Indian Journal of Animal Sciences **91** (2): 96–99, February 2021/Article
https://doi.org/10.56093/ijans.v91i2.113814

Cloning of cytadhesin protein gene (*pvpA*) and expression analysis of recombinant fusion protein of *Mycoplasma gallisepticum*

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Received: 14 February 2020; Accepted: 7 July 2021

ABSTRACT

Chronic respiratory disease (CRD) caused by *Mycoplasma gallisepticum* (MG) is one of the major respiratory tract infections of the poultry, resulting in significant economic loss to the poultry farmers. Diagnosis of such ailment is highly necessary for effective control measures. In addition, promising molecular tools are warranted for efficient epidemiological tracing of the outbreaks. The study was focused on the elucidation of phase variable cytadhesin protein gene (*pvpA*) of MG through cloning and expression analysis. A set of primers targeting the *pvpA* gene of MG was designed. The complete *pvpA* gene was amplified and cloned into pUC-derived expression vector *pRSETA*. Finally, the recombinant clones were examined through colony PCR and restriction endonuclease (RE) analysis with *Eco*R1 and *Bam*H1 enzymes followed by sequencing. The expression of the recombinant *pvpA* gene was optimized at 1.4mM/µl concentration of Isopropyl-β-D-thiogalactoside induction at 30°C. The recombinant fusion protein was purified by immobilized metal affinity chromatography and characterized by SDS-PAGE followed by confirmation of recombinant cytadhesin fusion protein through western blot analysis. The *pvpA* gene was successfully cloned and expressed. The deduced amino acid sequence analysis had shown the presence of two direct repeats (DR1 and DR2) along with predicted PRP motifs repeatedly with high proline encoding regions at the carboxy-terminal of *pvpA* gene indicating its scope for epidemiological studies.

Keywords: Chronic respiratory disease (CRD), Cloning, Expression, *Mycoplasma gallisepticum*, *pvpA* gene

Avian mycoplasmosis, caused by *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) in chickens imparts huge economic loss to the poultry industry. Chronic respiratory disease (CRD) caused by MG is characterized by respiratory tract involvement in poultry manifested by sneezing, coughing, rattling sound with reduced egg production and hatchability (Nascimento *et al.* 2005). Both broiler as well as and layer chickens of various age groups are susceptible to CRD (Manimaran *et al*. 2019). Thorough epidemiological investigation along with accurate diagnosis and suitable therapeutic measures are highly sought for controlling the disease. Understanding the epidemiology of a disease is important factor for an effective control strategy. Study of the novel genetic markers as potential targets for molecular typing of the pathogen is highly sought for investigating the nature of the disease outbreaks. On the surface of the *Mycoplasma,* lipoproteins are found, which are involved in attachment of the bacteria. These lipoproteins are involved in considerable variation in the expression of the surface antigens of the organism (Lockaby *et al*. 1999, Lam 2000, Bradbury and Morrow 2008). The present study was focused on selection of a suitable candidate *Mycoplasma* membrane protein (MP) gene, i.e. phase variable cytadhesin protein gene (*pvpA*) for further elucidation through cloning and expression analysis.

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MATERIALS AND METHODS

Designing of primers: The primers were designed from *pvpA* gene (NCBI GenBank Accession No. AF224059.1) with restriction sites of *Bam*H1 and *Eco*R1 in forward and reverse primers, respectively. The primers were as follows. Forward primer: 5′- CATAGC *GGA TCC* ATG GGG CAA GAG TTA AAT AAA TTA -3′ and the reverse primer: 5′- AGATGC *GAA TTC* TTA AGC TCG GTT TGG CCC ACC ATT TG -3′ (the italicized portions designate the restriction sites).

DNA extraction: Mycoplasma gallisepticum (MG) DNA was extracted from the MG isolate available in the laboratory [isolated from chicken (Lohmann Brown) from

Namakkal, Tamil Nadu state of India in 2014] using DNA Mini kit (Qiagen, Germany) as per the manufacturer's instruction.

Polymerase chain reaction (*PCR*)*:* The *pvpA* gene was amplified from MG genomic DNA in a thermal cycler (Eppendorf Pro-S, Germany). The PCR reaction mixture of 25 µl was composed of 12.5 µl of EmeraldAmp® Max PCR master mix (Takara Bio Inc., Japan), 1 µl each of forward and reverse primers of 10 picomol/µl concentration (0.4 µM final concentration of each primers) and appropriate amount of template DNA and nuclease-free water to adjust the volume. The standardized amplification protocol includes initial denaturation at 94°C for 5 min. followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min and a final extension phase of 72°C for 7 min. The amplified products were separated in agarose gel (1.5%) stained with ethidium bromide (0.5 µg/ml) in 1 × TAE buffer at a constant voltage of 80V and was visualized under Ultraviolet (UV) light using molecular imager® Gel Doc™ XRT with Image Lab™ software and recorded by gel documentation system (Universal Hood II, BioRad, USA). The amplified product was further purified for subsequent studies.

