Multiplex nested PCR for Brazilian Alphavirus diagnosis

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Summary A reverse-transcriptase PCR (RT−PCR) and a multiplex nested PCR were developed for the rapid detection and identification of 14 Brazilian alphaviruses. Using Alphavirus genus-specific primers in a RT−PCR, we obtained amplified products of 434bp. Species-specific primers were selected and simultaneously tested in a multiplex nested PCR. The nested PCR increased the test sensitivity 1000-fold and was capable of identifying Brazilian Alphavirus showing the expected bands with diagnostic sizes for Venezuelan (400bp), Eastern (124bp), and Western (208bp) equine encephalitis, Aura (86bp), and Mayaro (270bp) viruses. This strategy for diagnosis is fast, sensitive, specific and it can be used as a reliable alternative for routine Brazilian Alphavirus diagnosis. © 2004 Royal Society of Tropical Medicine and Hygiene. Published by Elsevier Ltd. All rights reserved.

1. Introduction
The Alphavirus genus of the Togaviridae family includes 27 species that are classified in seven serogroups (Calisher and Karabatsos, 1988). The Alphavirus genome is composed of single-stranded, positive-polarity RNA. This RNA encodes both the non-structural (nsP1−4) and the structural proteins (capsid, E3, E2, 6K, and E1) (Strauss and Strauss, 1994). Alphaviruses are the causative agents of a wide range of diseases in humans and animals in Brazil (Figueiredo, 1994). Mayaro virus (MAY) causes a febrile illness and arthralgia syndrome in humans in the Amazon region, while Eastern, Venezuelan, and Western equine encephalitis (EEE, VEE, and WEE) viruses regularly cause encephalitis in humans and horses in the Americas (Strauss and Strauss, 1994; Vasconcelos et al., 1998). Most human Alphavirus infections produce non-specific clinical symptoms that are similar to those caused by other arboviruses such as Oropouche or dengue. Thus, an early and specific diagnosis of alphaviruses is important for patient management and adoption of efficient preventive measures. In addition, due to the high frequency of travel to endemic regions, the wide variety of reservoirs and the potential for vector spread, a feasible and inexpensive diagnostic method is needed for the surveillance of these alphaviruses.
Diagnosis of alphavirus infection has been done by virus isolation or serological testing. However, these methods are relatively time consuming and considerably less sensitive than newer molecular biology methods, such as PCR (Erlich et al., 1991). PCR assays have been used for the detection of some alphaviruses such as chikungunya, EEE, WEE, and Mayaro (Hasebe et al., 2002; Linssen et al., 2000; Monroy et al., 1996; Talarmin et al., 1998; Vodkin et al., 1993, 1994).

In the present study, we used a genus-specific reverse-transcriptase PCR (RT—PCR) followed by a multiplex nested PCR for detection and identification of Brazilian alphaviruses. This strategy associates the high sensitivity of the nested PCR assay with the speed, facility, and the low cost of multiplex systems. These advantages are important in both clinical diagnosis and surveillance programmes of a wide variety of viruses.

2. Materials and methods

2.1. Virus and RNA extraction

Brazilian alphaviruses used in this study are listed in Table 1. Flavivirus yellow fever 17D (YF), Orthobunyavirus Oropouche BeAn19991 (ORO), Phlebovirus Bujaru BeAn47693 (BUJ), and uninfected mouse brain tissue extracts were used as negative controls for testing the specificity of the technique.

The viruses were propagated by intracerebral inoculation of suckling mice. Viral RNAs were extracted from 140 μl of a 1 in 20 suspension of mouse brain tissue, macerated in PBS, using the QIAamp Viral RNA Mini Kit (QIAGEN Inc., USA), according to the manufacturer’s instructions. This yielded 60 μl of final volume.

2.2. Reverse-transcriptase PCR

For amplification of alphaviruses, we used the genus-specific primers M2W and cM3W described by Pfeffer et al. (1997). These primers anneal to the nsP1 gene of Alphavirus producing amplicons of approximately 434 nucleotides. The reverse transcriptase mixture contained 50 μM of each dNTP, 36 units of RNase inhibitor, 5 μM of cM3W primer, 50 mM dithiothreitol, 200 units of Superscript reverse transcriptase (Invitrogen, USA) in the manufacturer’s reaction buffer, and 5 μl of RNA sample in a final volume of 20 μl. The mixture was incubated at 42°C for 50 min and then at 70°C for 15 min in order to inactivate the reverse transcriptase. The PCR reaction mixture contained 5 μl of reverse transcriptase mixture, 50 μM of each dNTP, 1 μM of M2W primer, 2.5 unit of Platinum Taq DNA polymerase (Invitrogen, USA), in the manufacturer’s reaction buffer, and 2 mM MgCl₂ in a final volume of 50 μl. The mixture was submitted to 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min. A final extension step was at 72°C for 10 min. Ten microlitres of the PCR products were electrophoresed on a 2% (w/v) agarose gel, stained with ethidium bromide and visualized under UV light.

