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Full Length Article

Impacts of *lmo2672* Gene Deficiency on Pathogenicity of *Listeria monocytogenes*

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Abstract

Listeria monocytogenes (LM) is an important food-borne pathogen, which is severely harmful to human and animals. In order to explore the roles of lmo2672 on pathogenicity of LM, a *⊿lmo2672* deletion strain was generated using homologous recombination technique, and then the virulence of *⊿lmo2672* mutant was analyzed in macrophage cells and mice. The transcription of virulence-related genes was also determined by real-time RT-PCR. Besides, the interaction between lmo2672 and the upstream DNA sequence of *prfA* gene was investigated by electrophoretic mobility shift assay (EMSA). The results showed that 1) the hemolytic ability of the *⊿lmo2672* mutant was significantly weaker than that of wild-type strain LM EGD-e ($P < 0.05$); 2) the invasion and intracellular proliferation in macrophage cells RAW264.7 of LM-*⊿lmo2672* were significantly decreased ($P < 0.05$); 3) the 50% lethal dose (LD_{50}) to mice for the *⊿lmo2672* mutant was significantly higher than wild-type strain ($P < 0.05$); 4) the bacterial loads of the *⊿lmo2672* strain in mouse liver and spleen were significantly less than that of wild-type strain ($P < 0.05$); 5) the transcription levels of virulence-related genes *prfA*, *actA* and *hly* in *⊿lmo2672* mutant declined significantly ($P < 0.05$) as compared with LM EGD-e. Furthermore, EMSA confirmed that there existed the interaction between lmo2672 and the upstream DNA sequence of *prfA* gene. These findings suggested that AraC family transcription regulator lmo2672 is involved in pathogenicity of LM, which provided an insight into the biological function of lmo2672 in LM virulent regulation.

Keywords: *Listeria monocytogenes*; *lmo2672*; Pathogenicity; AraC family

Introduction

Listeria monocytogenes (LM) is a Gram-positive zoonotic pathogen that infects humans through ingestion of contaminated food (Pérez-Trallero *et al.* 2014; Fagerlund *et al.* 2016), which can cause abortion in pregnant women, meningoencephalitis in infants, gastroenteritis and other fatal illnesses (Lotfollahi *et al.* 2017). Likewise, livestock population is mainly infected after fed with LM-contaminated feed, leading to abortion and a high mortality rate of 20–30% (Lomonaco *et al.* 2015; Boqvist *et al.* 2018; McDougal and Sauer 2018; Yao *et al.* 2018). What's more, as an important food-borne pathogen, LM can survive in a variety of complex environmental stress conditions, such as low temperature, high temperature, high concentration of salt and low pH, causing serious harm to food safety and animal husbandry (Colagiorgi *et al.* 2017; Boqvist *et al.* 2018; Chaturongakul *et al.* 2008; Hurley *et al.* 2019).

As a facultative intracellular pathogen, the process of LM infection requires the participation of many virulence factors and genes, such as internalin A (inlA), internalin B (inlB), adhesion protein (LAP), phospholipase C (plcA and plcB), listeriolysin O (LLO) and actin polymerizing factor (actA) (Chen *et al.* 2017; Drolia *et al.* 2018). However, the expression of these virulence factors is regulated by PrfA, SigB and other regulators, enabling LM to survive and reproduce in complex intracellular environments (O'Byrne and Karatzas 2008; Heras *et al.* 2011).

So far, many studies have found that AraC family proteins play important roles in regulating virulence, multiple drug resistance and metabolic reactions in bacteria (Gallegos *et al.* 1997; Bailey *et al.* 2010). lmo2672, as a member of AraC family protein, is encoded by a novel differential gene *lmo2672* between virulent and avirulent

strains of LM (Roche *et al.* 2008; Hadjilouka *et al.* 2016), However, the biological function of lmo2672 is still unclear. The purpose of this study is to explore the role of *lmo2672* on pathogenicity by construction of *⊿lmo2672* deletion strain and its comparison with that of the wild-type strain, which would provide insights into the biological function of lmo2672 in LM virulent regulation.

