anti-86079/7NS chicken sera in ELISA buffer. After washing as before, 100 µl horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG (Millipore, Merck, Australia) diluted 1/1000 in ELISA buffer was added to each well and the plate was incubated for 1 h at room temperature. The wells were washed as before and had 100 µl substrate added (3, 3′, 5, 5′-tetramethylbenzidine, Bio-Rad, Australia) and the plate was incubated for 5 min at room temperature. The enzymatic reaction was stopped after 5 min by adding 25 µl 2 M H_2SO_4 to each well and the plates were read at a wavelength of 450 nm (Multiscan FC ELISA reader, Thermo scientific, Australia). Two wells of conjugate control (containing only ELISA buffer instead of first antibody) were included in each plate. The optimal absorbance of antigens reactions against both anti-86079/7NS and anti-MS-H sera were obtained at concentration of 2.5 µg/ml for antigens and dilution of 1/100 for sera. Moreover, the HRP conjugated anti-sera was used at dilution of 1/1000. The reaction of purified recombinant proteins (OppF, OppF-N and OppF-C) with a panel of sera from chickens inoculated with MS-H or its reisolates, or with 86079/7NS and a field MS strain 94011/18d (Noormohammadi et al., 2003) were tested in ELISA. Recombinant MSPB protein (Noormohammadi et al., 1999) was also used in ELISA for comparison with recombinant OppF, OppF-N and OppF-C proteins. All sera were tested in duplicates against all antigens. The average OD from conjugate control wells was subtracted from that of other wells and for each serum ratios of OD between MSPB, OppF, OppF-N and OppF-C ELISAs were calculated and compared using student *t*-test (Microsoft Excel).

3. Results

3.1. Full length and two truncated versions of OppF of 86079/7NS were successfully expressed in E. coli

In order to express truncated forms of OppF protein, the upstream and downstream (N terminus and C terminus) of the mutation point of 86079/7NS *oppF* gene (Fig. 1a) were amplified using two pairs of oligonucleotide primers (S1 Table) and the synthetised pET151D/TOPO harbouring *oppF* gene as template. The PCR products were ligated to the expression vector pET151D/TOPO, and the resultant constructs in addition to pET151D-oppF were used to transform *E. coli* Rosetta (DE3) cells. The full length *oppF*, *oppF-N* and *oppF-C* were predicted to encode polypeptides of 834, 193 and 680 amino acids, respectively, in *E. coli* cells. SDS-PAGE of the whole-cell proteins of *E. coli* cells containing recombinant plasmids demonstrated the expression of fusion proteins of approximately 97, 21 and 79 kDa from the plasmids containing *oppF*, *oppF-N* and *oppF-C*, respectively (Fig. 1b). Immunostaining of Western blots of the whole-cell proteins of the clones expressing three lengths OppF polypeptides showed that His-tag antibody detected faint bands of ˜ 97 kDa, 21 kDa and 79 kDa in transformed *E. coli* cells with pET151D-oppF, pET151D/-oppF-N and pET151D/-oppF-C, respectively but not in untransformed cells (Fig. 1c). The anti-86079/7NS chicken

