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Evaluation of diagnostic methods for the detection of pestiviruses in clinical samples

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Abstract: There are several commonly used diagnostic methods to detect pestiviruses for routine diagnostic purposes. In the present study, we aimed to compare virus isolation-indirect immunoperoxidase monolayer assay (VI-IIPMA), antigen capture ELISA (ACE), and RT-PCR for the detection of pestiviruses in clinical samples. Out of 246 samples tested (11 serum, 119 swab, 116 tissue), 28 samples (11.39%) were positive and 218 (88.61%) were negative using the VI-IIPMA method. Using ACE, 70 samples (28.46%) were positive and 176 (71.54%) were negative. Finally, using RT-PCR analysis, we detected 19 (7.72%) positive and 227 (92.28%) negative samples. Inconsistencies were observed among results of the three methods: 8 samples were positive using VI-IIPMA but negative using ACE and RT-PCR. In addition, 4 samples that were found to be negative by VI-IIPMA were found to be positive by ACE and RT-PCR. Five samples were positive by ACE and VI-IIPMA. However, 46 samples were found to be positive only by ACE. These results show that the number of positive results detected by ACE is higher than that by VI-IIPMA and RT-PCR. Although ACE may prove advantageous for diagnosing pestiviruses, using a second method in combination with ACE will improve the validity of the results.

Key words: Pestiviruses, ELISA, immunoperoxidase monolayer assay, virus isolation, RT-PCR

1. Introduction

Pestivirus infections cause diseases in ruminants worldwide and are characterized by enteric, respiratory, and reproductive problems. Because the virus causes an immunotolerant persistent infection, it can circulate in a herd until the persistently infected animals are eliminated (1). Economic losses due to these viruses are substantial; for example, the bovine viral diarrhea virus (BVDV) reportedly causes a loss of between 760 million and 2.2 billion dollars in the United States each year (2).

Pestiviruses have a linear, positive-sense, single-stranded RNA genome and are classified in the genus *Pestivirus* of *Flaviviridae*. The genus *Pestivirus* contains BVDV, border disease virus (BDV), and classical swine fever virus (CSFV). BVDV has two genotypes, BVDV-1 (BVDV 1 a-t) (3) and BVDV-2 (BVDV 2 a-d) (4), while BDV has seven genetic clusters (BDV 1–7) (5). The *Pestivirus* genotypes can be classified into two biotypes, namely cytopathogenic (cp) and noncytopathogenic (ncp), based on the effects they have on infected cells (6). Genomic RNA encodes a polyprotein, which has one open reading frame and two untranslated regions (5'-N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A, NS5B-3'). The protein E2 is especially significant because neutralizing antibodies develop mainly against this protein; unfortunately it is often affected by mutations (7).

Serology, virus isolation (VI), detection of viral antigens, and detection of viral RNA are the approaches most commonly used in the laboratory to diagnose pestivirus infections (8,9). The indirect immunoperoxidase monolayer assay (IIPMA) and immunofluorescence assay (IFA) are used to detect ncp strains (10,11). VI is the currently accepted gold-standard method for diagnosing pestiviruses, but it is becoming less popular because of the expense and the response time (12). At present, VI, IIPMA, antigen capture ELISA (ACE), and reverse transcriptase polymerase chain reaction (RT-PCR) are the preferred methods for diagnosing pestivirus infections. An important problem in the standardization of diagnostic methods arises from the probability of a high mutation rate in the viral RNA genome, which has no proofreading mechanism, leading to enhanced antigenic differences between pestivirus field strains.

It is known that most pestivirus field isolates are noncytopathogenic (13). Therefore, in this study, VI and IIPMA were utilized as a single method in two stages (VI-IIPMA). We compared VI-IIPMA, ACE, and RT-PCR for detection of pestiviruses in field-originated clinical samples including serum, swab, and tissue materials.

