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Development of a DNA microarray for simultaneous detection and genotyping of lyssaviruses

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ABSTRACT

The lyssavirus genus of the *Rhabdoviridae* family of viruses includes 7 genotypes and several non-assigned isolates. The source of lyssavirus infections is diverse with numerous reservoirs in a wide geographical area. In many parts of the world reservoir hosts can potentially be carrying one of several lyssavirus strains and possibly new divergent isolates await discovery. Accordingly, generic detection methods are required to be able to detect and discriminate all lyssaviruses and identify new divergent isolates. Here we have allied a sequence-independent amplification method to microarray to enable simultaneous detection and identification of all lyssavirus genotypes. To do so, lyssavirus RNA was converted to cDNA and amplified in a random PCR, labelled and hybridized to probes on the microarray chip before being statistically analysed. The probes were to a 405 bp region of the relatively conserved N gene. Here we demonstrate a microarray capable of detecting each of the seven lyssavirus genotypes. The random amplification of lyssavirus RNA and the numerous oligonucleotide probes on the microarray chip also offer the potential to detect novel lyssaviruses.

1. Introduction

Rabies is a zoonotic disease of the central nervous system that is invariably fatal in humans. The rabies virus (genotype 1) is the type virus of the lyssavirus genus within the family *Rhabdoviridae*. The genus also includes six other viruses or genotypes namely: genotype 2, Lagos bat virus; genotype 3, Mokola virus; genotype 4, Duvenhage virus; genotype 5, European bat lyssavirus type 1; genotype 6, European bat lyssavirus type 2; and genotype 7 Australian bat lyssavirus (Bourhy et al., 1993). A further four non-assigned viruses from central Asia, Aravan, Khujand, Irkut, and West Caucasian bat virus are classified within the genus (Botvinkin et al., 2003; Kuzmin et al., 2003, 2005). All members of the genus cause rabies and all, with the exception of Lagos bat virus and the non-assigned viruses have caused rabies in humans.

All lyssaviruses have a 12 kb non-segmented, negative strand, RNA genome that encodes 4 structural proteins in the order: nucleoprotein (N), phosphoprotein (P), matrix protein (M) and the glycoprotein (G). A fifth protein, the RNA-dependent RNA polymerase or large protein (L) is also encoded (Tordo et al., 1986, 1988). The genomic region coding for the amino terminus of the N, which is relatively conserved among genotypes, has been used extensively for phylogenetic study of lyssaviruses (Kissi et al., 1995; Bourhy et al., 1999).

Genotype 1 is found worldwide except for a few countries that are declared rabies-free. However, even in these countries occasional cases of human rabies arise from contact with rabid animals in endemic areas (Johnson et al., 2002; Smith et al., 2003) or from contact with bats (Fooks et al., 2003). Genotypes 2–4 are widely distributed throughout Africa (Nel and Rupprecht, 2007) whereas genotypes 5 and 6 are distributed throughout Europe (Schneider and Cox, 1994; Davis et al., 2005). Genotype 7 has only been isolated within Australia although serology suggests that it may have a wider distribution (Arguin et al., 2002). Lyssavirus genotypes usually infect specific reservoir hosts; however, they can occasionally infect species other then their reservoir (Müller et al., 2004). Rabies virus is associated with carnivore species (dog, fox or wolf) or with a large number of bat species in North and South America (Finnegan et al., 2002). The reservoir host of Mokola virus is unknown. Bats are known to harbour the remaining viruses of the lyssavirus genus (Botvinkin et al., 2003; Kuzmin et al., 2003, 2005). The EBLVs are predominantly found in Serotine (*Eptesicus serotinus*) bats (EBLV-1) or Daubentons (*Myotis daubentonii*) bats (EBLV-2) (Fooks et al., 2003). EBLV infected bats have been reported in several European countries, from Russia to Spain. The bat species of the remaining lyssaviruses also have a broad geographical distri-

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bution covering extensive areas of Europe and Africa (Davis et al., 2005; Nel and Rupprecht, 2007). Consequently, in cases of rabies, the causative agent may not necessarily be the rabies virus. Furthermore, imported cases of lyssaviruses into disease-free countries, the occasional dislocation of bats (i.e. by illegal trade) and emergence of new lyssaviruses necessitate the use of diagnostics that detect all genotypes and have the potential to identify novel divergent strains. The direct fluorescent antibody test is the WHO recommended gold standard and the test of choice for most laboratories. This assay detects lyssavirus antigen in solid tissue samples and has adequate levels of sensitivity and specificity (Dean, 1996). However, the test utilizes brain biopsies, which cannot be acquired in case of live subjects, is ineffective for decomposed samples and non-transferable to saliva and cerebrospinal fluid (Crepin et al., 1998). Furthermore, the test does not discriminate between lyssavirus genotypes. The polymerase chain reaction (PCR) has been employed as a sensitive and quick method for detection of lyssaviruses in recent years (Black et al., 2002; Vazquez-Moron et al., 2006; Wakeley et al., 2005). While PCR is a rapid and accurate test for virus detection, genotyping requires a separate PCR for each genotype. A further problem is the emergence of new lyssaviruses in which sequence divergence might impair binding of PCR primers. The requirement for genomic sequencing in order to achieve strain identification for generic PCR assays also increases the time and cost with which a result can be obtained.