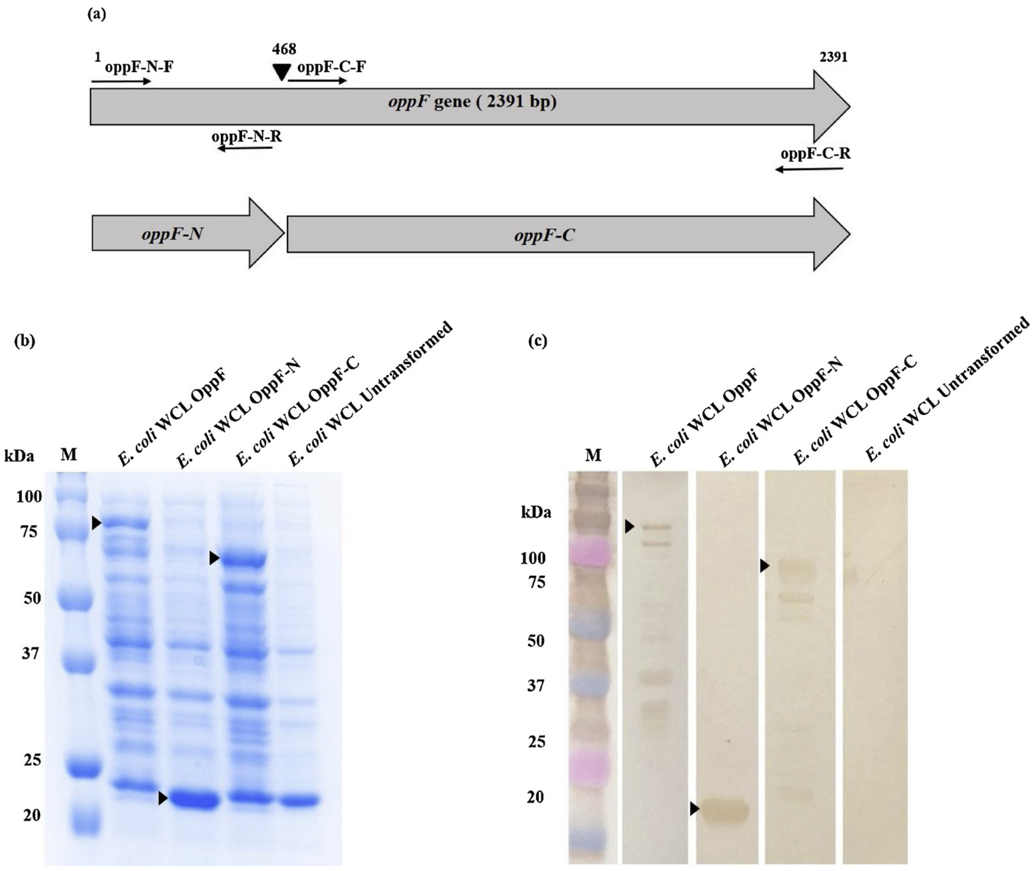

Fig. 1. Expression and detection of the full length and two truncated versions of OppF proteins. (a) Schematic presentation of *oppF* gene and location of the primers, *oppF-N* and *oppF-C*. The largest arrow shows the full length *oppF* encoded in forward orientation while the two smaller arrows indicate *oppF-N* and *oppF-C*. Thin arrows represent primer locations and the vertical arrow head indicates location of the SNP at position 468. (b) SDS-PAGE followed by Coomassie brilliant blue staining of whole-cell lysate (WCL) from induced *E. coli* with IPTG transformed with pET151D-oppF, pET151D-oppF-N and pET151D-oppF-C and induced *E. coli* with IPTG untransformed cells. Bands of expected sizes for OppF, OppF-N and OppF-C are indicated with arrow heads. M is Precision Plus protein TM Dual Color marker (Bio-Rad). (c) Immunoblots of WCL of the *E. coli* cells expressing OppF fusion proteins and untransformed *E. coli* against His-tag antibody. The arrow heads show the location of each of OppF proteins. M is Precision Plus protein ^{TM}, Dual Color marker (Bio-Rad) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

