Prokaryotic Expression and Polyclonal Antibody Preparation of Cdtc Protein from Haemophilus Parasuis

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Abstract: Haemophilus parasuis is the pathogen of swine Glasser's disease which responsible for enormous economic losses in the swine industry. Cytolethal distending toxin (CDT), containing three subunits: CdtA, CdtB and CdtC, is an important virulence factor of H. parasuis. With the character of decreased cell toxicity by mutation, CdtC is suitable for production of antibodies and it can be successfully expressed in soluble form in Escherichia coli when the signal peptides were removed. In this study, CdtC expression plasmid was constructed and transformed into E. coli BL21 (DE3). CdtC protein was expressed in large quantities and purified by nickel affinity chromatography. We obtained and verified CdtC protein with purity > 85% by SDS-PAGE and Western Blot. Purified CdtC protein then was used for animal immunization. Specific antibodies produced by the experimental animals are collected. Finally, polyclonal antibodies with a titer ⩾ 6.4×104 was obtained and confirmed by ELISA.

1. Introduction

Haemophilus parasuis (*H. parasuis*), an NAD dependent member of the *Pasteurellaceae* family, is a pathogen of swine Glasser's disease which characterized by polyarthritis, fibrinous polyserositis and meningitis [1]. Be known as one of the most important infectious swine diseases, Glasser's disease is distributed worldwide and responsible for enormous economic losses in the swine industry, affecting 1-4 month-old pigs especially under conditions of stress [2]. The cytolethal distending toxins (CDTs) composed of a family of bacterial protein exotoxins and were produced by varieties of Gram-negative pathogenic bacteria, e.g. *Campylobacter jejuni, Haemophilus ducreyi*, *Aggregatibacter actinomycetemcomitans*, *Helicobacter hepaticus, Escherichia coli, Shigella dysenteriae* and *H. parasuis* [3-7]. CDT has been described as the first bacterial genotoxin whose main action is activating the DNA damage responses, inducing cell cycle arrest and apoptosis of host cells [8]. Moreover, as a related pathogenic factor that can cause a variety of bacterial diseases, it is reported that cytotoxic lethal toxin (CDT) affects *Haemophilus parasuis* by promoting attachment to host cells and evading the immune system. All CDT holotoxins are tripartite complexes comprising CdtA, CdtB, and CdtC subunits. No matter which signal peptide is removed, they can be successfully expressed in soluble form in *E. coli.* [9]. CdtA and CdtC subunits are essential proteins that mediate toxin binding to the plasma membrane of target cells, allowing the internalization of the main active subunit CdtB which is functionally homologous to mammalian deoxyribonuclease I. It was reported that CdtA and CdtB form a more active toxin than CdtB and CdtC [10]. Therefore, CdtC may be suitable for introduction antibodies production. In the present study, a plasmid was designed for CdtC protein production. CdtC protein was expressed and purified by nickel affinity chromatography. By means of immunizing animal, polyclonal antibodies of *H. parasuis* CdtC were prepared.

2. Materials and Methods

2.1 Bacteria Strains and Materials

Escherichia coli was grown on LB. When required, media were supplemented with kanamycin (30 μg/mL). Isopropyl-β-D-thiogalactopyranoside (IPTG), Non-stained protein Marker, Pre-stained

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protein Marker, Protein concentration quantification kit and TMB Chromogenic kit were from Sangon Biotech company, China. HRP-labeled goat anti-rabbit IgG were from Jackson Immuno Research Laboratories.

2.2 Animal

Healthy female, 4.1 kg New Zealand white rabbits at 4 months were used in this study. Rabbit experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals Centre of South China Agricultural University. Experimental procedures for rabbits were approved by the Scientific Ethic Committee of South China Agricultural University (No. SCAU-2019-027). Following transferring to Laboratory Animals Centre of South China Agricultural University, the rabbits were raised in isolated and ventilated animal rooms with specific pathogen free conditions.

2.3 Construction of a Plasmid and Validation

The construction of the plasmid adopts the double enzyme digestion method. Primers for CdtC with restriction sites are GTATACCTTTGTGGTGGCTAC and TGGATCATCCAATAATATTGAGCTC. The PCR program was carried out as follows: 95˚C for 10 min, 40 cycles of 95˚C for 15 s, 55 ˚C for 20 s and 72˚C for 25 s. The target gene fragment with digestion sites were amplified by PCR. The pET-28a (+) were ligated to PCR product by ligase according to the complementary tails designed. The ligation solution was transferred into Top10 competent cells, then the positive clones were confirmed by PCR and sequencing. The detection primers of pET-28a (+) are TAATACGACTCACTATAGGG and GCTAGTTATTGCTCAGCGG.

