RT-PCR for detection of bovine parainfluenza virus type 3 (bPIV-3)

RT-PCR para detecção do vírus parainfluenza bovino tipo 3 (bPIV-3)

Rodrigo de Almeida Vaucher¹, Amauri Braga Simonetti² & Paulo Michel Roehe²,³

ABSTRACT

The RT-PCR technique has been frequently used for detection of the human parainfluenza virus type 3 (hPIV-3) but the literature is scarce in relation to the bovine parainfluenza virus type 3 (bPIV-3). The aim of this study was to describe a reverse transcriptase polymerase chain reaction (RT-PCR) for detection of bovine parainfluenza virus type 3 (bPIV-3) using degenerate oligonucleotides targeting a conserved region of hemagglutinin-neuraminidase (HN) gene. Reference strain SF-4 and three different brazilian bPIV-3 isolates, besides five viral strains from different sources, were included in this study. Viruses were cultured in MDBK cells under standard conditions. Hemagglutination (HA) test was used for viral titration and a direct immunofluorescence test (DFA T) for isolate screening. In RT-PCR all bPIV-3 isolates showed amplification of an expected 1009 bp fragment of HN gene, as oposed to non PIV-3 viral samples where no amplification was detected. Using SF-4 as positive control, sensitivity of 95 pg cDNA was achieved. In spite of the low number of bPIV-3 isolates tested, the results obtained in this study point out the potential use of this technique for detection of bPIV-3 in bovine clinical specimens.

Keywords: bovine parainfluenza type 3 virus; bPIV-3; RT-PCR; hemagglutinin-neuraminidase (HN) gene, detection.

RESUMO

A técnica de RT-PCR tem sido freqüentemente utilizada para a detecção do vírus parainfluenza humano tipo 3 (hPIV-3), mas a literatura é escassa em relação ao vírus parainfluenza bovino tipo 3 (bPIV-3). O objetivo deste trabalho foi descrever uma técnica de reação em cadeia pela polimerase, precedida de transcrição reversa (RT-PCR), para a detecção do vírus parainfluenza bovino tipo 3 (bPIV-3), usando oligonucleotídeos degenerados para uma região conservada do gene da hemaglutinina-neuraminidase (HN). A amostra-referência SF-4 e três diferentes isolados brasileiros de bPIV-3, além de cinco amostras virais de diferentes origens, foram incluídos neste estudo. Os vírus foram cultivados em células MDBK sob condições padrionizadas. O teste de hemaglutinação (HA) foi utilizado para a titulação viral, e o teste de imunofluorescência direta (DFTA) para a triagem dos isolados. Na RT-PCR, todos os isolados de bPIV-3 mostraram amplificação de um fragmento esperado de 1009 pb do gene HN, ao contrário do que aconteceu com as amostras virais não bPIV-3, onde não foi detectada amplificação. Empregando a amostra SF-4 como controle positivo, foi obtida uma sensibilidade de 95 pg de cDNA. Apesar do pequeno número de amostras de bPIV-3 testadas, os resultados obtidos neste estudo apontam para o uso potencial desta técnica na detecção de bPIV-3 em amostras clínicas bovinas.

Descritores: vírus parainfluenza tipo 3; bPIV-3; RT-PCR; gene da hemaglutinina-neuraminidase (HN), detecção.
INTRODUCTION

Bovine parainfluenza virus type 3 (bPIV-3) is a Respirovirus, member of Paramyxoviridae family, antigenically related to human parainfluenza virus type 3 (hPIV-3) [7]. It is an important pathogen for animals but can cause disease in humans. In cattle it is associated to the clinical picture known as “shipping fever” [13]. Most cases of respiratory infection with bPIV-3 occur with mild clinical signs. However, more severe infections may be associated with bacterial or virus agents [15]. bPIV-3 has also been associated to abortion in cattle [18].

Laboratory detection of bPIV-3 infections is classically performed by virus isolation in cell culture (gold standard technique). The virus induces a typical cytophatic effect with formation of syncytia and cytoplasmic inclusions [21]. Confirmation of the identity of the virus is usually achieved by HA [4,14] or DFAT [7] tests, but they are not capable to discriminate viral serotypes and may produce false-negative results.

