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Simultaneous detection of enteropathogenic viruses in buffalos faeces using multiplex reverse transcription-polymerase chain reaction (mRT-PCR)

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ABSTRACT: A multiplex reverse transcription- polymerase chain reaction (mRT-PCR) assay that detects Bovine Viral Diarrhoea Virus, Bovine Coronavirus, and Group A Rotaviruses in infected cell-culture fluids and clinical faecal samples is described. One hundred twenty faecal samples from buffalo calves with acute gastroenteritis were tested. The mRT-PCR was validated against simplex RT-PCR with published primers for Pestivirus, Coronavirus and Rotavirus. The multiplex RT-PCR was equally sensitive and specific in detecting viral infections compared with simplex RT-PCR. The mRT-PCR readily identified viruses by discriminating the size of their amplified gene products. This mRT-PCR may be a sensitive and rapid assay for surveillance of buffalo enteric viruses in field specimens. This novel multiplex RT-PCR is an attractive technique for the rapid, specific, and cost-effective laboratory diagnosis of acute gastroenteritis.

Key words: BVDV, BCoV, Rotavirus, mRT-PCR.

INTRODUCTION - Neonatal diarrhoea is one of the major disease syndromes in several species of domestic animals, especially bovine and buffalo calves, and is a cause of important economic losses particularly in the first few months of life (Reinhardt G. *et al.*, 1989).There is a consensus that control and prophylaxis of neonatal diarrhoea is essential in high technology rearing systems (Paul PS *et al.*, 1993). Among viral pathogens Coronavirus, Rotavirus and Bovine Viral Diarrhoea Virus play a key role in neonatal calf diarrhoea. Bovine Coronavirus (BCoV) causes severe diarrhoea in newborn calves and is associated with winter dysentery in adult cattle. Although diarrhoea is recognized as the main clinical feature of BCoV infections, it also causes respiratory tract infections in calves (Clark MA *et al.*, 1993). Bovine Viral Diarrhoea Virus (BVDV) causes significant disease in cattle worldwide like gastrointestinal disease, reproductive and respiratory disorders and persistent infections. In addition to the above-mentioned diseases, some virulent strains cause severe thrombocytopenia with haemorrhage and a severe acute disease resembling mucosal disea-

se (Hamers C *et al.*, 2001). Rotavirus is considered the most important etiological agent of acute viral gastroenteritis in young animals, and infants and young children throughout the world (Bern C *et al.*, 1992). These viruses are classified serologically in seven distinct groups (A–G) and serotypes/ genotypes (G and P types). Over the past decade, PCR assays for the rapid and sensitive detection of viral agents of acute gastroenteritis have been developed. These assays are used widely for the laboratory diagnosis of viral acute gastroenteritis. Direct Rotavirus, Coronavirus and Bovine Viral Diarrhoea Virus testing with commercially available antigen detection enzyme immunoassay is also currently used. These methods often yield variable results because of insufficient sensitivity and/or specificity and requires confirmation by PCR assay or virus isolation. Therefore, a multiplex reverse transcription-polymerase chain reaction (mRT-PCR) assay allowing simultaneous testing for BVDV, BCoV and Rotavirus in a rapid and cost-effective method is of interest. In this study, a mRT-PCR assay was developed and evaluated for the direct and simultaneous identification of these agents in faecal samples from buffalo calves with diarrhoea.

MATERIAL AND METHODS - Faecal samples: One hundred twenty faecal samples from buffalo calves with diarrhoea were collected from five dairy buffalo herds located in the Caserta and Salerno province in Campania Region and were stored at -80 °C until analysis; **Viruses and cell cultures:** The reference strains for BVDV, BCOV and Rotavirus were NADL strain, a field isolate and Mebus strain respectively. The first were propagated on Madin-Darby Bovine Kidney Cells (MDBK), the second on Human Rectal Adenocarcinoma (HRT-18) cells and the last on African Monkey Kidney (MA-104) cells. The growth medium was Dulbecco minimum essential medium (DMEM) supplemented with 1% antibiotics (penicillin, dihydrostreptomycin, and mycostatin), 1% L-glutamine and 5% Pestivirus free foetal calf serum; **Virus titration:** Virus titration was performed using MDBK, HRT-18 and MA-104 cell cultures grown in 96 well microplates. For virus titration, serial 10-fold dilutions of viruses were made in maintenance medium. Four wells of cells were each inoculated with 0.1 ml of each dilution, incubated for 4 to 6 days at 37°C and examined for cytopathic effects (CPE). Infectivity titers were expressed as median tissue culture infectious doses ($TCID_{50}$)/0.1 ml; **RT-PCR**: Viral RNA was extracted from faecal samples by the Trizol method (Invitrogen, Milano, Italy) according to the manufacturer's instructions. 2 µl of extracted RNA and 1 µl of Random-hexamers (50 ng/µl) were denaturated for 5 min at 65°C; then cDNA was synthesized at 55°C for 40 min in a volume of 20 µl containing 4 µl of 5x cDNA Synthesis Buffer, 1 µl of DTT (0,1 M), 2 µl of RNaseOUT™ (40U/µl), 2 µl of dNTP (10 mM), and 1 µl of ThermoScript™ RT (15 U/µl). The reverse transcription was performed using ThermoScript™ RT-PCR System (Invitrogen, Milan, Italy). PCR assays for the detection of BVDV, BCoV and Rotavirus, were carried out as described by Letellier *et al*., (1999), Hoet *et al*. (2001) and Martella *et al*. (2001), respectively. The mPCR assay was carried out using 34,1 µl of DEPC-treated water, 5 µl of PCR reaction buffer 10X, 1,5 µl of 50 mM $MgCl_2$, 1 µl of dNTP mix (10 mM), 1 µl of each primer (10 mM) BE, B2, BCoVs, BCoVas and Con2, Con3, 0.4 µl of Platinum© Taq DNA polymerase (5 U/µl)(Invitrogen, Milan, Italy) and 2 µl of cDNA. For m-PCR cycling conditions were as follow: 94 °C for 3 min, then 30 cycles of 94 °C for 30 seconds, 57 °C for 45 seconds, 72 °C for 1 min and 30 seconds and a final extension of 72 °C for 7 min. RT-PCR products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light;

