



Rapid Detection of Bovine Herpes Virus Type 1 (BoHV-1) in Egyptian Dromedary Camels

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ABSTRACT

Bovine herpesvirus 1 (BoHV-1) is known to cause reproductive disorders in Sudanese camels. Egypt imports about 90% of its camels from Sudan, and the rest from Somalia. The BoHV1 is a viral disease of bovines that can be transmitted to camel, sheep, and goat. Due to the absence of anti-camel conjugated with fluorescein isothiocyanate (FITC) in the market, we used protein-A conjugated with FITC which binds to the Fc region of IgG of many animal species. We, therefore, prepared rabbit anti-camel IgG conjugated with FITC and compared it with protein-A conjugated with FITC to the specificity and sensitivity of these compounds in IBR detection from 35 nasal swabs in imported Egyptian dromedary camels. The sensitivity and specificity of the prepared anti-camel IgG FITC and protein-A FITC were compared using Virus Neutralization Test. The labeled protein concentration in the prepared anti-camel conjugate was 2 mg/ml which was considered as an acceptable value. The degree of labeled protein (DOL) was 5.74 cm⁻¹M⁻¹ and optimal DOL usually fell between 2 and 10. The titer of the prepared anti-camel IgG-FITC was 3,125. The prepared anticamel IgG-FITC and protein-A-FITC showed a sensitivity of 93.75 and 90.9%, and a specificity of 71.43% and 62.5%, respectively. Our findings show no significant difference between protein-A conjugated FITC and prepared anti-camel IgG-conjugated FITC in the rapid diagnosis of BoHV-1 in Egyptian dromedary camels.

Keywords: Anticamel FITC, BoHV-1, dromedary , IBR, SNT

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INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) is a member of the genus Varicellovirus in the Alphaherpesvirinae subfamily which belongs to the family Herpesviridae, order Herpesvirales (Andrew, 2010) Globally, BoHV-1 is known to cause various infectious diseases in cattle including rhinotracheitis, vaginitis, balanoposthitis, abortion, conjunctivitis, and enteritis. It also contributes to shipping fever, also known as bovine respiratory disease (BRD) (Raaperi *et al.*, 2014). The virus spreads through sexual contact, artificial insemination, aerosol transmission, or even through the placenta (Sunil, 2000). BoHV-1 can cause both clinical and subclinical contaminations, depending on the

pathogenicity of the viral strain. Although the virus is rarely life-threatening, it constitutes a financial liability for cattle farmers, as pollution can cause the spread of infection and a drop in production because of increasing the abortion rate (Van *et al.*, 1993).

Like other herpesviruses, BoHV-1 causes deep-rooted inactive contamination and scattered shedding of the virus in all animal discharges (OIE, 2018). Egypt is one of the most important camel-rearing countries, with hundreds of thousands of camels imported yearly as 'butcher' animals to provide camel meat for the Egyptian market. These camels are primarily imported from Sudan and Somalia. These vehicles were categorized by street, foot, or ocean

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(Helena 2018). Previous research has shown that camels coming from Sudan are naturally exposed to BoHV-1 and they confirmed the infection by using the fluorescent antibody test (FAT) and enzyme-linked immunosorbent assay (ELISA) (Intisar *et al.*, 2009). Serological surveys were also performed in Iran to detect antibodies against (BoHV-1) in dromedary camels. (Raofi *et al.*, 2012).

A rapid diagnosis can effectively control disease spread and decrease economic losses associated with abortion. The indirect fluorescent antibody test (IFA) is a semi-quantitative, sensitive, and rapid serum antibody detection test for immunoglobulin M (IgM) and G (IgG) (Messenger and Rupprecht, 2015). Conventional methods for detecting the herpes virus have been compared, such as fluorescent antibody testing (FAT) and virus isolation (VI). Although FAT is less sensitive, it should always be used as an early diagnosis routine (Madhavan *et al.*, 2003).

