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SEROLOGICAL DIAGNOSIS OF BOVINE CORONAVIRUS IN CALVES USING A DEVELOPMENT NATIVE ELISA PREPARATION

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ABSTRACT

Bovine coronavirus (BCoV) infection has found worldwide among cattle of all ages, causing diarrhea in newborn calves, winter dysentery in adult cattle and respiratory tract diseases results in major economic losses in dairy herds. The objective of this study was to establish an ELISA kits as patent prepared in Lab. with assessment its applicability in the field samples. Sixty-five pneumonic calves 2- to 4-month-old, used in this study to detect BCoV antibodies in serum of infected calves, where it assayed against BCoV antibodies by using prepared ELISA plates and SNT. The results were positive for BCoV antibody 72.3% and 53.9% by ELISA and SNT respectively. The prepared ELISA showed high sensitivity of over SNT, where sensitivity, specificity and correlation were 91.4%, 50% and 72.3% respectively, and there is a positive correlation between both tests. The test has been successfully used in longitudinal field studies on virus dynamics and evaluation of management routines. In conclusion the patent prepared ELISA is more sensitive, accurate and specific assay than SNT for detection of BCoV antibodies, in addition to saving time and commercial kits cost.

Keywords: BCoV - newborn calves- ELISA - SNT – respiratory manifestations.

INTRODUCTION

In the dairy industry worldwide, bovine coronavirus (BCoV) is a widely spread pathogen causing disease and economic losses. The prevention of the spread of viruses is hampered by a lack of basic awareness of the potential for viral shedding and transmission in individual animals (Oma *et al.*, 2016; Johnson and Pendell, 2017 and Amer, 2019). BCoV has been associated with gastrointestinal and respiratory diseases in cattle including diarrhea in neonatal calves, winter dysentery and respiratory tract illness (Storz *et al.*, 2000). In dairy and beef calves, BCoV can cause enteritis in healthy and diarrheal calves, BCoV can often be identified, that complicating the assessment of its role as a primary pathogen (Bartels *et al.*, 2010).

BCoV may be life threatening, due to loss of electrolytes and malnutrition (Francoz *et al.*, 2015). BCoV infects the upper, lower respiratory tract and intestine also, It is not unexpected that cattle would have a coronavirus with tropism for the respiratory tract. Coronaviruses infect the respiratory tract of other species including pigs, turkeys, and chickens. Thus, this virus

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has a role in both respiratory tract disease and enteric diseases (Fulton, 2009). Subsequently, BCoV was also associated with the occurrence of respiratory distress in calves and adults (Lathrop *et al.*, 2002).

The respiratory syndrome is often observed during or after transportation because the shipping of cattle represents a stressing factor that may facilitate the onset of BCoV induced respiratory disease, mainly in calves (Decaro *et al.*, 2008). Ohlson *et al.*, (2010b) recorded nasal shedding within a herd form respiratory BCoV clinical or subclinical infected calves and young adult cattle. Gomez *et al.* (2017) observed shedding of BCoV in the absence of other important respiratory tract pathogens in cattle with naturally occurring BRD.

BCoV is consist of a single stranded, non-segmented positive sense genomic RNA, 32 kb long. It belongs to the Beta coronavirus genus cluster within the Coronavirinae subfamily, Coronaviridae family, and the order Nidovirales (http://ictvonline.org/virusTaxonomy.asp). (Hasoksuz *et al.*, 2008). BCoV possesses five major structural proteins have various functions in the viral cycle. Which spike (S), hemagglutinin/esterase (HE), nucleocapsid (N), transmembrane (M) and the small membrane (E) (Lai and Cavanagh, 1997). The S protein is responsible for the interaction between the virus and the cellular receptor eliciting antibodies with high neutralizing efficacy in its host (Yoo and Deregt, 2001). BCoV consists of one serotype with some antigenic variation between different strains. Acutely infected animals produce long-lasting antibodies, probably for many years (Lin *et al.*, 2002).

The seroprevalence of anti-BCoV antibodies among newborn calves ranged from 20% (17/82) in Algeria (Ammar *et al.*, 2014) to 93.9% (172/183) in Turkey (Yavru *et al.*, 2018). Molecular detection of the virus by qRT-PCR was reported from several countries leading to variable prevalences. Recently, the occurrence of BCoV in neonatal calf was estimated to 7.2% (14/194) in Iran using antigen-capture ELISA (Lotfollahzadeh *et al.*, 2020).