Materials and Methods

Strain and plasmid

LM EGD-e strain (serotype 1/2a) was kindly donated by Dr. W. Goebel, University of Woodsburg, Germany. A temperature-sensitive plasmid pKSV7 was a gift from Professor Zhu Guoqiang of Yangzhou University. *Escherichia coli* (*E. coli*) DH5α (TaKaRa, Japan) and BL21 (DE3) (TaKaRa, Japan) were grown in Luria-Bertani (LB) broth (Difco, USA). LM strain was cultured in brain-heart infusion (BHI) broth (Difco, USA) or tryptic soy agar (TSA) plate (Difco, USA) at 37ºC

Primer design and synthesis

Eleven pairs of primers were designed by Primer Premier 5.0 (Premier Inc., Canada) according to the genome sequence of LM EGD-e in GenBank (accession number: AL591824.1) (Table 1). The primers were synthesized by Huada Biotechnology Co., Ltd. (Beijing, China).

Generation of LM-*⊿lmo2672* deletion strain

A *⊿lmo2672* deletion strain was constructed using homologous recombination technique. Briefly, the DNA fragments containing upstream and downstream regions flanking the *lmo2672* gene in LM EGD-e strain were amplified using PCR with primers F1/F2 and F3/F4, respectively. The PCR products were recovered and the deletion fragment of the *lmo2672* gene was generated using overlap extension PCR (SOE-PCR). The deletion fragment was then cloned into the plasmid pKSV7 to generate pKSV7-*⊿lmo2672*. The recombinant shuttle plasmid (pKSV7-*⊿lmo2672*) was introduced into a competent strain LM EGD-e by electroporation (2.5 kV, 100 Ω and 25 μF). Then, the transformed bacteria were selected on BHI agar (Difco, USA) with chloramphenicol (12.5 μg/mL) (Amresco, USA) at 42ºC for several passages. The recombinant strain LM-*⊿lmo2672* was further screened by PCR with the primer pair F7/F8, and PCR product was sequenced for molecular identification.

Assay of hemolytic activity

In brief, overnight cultures of wild-type strain and *⊿lmo2672* mutant were diluted 1:10 in fresh medium, respectively, and 100 μL of the diluted bacterial suspension was cultivated on tryptic soy agar (TSA) plate (Difco, USA) with 5% sheep red blood cells (SRBCs) and incubated at 37ºC for 24 h. The size of transparent hemolytic ring around the colony was recorded. The hemolytic activity was determined as previously reported with slight modifications (Alonzo *et al.* 2009). The optical density at 600 nm (OD_{600}) was measured and normalized for each strain (OD_{600} = 0.5), followed by the addition of 1 mL PBS (pH=5.6) containing SRBCs, then 30 min of incubation at 37ºC. The mixtures were then centrifuged to pellet unlysed cell and the hemoglobin absorbance in the supernatants was measured at 543 nm.

Macrophage adhesion, invasiveness and intracellular survival assays

Briefly, mouse macrophages cells RAW264.7 were seeded in the 6-well plates (Gibco, USA) at 37ºC in 5% CO_2. Wild-type strain and *⊿lmo2672* mutant were collected at the mid-log phase, respectively. Then washed three times with PBS (pH=7.2) and re-suspended in DMEM (Gibco, USA). The cells were subjected to infection with the wild-type strain and *⊿lmo2672* mutant at a multiplicity of infection (MOI) of 10:1. After infection, the cells were washed three times with PBS, and lysed with 500 μL 0.1% Triton X-100 (Amresco Inc., USA) for 10 min. Then the bacteria in the lysate were counted. The adhesion rate, invasion rate and survival rate were calculated as previously reported (Zhang *et al.* 2013). All the infections were performed three replicates.

Mouse virulence assay

The overnight cultures of wild-type strain and LM-*⊿lmo2672* were plated on BHI agar to calculate the colony number. Three groups of Six-week-old BALB/c mice, 10 mice per group, were inoculated intraperitoneally with 200 μL different dilutions of bacteria (10^5, 10^6, 10^7, 10^8, and 10^9 CFU/mouse, respectively), one group was inoculated with 200 μL sterile PBS (pH=7.2). Then mice were assessed 10 days after inoculation and recorded for the number of deaths daily and the LD_{50} was calculated using the Karber method. Besides, according to the death of mice in the group of 10^7 CFU injection dose, the Kaplan-Meier survival curve was drawn. Meanwhile, the mice were divided into three groups randomly and intraperitoneally injected with 2×10^5 CFU of LM EGD-e, LM-*⊿lmo2672* and sterile PBS (pH=7.2), respectively. Then, the spleens and livers of mice were homogenized, and bacteria loads were determined through enumeration of CFU. On the fifth day after infection, the organs were fixed in formalin and tissue sections were prepared for histopathological analysis.