An optimal fluorescein protein ratio (F:P) was calculated to know the maximum labeled antibody with fluorescein. This procedure can be used to prepare functional, labeled antibody reagents with a defined activity and can help in quantitative applications where the stoichiometry and functionality of the labeled antibody are critical (Vira *et al.*, 2010). Therefore in this study, we prepared rabbit anti-camel IgG conjugated with Fluorescein isothiocyanate to diagnose BoHV-1 in Egyptian dromedary camels.

MATERIALS AND METHODS

Animals

Animal maintenance and care conformed with recommended International Guiding Principles for Biochemical Research Involving Animals (National Research Council ,US, Institute for Laboratory Animal Research, 2004).

1. Camels

- Three liters of blood were collected in a sterilized capped bottle from three camels in a slaughterhouse. They were stored in the refrigerator overnight at 4°C. The serum was separated and tested against BoHV-1 antibodies using the serum neutralization test.
- Nasal swabs were collected from 35 pasture camels on the desert road of El Sadat city.

2. Rabbits

Ten unvaccinated male rabbits aged between 1.5-2 months and weighing between 2-3 Kg were obtained from the laboratory animals unit at the Animal Reproduction Research Institute (ARRI), Haram, Giza.

Virus

A local strain of BoHV-1 propagated in Madin-Darby bovine kidney cells (MDBK) with a titer 10⁶ TCID₅₀/ml was used for antibody detection in the serum neutralization test. It was provided by Virology

Unit, Department of Animal Reproduction Diseases, Animal Reproduction Research Institute (ARRI)

Virus Neutralization Test (VNT)

Two-fold serial dilutions of the sera (1:2–1:128) were performed in the presence of 100 TCID₅₀/ml of BoHV-1 in the presence of a positive reference control, as previously described by Martinelle *et al.*, (2011). The neutralizing antibody titer of the collected camel sera was defined as the reciprocal of serum dilution, causing a 50% reduction in cytopathic effect in (MDBK) cells.

Preparation of camel IgG

Five hundred mg of ammonium sulfate was added to 1L camel serum to reach 50% saturated ammonium sulfate (SAS) solution to precipitate immunoglobulins, which were dialyzed by 15 mM of phosphate-buffered saline (PBS). The absence of SAS in the final product was tested using barium chloride. Immunoglobulins were concentrated using Polyethylene glycol according to the method of Abd El Hafez *et al.* (2010).

Purification of immunoglobulins

The purity of IgG was confirmed using protein-A sepharose gel with eluting buffer 0.1 M glycine. It was carried out according to the methods of Abd El Hafez *et al.* (2010). The protein contents of immunoglobulins were estimated according to the methods described in Lowry *et al.* (1951). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide gels according to Laemmli (1970). Buffer containing 2-mercaptoethanol was mixed with camel IgG and loaded to the gel. Then, the gel was stained with Coomassie Brilliant Blue dye and molecular weights of bands were calculated using Protein marker supplied by Sigma-Aldrich.

Mixing camel IgG with an adjuvant

Montanide™ oil ISA 206 was obtained from the Central Laboratory for the Evaluation of Veterinary Biologists (CLEVB), Agriculture Research Center (ARC), and Giza, Egypt. Montanide adjuvant ISA 206 was added in a 1:1 (v/v) ratio into the prepared camel IgG and was mixed by homogenization according to Aslam *et al.*, (2013).

Rabbit immunization

The purified camel IgG was mixed with an equal volume of ISA 206 montanide oil and 80µg/kg was used to immunize the rabbits four times weekly. Injections were done subcutaneously at different locations. The last dose was given without adjuvant according to Tendler *et al.*, (1991). Anti-camel IgG was titrated against camel serum using the AGID test according to Ouchterlony (1962).

Labeling of anti-camel with FITC

This procedure is according to **Hanan et al., (2004)**

1. Two mg/ml of protein was prepared in 0.1 M carbonate sodium buffer, (pH 9). The buffer was kept at 5 ° C for no more than one week (freshly prepared).

