

SHORT REPORT

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A novel protein-coding ORF72.2 gene was identified from Marek's disease virus strain CVI988

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Abstract

Marek's disease is a highly contagious disease of poultry characterized by rapid-onset of T-cell lymphomas, which is caused by Marek's disease virus (MDV), but its pathogenic mechanism is still not very clear. Recently, some new progress was achieved in molecular character of MDV. Along with the genomic sequencing of MDV serotype 1, some novel open reading frames (ORFs) were predicted, and ORF72.2 was one of them which have no homologues in other MDV serotypes or in other alphaherpesvirus. In the study, ORF72.2 was firstly identified as a protein-coding gene by the method of reverse transcription polymerase chain reaction (RT-PCR), western blotting and indirect immunofluorescence assay. This study paved the way to conduct further studies to determine whether ORF72.2 plays a role in MDV replication and pathogenicity.

Findings

Marek's disease (MD) is a highly contagious disease of poultry characterized by mononuclear cellular infiltrates in peripheral nerves and various other organs and tissues including iris and skin. The disease has a worldwide distribution and remains a major concern for the poultry industry even though vaccines are used widely today [1]. The causative agent of the disease is Gallid herpesvirus 2, which is also called Marek's disease virus (MDV) serotype 1. This virus is an alphaherpesvirus of the genus *Mardivirus* [2], which also includes the antigen-related Meleagrid herpesvirus 1 (HVT), a strain used widely as a vaccine against MD since the late 1960s [3,4], as well as Gallid herpesvirus 3 (MDV-2), which includes apathogenic strains some of which are used as live vaccines against MD [5].

The complete genomic sequence of the MDV-1 vaccine strain CVI988 was determined in 2007 which consisting of 178311 bp with an overall gene organization identical to that of the oncogenic strains such as GA, RB1B, Md5, Md11, 584A. The genome of

CVI988-BAC contains over 478 ORFs encoding proteins with more than 50 amino acid (aa) residues [6]. Among the ORFs, the putative functions of some ORFs were predicted based on the comparison with homologous genes of other alphaherpesvirus whose functions had already been well known [7,8], however, some ORFs are found to have no homologues in other alphaherpesvirus. ORF72.2 in vaccine strain CVI988 was one of those ORFs and consists of 621 nucleotides, encoding 206 aa residues. In this study, the ORF72.2 protein was firstly identified, expressed and localized in cell by western blotting and immunofluorescence assay, which laid the foundation for the study of pathogenic mechanism of MDV.

The MDV CVI988 strain used in this study was grown in chicken embryo fibroblast (CEF) cells. Cell cultures were maintained in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS), 0.22% NaHCO₃, 100 IU/ml penicillin and streptomycin [9]. The RNA was extracted using RNeasy pure Cell Kit (TIANGEN, Beijing, China) from normal CEFs and MDV-infected CEFs at 24 h, 48 h, 72 h, 96 h post-infection. Then a pair of primers were designed based on the bioinformatics analysis (Primer Premier 5.0 software) of the ORF72.2 gene and used to amplify a 590

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base pair (bp) fragment spanning nt 22 through 612 of ORF72.2 gene, and the amplification was performed with reverse transcription polymerase chain reaction (RT-PCR) (Figure 1). The sequences of the primers used for this purpose were R1: 5'-CCGGAATTCCTGATACTGCTAAGAGATCAC-3' with *EcoRI* site (underlined) and R2: 5'-CCCAAGCTTCGTATACAGCCGAA CATAAT-3' with *Hind III* site (underlined). The result showed that the ORF72.2 gene was successfully amplified from MDV-infected CEFs by RT-PCR at all time points tested, which suggested that the ORF72.2 gene was transcribed persistently in middle and late time of viral infection.

The amplified product was cloned into pET32 (+) plasmid to get a recombinant plasmid named pET32-ORF72.2. *Escherichia coli* BL21 (DE3) were transformed with the recombinant plasmid, and protein expression was induced with 1 mM IPTG at 37°C for 4 h. The bacterial proteins were analyzed by 12% SDS-PAGE under denaturing conditions. Protein bands were visualized after staining with 0.1% Coomassie blue R250, and the protein concentration was determined using program BandScan 5.0 [10]. The recombinant ORF72.2 protein with molecular weight of about 41 KDa by SDS-PAGE

analysis was successfully expressed in the transformed cells (Figure 2).