Transcriptional analysis of virulence genes

Briefly, LM-*⊿lmo2672* and LM EGD-e were incubated to the logarithmic growth phase. At this point, bacteria were harvested by centrifugation, and total RNA were extracted

for using Trizol agent (Invitrogen, USA). Reverse transcription into cDNA was performed using the AMV reverse transcription kit (TaKaRa, Japan). The qRT-PCR was performed using the reversely transcripted cDNA using SYBR Premix Ex TaqTM kit (TaKaRa, Japan) according to the manufacturer's instructions on LightCycler 480 instrument (Roche, Swiss). qRT-PCR analysis was conducted to determine the effects of lmo2672 on the transcription of genes associated with virulence, *sigB*, *prfA*, *hly*, *actA* and *inlB* genes were determined using the $2^{-\Delta\Delta CT}$ method with the housekeeping gene *rpoB* as internal reference.

Electrophoretic mobility shift assay (EMSA)

Eectrophoretic mobility shift assay (EMSA) was carried out to further confirm the interaction between lmo2672 and the upstream DNA sequence of *prfA* gene (containing promoter P1 and P2). Briefly, the full length *lmo2672* gene was cloned into pET32a vector, and then the recombinant plasmid (pET32a- *lmo2672*) was transformed into DE3 for the expression under the induction of 0.5 m*M* IPTG for 8 h at 37ºC. The recombinant protein lmo2672 was purified using Ni-NTA affinity chromatography (Invitrogen, USA). The promoter fragment of *prfA* gene was generated by PCR and purified using a Gel Extraction Kit (Omega, USA). The lmo2672 protein was mixed with 100 ng DNA in 20 μL of the gel-shift binding buffer (100 m*M* NaCl, 100 m*M* Tris-HCl, 1 m*M* DTT, 10% glycerol). Meanwhile, Bovine serum albumin (BSA) was used as negative control. After incubation at 25ºC for 30 min, the sample was analyzed by 8% non-denaturing polyacrylamide gel electrophoresis, then the polacrylamide gel was stained with ethidium bromide (EB) and visualizaed under ultraviolet light.

Statistical analysis

One-way analysis of variance was performed using GraphPad Prism software, version 5.0 (GraphPad Software Inc., USA). The analyses were expressed as the mean ± standard error of the mean. The statistical comparison between different variables was done using $P < 0.05$ and $P < 0.01$ as level of significance.

Results

Using the flanking primer pair F5/F6, a 1502 bp fragment was generated from LM-*⊿lmo2672*. Compared with the wild-type strain LM EGD-e, 807 bp was lost from LM-*⊿lmo2672* (Supplementary Fig. 1A). After 20 passages, a fragment of expected size was amplified by PCR (Supplementary Fig. 1B). Sequence analysis showed that the *lmo2672* gene was successfully deleted (Supplementary Fig. 2).

The hemolytic ring (Fig. 1A, Fig. 1B) and erythrocytolysis (Fig. 1C, Fig. 1D) of LM-*⊿lmo2672* were smaller or significantly weaker than these of wild-type strain ($P < 0.05$), indicating that the deletion of *lmo2672* reduces the hemolytic ability of LM, which is consistent with the results of transcription level.

Compared with wild-type strain LM EGD-e, there was no significant impacted adhesion rate of*⊿lmo2672* mutant ($P > 0.05$) (Fig. 2A), but the invasion rate was significantly lower ($P < 0.01$) (Fig. 2B). Within 12 h after infection, the proliferation capability to RAW 264.7 cells in *⊿lmo2672* mutant strain was significantly lower than that in wild-type strain LM EGD-e ($P < 0.01$) (Fig. 2C), suggesting that *lmo2672* gene deficiency can reduce the ability of LM infection.