Sequence-independent PCR, when allied to microarray has the potential to simultaneously detect and identify pathogenic viruses, both defined or unknown, in diseased tissue. Microarray also can provide a platform for multiplexed differential diagnosis of infectious diseases. The number of probes per microarray far exceeds those of any other known detection technology. Furthermore, probes with lengths of 60–70 nucleotides permit hybridization even in the absence of a complete homology between probe and target. The application of microarray for the detection of viruses particularly in clinical samples is not widely used at present. However, growing evidence demonstrates its applicability for detection and typing of human, animal and plant viruses (Boonham et al., 2007; Dankbar et al., 2007; Quan et al., 2007; Jack et al., 2009). The platform has contributed to identification of several viruses in diseased animals with unknown aetiology in recent years (Kistler et al., 2008; Mihindukulasuriya et al., 2008).

The aim of this study is to demonstrate the ability of a virus microarray to simultaneously detect and genotype lyssaviruses. Lyssavirus RNAs, representing the seven recognised genotypes, were reverse transcribed and amplified in a sequence-independent PCR. Amplified products were labelled and identified to genotype by hybridization to an oligonucleotide microarray and by analysis with DetectiV Software.

2. Materials and methods

2.1. Virus strains and RNA isolation

The details of each lyssavirus isolate used are given in Tables 1 and 3. Each virus was passaged by intracranial inoculation into 3-week-old mice (either Swiss OF1 or CD1). On the development of disease, mice underwent euthanasia and RNA was extracted from the brain using Trizol (Invitrogen). The RNA was resuspended in water and quantified using a Nanodrop (Thermo Scientific) and diluted to 0.5–1 μg/ml. Vesicular stomatitis virus (VSV) was grown in cell culture and used as an example of a non-lyssavirus member of the family *Rhabdoviridae*.

The DNA contaminant in the RNA sample was digested with Amplification grade DNase I (Invitrogen) following the manufacturers protocol. In brief, 1–2 μl of DNase I (1 units/μl) and 1 μl of 10× DNase Buffer were added to 7 μl of RNA and the final volume was adjusted to 10 μl. The mix was incubated for 15 min at room temperature. DNase I was then inactivated by the addition of 1 μl of 25 mM EDTA solution to the reaction mixture and heating for 10 min at 65 °C.

2.2. cDNA synthesis and random nucleic acid amplification

DNase I digested RNA (11 μl) was added to 1 μl of 25 mM dNTP and 1 μl of 40 μM primer A, 5′ GTT TCC CAG TCA CGA TAN NNN NNN NN 3′ (J.L. DeRisi, personal communication). The mixture was heated at 65 °C for 5 min and chilled on ice immediately. 4 μl of 5× RT buffer (Invitrogen), 1 μl of 0.1 M DTT, 1 μl RNasin Ribonuclease inhibitor (20–40 units/μl, Promega) and 1 μl of Superscript III (200 units/μl) were added to the mix, incubated at 42–50 °C for 60 min and then heated to 70 °C for 15 min.

Second strand DNA synthesis and nucleic acid amplification was conducted following the methods of Wang et al. (2002). The cDNA was heated to 94 °C for 2 min and was rapidly cooled to 10 °C for 5 min. 2 μl of 5× Sequenase buffer (USB, Ohio) and 0.3 μl of Sequenase DNA polymerase were added to the mix and the total volume was adjusted to 30 μl. The mixture was heated slowly from 10 °C to 37 °C over an 8-min period and held at 37 °C for another 8 min. The cycle was repeated with 0.3 μl of fresh Sequenase and terminated by heating the reaction at 94 °C for 8 min.

Double-stranded DNA template (5 μl) was amplified in a PCR which was composed of 5 μl of 10× KlenTaq PCR buffer (Sigma), 0.5 μl of 25 mM dNTP mix, 1 μl of primer B, 5′ GTT TCC CAG TCA CGA TA 3′, 1 μl of KlenTaq® LA DNA polymerase (5 units/μl) and the final volume was adjusted to 50 μl with water. The PCR mix was initially heated to 94 °C for 4 min, 68 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 68 °C 1 min and a final extension of 2 min at 68 °C and then held at 10 °C.

2.3. Real time PCR

Real time PCR was composed of 10 μl of Brilliant® II SYBR® Green QPCR Master Mix (Stratagene), 0.04 μl of reference dye (ROX, 25 μM, Invitrogen), 1 μl of primer JW12 (20 μM), 1 μl of primer N165-146 (20 μM), 3 μl of template DNA and water to a final volume of 20 μl (Wakeley et al., 2005). The PCR was performed at 94 °C for 2 min, followed by 45 cycles of 95 °C for 1 min, 55 °C for 30 s and 72 °C for 20 s. Randomly amplified PCR products were diluted 1/10 with deionised water before being measured by real time PCR.

2.4. Labelling DNA with fluorescent dye

Indirect labelling of amplified products was done in a second round of PCR and subsequent coupling of dye. The PCR consisted of 5 μl of 10× KlenTaq PCR buffer, 1 μl of KlenTaq® LA DNA polymerase (5 units/μl), 1 μl of amino allyl dNTP mix [10 mM amino allyl dUTP (Ambion), 2.5 mM dTTP, and 12.5 mM of each dGTP, dCTP and dATP], 1 μl of 100 mM amino-primer B, amino-C6 5′ GTT TCC CAG TCA CGA TA 3′ and 5 μl of first round PCR product. The total volume was adjusted to 50 μl with water. The PCR conditions were as before but run for 25 cycles. The PCR product was purified with MinElute PCR Purification kit (Qiagen) and DNA was eluted in 10 μl of water. The eluted DNA was coupled to Alexa Fluor 555 reactive dye (Invitrogen), dissolved in 4 μl of DMSO, in the presence of sodium bicarbonate for 1 h at room temperature in the dark. After the incubation the final volume of coupling reaction was adjusted to 50 μl with water and the unincorporated dye was removed using illustra AutoSeq™ G-50 columns (GE Healthcare) according to the manufacturer's instructions. The final product is labelled target DNA.