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Original Communication

Isolation and enhanced genetic analysis of rubella virus obtained from infants with congenital ocular defects

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ABSTRACT

Rubella virus (RV) continues to circulate in the Indian population, as rubella vaccination is not widespread. Congenital cataract due to maternal rubella constitutes a significant cause of visual loss among Indian infants. The aim of this study was to isolate and characterize RV from a range of specimens obtained from infants with serologically confirmed congenital rubella infection (CRI). Vero and RK13 cell lines were used for RV isolation, and RV strains were identified by indirect immunofluorescence (IFA), real-time and nested PCR assays. In total, 30 specimens from 17 infants were investigated. Twenty-two samples including two oral fluids (OF), four throat swabs and 16 lenses were simultaneously cultured in two cell lines. A total of 26 viruses were successfully isolated, 14 in Vero cells and 12 in RK13 cells. All 26 isolates were confirmed by PCR and 21/26 by IFA. For the first time, RV has been isolated from an oral fluid specimen. Higher viral loads were detected in Vero cell cultures, but a cytopathic effect was observed only in the RK13 cells when inoculated with lens material. In addition, two infants (case 9 and 12) were confirmed by PCR assay direct from their lens specimens. The generated sequences of the 739 nucleotide of E1 gene confirmed the presence of RV genotype 2B in South India. The point mutations observed in these RV strains may reflect geographic and temporal differences, adaptation to cell culture or the long period of incubation in CRS patients.

KEYWORDS: CRS, rubella virus isolation, PCR assays, genotyping, viral characterization

INTRODUCTION

Rubella virus (RV) is a positive single stranded RNA virus of the family *Togaviridae* and the only member of the genus *Rubivirus*. It has a genome approximately 10,000 nt in length which encodes two nonstructural proteins P150 & P90 at the 5' end and three structural proteins at the 3' end. The structural proteins consist of capsid and two envelope glycoproteins, E1 and E2. RV can be isolated in a variety of cell lines such as RK13, SIRC, BHK and Vero [1]. The replication cycle of RV is slow and limited cytopathic effects (CPE) have been observed in RK13, SIRC and in some Vero sub-lines [2]. Virus isolation is time consuming and laborious and is seldom used by diagnostic laboratories; however, it is valuable for virus characterization and defining antigenic variations, which may contribute to rubella control and eradication.

Women who become infected with RV during the first four months of pregnancy run a significant risk of giving birth to infants with a range of

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congenital malformation including cataracts [3, 4]. Rubella mediated congenital ocular defects are rarely seen in countries which have adopted universal vaccination for rubella [5]. The present study reports the isolation of RV using Vero and RK13 cell lines from a range of clinical specimens obtained from infants with ocular anomalies. Confirmation of RV replication was confirmed by indirect immunofluorescence assay (IFA), real-time PCR and virus characterised sequence analysis.

MATERIALS AND METHODS

Study subjects and sample collection

Thirty specimens from 17 infants with ocular anomalies, age range 10 days to 9 months, collected between 2004 and 2006 were investigated (Table 1), including four oral fluid (OF), seven throat swabs (TS) and 19 lenses. Oral fluid specimens were collected using Oracol collection devices (Malvern Medical Developments Ltd., Worcs, UK) and processed using 1 ml of Dulbecco's modified Eagles medium (DMEM). The OFs were extracted from the Oracol devices following centrifugation at 1800 rpm for 8 min. Throat swabs were collected with Virocult swabs (Corsham, UK) and processed using 500 µl of DMEM. Lenses were collected in Ringer's lactate solution during cataract surgery. All samples were stored at -70ºC prior to testing. Serum was collected from 16 of the 17 infants and was tested for anti-rubella IgM and IgG using ELISA assays (Dade Behring Enzygnost, Marburg, Germany).

