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Research Article

# Development and Preliminary Application of an Indirect ELISA to Detect Infectious Bovine Rhinotracheitis Virus Using Recombinant Glycoprotein D of IBRV Strain SD [1]

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## Abstract

Glycoprotein D (gD) is the major structural protein of infectious bovine rhinotracheitis virus (IBRV). It can induce both humoral and cellular immunity thus it is a preferable protein for IBR diagnostic reagent. Regarding to DNAstar analysis, the major antigenic region of the gD fragment was amplified by PCR using the IBRV genomic DNA as a template, and subsequenly constructed into the recombinant plasmid pET32a-gD. The fusion protein was expressed upon IPTG induction. The fusion protein was purified by immobilized Ni ion affinity chromatography with a Ni-NTA Kit after verification by SDS-PAGE and Western-blotting analysis, then was utilized as a coating antigen to detect antibodies to infectious bovine rhinotracheitis virus (IBRV) in an indirect ELISA method. Cross-reactivity examinations have showed that the recombinant antigen had no cross reaction with positive sera of other common viral diseases (bovine ephemeral fever, bovine viral diarrhea-mucosal disease, calf diarrhea, bovine intestinal virus infection, bovine coronavirus disease) which indicating a strong specificity. Application of this diagnostic method in 1315 clinical serum samples displayed an antibody positive rate of 23.7% (311/1315), with respect to a coincidence rate of 96.8% as compared to a commercialized IBRV whole virus ELISA. Our method is stable and sensitive hence provides a quick and convenient serological diagnosis favoring epidemiology and disease identification of domestic IBR.

***Keywords:*** *gD, Indirect ELISA, Infectious bovine rhinotracheitis virus, Serology*

# Enfeksiyöz Bovine Rhinotracheitis Virusun Tespitinde IBRV Suş SD Rekombinant Glikoprotein D Kullanılarak İndirekt ELISA Yönteminin Geliştirilmesi ve Uygulanması

## Özet

Glikoprotein D (gD) sığır enfeksiyöz rhinotracheitis virus (IBRV)'ün majör yapısal proteinidir. Glikoprotein D hem humoral hem de hücresel bağışıklığı uyarması nedeniyle IBR'nin tespitinde tercih edilen bir proteindir. DNAstar analizi ile ilgili olarak gD fragmanının majör antijenik bölgesi BRV genomik DNA'sı şablon olarak kullanılmak suretiyle PCR ile amplifiye edildi; sonrasında rekombinant plazmid pET32a-gD içinde yapılandırıldı. Füzyon proteini IPTG oluşturulmak suretiyle ifade edildi. Füzyon proteini; SDS-PAGE ve Western Blotting analizleriyle onaylandıktan sonra Ni-NTA Kit kullanılarak immobilize Ni iyon affinite kromotografi ile saflaştırıldı. Sonrasında, saflaştırılan protein indirekt ELISA metodunda enfeksiyöz bovine rhinotracheitis virüs (IBRV) antikorlarını belirlemek amacıyla örtü antijeni olarak kullanıldı. Çapraz reaksiyon incelemeleri ise diğer yaygın viral hastalıkların (bovine ephemeral fever, bovine viral diare-mukozal hastalık, buzağı dizanterisi, bovine intestinal virüs enfeksiyonu, bovine coronavirus hastalığı) pozitif serumları ile çapraz reaksiyon oluşturmayarak güçlü bir spesifiteye sahip olduğunu gösterdi. Tanı amaçlı olarak metodun 1315 serum örneğinde uygulanması neticesinde antikor pozitif oranı %23.7 (311/1315) olarak belirlendi. Bu sonuçla güvenli kullanım oranı ticari IBRV tüm virüs ELISA ile karşılaştırıldığında %96.8 olarak tespit edildi. Çabuk ve kullanışlı serolojik tanı sağlaması nedeniyle mevcut yöntem IBR'nin hastalık tanısı ve epidemiyolojisinde istikrarlı ve duyarlı bir test olarak kullanılabilir.