Compared with wild-type strain LM EGD-e, the LD_{50} of *⊿lmo2672* mutant increased by 6.3 times ($P < 0.05$) (Fig. 3A). The average survival time of mice infected with *⊿lmo2672* mutant was significantly increased ($P < 0.05$) (Fig. 3B). The bacterial load of LM-*⊿lmo2672* was significantly less than that of wild-type strain ($P < 0.05$) in liver (Fig. 3C), while, the bacterial load was significantly less than that of wild-type strain ($P < 0.05$) in spleen (Fig. 3D), suggesting that the gene deletion of *lmo2672* significantly reduced survival and reproduction ability of wild-type strain in liver and spleen.

Compared with normal mice (injected with PBS) (Fig. 4E, Fig. 4F), histopathological changes in liver of the mice injected with wild-type strain and *⊿lmo2672* mutant were characterized by vacuoles in hepatocytes, degeneration and necrosis, and hemorrhagic necrosis, while the structure of splenic cord disappeared with hemorrhage and lymphocyte infiltration. Compared with wild-type strain LM EGD-e (Fig. 4A and Fig. 4B), however, the pathological changes of liver and spleen in LM-*⊿lmo2672* infected mice were significantly reduced (Fig. 4C and Fig. 4D).

The qRT-PCR analysis revealed that *lmo2672* deletion down-regulated the expression of five genes associated with virulence. Compared with wild-type strain, the transcription levels of virulence genes *prfA*, *hly* and *actA* in *⊿lmo2672* mutant except *inlB* and *sigB* were significantly lower ($P < 0.05$) (Fig. 5).

The recombinant protein lmo2672 could bind to upstream DNA sequence of *prfA*, (Fig. 6, Supplementary Fig. 3), which implied that there was an interaction between lmo2672 and the upstream DNA sequence of *prfA* gene.

Discussion

AraC family transcription regulators (AFTRs) have been shown to be an important class of regulators in bacteria. They are generally composed of 200 and 300 amino acids arranged in two characteristic domains: one is the conserved DNA binding domain at the C-terminal (CTD), the other is the variable N-terminal domain (NTD) (Gallegos *et al.* 1997; Yang *et al.* 2011), which are necessary for *in vivo* transcriptional activation (Porter and Dorman 2002).

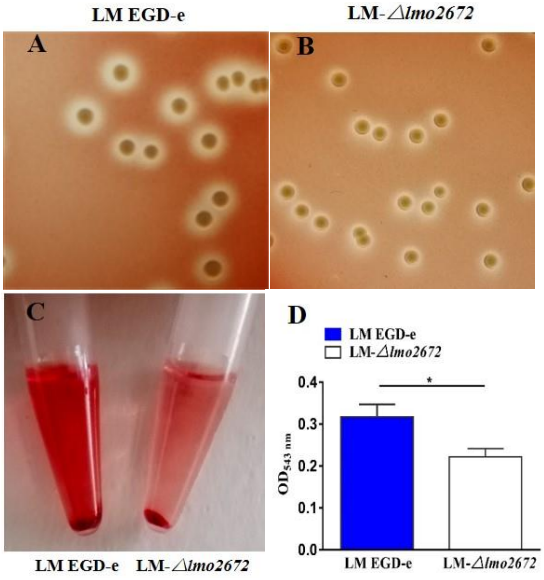

Fig. 1: Determination of hemolytic activity of wild-type strain LM EGD-e and LM-*⊿lmo2672*

(**A**) and (B): LM EGD-e and LM-*⊿lmo2672* cultured on sheep blood agar for 24 h, respectively. (**C**): Hemolytic activity of mutant and LM-*⊿lmo2672*. (**D**): OD_{543} nm value of the supernatant was measured. Error bars represent the mean ± standard error (SE) of triplicate experiments. Stars indicate *P*-values < 0.05 (*)

