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Research Article 研究报告

重组 IBV 多抗原肽疫苗和鸡卵黄抗体的制备及效应分析

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摘　要：【目的】设计并表达融合传染性支气管炎病毒(infectious bronchitis virus, IBV)已证实抗原表位的重组蛋白 rMKIBV，作为疫苗以期提供全方位保护，同时探讨经 rMKIBV 疫苗免疫蛋鸡收获的多克隆抗体卵黄抗体(IgY)在 IBV 防治中的潜力。【方法】通过网站获取的 IBV 已证实抗原表位序列与 GenBank 数据库收录的具有代表性的 IBV 毒株序列进行比对，设计柔性肽连接所有抗原肽。构成的氨基酸序列经过分析处理后，逆翻译并进行密码子优化，随后插入 pET-28a(+)克隆载体，导入大肠杆菌进行表达。纯化脱盐、去内毒素处理后的 rMKIBV 蛋白作为疫苗免疫动物，探究其免疫原性及是否可以刺激蛋鸡产生特异性 IgY。【结果】检索的 IBV 抗原表位序列与代表性 22 株 IBV 毒株的公开 N 蛋白和 S 蛋白序列在相应区域均高度相似。rMKIBV 蛋白预测的等电点和分子量分别为 10.25 和 63.39 kDa；二级结构显示无规则卷曲占比最高，表明抗原性较强。成功构建重组质粒 pET-28a-mkibv，并在大肠杆菌中表达出高纯度的 rMKIBV 蛋白。该蛋白能与抗 His-tag 抗体、N 蛋白抗体、S 蛋白抗体特异性结合。免疫小鼠后，脾脏指数增大(P<0.05)，血清特异性 IgG 抗体水平显著升高(P<0.01)，IFN-γ 水平升高(P<0.05)，IL-2 水平无明显变化。免疫蛋鸡后，成功提取蛋黄中 IgY，其特异性 IgY 抗体水平均显著升高，且 IgY 抗体滴度在免疫后逐步增大，至 50 d 左右达到最高，而后缓慢下降，达到稳定水平。【结论】本研究成功构建并表达了融

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合 IBV 已证实抗原表位的重组蛋白 rMKIBV，该蛋白具有较好的免疫原性，能够刺激小鼠和蛋鸡产生特异性抗体。特别是从免疫蛋鸡的蛋黄中获取的 IgY，为 IBV 的防治提供了新的思路，对开发针对 IBV 的疫苗具有重要的科学意义和应用价值。

关键词：传染性支气管炎病毒；疫苗；重组融合；抗原表位；卵黄抗体

Preparation and effectiveness assessment of a recombinant IBV multiple antigenic peptide vaccine and laying hen yolk antibodies

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Abstract: [Objective] To design and express a recombinant protein rMKIBV incorporating confirmed antigenic epitopes of infectious bronchitis virus (IBV) as a vaccine to provide comprehensive protection. Additionally, it explores the potential of polyclonal yolk antibodies (IgY) harvested from laying hens immunized with the rMKIBV vaccine in the prevention and control of IBV. **[Methods]** The antigenic epitope sequences of IBV, obtained from online databases, were compared with sequences of representative IBV strains from GenBank. Flexible peptides were designed to link all antigenic peptides. The constructed amino acid sequence was analyzed, reverse-translated, codon-optimized, and then inserted into the pET-28a(+) cloning vector. The recombinant vector was introduced into *Escherichia coli* for expression. The purified, desalted, and endotoxin-removed rMKIBV protein was used as a vaccine to immunize animals for investigation of its immunogenicity and ability to stimulate specific IgY production in laying hens.**[Results]** The retrieved IBV antigenic epitope sequences showed high similarity with the published N and S protein sequences of 22 representative IBV strains. The predicted isoelectric point and molecular weight of rMKIBV were 10.25 and 63.39 kDa, respectively. The secondary structure of rMKIBV included a high proportion of random coils, which suggested strong antigenicity. High-purity rMKIBV was obtained from *E. coli* transformed with the recombinant plasmid pET-28a-mkibv. This protein specifically bound to anti-His-tag antibodies, N protein antibodies, and S protein antibodies. The mice immunized with this protein showed increases in the spleen index (P<0.05), elevations in the levels of serum-specific IgG antibodies (P<0.01) and IFN-γ (P<0.05), and no significant change in the IL-2 level. Immunized laying hens successfully produced IgY in egg yolks, with specific IgY antibody levels significantly increasing. Moreover, the IgY antibody titer gradually rose after immunization, reaching the peak after about 50 days and then gradually

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declining to reach a stable level. **[Conclusion]** We successfully constructed and expressed the recombinant protein rMKIBV. The protein demonstrated good immunogenicity, stimulating specific antibody production in both mice and laying hens. Notably, the IgY extracted from the yolks of immunized laying hens offers a novel approach to IBV prevention and control. These findings hold significant scientific and practical value for the development of vaccines against IBV.
Keywords: infectious bronchitis virus (IBV); vaccines; recombinant fusion; antigenic epitopes; yolk antibody

Infectious bronchitis virus (IBV) belongs to the genus Gammacoronavirus[1] and is a highly variable coronavirus[2]. Its genome can encode various structural proteins, such as spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins[3]. These proteins play different roles in the processes of viral infection, pathogenesis, and immune escape[4-5]. Among them, the S1 subunit of the S protein is crucial for inducing neutralizing antibodies and triggering hemagglutination[6]. However, its high mutation and recombination rates lead to frequent antigenic variations. The S2 subunit is relatively conserved and mainly participates in membrane fusion[4]. The N protein is highly conserved and is a major immunogenic protein. It contains cytotoxic T lymphocyte (CTL) epitopes and can induce protective immunity[7-9]. Nevertheless, it also participates in immune evasion and antagonizes type I interferon (IFN-I)[10].

