Development of a potential diagnostic monoclonal antibody against capsid spike protein VP27 of the novel goose astrovirus

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ABSTRACT Goose astrovirus (**GAstVs**) is an emerging pathogen of goslings that causes fatal gout, kidney hemorrhages, renomegaly, and high mortality. The GAstVs VP27 protein is an important capsid protein and a candidate for the development of diagnostic reagents. The aim of this study was to clone and express the VP27 gene for preparation of a specific monoclonal antibody (**mAb**). The VP27 protein was expressed and purified in the supernatant of Escherichia coli BL21. Then, the mAb was obtained with the hybridoma technique and named 2AF11. It was differentiated as IgG1 with the help of immunoglobulin subclass tests. This mAb can specifically recognize the VP27 protein in GAstVs-infected cells, as evidenced by western blot analysis and immunofluorescent assay. Furthermore, this mAb could also detect the VP27 protein in GAstVs-infected tissues, as demonstrated by immunohistochemistry. These findings indicate that this mAb has high diagnostic potential. Therefore, the newly produced anti-VP27 mAb, 2AF11, could be a useful tool as a specific diagnostic marker for GAstVs.

Key words: goose astrovirus, VP27, 2AF11, mouse monoclonal antibodies, immunohistochemistry

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INTRODUCTION

Since 2017, there has been a new contagious disease outbreak in goslings that is characterized by gout, kidney enlargement, and hemorrhage. It has quickly spread to many provinces of China and has resulted in high morbidity and mortality rates among infected goslings (Niu et al., 2018; Yang et al., 2018). The causative agent has been identified as a novel type of astrovirus, named goose astrovirus (**GAstVs**), which is different from previously reported avian astroviruses (Jin et al., 2018; Wei et al., 2020). In the field of research, GAstVs has attracted much attention due to the severe economic losses it has caused in China's goose industry (Zhang et al., 2018). However, no GAstVs vaccine is currently available for clinical use. Therefore, in order to prevent GAstVs infection and curb the associated economic losses, there is an urgent need to develop accurate and rapid approaches for prevention and early diagnosis of GAstVs infection.

GAstVs infection can be diagnosed by viral isolation and RNA detection in tissue or fluid samples or products of their culture (Lee and Kurtz, 1981; Ji et al., 2020a,b). However, these techniques involve time-consuming and labor-intensive processes and are, therefore, unsuitable for clinical practice (Willcocks et al., 1990; Yuan et al., 2018). Real-time PCR tests that target the viral genome have good sensitivity, but can only be applied to samples from goslings with acute or subclinical infection. Instead, immunological methods, such as enzyme-linked immunosorbent assay (**ELISA**) and gold immunochromatography assay, are the preferred procedures for viral antigen detection and viral serotype identification in the clinical setting (Wilson and Cubitt, 1988; Hudson et al., 1989; Herrmann et al., 1990). However, in most cases, immunology-based diagnostic methods are limited by the lack of commercially available monoclonal antibodies (**mAbs**) against GAstVs. Therefore, it is essential to generate anti-GAstVs mAbs with high sensitivity and specificity that can recognize GAstVs to improve the diagnosis of this disease.

Astroviruses are nonenveloped positive-sense single-stranded RNA viruses belonging to the *Astroviridae* family (Madeley and Cosgrove, 1975). Astroviruses have 3 open reading frames (**ORFs**): ORF1a and ORF1b encode nonstructural proteins that are involved in viral

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replication, while ORF2 encodes the capsid protein (De Benedictis et al., 2011). Previously, ELISA based on mAbs against the capsid protein of Mamastrovirus was designed for the detection of astroviruses in the stool of patients with gastroenteritis (Herrmann et al., 1990). With regard to chicken astroviruses, Lee et al reported that capsid proteins produced by the baculovirus expression vector system can induce virus-specific antibodies, including virus neutralizing antibodies, and these proteins may therefore have potential for use in diagnosis and vaccine production (Lee et al., 2013). VP27 is a capsid spike protein of astroviruses that not only interacts with the host to define cell tropism and mediate cell entry, but also triggers the host immune response (Krishna, 2005; York et al., 2015). Thus, the GAstVs VP27 protein is an accurate, safe target for the development of anti-GAstVs mAbs with high binding ability and specificity to examine astroviruses.