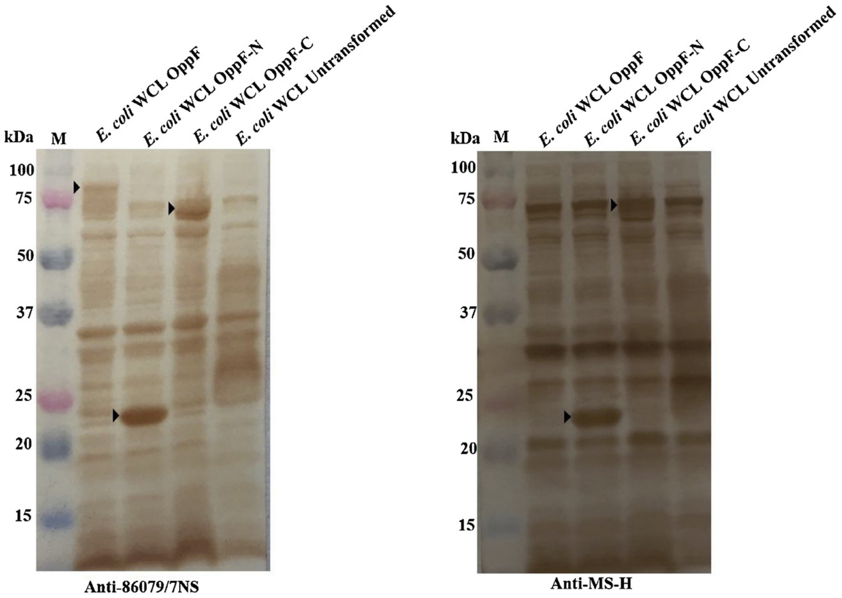

Fig. 2. Evaluation of recombinant OppF, OppF-N and OppF-C proteins reaction against anti-86079/7NS and anti-MS-H chicken sera. Immunoblots of whole-cell lysate (WCL) of the *E. coli* cells expressing OppF fusion proteins and untransformed *E. coli* against anti-86079/7NS and anti-MS-H chicken sera. The arrow heads show the location of each of OppF proteins. M is Precision Plus protein ^{TM}, Dual Color marker (Bio-Rad).

serum stained expected bands for OppF, OppF-N and OppF-C, while did not stain any protein of similar size in untransformed cells. The anti-MS-H chicken serum strongly stained the OppF-N and moderately stained OppF-C but did not stain the OppF (Fig. 2). Both anti- 86079/7NS and anti-MS-H chicken sera also detected several presumably non-specific bands of similar sizes in all *E. coli* lysates tested (Fig. 2).

3.2. Full length OppF was not detected in MS-H whole-cell proteins by specific anti MS-H sera

SDS-PAGE analysis of the MS-H and 86079/7NS whole-cell proteins did not reveal any major differences in banding profiles of the two strains (results not shown). Immunoblotting experiments revealed that antibody Rabbit-anti-OppF-N detected the OppF protein of expected size of ˜ 94 kDa in 86079/7NS, ˜18 kDa in MS-H, ˜97 kDa in *E. coli* transformed with pET151D-OppF, and of ˜22 kDa in *E. coli* transformed with pET151D-OppF-N. The same antiserum detected similar size bands

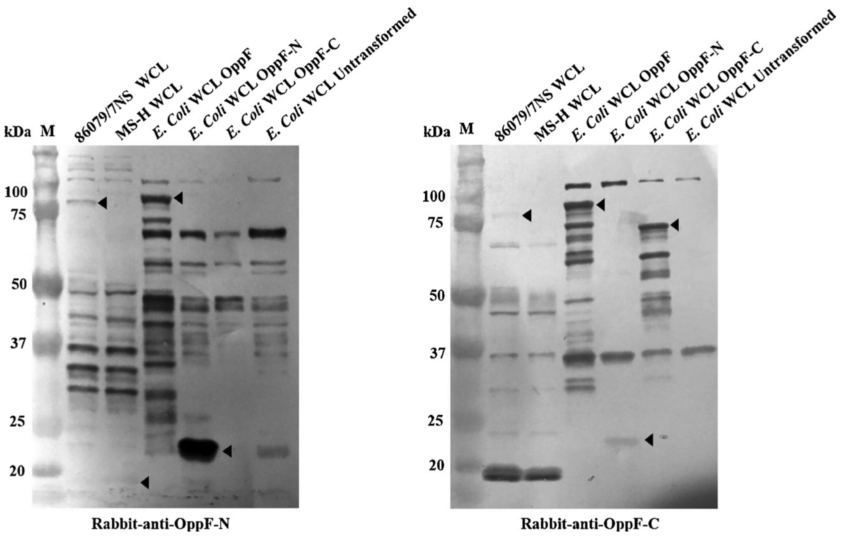

Fig. 3. Analysis of OppF expression in 86079/7NS and MS-H. Western Immunoblots of whole-cell lysate (WCL) of 86079/7NS, MS-H and *E. coli* transformed with OppF, OppF-N and OppF-C constructs. The blots were probed with antibody against N terminus of OppF (Rabbit-anti-OppF-N) or C terminus of OppF (Rabbit-anti-OppF-C). Bands of expected sizes for OppF, OppF-N and OppF-C are indicated with arrow heads. M is Precision Plus protein TM, Dual Color marker (Bio-Rad).

Table 1
Optical densities obtained in indirect ELISA using OppF, OppF-N, OppF-C and MSPB as coating antigens and chicken sera against MS strains/isolates with wild type (group W) or vaccine type (group V) OppF.

