Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqMan™ technology

Elizabeth M. Black ¹, J. Paul Lowings, Jemma Smith, Paul R. Heaton, Lorraine M. McElhinney *

Rabies Research and Diagnostic Group, Department of Virology, Veterinary Laboratories Agency (Weybridge), New Haw, Addlestone, Surrey, KT15 3NB, UK

Received 13 September 2001; received in revised form 18 March 2002; accepted 18 March 2002

Abstract

A rapid and sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay incorporating TaqMan™ probes has been developed that can distinguish among the six established rabies and rabies-related virus genotypes. TaqMan™ probes were designed and validated against 106 rabies and rabies-related virus isolates, one isolate of the Australian bat Lyssavirus (genotype 7), and 18 other non-rabies viruses important in the veterinary field. The N gene was used as the target for the probes as it is well conserved and has been intensively used to genotype rabies isolates. Additionally, it was found to contain regions specific to each genotype conducive to probe design. The RT-PCR assay described amplifies a portion of the nucleoprotein gene of all 107 rabies and rabies-related viruses, but none of the other viruses tested. Inclusion of TaqMan™-genotype-specific probes in the RT-PCR assay permits rapid identification of the virus present. By combining RT-PCR with TaqMan™ genotyping probes suspect rabies virus isolates can be identified in a single closed tube system that prevents potential PCR-product carry over contamination.

Keywords: Rabies virus; Rabies-related viruses; RT-PCR; TaqMan

1. Introduction

Rabies viruses are in the order Mononegavirales (enveloped negative strand RNA viruses with unsegmented genomes), family Rhabdoviridae, and belong to the Lyssavirus genus. Based on the nucleotide sequence and deduced percentage of amino acid similarity in the nucleoprotein (N), the Lyssavirus genus is divided into six established genotypes (Bourhy et al., 1992, 1993). The genus

* Corresponding author. Tel.: +44-1932-357-722; fax: +44-1932-357-239.
E-mail address: l.mcelhinney@vla.defra.gsi.gov.uk (L.M. McElhinney).
¹ Present address: Dstl Chemical and Biological Sciences, Porton Down, Salisbury, Wiltshire, SP4 0JQ, UK.
is composed of classical rabies virus (genotype 1) and the rabies-related viruses: Lagos Bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), and European Bat Lyssaviruses (EBL) 1 and 2 (genotypes 5 and 6, respectively) (Familusi et al., 1972; Crick et al., 1982; Foggin, 1983; King and Crick, 1988; Bourhy et al., 1993). The more recent Australian Bat Lyssavirus, first reported in 1996 (Fraser et al., 1996), has been classified as a separate and distinct genotype (genotype 7) within the Lyssavirus genus (Gould et al., 1998) (Fig. 1).

All mammals are susceptible to rabies, but the major hosts are wild and domestic canines and felines, viverrids, mustelids, racoons and Microchiroptera (insectivorous and haematophagous bats). All genotypes, with the exception of genotype 2, are known to cause human disease. The illness caused by the rabies-related viruses is virtually indistinguishable from classical rabies (Smith et al., 1992). Classical rabies viruses have a worldwide distribution except for a few island nations such as Great Britain, Ireland, New Zealand and Hawaii, the continents of Australia and Antarctica and an increasing number of Western European countries. The Lagos Bat, Mokola and Duvenhage viruses of genotypes 2–4 are mainly restricted in distribution to the African continent. However, a genotype 2 isolate has been isolated recently from an imported fruit bat in France (Aubert, 1999).

The bat viruses of genotypes 5 and 6 have only been isolated in Europe. European bat Lyssavirus 1 (EBL1) isolates are found primarily in Eptesicus bats, whilst EBL2 isolates have only been identified in Myotis bats (Brass, 1994). The EBLs have been further classified into the phylogenetic lineages EBL1a, EBL1b, EBL2a and EBL2b (Amenegual et al., 1997).