In the present study, we successfully expressed the VP27 protein of GAstVs in *Escherichia coli* and developed an mAb against it via hybridoma systems. This mAb was characterized and used to detect VP27 expression during GAstVs infection in goslings. Our findings indicate that this mAb may be an effective tool for further research on and improvement in the diagnosis of GAstVs.

MATERIALS AND METHODS

Vectors, Cells, and Animals

The pCold-SUMO vector, a prokaryotic expression vector containing a 6 × His tag, was obtained from Novagen (Madison, WI). E. coli BL21 (DE3)-competent cells were obtained from TransGen BioTech (Beijing, China). The SP2/0 cell line was purchased from ATCC. Six-wk-old female BALB/c mice were purchased from the Experimental Animal Center of Anhui Medical University (Hefei, China). Rabbit polyclonal antibody against VP27 protein was prepared by our lab.

Expression and Purification of Recombinant His-Tag *VP27 Protein*

The full length of the VP27 gene was amplified according to the available genome sequence of the AAstV/Goose/CHN/2019/DY-19 strain (GenBank Accession No. MT708902.1) using the following primers: VP27-F, 5'-CGGGATCCCAGGTTACTCCCTCGCT TGT-3'; VP27-R, 5'-CCCAAGCTTAGAGGTCTTGA GCGAGACT-3′ (the underlined sequences are the BamHI and HindIII sites, respectively). The PCR products were purified with the Gel Extraction Kit (Omega; Doraville, GA), digested with the restriction enzymes BamHI and HindIII (TaKaRa, Tokyo, Japan), and subsequently cloned into the pCold-SUMO expression vector using T4 DNA ligase (Vazyme Biotech Co. Ltd., Nanjing, China).

The recombinant pCold-SUMO-VP27 plasmid was transformed into DE3 cells and induced with 1 mM IPTG for 20 h at 16°C. Expression of the VP27 proteins was confirmed by SDS-PAGE and western blot analysis. The recombinant protein was purified using Ni-NTA agarose affinity resin (Beyotime, Shanghai, China) according to the manufacturer's instructions. The protein concentration was determined with a BCA Assay Kit (Beyotime). The purified VP27 protein was stored at −70°C.

Mouse Immunization

Female BALB/c mice (aged 6 wk) were purchased from the Experimental Animal Center of Anhui Medical University (Hefei, China). Mice were housed under standard conditions (12 h light-dark cycle, 25−7°C, ∼40% humidity) with free access to food and water throughout the duration of the experiments. Female BALB/c mice aged 6 wk were immunized subcutaneously 3 times at 10-d intervals with purified recombinant VP27 protein (100 μg VP27 per mouse). Booster immunization was performed 10 d after the third immunization via intraperitoneal injection of purified recombinant VP27 protein. Antibody titers were measured with ELISA, and the mice with the highest antibody titers for spleen cell fusion 3 d after the final booster injection were chosen. Sacrifice was performed by intraperitoneal injection of pentobarbital sodium (50 mg/kg) followed by cervical dislocation, and mortality was confirmed by observation. All the animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals by Anhui Agricultural University. Ethical approval was obtained from the Animal Care and Use Committee of Anhui Agricultural University.(Ethical Approval No.: AHAU2020-022).

Cell Fusion and Hybridoma Clone Screening

Cell fusion was carried out as described previously (Wang et al., 2017). Hybridoma cells were screened by indirect ELISA with recombinant VP27 protein as the coated antigen. The immunoglobulin class of the hybridoma antibodies was determined with the ELISA Mouse mAb Isotyping Kit (Southern Biotechnology Associates, Birmingham, AL), according to the manufacturer's instructions.