***Anahtar sözcükler:*** *gD, İndirekt ELISA, Enfeksiyöz Bovine Rhinotracheitis Virus, Seroloji*

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# INTRODUCTION

Infectious bovine rhinotracheitis (IBR) is an acute, feverish and contagious disease caused by infectious bovine rhinotracheitis virus (IBRV) [1-3]. IBRV is one of critical pathogens causing bovine respiratory diseases (also known as shipping fever). Apart from direct pathogenicity, IBRV also induces immune suppression resulting in secondary bacterial infection, which subsequently weakens cattle reproductivity and causes mortality in severe cases. This virus has a typical pantropism enabling it to attack a variety of tissues and organs with diverse symptoms. The cattle are characterized with latent and persistent infections after virus invasion and can be a long-term, or even life-long carrier [4,5]. The potential carriers intermittently release viruses during a period of up to one and half years which rapidly spread around in a high density stock causing exclusive infections. Meanwhile, certain amounts of IBRV in the semen also lead to a widespread of viruses making it extremely difficult to control and eliminate the disease.

From the 1850s when the disease was identified for the first time in the US, so far there have been IBR infections reported worldwide and thus it became a prominent global disease. Since IBR was isolated in China in 1980 from the cattle imported from New Zealand, the presence of this disease in most Chinese provinces is rising up and impacting greatly on the rates of cattle fattening, milk production and reproductivity [6,7]. IBR is classified as a class B infectious disease by the World Organization for Animal Health (OIE), and is a key quarantine by the Law of the People's Republic of China on the entry and exit animal [8]. Therefore, further development of diagnostic method for IBR is of great importance for prevention and control of the disease.

IBRV also called bovine herpesvirus type I (BHV-1). The BHV-1 genome is an approximately 138 kb linear double-stranded DNA [3]. It encodes about 30 to 40 structural proteins including 11 glycoproteins among which, the gD is a major one located at the surface of virus particle associated with virus infection [9]. It is mainly involved in virus entry and can induce humoral and cellular immunity in host cells [10]. The whole virus ELISA method experiences a risk of virus contamination although with a better antigenicity. The neutralization antibody against gD largely produced in the host has the best performance to antagonize IBRV, hence is favored as the top choice for manufacturing diagnostic reagents and subunit vaccines [11]. This study utilizes a prokaryotic system to express recombinant gD. Preliminary data showed that the recombinant product of the whole gD gene mainly existed as an inactive form in inclusion body and needed further complicated re-activation after purification. Hence in this study, a fragment of the gD gene centralized with antigen clusters was truncated depending on the outcome of biological software analysis. The majority product of this truncated gD gene was in a soluble form. In addition, Western-blot analysis of purified protein indicated that this shortened gD protein exhibited a relative high reactivity to be used as the antigen in ELISA [12].

This study aims to establish an indirect ELISA method to detect IBRV using the recombinant gD protein expressed in prokaryotic cells as the coating antigen. It provides a quick and convenient technique for IBRV serological and epidemiological investigation.

# MATERIAL and METHODS

### *Virus, Bacterial Strains, Plasmids and Serum Samples*

The IBRV-SD strain was isolated from a dairy farm in Jinan, Shandong, China. The cloning vector pEASY-T3, and DH5a, BL21(DE3) competent cells were purchased from Transgene Biotechnology Co., Ltd, Beijing, China. The pET32a (+) expression plasmid, IBRV positive/negative sera and 1315 blood samples collected from dairy cattle in some Shandong farms were stored in the Dairy Cattle Research Center, Shandong Provincial Academy of Agricultural Sciences, Jinan, China.

### *Enzymes and Reagents*

The enxymes and reagents were purchased from different companies *(Table 1)*.

### *Extraction of Virus Genomic DNA*

Monolayer MDBK cells were inoculated with 100$TCID_{50}$ IBRV ($TCID_{50}$ = $10^{-7.62}$/0.1 mL) and underwent 3 freeze-thaw cycles once 80% of lesion achieved. The IBRV genomic DNA was purified with the virus genomic DNA extraction kit (Takara Biotechnology Co., Ltd (Dalian, China) following manufacturer's instructions.

