We describe a reverse transcription-polymerase chain reaction (RT-PCR) and a nested-PCR for diagnosis of Piry, Carajás, Cocal, and Alagoas vesiculoviruses from Brazil. The RNA extracts of viral and clinical samples were submitted to a RT-PCR using Vesiculovirus G primers that amplify part of the glycoprotein gene. The RT-PCR produced amplicons of expected size, 290 base pair, for the four studied viruses. The RT-PCR showed a high sensitivity being 151.3 times (2.18 log) more sensitive for the detection of Piry virus than the classical procedure for virus detection in tissue culture based on the viral cytopathic effect. Amplicons had nucleotides sequenced and were aligned in order to select internal primers for a nested-PCR to confirm the origin of Piry, Carajás, Cocal, and Alagoas Vesiculovirus. Ten blood and tarsal pad epithelial samples of infected Guinea-pigs had Vesiculovirus genome amplified by RT-nested-PCR.

Key words: Vesiculovirus - vesicular stomatitis virus - reverse transcription-polymerase chain reaction - diagnosis - nested-PCR - Brazil
isolate are performed (Tavares Neto 1992). Monoclonal antibodies for use in immunofluorescent and immunoenzymatic tests have been also used (Vernon et al. 1985). The detection of VS virus antigens in clinical samples using enzyme-immunoassay was successfully tested for New Jersey and Indiana-1 viruses (Fernandez 1988).

The RT-PCR was tested for diagnosis of New Jersey virus in clinical samples of sick animals and the nucleotide sequence of the amplicons allowed a phylogenetic study of these viruses (Rodriguez et al. 1993). A RT-PCR was also used in pigs for elucidating the differential diagnosis of VS, FMD, Exanema Vesicular (EV) and Swine Vesicular Disease (SVD) (Nunez et al. 1998). In this paper, we show the development of a RT-PCR and a nested-PCR for diagnosis of four Brazilian vesiculoviruses (Carajás, Piry, Indiana 2-Cocal/Registro and Indiana 3-Alagoas).

MATERIALS AND METHODS

The viruses - Four Brazilian vesiculoviruses Piry, Carajás, Cocal, and Alagoas were tested in the study. Piry virus Bean 41191 and Carajás virus BeAn 411459 were kindly supplied by Dr Pedro Fernandes Vasconcelos from the Evandro Chagas Institute in Belém, Brazil. Alagoas virus Bn/64 was kindly supplied by Dr Cláudio Andrade from the School of Veterinarian of the Federal Fluminense University, Niterói, Brazil and Cocal virus Registro/SP was kindly supplied by Dr Aramis Augusto Pinto from the School of Agrarian and Veterinarian Sciences of the University of State of São Paulo, Jaboticabal, Brazil. Suspensions of the studied viruses were obtained from macerated brains of infected baby-mice and from supernatant fluid of C6/36 (Aedes albopictus) tissue culture, after confirmation by immunofluorescent test using mouse immune ascitic fluids (Figueiredo 1990). Clinical materials including 5 blood samples and 5 skin tarsal pad epithelial samples of guinea pigs infected with Indiana 3-Alagoas virus were also analyzed. The RNA was extracted from the virus suspensions and clinical samples using the Qiamp DNA Blood Mini Kit (Qiagen, US).

RT-PCR - Primers for use in the RT-PCR were selected from high homology regions of the G gene of Piry (Genbank D26175), New Jersey (Genbank AF170602), Indiana 1 (Genbank X03633), Indiana 2-Cocal (Genbank AF045556) and Chandipur (Genbank J04350) viruses after align their nucleotide sequences using the software DNAse (Hitachi, Japan, 1995). Thus, Vesiculovirus G complementary (5' - CAGATGTAT TGACCCAATA-3') and sense (5' - CCAACCCGATGAA TTGTTGAC-3') primers were selected.

The RT and the PCR were carried out in the same tube. The reaction mixture included 5 µl RNA extracts, 0.3 mM Vesiculovirus G reverse primer, 0.1 mM dNTPs, 10 U de inibidor de RNAase (Phamacia, US), 10 U reverse transcriptase (Pharmacia, US), and 2.1 µl of the corresponding 5 x buffer in a final volume of 13 ml. The reaction mixture was incubated at 41°C for 1h. The PCR mixture included 1U of Taq DNA polymerase (Phamacia, US), 4 µl of the corresponding 10 x buffer, 0.3 µM of sense and reverse primers in a final volume of 50 µl completed with DEPC treated water. Forty cycles were carried out at 93°C for 90 s, 50°C for 2 min and 72°C for 4 min (Rodriguez et al. 1993).