PCR detection thresholds: Detection thresholds of the RT-PCR/BVDV, RT-PCR/BCoV, RT-PCR/Rota and mRT-PCR were evaluated by experimental contamination of previously negative clinical samples by virus isolation and PCR. The concentrations of the reference strains were previously titrated as described above as $TCID_{50}$/0,1 ml, in order to perform the experimental contaminations.

RESULTS AND CONCLUSIONS - An mRT-PCR assay, which amplified three differently sized virus-specific products, was developed to detect bovine enteropathogen viruses. Six primers are included in this assay, as follows (see Table 1): BE/B2 was designed on sequence located in a highly conserved region of BVDV from the 5'UTR, BCoVs/BCoVas amplify a 730 bp fragment from the start codon of the nucleocapsid gene of BCoV and Con2/Con3 a 876 bp fragment for the partial Rotavirus gene VP4.

Table 1. Sequence of the oligonucleotide primers used in the RT-PCR reactions.

	Primer Sequence	Genome position
BE[1]	5'- CAT GCC CTT AGT AGG ACT AGC - 3'	108- 127
B2[1]	5'- TCA ACT CCA TGT GCC ATG TAC - 3'	395-375
BCoVs[2]	5'-GCA ATC CAG TAG TAG AGC GT-3'	21– 40
BCoVas[2]	5'- CTT AGT GGC ATC CTT GCC AA -3'	731–750
Con2[3]	5'-ATT TCG GAC CAT TTA TAA CC -3'	887–868
Con3[3]	5'- TGG CTT CGC TCA TTT ATA GAC A - 3'	11–32

[1]Letellier C et al., 1999; [2]Hoet AE et al., 2003; [3] Martella V et al., 2001.

PCR products of the expected size were obtained for all viruses tested. To confirm that the assay was capable of detecting multiple viruses in a single reaction, mRT-PCR was carried out using templates in which BVDV, BCoV and Bovine Rotavirus were mixed. The result demonstrated that the assay was capable of discriminating among these viruses, as well as identifying single viruses (see Fig. 1). The mRT-PCR was able to detect a minimum of 2 x 10^3 $TCID_{50}$ / 0,1 ml of BCoV, 10^3 $TCID_{50}$ / 0,1 ml of Rotavirus and 10^1 $TCID_{50}$ / 0,1 ml of BVDV per reaction of the virus.

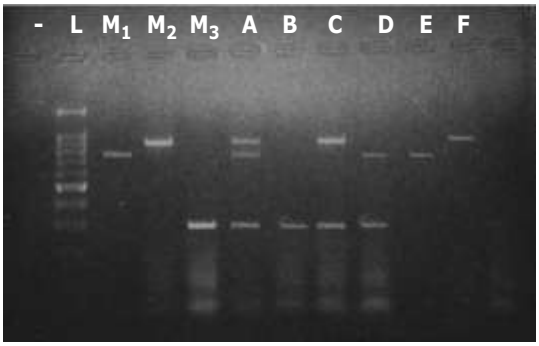

Figure 1.

mRT-PCR for BCoV (D, E); Bovine Rotavirus (C, F), BVDV (B, C, D): -: negative; L: ladder 100 bp; M_1: BCoV molecular marker (730 bp); M_2: Bovine Rotavirus molecular marker (876 bp); M_3: BVDV molecular marker (287 bp); sample 23; sample 54; sample 51; sample 52; sample 54

The laboratory diagnosis of acute gastroenteritis is based upon the identification of the infectious agent in stool specimens from acutely ill animals. Over the past decades, virus isolation and electron microscopy have been widely used for diagnosis of viral gastroenteritis (Saif LJ *et al.*, 1991). Virus isolation is inadequate for rapid therapeutic decisions as it requires several days or weeks and electron microscopy is not suitable for routine diagnostics, being a difficult and insensitive method (Buesa J *et al.*, 1996). Other methods like antigen detection enzyme immunoassay have the convenience of easy and rapid handling, but exhibit a lack of sensitivity and specificity (Kurokawa M *et al.*, 2004). In comparison, RT-PCR and PCR assays are powerful alternatives for the laboratory diagnosis of viral acute gastroenteritis (Gunson RN *et al.*, 2003; Cho KO *et al.*, 2001). Gastroenteritis causes economic losses and environmental contamination that determines the constant maintenance of the infections inside the premises, if not correctly and timely diagnosed: this mRT-PCR may be a sensitive and rapid assay for surveillance of buffalo enteric viruses in field specimens. mRT-PCR is an attractive technique for the rapid, specific, and cost-effective laboratory diagnosis of acute gastroenteritis and it is more sensible, less laborious and expensive than RT-PCR.

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