Virus isolation

Vero cells obtained from the European Collection of Cell Cultures (ECACC, Porton Down, UK) were cultured in DMEM containing 5% fetal calf serum (FCS), 200 mM L-glutamine and 50 mg/ml Gentamicin. RK13 cells (ECACC, Porton Down, UK) were cultured in Minimum Essential Medium (MEM) containing 200 mM L-glutamine, 10% FCS and 50 mg/ml Gentamicin. The cells were seeded in glass tubes at a concentration of $5x10^5$ cells/ml with the respective growth medium and incubated at 37ºC. Specimens (100 µl/tube) were inoculated, in duplicate, at 24 hours, by which time cell monolayers had formed. After 1 hour adsorption, 0.9 ml maintenance medium (MM), DMEM containing 2% FCS, 200 mM L-Glutamine and Gentamicin with additional Penicillin-Streptomycin (2 ml/100 ml) and Fungizone (1 ml/100 ml) was added. Uninfected cells were used as controls and the cells were observed daily. The tubes with primary infection (zero passage-P0) were harvested 10 days after inoculation and further passages (P1-P5) were carried within one week.

Immunofluorescence assay (IFA)

Cell monolayers in the tubes were trypsinized using 0.25% Trypsin-EDTA. The cell density was adjusted with PBS and 25 ul/well were placed on 8-well multitest slides (Flow laboratories, UK), which were allowed to dry and then fixed with cold acetone (stored at -20ºC) for 15 min at 4-8ºC. The slides were stained with RV specific monoclonal antibodies anti-C and anti-E1 (CDC, Atlanta, USA), the 14B/F (gifted by Dr. Dhan Samuel, PHE, UK). Following washing with PBS, binding of the monoclonals was detected by addition of FITC conjugated anti-mouse antibody (Chemicon, UK) and specific fluorescence observed using a fluorescent microscope (ZEISS, Germany).

PCR assays for confirmation of RV isolates

Viral RNA was extracted from clinical samples using Qiagen spin columns (Qiagen, UK) following the manufacturer's protocol using the cell culture supernatants stored at -70ºC. Extraction of viral RNA from cell culture supernatant was carried out by Magnapure Extraction (Roche Diagnostics, UK). The RNA was reverse transcribed to produce cDNA and amplified by the real-time PCR-Prob-56, nested PCR-E317 and PCR-E820 according to previously published protocols [6-8]. The viral load quantification was carried out by the real-time PCR [6].

Genotyping and viral characterization

PCR products were purified using PCR purification kits (Qiagen, UK) and sequenced with the nested primers of PCR assays [7, 8]. Genetic characterization of RV isolates was carried out by analysis of the sequences according to WHO criteria [9]. Phylogenetic trees were derived using the Neighbor-Joining of MEGA or DNAStar software.

Table 1. Virus isolation results for the 17 CRS infants (listed chronologically based on the dates of sample collection).

Case (Age)	Location Town/ State-Case	Ocular defect/ Other Abnormalities/ serological data	Vero culture		RK13 culture			RV isolate (sample no.)	RV strain name GB accession no.
			PCR	IF	PCR	IF	CPE		
1 (3 mon)	Trivandrum /Kerala	Nuclear cataract, Microcornea/Cardiac (PDA, VSD)/Rubella IgM+, IgG+	- +	- ++	+ +	- +	- ++	TS (C025) L-Lens (C040)	RVi/Kerala. IND/38.04[2B]CRS KC618667
2 (4 mon)	Sivagangai/ TN	Nuclear Cataract, Microphthalmos, Microcornea, Nystagmus/ Microcephaly, Mental retardation /Rubella IgM+, IgG+	NT + NT	NT +++ NT	+ + +	- + +	- ++ +++	TS (C055) R-Lens (C056) L-lens (C057)	RVi/Tamilnadu. IND/47.04[2B]CRS KC618668
3 (2 mon)	Ernakulam/ Kerala	Cataract, Microphthalmos (RE), Microcornea , Retinopathy, strabismus, nystagmus (BE)/Rubella IgM+, IgG+	NT +	NT +++	+ +	- +	- ++	TS (C043), R-Lens (C041)	RVi/Kerala. IND/49.04[2B]CRS KC618669
4 (10 days)	Ramanathap uram/TN	Microphthalmos, cloudycornea, Glaucoma (BE)/Cardiac disease, Splenomegaly, hepatomegaly, Microcephaly/Rubella IgM+, IgG+	NT	NT	+	-	-	OF (C035)	RVs/Tamilnadu. IND/02.05[2B]CRS** KC618670
5 (6 mon)	Thanjavur/ TN	Total Cataract, Microphthalmos (LE), Microcornea, Iris hypoplasia (LE), pupil rigidity (RE), nystagmus /Rubella IgM+, IgG+	- +	- ++	+ +	- +	- +	TS (C098) L-Lens (C095)	RVi/Tamilnadu. IND/03.05[2B]CRS KC618671
6 (2 mon)	Sivagangai/ TN	Total Cataract (LE), Microphthalmos, Microcornea, Retinopathy/Cardiac (PDA, VSD)/Rubella IgM+, IgG+	NT - +	NT - +++	- NT +	- NT +	- NT +++	OF (C100) TS (C101) L-Lens (C105)	RVi/Tamilnadu. IND/20.05[2B]CRS KC618672
7 (9 mon)	Prakasam/ AP	Cataract (BE), Microcornea (BE)/ Cardiac (PDA)/Rubella IgM+, IgG+	-	-	-	-	-	Lens (C205*)	RVs/Andhra Pradesh.IND/ 33.05[2B]CRS KC618673
8 (3 mon)	Cuddapah/ AP	Cataract (RE)/Low birth weight, small for the age/Rubella IgM+, IgG+	+	++	-	-	-	R-Lens (C134)	RVi/Andhra Pradesh.IND/ 40.05[2B]CRS KC618674