The RT-PCR has been used as an alternative procedure for detection of human PIV-3 (hPIV-3) in nasal secretions or for confirming the identity of isolates [9]. Oligonucleotides based on the highly conserved sequences of the hemagglutinin (HN) and fusion protein genes have generally been used for diagnostic purposes [10,16,21]. RT-PCR for hPI-3 has been associated to other diagnostic PCRs in multiplex [1,8] and hexaplex [11] formats and have been used to detect a number of viruses and other agents in clinical specimens. However, to our knowledge, one report was published for detection and differentiation of bPIV-3 strains by amplification and sequencing of the HN gene studying Northern American viral strains and Russian isolates [22]. The aim of the present study was to develop a RT-PCR for detection of bPIV-3 and evaluate the test on three Brazilian bPIV-3 isolates.

MATERIALS AND METHODS

Viruses and cells

The bPIV-3 strain (National Veterinary Laboratories Service - USA), named SF-4, was kindly provided by Dr. Silvia Sardi from Federal University of Bahia (Salvador, BA, Brazil). After four passages in MDBK cells, the virus was used as positive control in all tests performed. As negative control uninfected MDBK cells were used.

Other five viral strains were included in the RT-PCR for specificity tests: human parainfluenza virus 1 and 2 (hPIV-1 and hPIV-2), canine distemper virus (CDV - Lederle strain); bovine herpesvirus type-1 (BHV-1, kindly supplied by Instituto Nacional de Tecnologia Agropecuária, Balcarce-Argentina, and equine influenza virus (H2N2) provided by the Laboratory of Virology-Oswaldo Cruz Foundation (FIOCRUZ/RJ-Brazil).

Three Brazilian isolates were analysed by RT-PCR in this study. Two isolates (DIO and PG1775) were originated from nasal secretions of bovines with clinical symptoms from different states of Brazil (Rio Grande do Sul and Goiás, respectively). Viruses were multiplied in Madin-Darby Bovine Kidney cells (MDBK, ATCC, CCL-22) following standard procedures [12,19]. The third brazilian isolate (CRIB) was obtained from an unintentionally contaminated culture of bovine cells, gently provided by Dr. Eduardo Flores (Laboratório de Virologia – Faculdade de Veterinária da Universidade Federal de Santa Maria – Santa Maria, RS).

Viral multiplication and titration

Viruses were cultured in MDBK cells in 25 cm² flasks in Eagle’s Minimal Essential Medium (E-MEM) supplemented with 8% fetal bovine serum (FBS) and 10 mg/L enrofloxacin. Cells were maintained in closed bottles at 37°C according to usual methods [3]. Titrations were carried out as described [5], adapted to 96 wells plates incubated in a 5% CO₂ atmosphere. Haemagglutination tests (HA) were performed as reported previously [6] with the reference strain SF-4 as positive control. Titrations were expressed as tissue culture 50% infectious doses (TCID₅₀).

Hemagglutination test (HA)

Hemagglutination test was performed as described previously [12]. Results were expressed as hemagglutinating units/50 µL (HAU/50 µL).

Direct fluorescence antibody test (DFAT)

Isolates were first tested by DFAT as described previously [12] with few modifications. Briefly, in 6 well plates containing sterile coverslips (24 x 24 mm) 200 µL of trypsinized (0.1% trypsin) MDBK cells and 800 µL of serum-free E-MEM were added. After incubation for 24 hours at 37°C in 5% CO₂, 50 µL of viral samples were inoculated at concentration of approximately 10⁵.⁰ TCID₅₀ and the plate was incubated for 72 hours to produce cytopathic effect.
Vaucher R.A., Simonetti A.B. & Roehe P.M. 2008. RT-PCR for detection of bovine parainfluenza virus type 3 (bPIV-3). Acta Scientiae Veterinariae. 36(3): 215-220.

Supernatants were discarded and cells were fixed in acetone for 10 minutes. Following 3 washes in PBS (116 mM NaCl, 20.8 mM NaH₂PO₄, 2.9 mM KH₂PO₄, pH 7.4), coverslips were incubated with 20 µL of fluorescein labelled anti-bPIV-3 monoclonal antibody diluted 1:5 in PBS, for 30 minutes at 37°C. Subsequently, coverslips were washed 3 times with PBS and observed under ultraviolet illumination. SF-4 reference strain was used as positive control for bPIV-3 and an anti-bovine herpesvirus monoclonal antibody as negative control.