IBV is mainly transmitted through aerosols or direct contact[11] and can damage the respiratory tract[12-13], kidneys, and reproductive systems of poultry[14-17], causing huge economic losses to the poultry industry. Vaccines are key means of preventing and controlling IBV. Effective vaccination can reduce the infection rate and incidence of poultry[18]. Currently, there is a wide variety of IBV vaccines, including live-attenuated vaccines, inactivated vaccines, vector vaccines[9,19], DNA vaccines[20-21], and subunit vaccines[22]. Commercially available vaccines mainly consist of live-attenuated vaccines and inactivated vaccines. Although live-attenuated vaccines are widely used, they have risks such as significant differences in cross-protection ability, the potential to cause the emergence of new variant strains, and virulence reversion[23-26]. Inactivated vaccines have high safety but weak immune responses and high production and management costs[26-27]. DNA vaccines have issues of low efficiency and easy degradation[28]. Vector vaccines often use Newcastle disease virus (NDV), fowl adenovirus (FAdV), etc. as vectors and can induce the production of specific neutralizing antibodies and provide dual protection[29]. Subunit vaccines are made from specific parts of pathogens and can induce better immune protection[30-31]. As emerging vaccines, nanoparticle vaccines require careful consideration of cost and safety issues in practical applications[22,32].

Yolk antibody (IgY) shows potential in the prevention and control of IBV. IgY is naturally deposited in egg yolks, providing immunity to chicks[33]. Its function is similar to that of mammalian IgG, but its structure is closer to IgM and IgE, without a hinge region[34], and its molecular weight is 180 kDa[35]. IgY has advantages such as non-invasive acquisition, high yield (about 50 mg in 15 mL of egg yolk), low cost, good stability (resistant to hypertonicity, repeated

freeze-thaw cycles, and acids and alkalis), and high safety[36]. It is a potential alternative to antibiotics, and there are already commercialized preparations used in the treatment of poultry diseases.

Due to the fact that the development speed of existing vaccines cannot keep up with the mutation rate of IBV, it is urgent to develop a safe and effective IBV vaccine that can provide comprehensive protection. This study integrated the advantages of epitope-based vaccines and protein subunit vaccines, designed and expressed the recombinant protein rMKIBV, aiming to evaluate its immunogenicity and explore the value of IgY harvested from immunized laying hens in the prevention and control of IBV.

1 Materials and Methods

1.1 rMKIBV sequence design and cloning vector construction

The antigenic epitope sequences of IBV were searched and analyzed in GenBank and IEDB. The antigenic epitope sequences were compared with those of 22 representative IBV strains. The flexible peptide sequence was designed to connect different epitope peptides to form the rMKIBV amino acid sequence. The amino acid sequence of rMKIBV was predicted by antigenic determinant, molecular weight prediction, isoelectric point prediction, hydrophilicity prediction and secondary structure analysis. Then the amino acid sequence of rMKIBV was transformed into the nucleotide sequence, and the selected site 5′ *Nco* I−3′ *Xho* I was used to inserting the sequence into the pET-28a(+) vector to construct the recombinant rMKIBV plasmid vector (Beijing Boxin Tongchuang Biotechnology Co., Ltd.), named pET-28a-mkibv.

1.2 Purification, desalting and endotoxin removal of rMKIBV

1.2.1 Expression and purification of rMKIBV

The obtained pET-28a-mkibv plasmid was thermally transformed into competent *E. coli* Rosetta(DE3) to induce rMKIBV protein expression. The pET-28a-mkibv plasmid powder was dissolved and mixed with melted *E. coli* Rosetta(DE3) competent cells in an ice bath. After heat shock transformation, the recombinant plasmid was added to the LB medium for recovery culture. The bacterial solution cultured to the logarithmic growth phase was verified by sequencing and used for sequencing verification of target genes. After sequence alignment using DNAMan software, the bacterial solution was induced with 1 mmol/L IPTG, centrifuged at 12 000 r/min for 1 min at room temperature, and the supernatant was discarded and weighed. The precipitate was washed twice with buffer I (Beijing Solebo Technology Co., Ltd.) and added with appropriate buffer I (10 mL/0.5 g), lysozyme (10 μg/mL) and sodium deoxycholate (0.05 mol/L) according to the weight. The supernatant and precipitate were collected by centrifugation at 12 000 r/min for 20 min at 4 ℃ and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results of electrophoresis were used to determine whether the induction conditions were suitable and whether rMKIBV protein was soluble in the supernatant or existed in the form of inclusion bodies in the precipitate.

The purification was preliminarily carried out by washing with 0−8 mol/L urea gradient. The washing solutions I and II (Beijing Solaibao

Technology Co., Ltd.) were washed and precipitated once according to the concentration gradient, and the supernatant was removed by centrifugation at 12 000 r/min at 4 ℃ for 5 min. Washing solution III three times, centrifuged to remove the supernatant; Wash twice with washing solution IV and centrifuged to remove the supernatant; The remaining inclusion body precipitation was dissolved in 8 mol/L urea to obtain inclusion body extract, and the concentration was measured. The inclusion body extract was further purified by basic protein gel recovery technology, and then SDS-PAGE was used to verify the purification effect. The purified rMKIBV protein was stored at −80 ℃. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), basic protein active gel separation and recovery of rMKIBV protein (native PAGE), Western blotting were performed according to Molecular Cloning: A Laboratory Manual (Fourth Edition)[37].