ELISA

Recombinant VP27 protein was diluted in carbonate-bicarbonate buffer (pH 9.5) to a final concentration of 2 μg/mL and coated on 96-well plates (100 μL per well). Following adsorption of the antigen after incubation at 4°C overnight, the plates were washed with PBST 3 times and then blocked with 5% non-fat milk at 37°C for 1.5 h. Next, 100 μL of serum from immunized mice was incubated in the plate at a 1:1,000 dilution at 37°C for 1 h, and subsequently washed 3 times with PBST.

Then, 100 μL of HRP-conjugated goat anti-mouse IgG (1:10,000; Solarbio, Beijing, China) was added into the plates and incubated at 37°C for 1 h. After washing with PBST 3 times, 100-μL TMB solution (TIANGEN, Beijing, China) was added into the plates at 37°C for 15 min. The reaction was stopped with 50-μL sulfuric acid (2 mol/L; Sangon Biotech Co., Ltd, Shanghai, China), and absorbance was measured at 450 nm using a microplate reader (Model550; Bio-Rad, Hercules, CA).

Western Blot Analysis

DE3 cells expressing recombinant His tag-VP27 were centrifuged at 6,000 *g* for 10 min. *E. coli* BL21 (DE3) cells containing the empty vector pCold-SUMO served as the control. LMH cells infected with GAstVs were collected and lysed in lysis buffer (Servicebio, Wuhan, China) on ice. The cells or the cell lysates were mixed with sample loading buffer, boiled for 5 min, and centrifuged at 10,000 *g* for 10 min. The supernatants were then separated by 12% SDS-PAGE gel and transferred onto PVDF membranes (0.2 μm; Millipore, Billerica, MA). The membranes were blocked with 5% skimmed milk for 1.5 h at room temperature and then rinsed with PBS 3 times. The membranes were then incubated with a His-tag mAb (1:1,000; Cat. No.: AH367, Beyotime) or polyclonal antibody against the VP27 protein (1:100) or VP27 mAb in ascitic fluid (1:5,000) in primary antibody dilution buffer (Beyotime) for 1 h at room temperature. Subsequently, the membranes were incubated with HRP-conjugated secondary anti-mouse (1:5,000; Cat. No.: G1214, Servicebio) or anti-rabbit IgG (1:5,000; Cat. No.: A0208, Beyotime) antibody in PBS for 1 h at room temperature. The primary antibodies were diluted with Primary Antibody Dilution Buffer (Beyotime) and the secondary antibodies were diluted with blocking buffer mentioned as above. The proteins were visualized with DAB as the substrate (Servicebio) under the conditions recommended by the manufacturer.

Indirect Immunofluorescence Assay

When LMH cells reached a confluence of 80 to 90%, they were infected by GAstVs at an MOI of 0.1 and incubated at 37°C for 48 h. The cells were fixed with 4% paraformaldehyde (Sangon Biotech Co., Ltd, Shanghai, China) and washed 3 times with PBS. The fixed cells were incubated with VP27 mAb in ascitic fluid (1:500) in PBS for 1 h at 37°C. After the cells were washed 3 times with PBS, they were incubated with 1:1,000 diluted FITC-conjugated anti-mouse IgG secondary antibody (1:1,000; Cat. No.: SF131, Solarbio) for 1 h at 37°C. Fluorescence intensity was detected under a confocal microscope (TCS SP5II; Leica, Germany).

In Vitro Transfection

To further analyze the reaction of the mAbs with VP27 protein, the VP27 gene was constructed into pCMV-14 and named pCMV-14-VP27. Next, 4 μg of the plasmid in 10 μL of Lipofectamine 2000 (Invitrogen, Waltham, MA) was diluted in 1.5 mL of DMEM without serum and then added to 293T cells in a 6-well cell culture plate. At 24 h after transfection, the cells were analyzed by western blotting to determine the reaction of VP27 with the mAbs.