**Selection and synthesis of oligonucleotides**

The sequences of the gene coding for hemagglutinin-neuraminidase (HN) protein of SF-4 strain, bPVI3 and hPVI-3 were obtained from GenBank database (accession numbers: AF178655, AF178654, D84095, NC001796, ABO12132, M21649 and Z11575). These sequences were aligned using the program CLUSTAL W - Version 3.0 [20]. The degenerate oligonucleotides were selected using the program VECTOR NTI - Version 4.0 (InforMax, Inc.) and synthesized (50 nmol) by Invitrogen. Data on primers selected for RT-PCR are shown in Table 1.

**DNA extraction from bovine herpesvirus type 1 (BHV-1)**

To test RT-PCR specificity, DNA extraction of BHV-1 was carried out with phenol-chloroform as described elsewhere [17].

**RNA isolation, cDNA synthesis and PCR amplification**

Total viral RNA was extracted from viral stocks with Trizol according to manufacturer’s instructions. cDNA synthesis of the genomic RNA and PCR amplification were performed with P3 and P6 primers (Table 1). cDNA concentration was estimated spectrophotometrically at 240 nm.

Viral RNA (3–5 µg) was reverse-transcribed using 200 units of M-MLV reverse transcriptase according to the manufacturer’s instructions and amplified by PCR using Taq DNA polymerase (5 units/µl) in PCR buffer (3 mM MgCl₂, 75 mM KCl, 50 mM Tris–HCl, pH 8.3, 1 mM each dNTP) in a final volume of 50 µl. Thermal cycling conditions were: 1 cycle at 96°C for 2 min; 35 cycles at 94°C for 1 min, 44°C for 1 min and 72°C for 1 min, followed by 1 final incubation at 72°C for 7 min. PCR products were examined by electro-phoresis in 1.5% agarose gels and visualized under a UV light after ethidium bromide staining. As controls, cDNA from SF-4 reference strain (positive) and cDNA from uninfected MDBK cells (negative) were used.

**Estimation of RT-PCR detection limit**

The detection limit of RT-PCR was evaluated by: 1) Titration of the reference strain SF-4 followed by individual RNA isolation, cDNA synthesis and PCR amplification; 2) Amplification of cDNA obtained from SF-4 strain using dilutions from 1:10 to 1:20000 with initial cDNA concentration of 475 ng/µL. Amplicons were visualized in 1.5% agarose gels.

**Estimation of RT-PCR analytical specificity**

The specificity of RT-PCR was analysed by RT-PCR amplification of SF-4 strain (positive control) and hPIV-1, hPIV-2, Lederle, BHV-1 and H2N2 strains, at the same conditions described earlier.

**RESULTS**

Titration results for DIO, PG1775 and CRIB isolates were 10^5.25 TCID₅₀, 10^6.0 TCID₅₀ and 10^4.75 TCID₅₀ respectively. When performed by HA titration results were 16 HAU/50 µL, 32 HAU/50 µL and 8 HAU/50 µL, respectively.

The RT-PCR described here, using primers representing part of bPIV-3 hemagglutinin-neuraminidase gene (HN), amplified a fragment of approximately 1009 bp with material extracted from SF-4

---

**Table 1. Primers representing part of bPIV-3 hemagglutinin-neuraminidase gene (HN) used for RT-PCR and sequencing.**

| Oligonucleotides | Nucleotide Location * | Sequence (5' → 3') * | References | Amplicon |
|------------------|----------------------|----------------------|------------|----------|
| P3 (+)           | 7088–7108            | ACAAGYRCTTCTYYAACAATTCAG | *Stokes et al., 1992 | 1009 bp  |
| P6 (-)           | 8076–8096            | GTAATTTYRCTYRTGCCAACTTG | *Storey et al., 1987 |         |

(*+) Direct and (-) reverse oligonucleotides. * Adapted oligonucleotides. Location of the nucleotides was based on the sequence of the complete gene of the bPIV-3, SF-4 strain. YR=A/G and YY=C/T.
To test the limit of detection by titration of the reference strain SF-4, the RT-PCR displayed amplification up to 140 TCID\textsubscript{50} (not shown). After serial dilutions of an initial SF-4 cDNA concentration of 475 ng/µL, visible amplification was observed up to the dilution 1:5000 corresponding to approximately 95 pg/µL of cDNA (Figure 2).

