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Short communication

Phylogenetic analysis of rubella viruses found in Morocco, Uganda, Cote d'Ivoire and South Africa from 2001 to 2007☆

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Abstract

Background: Rubella virus (RV) causes a mild disease, but maternal infection early in pregnancy often leads to birth defects known as congenital rubella syndrome (CRS). Rubella remains poorly controlled in Africa.
Objectives: To identify RV genotypes found in Africa to help establish a genetic baseline for RV molecular epidemiology.
Study design: Urine and nasopharyngeal specimens were collected between 2001 and 2004 during measles surveillance in Morocco, Uganda and South Africa, and from two persons in the United States who contracted rubella in Cote d'Ivoire and Uganda in 2004 and 2007, respectively. RV RNA was obtained directly from specimens or from RV-infected cell cultures, amplified by reverse transcriptase polymerase chain reaction, and the resulting DNAs sequenced. Sequences were assigned to genotypes by phylogenetic analysis with RV reference sequences.
Results: Nine RV sequences were assigned as follows: 1E in Morocco, 1G in Uganda and Cote d'Ivoire, and 2B in South Africa.
Conclusions: Information about RV genotypes circulating in Africa is improved which should aid in control of rubella and CRS in Africa.

Keywords: Rubella virus; Genotype; Molecular characterization; Sequences

1. Introduction

Rubella virus (RV) infection during the early stages of pregnancy can lead to serious birth defects, known as congenital rubella syndrome (CRS). Although a vaccine-preventable disease, rubella still accounts for an estimated 100,000 CRS cases annually worldwide (Robertson et al., 2003). Rubella remains endemic in most of Africa; as of 2004, only Morocco, Tunisia, Libya, and Egypt included rubella vaccine in their national immunization programs (World Health Organization (WHO), 2006a). For most of Africa, little surveillance data for rubella or CRS are available (Bloom et al., 2005).

RV is a positive sense, single-stranded RNA virus in the genus *Rubivirus* within the Togaviridae family. Although known RVs are a single serotype, sufficient genetic variation exists to allow the molecular epidemiology of wild-type RVs to be used in control and elimination efforts (Icenogle et al., 2006). A systematic nomenclature for wild-type RVs (WHO, 2005, 2007) divided rubella viruses into two clades containing nine genotypes (designated by clade and a letter

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designation: 1B, 1C, 1D, 1E, 1F, 1G, 2A, 2B and 2C), and four provisional genotypes (1a, 1h, 1i, and 1j).

General summaries of RV genotypes found in the world, including limited information from Africa, have been reported (WHO, 2005, 2006b); the sequence information, phylogenetic analysis, and virus circulation information reported here applies to four countries in Africa.

2. Materials and methods

2.1. Specimens

Four urine specimens from Morocco and two from South Africa were collected from rubella IgM-positive persons with rash illness. A nasal aspirate from Uganda was obtained from a suspected measles case from which measles virus could not be isolated. A throat swab and a urine specimen collected in NH, USA were from an infant with CRS whose mother had been infected with rubella early in her pregnancy in Cote d'Ivoire (Centers for Disease Control and Prevention (CDC), 2005; Plotinsky et al., 2007). A throat swab specimen was collected in 2007 in MA, USA, from a person who developed rash illness 1 week after returning from a trip to Uganda.

