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A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqMan™ technology

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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*es (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

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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 *C*ontinent. 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 (Amengual et al., 1997).

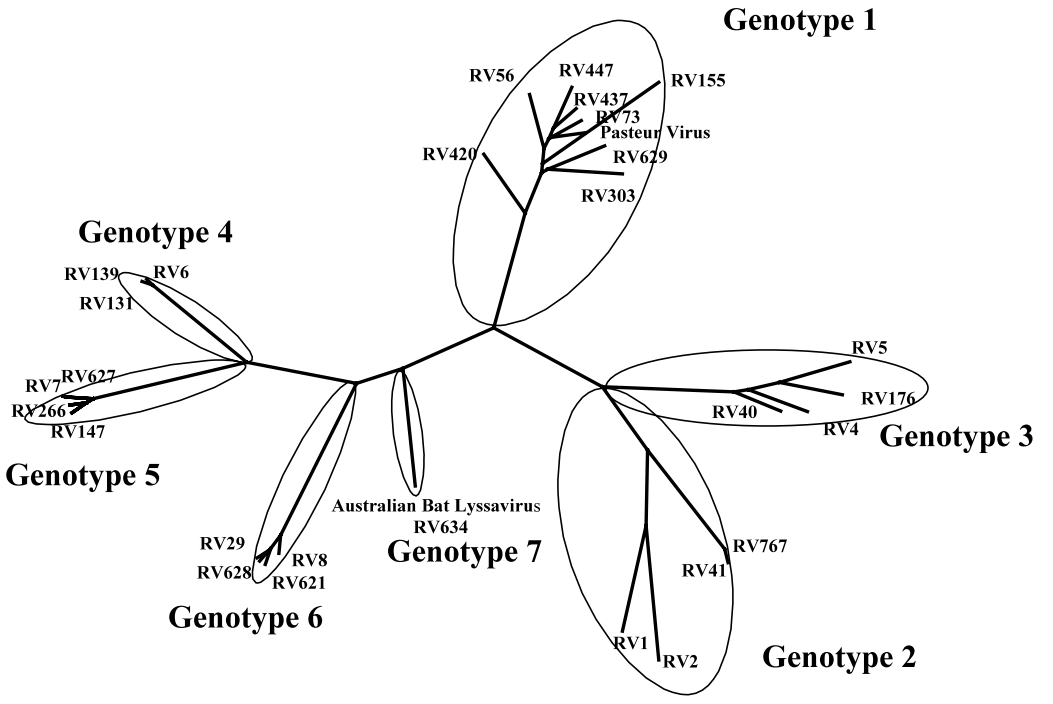

Fig. 1. Phylogenetic tree to demonstrate the genetic distances between the 405 bp region from the amino terminus of the Nucleoprotein gene of selected rabies and rabies-related virus isolates. (Multiple sequence alignments were created using the CLUSTAL W programme, The tree was generated using the DNADIST (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.

Table 1
Origin of Rabies and Rabies-Related Viruses

RV number	Species isolated from	Country of isolation
Genotype 1		
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
RV519	Bovine	Africa
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	*Felis libyca*	South Africa
RV56	Fox	USA
RV313, RV318	Fox	Germany
RV62	Horse	Nigeria
RV629	Human	Nigeria
RV137	Meercat	South Africa
RV54	Racoon	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 Mongoose	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
RV610	Cat	Ethiopia
RV40	Harsh-furred mouse	South Africa
RV4	Shrew	Nigeria
RV39	Shrew	Cameroon
Genotype 4		
RV131	Bat	Zimbabwe
RV139	Bat	South Africa
RV6	Human	South Africa
Genotype 5		
RV66	Bat	Poland
RV154	Human	Ukraine
RV264	*N. noctula*	Ukraine
RV265	*V. murinus*	Ukraine
RV266	*E. serotinus*	France
RV627	*Fruit Bat*	Denmark
RV7	*E. serotinus*	Denmark
RV19, RV20, RV24, RV25	Bat	Denmark
RV9, RV144, RV146,	*E. serotinus*	Germany
RV347, RV345, RV350	*E. serotinus*	Germany
RV10, RV11, RV145, RV147,	Bat	Germany
RV148, RV346, RV348, RV349	Bat	Germany
RV31, RV32, RV33,	*E. serotinus*	Netherlands
RV36, RV37, RV38	*E. serotinus*	Netherlands
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 Mg^{2+} 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) (Mg^{2+} 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/ genomic sense	Position in genome[a]	Use of primer	
(*a*) *oligonucleotide primers for RT-PCR*					
JW12	ATG TAA CAC CYC TAC AAT G	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 TG	G	660–641	PCR	
JW6(M)	CAG TTA GCG CAC ATC TTA TG	G	660–641	PCR	
JW6(E)	CAG TTG GCA CAC ATC TTG TG	G	660–641	PCR	
Probe	Sequence (5′–3′)	Messenger/ genomic sense	Position in genome[a]	Mg^{2+} Concentration (mM)	Annealing temperature (°C)
(*b*) *TaqMan™ Probes*					
TQM1 (a)	CCC AAT TCC CTT CTA CAT CAG TAC GT	G	360–335	6.0	55
TQM1 (b)	CCC AGT TCC CTT CTA CAT CAG TAC GT	G	360–335	6.0	55
TQM1 (c)	CCC AAT TTC CTT CTA CAT CAG TAC GT	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).

re-optimised for each TaqMan™ probe with respect to magnesium concentration and annealing temperature to obtain the highest intensity of reporter fluorescence whilst maintaining the highest specificity possible. Agarose gel electrophoretic analyses of the RT-PCR products in the TaqMan™ assays gave weaker specific and often secondary non-specific bands, but were useful for confirming negative TaqMan™ results during assay development. None of the eighteen non-rabies viruses tested gave any bands when examined by agarose gel electrophoresis.

