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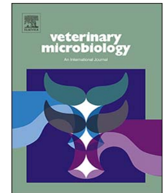

Original article

Identification of *Lawsonia intracellularis* putative hemolysin protein A and characterization of its immunoreactivity

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ABSTRACT

Despite the recent global increase in fatal endemic outbreaks of proliferative enteropathy (PE) caused by the obligate intracellular bacterium *Lawsonia intracelluralis* (LI) in the swine industry, development of effective prevention strategies or immunodiagnostic tests has been delayed due to the difficulty of cultivating this pathogen *in vitro*. Although several genetic analyses have been performed at the level of gene transcription after the complete genome sequence of LI was made available, the mechanism of LI infection and virulence genes remain unidentified. In the present study, we assessed the antigenic features of the LI0004 protein, which we putatively defined as *Lawsonia* hemolysin A (LhlyA), by employing bioinformatics tools and *in vivo* and *in vitro* protein-based molecular assays. The amino acid sequence of LhlyA showed approximately 60% homology to the hemolysin-like proteins of *Bilophila wadsworthia* and *Desulfovibrio piger*. Presence of computationally predicted linear antigenic B-cell epitopes on the LhlyA protein was demonstrated by immunoblotting; a band with a molecular mass corresponding to the predicted size of the protein was strongly recognized by sera collected from artificially infected mice. Further, in an *in vivo* cytotoxicity assay, no splenomegaly was observed in mice inoculated with purified LhlyA. Collectively, the data presented here suggest that the LhlyA protein is a highly immuno-reactive antigen of *L. intracellullaris* and can potentially be used to develop effective protection strategies against PE.

1. Introduction

Lawsonia intracellularis (LI) is a gram-negative, microaerophilic, obligate, intracellular bacterial pathogen and a causative agent of proliferative enteropathy (PE) (McOrist et al., 1995). The pathogen has a tropism for intestinal epithelial cells and only replicates in the cytoplasm of infected intestinal crypt, which induces enterocyte proliferation (Jacobson et al., 2010). PE poses a substantial economic burden in the swine industry worldwide and is occasionally reported in a variety of other animals including horses, dogs, rabbits, rats, hamsters, deer, and ostrich (Cooper et al., 1997; Pusterla et al., 2012; Watarai et al., 2008). Two major forms of PE are recognized: porcine intestinal adenomatosis (PIA) and proliferative hemorrhagic enteropathy (PHE) (Huerta et al., 2003). PIA, a chronic form of PE, causes mild diarrhea and weight loss in growing pigs aged 8 to 20 weeks (Guedes and Gebhart, 2003). PHE, an acute form of PE, causes bloody diarrhea and sudden death in finishers or breeder pigs aged 4 to 12 months, with high morbidity and mortality.

Despite endemic outbreaks and the large impact of PE on pig production, difficulties related to *in vitro* cultivation of LI have hindered the use of genetic approaches to elucidate the pathogenesis of PE (McOrist et al., 1995). Considering its entero-invasive characteristic, it has been speculated that LI escapes from the vacuolar compartment into the cytoplasm after internalization, multiplies, and spreads from cell to cell, resulting in the proliferation of immature enterocytes (Vannucci and Gebhart, 2014). However, it is not known how this pathogen disseminates beyond the infected intestinal epithelium, causes pathogenesis, and result in the clinical symptoms. This lack of knowledge has restricted the development of prevention strategies such as vaccines and diagnostic methods. Before completion of the genome sequence of LI, a few proteins expressed by infected in vitro cell cultures were elucidated using a PCR-based molecular technique (McCluskey et al., 2002) or a mass spectrometry approach (Watson et al., 2011). Recently, the pathogenic characteristics of LI were elucidated at the gene tran;1;scriptional level using genome sequence information (PHE/MN1-00; NCBI accession #NC_008011) (Vannucci et al., 2012). Although the availability of the complete LI genome sequence can facilitate identification of putative antigens, few studies have per-

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formed antigenic characterization of LI genes.

In this study, we report the antigenic properties of the LI0004 protein (accession: NC_008011), putatively defined as *Lawsonia* hemolysin A (LhlyA). Antigenic properties were characterized by computational bioinformatics analysis of amino acid sequences and computational prediction of epitopes. Potential cytotoxicity and hemolytic activity of the protein were also assessed. To assess this protein's immunological properties, it was reacted with immune serum raised against an avirulent LI strain. Overall, we established that this protein is a highly immuno-reactive antigen that might have the ability to induce the production of protein-specific antibodies. This could contribute to the development of effective prevention strategies against proliferative enteropathy.

2. Materials and methods

2.1. Bioinformatics analysis

Homology of the *Lawsonia* hemolytic protein (LhlyA) to other proteins was assessed using NCBI BLASTP and PSI-BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and GeneDoc software (Nicholas et al., 1997). To predict linear antigenic B-cell epitopes in the protein, the peptide property calculator on the BepiPred server (http://www.cbs.dtu.dk/services/BepiPred) was used with an automated threshold score based on a hidden Markov model and a propensity scale method (Larsen et al., 2006). Further, three different *in silico* B-cell epitope predictors, BCPred, AAP, and FBCPred, were used to determine linear B-cell epitopes of the protein according to the protocol described previously (EL-Manzalawy et al., 2017; Singh et al., 2013).