The importance of good colostrum management, leading to an adequate passive transfer in the prevention of calf diarrhea is without debate (Dunn *et al.*, 2017). It is unclear if vaccinating cows late in gestation improves calf antibody titers or whether the practice improves resistance of calves to disease. To provide optimal immunity, vaccine antigens should be as similar as possible to the circulating strains. Therefore, future studies should focus on epidemiological surveillance in order to avoid potential causes of vaccination failure (Mihai *et al.*, 2006).

Rapid and reliable diagnostic methods are the primary prerequisite for timely and effective implementation of therapeutic and preventive measures (Reschová *et al.*, 2001). The diagnosis of BCoV can be achieved using viral culture, antigen-capture ELISA, hemagglutination assay using mouse erythrocytes, and PCR (Boileau and Kapil, 2010).

The enzyme linked immunosorbant assay (ELISA) for antibody detection is fast, simple and precise method for screening large numbers of tested samples (Nylin *et al.*, 2000). The BCoV commercial ELISA kits is so expensive and not always available, so the objective of this study was to establish an ELISA kits for BCoV antibody detection as a patent prepared in Lab. with assessment its sensitivity and applicability in the field samples.

MATERIALS AND METHODS

1. Clinical samples:

Sixty-five pneumonic calves 2- to 4-month-old were included in this study. These calves were carefully examined and excluded other agents that cause respiratory

manifestation. Blood samples were taken into sterile vacuum tubes collected for serological testing were brought into laboratory under cold chain and centrifuged at 720 xg for 15 minutes and taken into sterile eppendorf tubes. Serum samples inactivated in (56ºC, 30 min) then kept at -80ºC until testing.

2 Virus and cell culture:

Madin Darby bovine kidney (MDBK) cells tested against latent infection with mycoplasma and bovine viral diarrhea virus were supplied by National Animal Disease Center, Ames Iowa, USA. Eagle's minimum essential medium (EMEM; Gibco BRL, life technologies, Scotland, UK) supplemented with Fetal bovine serum (FBS) was obtained from Sigma, Chemical CO.USA.A commercial antibiotic/ antimycotic mixture (Sigma, Chemical CO. USA). An international Mebus reference strain was used as tested antigen all over the study.

3. Quantitation of the virus:

After serial passage of the virus in MDBK cells, the tissue culture infected dose $_{50}$ ($TCID_{50}$) was calculated according to (Reed and Muench, 1938) method.

4. Virus concentration by polyethylene glycol-6000 (PEG-6000) precipitation:

Virus concentration was done as illustrated in Mahy and Kangro (1996). At complete cytopathic effect (CPE), the cells and medium are harvested by scraping with a rubber policeman. Multiple cycles of freeze-thawing can effectively break up the cells. The pooled harvest was clarified by centrifugation at 5000xg for 5 minutes. To the supernatant and at a final volume slowly add 2.3% NaCI and 7.0% PEG-6000 with constant and gentle stirring. Cover the beaker and stir for about 1 h more to ensure complete solubilization of the PEG. Transfer the beaker and ice bath to a refrigerator, and allow the virus and other proteins to precipitate overnight at 4ºC. In the next day, the precipitate was collected by centrifugation at 10000xg for 10 minutes in cooling centrifuge. To the precipitate, 3ml of TES buffer (0.01 M Tris-HCI, pH 7.2, 0.002 M EDTA, 0.15 M NaCI) was added and the precipitate was thoroughly suspended. Finally, the PEG is removed (pelleted) by centrifugation of this pooled suspension at 13,000 xg for 4 min and the supernatant contains the concentrated virus in isotonic TES buffer was collected.

Estimation of antigen concentration:

Antigen concentration was determined as in Bradford (1976). A mixture of 100 µl of antigen and 5 ml of reagent (100 mg coomassie brilliant blue G-250, 50 ml absolute ethanol, 100ml orthophosphoric acid 85% and water to 1 liter was incubated for minutes before reading the absorbance in spectrophotometer at wave length 595nm. A stander curve was made using bovine serum albumin. (He, 2011).