2.3. Nucleotide sequencing

Five Alphavirus species belonging to different antigenic complexes were sequenced: VEE BeAr40403 belonging to the VEE complex, EEE SPAn14723 belonging to the EEE complex, WEE Rio1257 and Aura BeAr10315 belonging to the WEE complex, and MAY BeAr20290 belonging to the Semliki Forest complex. The amplicons of 434 bp were recovered from the gel and purified by using the QIAquick Gel Extraction Kit (QIAGEN, USA) as recommended by the manufacturer. Sequencing reactions were performed with the Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, USA), using M2W primer and analysed by a SEQ 4 × 4 Personal Sequencing System (Amersham Pharmacia Biotech, USA).

Table 1: Brazilian alphaviruses used in the study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
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<tr>
<td>Venezuelan equine encephalitis</td>
<td>BeAr 40403*</td>
</tr>
<tr>
<td>(VEE)</td>
<td>BeAn 10967</td>
</tr>
<tr>
<td></td>
<td>MUC BeAn 8</td>
</tr>
<tr>
<td></td>
<td>MUC BeAn 19991</td>
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<tr>
<td>Eastern equine encephalitis</td>
<td>SPAn 14723*</td>
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<tr>
<td>(EEE)</td>
<td>BeAn 7526</td>
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<tr>
<td></td>
<td>BeAr 126450</td>
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<td></td>
<td>BeAn 416361</td>
</tr>
<tr>
<td>Western equine encephalitis</td>
<td>Rio 1257*</td>
</tr>
<tr>
<td>(WEE)</td>
<td>BeAn 70100</td>
</tr>
<tr>
<td>Aura</td>
<td>BeAr 10315*</td>
</tr>
<tr>
<td>Mayaro (MAY)</td>
<td>BeAr 20290*</td>
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</table>

* Virus whose nucleotide sequence was used for the selection of species-specific primers.
2.4. Selection of primers for the multiplex nested PCR

Nucleotide sequences obtained from the five Alphavirus amplicons, including other published sequences such as VEE 78V 3531, PIX BeAr 35645, and MUC BeAn8 from VEE complex (Kinney et al., 1998), were aligned using the Clustal W software (Informax, USA) and low homology regions were selected as specific inner primers for VEE, EEE, Aura, and MAY viruses.

2.5. Multiplex nested PCR

A multiplex nested PCR reaction was carried out in a final volume of 50 μl and contained 1 μl (1:50 dilution) of the first amplification reaction, 50 μl each dNTP, 1 μl of each primer (cM3W, nVEE, nEEE, nWEE, nAURA, nMAY), 2.5 units of Platinum Taq DNA polymerase (Invitrogen, USA) in the manufacturer’s reaction buffer adjusted to 2 mM MgCl₂. The mixture was then subjected to 25 cycles of 94 °C for 1 min; 53 °C for 1 min; 72 °C for 2 min. A final extension step was carried out at 72 °C for 10 min. Analysis of the amplicons was performed as described above.

2.6. Determination of sensitivity

The sensitivity of the RT–PCR and of the multiplex nested PCR was determined using a stock seed of MAY (BeAr 20290) having 10⁻⁵ TCID₅₀/ml. Serial 10-fold dilutions of this virus were prepared in phosphate-buffered saline (PBS) or in human serum, to mimic viraemic serum samples. The RNA was extracted from each virus dilution and submitted to RT–PCR and multiplex nested PCR as described above.
3. Results

3.1. Reverse transcriptase PCR and multiplex nested PCR

The RT—PCR, developed to detect Brazilian alphaviruses using the genus-specific primers reported by Pfeffer et al. (1997), was able to amplify the genomes of all the viruses tested (Table 1), producing amplicons with the expected size (∼434bp).