The expressed recombinant ORF72.2 protein was trapped in inclusion bodies. The cells were harvested by centrifugation and resuspended in Phosphate-Buffered Saline (PBS) (pH 8.0) containing lysozyme (0.1 mg/mL) by 1/10 (v/v), after ice bathing for 30 min, the suspension were sonicated and centrifuged at 12000 g for 10 min. The pellets were homogenated and washed with washing buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2% Triton X-100, 2 M urea] for three times at 10 min/times and centrifuged at 12000 g for 10 min. The pellets were dissolved by denaturation buffer [50 mM Tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 8 M urea] and supernatant were collected after centrifugation. The supernatant was treated with renaturation buffer [50 mM Tris-HCl (pH 8.0), 0.1 mM oxidized glutathione, 1 mM reduced glutathione, 0.5 M urea] for overnight at 4°C and filtrated through 0.45 μm filtration membrane. Then the solution was purified on a column packed with Ni-NTA His Bind superflow according to the manufacture's instruction (Merck, Darmstadt, Germany). Bound protein fractions were pooled, dialyzed and concentrated, and the protein expression yield was analyzed by Bradford assay [11]. For the purified fusion protein, a single objective band was detected by SDS-PAGE (Figure 2).

For production of chicken antiserum against MDV, 1-day-old specific pathogen-free (SPF) chicken were injected intraperitoneally (i.p) with 0.2 ml of MDV CVI988 (Intervet) containing 4000 plaque forming unit (PFU), and booster injection was done at days 10 using the same method [12]. Two weeks after immunization, the blood was harvested from jugular vein and the antiserum was isolated. The serum of non-immunized chicken was also isolated as control.

For production of rabbit antiserum against recombinant ORF72.2 protein, New Zealand white rabbits were first immunized intradermally with 1 mg purified recombinant protein mixed with an equal volume of complete Freund adjuvant (Sigma, Shanghai, China) and with purified recombinant protein mixed with an equal volume of incomplete Freund adjuvant on days 14 and 28 later as booster injection, respectively. The serum was collected at 10 days after the final booster injection. The antiserum was purified using ammonium sulfate precipitation and High-Q anion-exchange chromatography [13].

To testify indirectly whether the ORF72.2 protein can be expressed in chickens after MDV CVI988 infection, the chicken antiserum against MDV produced above was used to make a western-blot. Firstly, purified recombinant proteins were separated by SDS-PAGE, and then the proteins were transferred to Polyvinylidene Difluoride (PVDF) membrane with 0.25 μm pore size (Millipore Corp., USA) at 15 V for 1.5 h. Then the