In RT-PCR specificity tests it was observed amplification of 1009 bp fragments corresponding to SF-4 reference strain and DIO, PG1775 and CRIB isolates, whereas no amplification was detected in cDNA from five different viruses (Figure 3).

**DISCUSSION**

In human clinical specimens the RT-PCR has been used as an alternative procedure for detection of PIV-3 using oligonucleotides based on highly conserved sequences of structural genes of hPIV-3 coding for the HN protein\cite{9,11,16}. Corne et al.\cite{8} achieved a sensitivity of 1 TCID\textsubscript{50} for a multiplex RT-PCR whereas Aguilar et al.\cite{1} reported 32 TCID\textsubscript{50}\textsuperscript{p} both for hPIV-3. In our study, sensitivity of RT-PCR corresponded to 140 TCID\textsubscript{50}\textsuperscript{p} and the detection limit...
was approximately 100 pg/µL after cDNA dilution. This apparent discrepancy is probably due to the different experimental conditions used by the authors including cell lineages and methods for sensitivity determination.

Within the conditions employed in this study the specificity of the RT-PCR was 100%, compared to that obtained for hPIV-3 by Fan et al. [11] and Corne et al. [8] who found 98% and 100%, respectively. Although including only five viral samples to check the specificity of our method, it is worthy to stress that two of them were human parainfluenza viruses (hPIV-1 and hPIV-2) which emphasizes the specificity of the oligonucleotides used, since there is a great similarity among PIV-3 strains [2].

Recently, amplification of gene HN fragment was utilized for identification and strain differentiation of bovine parainfluenza-3 virus in Northern American viral strains and Russian isolates [22]. This study revealed 2 genetic groups among the investigated virus strains and isolates: group 1 was made up of Northern American viral strains and of Russian isolates, whose primary structure has a high level of homology to the primary SF-4/32 strain structure; group 2 including the virus’ Russian isolates with high level of homology to Japanese strains’ sequences. With the improvement of the RT-PCR technique described here it could be possible to carry out similar studies in Brazil, comparing viral samples from different regions.

CONCLUSION

The RT-PCR presented in this study is adequate for detection of bPIV-3 from cell cultures and this technique can be improved for viral detection in aspirates or swabs from animals, contributing to diagnosis and epidemiological studies on bPIV-3.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. Sílvia Sardi and Eduardo Flores for supplying the SF-4 and CRIB isolates, respectively. Our gratitude to MSc Celir Straliotto from Laboratório Central do Rio Grande do Sul (LACEN/RS-Brazil) for supplying the human parainfluenza virus samples. P.M. Roehe is a CNPq research fellow. R.A.Vaucher was recipient of MSc. scholarship from CAPES. The authors wish to thank the Brazilian Institutes CNPq and CAPES for financial support.

REFERENCES

1 Aguilar J.C., Pérez-Breña M.P., Garcia M.L., Cruz N., Erdman D.D. & Echevarria J.E. 2000. Detection and identification of human parainfluenza viruses 1,2,3 and 4 in clinical samples of pediatric patients by multiplex reverse transcription-PCR. Journal of Clinical Microbiology. 38: 1191-1195.

2 Bailly J.E., McMaulliff J.M., Skiadopoulos M.H., Collins P.L. & Murphy B.R. 2000. Sequence determination and molecular analyses of two strains of bovine parainfluenza virus type 3 that are attenuated for primates. Virus Genes. 20: 173-182.

3 Butler M. & Dawson M. 1992. Cell culture. London: Blackwell Scientific, pp.247-263.

4 Canchola J.G., Chanock R.M., Jeffries B.C., Christmas E.E., Kim H.W., Vargosko A.J. & Parrott R.H. 1965. Recovery and identification of human myxoviruses. Bacteriological Reviews. 29: 496-503.

5 Carbrey E.A., Stewart W. & Kresse J.L. 1971. Recommended standard laboratory techniques for diagnosing infectious bovine rhinotracheitis, bovine diarrhea virus and shipping fever (Parainfluenza 3). Proceedings of U. S. A. Health Association. 75: 629-648.