Fig. 1. Phylogenetic tree to demonstrate the genetic distances between the 405 bp region from the amino terminus of the Nucleoprotein gene of selected rabies and rabies-related virus isolates. (Multiple sequence alignments were created using the CLUSTAL W programme. The tree was generated using the DNADIST (maximum likelihood option) and Neighbour programmes of the PHYLIP package using an estimated transition/transversion ratio derived from the PUZZEL 32 programme.)
The accepted diagnostic standard for rabies detection, the fluorescent antibody test (FAT), detects virus antigen in brain smears using fluorescently-labelled anti-rabies antibodies (Dean and Abelseth, 1973). The sensitivity of this detection method is good with classical rabies isolates, but may be reduced with isolates from the rabies-related viruses (Perrin et al., 1992). In addition, FAT cannot be used to distinguish between the genotypes.

PCR-based assays have the added advantage over the standard diagnostic assays that they produce a product that can be further analysed by sequencing. The resultant data can then be used for phylogenetic analyses that enable a highly accurate characterisation of virus isolates. A hemi-nested reverse transcriptase PCR (hnRT-PCR) assay that uses a cocktail of primers capable of detecting the six established genotypes of rabies and rabies-related viruses has been described (Heaton et al., 1997). The hnRT-PCR offers a higher level of sensitivity than the FAT for normal and decomposed tissues (Heaton et al., 1997), but it does not distinguish between the genotypes. However, the resultant N-terminal 405 bp sequence data is sufficient to distinguish between the various genotypes (Fig. 1). More recently, an assay using PCR-ELISA was described which could amplify and distinguish genotype 1, 5 and 6 viruses (Black et al., 2000).

A technique that gives rise to phylogenetic data much more rapidly than sequencing involves the use of TaqMan™ probes. TaqMan™ is an adaptation by Perkin–Elmer (Livak et al., 1995) of the 5′ nuclease PCR assay described by Lee et al. (1993). A result confirming the genetic lineage of an isolate can be achieved using TaqMan™ probes within a few minutes of completing a standard PCR or reverse transcriptase-polymerase chain reaction (RT-PCR) cycle. A closed tube system can be used for the PCR and to measure the fluorescence emitted by the TaqMan™ probes, resulting in minimal post-PCR manipulation and therefore, a reduction in potential cross contamination. Since its development, TaqMan™ has found many applications in research, viral and bacteriological diagnostics and epidemiological studies (Chen et al., 1997; Sharma et al., 1999; McGoldrick et al., 1998; Kimura et al., 1999; Laue et al., 1999).

Using the sequence data from a number of isolates from each of the rabies and rabies-related virus genotypes, TaqMan™ probes were designed to distinguish the six established genotypes of rabies and rabies-related viruses. Due to the genetic diversity of genotype 1 isolates, a combination of three probes was found to be necessary to detect all of the classical isolates tested. The remaining five genotypes required only a single probe each. Using these probes rapid identification and classification of suspect rabies virus isolates can be made within a few hours and provide additionally useful epidemiological data in general research.