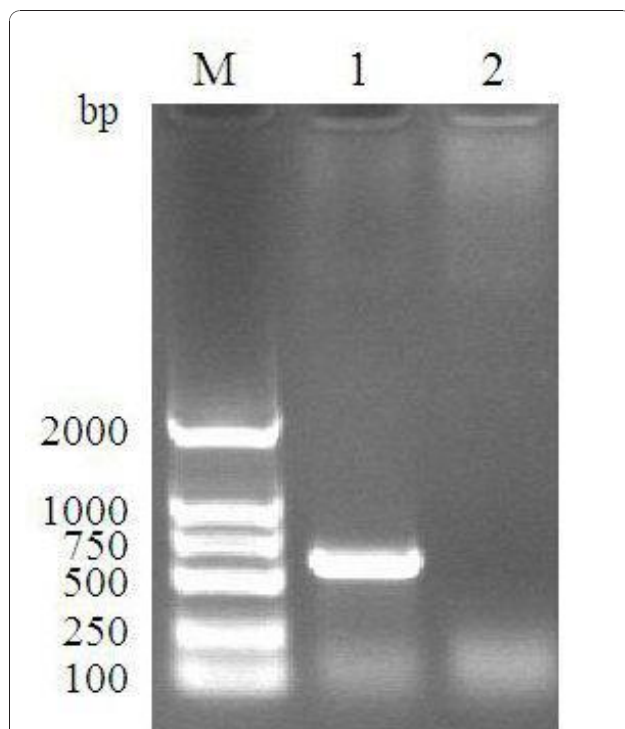
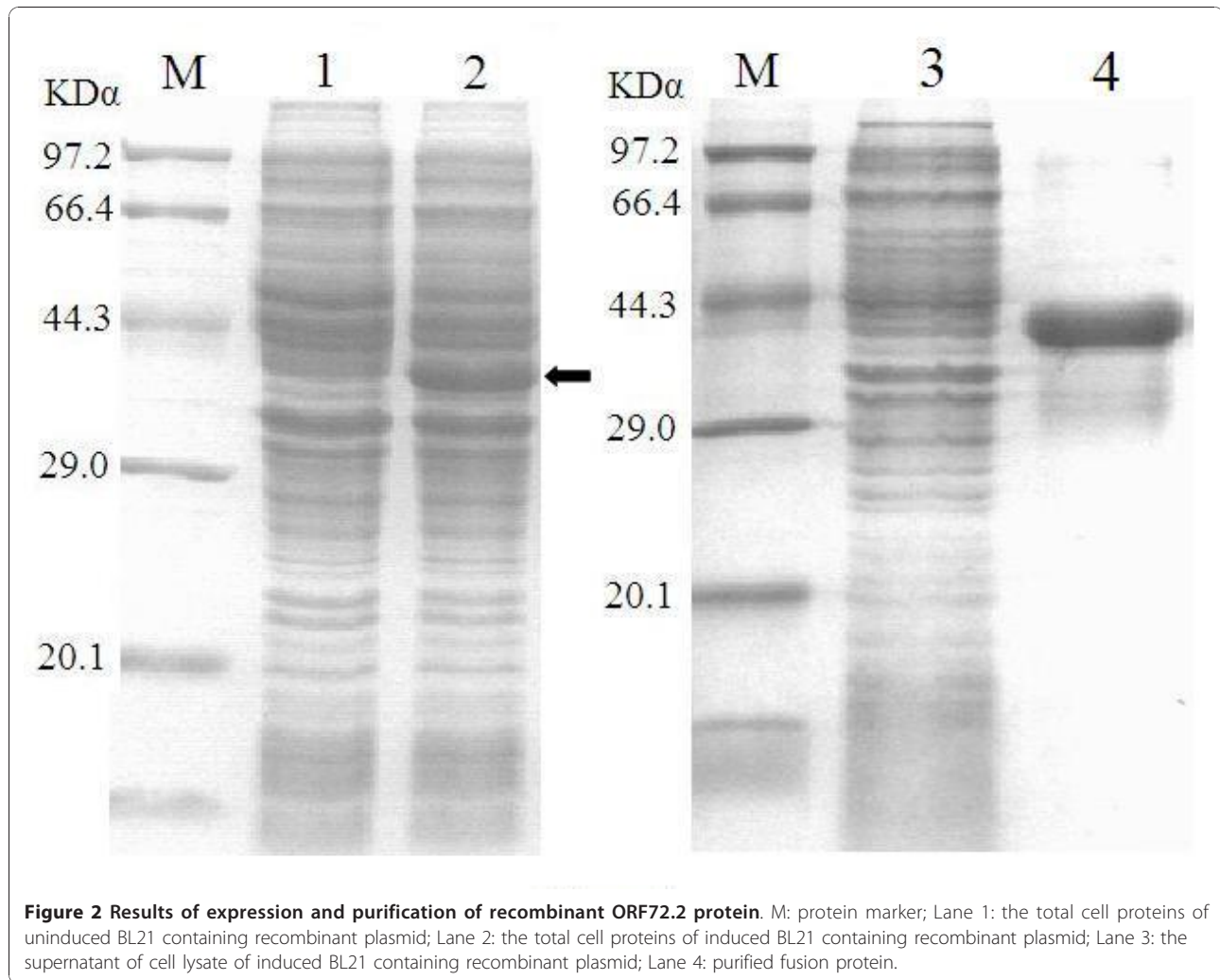


Figure 1 Amplification results of ORF72.2 gene by RT-PCR. M: DNA Marker DL2000; Lane 1: RT-PCR result of ORF72.2 gene from MDV-infected CEFs; Lane 3: RT-PCR result of ORF72.2 gene from normal CEFs.