Groups	N[a]	Mean (SD[b])			
		Full OppF	OppF-C	OppF-N	MSPB
W	55	0.55 (0.3)	0.43 (0.4)	1.18 (0.53)	1.88 (0.21)
V	33	0.04 (0.01)	0.18 (0.14)	0.54 (0.44)	1.16 (0.74)

[a] Number of serum samples in each group.

[b] Standard deviation.

in both MS-H and 86079/7NS, and in all *E. coli* lysates examined but not any band comparable to the size of OppF in MS-H or OppF-C in *E. coli* transformed with pET151D-OppF-C (Fig. 3). Similar experiments using Rabbit-anti-OppF-C serum detected a band of ~80 kDa OppF-C in *E. coli* transformed with *oppF-C,* ~ 94 kDa OppF in 86079/7NS and *E. coli* transformed with *oppF*, but not in any other lysate used (Fig. 3). Similar to Rabbit-anti-OppF-N, Rabbit-anti-OppF-C also detected similar bands in both MS-H and 86079/7NS, and in all *E. coli* cell lysates examined.

3.3. Fusion proteins OppF, OppF-N and OppF-C were successfully purified

His-tagged recombinant proteins OppF, OppF-N and OppF-C were affinity purified from transformed *E. coli* cell lysates using Profinity™ IMAC Resins under denaturing conditions as described in Methods. All proteins were eluted from the IMAC column at 200–250 mM Imidazole. SDS-PAGE of the collected fractions revealed abundant proteins of ~97 kDa, 22 kDa and 80 kDa, for the OppF, OppF-N and OppF-C respectively (S1 Figure). Immunostaining of the Western blots of the purified proteins using anti-86079/7NS chicken sera strongly stained the three lengths of OppF proteins. Sera from vaccinated chicken with MS-H also strongly bound to OppF-N but very weakly to OppF and OppF-C (S1 Figure).

3.4. The field strain 94011/18d and all ts^- MS-H reisolates except for MS-H^5 had oppF sequence consistent with that of the 86079/7NS

PCR amplicons of the anticipated size of 627/628 bp were generated from all MS strains/isolates examined in this study. Analysis of sequences of the amplicons revealed adenine deletion at nucleotide position 203 (position 468 in the whole gene of *oppF*) in MS-H and MS-H reisolate MS-H^5. The other MS-H reisolates (MS-H^1 MS-H^3 and MS-H^4), the vaccine parent strain 86079/7NS and the field MS strain 94011/18d did not have this deletion.

3.5. MS strains/isolates having vaccine type OppF had proportionally higher antibodies titres to OppF-N than OppF-C

The optimal absorbance of the OppF, OppF-N, OppF-C and MSPB antigens reactions against both anti-86079/7NS and anti-MS-H sera were obtained at a concentration of 2.5 μg/ml for antigens and dilution of 1/100 for sera. Furthermore, the HRP conjugated anti-sera was used at dilution of 1/1000.

Using optimum conditions described above, groups of chicken sera raised against MS strains/isolates with wild/vaccine type *oppF* gene were tested in OppF, OppF-N, OppF-C and MSPB ELISAs. Average ODs obtained from group W sera were 0.55, 0.43, 1.18 and 1.88, while those obtained from group V were 0.04, 0.18, 0.54, 1.16 in OppF, OppF-C, OppF-N and MSPB ELISAs, respectively (Table 1).

Ratios were calculated between ODs obtained in the three ELISAs using of OppF, OppF-C, OppF-N and those obtained in MSPB ELISA. The

Table 2
Ratios calculated from the OppF, OppF-N, OppF-C and MSPB ELISAs using chicken sera against MS strains/isolates with wild type (group W) or vaccine type (group V) OppF.

Groups	N[a]	Mean (SD[b])					
		MSPB/OppF	MSPB/OppF-C	MSPB/OppF-N	OppF/OppF-C	OppF/OppF-N	OppF-C/ OppF-N
W	55	4.49 (2.30)	8.87 (7.83)	2.39 (2.93)	1.44 (0.47)	0.41 (0.10)	0.34 (0.22)
V	33	17.10 (12.90)	24.00 (33.70)	3.49 (2.94)	2.01 (1.41)	0.30 (0.14)	0.26 (0.29)
P value[#]		0.01	0.01	0.09	0.24	0.05	0.18

[a] Number of serum samples in each group.

[b] Standard deviation.

[#] *P* value < 0.05 significantly different. Comparison was made between the same ratio in the two groups.