1.2.2 Endotoxin was removed by Triton X-114 phase separation

1% Triton X-114 was added to the recovered protein solution and mixed thoroughly, allowed to stand at 4 ℃ for 5 min, and then incubated at 37 ℃ until the solution was delaminated, 12 000 r/min, room temperature, centrifuged for 5 min, and the supernatant was aspirated into EP tubes. Thereafter, 1% Triton X-114 was added again and the above procedure was repeated. The dialysis membrane (Yisheng Bio-Co., Ltd.) (10 kDa) was cleaned by boiling with solution II and solution III (Beijing Solaibao Technology Co., Ltd.). The endotoxin-free rMKIBV protein solution was injected into the dialysis bag into PBS buffer and dialyzed by rotation for 2 h at 4 ℃, followed by replacement with a new PBS buffer and overnight dialysis by rotation. The dialysed protein solution was centrifuged at 12 000 r/min at 4 ℃ for 1 min to remove the precipitate and the protein content was determined.

1.2.3 Endotoxin was detected by limulus amebocyte lysate

According to the instructions of the gel method Limulus Lysate Kit (Zhanjiang Bokang Marine Biological Co., Ltd.), the endotoxin standard was diluted to different concentrations, mixed with limulus lysate and incubated in a 37 ℃ water bath for 60 min. The results were judged by the formation of the gel. For the detection of endotoxin in protein solutions, ampules containing limulus amebocyte lysate were prepared and positive and negative controls were set, and the results were judged by observing the formation of the gel.

1.2.4 Determination of rMKIBV concentration by BCA protein quantification

rMKIBV protein concentration was determined using BCA Protein Quantification Kit (Shanghai Biyuntian Biological) according to the manufacturer's instructions. The protein samples were set up in three multiple wells, and the average OD_{562} value measured was substituted into the standard curve linear regression equation drawn by the standard substance to calculate the concentration of rMKIBV protein.

1.3 Immunogenicity evaluation in mice and laying hens

Ten female BALB/c mice aged 5−6 weeks and ten laying hens were completely randomly divided into the immunization group and the control group. PBS was used in the control group. The mice in rMKIBV group were injected with the same amount of Freund's complete adjuvant for the first time, and the mice in the rMKIBV group were

injected with the same amount of Freund's complete adjuvant for the second time after 14 days. The mice in the rMKIBV group were injected with the same amount of Freund's incomplete adjuvant for the third time after 14 days. The normal control group was injected with the same dose of PBS (pH 7.4). Eggs were collected continuously for 2 months after the second immunization and stored at 4 ℃ (Figure 1). All experimental mice were raised in the animal experiment center of Yan'an University (number: YAU-G20240122).

Samples were collected and weighed on the 14th day after the last immunization. The mice were anesthetized by intraperitoneal injection of 1% sodium pentobarbital solution, the eyeballs were clamped and quickly removed, the blood of the mice was collected, and the mice were centrifuged at 3 000 r/min for 15 min at room temperature. Spleen index=Weight of the spleen (g)/ Weight of the body (g) was calculated. The spleen index data of the two groups of mice were expressed as mean±standard deviation (mean±SD) and statistically analyzed by two independent samples *t*-test in GraphPad Prism 8. Western blotting was used to detect the production of protective antibody IgG against rMKIBV in mouse serum. Immunoassays were performed with specific primary antibodies (serum diluted in PBS (1∶100)) and secondary antibodies (sheep anti-mouse IgG (H+L) (1∶500), Abcam), and signal detection and imaging was performed by chemiluminescence. Data analysis: Band gray values were analyzed by ImageJ software and statistically analyzed by two independent samples *t*-test in GraphPad Prism 8. The levels of

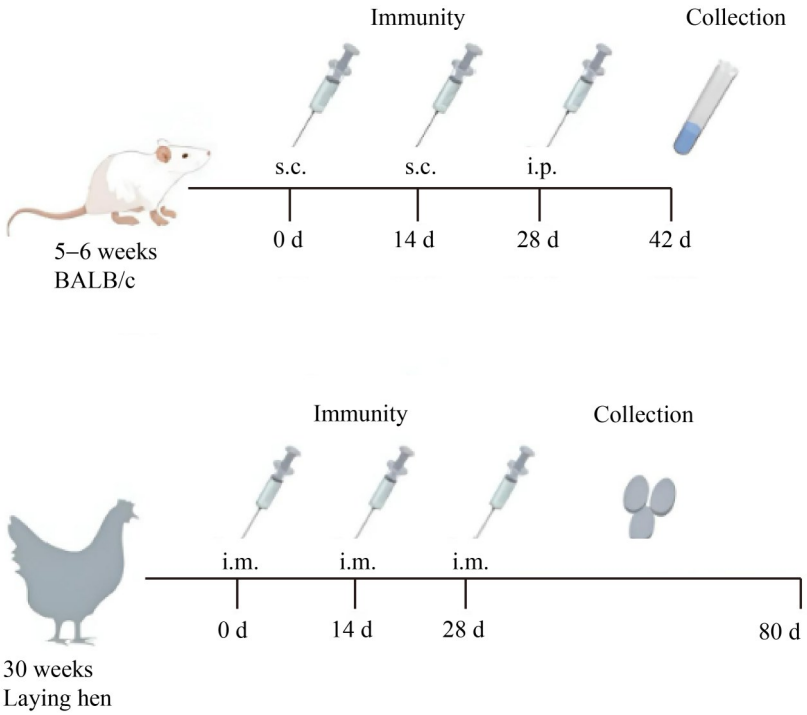

Figure 1 Immune timeline of mice and laying hens. s.c.: Subcutaneous; i.p.: Intraperitoneal; i.m.: Intramuscular.

interleukin-2 and IFN-γ in the serum of immunized mice were measured using the "Cytokine ELISA Quantitative Kit" according to the manufacturer's instructions. The microplate reader was used to detect the wavelength at 450 nm, and the sample concentration was calculated according to the standard curve. Two independent sample *t*-test was used for statistical analysis in GraphPad Prism 8.

