



COMPARISON OF THREE DIAGNOSTIC TECHNIQUES FOR DETECTING BOVINE VIRUS DIARRHEA VIRUS (BVDV) IN BUFFY COAT SAMPLES

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ABSTRACT

Three diagnostic techniques were compared in terms of their ability to detect BVDV in Buffy coats. Two hundred twenty seven BVDV Buffy coats were used. The techniques used were antigen captures ELISA (ACE), virus isolation-immunofluorescent test (IFX), and one step real time reverse transcription-polymerase chain reaction (RRT-PCR). Out of 227 Buffy coat samples, 35 (15.4%) were found to be positive with RRT-PCR, 23 (10.1%) with ACE, and 20 (8.8%) with virus isolation-IFT. The results obtained indicated that RRT-PCR is more suitable for the detection of BVDV in Buffy coats when compared to other techniques, and ACE can be used for the routine diagnosis of BVDV infection as an alternative technique to virus isolation-IFT

Key Words: BVDV, ACE, IFX, RRT-PCR

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1. INTRODUCTION

BVDV, a small (12.5 kb genome) positive sense RNA virus, is a member of the genus *Pestivirus* in the Family *Flaviviridae*. Since its discovery in the late 1940s, BVDV has become the most economically important viral disease of cattle [1]. Based on the presence or absence of visible cytopathic effect (CPE) in infected cell cultures, BVDV is distinguished into two biotypes: cytopathic strain (CP) and noncytopathic strain (NCP) [2].

As instances of BVDV infection and subsequent economic losses increase, detection of the pathogen becomes essential to prevent and eradicate BVDV infections. Different diagnostic methods have been developed based on antibodies, antigens, and nucleic acid at both the herd and individual level. BVDV has been detected through spot hybridization with probes prepared from cloned cDNA sequences and

RNA hybridization [3]. BVDV has also been detected using enzyme-linked immunosorbent assay (ELISA), virus isolation (VI), immunohistochemistry (IHC), serum neutralization (SN), and direct and indirect fluorescent assay (FA) [4, 5]. PCR amplification was developed to detect BVDV from bulk milk samples; and single-tube single-enzyme RT-PCR, TaqMan PCR, and RRT-PCR were used to identify BVDV in tissue, serum, semen, and milk [6, 7].

In this study, the reliability of two test systems, namely the antigen capture ELISA (ACE), and real time reverse transcription-polymerase chain reaction (RRT-PCR) to the conventional virus isolation-immunofluorescent test (IFX), were compared in the detection of bovine pestivirus in Buffy coat samples from suspected cattle and buffaloes.

2. MATERIALS AND METHODS

2.1. Virus strain:

The standard reference BVDV genotype 1, NADL cytopathic strain, as tissue culture adapted virus was kindly obtained from the Department of virology, Animal health Research Institute, Dokki, Giza. The virus had a titer of 10^6 log₁₀ TCID₅₀/ ml in MDBK cells. It was used as positive control for RRT-PCR.

2.2. Buffy coat samples:

A total of 227 Buffy coat samples were collected from a herd of unvaccinated cattle and buffaloes. These samples were preserved at - 20 °C till used in direct detection of BVDV using antigen capture ELISA (ACE), conventional virus isolation-immunofluorescent test (IFX) and real time reverse transcription-polymerase chain reaction (RRT-PCR).

2.3. Cell Line:

BVDV-free Madin Darby Bovine Kidney (MDBK) Cells were grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 5% Fetal Calf Serum were used in conventional virus isolation-immunofluorescent test (IFX).

2.4. BVDV Reference Antiserum:

BVDV polyclonal antiserum was supplied by the Department of virology, Animal health Research Institute, Dokki, Giza. It was used in viral detection by isolation – IFX.

2.5. Rabbit Antibovine IgG conjugated with fluorescein isothiocyanate:

It was supplied by Sigma and used in IFX.

2.6. Primers and probe:

Oligonucleotides primer and probe sequences for BVDV was synthesized by Metabion international, Germany as described by [8]. They were used in RRT-PCR

P15'-GGGNAGTCGTCARTGGTTCG-3' (forward primer), P25'-GTGCCATGTAC

AGCAGAGWTTTT-3' (reverse primer) and Probe 5'-FAM-CCAYGTGGACGAGGGCAYGC-BHQ-1-3'.

2.7. Antigen captures ELISA (ACE):

It was performed using IDEXX BVDV-Ag/leukocytes test kit following manufacture instructions.

2.8. Isolation of BVD Virus (Isolation – IFX):

After seeding culture tubes with MDBK cell culture, they were inoculated with the test materials. The culture medium was changed at 24 hour post-inoculation and cells were maintained for a further 4 days to obtain efficient viral growth. At the end of the period, the culture drums were frozen and thawed twice and culture fluids were subsequently inoculated onto MDBK cells grown in 24- well plates. Isolations were proved by immunofluorescence (IFX) staining as described by [9].