### *Construction and Verification of the gD Vector for Prokaryotic Expression*

The gD ORF was amplified by PCR from the IBRV SD strain genome. The primer sequences for the gD gene were gD-F and gD-R. The primers were designed specifically using a primer design software *(Table 2)* and synthesized by Sangon Biotechnology Co., Limited (Shanghai, China). The truncated gD fragment was amplified by PCR and digested with BamH I/Hind III after purification. Afterwards it was cloned into the pET32a (+) backbone to generate the recombinant plasmid pET32a-gD which was transformed into DH5α competent cells. The plasmids were purified from a bulk culture and verified by restriction analysis and sequencing.

### *Expressionand Activity Evaluation of Recombinant gD Protein*

BL21 (DE3) cells were transformed with positive recombinant pET32a-gD plasmids and a single colony was picked

**Table 1.** *Enzymes and reagents used in this study*
**Table 1.** *Çalışmada kullanılan enzim ve maddeler*

| Enzymes and Reagents | Sellers |
|---|---|
| LA Taq enzyme | Takara Biotechnology Co., Ltd (Dalian, China) |
| T4 DNA ligase | Takara Biotechnology Co., Ltd (Dalian, China) |
| restriction endonucleases | Takara Biotechnology Co., Ltd (Dalian, China) |
| viral genomic DNA extraction kit | Takara Biotechnology Co., Ltd (Dalian, China) |
| protein loading buffer and marker | Sangon Biotech Company (Shanghai, China) |
| Ni-NTA spin kit | QIAGEN (Hilden, Germany) |
| IPTG | Sangong Biotechnology Co.,Ltd (Shanghai, China) |
| X-Gal | Sangong Biotechnology Co.,Ltd (Shanghai, China) |
| plasmid extraction and gel extraction kits | Tiangen Bioech (Beijing) Co.,Ltd (Beijing, China) |
| mouse anti-6-histidine (His) monoclonal antibody | Sigma-Aldrich Co. LLC (Saint Louis, USA) |
| horseradish peroxidase-conjugated goat anti-mouse IgG antibody | Sigma-Aldrich Co. LLC (Saint Louis, USA) |
| 96-well plates | Sigma-Aldrich Co. LLC (Saint Louis, USA) |

**Table 2.** *Primers for prokaryotic expression*
**Table 2.** *Prokaryotik ifade tespitinde kullanılan primerler*

| Primer Name | Primer Sequences (5′-3′) |
|---|---|
| gD-F | ATGGATCCTTCGCCTACCCCACGGAC |
| gD-R | GCAAGCTTGTTGACGTTGCCAAAGGCC |

and inoculated into 5 mL of LB containing 0.1% ampicillin followed by overnight growth at 37°C, 200 r/min in a shaking incubator. The next morning the overnight culture was inoculated into 5 mL of LB containing 0.1% ampicillin at 1 in 100 dilution and shake at 200 r/min, 37°C until OD600 to be 0.6 to 0.8. Then IPTG was added to a final concentration of 1 mmol/L and the bacteria were induced at 30°C for 6 h before harvesting. The pellet from 1 mL of broth was vortexed and boiled in 80 μL 1% SDS plus 20 μL of loading buffer for 5 min, after short spin, 10 μL of the supernatant was loaded on a 12% SDS-PAGE gel for analysis.

The induced bacteria were harvested and washed for three times with ice-cold PBS. The cells were disrupted by sonication and centrifuged at 1200 r/min for 15 min. Then the target protein expression in the supernatant and cell pellet were separately analyzed by SDS-PAGE electrophoresis. The target protein was purified with the Ni-NTA protein purification kit according to manufacturer's instructions.

The proteins separated by 12% SDS-PAGE gel were transferred onto a PVDF membrane. The membrane was blocked in PBST containing 5% of skimmed milk, 4°C for overnight, followed by sequential staining with primary mouse anti-6-Histidine (His) monoclonal antibody (1:5000 dilution) and secondary HRP-conjugated goat anti-mouse IgG antibody (1:5000 dilution). The immunoactivity of recombinant gD protein was detected through X-ray spectroscopy.