5. Enzyme linked immunosorbant assay (ELISA):

At antigen concentration 8 µg/well, ELISA plates (Nune immune-11 96wells) were coated at 4ºC overnight with coating buffer (1.59 gm $NaCo_3$ and 2.9 gm $NaHCo_3$ per 1000 ml deionized water. The upper two left well will act as blank and the next three one as negative control by FBS. A positive control was also included in 3 wells. In the next day and after each step the plates were washed 3 times with washing buffer (phosphate buffer saline (PBS) and 0.05% tween 20). Also the plates were incubated at 37ºC for one hour after each step. The plates were blocked with 200µl /well blocking buffer (PBS and 5% skimmed milk). Sera samples were diluted as 1/40 in diluting buffer (PBS, 0.5% Skimmed milk and 0.05% tween 20) and loaded as duplicated. Peroxidase labeled anti bovine IgG 1:20000 (Bethyl laboratories, INC Germany) was used as 100ml/ well. The substrate (0.4 mg o-phenylenediamine – OPD and 0.4 µl of 30% H_2O_2 per ml of 0.01 M citrate buffer PH 5) was added as 100µl/well. After color development the reaction stopped by 1:9 sulfuric acids in water and the optical density (OD) At (492)

nm wavelength was read immediately after stopping the reaction with the stop solution.

Cutoff endpoint calculation:
By divided OD summation of the 3 negative control and 3 positive samples on the number 6, the samples, which their OD above the cutoff endpoint was considered positive. While the samples, which their OD equal or lower than the cutoff endpoint was considered negative.

6. Serum neutralization assay:
A pool of infectious virus suspension was prepared, clarified and free aggregates by centrifugation. The aliquot of virus was diluted to obtain 100 $TCID_{50}$ per inoculum. The sera samples were heat inactivated at 56ºC for 30 min. Equal volume of serial dilution sera samples and100 $TCID_{50}$ virus suspension were mixed and incubated at 37ºC for one hour. In 96 wells tissue culture plates 50 µl of serum- virus mixture in each well was added. Cellular control and virus control were also included. A cellular suspension was prepared and added to all wells and the plates were incubated at 37ºC, 5% Co_2 with daily microscopic examination for cytopathic changes.

RESULT

1. The obtained result in virus and cell culture showed characteristic CPE of an international Mebus reference strain 72 hrs post inoculation on MDBK cells as shown in (Fig.1&2).

Fig. (1): CPE consisted of cell rounding, detachment, and complete monolayer destruction, 100x.

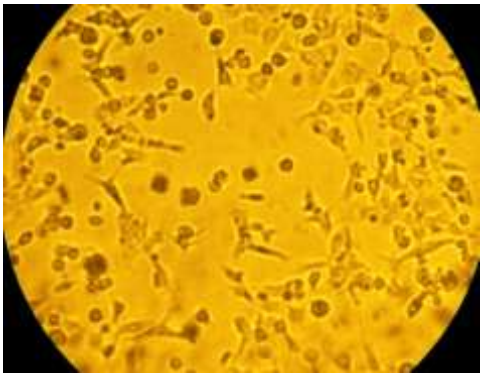

Fig. (2): CPE consisted of cell rounding, detachment, and complete monolayer destruction, 200x.

2. Concentration of BCoV antigen is 4.3 g/dl.
The results showed by Checkerboard titration finding for the prepared BCoV viral antigen and antibody optimization of ELISA are that the optimal amount of tissue culture polyethylene glycol concentration antigen coating level at 8 µg/well and the working dilution in case of serum is 1:40. The Result of concentrated virus indicated that the major peak of the viral infectivity was found to be 8µg/well. At the 1:2000 dilution Conjugate was determined and used for the detection of BCoV antibodies using ELISA assay.

3. Detection and titration of antibodies to BCoV in field serum sample using ELISA and SNT.

The obtained results were as showed in table (1).

Table 1: BCoV antibodies in calves serum by ELISA and SNT.