2.2. Laboratory methods

Virus isolations and RNA extractions were done as previously described (Frey et al., 1998). The presence of virus in cultures of Vero cells was detected by a reverse transcription-polymerase chain reaction (RT-PCR) which amplified a 185-nucleotide (nt) fragment of the E1 coding region. The detailed description of this 185-nt RT-PCR including its sensitivity, specificity, and a comparison of it with other assays including an immunofluorescent assay has been described (Zhu et al., 2007). A 1124-nt fragment of the E1 coding region containing the 739-nt WHO-recommended sequence window (nts 8731–9469) was amplified from positive cultures using the Titanium One Step kit (BD Bioscience, San Jose, CA) and the resultant DNA used for sequencing. The sensitivity of the 1124-nt reaction was determined to be 3000 copies of rubella RNA using known copy number transcribed RNA as the template. The 1124-nt reaction mix consisted of 1× kit buffer, 1× dNTP mix, 20 units RNase inhibitor, 1× thermostabilizing reagent, 1× GC-melt, 1× RT-Titanium *Taq* enzyme mix, 0.45 μM of primers RV8656 (5′ CCCCACCGACACCGTGATGAG) and RV3′ (5′ TTTTTTTTTTTTTTTTTTCTATACAGCAACAGGTGC), and 1/50 of the extracted RNA. The parameters for amplification were 50 °C for 1 h, 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 68 °C for 1 min.

For RNA extracted directly from clinical specimens, either 250 μl of specimen and Tri-Reagent for liquid samples (Molecular Research Center, Cincinnati, OH) or 140 μl of specimen and the QIAamp Viral Mini Kit (Qiagen, Valencia, CA) was used. The small amounts of RV RNA present in clinical specimens made nested RT-PCR reactions necessary for amplification of sufficient DNA for sequencing. Two pairs of primers were chosen to amplify a 722-nt fragment of the E1 coding region containing a 601-nt region to be sequenced (nts 8869–9469) using the Titanium One Step kit as described above. One-tenth of the extracted RNA was added to the 50-μl reaction mix containing 0.45 μM of primers RV8812 (5′ CAACACGCCGCACGGACAAC [Bosma et al., 1995]) and RV3′. After amplification, 3 μl of the first round product was transferred to the second round PCR reaction containing 0.45 μM of primers RV8823 (5′ ACGGACAACTCGAGGTCC) and RV9545 (5′ TGGTGTGTGTGCCATAC); the 1-h reverse transcription segment was omitted. Sensitivity of this nested set was determined to be 30 RNA copies, using transcribed RV RNA.

2.3. Sequencing and data analysis

RT-PCR products, purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) were sequenced using Big Dye fluorescent dye terminators (Applied Biosystems (ABI), Foster City, CA). Reaction

Table 1
Wild-type rubella viruses from Africa

WHO name/genotype[a]	Country of origin	Age at onset	Specimen type	IgM results	Accessions Number
RVs/Berkane.MAR/24.02[1E]	Morocco	7 years	Urine	Positive	EF588973
RVs/Oujda.MAR/23.04[1E]	Morocco	7 years	Urine	Positive	EF588971
RVs/Taroudant.MAR/22.04[1E]	Morocco	5 years	Urine	Positive	EF588972
RVs/Tetouan.MAR/29.04[1E]	Morocco	10 years	Urine	Positive	EF588974
RVi/UGA/20.01[1G][b]	Uganda	2 years	Nasal aspirate	Unknown	EF588978
RVi/Boston.MA.USA/13.07[1G]	Uganda	38 years	Throat swab	Positive	EF588977
RVs/Westonaria.ZAF/47.03[2B]	South Africa	5 years	Urine	Positive	EF588976
RVs/Vereeniging.ZAF/2.04[2B]	South Africa	46 years	Urine	Positive	EF588975
RVi/Lebanon.NH.USA/3.05[1G]	Ivory Coast	Birth[c]	Throat swab, urine	Positive	EF588979

[a] Date of specimen collection is indicated by week and year (e.g., 24.02).
[b] This virus is a WHO approved reference virus for genotype 1G. Other indicated genotypes are based on figure.
[c] Specimens collected 3 months after birth; CRS infants can remain virus positive for up to 1 year of age (Bellini and Icenogle, 2007).

products were analyzed with an ABI 3100 (ABI, Foster City, CA) automatic sequencer. Sequence data were aligned by using Version 10.3 of the Genetics Computer Group Package (Accelrys, San Diego, CA). Phylogenetic analyses were performed using the Bayesian analysis program, MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). Sequences obtained were compared with WHO-recommended reference virus sequences and candidate reference sequences (WHO, 2007).