### *Development of Indirect ELISA (iELISA) to Detect Recombinant gD*

*- Procedure of Indirect ELISA*

The purified protein was appropriately diluted in carbonate buffer (pH 9.6) and coated onto a 96-well ELISA plate at 4°C, overnight. The plate was washed for 3 times with 0.5% PBST buffer for 3 min each, and blocked at 37°C followed by repeated washes as above. The diluted serum samples were added into the reaction plate and incubated at 37°C. Then the plate was washed again as above. The secondary HRP-conjugated antibody was added into the reaction plate after appropriate dilution. After washing, the TMB chromogenic substrate solution was added and reacted for certain time before stopped by 2 mol/L of $H_2SO_4$. The absorbance of degraded substrate was measured at 450 nm in a microplate reader.

*- Optimization for Indirect ELISA*

The main factors impacting on the outcome of indirect ELISA include the concentration of coating antigen, the blocking buffer and time, serum dilution, serum-antigen reaction time, the secondary antibody dilution and incubation time. The optimized conditions were determined by the square matrix titrimetry. Under the same reaction conditions, the plate was coated with different concentrations of antigen (0.5 μg/well, 1.0 μg/well, 1.5 μg/well, 2.0 μg/well, 2.5 μg/well, 5.0 μg/well, 7.5 μg/well, 10.0 μg/well, 12.5 μg/well, and 15 μg/well), added in serially diluted serum samples (1:20, 1:40, 1:80, 1:100, 1:120, 1:140, 1:160, and 1:320), blocked with different blocking solution (5% horse serum, 10% horse serum, 20% horse serum in PBST and the PBST control) for various duration (30 min, 60 min, 90 min, 120 min, and 150 min), incubated with serially diluted secondary antibody solution (1:1000, 1:2000, 1:3000, 1:4000, 1:5000, 1:6000, 1:7000, and 1:8000) with a range of reaction time (30 min, 60 min, 90 min, 120 min,

and 150 min). All these conditions made up of a square with which the negative and positive OD values at 450 nm were measured via a plate reader, and subsequently the maximum P/N value was selected as the best combination.

*- Determination of Positive and Negative Thresholds of iELISA*

Indirect ELISA was performed with 40 bovine serum samples in duplicate (all samples were negative in serum neutralization test and IBRV whole virus analysis). The final results of OD450nm were calculated as mean (x) and standard deviation (S). In accordance with statistical principles, the thresholds were set up based on the criteria as follows: a sample OD450nm value ≥ x + 3S was considered as positive; a sample OD450nm value ≤ x + 2S was considered as negative; and the values between x + 3S and x + 2S were regarded as suspicious. The suspicious samples were double examined and considered as positive if the value still questionable. In this circumstance, clinical symptom was taken into account for diagnosis if applicable, and follow-up monitoring was carried out [13].

*- Specificity Analysis*

Under the same conditions, the bovine sera positive of other viral diseases (bovine ephemeral fever, bovine viral diarrhea-mucosal disease, calf diarrhea, bovine intestinal virus infection, and bovine coronavirus disease) were examined by indirect ELISA in parallel with standard IBRV positive and negative serum samples as reference. Cross-reactivity is determined according to the standard.

*- Experiment Reproducibility*

*Intra-batch experiment reproducibility:* under the same experimental conditions, 6 serum samples were chosen randomly with each examined for 6 times. The average coefficient of variation was calculated.

*Inter-batch experiment reproducibility:* under the same experimental conditions, 6 serum samples were chosen randomly with each examined by ELISA for twice using 4 batches of proteins purified at different time. The average coefficient of variation was calculated (C.V %).

$$C.V\ \% = S/x \times 100\%$$

*- Evaluation of Clinical Samples*

In total 1315 bovine serum samples were examined simultaneously using the indirect ELISA method with IBRV recombinant gD developed in this study and commercialized IBRV whole virus ELISA diagnostic kit. The results from whole virus ELISA were used as reference to evaluate the coincidence rate, sensitivity, specificity of gD-ELISA.