1.4 Ig Y identification and titer determination of laying hens

Eggs of different days (20, 35, 50, 65, 80 d after the first immunization) were disinfected, the yolk was separated, and the yolk fluid was collected. The egg yolk solution was diluted 1:8 with ultrapure water, stirred at 4 ℃ for 10 min, adjusted to pH 5.2, left at 4 ℃ for 12 h at 10 000 r/min, centrifuged at 4 ℃ for 20 min, and the supernatant was collected. The precooled ethanol was added to the supernatant to make the final concentration of ethanol reach 60% (*V*/*V*) at 10 000 r/min. After centrifugation at 4 ℃ for 20 min, the precipitate was dissolved in an appropriate amount of 0.16% NaCl, left to fully dissolve, and filtered by filter paper to remove lipoprotein precipitate. Pre-cooled 95% ethanol was slowly added to the collected filtrate to achieve a final ethanol concentration of 30% (*V*/*V*), and the precipitate IgY was obtained by centrifugation at 10 000 r/min at 4 ℃ for 20 min. The precipitate was added to 5 mL of 1×PBS and stored at −20 ℃.

The presence and purity of IgY on different days were determined by SDS-PAGE. 12 μL of IgY samples from different days after dissolution were sucked, mixed with 3 μL of 5×loading buffer, boiled at 100 ℃ for 10 min, centrifuged at 12 000 r/min for 5 min at room temperature. Water soluble components of egg yolk were used as primary antibody, and rabbit anti-chicken IgY (H+L) was used as secondary antibody. The results were observed after staining and decolouring.

A 96-well polystyrene plate was coated with 1 μg/mL rMKIBV protein in 100 μL per well and placed for 20 h at 4 ℃. The next day, discard the liquid in the hole and pat it dry on absorbent paper. Then 200 μL 3% blocking solution was added to each well, incubated at 25 ℃ for 2 h, washed with washing solution 3 times (3 min each time), and patted dry. IgY extracted at different times (20, 33, 50, 65, 80 d) was diluted with PBS from 1:1 000 to 1: 64 000. Negative IgY extract (eggs of control group) was used as negative control. Add 100 μL/well to the plate, incubate at 25 ℃ for 2 h, wash with washing solution 3 times (3 min/time), and pat dry. Hrp-labeled rabbit anti-chicken IgY (H+L) antibody was diluted at an appropriate ratio (1:8 000), 100 μL/well, incubated at 37 ℃ for 1 h, washed with washing solution 3 times (3 min/time), and patted dry. TMB chromogenic solution (100 μL/well) was added to the plate and incubated at 37 ℃ in the dark for 15 min, then the reaction was terminated by adding termination solution. The antibody titer was defined as the maximum dilution of the sample when the ratio of the OD_{450} value of the sample to the negative control OD_{450} value was ≥2.1.

According to the manufacturer's instructions, the "Chicken IBV-Ab ELISA Kit" was used to detect the levels of IBV-Ab in the extracts of egg yolk liquids on the 50th and 80th days after the first immunization. The microplate reader was used to detect the wavelength at 450 nm, and the sample concentration was calculated according to the standard curve. Two independent sample *t*-test was

used for statistical analysis in GraphPad Prism 8.

2 Results and Analysis

2.1 Expression of high-purity rMKIBV

The retrieved IBV epitope sequences were compared with the representative IBV sequences after eliminating duplicates. It was shown that the retrieved IBV epitope sequences were similar to the corresponding regions in different IBV (Figure 2). A flexible peptide sequence (APAPAPAPAPAPAP) was designed to link the retrieved epitopes to form the rMKIBV amino acid sequence (accession number: PQ449047). Subsequently, the isoelectric point and molecular weight of the amino acid sequence of rMKIBV were predicted to be 10.25 kDa and 63.39 kDa, respectively. The more hydrophilic segments of protein antigens are often strongly associated with dominant epitopes, and peptides that are easily soluble in water phase are preferred in the preparation of recombinant proteins. We performed hydrophilicity analysis of

Figure 2 Amino acid sequence alignment results of rMKIBV PQ449047, strain Beaudette spike glycoprotein (UniProt: P11223), and strain Beaudette nucleoprotein (UniProt: P69596). ★ represents residues that are identical.

the designed rMKIBV protein and concluded that the rMKIBV protein was more hydrophilic. In addition, there are protrusions, turns, and coils on the surface of the antigen in the secondary structure of the protein, which are usually easy to chimeric with antibodies. By analyzing the secondary structure of the protein, it can be obtained that the secondary structure of rMKIBV protein showed the highest proportion of Random coil, indicating that the antigenicity was strong (Figure 3).

After prediction evaluation, the rMKIBV amino acid sequence was converted to a nucleotide sequence by reverse translation, followed by *Escherichia coli* codon optimization. The optimized rMKIBV nucleotide sequence was inserted into the plasmid pET-28a(+), and the target restriction sites (*Nco* I−*Xho* I) were used to form the recombinant rMKIBV plasmid vector pET-28a-mkibv. To prevent the nucleotide sequence of rMKIBV from being cleaved by enzymes, the restriction site analysis was performed, and the *Nco* I−*Xho* I restriction site codon was replaced to obtain the nucleotide sequence (accession number: PQ449047).