3. Results

Virus isolations from cell culture were successful for the two specimens from Uganda and the one from Cote d'Ivoire. Attempts to isolate virus from the four Moroccan and two South African specimens were unsuccessful, but sufficient amounts of RV RNA were obtained directly from the specimens to produce fragments for sequencing using the nested RT-PCR (Table 1).

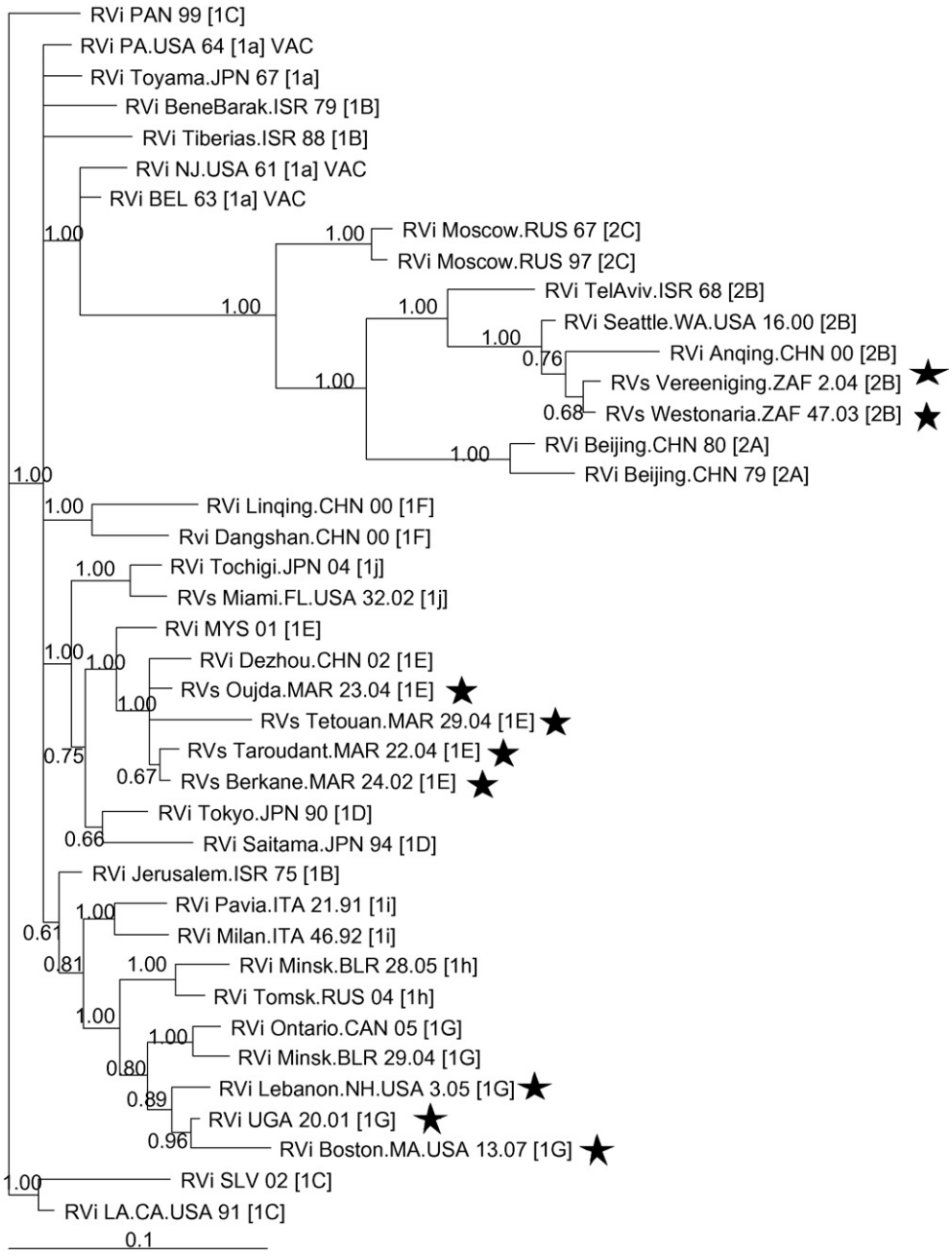

Fig. 1. Phylogenetic tree of sequences of 601 nts (8869–9469) of the E1 coding region of wild-type rubella viruses from four African countries. The unrooted tree was made using the MrBayes 3.0 program with 200,000 genes, burnin of 400, and the gamma-distributed rate variation model of DNA substitution. Nodes with clade credibility values ≥0.6 are indicated on the trees. The sequences used to construct the trees are the 32 WHO reference strains (WHO, 2007) and the African sequences (marked). One African virus (RVi UGA 20.01[1G]) is a WHO reference virus. The Canadian IG reference virus was imported into Canada from the Netherlands.

The 601-nt region was sequenced because this region has been used effectively for RV phylogeny (Reef et al., 2002). Analysis of the 601-nt sequences showed that the nine African sequences fell into three different genotypes; these assignments were supported by high clade credibility values (Fig. 1). The Uganda and Cote d'Ivoire sequences branched with the two 1G reference sequences; the 2001 Uganda sequence is also a 1G reference sequence (WHO, 2007). The four Moroccan sequences grouped with sequences of genotype 1E reference viruses. The two South African sequences grouped with sequences of genotype 2B reference viruses.

The recent update of the WHO-recommended nomenclature for wild-type rubella viruses did not affect genotypes 1E or 2B (WHO, 2007). However, it did reorganize viruses from provisional genotype 1g into 1G, 1h, and 1i. Sequences for the WHO-recommended 739-nt sequence region were obtained for the three 1G RVs (UGA/01, Lebanon.NH.USA/05, and Boston.MA.USA/07) and were compared with this region of reference and candidate reference sequences for 1G, 1h and 1i. All three were confirmed to be genotype 1G (data not shown).

4. Discussion