	ELISA	SNT
Positive	47	35
Negative	18	30

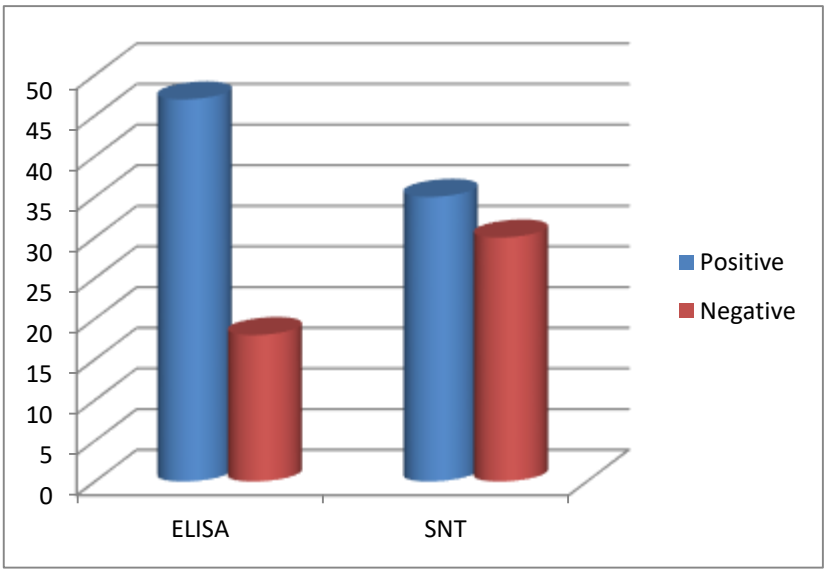

Fig. (3): Comparison between ELISA and SNT in detection of BCov antibodies

4. Determination of sensitivity, specificity and correlation of detection of BCoV antibodies by ELISA and SNT.
The results showed in table (2).

Table 2: BCoV antibodies sensitivity, specificity and correlation by ELISA and SNT

ELISA	SNT		Total
	Positive	Negative	
Positive	32 [a]	15 [b]	47
Negative	3 [c]	15 [d]	18
Total	35	30	65

Sensitivity = a/(a+c) = 91.4%.
Specificity =d/(b+d) = 50%.
Correlation= (a+d) /total = 72.3%.

DISCUSSION

Newly born calves are an important source in livestock production worldwide for meat or breeding as replacement stock (Radostitis *et al*., 2007). BCoV is usually associated with the occurrence of diarrhea in calves and adult cattle, but it is also responsible for respiratory distress, being implicated as an etiological agent of bovine respiratory disease (BRD) (Bok *et al.*, 2015; Johnson and Pendell, 2017). Bovine respiratory disease is a complex multifactorial disease caused by multiple viruses such as BRSV, BoHV-1, BPIV-3 and BVDV. Also concurrent bacterial and mycoplasmal infections can exacerbate the course of viral diseases, increasing morbidity and mortality rates (Ha¨gglund *et al*., 2007). The disease results in major economic losses in dairy herds that result from treatment costs and calf deaths (Sibel

et al., 2016). Decaro *et al*. (2008), found in their study BRD occurred only in 2–3-month-old calves as a result of a single BCoV infection without evidence of classical respiratory pathogens, such as BRSV, BoHV-1, and BVDV

Diagnosis of BCoV usually occurs by isolation of the virus or demonstration of an elevated serum antibodies titer. Because of time consuming of virus isolation methods, serum neutralization test and various ELISA are usually used for detection of antibodies against BCoV in enzootic regions and potentially respective extension zone (Lin *et al.,* 2000). We aimed to prepare a highly sensitive antigen to be used as early diagnostic tools for detecting of specific BCoV antibody elevation. For this purpose, BCoV antigen was prepared by propagation of the virus in MDBK cell line and development of CPE 72 hrs post-inoculation. The titer of the virus was $10^{6.4}$ TCID50. BCoV antigen was successfully prepared from reference Mebus BCoV strain by concentration with polyethylene glycol (PEG) 6000 used for examination of sera samples of cattle by ELISA technique. The result of concentrated virus indicated that the major peak of the viral infectivity was found to be 8µg/well. At the 1:2000 dilution, conjugate was determined and used for the detection of BCoV antibodies using ELISA assay. The same methodology was successfully used for preparing antigen by (Li *et al*., 2013).

The obtained results in virus and cell culture showed characteristic CPE of an international Mebus reference strain 72 hrs post inoculation on MDBK cells CPE consisted of cell rounding, detachment, and complete monolayer destruction, (Fig.1&2) these results agreed with that mentioned by (Saif *et al.,* 1988) who successfully propagate BCoV at MDBK cells with high titres.