The multiplex nested PCR was developed to identify Brazilian Alphavirus species by using virus-specific primers. The nsP1 gene nucleotide sequence of the five viruses shown in Table 1 were determined and submitted to GenBank (EEE, VEE, WEE, MAY, and Aura accession numbers AY348558–AY348562). The identities of these virus sequences with those of viruses of the same species from GenBank exceeded 94% except for EEE virus (89%) that was compared with a non-Brazilian EEE virus. These sequences and others, previously published, were aligned and five virus-specific primers were selected as shown in Figure 1 and Table 2. Using these primers in a multiplex nested PCR, amplicons having distinct sizes were observed for VEE (400bp), EEE (124bp), WEE (208bp), Aura (86bp), and MAY (270bp), as shown in Figure 2. There was no cross-reactivity between specific primers and heterologous viruses and no amplicon was obtained for YF, ORO, BUJ, or uninfected mouse brain tissue extract (Figure 2), indicating that the assay was virus species-specific. Serial 10-fold dilutions of a MAY stock (10^{−3.5} TCID_{50}/ml) were prepared in either PBS or human serum. RNA extracts of each one of these virus dilutions were submitted to RT—PCR and multiplex nested PCR. Amplicons having the expected sizes were visible at 10^{−3} and 10^{−6} dilutions, which corresponds to detection limits of 10^{−3.5} TCID_{50}/ml and 10^{−6.5} TCID_{50}/ml for RT—PCR and multiple nested PCR, respectively. The presence of human serum did not reduce the sensitivity of the test (Figure 3).

<table>
<thead>
<tr>
<th>Table 2 Species-specific primers used in the multiplex nested PCR</th>
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<tr>
<td>Primer (sense)</td>
</tr>
<tr>
<td>nVEE</td>
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<tr>
<td>nEEE</td>
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<tr>
<td>nWEE</td>
</tr>
<tr>
<td>nAURA</td>
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<tr>
<td>nMAY</td>
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</table>

* With cM3W primer.

Figure 2 Agarose gel electrophoresis of products from reverse transcriptase PCR and multiplex nested PCR of 14 alphaviruses. Key: MW, molecular weight marker (DNA ladder = 100 or 50bp); lane 1, VEE BeAr 40403; lane 2, VEE 78V 3531; lane 3, MUC BeAn 8; lane 4, MUC BeAn 10967; lane 5, MUC BeAn 484223; lane 6, PIX BeAr 35645; lane 7, EEE 89 14723; lane 8, EEE BeAn 7252; lane 9, EEE BeAn 126550; lane 10, EEE An416361; lane 11, WEE BeAn 1257; lane 12, WEE An 70100; lane 13, Aura; lane 14, Mayaro; lanes 15–19, negative controls (15, RNA extract from uninfected mouse brain tissue; 16, yellow fever; 17, Oropouche; 18, Bujaru; 19, water).
4. Discussion

Periodic outbreaks or sporadic Alphavirus infection cases of MAY, EEE, WEE, and VEE have been reported in humans and horses in South America, including different regions of Brazil (Figueiredo, 1994). Because alphaviruses have a wide geographic distribution, produce severe diseases without specific clinical symptoms and have an important social and economic impact in these regions, specific and fast diagnostic methods should be useful tools for surveillance of these diseases and control of Alphavirus dissemination. Therefore, we developed a strategy for detection of alphaviruses using RT-PCR, followed by a multiplex nested PCR for specific virus identification.

The RT-PCR was based on the study of Pfeffer et al. (1997) that used genus-specific primers for Alphavirus detection, modified to use a lower concentration of reagents and a smaller volume of reaction in order to reduce costs. The sensitivity and specificity observed with our test was similar to that reported for the original protocol.

We selected inner primers for identification of the most common Brazilian alphaviruses using a multiplex nested PCR. The assay was able to amplify the genome of all alphaviruses tested. The amplicons, being of different sizes, allowed a simple and specific diagnosis of each virus.

Pfeffer et al. (1997) and Sanchéz-Seco et al. (2001) developed a nested PCR using genus-specific primers for alphaviruses. Other authors used species-specific primers for Ockelbo (Hör ling et al., 1993) and Ross river (Selin er et al., 1994) viruses. In these studies the nested PCR was only used to confirm and to increase the sensitivity of the PCR. The multiplex nested PCR described in this study has been used for both virus identification at species level and improvement of the sensitivity. The multiplex nested PCR was 1000-fold more sensitive than the RT-PCR and this sensitivity was not changed when the virus RNA was extracted from human serum, thus making this test suitable for use in viraemic individuals. To overcome the risk of contamination when using the nested PCR system, this study was developed by following common sense guidelines: separation of pre- and post-PCR work areas and use of dedicated pipettes.

In short, the strategy of diagnosis using RT-PCR for detection and multiplex nested PCR for virus identification was fast, sensitive, specific and could be used as a reliable alternative for Brazilian Al phavirus routine diagnosis and surveillance.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.
Acknowledgements

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