2. Methods

2.1. Cells and viruses

Virus isolates (n = 106) from the six established genotypes of rabies and rabies-related viruses and one Australian bat Lyssavirus isolate were selected for this study. The rabies virus isolates were propagated and passaged in mice or wherever possible in BHK-21 cells as described (King, 1996; Heaton et al., 1997; Black et al., 2000). The first 405 bases of the N gene had previously been sequenced to confirm the genotype of the strain. Forty-three genotype 1 viruses and all available genotype 2–6 viruses were tested. The original hosts and geographical sources are summarised in Table 1. The RNA from eighteen other non-rabies viruses important in the veterinary field was also tested to assess the specificity of the primers and probes. These were, Classical Swine Fever Virus, Border Disease Virus, Bovine Viral Diarrhoea Viruses types 1 and 2, Porcine Epidemic Diarrhoea Virus, Porcine Respiratory Coronavirus, Porcine Reproductive & Respiratory Syndrome Virus, Bromo Mosaic Virus, Newcastle Disease Virus, Avian Paramyxovirus, Feline Calicivirus, Feline Infectious Peritonitis Virus, Feline Coronavirus, Feline Herpes Virus, Feline Poxvirus, Feline Immunodeficiency, Virus Feline Leukaemia Virus and Feline Spumavirus.
| RV number | Species isolated from | Country of isolation |
|-----------|-----------------------|---------------------|
| RV44      | Bat                   | South America       |
| RV110, RV114 | Bat               | Chile               |
| RV155     | Bat                   | Brazil              |
| RV166, RV167 | Bat              | Canada              |
| RV255     | Bat                   | Russia              |
| RV630     | Bat                   | Yugoslavia          |
| CVS11, RV651 | Bovine        | France              |
| RV307     | Bovine                | Georgia             |
| RV338     | Bovine                | China               |
| RV68, RV69, RV71 | Canine          | Switzerland         |
| RV73      | Canine                | Belize              |
| RV202     | Canine                | Turkey              |
| RV234     | Canine                | Russia              |
| RV305     | Canine                | Georgia             |
| RV341     | Canine                | China               |
| RV447     | Canine                | Botswana            |
| RV409     | Cape Wild Cat         | South Africa        |
| RV484     | Duiker                | Botswana            |
| RV490     | Felis sp.             | South Africa        |
| RV498     | F. libyca             | South Africa        |
| RV56      | Fox                   | USA                 |
| RV313, RV318 | Fox               | Germany             |
| RV62      | Horse                 | Nigeria             |
| RV629     | Human                 | Nigeria             |
| RV137     | Meercat               | South Africa        |
| RV54      | Raccoon-Dog           | USA                 |
| RV303     | Raccoon-Dog           | Russia              |
| RV437     | Raccoon-Dog           | Estonia             |
| RV57, RV58 | Skunk                | USA                 |
| RV521     | Simian Jackal         | Ethiopia            |
| RV334     | Vaccine Strain        | China               |
| RV253     | Wolf                  | Russia              |
| RV420     | Yellow                | South Africa        |
| RV100     | Data Unknown          | Morocco             |
| RV463     | Data Unknown          | Kenya               |

**Genotype 2**

| RV1       | Bat                   | Nigeria             |
| RV2, RV3, RV43, RV190 | Bat               | South Africa        |
| RV41      | Bat                   | Senegal             |
| RV133     | Cat                   | Zimbabwe            |
| RV611     | Dog                   | Ethiopia            |
| RV767     | Bat                   | France              |

**Genotype 3**

| RV16      | Bat                   | Data Unknown        |
| RV42      | Bat                   | South Africa        |
| RV5       | Cat                   | South Africa        |
| RV174-RV177 | Cat              | Zimbabwe            |

**Genotype 4**

| RV131     | Bat                   | Zimbabwe            |
| RV139     | Bat                   | South Africa        |
| RV6       | Human                 | South Africa        |

**Genotype 5**

| RV66      | Cat                   | Ethiopia            |
| RV40      | Harsh-furred mouse    | South Africa        |
| RV4       | Shrew                 | Nigeria             |
| RV39      | Shrew                 | Cameroon            |

**Genotype 6**

| RV8       | Human                 | Finland             |
| RV29, RV30, RV228, RV229 | M. dasycneme    | Netherlands          |
| RV594     | M. daubentonii        | Switzerland         |
| RV621     | Bat                   | Switzerland         |
| RV628     | M. daubentonii        | UK                  |

**Genotype 7**

| RV634     | Fruit Bat             | Australia           |

### 2.2. RNA extraction and cDNA synthesis

Total RNA was extracted directly from rabies-infected BHK-21 monolayers or infected mouse brain tissue using Trizol® (Gibco BRL) according to manufacturer’s instructions. Complementary DNA was produced using the RNA extracted from each of the infected cultures or mouse brains and the messenger sense RT primer JW12 as described (Heaton et al., 1997).
2.3. Primers and TaqMan™ probes

The primer sets for PCR amplification were based on those of Heaton et al. (1997) although a new messenger sense primer BB6 was found to be more efficient than JW12 for PCR amplification. The TaqMan™ probes were designed by eye using multiple alignments (MegAlign, Dnastar) following the recommended criteria (Applied Biosystems, UK). They were based on regions of high homology that were specific to each genotype and were synthesised by Applied Biosystems. A list of primers and probes is given in Table 2.