2. The protein to be conjugated should be free from contaminating proteins; therefore, it was first tested. The protein solutions were prepared in buffers that excluding sodium azide or amines such as Tris or glycine, as these compounds inhibit the reaction to the mark. If amines or sodium azide were present in the buffer, the protein solution was dialyzed against PBS (pH 7.4) overnight between 0 and 5 ° C. Note that dialyzing at high pH values (> 8.0-8.5) may be harmful to some proteins.

3. The FITC was dissolved to 1 mg/ml in anhydrous DMSO and was freshly prepared throughout the experiment.

4. In total, 50 ml of FITC were added very slowly in 5 ml aliquots for every 1 ml of protein solution, while gently and continuously stirring the protein solution.

5. After adding the entire required amount of FITC, the reaction was incubated in the dark at 4 ° C for 8 hours.

6. Ammonium chloride (NH₄Cl) was added to the solution to achieve a final concentration of 50 mM and incubated for 2 hours at 4 ° C.

7. Through gel filtration, the unbound FITC was separated from the conjugate using a fine-sized gel matrix with an exclusion limit of 20,000 to 50,000 (for globular proteins such as antibodies). With the column flow stopped, the reaction mixture was carefully layered to the top of the column. Then, the column was opened, allowing the reaction mixture to flow into the column. As the mixture entered the column bed, PBS was carefully added to the top of the column and connected to a supply buffer. Two distinct bands formed on the column. The first, fast-moving band consisted of the elutes of the conjugated protein and could be observed under normal lighting conditions. The second, slower, moving band consisted of the unreacted FITC.

8. The conjugate was stored in a light-proof container at 4 ° C. Sodium azide was added as a preservative (final concentration 15 mM). FITC was supplied by the Pet Animals Department of the Veterinary Serum and Vaccine Research Institute (VSVRI).

9. The final product was re-titrated after conjugation using the AGID test.

Protein-A- FITC

Protein-A-FITC was commercial and lyophilized. Louis SIGMA CHEMICAL. It was used in

conjunction with prepared anti-camel IgG-FITC as the control reagent.

Validation of the prepared anti-camel IgG- FITC

● Determination of protein labeling degree (DOL): The conjugates were analyzed using a UV-VIS spectrophotometer (type SPV-72, 220V, 50/60Hz, S.N.11124, Federal Republic of Germany). Quartz cuvettes were available for the 2-ml sample. In the conjugates, labeled anti-camel IgG was measured by recording the absorbance at two wavelengths, 280 nm and 494 nm (A₂₈₀ and A₄₉₄), which are the maximum antibody and dye absorbance.

● The (FITC) molar substitution ratio (MSR) to protein (anti-camel IgG) is called the F/P ratio. The F/P molar ratio in the conjugate, accordingly, refers to the rate of fluorescein (F) moles to protein (P) moles. The three equations listed below were used to calculate the F/P molar ratio in our samples.

1. Protein concentration (mg/mL) = [A₂₈₀ - (0.11(A₄₉₄))] × dilution factor/A₂₈₀ of protein at 1 mg/mL

Where A₂₈₀ and A₄₉₄ are the absorbance values of the conjugated peptides at 280 and 494, respectively. 0.11 is the correction factor, and the sample diluted 1:10 and 1.4 are the extinction coefficient of the antibodies or A₂₈₀ of protein at 1 mg/ml.

2. Protein concentration (M) = answer from Eq. A/ protein molecular weight (Da)

Where the labeled IgG is (MW 150,000).

3. DOL (degree of labeling) = (moles dye)/(mole protein) = A₄₉₄ × dilution factor/ 71,000 × protein concentration (M)

Where 71,000 cm⁻¹M⁻¹ is the approximate molar extinction coefficient.

Protein A-FITC and the prepared rabbit anti-camel IgG conjugated with FITC validated with seropositive camel anti-sera against IBR which previously titrated using SNT and are 128. Thirty-seven coverslips were cultured with (MDBK) cells overnight. The coverslips were injected with camel nasal swaps, bovine herpesvirus as positive control and one kept as negative then incubated for 1hr at 37°C.