2.4 Expression of Cdt Genes

Recombinant plasmid was transformed into *E. coli* BL21 (DE3) by a heat shock method, then screening was performed on LB agar plate containing kanamycin (30 μg/ml). After overnight cultivation, clones were picked out and confirmed by PCR. A positive clone was selected and cultivated for further expression. When the OD600 reached 0.6, Isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the cells were continued to culture at 20 ˚C for 16 h or 37 ˚C for 4 h. The condition without IPTG was considered as the negative control. Cells were harvested by centrifugation at 5, 000 × g for 15 min at 4 ˚C, then cells were resuspended and lysed in Tris-HCl buffer (8 M Urea, 50 mM Tris-HCl, 300 mM NaCl, pH 8.0) by ultrasonication. The supernatant and insoluble material were treated separately and detected by SDS-PAGE.

2.5 Purification and Detection of Proteins

Cells lysed in Tris-HCl buffer were sonicated and centrifuged to collect the crude protein. 5 mL Ni-NTA was equilibrated by 25 ml Binding buffer (8 M Urea, 50 mM Tris, 300 mM NaCl, pH 8.0), the flow rate was 5 ml/min. The equilibrated column packing was incubated in 1 h with crude protein, then the production was collected by column. The equilibrated column was washed again by binding buffer. The outflow was collected by washing with 20 mM imidazole washing buffer (8 M Urea, 50 mM Tris, 300 mM NaCl, pH 8.0), which repeated with 50 mM imidazole washing buffer. The outflow was collected by eluting replace with elution buffer (8 M Urea, 50 mM Tris, 300 mM NaCl, 500 mM Imidazole, pH 8.0). Finally, crude protein, washed-outflow and eluted-outflow were prepared alone for detection by SDS-PAGE. Four groups with better purity were dialyzed in 0.1% SKL buffer (500mM L-Arginine, 2 mM DTT, pH 7.3) and transferred into 0.2% SKL (50 mM Tris, 300 mM NaCl, 2mM DTT, pH 7.3). After being concentrated with PEG 20000, the protein was filtrated with a 0.22 μm filter membrane and aliquoted 1 mL/tube and stored at -80 ˚C. The predicted molecular mass of the purified recombinant proteins were confirmed by SDS-PAGE and western blotting.

2.6 Immunity

The immunization program consists of four times: day 1, day 14, day 28 and day 42. Freund's

complete adjuvant and recombinant protein were the first day's antigen. Freund's incomplete adjuvant and recombinant protein were the antigen used for the next three immunizations. In this program, 1 ml blood was collected from the ear veins of rabbits for ELISA detection on the 35th day. After the anti-serum titer reached the requirements on the 49th day, all the bloods from rabbits were collected through the carotid artery.

2.7 Indirect Elisa

Indirect ELISA was performed according to the basic protocol. The antigen that diluted to 6 μg/ml with 0.05 mol/L carbonate buffer (pH 9.6) was added to an ELISA plate (100 ul/well). After 4 ˚C overnight of laying, ELISA plates were washed three times (3 min/times) with PBST (PBS including 0.05% Tween 20). ELISA plates were blocked with PBST containing 5% skim milk (60 μl/well) at 37 °C for 2 h, then washed three times (3 min/times) with PBST. The antiserum diluted (1:1000) were serial diluted and incubated for 1 h at 37 °C. The same washing steps with PBST were repeated. HRP-labeled goat anti-rabbit IgG was added which diluted 1:8000 in PBST and incubated for 45 min at 37 °C. After repeating the washing steps with PBST, the reaction of ELISA plates was started by adding tetramethylbenzidine (TMB) peroxidase substrate (100 μl/well) for 5-10 min and stopped by 2 mol/H_2SO_4 (100 μl/well). The optical density at 450 nm (OD450) was measured by Microplate reader (KHB ST-360).

3. Results and Discussion

3.1 Construction of a Plasmid and Validation

The constructed vector is calculated for 5369 bp. The DNA and amino acid sequences of cdtC gene is showed in (Figure 1A). The plasmid was transformed into *E. coli* BL21 (DE3), then validated by restriction enzyme cutting and PCR. The results showed that a single fragment was cut and appeared at about 500 bp, which proved the correction of the plasmid (Figure 1C).