The recombinant plasmid pET-28a-mkibv was transformed into competent *E. coli* Rosetta(DE3) by heat shock, and the activated culture solution was sent to sequencing to obtain the target gene sequence. Then the target gene sequence was aligned with the designed rMKIBV protein sequence to ensure the correct sequence of the target gene in the plasmid. The sequence of the target gene in the plasmid was consistent with that of the designed rMKIBV protein (identity=99.95%; gap=0.00%). After the sequence alignment was correct, the cultured bacterial liquid was induced by IPTG for expression and verified by SDS-PAGE. The results are shown in Figure 4A. After induction, the band at 85 kDa was significantly thicker, although there was a certain gap with the predicted molecular weight of the protein, which may be due to the modification after translation and expression, the presence of His tag, etc. After fragmentation, the protein was present in the precipitate as inclusion bodies.

The large amount of expression can provide raw materials for subsequent purification, etc., and obtain a large number of bacterial precipitates after fragmentation. After preliminary purification with urea gradient washing, insoluble impurities were washed away and some miscellaneous proteins were removed to obtain the extract of inclusion

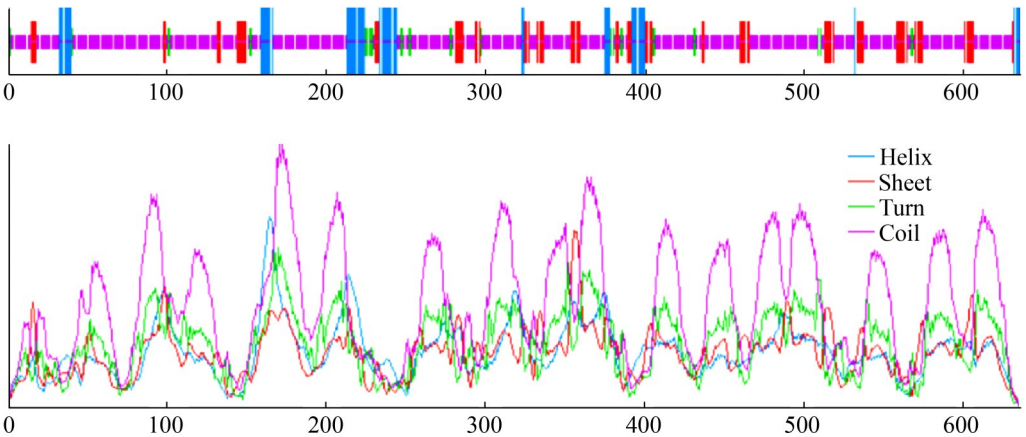

Figure 3 Results of rMKIBV protein secondary structure prediction.

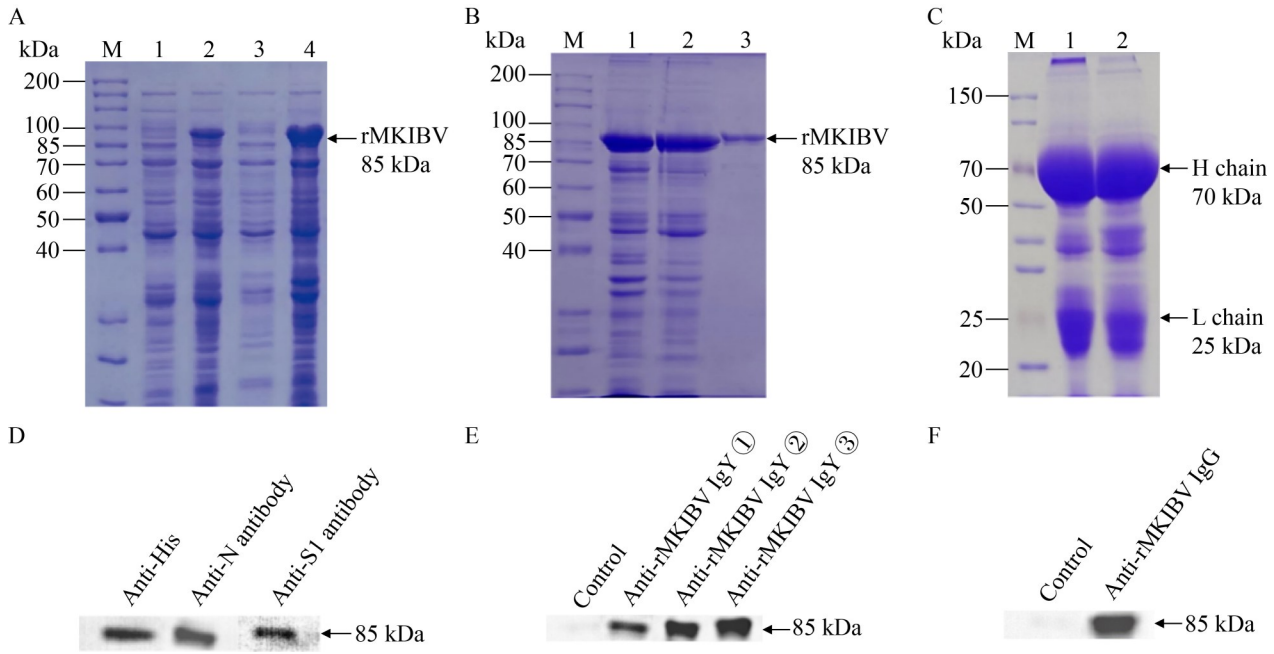

Figure 4 Expression and purification of rMKIBV and preparation and identification of its induced polyclonal antibody. A: Induced expression of rMKIBV protein (Lane M: Protein marker; Lane 1: Pre-induction strain; Lane 2: Induced strain; Lane 3: Supernatant of bacterial fragmentation after induction; Lane 4: Broken and precipitated after induction); B: SDS-PAGE of purified rMKIBV protein (Lane M: Protein marker; Lane 1: Bacterial fragmentation and precipitation after induction; Lane 2: Inclusion body after precipitation gradient washing; Lane 3: Cutting and recovering rMKIBV); C: Polyclonal antibody IgY extracted from eggs (Lane M: Protein marker; Lane 1 and lane 2: IgY); D: The rMKIBV protein was identified by Western blotting; E: The specificity of polyclonal IgY detected by Western blotting (①, ②, ③ refers to the 30−45 d, 46−60 d, 61−75 d, respectively); F: Western blotting for detection of murine polyclonal antibody IgG.