2.4. TaqMan™ reaction

The cDNAs were amplified using the specific primer set BB6 (messenger sense) and a cocktail of JW6(DPL)/JW6(M)/JW6(E) (genomic sense) (Table 2). For each probe the conditions were optimised with relation to Mg²⁺ concentration and annealing temperature. Amplification of 5 μl of the cDNA template was performed in a final volume of 50 μl of 1× PCR buffer containing 4.0–6.0 mM magnesium chloride (Applied Biosystems) (Mg²⁺ concentration for each probe given in Table 2), 200 μM each dNTP, 5.0 pmol of primer BB6, 7.5 pmol of each primer JW6(DPL), JW6(M) and JW6(E), 0.5 U of AmpliTaq GOLD (Applied Biosystems) and 5 pmol of the probes for genotypes 2–6 or 2.5 pmol of each of the three genotype 1 probes to be used in combination. The amplification was carried out in 0.2 ml TaqMan™ optical tubes on an Applied Biosystems Thermal Cycler 2400. The cycling conditions were as described (Black et al., 2000) with the exception of annealing temperatures (Table 2).

2.5. Fluorescence monitoring

Fluorescence monitoring was performed according to the Manufacturer’s directions (Perkin–Elmer, 1994). On completion of the PCR the samples were analysed in the closed PCR tubes using a Applied Biosystems LS50B luminescence spectrometer to quantify the fluorescence emitted by the TaqMan™ probes. The tubes were scanned at 518 nm (FAM) and then 582 nm (TAMRA), with an excitation wavelength of 488 nm. Data acquisition and analyses were carried out using the Fluorescence Data Manager (Applied Biosystems) and MICROSOFT EXCEL Spreadsheets. The increase in sample fluorescence was compared with the fluorescence of no template controls in triplicate. The magnitude of the generated signal (ΔRQ) represents the difference between the sample RQ (RQ +) and the mean value attained for the no template controls RQ (RQ −), where RQ is the emission intensity of the reporter divided by the emission intensity of the quencher. In this equation, the quencher acts as an internal standard to normalise fluctuations in fluorescent intensity due to non-specific effects such as concentration changes due to volume fluctuations. Any other fluctuations not due to PCR-related nuclease digestion are normalised by taking the RQ + value for a tube that contains all the components including template and subtracting the RQ − value for the no template control tubes. This final ΔRQ value reliably indicates the magnitude of the signal generated by the given set of PCR conditions. A final calculation, defined as the threshold ΔRQ, was carried out to obtain a numerical cut-off value above which a given ΔRQ should represent a positive result. This value is calculated at a 99% confidence interval using the standard deviation (S.D.) obtained from the three no template controls (Perkin–Elmer, 1994). For the purposes of this assay a higher threshold ΔRQ (1.0) than that calculated using this method (usually < 0.5) was adopted.

A confirmatory test for successful PCR amplification was carried out by ethidium bromide stained agarose gel electrophoresis during the development of the assay.

3. Results

3.1. TaqMan™ assay optimisation

Using 1.5 mM magnesium at an annealing temperature of 51 °C, the RT-PCR gave clear well-defined bands on agarose gels. However, these conditions produced poor ΔRQ readings in the TaqMan™ assay. The RT-PCR was therefore,
Table 2
Details of (a) oligonucleotide primers for RT-PCR and (b) TaqMan™ probes

| Primer | Sequence (5′–3′) | Messenger/ | Position in | Use of primer |
|--------|------------------|-------------|-------------|---------------|
|        |                  | genomic sense | genome |               |
|       |                  |             | a           |               |
| (a) oligonucleotide primers for RT-PCR | | | | |
| JW12  | ATG TAA CAC CYC TAC AAT CAC | M | 55–73 | RT |
| BB6   | GAT CAR TAT GAG TAY AAA TAT CC | M | 140–162 | PCR |
| JW6(DPL) | CAA TTC CGA CAC ATT TTG GTC | G | 660–641 | PCR |
| JW6(M) | CAG TTA GCC CAC ATC TTA TG | G | 660–641 | PCR |
| JW6(E) | CAG TCG GCA CAC ATC TTG TG | G | 660–641 | PCR |
| Probe | Sequence (5′–3′) | Messenger/ | Position in | Mg²⁺ | Annealing |
|       |                  | genomic sense | genome | Concentration | temperature (°C) |
|       |                  |             | a           | (mM)           |               |
| (b) TaqMan™ Probes | | | | | |
| TQM1 (a) | CCC AAT TCC CTT CTA CAT | G | 360–335 | 6.0 | 55 |
| TQM1 (b) | CCC AGT TCC CTT CTA CAT | G | 360–335 | 6.0 | 55 |
| TQM1 (c) | CCC AAT TCC CTT CTA CAT | G | 360–335 | 6.0 | 55 |
| TQM2   | ACA GAT GGG AAG AAA CCT GGT | M | 100–120 | 5.0 | 56 |
| TQM3   | TAG ATG GAA AGA AAC CAG GGA TAA C | M | 101–125 | 4.0 | 47 |
| TQM4   | TGT GTG TCC CGA AGA TTG GGT T | M | 249–270 | 4.0 | 55 |
| TQM5   | TTT ACG TGG ACG CAT GGT CTT GT | M | 219–241 | 6.0 | 55 |
| TQM6   | AGA GCT ACG GGA TTC TCA TTG CT | M | 269–291 | 4.0 | 55 |