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2. Materials and methods

2.1. Samples

The sampling area is located in the northwestern part of Turkey. During the sampling period (2010–2014), a total of 100 nasal and 19 conjunctival swabs were collected from calves showing clinical signs of a respiratory tract disease. At the same time, lung tissue was gathered from 39 cattle with or without clinical signs of pestivirus infection in a slaughterhouse in the city of Bursa. An additional 77 samples from ruminant abortion cases (56 calves, 2 kids, and 19 lambs) from the Marmara Region were obtained. Eleven blood samples that were sent to the laboratory for diagnosing pestiviruses during the same period were also included in the study (Table 1). Hence, the total number of clinical samples was 246. All the samples collected from live animals were obtained and processed according to local ethics committee approval (2010-02/01).

Blood samples were collected in vacutainer tubes by venipuncture. Serum was separated by centrifugation at 3000 rpm and 4 °C for 10 min and stored at –80 °C until testing.

Swab samples were collected in 2 mL of sterile white phosphate-buffered saline (W-PBS) and immediately delivered to the laboratory under cold conditions. After centrifugation at 4 °C and 3000 rpm for 20 min, supernatants were filtered using a 220-nm disposable filter and stored at –80 °C until testing.

Tissue samples were homogenized in W-PBS using a homogenizer (Sartorious, Germany). Supernatants were separated by centrifugation at 4 °C and 3000 rpm for 20 min before passing through a 220-nm filter. Prepared homogenates were stored at –80 °C until testing.

2.2. Cell line and viruses

For the VI and IIPMA methods, Madin-Darby bovine kidney (MDBK) cells, originating from the Virology Institute at Justus-Liebig University, Giessen, Germany, were used. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) was used for the cell cultures. The cell line and FCS were tested for the absence of pestivirus antigens and antibodies. BVDV TR-19, a ncp strain of BVDV, and the BVDV-NADL reference strain were used as positive controls in IIPMA and panpestivirus RT-PCR methods, respectively.

2.3. Virus isolation and indirect immunoperoxidase monolayer assay

All the samples were inoculated onto MDBK cell cultures, and IIPMA was performed on the third blind passage as described previously (3). For VI, 24-well plates were coated with MDBK cells at a concentration of 100,000 cells/mL. After 24 h, the medium was removed and 200 µL of inocula was added for 1 h at 37 °C. Then 1 mL of DMEM (without FCS) was added to the wells. The culture medium was changed 24 h after inoculation. For the next 5 days the cells were observed for cytopathogenic effects. At the end of this period, the cells were harvested by freezing at –80 °C. The culture fluids were used for the blind passage inoculations using the same processes. A total of 3 blind passages were applied to all samples. To ensure the proper isolation of the ncp *Pestivirus* isolates, IIPMA was used. During the protocol a negative control well, in which PBS was inoculated, was used.

On day 3 of the third blind passage, 24-well plates were washed with W-PBS and kept at 80 °C for 3 h to fix. In the next step, 200 µL of O-D-glucopyranoside (Sigma, 75081-5G) was added to each well to increase the permeability of the cells, and they were incubated at room temperature for 10 min. After each incubation step, plates were rinsed 3 times with W-PBS. The cells were then incubated for 90 min at 37 °C with primary mouse anti-*Pestivirus* monoclonal antibody 1/4/7 (14) diluted 1:40 in Tween-20 W-PBS. A second antibody, biotinylated antimouse antibody (Pierce, 31800), was added at a dilution of 1:400 and incubated for 90 min under the same conditions. The last incubation was carried out with a peroxidase-labeled streptavidin-biotin complex (1:300) (Pierce, 21124) under the same conditions of 90 min at 37 °C. The reaction was stopped 30 min after adding the substrate [3-amino-9 ethylcarbazole (Sigma, A5754), hydrogen peroxide, and sodium acetate]. The test was evaluated by checking for reddish-brown intracellular aggregates using an inverted light microscope (Nikon Eclipse TS100).

2.4. Antigen capture ELISA

The pestivirus ACE (IDEXX, Switzerland, Cat. No. 99-43830), which screens for the pestivirus E^{rns} structural protein, was applied to sera and inocula prepared from swabs and tissues for the detection of viral antigens. The test was performed according to the manufacturer's instructions.

Table 1. Number of samples tested.