After 48h, the coverslips were washed with PBS and fixed in cooled acetone at - 20°C for 20 min. The seropositive camel serum was incubated for 1hr at 37°C. The other coverslips were stained with protein-A conjugated FITC and the prepared rabbit anti-camel IgG-conjugated FITC. The slides were examined using a fluorescent microscope to identify the variation in the number of positive samples and image clarity.

The same samples were examined using SNT and its sensitivity and specificity were both established. The sensitivity is a true positive rate, where SNT positive samples divided by total positive samples for

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Methods used	SNT		
	positive	negative	Total LAT
IFAT protein-A			
IFAT positive	30	3	33 (80.48%)
IFAT negative	3	5	8 (19.5%)
Total SNT	33 (80.48%)	8 (19.5%)	41 (100%)

both SNT and IFAT. The specificity is a true negative rate, where SNT negative samples divided by total negative samples for both SNT and IFAT.

RESULTS

Serum Neutralization Test (SNT)

Camel sera samples were tested against BoHV-1. Only 1 liter was used has titer 4. The other 3 liters with a high level of BoHV-1 antibodies were neglected.

The agar gel immunodiffusion (AGID) test

After one week from the last injection in rabbits, the analysis was performed to estimate the titer of anticamel in the rabbit and check the immune level. The labeled anticamel IgG with FITC was also titrated and the result was 1000 and 625, respectively.

Validation of the prepared anti-camel IgG- FITC

1. Estimation the degree of labeling

Protein concentration (mg/mL) =
 $[0.34 - (0.11 \times 0.53)] \times 10 / 1.4 = 2 \text{ mg/ml}$

Protein concentration (M) =
 $2 / 150,000 = 1.3 \times 10^{-5} \text{ M}$

DOL (degree of labeling) =
 $0.53 \times 10 / 71,000 \times 1.3 \times 10^{-5} = 5.3 / 0.923 = 5.74 \text{ cm}^{-1} \text{M}^{-1}$

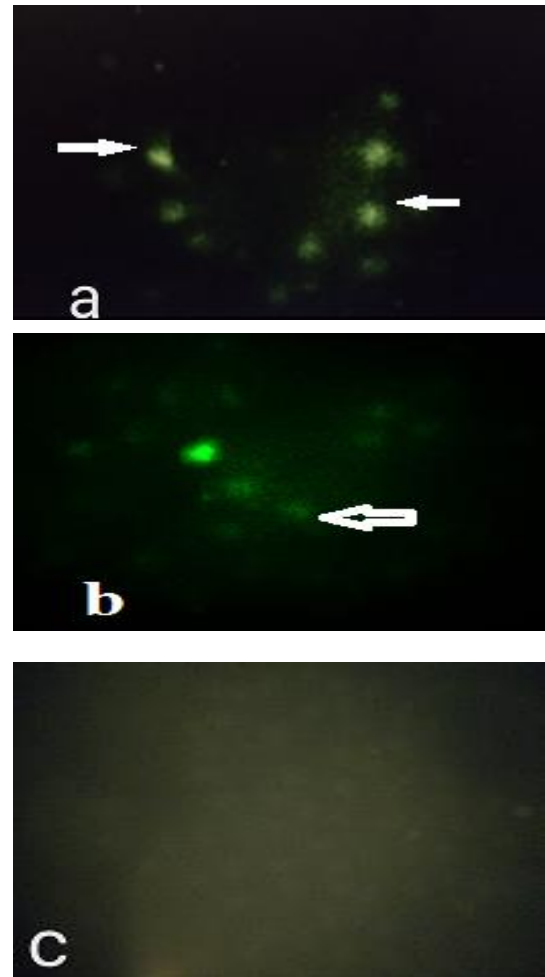
2. Differentiation between anticamel IgG and protein A-FITC

There was no significant difference between the slides stained with protein-A FITC and the prepared anti-camel IgG FITC either in the number of positive slides or in image clarity (figure 1). The IFAT results were compared with SNT to show the specificity and sensitivity of both anticamel IgG and protein A.