Key: Y = C or T; R = A or G.

*a* Nucleotide positions numbered according to the Pasteur Virus sequence (Tordo et al., 1986).

3.2. Discrimination of the rabies and rabies-related viruses

The ΔRQ values for each of the genotypes are summarised in Fig. 2. Fig. 2a–f displays the individual values returned for each isolate. The three genotype 1 probes (TQM1a, TQM1b and TQM1c) used in combination detected all of the genotype 1 isolates and none of the isolates from the other genotypes or viruses tested (Fig. 2a). Similarly, the probes used individually to detect genotypes 3–6 (TQM3, TQM4, TQM5 and
TQM6, respectively) specifically detected all isolates from their respective genotype panels and none of the other genotypes or viruses tested (Fig. 2c–f). The probe used to detect the genotype 2 isolates also detected four of the genotype 3 isolates (Fig. 2b). Fortunately, the genotype 3 probe detected only genotype 3 isolates (Fig. 2c). Thus, if an isolate tested positive with both the genotype 2 and genotype 3 probes it was classed as genotype 3. Isolates detected with the genotype 3

Fig. 2. Graphs demonstrating the ΔRQ values obtained for each virus isolate using the TaqMan probes (a) TQM1 a, b and c in combination for genotype 1 (b) TQM2 for genotype 2 (c) TQM3 for genotype 3 (d) TQM4 for genotype 4 (e) TQM5 for genotype 5 and (f) TQM6 for genotype 6.
probe only, were classed as genotype 3. Similarly, isolates detected with the genotype 2 probe only, were classed as genotype 2.

The genotype 3 isolates that gave positive results with the TQM2 probe were RV174–RV177. All four viruses were isolated from cats in Zimbabwe and have identical sequences in the 405 bp 5' region of the nucleoprotein gene (unpublished data). Using the RT-PCR primers developed by Heaton et al. (1997) as well as the RT-PCR primers used in the development of this TaqMan™ assay these four isolates gave two bands on agarose gel electrophoresis. With respect to the TQM2 probe region, RV174–RV177 have four base differences, whilst the remaining genotype 3 isolates contain five base differences from the probe. The maximum number of base differences between the TQM2 probe and the genotype 2 isolates is three for RV1, RV41 and RV767. These latter isolates gave relatively low ΔRQ results (2.43, 1.08 and 1.46, respectively) compared with the remaining isolates, which were all identical to the probe and, with the exception of RV2 (2.04), gave results in the range 7.07–7.87. The ΔRQ results with TQM2 for the genotype 3 isolates (RV174–RV177) were in the range 1.12–2.07. Examination of the remainder of the available sequence data for these isolates as well as sequencing of DNA from the second band on the agarose gels failed to reveal any further areas of possible homology to the probe.