membrane was blocked for 90 min with milk buffer [20 mM Tris-HCl (pH8.0), 150 mM NaCl, 0.05% Tween 20, 5% skinned dry milk] at 37°C and washed with Tris-Buffered Saline with Tween 20 (TBST) buffer [20 mM Tris-HCl (pH8.0), 150 mM NaCl, 0.05% Tween20] for three times. Then the membrane was incubated with chicken antiserum diluted 1:50 in 0.1% Bovine Serum Albumin (BSA)/PBS for 60 min at 37°C and washed with TBST, and then incubated with horseradish peroxidase (HRP)-labeled rabbit-anti-chicken IgG (Zhongshan Goldenbridge Biotechnology co., Ltd, Beijing, China) for 60 min at 37°C. Target proteins were visualized using 3, 3'-Diaminobezidine (DAB) (TIANGEN, Beijing, China). The result of western blotting showed the fusion protein can react positively with chicken antiserum against MDV (Figure 3), which proved that the ORF72.2 protein can be expressed in chickens after MDV infection and an immune response can be induced by natural ORF72.2 protein, but band line of western blotting was light, implying the expression level or immunogenicity of

natural ORF72.2 protein in chicken after MDVs infection is weak.

To further confirm the existence of ORF72.2 protein in MDV-infected CEFs, indirect immunofluorescence studies were performed with the rabbit antiserum against recombinant ORF72.2 protein produced above. CEFs were mock-infected or infected with MDV and were collected at 72 h post-infection when cytopathogenic effect (CPE) had just appeared. Cells on coverslips were fixed with 4% cold paraformaldehyde for overnight at 4°C and treated with 3% BSA to block the nonspecific staining, and then permeabilized with 0.2% (v/v) TrionX-100 in PBS for 20 min at room temperature. The samples were incubated with rabbit antiserum against recombinant ORF72.2 protein diluted in 1:100 for overnight at 4°C and washed with TBST for three times, and then incubated with fluorescein isothiocyanate (FITC)-conjugated sheep-anti-rabbit IgG (Zhongshan Goldenbridge Biotechnology co., Ltd, Beijing, China) for 1 h at 37°C [14,15]. The result showed that specific

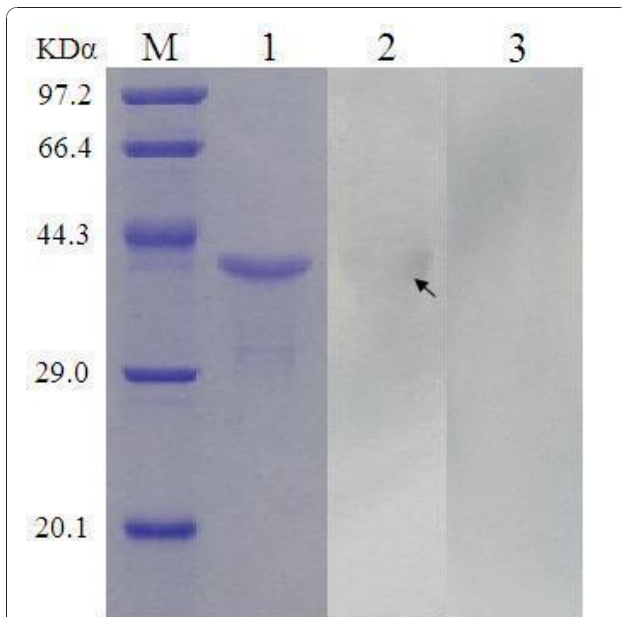


Figure 3 Demonstration of antibodies against ORF72.2 protein in serum of MDV-infected chickens by Western blotting. M: protein marker; Lane 1: purified fusion protein; Lane 2: Western-blot result of purified recombinant protein with chicken antiserum against MDV; Lane3: Western-blot result of purified recombinant protein with serum from normal chickens.

fluorescence appeared in cytoplasm and nuclear membrane region of infected cells at 72 h post-infection (Figure 4).

In this study, ORF72.2 gene was identified from MDV CVI988 in the level of transcription and expression. Firstly, mRNA was extracted from CEFs infected by CVI988 using RT-PCR, which proved that ORF72.2 gene is successfully transcribed in infected cells. Secondly, the highly antigenic region of ORF72.2 gene was successfully cloned and expressed in an *E. coli* pET32 (+) expression system. Thirdly, the existence of antibodies against natural ORF72.2 protein in chickens after MDV infection was verified by western blotting using recombinant ORF72.2 protein as the antigen. Meanwhile, the existence of ORF72.2 protein in MDV-infected CEFs was further verified by immunofluorescence assay using rabbit antiserum against recombinant protein. Generally, natural ORF72.2 protein was found in MDV infected cells and natural antibodies against ORF72.2 protein can be detected in chicken after MDV infection, all this proved that ORF72.2 gene is really a novel protein-coding gene of MDV.