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

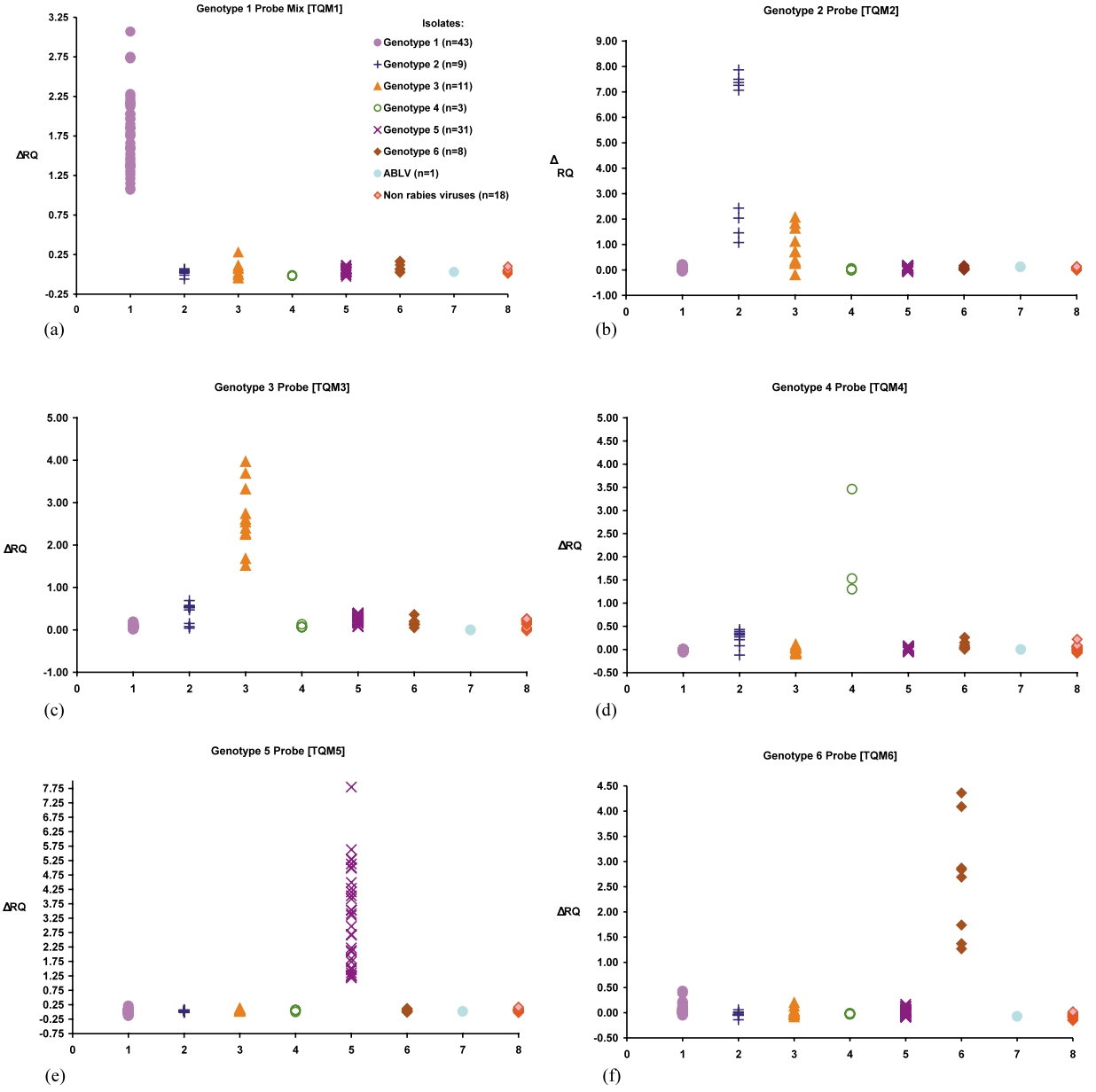

Fig. 2. Graphs demonstrating the ΔRQ values obtained for each virus isolate using the TaqMan probes (a) TQM1 a, b and c in combination for genotype 1 (b) TQM2 for genotype 2 (c) TQM3 for genotype 3 (d) TQM4 for genotype 4 (e) TQM5 for genotype 5 and (f) TQM6 for genotype 6.

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 TQM1 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 TQM1 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 Mg^{2+} 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 $TCID_{50}$ 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.

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