Possible patterns in the ΔRQ values were sought to determine if the number of differences between the target sequence and probe within a genotype was reflected in the ΔRQ value. All the genotype 1 isolates had from 0 to 4 differences between the TQMs1 probes and target sequence but there was no apparent pattern between the number of differences and ΔRQ. All of the other genotypes had at least five differences between the target sequence and TQMs1 probes. All of the genotype 3 isolates had no or one base difference to the TQM3 probe and again no pattern was seen between the differences and ΔRQ. Of the other genotypes, the closest to this probe was 5 + differences with the genotype 2 isolates and 7 + differences with the remaining genotypes. All the genotype 4 isolates were identical to the TQM4 probe, which had six or more base differences to the other genotypes. All the genotype 5 and 6 isolates had no or one base difference to their respective probes and again no patterns were seen between the differences and ΔRQ. The TQM5 probe had 7 + base differences and the TQM6 probe had 6 + base differences to the other genotypes. The only probe that seemed to show any pattern was the TQM2 probe as mentioned above, where differences in ΔRQ reflected the complementarity of probe to target sequences for all but one of the genotype 2 isolates. It appears that the lower discriminatory limit of probe detection is four base differences between probe and target sequence. This was seen with the genotype 1 probes and is the most likely explanation for the detection of the genotype 3 isolates RV174–177 by the TQM2 probe. No isolates with more than four base differences to the probe were falsely detected.

The genotype 7 isolate was tested with these probes and was negative with all of them. A genotype 7 probe was not designed as only one isolate was available for testing.

4. Discussion

Despite the availability of an effective vaccine, rabies is still a significant problem in many parts of the world. A rapid test that could distinguish between the rabies genotypes would be very advantageous for both epidemiological studies, for example in Africa and Europe where more than one genotype co-exist, and as a diagnostic test in the case of an outbreak. In the UK the most likely genotypes that could enter this country from mainland Europe are classical rabies and the EBLs. At present genotype differentiation relies on RT-PCR and subsequent sequencing or monoclonal antibody typing. Rapid genotyping of any strain found in the UK would be particularly important, as the outcome may dictate the control measures implemented. Classical rabies has the potential to establish within indigenous terrestrial animal populations and hence may require the implementation of extensive control measures, whereas, the EBLs are likely to be limited to
spread among the UK bat population and could prove difficult to control.

This report describes the development and application of eight TaqMan™ probes to differentiate between six genotypes of rabies and rabies-related viruses. Using this method a definitive result can be acquired within a few minutes of completion of a standard RT-PCR cycle. Monoclonal antibody analysis usually necessitates the growth of virus in cell culture, which may be impossible or result in a delay of several days with decomposed material. An additional problem encountered with monoclonal antibody typing is that the results are subject to individual interpretation. Sequence analysis gives a definitive answer and is thus more reliable, but can also take several days to achieve a result unless there is direct access to automated sequencing equipment. An additional advantage of TaqMan™ is that it is a closed tube system that significantly reduces the risk of cross contamination by PCR products and thus results in increased confidence in the results acquired.

The N gene was selected as the target for this assay as it is well conserved and has been intensively used to classify rabies isolates into their respective genotypes (Black et al., 2000; Bourhy et al., 1992, 1993; Kissi et al., 1995; Smith et al., 1992). There is a large amount of N gene sequence data available, which, on examination, revealed conserved regions specific to each genotype that could be used to design genotype specific TaqMan™ probes. The classical rabies virus isolates have a greater representation in our archive and due to extensive geographical distribution and host range, demonstrate a much broader genetic diversity than the other genotypes. For this reason, three probes were required in combination to detect all of the classical isolates tested. All three probes cover the same region of the gene, but have one base change from the others in either the fifth or sixth position. The remaining five genotypes had sufficient similarities in specific regions to enable the use of a single probe for their discrimination.

The TaqMan™ probes were optimised individually with relation to magnesium concentration and annealing temperature to yield the highest intensity of reporter fluorescent intensity without compromising specificity. Unfortunately, when relative specificities were compared, the probes were found to have different optimum Mg2+ concentrations. Using the Universal 2X master mix (Applied Biosystems), which became available more recently it may be possible to standardise the reaction mixes to ease panel preparation. Magnesium chloride affects annealing and the melting temperatures of both the TaqMan™ probe and the PCR primers as it help stabilise the hybridisation complexes. It is also required for Taq DNA polymerase activity and affects FAM quenching by TAMRA on the probe. Increasing the magnesium concentration can enhance TAMRA quenching in the intact probe (Livak et al., 1995), which is important for calculation of RQ−, that is, the lower the reporter emissions in RQ−, the greater ΔRQ will be in the presence of specific template. However, too little or too much magnesium can result in reduced amplification efficiency or amplification of non-target sequences. The PCR primers were known to produce (Heaton et al., 1997) specific product at a range of temperatures, although this was strongest and cleanest with respect to non-specific products at 51 °C. The annealing temperature was therefore optimised, again to achieve the best possible ΔRQ values. We observed that when transferring the technology to the newer ABI7200 reader, the ΔRQ values were much higher than obtained previously using the LS50B and the threshold value required re-evaluation. Hence, the actual values represented in Fig. 2 may be significantly different when alternative readers are used.