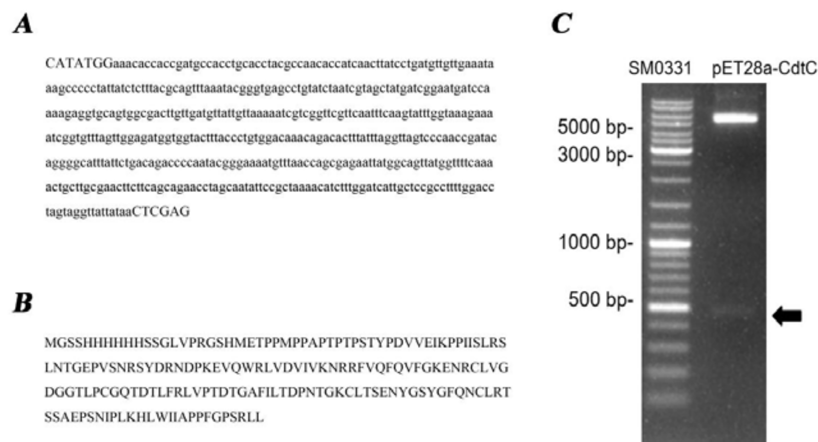

Figure 1 Sequence information and validation of expression plasmid.

Figure 1. A.The CdtC genes sequence with restriction sites. B. Amino acid sequence of CdtC. C. Restriction enzyme cutting analysis of CdtC expression vector.

3.2 Expression of Cdtc Gene and Detection of Protein

The CdtC protein was expressed through adding IPTG in the *E. coli*. Crude proteins were confirmed by SDS-PAGE and its bands were shown between the range 18.4-25 kDa, which matched the molecular weight of the protein of interest (Figure 2A). After crude proteins were confirmed to the targeted, the cells were cultured in large numbers. The CdtC protein were then purified and analyzed by SDS-PAGE also (Figure 2B).

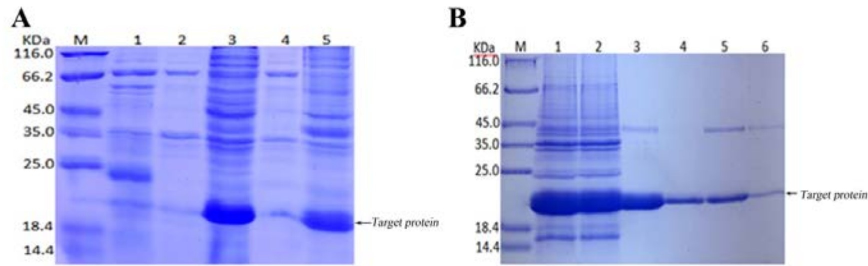

Figure 2 Expression and purification of CdtC protein

In Figure 2, A. SDS-SAGE analysis of fusion protein expression test. M: protein Marker; 1: Total protein before induction; 2: Supernatant of lysate at 20 °C; 3: Precipitation of lysate at 20 °C; 4: Supernatant of lysate at 37 °C; 5: Precipitation of lysate at 37 °C. B. SDS-PAGE analysis of CdtC protein nickel agarose affinity chromatography purification. M: Protein marker;; 1: loading; 2: elution; 3: 20 mM Imidazole elution component; 4-5: 50 mM Imidazole elution component; 6: 500 mM Imidazole elution component.

3.3 Validation and Quantification of Recombinant Protein

It showed obvious bands at the corresponding positions of the range 18.4-25 and theoretical molecular weight ± 5 kDa. It can be preliminarily determined that the CdtC protein was successfully purified (Figure 3A). To further determined the target protein, the purified protein was confirmed by western blot using a TMB development kit according to manufacturer procedure. The results showed that a clear band appear at the corresponding position, indicating that the purified protein is CdtC (Figure 3B). The protein loading volume was 20 μl. A non-interfering protein quantification kit was used to draw a standard protein curve and determined the protein concentration. Finally, 6 ml of the target protein (0.49 mg/ml) was obtained.

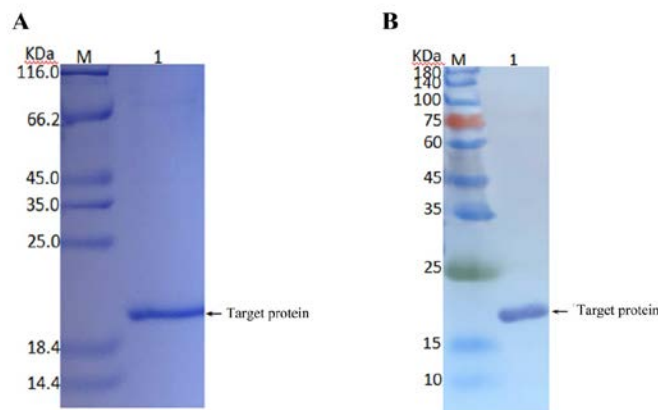

Figure 3. Validation and quantification of recombinant protein.