Table 1 continued..

9 (7 mon)	Kollam/ Kerala	RE-Cataract, ASD+PDA; LE-Myopic fundes, RE-Microcornea, Microphthalmos/Delayed milestones No neck contral still/Rubella IgM+, IgG+	NT					R-Lens(C235*)	RVs/Kerala. IND/42.05[2B]CRS KC618675
10 (5 mon)	Alapuzha/ Kerala	Cataract (RE), Microcornea (RE)/No /Rubella IgM+, IgG+	+	+	-	-	-	R-Lens (C188)	RVi/Kerala. IND/46.05[2B]CRS KC618676
11 (6 mon)	Theni/TN	Cataract (BE), soft systolic murmur, microcephaly, pupilrigidity (BE) /CDS, low birth weight/Rubella IgM+, IgG+	+	++	-	-	-	L-Lens (C213)	RVi/Tamilnadu. IND/47.05[2B]CRS KC618677
12 (8 mon)	Mysore/ Karnataka	BE-Cataract, RE-Retinopathy, LE-Microphthalmos, Microcornea/Low birth weight (1.75kg), PDA, ASD/Serum not available	NT					L-Lens (C336*)	RVs/Karnataka. IND/50.05[2B]CRS KC618678
13 (3 mon)	Trivandrum/ Kerala	Cataract (BE), CDS/Cardiac (PDA, small ASD), /Rubella IgM+, IgG+	+	++	-	-	-	R-Lens (C172*)	RVs/Kerala. IND/51.05[2B]CRS KC618679
14 (4 mon)	Dindigul/ TN	Cataract (LE), microphthalmos (LE), iris hypoplasia, microcornea, strabismus, nystagmus/ retinopathy, Cardiac (PDA)/Rubella IgM+, IgG+	+	++	-	-	-	L-Lens (C225)	RVi/Tamilnadu. IND/02.06[2B]CRS KC6186780
15 (5 mon)	Karur/TN	Cataract (BE), micropthalmos (BE), iris hypoplasia, CDS (LE)//Cardiac (ASD with PDA)/Rubella IgM+, IgG+	- - + -	- - ++ -	- - - -	- - - -	- - - -	OF (C491) TS (C492) R-Lens(C497*) L-Lens (C526*)	RVi/Tamilnadu. IND/36.06[2B]CRS KC618681
16 (8 mon)	Karur/TN	Cataract (BE), systolic murmur, microcephaly/Failure to thrive/ Rubella IgM+, IgG+	- - + +	- - + +	- - + -	- - - -	- - - -	OF (C566) TS (C567) L-Lens (C568*) R-Lens (C572)	RVi/Tamilnadu. IND/42.06[2B]CRS ll KC618682 RVi/Tamilnadu.IND/42.06[2B]CRS rl KC618683

Table 1 continued..