The assay appears to be highly specific and reproducible. All the rabies and rabies-related virus RNA samples used in the development of this assay had been amplified previously using the PCR protocol described by Heaton et al. (1997) and the products sequenced partially and genotyped. Due to the large number of available isolates, a representative group of genotype 1 isolates were carefully selected for this covering as wide a range of animal hosts, geographical isolation and sequence variation, as possible. All of the genotype 2–6 isolates available in our archive were tested and a selection of unrelated viruses were
also tested to ensure the specificity of the assays for rabies and rabies-related viruses. Seven of the eight probes were used directly to give a definitive result, whilst the remaining probe, that for detecting Lagos Bat isolates had to be used alongside the Mokola probe to determine whether a positive result is a Lagos Bat or Mokola isolate. Detection by the Mokola probe or both probes indicated a Mokola isolate, while detection with the Lagos Bat probe and not the Mokola probe indicated a Lagos Bat isolate. For complete confidence, automated sequencing may be used to confirm the genotype of an isolate. It was not deemed necessary to further discriminate between the EBL1a and b and EBL2a and b subgroups as generic EBL1 and EBL2 probes would be sufficient for the aims of this assay. In the case of an outbreak, the control measures introduced would be the same whether an ‘a’ or ‘b’ subgroup was responsible.

The material used during the development of the assay was passaged in either BHK cells or mice to obtain large quantities of RNA permitting uniform validation experiments. The assay has been used successfully on panels of original rabies infected material (data not included). The sensitivity of the assay with each probe was found to be similar to that reported by Heaton et al. (1997) for the RT-PCR alone, approximately 0.02 TCID₅₀ per ml (data not included).

The assay described above is a rapid and sensitive method for distinguishing between the rabies and rabies-related viruses with several advantages over traditional genotyping techniques. It is more rapid than traditional typing methods and reduces the need for further processing of the RT-PCR product, thereby reducing the risk of cross-contamination. It has the potential to be used in both diagnostic and research laboratories in the identification and classification of suspect rabies isolates in the case of a potential outbreak situation, or for generating routine epidemiological information. The probes may be used in the development of quantitative real time PCR assays using either the Light Cycler (Roche Applied Science) or ABI7700 (Applied Biosystems) technologies.

Acknowledgements

This work was supported by funding from the Ministry of Agriculture, Fisheries and Food and was carried out under ROAME project SE0415.