Immunohistochemical Staining

GAstVs-infected and uninfected kidney and spleen tissues were fixed with 4% formaldehyde and embedded in paraffin. The paraffin blocks were cut into 3 to 4-μm thick slices. Immunohistochemical (**IHC**) staining was performed as described previously (Li et al., 2018). Briefly, the tissue samples were fixed by 4% formaldehyde and embedded in paraffin. The slides were dewaxed by a Leica automatic dyeing machine and blocked with 3% peroxide-methanol f or 20 min at room temperature to inactivate endogenous peroxidase. After washing three times with PBS, antigen retrieval was carried out by incubating the tissue sections with a 0.1% of PBS diluted trypsin solution. After rinsing with PBS, they were blocked with PBS containing 8% skimmed milk and incubated at 37°C for 40 min in a moisture chamber. The mAb was diluted 1:800 with PBS and incubated at 4°C overnight. The slides were rinsed with PBS 3 times and then incubated with HRP goat anti-mouse IgG at 37°C for 1 h before being stained with hematoxylin for 10 s. Finally, the slides were analyzed using a 200 × microscope.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA). The results are presented as the means ± SD. Two groups were compared by Student *t* test, more groups were compared by one-way analysis of variance followed by Tukey's post hoc test. A *P* value of 0.05 was considered significant.

RESULTS

Preparation and Analysis of Recombinant GAstVs VP27 Protein

To induce expression of the VP27 protein of GAstVs in *E. coli*, the 723-bp VP27 gene fragment was amplified from the cDNA of GAstVs-infected LMH cells, constructed into a prokaryotic expression vector, and transformed into BL21 (DE3) cells for recombinant protein expression by induction with IPTG. SDS-PAGE analysis showed that the protein was mostly expressed in the supernatant of *E.coli* with the predicted molecular mass of around 45 kDa (Figure 1A). The recombinant VP27 was effectively purified from the supernatant of *E. coli* (Figure 1A). Western blot analysis indicated that the purified VP27 could react with both His-tag mAb and

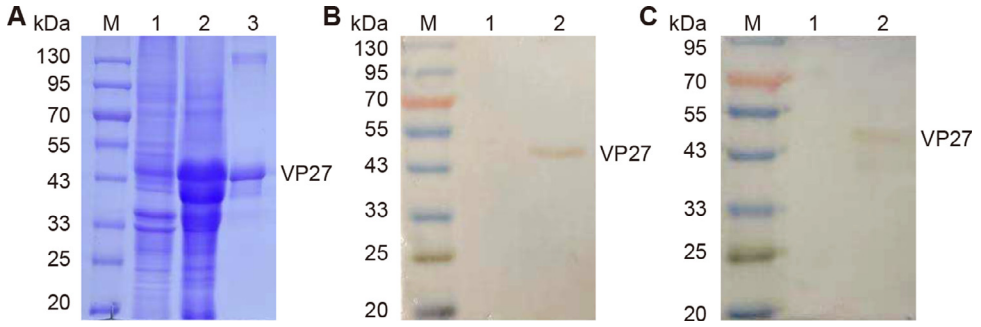

Figure 1. Expression, purification, and characterization of the VP27 protein in Escherichia coli system. (A) Expression of the recombinant GAstVs VP27 protein analyzed by SDS-PAGE and stained with Coomassie blue. Lane M, protein marker; Lane 1, non-IPTG-induced cell lysate of pCold-SUMO-VP27-transformed *E. coli* BL21 (DE3); Lane 2, IPTG-induced cell lysate of pCold-SUMO-VP27-transformed *E. coli* BL21 (DE3); Lane 3, purified VP27 protein. (B) Western blot analysis of purified VP27 protein using anti-His-tag antibody. (C) Western blot analysis of purified VP27 protein using polyclonal antibody against the VP27 protein. Lane M, protein marker; Line 1, non-IPTG-induced cell lysate of pCold-SUMO-VP27-transformed E. coli BL21 (DE3); Line 2, purified His-tag VP27 protein.

serum from mice immunized with VP27 (Figures 1B and 1C). These findings show that VP27 is successfully expressed and shows immunogenicity and reactivity, and can be used as an immunogen for the preparation of mAb.