In addition, bioinformatics analysis of the complete genomic sequence of MDV revealed that ORF72.2 gene existed both in mild virulent MDVs (such as CU-2) and high virulent MDVs (such as GA, RB1B, MD5, MD11, 584A), but it was only found in MDV serotype 1 and its homologous gene was not identified in MDV serotype 2 and 3 or in other alphaherpesvirus. Deduced amino acid sequence of ORF72.2 was searched for the existence of protein motifs in the PROSITE pattern database and the function of this protein was searched too, but no available database and protein motifs could be found.

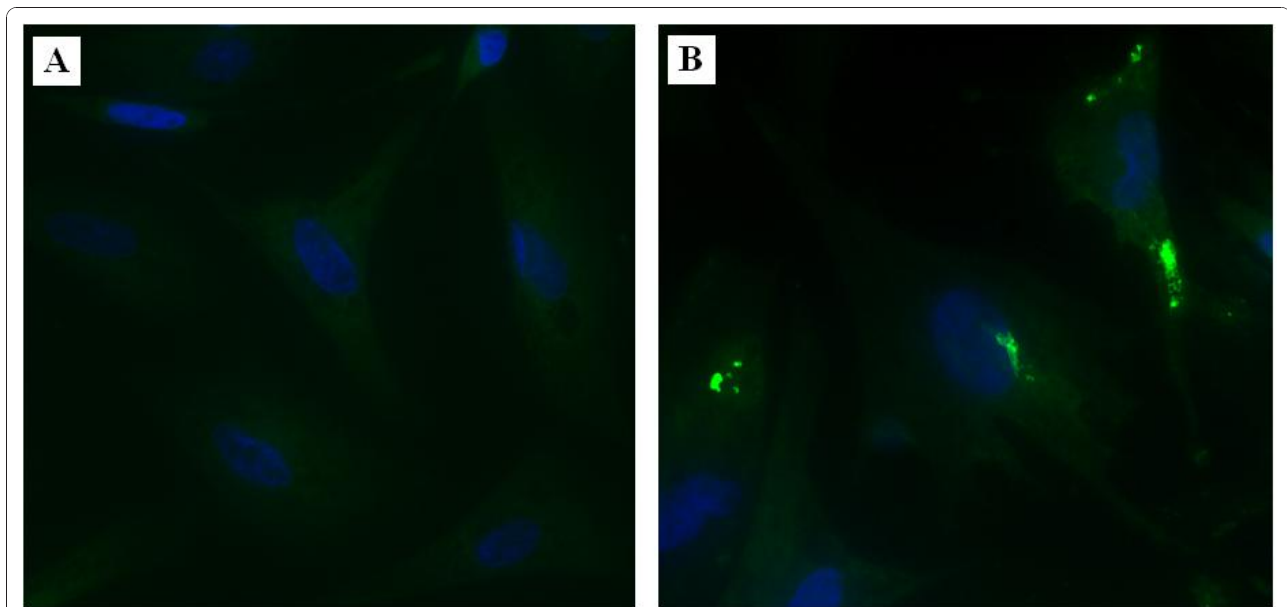


Figure 4 Detection of ORF72.2 protein by immunofluorescence assay in non-infected CEFs (A) and MDV-infected CEFs (B). CEFs were incubated with rabbit antiserum against recombinant ORF72.2 protein, and subsequently stained with fluorescein isothiocyanate (FITC)-conjugated sheep-anti-rabbit IgG. Nuclear were counterstained with DAPI (blue).

In conclusion, a novel protein encoded by ORF72.2 gene was identified and verified by method of western blotting and indirect immunofluorescence assay (IFA), however, it was not really clear whether this protein is important to the replication and pathogenicity of MDV, further study should be undertaken in the following study.

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Authors' contributions

MT: Study design, performed the experiments, interpreted the data and wrote the manuscript. YZ, MS, YL, NZ, PL, XW, SC and YH: helped in experiments and drafting the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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