Selection of primers for the nested-PCR - The amplicons obtained by the RT-PCR were purified using the PCR purification Kit (Qiagen, US) and submitted to the Thermo Sequenase CYS.5 terminator kit (Amersham, England) also including the Vesiculovirus G complementary primer. Reaction products were submitted to eletrophoresis in a 6% polyacrilamide gel, inside of the Personal seq 4 x 4 (Pharmacia, US) machine following manufacturer’s recommendations. These amplicon nucleotide sequences of Piry, Indiana 3-Alagoas and Carajás viruses were aligned in order to confirm their viral origin based on the high homology with Vesiculovirus previously known sequences. The amplicon nucleotide sequences of Piry, Indiana 3-Alagoas, and Carajás viruses were also aligned in order to select internal primers from high homology regions for a nested-PCR. Thus, Piry complementary (5' - CATCTGAGACCGACAACATC3') and sense (5' - TCACTTGGACAAACATGCCC3') primers were selected.

Nested-PCR - The nested-PCR was used for confirmation of the Vesiculovirus origin of amplicons obtained by RT-PCR. The reaction mixture contained 1 µl of the RT-PCR amplicon, 1U of Taq DNA polymerase (Pharmacia, US), 5 µl of buffer 10 x, 0.3 mM of Piry primers in a total volume of 50 µl. Thirty five cycles were performed at 93°C for 90 s, 50°C for 2 min and 72°C for 2 min.

Amplicons obtained by RT-PCR and nested-PCR were submitted to an eletrophoresis in 1.7% agarose gel stained with ethidium bromide, visualized under UV light. These sizes of the amplicons were determined by comparison with a 100 bp DNA ladder (Promega, US).

Sensitivity determination of the RT-PCR - The sensitivity of the RT-PCR was determined by comparing the Vesiculovirus amplicon detection with a method in Vero E6 (green monkey kidney) cell cultures based on the detection of cytopathic effect 24 h after viral infection. Ten-fold dilutions of the Piry virus seed were used to infect 8 microplate wells (50 ml/well) containing Vero E-6 cell monolayers. The TCID50/ml titer of Piry virus was measured by the Reed-Muench method (Reed & Muench 1938). The tenfold dilutions of Piry virus had the RNA extracted (140 µl/sample), in quadruplicate, and these RNA extracts were submitted to RT-PCR.

RESULTS

Amplicons having ~ 290 bp, the expected size, were obtained by RT-PCR for Piry, Indiana 3-Alagoas, Carajás, and Indiana 2-Cocal viruses, as shown in Fig. 1A. These amplicons were sequenced and aligned showing for Piry virus a 97% homology with the G genus nucleotide sequence of the same virus (Gen Bank D26175). Carajás and Indiana 3-Alagoas viruses were not previously sequenced and showed 85.2 and 80.6% homology rates, respectively, with the Piry virus amplicon. These high homologies with Piry virus confirm the viral origin of the amplicons. The amplicon nucleotide sequences of Piry, Indiana 3-Alagoas, and Carajás viruses were also aligned in order to select
the Piry internal primers in high homology regions for the nested-PCR. The nested-PCR with Piry primers produced amplicons having the expected size, ~130 bp, as shown in Fig. 1B.

The genome of Indiana 3-Alagoas virus was detected in the 10 clinical samples of guinea pig (blood and tarsal pad epithelial lesions tissue), and amplicons were also obtained by nested-PCR confirming the result of the RT-PCR, as shown in Fig. 2.

The titer of the Piry virus seed in Vero E-6 cells was $10^{7.82}$ TCID50/ml. Amplicons were obtained by RT-PCR until the $10^{-10}$ dilution of the Piry virus seed, as shown in Fig. 3.