Generation of mAbs *Against the VP27 Protein*

After immunization 3 times in five mice, the anti-sera titers were measured with ELISA with VP27 as the antigen. The results indicated that the antibody titers of the 5 immunized mice reached at least 1:16,000 compared with the negative control. Further, the antibody titers in mouse no. 2 and 3 were higher than those in the other mice (Figure 2A).

To establish mAbs, spleen cells were removed from mouse no. 2 and 3 and fused with SP2/0 myeloma cells. After 6 d, a few hybridoma cell clones appeared in most wells (Figure 2B). At 14 d, hybridoma cell lines secreting mAbs specific for VP27 were screened by indirect ELISA using purified VP27 protein. After at least 3 rounds of subcloning, one positive hybridoma clone producing VP27 mAb was successfully obtained and named 2AF11. The reactivity of the mAb was tested against purified recombinant VP27 or 293T cells transfected with the pCMV-14-VP27 plasmid. The presence of reaction bands with the expected molecular mass on the western blots indicated that the mAb reacted with purified recombinant VP27 or transfected 293T cell lysates (Figures 2C and 2D). These results imply that the mAb produced in this study has good, specific recognition capability for the VP27 protein.

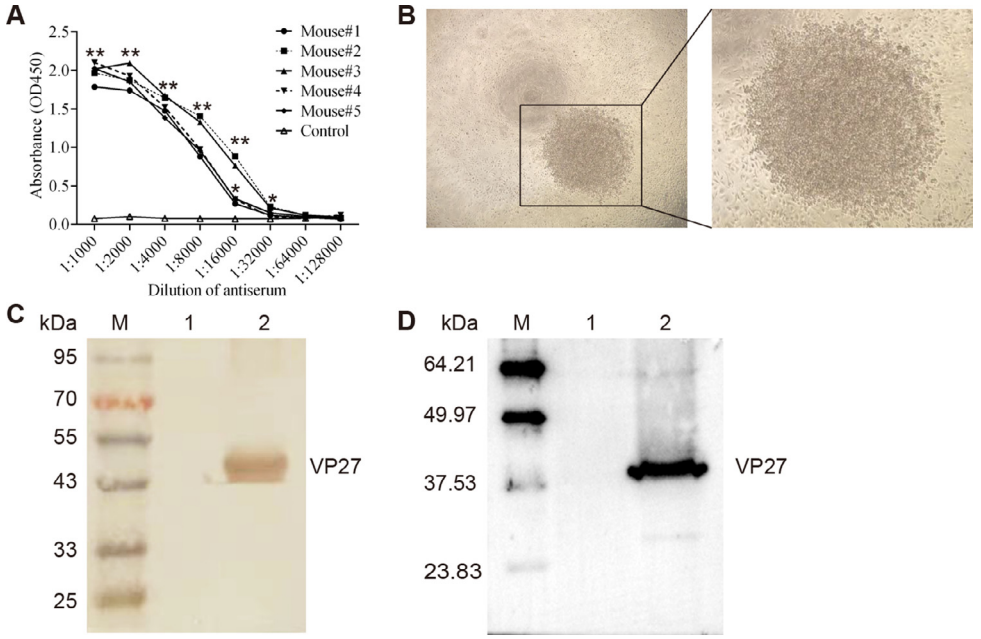

Figure 2. Screening and characterization of mAbs specific for VP27 Titers of VP27 antibody in the mouse sera after immunization with the VP27 protein were measured with ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Control group. (B) Cell clones and morphology of hybridoma cells. Original magnification 40 × (C) Reactivity of the mAb 2AF11 with recombinant VP27 protein by western blot analysis. Lane M, protein marker; Lane 1, non-IPTG-induced cell lysate of pCold-SUMO-VP27-transformed *E. coli* BL21 (DE3); Lane 2, purified VP27 protein. (D) Reactivity of the mAb 2AF11 with VP27 eukaryotic expression vector-transfected 293T cells. Lane M, protein marker; Lane 1, pCMV-14 empty plasmid; Lane 2: pCMV-14-VP27-transfected cell lysates.