In Figure 3, A. SDS-PAGE analysis of the purified protein. M: Protein marker; 1: Purified protein. B.Western Blot analysis of the purified protein. M: marker; 1: Purified protein.

3.4 Indirect Elisa of Antiserum or Antibody Titer

The antiserum titer test of final release was obtained by ELISA. According to the color reaction, it can be seen that ELISA reactions of rabbit-A and rabbit-B disappeared at 2.56×10^5 (Figure 4A). The Abs value was 0.179 and 0.228 by microplate reader (Figure 4B). It was concluded that the antiserum titer after final release: A $\geqslant 2.56 \times 10^5$, B $\geqslant 2.56 \times 10^5$. The antibody titer test of final release was also obtained by ELISA. From the color reaction, reaction color of rabbit-A disappeared at 1.28×10^5 and 5.12×10^5 for rabbit-B (Figure 5A). The absorbances were 0.288 and 0.3 by microplate reader (Figure 5B). It was concluded that the antiserum titer after final release: A $\geqslant$

1.28×10^5, B $\geqslant$ 5.12×10^5.

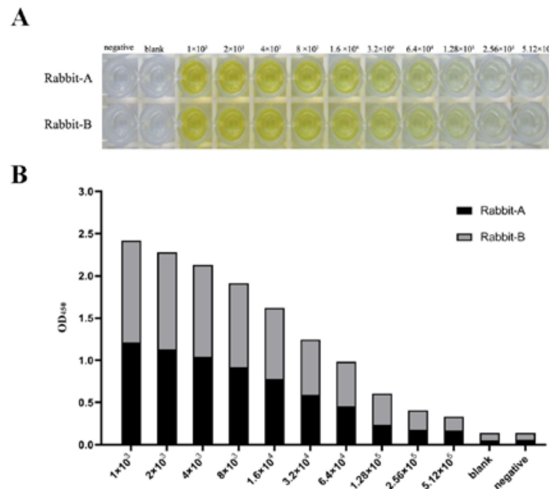

Fig.4 Indirect Elisa of Antiserum Titer.

In Fig.4, a. Elisa Analysis of Immune Sera Which from Experimental Rabbits. B. Absorbance Value Read at 450 Nm by Microplate Reader.

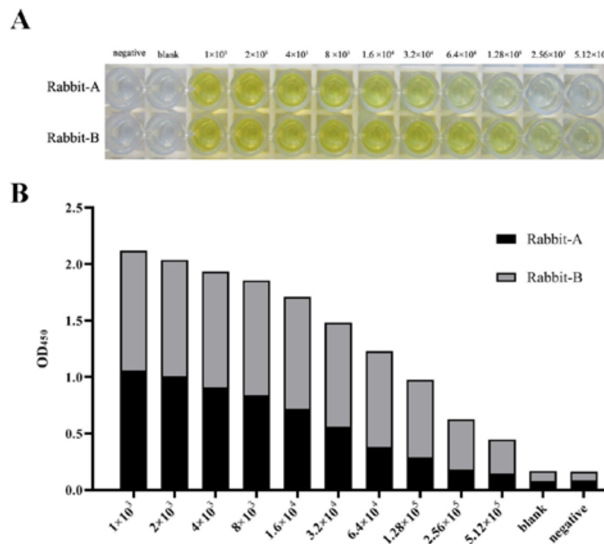

Fig.5 Indirect Elisa of Antibody Titer

In Fig.5, a. Elisa Analysis of Antibody Titer Which from Experimental Rabbits. B. Absorbance Value Read at 450 Nm by Microplate Reader.

4. Conclusion

The present study identified virulence factor CdtC of *H. parasuis* as a novel diagnostic antigen. With construction of the CdtC expression plasmid, CdtC proteins expressed a large amount and were obtained with purity > 85%. In addition, purified CdtC protein was used as antigen for animal immunization, polyclonal antibodies were developed and demonstrated polyvalent $\geqslant$ 6.4×10^4. This polyclonal antibodies of CdtC exhibits the potential to be applied toward Glasser's disease diagnosis.

Acknowledgments

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