17 (1 mon)	Kottayam/ Kerala	Cataract (BE)/Cardiac (ASD with PDA), microcornea (BE), Intra Uterine Growth Retardation (IUGR), Anaemia/Rubella IgM+, IgG+	+	++	-	-	-	R-Lens (C499)	RVi/Kerala. IND/52.06[2B]CRS KC618684

Notes: BE - Both eyes; RE - right eye; LE - left eye; PDA - patent ductus arteriosus; VSD - ventricular septal defect; ASD - atrial septal defect; CAHD - coronary artery heart disease; PAH - pulmonary arterial hypertension; NT - not tested; R - Right eye; L - Left eye; OF- oral fluid; TS - throat swab; Percentage of positivity: -, negative; + 25%; ++ 50%; +++ 75% of CPE/IFA;

* PCR positive for the original sample;

**Epidemic week number based on the estimated birth week and all the others based on the birthday.

RESULTS

CRS confirmation by serological tests

Sera collected from 16 of the 17 infants (excluding case 12 in Table 1) were all positive for anti-rubella IgM and anti-rubella IgG (Table 1).

RV isolation in RK13 and Vero cells

Virus isolation was performed on 28 specimens. Eleven specimens (two OF, five TS and four lenses) were cultured at PHE, UK and 17 specimens (two OF, two TS and 13 lenses) were cultured at the Aravind Medical Research Foundation (AMRF), India (Tables 1). All specimens were initially screened by the nested PCR-E317 as described previously [6], and only positives were submitted for RV isolation.

A total of 27 of 28 specimens (except for the TS from case 6) were inoculated to RK13 cells and 12 rubella viruses were isolated including one from OF, four from TS and 7 from lenses (Table 1). CPE was observed in RK13 cells from the 3rd passage in tubes inoculated with two then five lens specimens at passage 4, but CPE was less visible at passage 5 (Tables 1 and 2). IF was carried out on passage 3 cells and positives were observed for only the seven lens specimens. All 12 isolates were positive by the PCR-E317 [7], which confirmed the viral growth.

A total of 22 of the 28 specimens (16 lenses, four TS and two OF) were simultaneously inoculated into Vero cells, and 14 isolates were obtained from 14 lens specimens (Table 1). CPE was not visible in any of these isolates, but specific immunofluorescence was seen at passage 3 and 4 of Vero cells inoculated with the 14 lenses and virus growth was confirmed by the PCR-E317 [7] (Table 1).

Quantification of viral loads

Passages of both RK13 and Vero cells were harvested and viral loads were measured simultaneously for five isolates by the real-time PCR-Prob56 at PHE, UK. The results (Table 2) showed that the viral loads increased along with every passage up to the 5th in Vero cells. For RK13 cells the viral load decreased following the 4th passage (Table 2). In addition, the copy numbers in the five passages were generally much higher in the Vero cells compared to the RK13 cells. When the passage 4 (P4) of the RK13 cultures were passaged into Vero cells, the viral copy number increased approximately 10-50 times within 7 days of inoculation; meanwhile, the P4 of five RV positive Vero cultures were passaged into RK13 cells and CPE was observed 3 days after the inoculation (Results not shown).

Virus characterization

Figure 1 shows that the 280 nt of E1 sequences [6] generated directly from the five original specimens (C040, C041, C056, C095 and C0105) were identical to those generated following growth in Vero cells. All viruses belonged to genotype 2B. PCR amplification of the 739 nucleotide (nt) sequence of E1 gene [8] was successful for all 14 isolates from Vero cells and seven directly from lens specimens (those with * in Table 1).

Figure 2 was drawn based on the 739 nt sequences of the E1 gene and includes the 14 Vero isolates and the direct sequences for seven strains from clinical specimens (Table 1), and the recommended WHO reference strains [4]. Multiple sequences were generated from different specimens from two infants, cases 15 and 16, and identical sequences were only seen from samples collected in the same patient, e.g. case 15. There was one nt difference between sequences from the left lens and right lens of case 16. The most divergence within these 21 RV strains was 3.5% between sample C188i (case 10) and C213i (case 11), which originated in two different states (Table 1). When compared with the three genotype 2B reference strains the divergences ranged from 0.8% (5 nt) between C040i (case 1) and strain RVi/Seatle.Wash.USA/16.00[2B] (AY968220) to 6.5% between C235 (case 9) or C172 (case 13) and strain RVi/TelAviv.ISR/68[2B] (AY968219). All of the 21 sequences were most closely related to the reference strain RVi/Seatle.Wash.USA/ 16.00[2B] (AY968220) and the divergence was up to 2.5% from C041i (case 3). All 17 infants were infected with genotype 2B viruses. Minor diversities were observed between the isolates/ strains (Figure 2). The geographic origin of the 17 cases reported (Table 1) are shown on the map (Figure 3). Although these cases were collected

Table 2. RV growth in RK13 and Vero cell lines inoculated with five lens samples: Comparison of the viral loads.