Coincidence rate = sample number with the same result/total number of samples × 100 %

Sensitivity = sample number with positive results by gD ELISA/sample number with positive results by whole virus ELISA kit × 100%

Specificity = sample number with negative results by gD ELISA/sample number with positive results by whole virus ELISA kit × 100%

# RESULTS

***Expression and Purification of Recombinant Protein***

The recombinant plasmid pET32a-gD transformed into *E. coli* BL21 (DE3) displayed efficient expression under IPTG induction. There were a large amount of recombinant proteins in expected size in the induced group over the non-induced one as verified by 12% SDS-PAGE electrophoresis *(Fig. 1)*. The majority of expressed target proteins were present as inclusion bodies in the precipitates, whereas some were in a soluble form in the supernatant. The concentration of purified protein was measured by the BCA assay with an average of 0.210 mg/mL.

***Identification of Recombinant Protein by Western-blot***

Western-blot analysis was carried out to evaluate recombinant gD. As a result, there was a specific band presented at the desired location for the target protein only in the positive serum sample instead of the negative one *(Fig. 2)*. This demonstrates that the expressed recombinant protein could react specifically with the standard IBRV positive serum with a promising reactogenicity.

***Establishment of Indirect ELISA using Recombinant gD***

*- Confirmation of ELISA Conditions*

The final optimal reaction conditions were confirmed after the square titration experiments as follows: protein coating concentration: 2.5 μg/mL; blocking solution: 20% (v/v) horse serum, blocking time: 37°C for 2 h; serum sample dilution: 1:100; incubation time: 37°C for 1 h; optimal secondary antibody dilution: 1:5000; incubation time: 37°C for 1 h; chromogenic reaction time: 37°C for 5 min.

*- Establishment of Positive and Negative Thresholds*

The range of OD450nm values was between 0.138~0.338 with an average of 0.198 and a standard deviation of 0.055, as measured from 40 negative serum samples. The threshold between negative and positive samples was 0.308 which indicates that, a serum sample can be regarded as positive at OD450nm ≥0.363, or negative at OD450nm ≤0.308, or as suspicious with a value between 0.308-0.363. The suspicious sample was measured again and considered as positive with a questionable value.

*- Specificity Evaluation*

According to established iELISA conditions, the positive sera of other common viral diseases (bovine ephemeral

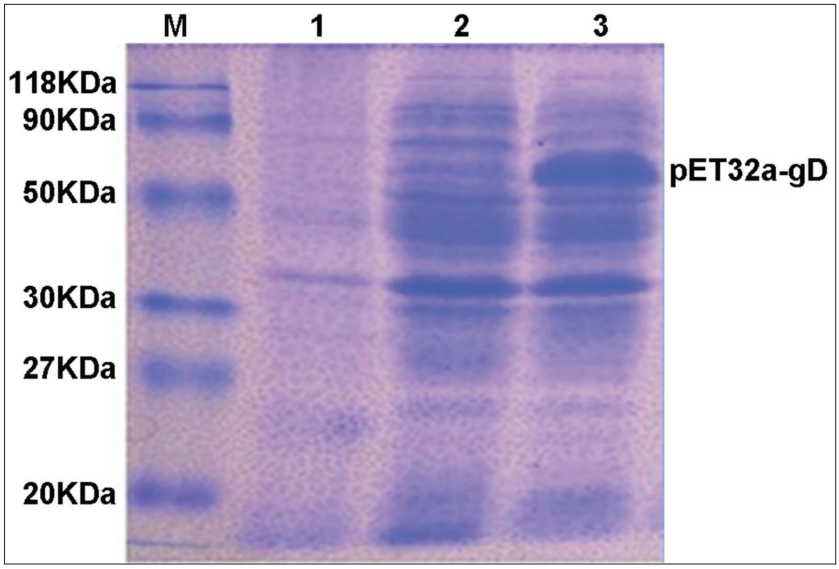


**Fig 1.** Induced expression of pET32a-gD. M. Protein marker; 1. Non-induced lysates from BL21 transformed with pET32a (+); 2. Non-induced lysate from BL21 transformed with pET32a-gD; 3. Lysates from BL21 transformed with pET32a-gD and induced with IPTG