References

Amengual, B., Whitby, J.E., King, A.A., Sera Cobo, J., Bourhy, H., 1997. Evolution of European bat Lyssavirus. J. Gen. Virol. 78, 2319–2328.
Aubert, M., 1999. WHO: Rabies Bulletin Europe. Second Quarter. April–June, p. 6.
Black, E.M., McElhinney, L.M., Lowings, J.P., Smith, J., Johnstone, P., Heaton, P.R., 2000. Molecular methods to distinguish between classical rabies and the rabies-related European bat Lyssavirus. J. Virol. Methods 87, 123–131.
Bourhy, H., Kissi, B., Lafon, M., Sacramento, D., Tordo, N., 1992. Antigenic and molecular characterization of bat rabies virus in Europe. J. Clin. Microbiol. 30 (9), 2419–2426.
Bourhy, H., Kissi, B., Tordo, N., 1993. Molecular diversity of the Lyssavirus genus. Virology 194, 70–81.
Brass, D.A., 1994. Rabies in Bats: Natural History and Public Health Implications. Livia Press, Connecticut, USA.
Chen, S., Yee, A., Griffiths, M., Larkin, C., Yamashiro, C.T., Behari, R., Paszko-Kolva, C., Rahn, K., De Grandis, S.A., 1997. The evaluation of a fluorogenic polymerase chain reaction assay for the detection of Salmonella species in food commodities. Int. J. Food Microbiol. 35, 239–250.
Crick, J., Tignor, G.H., Moreno, K., 1982. A new isolate of Lagos bat virus from the republic of South Africa. Trans. R. Soc. Trop. Med. Hyg. 76 (2), 211–213.
Dean, D.J., Abelseth, M.K., 1973. The fluorescent antibody test. In: Kaplan, M.M., Koprowski, E. (Eds.), Laboratory Techniques in Rabies, third ed. WHO, Geneva, Switzerland. pp. 73–84.
Familusi, J.B., Osunkoya, B.O., Moore, D.L., Kemp, G.E., Fabiyi, A., 1972. A fatal human infection with Mokola virus. Am. J. Trop. Med. Hyg. 21 (6), 959–963.
Foggin, C.M., 1983. Mokola virus infection in cats and a dog in Zimbabwe. Vet. Rec. 113, 115.
Fraser, G.C., Hooper, P.T., Lunt, R.A., Gould, A.R., Glesson, I.J., Hyatt, A.D., Russell, G.M., Kattenbelt, J.A., 1996. Encephalitis caused by a Lyssavirus in fruit bats in Australia. Emerging Infect. Dis. 2 (4), 327–331.
Gould, A.R., Hyatt, A.D., Lunt, R.A., Kattenbelt, J.A., Hengstberger, S., Blacksell, S.D., 1998. Characterisation of a novel Lyssavirus isolated from Pteropid bats in Australia. Virus Res. 54, 165–187.
Heaton, P.R., Johnstone, P., McElhinney, L.M., Cowley, R., O’Sullivan, E., Whitby, J.E., 1997. Hemi-nested PCR assay for detection of six genotypes of rabies and rabies-related viruses. J. Clin. Microbiol. 35 (11), 2762–2766.
Kimura, H., Motita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T., Morishima, T., 1999. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J. Clin. Microbiol. 37 (1), 132–136.

King, A.A., 1996. Cell culture of rabies virus. In: Meslin, F.-X., Kaplan, M.M., Koprowski, H. (Eds.), Laboratory Techniques in Rabies, fourth ed. WHO, Geneva, Switzerland, pp. 114–130.

King, A.A., Crick, J., 1988. Rabies-related viruses. In: Campbell, J.B., Charlton, K.M. (Eds.), Rabies. Kluwer Academic Publishers, Massachusetts, USA, pp. 177–179.

Kissi, B., Tordo, N., Bourhy, H., 1995. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology 209, 526–537.

Laue, T., Emmerich, P., Schmitz, H., 1999. Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. J. Clin. Microbiol. 37 (8), 2543–2547.

Lee, L.G., Connell, C.R., Bloch, W., 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. 21 (16), 3761–3766.

Livak, K.J., Flood, S.J.A., Marmaro, J., Giusti, W., Deetz, K., 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridisation. PCR Methods Appl. 4, 357–362.

McGoldrick, A., Lowings, J.P., Ibata, G., Sands, J.J., Belak, S., Paton, D.J., 1998. A novel approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic probe (TaqMan). J. Virol. Methods 72, 125–135.

Perkin Elmer, 1994. TaqMan™ PCR Reagent Kit Protocol, Part Number 401845, Rev. A.

Perrin, P., Gontier, C., Lecocq, E., Bourhy, H., 1992. A modified rapid enzyme immunoassay for the detection of rabies and rabies-related viruses: RREID-lyssa. Biologicals 20, 51–58.

Sharma, V.K., Dean-Nystrom, E.A., Casey, T.A., 1999. Semi-automated fluorogenic PCR assays (TaqMan) for rapid detection of Escherichia coli 0157:H7 and other shiga toxicogenic coli. Mol. Cell. Probes 13 (4), 291–302.

Smith, J.S., Orciari, L.A., Yager, P.A., Seidel, H.D., Warner, C.K., 1992. Epidemiologic and historical relationships among 87 rabies virus isolates as determined by limited sequence analysis. J. Infect. Dis. 166, 296–307.

Tordo, N., Poch, O., Ermine, A., Keith, G., 1986. Primary structure of leader RNA and nucleoprotein genes of the rabies genome: segmented homology with VSV. Nucleic Acids Res. 14, 2671–2678.