bodies. Verified by SDS-PAGE, the results showed Figure 4B that there were indeed fewer miscellaneous proteins after washing than in the fragmentation precipitate. According to the pI (10.65) of rMKIBV protein, the rMKIBV protein was further purified by alkaline protein active gel technology, and the results showed that the isolated rMKIBV protein was consistent with the target band.

After two cycles of treatment with Triton X-114 non-ionic surfactant, it was found that there was still residual Triton X-114 in rMKIBV protein, so the large molecules of rMKIBV protein were intercepted by dialysis method, and the small molecules slowly diffused through the concentration difference with the dialysis buffer, and the dialysis buffer was constantly replaced. The small molecular salt and LPS in the dialysis bag were removed. The high-purity rMKIBV protein solution after salt removal and endotoxin treatment was obtained. The endotoxin residual in the final product was detected by limulus amebocyte lysate. The results of limulus amebocyte lysate sensitivity recheck showed that the maximum concentration of 2λ tube was positive, the minimum concentration of 0.25λ tube was negative, and the negative

control tube was negative. The test method was effective. The geometric mean of the end-point concentration of the reaction was calculated as the measured value of limulus sensitivity λc.

For the determination of the residual endotoxin content of rMKIBV protein solution, the positive control containing 2λ endotoxin standard was positive, and the negative control was negative, indicating that the test was effective. The sample rMKIBV protein solution was positive when 2λ of endotoxin standard was added to the sample, while the sample treated with detoxification was negative. The results indicated that the endotoxin content in the protein samples treated with Triton X-114 and dialysis did not exceed the minimum limit of endotoxin that could be detected by this method.

The BCA protein content detection kit was used to detect rMKIBV protein content, and the standard curve was established according to the requirements of the kit. The results showed that the linear relationship between the concentration of the standard curve was good, R^2=0.995 7. According to the standard curve and OD_{562} value of the sample, the corresponding protein concentration was calculated. After calculation, the concentration of inclusion body extract, rMKIBV protein recovered from gel and rMKIBV protein after dialysis was 7.85, 1.62, 1.19 mg/mL, respectively.

2.2 rMKIBV protein detection

The prepared rMKIBV protein was actually a fusion of the confirmed antigenic peptides from the N and S proteins of IBV. It is necessary to verify whether rMKIBV protein can specifically bind to the relevant anti-N protein antibodies and anti-S protein antibodies *in vitro* to ensure that the prepared rMKIBV protein is consistent with expectations, that is, no degradation or extensive epitope folding occurs during the entire purification and salt removal process. The results of Western blotting of rMKIBV protein with His-tag antibody, N antibody and S antibody are shown in Figure 4D. The position of rMKIBV protein band in the figure is consistent with that in SDS-PAGE before. The specific binding with His-tag antibody further confirmed that the recombinant protein expressed in *E. coli* had intact structure (with His tag at the end). The specific binding of rMKIBV to N antibody and S antibody indicated that the prepared rMKIBV protein was exposed to antigenic peptide, that is, it could react with corresponding antibody.

2.3 Changes in spleen index of mice

As an important immune organ, the spleen plays an important role in the immune response. The spleen ratio is a commonly used measure to assess the size of the spleen relative to body weight. The spleen index of each group was analyzed after immunization, as shown in Figure 5A. Compared with the control group, the spleen index increased after immunization with rMKIBV vaccine, and the difference was statistically significant (P<0.05), which preliminarily indicated that it could stimulate the immune response of mice.

2.4 Detection of specific antibody levels in mice

rMKIBV vaccine is essentially a fusion of multiple IBV antigen peptides. Whether mice can be immunized with rMKIBV vaccine to stimulate the production of specific IgG antibodies is the core of vaccine immunogenicity evaluation. The specific binding of rMKIBV protein to the mouse serum polyclonal antibody was determined by Western blotting, and the results showed Figure 4F that a specific band at 85 kDa with similar size to