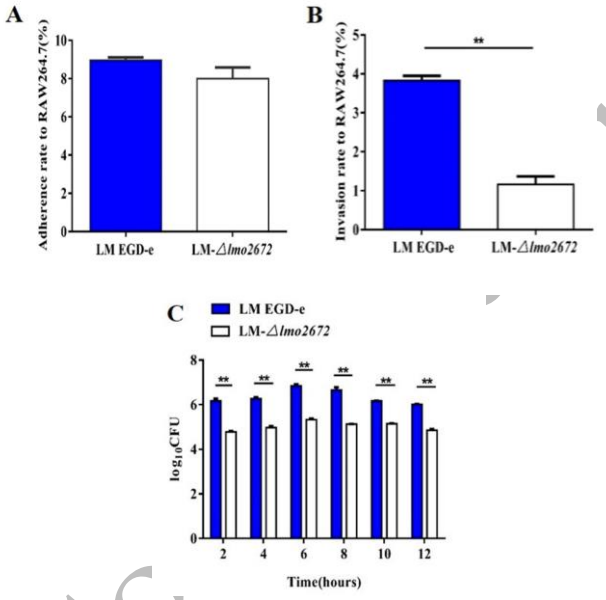

Fig. 2: Determination of adhesion, invasion and replication of wild-type strain LM EGD-e and LM-*⊿lmo2672* in RAW 264.7 cells

The macrophage RAW 264.7 is infected with bacterial suspension to a MOI of 10 bacteria per cell

(**A**): Adhesion rates of wild-type strain LM EGD-e and *⊿lmo2672* mutant; (**B**): Invasion rates of wild-type strain LM EGD-e and *⊿lmo2672* mutant; (**C**): The number of bacteria in live in different time. Error bars represent the mean ± standard error (SE) of triplicate experiments. Stars indicate *P*-values < 0.01(**)

Furthermore, most members of the AraC family have been demonstrated to be involved in virulence regulation in many bacteria such as *Vibrio cholerae* (Champion *et al.* 1997; Krukonis and DiRita 2003), *Citrobacter rodentium* (Kelly *et al.* 2006; Hart *et al.* 2008; Santiago *et al.* 2016) and *Shigella flexneri* (Koppolu *et al.* 2013; Martino *et al.* 2016). Garrity-Ryan *et al.* confirmed that the virulence of *Yersinia pseudotuberculosis* could be significantly reduced by inhibiting or knocking out the transcriptional regulatory genes of the AraC family (Gallegos *et al.* 1997; Martin and Rosner 2001; Garrity-Rya *et al.* 2010). However, the role of AraC family member lmo2672 in pathogenicity of LM is still unclear. Here, the impacts of *lmo2672* gene deficiency on LM pathogenicity were investigated using LM-*⊿lmo2672* decifiency strain, erythrocytolysis, macrophage infection and mouse infection assays. The results showed that the *⊿lmo2672* deletion strain has significantly reduced erythrocytolysis, adhesion, invasion and intracellular proliferation of macrophage RAW264.7, and significantly lowered virulence to mice, suggesting that *lmo2672* is involved in pathogenicity of LM.

LM can survive in complex environment inside hosts, which is due to its complex gene regulatory network (O'Byrne and Karatzas 2008; Heras *et al.* 2011; Guariglia-Oropeza *et al.* 2014). It has been shown that the AraC family transcription factors regulate the expression of bacterial genes (Bailey *et al.* 2010). Recently, Rowe *et al.* found that AraC-type regulator Rbf influences the *Staphylococcus epidermidis* biofilm formation by regulating the expression of the *icaADBC* gene, and subsequently influences its drug resistance and virulence (Rowe *et al.* 2016). Fang *et al*. found that BfvR directly or indirectly regulate the expression of *psaABC* and *psaEF* to inhibit the pathogenicity of *Y. pestis* in mice (Fang *et al.* 2018).

In this study, the qRT-PCR found that *⊿lmo2672* mutant repressed the expression of three deletion of virulence genes (*prfA*, *actA*, *hly*). PrfA is the member of the Crp/Fnr transcription regulator families in LM (Hall *et al.* 2016), which can directly or indirectly regulate the expression of many virulence genes in LM (Heras *et al.* 2011). ActA (actin aggregation factor) is involved in the cell adhesion and invasion of LM (Bruhn *et al.* 2007; Hadjilouka *et al.* 2018), which promotes the aggregation of actin to drive LM through the cytoplasm into adjacent cells (Milohanic *et al.* 2003; Hamon *et al.* 2012; Brenner *et al.* 2018). Hly encodes virulence factor LLO, enabling LM to escape from the phage of host cells and replicate inside cells (Chen *et al.* 2017). This study showed the significant decrease of *hly* gene transcription level is consistent with the phenotype of reduced hemolytic activity. Besides, the transcription levels of *prfA* and *actA* in LM-*⊿lmo2672* are significantly reduced, which is consistent with the results of LM macrophage cell and mouse infection assays. In addition, the interaction between lmo2672 and the upstream sequence of *prfA* gene was also confirmed by EMSA.