6 Chanock R.M. & Johnson M.D. 1989. Parainfluenza viruses. In: Schmidt N.J., Emmons R.W.(Ed). Diagnostic Procedures for Viral and Rickettsial Diseases. 6th edn. New York: American Public Health Association Publications, pp.470-483.

7 Collins P.L., Chanock R.M. & McChinstosh K. 1996. Parainfluenza virus. In.: Fields B.N.(Ed). Fields Virology. 3rd edn. Philadelphia: Lippincott-Raven, v.1, pp. 1205-1223.

8 Corne J.M., Green S., Sanderson G., Caul E.O. & Johnston S.L. 1999. A multiplex RT-PCR for the detection of parainfluenza viruses 1-3 in clinical samples. Journal of Virological Methods. 82: 9-18.

9 Echevarria J.E., Erdman D.D., Meissner H.C. & Anderson L. 2000. Rapid molecular epidemiologic studies of human parainfluenza viruses based on detect sequencing of amplified DNA from a multiplex RT-PCR assay. Journal of Virological Methods. 88:105-109.
10 Fan J. & Henrickson K.J. 1996. Rapid diagnosis of human parainfluenza virus type 1 infection by quantitative reverse transcription-PCR-enzyme hybridization assay. *Journal of Clinical Microbiology*. 34: 1914-1917.

11 Fan J., Henrickson K.J. & Savatski L.L. 1998. Rapid Simultaneous Diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza Virus Types 1, 2 and 3 by Multiplex Quantitative Reverse Transcription – Polymerase Chain Reaction – Enzyme Hybridization Assay (Hexaplex). *Clinical Infectious Diseases* 26: 1397–1402.

12 Gonçalves D.A., Spilki F.R., Chiminazzo C., Oliveira M.A., Franco A.C. & Roehe P.M. 2003. Isolamento do vírus parainfluenza bovino tipo 3 no Rio Grande do Sul, Brasil. *Ciência Rural, Santa Maria*. 33: 953-956.

13 Henrickson K.J. & Savatski L.L. 1997. Antigenic structure, function, and evolution of the hemagglutinin-neuraminidase protein of human parainfluenza virus type 1. *Journal of Infectious Diseases*. 176: 867-875.

14 Herrmann E.C. & Hable K.A. 1970. Experiences in laboratory diagnosis of parainfluenza viruses in routine medical practice. *Mayo Clinic Proceedings*. 45: 177-188.

15 Kapil S. & Basaraba R.J. 1997. Infectious bovine rhinotracheitis, parainfluenza-3, and respiratory coronavirus. *Veterinary Clinics of North America and Food Animal Practice*. 13: 455-469.

16 Karron R.A., Froehlich J.L., Bobo L., Belshe R.B. & Yolkken R.H. 1994. Rapid detection of parainfluenza type 3 virus RNA in respiratory specimens: use of reverse transcription–PCR-enzyme immunoassay. *Journal of Clinical Microbiology*. 32: 484-488.

17 Sambrook J., Fritsh E.F. & Maniatis T. 1989. Molecular Cloning: a Laboratory Manual. 2nd edn. New York: Cold Spring Harbor Laboratory. v.3., pp. E3-E7.

18 Sattar S.A., Bohl E.H. & Senturk M. 1965. Viral causes of bovine abortion in Ohio. *Journal of American Medical Association*. 147: 1207-1210.

19 Silva A.D., Gonçalves D.A., Spilki F.R., Chiminazzo C., Oliveira M.A., Franco A.C. & Roehe P.M. 2002. Isolamento do vírus parainfluenza tipo 3 em aborto bovino no Estado de Goiás (GO). In: *Congresso Brasileiro de Medicina Veterinária* (Gramado, RS), pp.29.

20 Thompson J.D., Higgins D.G. & Gibson T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*. 22: 4673-4680.

21 Waner J.L. 1999. Parainfluenza Viruses. In.: Murray P.R. (Ed.). *Manual of Clinical Microbiology*. 7th edn. New York American Society for Microbiology, pp. 1157-1197.

22 Vecherov A.E., Aiatot P.K., Timina A.M. & Lisitsin V.V. 2003. Detection and differentiation of the bovine parainfluenza-3 virus strains studied by amplification and sequencing of the HN gene. *Vopr Virusol*. 48:46-9. (russian)