**Şekil 1.** pET32a-gD ekspresyonu. M. Protein markırı; 1. pET32a (+) ile oluşturulan BL21'in uyarılmamış lizatları; 2. pET32a-gD ile oluşturulan BL21'in uyarılmamış lizatı; 3. pET32a-gD ile oluşturulan ve IPTG ile uyarılmış BL21'in lizatları

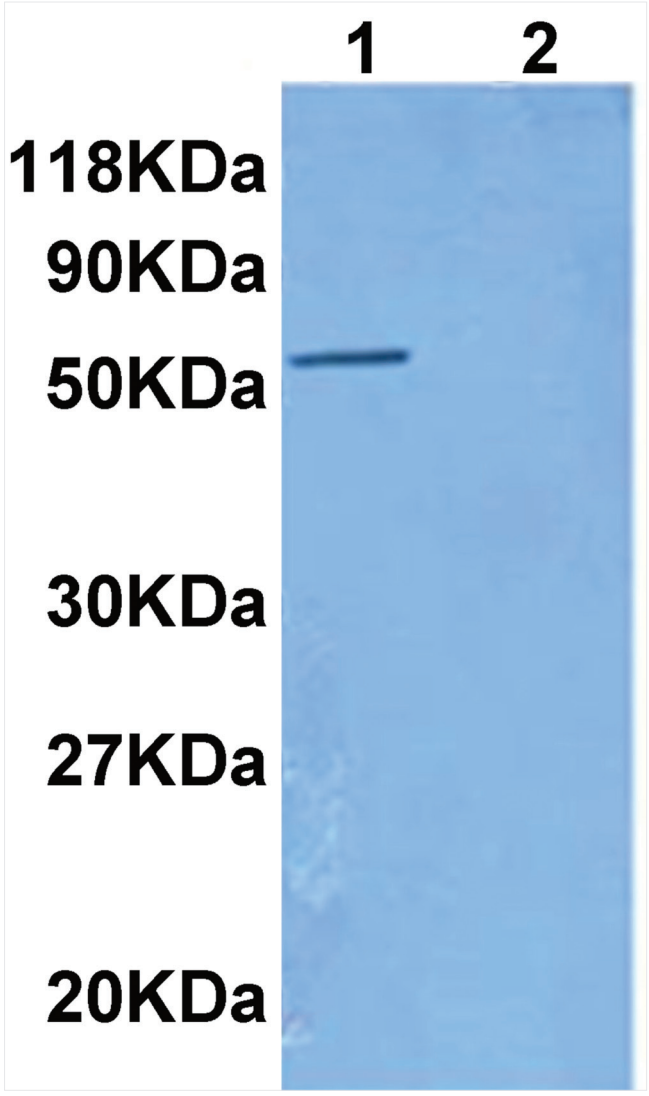


**Fig 2.** Western blot analysis of pET32a-gD. 1. Positive serum of IBRV; 2. Negative serum of IBRV

**Şekil 2.** pET32a-gD'nin Western blot analizi. 1. IBRV'nin pozitif serumu; 2. IBRV'nin negatif serumu

fever, bovine viral diarrhea-mucosal disease, calf diarrhea, bovine intestinal virus infection, and bovine coronavirus disease) all showed negative OD450nm values, indicating a negative cross-reactivity of this antigen with above sera.

### *Experiment Reproducibility*

The variation coefficient is less than 7% within intra-batch experiments, whereas less than 11% with inter-batch experiments using different batches of purified recombinant antigens. This result suggests a good reproducibility of this study.

### *Clinical Sample Evaluation*

Examination of serum samples from 1315 cows in Shangdong Province using the indirect ELISA method and commercialized IBRV whole virus ELISA kit (Laboratorios HIPRA, Girona, Spain) showed that, there were 311 samples with positive IBRV antibodies and a 23.7% of seropositivity rate by indirect ELISA. The coincidence rate is 96.8% in comparison to the results using commercialized whole virus ELISA kit *(Table 3)*.