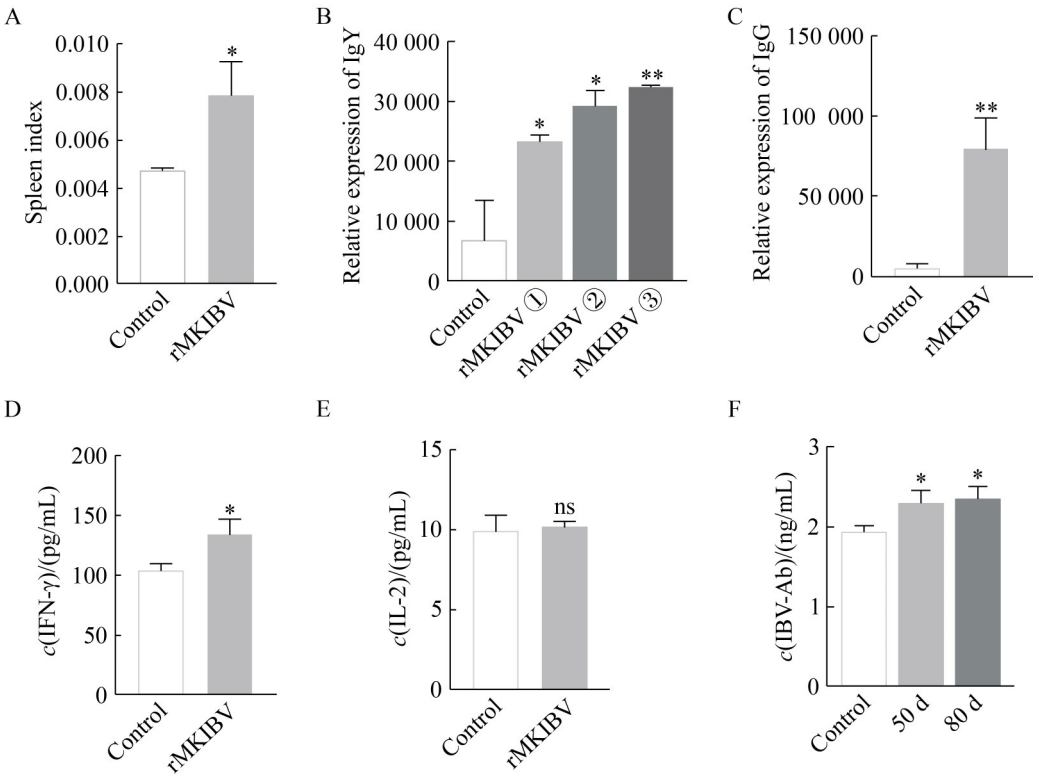

Figure 5 Effects of rMKIBV multiple antigenic peptide vaccine on immune responses in mice and specific antibodies in egg yolk. A: Effect of rMKIBV vaccine on spleen index in mice (*: P<0.05); B: Polyclonal IgY content in egg yolk at different stages (①, ②, ③ refers to the 30−45 d, 46−60 d, 61−75 d, respectively, *: P<0.05, **: P<0.01); C: Specific IgG content in mouse serum (Compared with the normal control group, **: P<0.01; D: IFN-γ levels in serum of immunized mice. *: P<0.05); E: IL-2 levels in serum of mice after immunization (Compared with the normal control group, ns: Not significant); F: The content of IBV-Ab in egg yolk liquid on the 50th and 80th days after the primary immunization (Compared with the normal control group, *: P<0.05).

the target band could be generated, while the mice serum from the control group failed to form a band. Compared with the control group, the level of specific IgG antibodies in rMKIBV group was significantly increased, and the difference was statistically significant (P<0.01) (Figure 5C).

2.5 Serum cytokine levels in mice

Detection of cytokine secretion level is one of the common means to reflect the body's cellular immune response. The levels of IFN-γ and IL-2 in the serum of mice in each group after immunization were shown in Figure 5D and 5E. Compared with the control group, the level of IFN-γ in the rMKIBV group was increased, and the difference was statistically significant (P<0.05). There was no significant change in IL-2 levels, suggesting that rMKIBV protein may induce cellular immunity.

2.6 IgY identification and titer determination

After egg collection, the two-step precipitation method of water dilution and cold ethanol was used for IgY extraction and preliminary purification, and the results are shown in Figure 4C. In SDS-PAGE,

67 kDa IgY heavy chain and 22 kDa IgY light chain can be seen, indicating successful extraction of IgY from yolk. The water-soluble components of egg yolk at 30−45 d, 46−60 d and 61−75 d were named as rMKIBV ①, ② and ③, respectively. The specific binding of rMKIBV protein to polyclonal IgY was determined by Western blotting, and the results showed (Figure 4E) that a specific band at 85 kDa with similar size to the target band could be generated, while the control group failed to form a band. Compared with control group, the specific IgY antibody levels in rMKIBV group were significantly increased, compared with control group at 30−45 d and 46−60 d, $P<0.05$; 61−75 d compared with control group, $P<0.01$ (Figure 5B).

To further validate the protective efficacy of the recombinant protein rMKIBV against IBV, assays were conducted using plates coated with standard commercial IBV antigens (Figure 5F). The findings revealed that the IB-Ab concentration in the control group remained at a relatively low level. In contrast, on the 50th and 80th days post-immunization with rMKIBV, the IBV-Ab concentrations in chickens were remarkably higher than those in the non-immunized control group ($P<0.05$). These results suggest that rMKIBV immunization can effectively enhance the IBV Ab concentration in chickens. This strongly implies that the recombinant protein rMKIBV has a high potential to offer a certain degree of protection against IBV infection in chickens.

The antibody titers of IgY extracted at different days (20, 35, 50, 65, 80 d after primary immunization) were measured, and the antibody titer change curve was drawn to monitor the trend of antibody growth and decline, as shown in Figure 6. The titer of IgY antibody gradually increased after immunization and reached the highest level (1:16 000) about 50 days after immunization, then slowly decreased and reached a stable level.

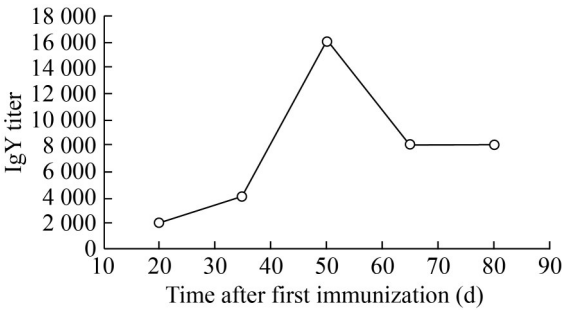

Figure 6 IgY antibody titer change.