2.2. Construction of recombinant plasmids harboring hlyA

The bacterial strains and plasmids used in this study are described in Table 1. The full-length gene (LhlyA,762 bp) encoding a putative hemolysin protein of LI, LI0004 was chemically synthesized (Bioneer, Korea). To assess the hemolytic activity of purified LhlyA protein, the enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (Ehly) was used as a positive control. The *Ehly* gene was amplified in JOL364 isolated from infected bovine using PCR with the primer pairs Ehly_F: 5′-GGATCCATGACAGTAAATAAAATAAAGAACA-3′ and Ehly_R:5′- GTCG-ACTCAGACAGTTGTCGTTAAAGTT-3′. The synthesized LhlyA and the PCR product, Ehly, were digested with the restriction endonucleases XbaI and BamHI, and the cleaved fragments were subcloned into plasmid pET28a (+), which bears a kanamycin-resistant maker (Kan^r) and a carboxy terminal 6-histidine-tag for protein purification using Ni-NTA resin. pET28a-*LhlyA* and pET28a-*Ehly* were individually electrophorated into *E. coli* BL21 (DE3) pLysS and designated JOL1742 and JOL1898, respectively. Target proteins were purified using an affinity purification procedure with a Ni^{2+}-nitrilotriacetic acid-agarose support (Qiagen, GmbH. Hilden, Germany) according to the procedure described previously (Xie et al., 2010). Proteins were quantified using the Bradford protein assay (Kruger, 1994). *E. coli* cells harboring the expression vector $pET28a^+$ were grown in Luria broth (LB) containing kanamycin (50 μg/ml) at 37 °C.

Table. 1
Bacterial strains and plasmids utilized in this study.

Strains/plasmids	Description	References
Strains		
BL21(DE3)pLysS	*F- ompT hsdSB (rB- mB-) dcm galλ(DE3) pLysS Cmr*	Promega
JOL364	Wild type Hly+ EHEC isolate from bovine	Lab stock
JOL1742	*E. coli* BL21(DE3) pLysS expressing *LhlyA via* pET28a (+) system	This study
JOL1898	*E. coli* BL21(DE3) pLysS expressing *E. coli* hemolysine gene via pET28a (+) system	This study
Plasmids		
pET28(+)	IPTG-inducible, T7 expression vector, C-terminal 6 x His tag, Kan^R	Novagen, USA
pET28(+)-*LhlyA*	pET28a derivative containing *LhlyA*	This study
pET28(+)-*EHly*	pET28a derivative containing *Ehly*	This study

2.3. Western blot analysis

Serum was collected from mice orally injected with a vaccine strain of *Lawsonia intracellularis* (Enterisol® Ileiti, Boehringer Ingelheim). Mice were inoculated with $5 \times 10^{6.9}$ TCID50 (tissue culture infectious doses) of the vaccine strain twice at two-week intervals to obtain immune sera against LI. Serum from a mouse inoculated with sterile PBS was used as the negative control. Purified LhlyA protein sample (100 μg total protein) was boiled in Laemmli SDS sample buffer for 10 min and separated on 15% SDS-PAGE gel and then transferred onto PVDF membrane. The membrane was blocked in 3% BSA and then incubated with the anti-LI mouse hyper immune serum at a 1:300 dilution for 2 h. After thoroughly washing the membrane with PBS- 0.1% Tween 20, the membrane was incubated with HRP conjugated anti-mouse IgG (Southern Biotechnology) at a 1:5000 dilution for 1 h and then developed by addition of Peroxidase Western Blotting Substrate (Sigma Co., St. Louis, Mo.).

2.4. In vivo cytotoxicity assay in a mouse model

Specific pathogen-free BALB/c inbred mice experimental procedures were approved (CBNU2015-00085) by the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, 2007; Article 13 (Experiments with Animals). The *in vivo* cytotoxicity of the putative antigenic protein, LhlyA, was evaluated by examining changes in spleen morphology in mice injected with the purified protein (Maamar et al., 2016). Five-week-old female BALB/C mice (n = 30) were divided into two groups (n = 15). Group A mice were inoculated with 100 mg/ml of LhlyA protein *via* an intraperitoneal (i.p.) route at week 0. Mice in group B were also inoculated with sterile PBS *via* i.p. route at week 0. Three mice in each group were aseptically sacrificed weekly up to week 4, and spleen samples were collected, weighed, and sized individually.

2.5. RBC lysis assay

Whole blood samples were collected from mice, chicken, and pig in a vacutainer tube with EDTA. Subsequently, RBCs were isolated from the whole blood and washed twice following a procedure described previously (Wang et al., 2007). A 1% suspension of the washed RBCs sampled from each animal was prepared by adding 100 μl of the washed RBCs to 900 μl sterile saline. Purified proteins, LhlyA and Ehly (500 ng per well), were mixed with each type of animal RBC preparation (1%), briefly centrifuged to establish physical contact with the RBCs, and then incubated for 24 h at room temperature. At the end of the incubation, cell suspensions were centrifuged to remove unlysed cells, and the absorbance of the supernatant containing released hemoglobin was measured at 540 nm in a spectrophotometer.

3. Results

3.1. Characterization of LhlyA as a putative antigen

Based on the genome sequence of *Lawsonia intracellularis*, LI0004 was identified as being homologous to the hemolysins of *Bilophila wadsworthia* and *Desulfovibrio piger*, and designated as a 'TlyA-like protein'. This protein showed greater than 60% similarity to other hemolysin proteins (Fig. 1) (64% identity to the hemolysin protein of

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