## DISCUSSION

Infectious bovine rhinotracheitis is prevalent worldwide as a globally important disease. Almost all countries had seropositive cattle (even including a small number of countries in South American yet without isolated viruses). World Organization for Animal Health (OIE) included infectious bovine rhinotracheitis virus in the statutory reporting list of animal diseases, whilst China also obliged IBRV assessment as an obligation for all imported and exported cattle. Since the disease was detected and reported in China for the first time from cows imported from New Zealand in 1980, its prevalence all over the country has been rising up. A seroprevalence survey in 2010 revealed 66.7% of positive serological antibodies in

***Table 3.*** *Comparison of the results obtained from gD-ELISA and the commercialized IBRV whole virus ELISA*
***Table 3.*** *gD-ELISA ve ticari IBRV tüm virus ELİSA'dan elde edilen sonuçların krşılaştırması*

| Detection Method and Results | | gD-ELISA Detection | | | | |
|---|---|---|---|---|---|---|
| | | Positive | Negative | Specificity/% | Sensitivity/% | Coincidence Rate/% |
| IBRV whole virus ELISA | Positive | 294 | 25 | 98.3% | 92.2% | 96.8% |
| | Negative | 17 | 979 | | | |
| Total | | 319 | 996 | | | |

cows distributing in five northern regions (Beijing, Liaoning, Jilin, Inner Mongolia, and Shanxi). There were also 1505 out of 5000 breeding bulls imported from Australia exhibiting positive reactions to IBRV antibodies during quarantine in 2005, with 2 of them having isolated viruses. Taken together, the epidemic situation is not optimistic on domestic herds under double pressure from both imported carries with hidden infection and also local cattle with high infectious bovine rhinotracheitis. Thus, it is of top importance to establish a fast and accurate method for diagnosis of the disease.

Serological detection of antibodies is an essential immunological technology for diagnosis of the disease. Serum neutralization assay and ELISA are two major serological methods and also commonly used techniques worldwide. Serum neutralization assay has high accuracy, yet requires a long period and complicated procedure to perform therefore is not suitable for a large number of samples with limited use. ELISA becomes a widely used serological diagnostic technique, as also recommended by OIE International Trade, in animals quarantine for infectious diseases and epidemical investigation due to several advantages including strong specificity, sensitivity and objective criteria, etc.

The specificity of ELISA can be affected by many factors, notably the antigen coating concentration and serum sample dilution factors. High antigen coating concentration leads to multi-layer formation of proteins so that some are detached from the support surface to be easily washed off during the wash process causing a non-specific effect. On the other hand, low concentration would leave some un-occupied spaces by antigen on the surface of the ELISA plate also contributing to high non-specificity. There are also some extra effects from various blocking buffer and time, reaction time with serum samples and secondary antibody, as well as chromogenic reaction time. In this study all optimal experimental conditions were probed by the square titration and the thresholds were established via statistical analysis. Evaluation of clinical samples suggested that, as compared to the commercialized IBRV whole virus ELISA kit, our method has 96.8% coincidence, 92.2% sensitivity and 98.3% specificity. Meanwhile, the variation coefficient of intra-batch experiments is less than 7%, and the one of inter-batch experiments is less than 11%, indicating that this methodology has decent reproducibility and stability. One of the important characteristics of BoHV-1 is establishment of life long latency in sensory neurons of the peripheral nervous system after replication in mucosal epithelium. Reactivation from latency can occur after natural stimulus exposure [14,15] or corticosteroid treatment [16] culminating in recurrent virus transmission to uninfected animals generally without clinical signs. Currently the most developed and widely used technique for IBRV detection includes virus isolation, virus neutralization test, ELISA, and PCR, etc.[17,18]. Rational use of these detection technologies in combination with regulatory procedures can greatly improve the efficiency of IBRV detection and consequently reduce the possibility of hidden IBRV. It applies particularly on the imported cattle in quarantine in order to reassure the quality of animals and keep carriers out of the country. Our study establishes an indirect ELISA method for detection of IBRV antibody which provides a promise for developing IBRV ELISA kit in the future and also technical support for quarantine, diagnosis, antibody surveillance, prevention and control of IBR.

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