Sample ID.	Cells used Confirmed by	Viral load (Copy no.)				
		P1	P2	P3	P4	P5
C040	RK13 CPE	6 -	47 -	113 -	1,045 ++	106 -/+
	Vero IFA	1,304 ND	1,501 ND	16,317 +	22,200 ++	40,913 ND
C041	RK13 CPE	26 -	47 -	406 ++	1,566 ++	544 ++
	Vero IFA	4,751 ND	3,976 ND	6,894 +++	17,862 +++	20,503 ND
C056	RK13 CPE	53	92	319 +	4316 ++	415 +
	Vero IFA	2,707 ND	7,429 ND	2,028 +++	23,104 +++	114,853 ND
C095	RK13 CPE	24 -	108 -	14 -	4,682 ++	1,370 +
	Vero IFA	1,640 ND	4,813 ND	6,327 +++	32,521 +++	21,318 ND
C105	RK13 CPE	11 -	6 -	ND -	2967 ++	488 +
	Vero IFA	4 ND	1279 ND	1926 +++	19739 +++	15297 ND
Mean	RK13 Vero	25 1951	65 3799	279 6698	3238 23085	650 42577

ND: not done

over two years and from four states, genetic variation was detected in the RV sequences. The maximum variation over the 739 nt sequenced was 3.5% (24 nt) between sample C213i (case 11) and C188i (case 10).

DISCUSSION

Rubella infection in India is endemic since vaccination for rubella is not part of the National Immunization Schedule [5]. In India, a number of studies have shown 21-25% of congenital cataract to be a consequence of rubella infection in pregnancy [10, 11].

In this study, 26 RV isolates from 14 out of 16 serologically confirmed CRS cases and one non serologically confirmed CRS case (Case 12) grew both in Vero cells (14 isolates) and RK13 (12 isolates) cells. Lens samples were found to be the best source for RV isolation, and RV was successfully isolated from 87.5% of lenses in Vero cells compared to 41.2% in RK13 cells. In an earlier report, RV was only isolated in BHK