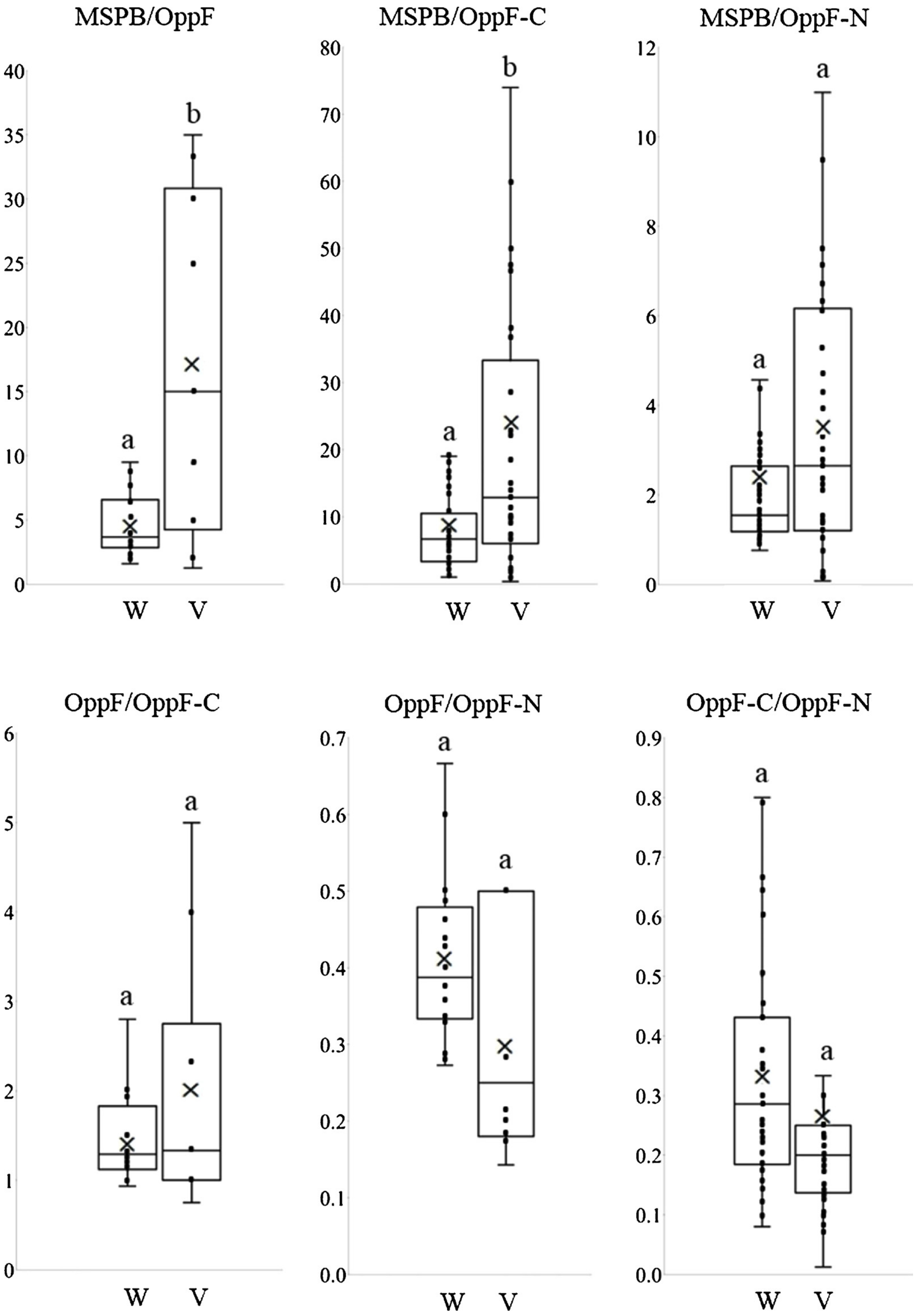

Fig. 4. Boxplots of ELISA OD ratios of MSPB/OppF, MSPB/OppF-C, MSPB/OppF-N, OppF-OppF-C, OppF/OppF-N and OppF-C/OppF-N. The OD ratios on vertical axis have been plotted against sera groups (chicken sera against MS strains/isolates with wild type (group W) or vaccine type (group V) OppF) on horizontal axis. Rectangles represent the interquartile range of each group. Whiskers have extended down to the minimum value and up to the maximum value. The circles represent the values of each group while the crosses show the mean. The horizontal lines inside the rectangles indicate the median. Comparing the same ratio, groups with different letters on top, are significantly different ($P = 0.01$).