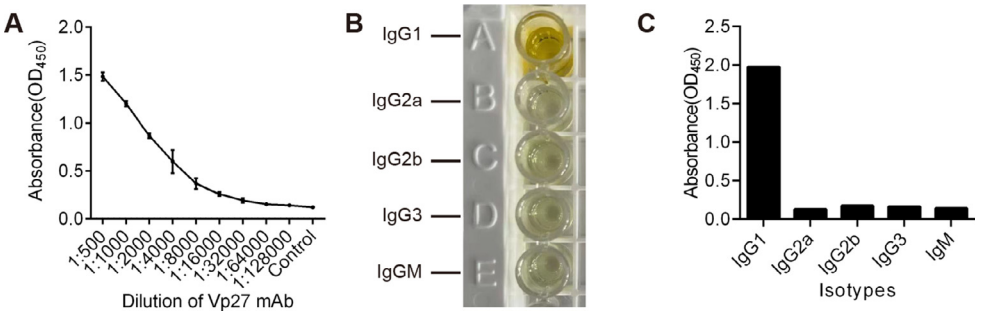

Figure 3. Characterization of the mAb against the VP27 protein of GAstVs. (A) The VP27 mAb titer was measured by ELISA. (B, C) The isotype of the VP27 mAb was identified as IgG1 with the isotyping ELISA kit.

Isotype Determination

In order to produce VP27 mAbs in large quantities, 2AF11 hybridoma cells were injected into the peritoneal cavity of mice, and 2AF11-containing ascitic fluid samples were collected on the seventh day after injection. The antibody titer in the fluid samples was measured by ELISA, as described above, and was found to be about 1:32,000 (Figure 3A). The isotypes of the mAb were identified with the ELISA Mouse mAb Isotyping Kit as the IgG1 isotype (Figures 3B and 3C).

Reactivity of the mAb toVP27 in GAstVs-Infected LMH Cells

To further verify if the VP27 mAb could detect the intracellular localization of GAstVs VP27, indirect immunofluorescence assay (IFA) was performed in GAstVs-infected LMH cells. As shown in Figure 4, VP27 mAb could recognize GAstVs VP27 in GAstVs-infected LMH cells, and the VP27 protein was obviously distributed in the cytoplasm of the GAstVs-infected cells. As expected, no immunofluorescence was observed in the normal LMH cells. Collectively, these data indicate that the VP27 mAb could discriminate GAstVs-infected cells from uninfected ones and, therefore, this mAb can be used for further diagnostic applications for the detection of GAstVs infection.

Diagnostic Application of the VP27 mAb for GAstVs Detection in Clinical Samples

The application of this mAb was evaluated by immunohistochemical staining analysis using kidney and spleen tissues from GAstVs-infected goslings. The results showed that the paraffin-embedded kidney tissues were positive for the antibody, as evidenced by the appearance of dark brown spots, which indicate strong immunoreactivity against the GAstVs antigen (Figure 5A). By contrast, there were no positive spots in the GAstVs-negative kidney and spleen samples. Similar immunoreactivity results were obtained for the spleen tissues (Figure 5B). These results indicate that the VP27 mAb was able to discriminate GAstVs-infected tissues or cells from uninfected tissue.

DISCUSSION

In the present study, we generated a new anti-GAstVs-VP27 mAb that could specifically bind to the VP27 protein in VP27 eukaryotic expression vector-transfected cells and recognize the virus in GAstVs-infected gosling tissues, as well as detect the location of VP27 in the cytoplasm of the infected cells. These findings indicate the high diagnostic potential of this antibody and its potential for the development of diagnostic methods for identifying GAstVs-infected goslings and further functional analyses of the VP27 protein.