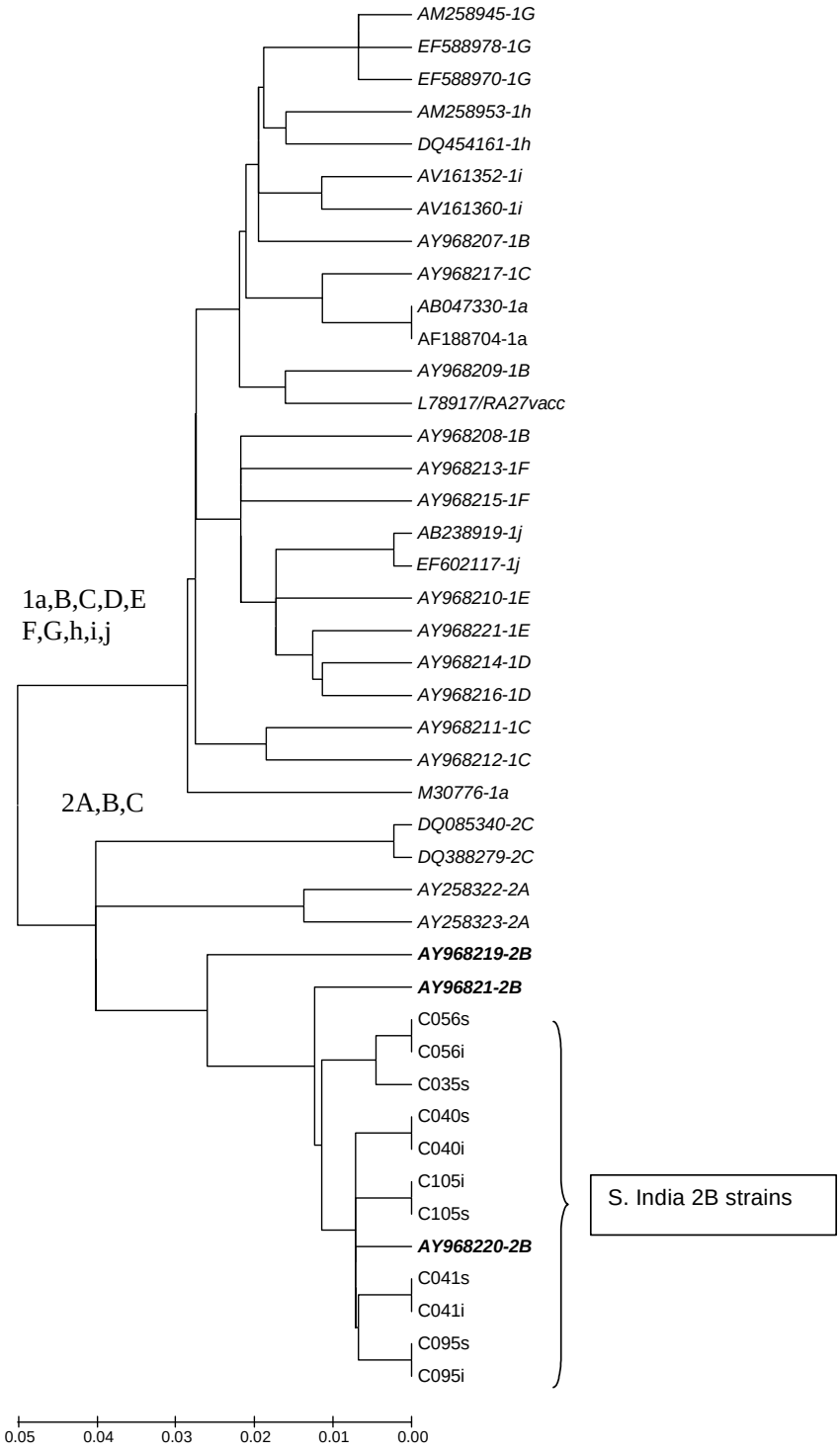

Figure 1. Comparison of RV sequences obtained from original lens specimens and the correlated isolates after three passages in Vero cells. Phylogenetic analysis was based on the 280 nt of E1 gene of the five lens specimens and the 820 nt of E1 gene of correlated isolates (indicated with either "s" or "I" at the end of sample name). All WHO reference strains in italics were included with genotype indicated at the end of GenBank accession numbers. The phylogenetic tree was drawn by bootstrap analysis (1000 times) using the Neighbor-Joining of MEGA software (Version 3.1).