2.9. Extraction of BVDV RNA:

It was performed using QIAamp® Viral RNA mini Kit, QIAGEN, USA according to the manufacturer's instructions.

2.10. Detection of BVDV RNA using one step real time RT-PCR (RRT-PCR)

One-step real-time RT-PCR was performed by using commercial reagents according to the One-Step RRT-PCR Master Mix, Qiagen Operon Technologies, Alameda, CA, manufacturer's instructions. The optimal annealing temperature for a given combination of primers and probes was determined by increasing the temperature from 55°C to 59°C in increments of 1°C. The optimal concentration of primers and probes were determined by titration between 0.1 mmol and 1 mmol. The optimal MgCl concentration was determined by titration between 1.5 mM and 2.5 mM. The final volume of real-time RT-PCR was 25 ml, which contained 8 ml of sample RNA and 17 ml of reagent mixture with 2.5 mM MgCl, 10 mM deoxyribonucleotide triphosphates, 0.2 mmol of forward primer, 0.2 mmol of

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reverse primer, 0.24 mmol of BVDV probe and 5 mmol of random hexamers, 10 U of RNase inhibitor, and 1 ml of enzymes. The reactions were carried out on a programmable thermocycler as follows: 48°C for 25 min; 95°C for 10 min; 50 cycles of 95°C for 25 sec, 56°C for 25 sec, 72°C for 30 sec; and final elongation at 72°C for 10 min.

3. RESULTS

From table (1), it is clear that the total number of positive Buffy coat for BVDV

varied with the used test. In RRT-PCR was more sensitive test detecting 35 samples (15.4%), ACE detecting 23 samples (10.1%) while Isolation-IFX detecting 20 samples (9.3%). The specific intracytoplasmic apple green fluorescence of BVDV antigen detected in MDBK cells by IFT was revealed in figure (1), and the amplification blot curve for positive BVDV samples and negative control was shown in figure (2). In correlation to RRT-PCR results (table 2) ACE was in agreement by 94.7% but Isolation-IFX was 88.9%.

Table (1): The sensitivity of tests used in terms of percentage of detection of BVDV in buffy coat samples.

| sample | Total tested sample | ACE | | Isolation-IFX | | RRT-PCR | |
|------------|---------------------|----------------|-----|---------------|-----|---------------|-----|
| | | +ve | -ve | +ve | -ve | +ve | -ve |
| Buffy coat | 227 | 23 (10.1%)* | 204 | 20 (9.3%) | 207 | 35 (15.4%) | 192 |

ACE=antigen capture ELISA, RRT-PCR= real time reverse transcriptase PCR, *Positive % from total sample

Table (2) correlation between RRT-PCR, ACE and Isolation-IFX for detection of BVDV in total examined samples

| RRT-PCR results | ACE | | Isolation-IFX | |
|-----------------|-----|-----|---------------|-----|
| | +ve | -ve | +ve | -ve |
| Positive(n=35) | 23 | 12 | 20 | 15 |
| Negative(n=192) | 0 | 192 | 0 | 192 |

Percentage of agreement = total No. of agreement/total No. of examined sample.

Agreement for ACE with RRT-PCR = $215/227 \times 100 = 94.7\%$.

Agreement for Isolation-IFX with RRT-PCR = $202/227 \times 100 = 88.9\%$.

From table (3), also there is a difference in the sensitivity of the test used for detection of BVDV in cattle and buffaloes. From 205 examined cattle, 30 (14.6%), 21(10.2%) and 18 (8.8%) were positive for BVDV

using RRT-PCR, ACE and Isolation-IFX respectively. While From 22 examined buffaloes, 5 (22.7%) were positive for BVDV using RRT-PCR and the same 2 (9.1%) buffaloes were positive by ACE and Isolation-IFX .

Table (3): The sensitivity of tests used in terms of percentage of detection of BVDV in cattle and buffaloes.

| Species | Total number | ACE | | Isolation-IFX | | RRT-PCR | |
|-----------|--------------|----------|------|---------------|-----|----------|------|
| | | positive | % | positive | % | positive | % |
| Cattle | 205 | 21 | 10.2 | 18 | 8.8 | 30 | 14.6 |
| Buffaloes | 22 | 2 | 9.1 | 2 | 9.1 | 5 | 22.7 |

ACE=antigen capture ELISA, RRT-PCR= real time reverse transcriptase PCR.

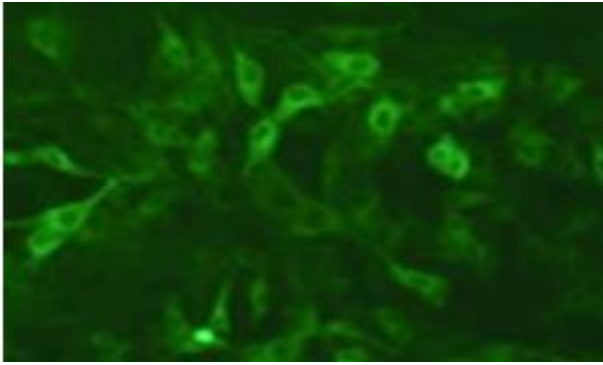


Figure (1) show intracytoplasmic apple green fluorescence as positive indirect FA technique on inoculated MBDK cell line indicative for presence of local BVDV isolate.