mean, standard deviation and P values of these ratios are shown in Table 2, while interquartile range, maximum and minimum values, and median are shown in Fig. 4. The average ratios of MSPB/OppF, MSPB/OppF-C and MSPB/OppF-N were 4.49, 8.87, 2.39 for group W and 17.10, 24.00, 3.49 for group V, respectively. The average ratios of OppF/OppF-C, OppF/OppF-N and OppF-C/OppF-N were 1.44, 0.41 and 0.34 for group W, and 2.01, 0.30 and 0.26 for group V, respectively.

Comparison of the same ratio between the two groups using student *t*-test revealed that the OD ratios of MSPB/OppF and MSPB/OppF-C of the group V were significantly ($P = 0.01$) higher than those of group W.

4. Discussion

This study confirmed that the frame shift mutation found in the *oppF* gene of MS-H is reflected in truncation of its deduced protein OppF. Immunoblotting of MS-H and 86079/7NS whole-cell proteins with mono-specific rabbit sera against amino- and carboxy terminal of the 86079/7NS OppF showed that only a truncated amino-terminal end of OppF was expressed in the MS-H, while as expected, the full length OppF (containing both amino- and carboxy terminal) was expressed in 86079/7NS. The same mono-specific rabbit sera also detected multiple other bands in MS-H and 86079/7NS but given that band sizes were identical in both strains, they probably reflected nonspecific reaction of the rabbit sera and that they were unrelated to OppF (Fig. 3). It is also notable that there are two copies of *oppF* gene in MS and that only one copy is mutated in MS-H. Although there is a relatively poor identity (approximately 25%) between the two copies, one of these reacting bands could have been the second copy of the *oppF* gene since it is matched approximately to the predicted size (66 kDa) of the second copy of the OppF. Similar Western immunoblotting experiments using the same rabbit sera used as probe against *E. coli* whole-cell proteins expressing three versions of recombinant OppF revealed that, as expected, recombinant OppF (full length) and recombinant OppF-N were detected by rabbit-anti-OppF-N, and recombinant OppF and recombinant OppF-C were detected by rabbit-anti-OppF-C (Fig. 3). Also, recombinant OppF-N was weakly stained with rabbit-anti-OppF-C (Fig. 3) but this was possibly due to relatively large amount of recombinant OppF-N found in transformed *E. coli* cells and/or perhaps partially related to non-specific reaction of the rabbit sera. The latter was further evidenced by detection of similar size bands in whole cell proteins of all *E. coli* clones including untransformed *E. coli* cells. Smaller sized bands detected in *E. coli* cells expressing recombinant OppF and OppF-C were potentially break-down products of the larger recombinant OppF (Fig. 3).

Also, as expected, staining of Western blots of whole-cell proteins of the *E. coli* expressing recombinant OppF proteins with anti 86079/7NS chicken serum detected all three recombinant OppFs. Anti MS-H chicken serum strongly stained recombinant OppF-N as expected but also weakly stained OppF-C (Fig. 2). It is possible that this weak reaction may have been due to the second copy of *oppF,* although they share very poor identity (see above).

OppF, an oligopeptide permease, is a highly conserved ATPase domain and belongs to ATP-binding cassette (ABC) systems (Nicolas et al., 2007). The ABC systems are mainly involved in cell nutrition, but some are indirectly associated with virulence through controlling the expression of virulence genes in pathogenic bacteria (Moraes et al., 2014; Nicolas et al., 2007). The attenuated virulence phenotype of *Staphylococcus aureus* and *Streptococcus pneumoniae* have been demonstrated in animal infection models through signature-tagged mutants in ABC transporter genes, one of which was *oppF* (Mei et al., 1997; Nicolas et al., 2007; Polissi et al., 1998). Given that MS-H only expresses very small section of the OppF, it can be used as a model to investigate the role of OppF in both *in vitro* and *in vivo* studies. Given that MS-H harbours several other mutations other that found in *oppF* gene, such investigations would require side by side comparison with MS-H complemented with wild type *oppF* using *oriC* plasmid constructs recently developed in our laboratory (Shahid et al., 2014a, 2014b). Alternatively, natural MS-H field reisolates which have reversion to wild type *oppF* may be used. The temperature sensitivity and pathogenicity of a number of MS-H field reisolates have been examined in previous studies (Noormohammadi et al., 2003) but their colony morphology or *in vitro* growth dynamics have not been investigated. Possible *in vitro* effects of *oppF* mutation that could be investigated of complemented MS-H with wild type *oppF* could be subjected to investigation include growth characteristics and temperature sensitivity, whereas *in vivo* effects can include pathogenicity in chickens under experimental conditions.