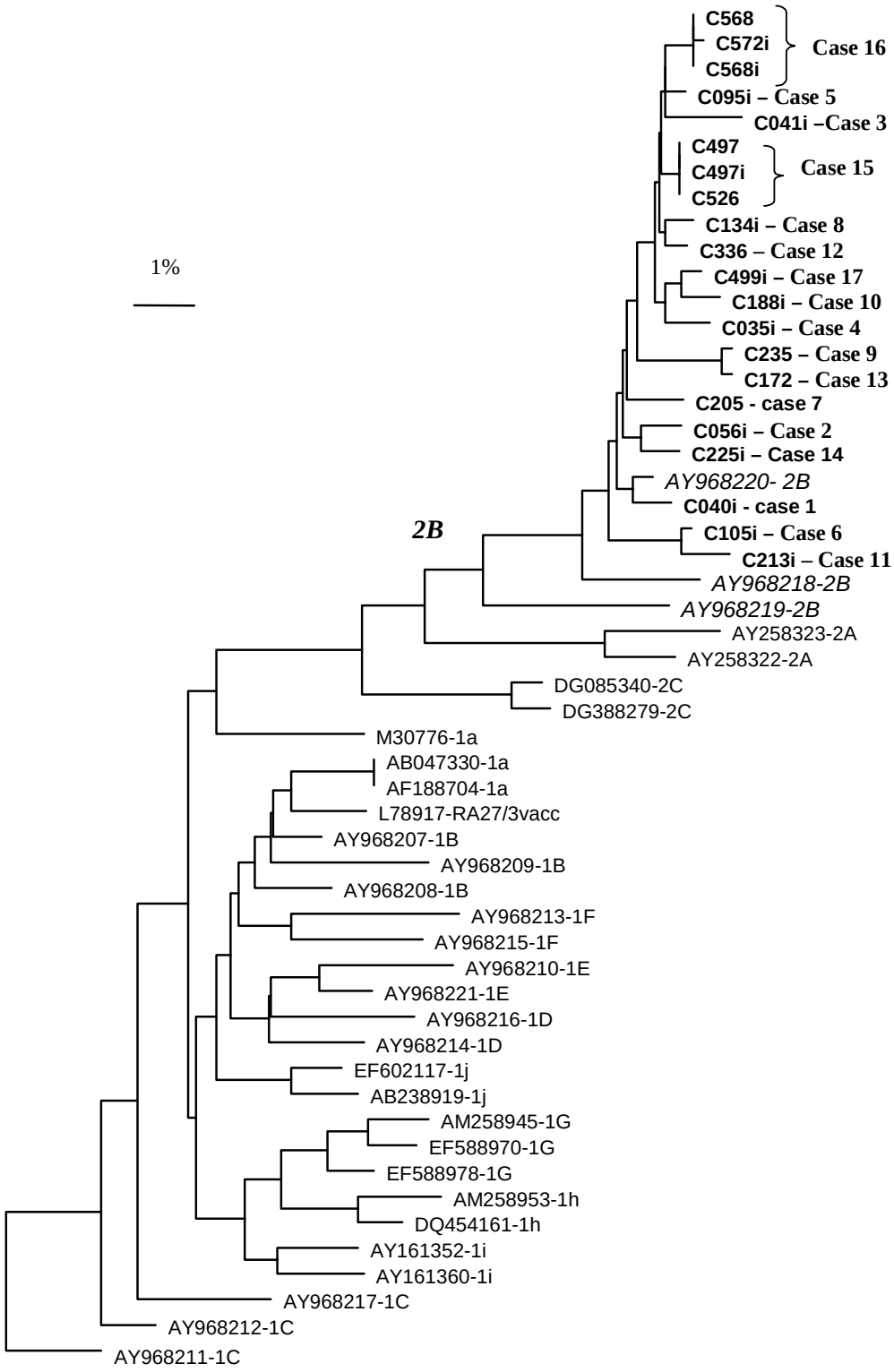

Figure 2. Genetic relationships between the 21 RV strains and the WHO reference strains. The 17 sequences generated from cell culture isolates were indicated with "i" at the end of strain name, otherwise were generated from original specimens. The analysis was based on the 739 nt of E1 gene and the phylogenetic tree was constructed using DNAStar software.

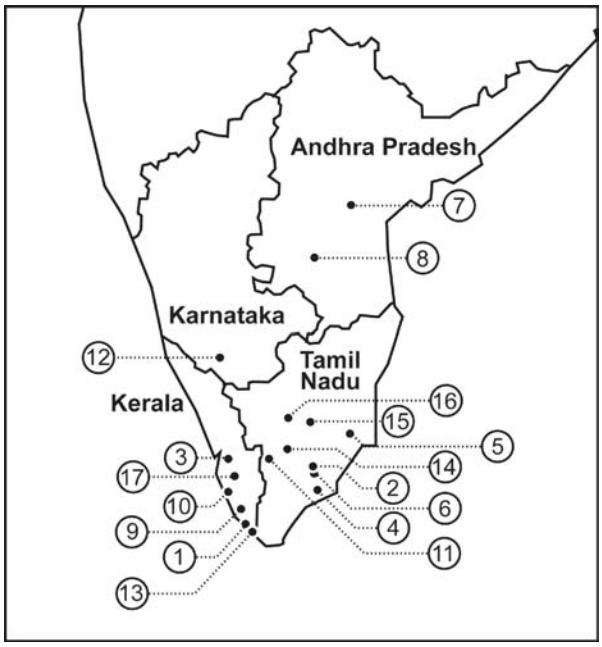

Figure 3. Geographical locations of the 17 cases in the Southern Indian. The 17 CRS infants were numbered chronologically based on the dates of their sample collections as showed in Table 1.

cells from 10% (7/70) of lens aspirates of infants aged between 0-11 months with congenital cataract [12], suggesting that Vero and RK13 cells could be more sensitive than BHK cells for RV isolation. However, the quality of sample collection and storage may also be critical.