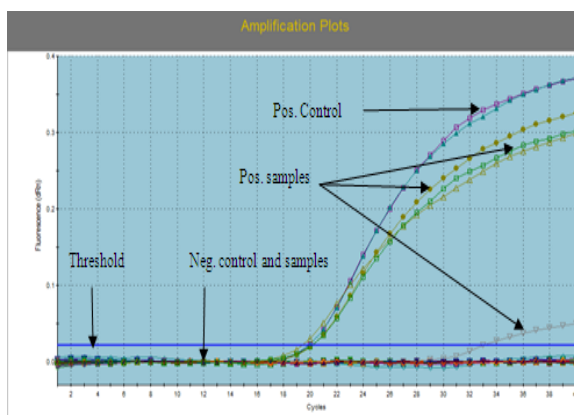


Figure (2) Amplification curves of sensitivity test of common BVDV by using real-time PCR that showing positive results for examined samples from buffy coat compared with negative and positive control samples. Negative CT value considered 40 cycles.

4. DISCUSSION

Accurate diagnosis of BVDV infection depends upon isolating the virus from blood of affected animals and [10] mononuclear cells obtained from whole blood are the ideal sample for BVDV isolation, because neutralizing antibodies to BVDV present in serum may interfere with virus isolation [11].

In this study, the ability of detecting the BVD virus by means of virus isolation-IFX, ACE, and RT-PCR techniques in buffy coat samples from cattle and buffaloes was investigated. While 35 of the 227 buffy coats examined for BVDV were found to be positive by RT-PCR, 23 of them were positive with ACE, 20 of them with virus isolation-IFX. In the research, RRT-PCR

gave better results than the other techniques. This result most probably arose from Real-Time allows one to visualize the amplification of the amplicon as it progresses in real time .also it can detect all BVDV strains and the reduced risk of carry-over contamination [12]. In addition, Real time PCR can detect the genome of neutralizing and semi neutralizing viruses; in contrast, neutralizing antibodies can mask viruses and make negative ACE results [13] and this might be the reason for different results between Real time RT-PCR and ACE.

This research demonstrated that ACE is more efficient than virus isolation-IFX but less efficient than RRT-PCR. Beyond the toxic effect caused by the substances of cells lysed during storage, or preparation affect the infective antigen of the virus and this affect the isolation on tissue culture ,while ACE detect both structural and nonstructural protein of the virus our results come in agreement with [14]. This data suggests that the ACE was selected in the best combination between a panel of monoclonal antibodies and BVDV field isolates.

In conclusion, the results obtained in this study indicated virus isolation-IFX is less sensitive than RRT-PCR and ACE. However, it is obvious that RRT-PCR has an advantage since it is faster and less laborious than the virus isolation-IFX technique. The ACE can be used as a time saving alternative for the routine diagnosis of BVD virus infection in laboratories without RRT-PCR technique facilities. However, it should be remembered that the MAbs used in the test should have the broadest range of reactivity.

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مقارنة ثلاثة اختبارات تشخيصية لكشف عن فيروس الإسهال البقري المعدي في عينات الطبقة الدموية

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الملخص العربي

تم مقارنة ثلاثة اختبارات تشخيصية في قدرتها للكشف عن فيروس الإسهال البقري المعدي في عينات الطبقة الدموية. استخدمت 227 عينة من الطبقة الدموية واجريت عليها ثلاثة اختبارات تشخيصية هي الاليزا، الماسك الانتيجيني والعزل الفيروسي متبعا بالوميض الفلورسنتي المناعي واختبار انزيم البلمرة المتسلسل الارتدادي العكسي حقيقي الزمن ذو الخطوة الواحدة. وقد تبين ان من إجمالي 227 عينة كانت هناك 35 (15.4%) عينة ايجابية باختبار انزيم البلمرة المتسلسل الارتدادي العكسي حقيقي الزمن ذو الخطوة الواحدة و 23 (10.1%) بالاليزا الماسك الانتيجيني و 20 (8.8%) بالعزل الفيروسي متبعا بالوميض الفلورسنتي المناعي. وقد اتضح من النتائج ان اختبار انزيم البلمرة المتسلسل الارتدادي العكسي حقيقي الزمن ذو الخطوة الواحدة الاكثر تناسبا للكشف عن فيروس الإسهال البقري المعدي في عينات الطبقة الدموية مقارنة بالاختبارات التشخيصية الاخرى وان اختبار الاليزا الماسك الانتيجيني يمكن استخدامه في التشخيص الروتيني لفيروس الإسهال البقري المعدي عوضا عن العزل الفيروسي متبعا بالوميض الفلورسنتي المناعي.

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