Phylogenetic analysis of RV sequences showed that at least three genotypes of RV were present in Africa during 2001–2007. Genotype 1E viruses were identified in Morocco in 2002 and 2004, suggesting that viruses of this genotype were circulating in this country. Viruses of genotype 1E have previously been found to have worldwide distribution (WER, 2005, 2006b). Viruses of genotype 1G were found in specimens from a case in Uganda from 2001 and from cases in the USA with exposures in Cote d'Ivoire in 2005 and Uganda in 2007. The importation from Cote d'Ivoire was linked to a documented rubella outbreak in that country (CDC, 2005; Plotinsky et al., 2007). These data indicate that viruses of genotype 1G were circulating in Uganda and perhaps throughout central Africa. Such viruses have previously been found in Europe, Brazil, and Israel (Hubschen et al., 2007; WHO, 2005, 2006b, 2007). Recently, 1G viruses collected in 2004 in another African country, Ethiopia, were reported (Jin and Thomas, 2007). Interestingly, the three African 1G sequences segregate on a branch separate from the other two 1G reference sequences. Collection of additional African viruses is needed to determine if African 1Gs can be tracked separately from other 1G viruses. The two genotype 2B viruses were found in South Africa 7 weeks and 60 miles apart, suggesting that this genotype was circulating in this country. Genotype 2B viruses were previously known to circulate in India, China and South Korea (WHO, 2005, 2006b).

Collection of genetic baseline data on RVs was identified by the 2004 WHO Steering Committee on Research Related to Measles and Rubella Vaccines and Vaccination as necessary to improve global control of rubella and CRS (Best et al., 2005). RV molecular epidemiologic data have contributed significantly to rubella control efforts (e.g., elimination of rubella from the United States) and also to the understanding of the worldwide genetic diversity of RVs (Best et al., 2005; Icenogle et al., 2006). As more molecular epidemiologic data become available for Africa, they could be effectively used in rubella and CRS control for this continent as well.

Conflict of interest statement

None.

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