It would appear that although lens specimens are optimal for RV isolation in CRS patients with ocular anomalies, other specimens such as TS and OF specimens may have some utility. In this respect, RV was isolated from the OF specimen from case 4, a 10-day old infant (the only specimens available at the time), and the four TS specimens from four infants aged between two and six months. Such findings may reflect chronic vial shedding via the respiratory system, which may last as long as 20 months [13]. OF and TS could be valuable specimens for viral isolation, however, prevention of contamination by anti-bacterial and anti-fungal treatment prior to cell culture inoculation is vital.

RK13 cells may be initially more sensitive than Vero cells in isolating RV as the two isolates from TS specimens, C025 of case 1 and C098 of case 5 (Table 1) did not grow in Vero cells; however, too few specimens were tested in this study to make such a conclusion.

The results showed that Vero and RK13 cells were both suitable for RV isolation; though RV replicated much faster in Vero cells. The quantification of viral loads demonstrated higher viral copy numbers in culture of Vero cells in every passage when compared to the cultures in RK13 cells (Table 2). Interestingly, CPE was observed only in RK13 cells inoculated with lens samples when the viral genome copies were over 300 (Table 2), but not in those with OF or TS samples (Table 1). RV may enter into lens before the development of the lens capsule, which would otherwise act as barrier to the virus [14].

RV growth was also confirmed in cell cultures by IFA which is recommended by the WHO (www.who.int/vaccines-documents) for confirming the viability of virus. The 14 RV isolates grown from lens specimens were all positive in the 3rd passage in Vero cells and six of seven RK13 cell cultures were positive by IFA (Tables 1 and 2). None of the non-lens specimens were positively confirmed by IFA which suggests that lens tissue is a good source of viable RV. Compared with confirmation by PCR, IFA is less sensitive (Table 2). The RV genome quantification in cell cultures suggests that the determination of viral genome copies may be an alternative to IFA for confirmation of viability of RV isolates; for example, Vero culture blind passaged for three or four times in Vero with a copy number over 3,000 could be proposed as preliminary confirmation of viable RV isolates. This study confirms the utility of Vero and RK13 cells in isolating RV from the various clinical specimens of serologically confirmed CRS cases. These findings provide evidence that Vero cell culture could be an alternative for RV isolation as this cell line is sensitive and easy to handle.

Successful RV culture in this study yielded RV isolates which then could be sequenced for genotyping. This allowed an additional 11 CRS cases to be confirmed which was not possible from the original clinical specimens (Table 1) [6]. In total, 21 RV strains from 17 CRS cases were confirmed and genotyped based on the window E1-739 nt sequences (Table 1) including seven

directly from lenses of six infants without cell culture, which suggests the PCR is more sensitive than cell culture for virus detection. Phylogenetic analysis based on the E1-739 nt window recommended by WHO [4] showed that all these 17 CRS cases were due to early gestational infection of genotype 2B viruses. The result confirmed the presence of genotype 2B in the south Indian population as reported previously [6].

The infants studied were aged between 10 days (case 4) and 9 months (case 7) when their specimens were collected. They had resided for varying times during 2004-2006, in 15 different towns of four states in Southern India (Table 1). None of the sequences were 100% identical at the E1-739 nt region analysed (Figure 2) except for sequences obtained from the same patients, e.g., C497, C497i and C526 from case 15, and C568 and C568i from case 16. The divergence within the 21 (17 individuals) sequences was up to 3.5%. One strain (C040i/case 1) was most closely related (99.2% homology, 5/739 nt differences) to the reference strain, RVi/Seatle.Wash.USA/16.00 [2B] (AY968220), previously detected in the USA in year 2000. There was a minimal 2 nt difference between sequences obtained from case 9 and 13, who were born 6-7 months apart in 2005 in the same state, Kerala. It is difficult to determine if multiple 2B strains were circulating during the study period or whether a single 2B strain was predominant; however, point mutation might occur in CRS patients during a long period of incubation, as this was unlikely to be due to technical factors (e.g. cell culture passages and molecular technical errors). Further investigation is required to clarify this.

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