Thirty out of 35 nasal swabs tested positive under SNT, but 27 and 28 out of 35 nasal swabs were positive for protein-A FITC and prepared anti-camel IgG FITC, respectively. The results of the prepared anti-camel IgG FITC for the IFAT and the SNT were compared, showing 93.75% sensitivity (true positive rate) and 71.43% specificity (true negative rate) (Table 1).

Table 1: Comparison between the results of (SNT) and (IFAT) using anti-camel IgG FITC to detect (BHV-1) in camel nasal swab samples.

Figure 1: MDBK cells infected with camel nasal swab samples and stained with both protein-A- FITC and prepared anti-camel-FITC, showing a diffused intranuclear yellowish-green color



a) Slide stained with anti-camel-FITC
b) Slide stained with protein-A FITC
c) Control MDBK cells

Furthermore, when comparing the results obtained by SNT and IFAT with protein-A FITC, these showed 90.9% sensitivity and 62.5% specificity (Table 2).

Table 2: Comparison between results of (SNT) and (IFAT) using protein-A FITC to detect (BHV-1) in camel nasal swab samples.

DISCUSSION

Camel serum with titer four was used and the others were neglected because the serum more than ten titers was considered positive for BoHV-1 **Fulton et al. (1995)**. Camel immunoglobulin was precipitated and purified using ammonium sulfate, given its low price and large scale use (**Mariam et al., 2015**). The rabbits used in this study produced highly specific antibodies, responding to experimental immunizations in peripheral sites, including in the spleen and lymph nodes. Rabbits are a major source of polyclonal and monoclonal antibodies (mAbs) used in research and therapy (**Esteves et al., 2018**).

Montanide oil 206 was used because it is very fluid, stable, well-tolerated and induces a short and long term immune response (**Mohi-ud-din et al., 2014**). They are also useful as traditional Freund's adjuvant in enhancing immune responses in mice (**Yang et al., 2010**). The subcutaneous route is better than the intramuscular one because there are no ulcerative lesions; it is, therefore, less painful to the experimental subjects and causes the slow release of camel IgG (**Blanc et al., 2009**).

AGID test was used in our study because not only is it available easy cheap test, but also it is the only test that can estimate the level of both anticamel IgG and labeled anticamel with FITC. SNT can't be used because fluorescein has toxic effects on cell culture (**Alford et al., 2009**). AGID sensitivity was 57% and specificity was 92.5%. The positive predictive value was calculated in 85.7% for a confidence interval of 95%. (**Ferreira et al., 2002**).

The concentration of protein labeled in the conjugate was 2 mg/ml and successful labeling was likely to occur if the starting concentration was > 1 mg/mL (**Molecular Probes 2006**). The degree of labeling conjugate was 5.74 and the optimal DOL usually falls between 2 and 10 (**AAT Bioquest, Inc., 2020**). The sensitivity of anticamel was 93.75% and 90.9% in protein A; these results agreed with (**Hanan et al., 2004**) which revealed that the sensitivity of protein-A less than anticamel IgG as protein-A conjugate failed to detect 3 cases of the positive sera. The highest prevalence of natural infectious bovine rhinotracheitis virus (BoHV-1) was observed in the sera of dromedary camels in Sudan (**Zhou et al., 2017**), so we should have a rapid test such as IFAT to diagnosis dromedary camels come from Sudan.

CONCLUSION

This study provides a suitable rapid method to diagnose BoHV-1 in camels, using prepared rabbit anti-camel IgG -conjugated with fluorescein and other commercial product. But, unfortunately, it will be used only in laboratories having a fluorescent microscope to

Methods used	SNT		
	positive	negative	Total LAT
IFAT anti-camel			
IFAT positive	30	2	32 (82%)
IFAT negative	2	5	7 (17.9%)
Total SNT	32 (82%)	7 (17.9%)	39 (100%)

prevent the spread of the BoHV-1 from the Sudanese and Somalia imported camels to the herds in Egypt, causing abortion and economic losses.

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