Also, Western immunoblotting experiments confirmed for the first time that OppF is antigenic, inducing antibody response in the host (chickens), although this was not unexpected since preliminary *in silico* analysis of OppF had indicated that it had antigenic domains (result not shown). The lack of expression of the major C terminal region of the OppF in MS-H was further evidenced in ability of recombinant OppF-C in an indirect ELISA to discriminate antibody responses induced by MS-H and its reisolate with *oppF* mutation from those induced by strains that have intact *oppF* gene. This indicated that OppF-C can potentially serve as an antigen in a Differentiating Infected from Vaccinated Animals (DIVA) serological assay. A ratio between OppF-C and *M. synoviae* major membrane antigen MSPB was used in this study to adjust the antibody titres induced in individual birds by MS, so that OppF-C antibodies could be compared between birds. To our best knowledge, this is the first time such system has been used to develop a DIVA assay. The true DIVA potential of the ELISA system developed here require examination of sera from field birds vaccinated with MS-H versus those obtained from birds infected with field strains. In particular, potential cross reaction of OppF from other pathogenic or non-pathogenic avian mycoplasmas that may coinfect the birds in the field should be investigated. These investigations should focus on detailed sequence analysis of OppF from common avian mycoplasmas including *M. gallisepticum*, *M. gallinarum*, *M. gallinaceum*, *M. pullorum* and selection of a *M. synoviae* specific domain for further examinations.

Partial *oppF* gene sequence analysis of several ts- MS-H reisolates in this study revealed that only one reisolate (MS-H^5) had the mutation consistent with MS-H (adenine deletion at position 468) while three others (MS-H^1, MS-H^3 and MS-H^4) did not contain this mutation. This finding suggests that adenine deletion at position 468 may not be stable. However, a recent study in our laboratory has shown that *oppF* mutation is stable in MS-H reisolates at least after 5 consecutive passages *in vivo* in experimentally inoculated SPF birds (Zhu et al., 2017). It should be noted however that the isolates used in our study represent only a small number of isolates from MS-H vaccinated field birds and that were confirmed as MS-H through RFLP of their genomic DNA and further selected because of their ts- phenotype(Morrow et al., 1998). In order to obtain a clear perspective of the frequency of reversion of this *oppF* gene mutation under field condition, a large number of MS-H field reisolates selected irrespective of their ts phenotype, need to be examined. Also, such reisolates need to be subjected to full molecular characterisation (i.e. through whole genome sequencing) to ensure that they are true MS-H reisolates, and not field strains. Moreover, to demonstrate the true value of the specific diagnostic method based on *oppF* gene, the frequency of other mutations at *oppF* should be investigated (Felde et al., 2018).

Interest in studying ABC transporters is increasing as evidenced by these molecules being targeted for the development antibacterial vaccines, antitumor agents and antimicrobials (Garmory and Titball, 2004; Nicolas et al., 2007). Mutation found in the MS-H OppF and some of its reisolates can present an invaluable model that can be readily used under *in vitro* and *in vivo* conditions to study the role of ABC transporters in bacterial microorganisms.

5. Conclusion

Results of this study shows that deletion found in the MS-H *oppF* gene causes truncation of OppF in this strain. The indirect ELISA based on OppF-C has the potential to differentiate between MS-H and field strain antibody responses.

Conflict of interest

The authors have declared no conflict of interest.

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Authors’ contributions

Amir H. Noormohammadi and Somayeh Kordafshari conceived the idea, designed the study and interpreted the results. Somayeh Kordafshari conducted the laboratory work and analysed the data, and drafted the manuscript. Marc S. Marenda provided advice on bioinformatics. Denise O’Rourke provided technical assistance for ELISA and Pollob Shil provided advice on the structure of the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.02.029.

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