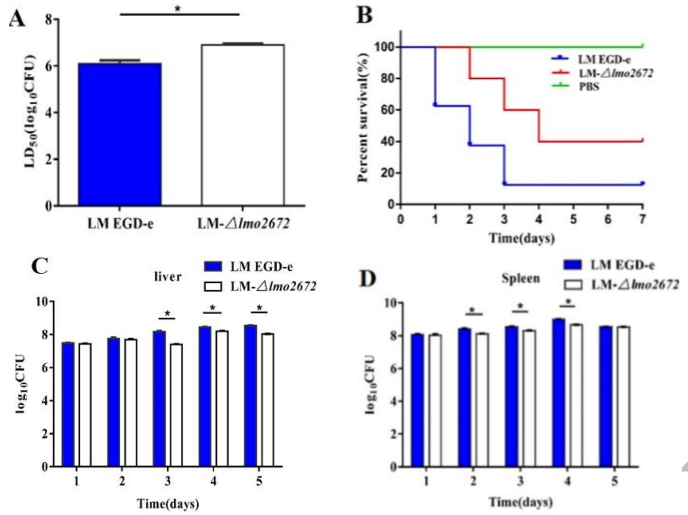

Fig. 3: Effects of *lmo2672* gene deletion of LM on virulence in mice
(**A**): The LD_{50} of wild-type strain LM EGD-e and LM-*△lmo2672*; (**B**): Survival curves of mice infected by intraperitoneal injection of wild-type strain LM EGD-e and LM-*△lmo2672*; (**C**): Bacteria count in liver; (**D**): Bacteria count in spleen. Error bars represent the mean ± standard error (SE) of triplicate experiments. Stars indicate *P*-values < 0.05 (*)

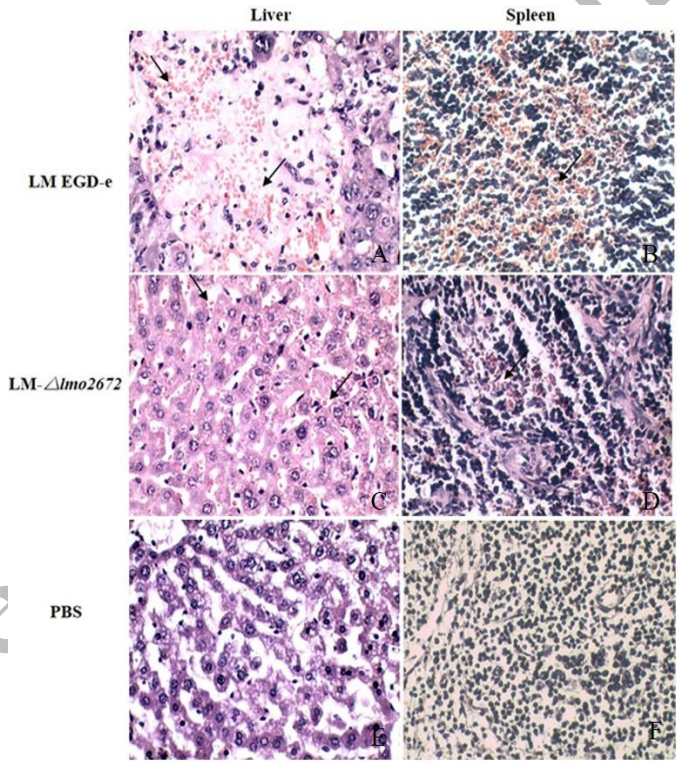

Fig. 4: Histopathological analysis of liver and spleen of mice infected by wild-type strain LM EGD-e and LM-*△lmo2672* (HE staining, × 400)
(**A**) and (**C**): The liver of the mice injected with LM EGD-e and LM-*△lmo2672*. Hepatocytes showed vacuol-like appearance, degeneration necrosis and hemorrhagic necrosis (indicated by arrows); (**B**) and (**D**): The spleen of the mice injected with LM EGD-e and LM-*△lmo2672*. The structure of splenic cord disappeared with hemorrhage and lymphocyte infiltration (indicated by arrows); (**E**) and (**F**): Liver and spleen tissue's slices of mice inoculated with PBS (control)

Conclusion

We for the first time demonstrated that *lmo2672* gene deficiency could reduce the virulence of LM, indicating that *lmo2672* was involved in the pathogenicity of LM, which provided an insight into the biological function of *lmo2672* in LM virulent regulation.