**DISCUSSION**

The RT-PCR and the nested-PCR have been used for diagnosis of vesiculovirus infections (Bilsel PA, Nichol ST 1990, Rodriguez et al. 1993, Nunez et al. 1998). In the present study we used primers (VSVG) that anneal in the G gene of Brazilian Vesiculovirus (Piry, Indiana 3-Alagoas, and Carajás) in a RT-PCR. G is a transmembrane N-glycosil protein present in the spikes of the viral surface. Thus G is the viral linker to cell receptors and a fundamental piece for viral endocytosis and envelope fusion to cell endosomes. G is also an important Vesiculovirus antigen that induces neutralizing antibodies by the infected animal (Robert & Rose 1996). The G gene of Vesiculovirus has a variable nucleotide sequence but as is it was possible to find high homology regions in the G genus for primer selection.

The use of primers that anneal in the G gene for diagnosis has not been previously reported. However, Vesiculovirus G primers allowed the amplification in the RT-PCR of the Brazilian Piry, Carajás, Indiana 2-Cocal, and Indiana 3-Alagoas viruses. The amplified products were sequenced and had the viral origin confirmed. The previously unknown sequences of Indiana 3-Alagoas and Carajás virus were registered in the GenBank (AY335184 and AY335185, respectively).

**Fig. 1 - A:** agarose gel stained with ethidium bromide showing 290 bp amplicons of a RT-PCR with *Vesiculovirus* G primers testing Piry (1), Carajás (2), Cocal (3) and Alagoas (5) viruses; B: it shows 130 bp amplicons obtained in a nested-PCR with Piry primers testing Piry (1), Carajás (2), Indiana 3-Alagoas (3) and Indiana 2-Cocal (4) viruses. Water was used as negative control (4) and M is a DNA 100bp ladder.

**Fig. 2 - A:** agarose gel stained with ethidium bromide of RT-PCR with *Vesiculovirus* G primers testing clinical samples of Guinea pig infected with Indiana 3-Alagoas virus. Lines 1 to 5 include RNA extracts of epithelial tissues from the tarsal pad of 5 animals collected 3 days after virus inoculation and lines 5 to 10 include RNA extracts of blood samples of the same animals. B: agarose gel stained with ethidium bromide of nested-PCR with Piry internal primers testing clinical samples of Guinea pig infected with Indiana 3-Alagoas virus. Lines 1 to 5 include RNA extracts of epithelial tissues from the tarsal pad of 5 animals collected 3 days after virus inoculation and lines 5 to 10 include RNA extracts of blood samples of the same animals. M is a 100 bp DNA ladder.

**Fig. 3:** agarose gel stained with ethidium bromide of the RT-PCR with *Vesiculovirus* G primers testing ten fold dilutions of an Alagoas virus sample. The test was able to amplify the virus genome (290 bp amplicons) until the $10^{-10}$ dilution. M is a 100 bp DNA ladder.
The diagnosis of Piry virus infection in Vero E6 cells using the RT-PCR was 151 times more sensitive (2.18 log) than that based on the detection of cytopathic effect. The RT-PCR was able to detect Piry virus when the sample was diluted 10 billion times (10^{-10}). It indicates that this RT-PCR for Piry virus is a highly sensitive method.

The nested-PCR was developed for confirmation of the RT-PCR results. The selected internal primers were able to amplify the genome of the four Brazilian Vesiculovirus, Piry, Indiana 3-Alagoas, Indiana 2-Cocal and Carajás.

The genome of Indiana 3-Alagoas virus was amplified from the clinical samples of infected guinea pig, detecting viremia and virus presence in the animal tarsal pad epithelial samples. Therefore this RT-nested-PCR could be used for diagnosis of VS acute infection in domestic animals since on several occasions serologic survey and virus isolation, revealed the presence of the SV virus in horse and cattle in Brazil (Federer et al. 1966, Andrade et al. 1974). Carajás and Marabá virus and specially Piry virus are also common causatives of human infection in Brazil. Serologic surveys carried out in Northeastern and South-eastern inland towns in Brazil showed that 16 and 14.3% of the participants had neutralizing antibodies to Piry virus, respectively (Figueiredo et al. 1985). Knowing that Piry infections are common and that Piry fever is probably under reported because only 3 human cases were described, this RT-PCR could be used for diagnosis of infections by this virus in Brazilian patients having acute febrile illness. Virus identification could be done by nucleotide sequencing of the amplicons.

REFERENCES