Blood	Swab		Tissue		Total
Serum	Nasal	Ocular	Lung	Abortus	246
11	100	19	39	77	

2.5. RNA isolation and RT-PCR

Total viral nucleic acid extraction was performed using a commercial kit (Axygen, Canada). Synthesis of cDNA was performed with panpestivirus primer p324 (5'-ATG CCC WTA GTA GGA CTA GCA-3') using a cDNA synthesis kit (Biomatik, Canada) according to the manufacturer's instructions. For detecting panpestivirus nucleic acid, RT-PCR was performed with primers p324 and p326 (5'-TCA ACT CCA TGT GCC ATG TAC-3') (15) according to the following protocol: 35 cycles of 1 min at 94 °C, 1 min at 56 °C for annealing, and 1 min at 72 °C for elongation. This cycle amplified the 288-bp product from the 5' untranslated region (UTR). RT-PCR products were visualized on 1% agarose-ethidium bromide gels by electrophoresis.

2.6. Statistical analysis

Fischer's exact test was used to compare the applied methods.

3. Results

Among the 246 clinical samples, 28 (11.39%) were positive while 218 (88.61%) were negative according to the VI-IIPMA results. With ACE, 70 samples (28.46%) were positive and 176 (71.54%) were negative. The RT-PCR method detected only 19 positive samples (7.72%) and 227 samples (92.28%) were negative. There was no statistically significant difference between the VI-IIPMA and RT-PCR results (P = 0.2195), but ACE results were statistically significant in comparison to the VI-IIPMA and RT-PCR methods (P < 0.0001) (Table 2).

There were inconsistencies in the results from the samples gathered from the different methods. Using VI-IIPMA, 8 samples were determined as positive, but they were negative according to the ACE and RT-PCR methods. In addition, 4 samples determined to be negative by VI-IIPMA were positive by ACE and RT-PCR. Five samples were detected positive by ACE and VI-IIPMA. Another 46 samples tested positive by only the ACE method. Of the remaining 183 samples, 15 samples tested positive while 168 samples tested negative with each method. A second ACE test was applied to supernatants from MDBK cell cultures inoculated at passage 3 with 78 samples, which were determined to be positive by at least one of the methods. An additional 25 samples tested positive in this second ACE test. The comparison of the results is shown in Table 3.

Table 2. The result of VI-IIPMA, ACE and RT-PCR methods (n = 246).

	Number of positives	Number of negatives	Statistics*
ACE	70 (28.46%)	176 (71.54%)	
VI-IIPMA	28 (11.39%)	218 (88.61%)	P < 0.0001
RT-PCR	19 (7.72%)	227 (92.28%)	P < 0.0001

*P-values represent a comparison of ACE and the other tested methods.

Table 3. The pattern of results for 246 samples with the three applied methods.

Number of samples	ACE applied to the inocula	VI-IIPMA	RT-PCR	ACE applied to cell culture supernatants
46	+	-	-	-
4	+	-	+	-
3	-	+	-	-
5	-	+	-	+
5	+	+	-	+
15	+	+	+	+
168	-	-	-	-
Total (246)	70 (+), 176 (-)	28 (+), 218 (-)	19 (+), 227 (-)	25 (+), 221 (-)

ACE: Antigen capture ELISA.
VI-IIPMA: Virus isolation-indirect immunoperoxidase monolayer assay.
RT-PCR: Reverse transcriptase polymerase chain reaction.
+: Positive; -: Negative.

4. Discussion

Each diagnostic method has its own advantages and disadvantages including high sensitivity, cost, and response time. VI-IIPMA is known as the gold standard of virus detection but has become less popular because of its expense and long processing time (12). ACE, on the other hand, is quick, easy to perform, and sensitive as long as a sufficient amount of viral protein is present in the test sample. False negative results can be obtained when incorrect tissue samples are used or when hemolysis is present in the blood samples. Despite these issues, ACE has become the most commonly used diagnostic technique in veterinary diagnostic laboratories. PCR protocols have developed rapidly over the past few decades and are becoming one of the most frequently used tools for diagnostic work. The three methods studied in this study (VI-IIPMA, ACE, and RT-PCR) are frequently used for diagnosing pestivirus infections under field conditions. Here, we evaluated each method for its ability to detect pestiviruses in routine diagnostic samples.