Acknowledgements

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Table 1: List of primer sequences used in this study

Target gene	Primer name	Nucleotide sequence (5'→3')	Product size (bp)
lmo2672-L	F1	GGGGTACCCTGTCATTTTTTCTCCTCCT	638
	F2	CAAAGCATTTACGTTTTAAAGAGACCCCCTTTTC	
lmo2672-R	F3	GAAAAGGGGGTCTCTTTAAAACGTAAATGCTTTG	698
	F4	AACTGCAG GCGAATCAAGTCTTTATCTC	
lmo2672-I	F5	ATAACGTCGCAAGGTGCATG	2309/1502
	F6	GGTCCATACAGAAAACCACGA	
lmo2672-E	F7	ATGATTAATGAATTTGTTTGTA	840
	F8	TTAGAGTTTTTCGACAGTCT	
rpoB	*rpoB*-F	TGCCATTTATGCCAGAC	188
	rpoB-R	TTCTTCCACTGTGCTCC	
prfA	*prfA*-F	TTAGCGAGAACGGGACCAT	392
	prfA-R	TGCGATACCGCTTGAATAG	
actA	*actA*-F	GGCGAAAGAGTCAGTTGC	492
	actA-R	GTTGGAGGCGGTGGAAAT	
hly	*hly*-F	TGTAAACTTCGGCGCAATC	462
	hly-R	TAAGCAATGGGAACTCCTG	
sigB	*sigB*-F	TCATCGGTGTCACGGAAGAA	310
	sigB-R	TGACGTTGGATTCTAGACAC	
inlB	*inlB*-F	TATGCAGCATGGCTTGTAACC	200
	inlB-R	GTTCTTGCAGAGATGGCACG	
prfA-EMSA	*prfA*-1	AGTATATCTCCGAGCAACCTCG	243
	prfA-2	GTCTCATCCCCCAATCGTT	

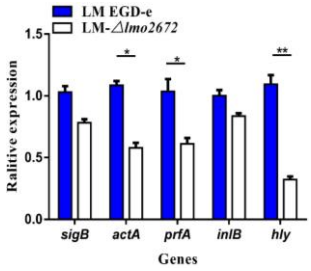

Fig. 5: Determination of transcriptional levels of virulence related genes in LM EGD-e and LM-*⊿lmo2672*

Error bars represent the mean ± standard error (SE) of triplicate experiments. Stars indicate *P*-values < 0.05 (*) and < 0.01(**)

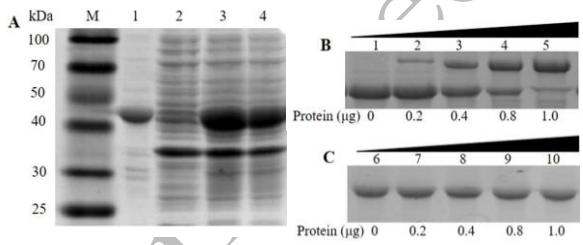

Fig. 6: Analysis of the interaction between lmo2672 and the upstream DNA sequence of *prfA* gene using EMSA

(**A**); Analysis of expressed lmo2672 by SDS-PAGE

Lane 1, Purified lmo2672 protein; lane 2, Expression of pET32a vector in *E. coli* BL21(DE3); lanes 3–4, Expression of recombinant lmo2672 in *E. coli* BL21(DE3)

(**B**) The interaction between lmo2672 and the upstream DNA sequence of *prfA* gene

Lane 1-5, The upstream DNA sequence of *prfA* gene incubated with an increasing protein lmo2672;

(**C**) The interaction between BSA and the upstream DNA sequence of *prfA* gene

Lane 6-10: an increasing BSA (unrelated protein) binds to the upstream DNA sequence of *prfA* gene as negative control; Shifted bands are indicated by arrow heads

middle-aged leading science and technology innovation talents plan of Xinjiang Corps (No. 2016BC001), International Science & Technology Cooperation Program of China (No. 2014DFR31310) and Outstanding young and middle-aged talents Training Project of State Key Laboratory for Sheep Genetic Improvement and Healthy Production (SKLSGIHP2017A03).

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