The results of indirect ELISA and SNT, respectively for detection of antibody titer in 65 bovine serum samples, Table (1), showed that 47 out of 65 (72.3%) were positive for BCoV antibody detected by ELISA while only 35 out of 65 (53.9%) detected by SNT. a total of 15 samples were positive by ELISA and negative by SNT. These results agreed with that mentioned by (Cho *et al.,* 2001) who indicated approximately 90% of the worldwide cattle population has antibodies against BCoV and that BCoV antibodies-positive herds remained persistently high (75-100%) (Ohlson *et al*., 2013). Also, (Clark, 1993) found that the BCoV prevalence in calves developing diarrhoea and clinically normal calves ranges from 8 to 69% and from 0 to 24%, respectively. These results may be due to chronic virus shedding by affected animals (Ohlson *et al.,* 2010b) who recorded nasal shedding within a herd form respiratory BCoV clinically or subclinically infected calves and young adult cattle. Shedding of BCoV in the absence of other important respiratory tract pathogens has been observed in cattle with naturally occurring BRD (Gomez *et al.,* 2017).

Our results revealed high sensitivity of ELISA over SNT, where sensitivity, specificity and correlation were 91.4%, 50% and 72.3% respectively, (Table 2). This was agreed by (Alenius *et al.,* 1991) who concluded that ELISA is more sensitive than SNT in detection of antibodies in bovine sera. They expected that ELISA which detects antibodies against all viral components is more sensitive than SNT which detects only antibodies against viral neutralizing antigens, and observed that sera with high titer of antibody to BCoV by SNT had high titer by ELISA. Also, (Ohlson *et al.,* 2010a) mentioned that one of the most important factors for using ELISA is its reliability for examination of serum samples with cytotoxic nature which cannot be examined by SNT. In addition to the indirect ELISA does not require any reagent which difficult to obtain or prepare. (Aspen, 2017) found BCoV ELISA was an effective procedure and simple technique yielded a high level of antigen and produced results at least

equivalent to the standard SNT with considerable saving in time and labor cost.

The ELISA used to measure anti-BCoV antibodies in the present study detected total reactive rather than neutralizing antibodies. It is also possible that anti-BCoV antibodies generated in response to a natural infection might have had a weak protective effect against viral replication, which would protect against the development of clinical signs of disease but not against virus shedding.

The study concluded that, the patent prepared ELISA is more sensitive, accurate and specific assay than SNT for detection of BCoV antibodies. Benefits to control programs include the test´s ability to detect changes in antibody levels by analyzing paired samples and thereby identifying recent exposure to BCoV, in addition to saving time and commercial kits cost.

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التشخيص السيرولوجى لفيروس الكورونا البقري في العجول باستخدام إليزا مطورة محليا

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يعتبر فيروس الكورونا البقري من الفيروسات المنتشرة بين الماشية في جميع الاعمار فى مختلف انحاء العالم حيث تتسبب في الاسهالات والاعراض التنفسية في العجول الصغيرة وفي الابقار البالغة. وقد صممت هذه الدراسة لتحضير أطقم إليزا محليا لإستخدامها على العينات الحقلية حيث تم اكثار العترة العالمية المرجعية ميبوس (Mebus) وتمريرها علي خلايا الزرع النسيجى وتم تجميع الفيروس وتركيزه باسخدام البولى اثيلين جليكول ٦٠٠٠ واستخدامه كانتيجين لاختبار الاليزا . ومن ثم اجراء اختبار الاليزا الغير مباشر واختبارالسيرم المتعادل علي عدد ٦٥ عينة سيرم للعجول الصغيرة باعراض تنفسية للكشف عن الأجسام المضادة للفيروس. وكانت نتيجة الاليزا الغير مباشرة ٧٢,٣% والسيرم المتعادل ٥٣,٩%. وأظهرت النتائج ان حساسية وخصوصية وقوة الارتباط عالية لاختبار الاليزا الغير مباشر مقارنة مع اختبار السيرم المتعادل وكانت٩١,٤٪ و ٥٠٪ و ٧٢,٣٪ على التوالي. حيث ان اختبار الاليزا يكشف عن الاجسام المضادة لكل مكونات الفيروس بينما اختبارالسيرم المتعادل يكشف عن الاجسام المضادة الخاصة بتعادل الفيروس فقط. واجمالا فان الاليزا المطورة بالمعمل ذات حساسية ودقة وخصوصية عالية مقارنة باختبار السيرم المتعادل للكشف عن الاجسام المضادة للفيروس كما انها توفر الوقت وتكاليف استيرادها من الخارج.