Cloning: The insert as well as the cloning/expression vector *pRSETA* were digested with *Eco*R1-HF and *Bam*H1-HF restriction enzymes and ligated using T4 DNA Ligase (5 U/µl) (0.3 µM final concentration). The ligated product was transformed into chemically competent expression host *E. coli* strain *BL21* cells. The transformed colonies were streaked on the LB agar plate containing the antibiotic Ampicillin (Amp^+). The recombinant clones, selected on LB agar plate were screened through colony PCR and further analyzed by restriction enzyme digestion analysis to release the insert from the recombinant plasmid. The recombinant plasmid was analyzed by sequencing using the forward primer (T7-universal forward): 5′-TAA TAC GAC TCA CTA TAG GG-3′ and reverse primer (T7-terminator): 5′-GCT AGT TAT TGC TCA GCG G-3′ (each 0.4 µM final concentration) prior to expression studies for the correct orientation of the gene and absence of any mutation.

Expression: A single colony of *pvpA* recombinant clone was inoculated into 5 ml of Luria-Bertani (LB) + Ampicillin (Amp^+; 50 µg/ml) broth incubated overnight at 37°C at a constant speed of 150 rpm. Overnight culture (2 ml) was inoculated into 50 ml of LB broth and incubated at 37°C at a constant speed of 180 rpm till the optical density reached 0.6. The expression of recombinant protein was optimized at various concentration of Isopropyl-β-D-thiogalactoside (IPTG) at a wide range of incubation temperature, i.e. 28°C to 35°C.

Blotting: Western blotting was carried out for confirmation of the expressed protein. The recombinant protein was purified through affinity column chromatography using nickel-nitriloacetic acid (Ni-NTA) resin. The purified protein was transferred to nitrocellulose membrane at 100V and 150 mA for 1 h.

RESULTS AND DISCUSSION

Chronic respiratory disease of chickens, caused by *M. gallisepticum* is a disease of major concern among the poultry farmers. Interestingly, these causative agents exhibit a high degree of phenotypic variation as well as variation in the surface topology which ultimately results in altered pathogenicity. Considerable variation among the surface epitopes in Mycoplasmas is a major concern (Bencina *et al*. 1994, Garcia *et al*. 1994, Levisohn *et al*. 1995). The *pvpA* gene encodes the phase-variable putative adhesin protein in Mycoplasma (Boguslavsky *et al*. 2000). The importance of adhesin-encoding *pvpA* gene in epidemiological studies was well-emphasized among the *Mycoplasma gallisepticum* strains (Liu *et al*. 2001, Bencina 2002). Interestingly, this PvpA protein is an integral membrane protein with N-terminal buried inside the membrane and the C-terminal exposed to surface (Yogev *et al*. 1994) containing a proline-rich region (Boguslavsky *et al*. 2000, Bencina, 2002). The region interestingly contains two 52 amin acid long repeat sequences which were designated earlier as DR-1 and DR-2 showing sequence variation in between the repeats due to deletions (Boguslavsky *et al*. 2000). The *pvpA* gene size polymorphism was also well-investigated (Ferguson *et al*. 2005). The molecular variation of gene was also studied among the field isolates through sequencing along with restriction fragment length polymorphism (RFLP) analysis (Pillai *et al*. 2003). Hence, this gene possesses a greater importance as the suitable molecular typing tool (Liu *et al*. 2001). Furthermore, the multiple gene-targeted sequencing (GTS) analysis of various surface proteins including the PvpA was found suitable for epidemiological studies, which were found even more promising than random amplified polymorphic DNA (RAPD) analysis (Ferguson *et al*. 2005).

In the present study, the 1149 bp of *pvpA* gene along with TAA stop codon was successfully amplified (Fig. 1)

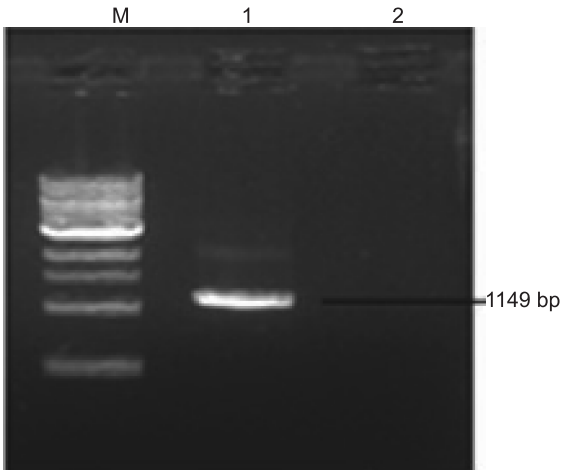

Fig. 1. Visualization of agarose gel showing amplified product of *pvpA* gene of *Mycoplasma gallisepticum* [Lane M, 1kb DNA Ladder (New England Biolabs); Lane 1, *Mycoplasma gallisepticum* DNA showing band at 1149 bp DNA fragment; Lane 2, Negative control]