3 Discussion and Conclusion

The N and S proteins of IBV are rich in antigenic epitopes and play a crucial role in stimulating the host's immune response. Protein subunit vaccines based on antigenic epitopes ingeniously fuse multiple antigenic epitopes to construct subunits, which greatly enhances antigen specificity and significantly improves vaccine safety, thus showing promising application prospects. However, there are still limitations in the current application of IBV epitopes. Most studies only use partial antigenic epitopes from some strains. Given that IBV has extremely rich genetic diversity, with as many as 39 lineages, and each lineage contains numerous different strains, the existing vaccine strategies based on partial epitopes are difficult to provide comprehensive and effective immune protection against all IBV strains for the host. This study focuses on addressing this key issue. By constructing the rMKIBV expression vector and applying a series of sophisticated separation and purification techniques, high-purity rMKIBV protein was successfully obtained. This achievement not only lays a solid foundation for

in-depth exploration of the immunological characteristics of the rMKIBV protein but also provides important material support and research directions for the subsequent development of more efficient and comprehensive IBV vaccines, holding the promise of promoting breakthroughs in the field of IBV immune prevention and control.

BALB/c mice were selected to evaluate the immunogenicity of the rMKIBV protein. Under the current immunization dose and method, the rMKIBV protein can induce the production of specific protective IgG antibodies in mice. However, there is still room for optimizing the immunization method and dose. Cellular immunity is equally important in the host's immune response. The designed rMKIBV protein contains N-protein antigenic epitopes, and N-protein epitopes contain T-cell epitopes, which can induce cellular immunity[7-9]. Detecting the levels of cytokines can effectively reflect the situation of cellular immunity. IFN-γ is mainly secreted by Th1 cells, $CD8^+$ T cells, and natural killer cells and has functions such as antiviral, anti-infection, and immune regulation[38]. IL-2 is produced by $CD4^+$ T cells and NK cells and is of great significance in immune memory[39]. In this study, it was detected that compared with the control group, the level of IFN-γ in the serum of mice immunized with the rMKIBV protein increased significantly, and the difference was statistically significant ($P<0.05$). However, the level of IL-2 did not change significantly. In the follow-up, it is necessary to more comprehensively detect the levels of multiple cytokines (such as IL-6, IL-4, TNF-α, IL-10) and conduct splenic lymphocyte proliferation experiments to more deeply clarify the situation of cellular immunity.

In the laying hen experiment, laying hens were immunized with a protein dose of 500 μg supplemented with Freund's adjuvant, and specific polyclonal antibody IgY was successfully obtained. The detection of IgY by Western blotting and ELISA showed differences between the two methods. The Western blotting results indicated that the IgY content gradually increased, while the ELISA results showed that IgY reached a peak at 50 days and then slowly decreased. This difference may be due to the different purposes, sample processing methods, and selected time points of the two detection methods. Western blotting is mainly used to detect specific recognition, and a mixed sample of IgY at a certain time period was selected in the experimental design. In contrast, ELISA uses a single IgY sample at a certain time point to determine the antibody titer at that time. In addition, Western blotting incubates with the water-soluble components of egg yolk, while the IgY samples at each time point in ELISA are extracts of the water-soluble components of egg yolk after step-by-step purification with cold ethanol, and the time points selected for ELISA determination are not comprehensive enough. In subsequent studies, more time points can be added to draw a more detailed change trend graph.

Although certain achievements have been made in this study, there are still limitations. In practical applications, more factors need to be considered, such as the cross-protection effect of the rMKIBV vaccine against different strains and the long-term immune effect. The current immunization procedure for laying hens can be further optimized to ensure more efficient and sustainable acquisition of IgY. The preventive and therapeutic effects of specific IgY antibodies against different IBV strains should be further evaluated in SPF-grade chicks to provide a more

reliable basis for their development into emerging products for the prevention and control of IBV. In addition, the rMKIBV vaccine can be used in combination with probiotics, prebiotics, traditional Chinese medicine, and their natural active products to enhance the prevention and control effect by synergistically regulating the intestinal microbiome. At the same time, the extraction and purification methods of IgY antibodies in this study need to be optimized to improve the yield and purity.

Overall, this study successfully constructed and expressed the rMKIBV protein. This protein exhibited good immunogenicity in both mouse and laying hen experiments, providing important theoretical and practical bases for the development of IBV vaccines and the optimization of prevention and control strategies. Subsequent research will focus on in-depth exploration of the above-mentioned deficiencies to promote the development of the IBV prevention and control field.

In conclusion, the findings of this study carry significant scientific implications and practical value for the development of vaccines against IBV. The excellent immunogenicity of the rMKIBV protein serves as a crucial reference for the research and development of novel vaccines. The IgY extracted from the egg yolks of immunized laying hens paves a new way for the prevention and control of IBV, offering the potential to provide more effective measures for the poultry industry to combat the detrimental effects of IBV.

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Availability of Data

The original contributions presented in the study are publicly available. GenBank accession number: PQ449047.

Credit Authorship Contribution statement

CHENG Jiahua: Original draft; WANG Zhuoling: Methodology; ZHENG Min: Validation; HE Xiaochan: Supervision; TIAN Yezi: Methodology; WANG Shuo: Project administration; WANG Ruirui: Technology support; WU Zhuoxuan: Assisting with research and experimentation programs; FENG Yuanrui: Participate in the thesis discussion; ZHANG Yuemeng: Funding acquisition and experimental method guidance; YUE Changwu: Writing – review & editing, Funding acquisition, Supervision and Project administration.

Declaration of Competing Interest

The authors declare no competing financial interest.

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