In 2017, GAstVs was first isolated from diseased goslings in China and was characterized by gout, and hemorrhage and swelling of the kidneys (Niu et al., 2018; Chen et al., 2020; Chen et al., 2021). It has spread to the major goose-producing regions of many provinces of China and is associated with high mortality and morbidity rates in goslings (An et al., 2020; Yin et al., 2021). According to one report, the economic losses caused by this disease are estimated to be between 1.2 and 1.5 billion yuan. Presently, there is a lack of vaccines, diagnostic approaches, and antiviral drugs for the treatment of this disease, as the development of novel virus-specific vaccines and antiviral drugs against GAstVs can be generally time-consuming and labor-intensive (Bogdanoff et al., 2018). Thus, accurate and rapid diagnostic methods that can be used to detect GAstVs infection early on are readily available options to prevent GAstVs infection. mAbs are increasingly being recognized as a promising diagnostic agent for determining viral titers (Chen et al., 2014; Huang et al., 2017; Liu et al., 2017). Therefore, it is essential to generate a new GAstVs-specific mAb with high sensitivity and specificity as a diagnostic agent.

As observed with all astroviruses, the spike protein is the key player in receptor recognition, virus attachment and entry, as well as the host immune response and, therefore, the key antigenic protein of the outer particle surface in astroviruses (Bass and Upadhyayula, 1997). Antigenicity studies have indicated that antiastrovirus mAbs against the spike protein could block virus attachment to host cells and/or provide protective immunity against virus infection (Sanchez-Fauquier et al., 1994; Bass and Upadhyayula, 1997). In this study, we successfully induced the expression of recombinant VP27

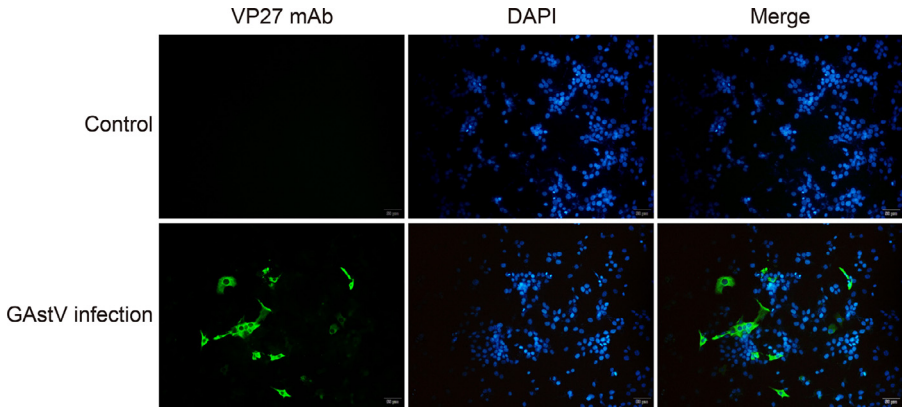

Figure 4. Immunofluorescence assay for expression and intracellular localization of the VP27 protein. The VP27 mAb 2AF11 was used as the primary antibody for recognizing the VP27 antigen in GAstVs-infected LMH cells at postinfection 48 h. The fluorescent agent 4′,6-diamidino-2-phenylindole (DAPI) was used to counterstain the cell nuclei of the cultures. Original magnification 200 ×.

protein in the supernatant of *E. coli* in which the activity of the protein was preserved as much as possible. With the purified VP27 as an immunogen, one mAb (named 2AF11) was obtained. This mAb exhibited clear reactivity with GAstVs-infected cells and kidney and spleen tissues in western blot and IHC analyses. Thus, this newly produced mAb, 2AF11, could be useful for the development of antigen detection tests and serological assays targeting GAstVs. In addition, this mAb could be useful for understanding the pathogenic mechanisms of GAstVs in detail. For example, visceral gout is a typical characteristic of goslings infected with GAstVs, which is distinct from known astroviruses that cause gastroenteritis in most mammals and both intestinal and extraintestinal diseases in birds (Dong et al., 2011; Wu et al., 2020). However, the mechanism by which gout is induced by GAstVs remains unclear, but could be explored with the help of the antibody developed in this study.