and cloned in *pRSETA* vector. Further, the sequence of the target gene was deposited in NCBI GenBank with accession no. KY745775.1. The expression of recombinant protein was optimized at 1.4 mM/µl concentration of IPTG at 30°C incubation temperature. The expression was found optimum in 3 h of induction period after which it was found decreasing (Fig. 2). The PvpA protein with molecular weight of approximately 40 kDa was detected by western blot analysis (Fig. 3). An open reading frame of predicted 1149 nucleotide length of *pvpA* gene size was analyzed within the sequenced fragment. The PvpA protein size of 383 amino acid was analyzed *in silico* containing highly repeated proline rich regions at the carboxy-terminal comprising of repeated PRP (proine-arginine-proline) motifs (Boguslavsky *et al*. 2000). Similarly, two direct repeat sequences (termed as DR1 and DR2) comprising of 55 amino acids (QPRPQQAGPRPMGAGGSNQPRPM PNGPQNPQGPRPMNPQGDPRPQPAGVRPNSPQ) were found from 222nd to 276th (DR1) and 299th to 353rd (DR2) amino acid position respectively. There was a single amino acid residue mismatch at 247th position of DR1 (glycine) to that of 324th position of DR2 (arginine) as revealed from deduced amino acid sequence analysis. Similar to this study, the DR1 and DR2 sequences of 52 amino acids were reported in PvpA protein of MG (Boguslavsky *et al*. 2000).

Interestingly, variation of the PvpA protein size was observed among various strains of *M. gallisepticum*, as a result of several kinds of mutations (deletions/frameshift mutations) at the C-terminal of the gene and within DR1 and DR2 (Boguslavsky *et al*. 2000). Similarly, a premature termination of *pvpA* gene expression was reported in *Mycoplasma gallisepticum* strain R_{low} earlier due to frameshift mutation as a result of nucleotide duplication (Papazisi *et al*. 2003). One of the earlier studies suggested that lack of PvpA expression or its expression in a truncated protein adds to the nature of the MG in its adherence

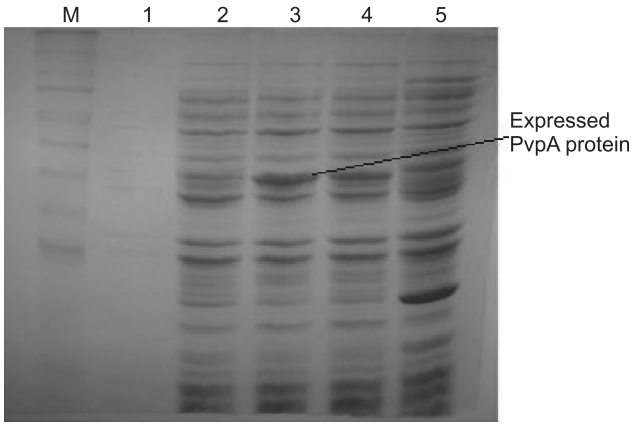

Fig. 2. Visualization of SDS-polyacrylamide gel showing *E.coli* expressed recombinant PvpA protein [Lane M, Broad Range Protein Marker; Lane 1, PvpA at zeroth hour (without IPTG); Lane 2, PvpA after overnight incubation (without IPTG); Lane 3, PvpA at third hour (1.4 mM IPTG at 30°C); Lane 4, PvpA at sixth hour (1.4 mM IPTG at 30°C); Lane 5, PvpA after overnight incubation (1.4 mM IPTG, 30°C)].

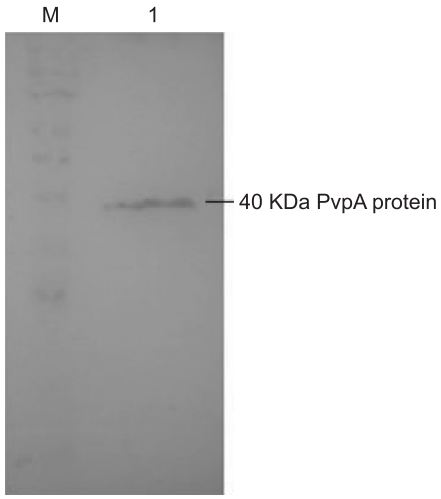

Fig. 3. Western blot analysis showing approximately 40 kDa PvpA protein [Lane M, Broad Range Protein Marker; Lane 1, Purified PvpA protein].

capabilities to the host (Boguslavsky *et al*. 2000). Even the GTS analysis described earlier reported the sequence stability over the period of *in vitro* passages (Ferguson *et al*. 2005). It indicates importance of the *pvpA* gene along with other cytadhesin protein genes as the molecular typing targets in investigating the field isolates.

In the present study, the *pvpA* of MG was successfully cloned and expressed in prokaryotic expression system. The study may further help in investigation of the disease outbreaks in the field for understanding the nature of the incidence and epidemiological tracking.

ACKNOWLEDGEMENTS

The funding provided by the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi to conduct the research work is duly acknowledged. The authors are also thankful to the Director (Centre for Animal Health Studies, TANUVAS) as well as the Professor and Head (Central University Laboratory, TANUVAS) for providing necessary facilities for the research work.

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