Our findings show that the highest number of positive samples was detected by ACE (28.46% positive) compared to VI-IIPMA (11.39% positive) and RT-PCR (7.72% positive). For the 15 samples that tested positive using all three methods, it is conceivable that an abundance of infectious virus existed (Table 3). The presence of infectious virions may also account for why 8 samples were negative in the first round of ACE and RT-PCR testing but were positive when tested by VI-IIPMA. The fact that 50 samples tested positive in the first ACE and ACE / RT-PCR tests but tested negative in the VI-IIPMA test may be explained by the existence of noninfectious or defective virions. Inactivation of the virus in these samples can occur during transport and storage conditions. Very small amounts of infected cells in the inoculated cultures were determined by VI-IIPMA (Figures 1A and 1B) and these were negative by both ACE and RT-PCR for three samples. These findings suggest that the ACE method may overlook infectious virions when the amount of antigen is small. Saliki and Dubovi (13) stated that ACE is preferably used for examinations of persistently infected animals. In another study it was shown that ACE, VI and RT-PCR are reliable methods for diagnosing BVDV in animals with persistent infection (16). These animals have a large load of virions that can be easily detected; therefore, there are likely not enough detectable virus particles in acutely infected animals, especially if they are at the beginning stages of the infection.

RT-PCR could not detect the virus in 59 samples that were declared positive by either ACE or VI-IIPMA (Table 3). This may have been due to inhibitors that existed in the samples, RNA proteases that can degrade the RNA during storage (8). The chosen primers could be another reason for this result. The 5' UTR and the *NS3* gene of pestiviruses have a large number of regions with preserved nucleotide sequences; for this reason, RT-PCR assays performed best with primers that were specific for these regions (17). However, certain single base changes may affect the success of primer annealing. In addition, new pestivirus variants may be missed by these protocols. Thus, further studies will be conducted using the HoBi-like class of BVDV isolates, a newly discovered variant.

In conclusion, the results of the present study show that ACE is capable of yielding a higher number of positive results for the detection of pestiviruses in clinical

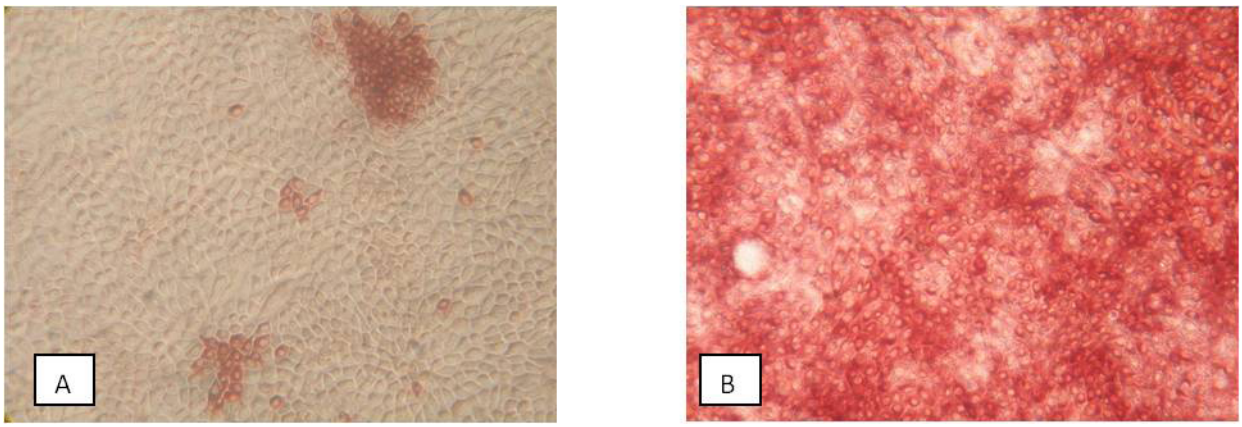

Figure 1. Microscopic appearance from selected wells from VI-IIPMA method: A) a sample detected as positive by VI-IIPMA (10×), B) a sample detected as positive by ACE and VI-IIPMA (10×).

samples than other commonly used methods. Moreover, a combination of virus isolation and ACE could improve the validity of the diagnostic results.

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