Immunological diagnostic methods that use antibodies are ideal for large-scale screening and quantitative analysis of viral antigens on account of their high sensitivity, reproducibility, and specificity. mAbs are widely used in immunology-based diagnostic methods, such as colloidal gold-immunochromatography assay, sandwich-ELISA, and IHC. For example, the Nsp10-specific mAb of porcine reproductive and respiratory syndrome virus (**PRRSV**) may facilitate the establishment of diagnostic methods to discriminate between genotype 1 and genotype 2 PRRSV infection (Zhang et al., 2017). Additionally, Tesfagaber et al developed mAb-based ELISA for African swine fever virus antibody detection (Tesfagaber et al., 2021). mAb-based methods have also been developed for the diagnosis of astrovirus and are based on several techniques, including ELISA, IFA, and IHC (Herrmann et al., 1988, 1990; Sanchez-Fauquier et al., 1994). For example, Katja Schmidt et al. successfully induced the expression of VP70 long, VP70 short, VP34, and VP27 in E. coli and compared their reactivities through a bead-based multiplex serology method. The result showed that VP27 capsid protein showed best antibody reactivity between positive and negative mice (Schmidt et al., 2017). In our study, the mAb was used to successfully detect the native form of the VP27 protein in infected cells and tissues. Therefore, this mAb may be useful in the development of high-sensitivity methods to detect GAstVs, such as immunoblot assays, immunofluorescence assays, and antigen-capture ELISA. However, there are still some limitations in the present study. For example, this study

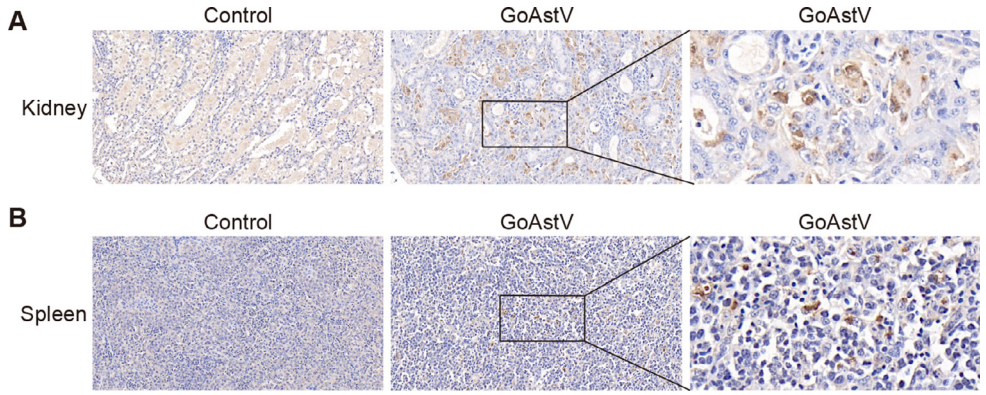

Figure 5. Immunohistochemical staining of GAstVs-infected gosling tissues. The mAb 2AF11 was used as the primary antibody for recognizing the GAstVs antigen in the kidney (A) and spleen tissues (B). Noninfected gosling kidney samples and spleen samples were used as controls. Original magnification 400 ×.

lacks cross reactivity and specificity data of the mAb against other avian astroviruses such as turkeys (TAstV), ducks (DAstV), and chicken (CAstV). Moreover, large clinical samples should be used to validate the diagnostic value and the sensitivity of this mAb.

In summary, in the present study, a mAb against the GAstVs VP27 protein was successfully prepared using a mouse hybridoma system, and our results demonstrate that this mAb shows a strong reaction with purified recombinant VP27 or VP27 expression vector-transfected LMH cells, as well as tissues infected with GAstVs. Thus, the mAb 2AF11 might be useful for the development new diagnostic approaches for GAstVs that could help prevent the spread of GAstVs in the future.

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DISCLOSURES

The authors declare no competing interests.

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