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Phylogenetic Analysis of Rubella Viruses Identified in Uganda, 2003–2012

Prossy Namuwulya[1,*], **Emily Abernathy**[2], **Henry Bukenya**[1], **Josephine Bwogi**[1], **Phionah Tushabe**[1], **Molly Birungi**[1], **Ronald Seguya**[1], **Theopista Kabaliisa**[1], **Vincent P. Alibu**[3], **Jonathan K. Kayondo**[1], **Pierre Rivailler**[2], **Joseph Icenogle**[2,**], and **Barnabas Bakamutumaho**[1]

[1]Uganda Virus Research Institute, Entebbe, Uganda [2]Division of Viral Diseases, National Center for Immunizations and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia [3]Makerere University, Kampala, Uganda

Abstract

Molecular data on rubella viruses are limited in Uganda despite the importance of congenital rubella syndrome (CRS). Routine rubella vaccination, while not administered currently in Uganda, is expected to begin by 2015. The World Health Organization recommends that countries without rubella vaccination programs assess the burden of rubella and CRS before starting a routine vaccination program. Uganda is already involved in integrated case-based surveillance, including laboratory testing to confirm measles and rubella, but molecular epidemiologic aspects of rubella circulation have so far not been documented in Uganda. Twenty throat swab or oral fluid samples collected from 12 districts during routine rash and fever surveillance between 2003 and 2012 were identified as rubella virus RNA positive and PCR products encompassing the region used for genotyping were sequenced. Phylogenetic analysis of the 20 sequences identified 19 genotype 1G viruses and 1 genotype 1E virus. Genotype-specific trees showed that the Uganda viruses belonged to specific clusters for both genotypes 1G and 1E and grouped with similar sequences from neighboring countries. Genotype 1G was predominant in Uganda. More epidemiological and molecular epidemiological data are required to determine if genotype 1E is also endemic in Uganda. The information obtained in this study will assist the immunization program in monitoring changes in circulating genotypes.

Keywords

genotype; molecular characterization; sequences; rubella epidemiology

*Correspondence to: Prossy Namuwulya, EPI-LAB, Uganda Virus Institute, 51-59 Nakiwogo Rd., P.O. Box 49, Entebbe, Uganda (rubella epidemiology and virus characterization). pnamuwulya@uvri.go.ug. **Correspondence to: Joseph Icenogle, PhD, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, Georgia 30333 (sequence analysis and interpretation). jci1@cdc.gov.

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INTRODUCTION

Rubella, a disease caused by the rubella virus (RV), is characterized by fever and rash and is easily passed from one person to another through respiratory secretions. Its public health importance arises from the ability of RV to infect the fetus. Of infants born to mothers, 75–90% infected with RV during the first trimester of pregnancy have congenital defects [Andrade et al., 2006; Reef et al., 2011]. The spectrum of defects includes hearing loss, cardiovascular abnormalities, cataracts, and developmental delays and is known collectively as congenital rubella syndrome (CRS). The global burden of CRS is estimated at 110,000 cases per year [Robertson et al., 2003].

Many persons residing in African countries, including Uganda, remain susceptible to rubella infection due to low levels of rubella vaccination and approximately 5% of reported rubella cases occur in women of reproductive age, suggesting that CRS is a public health burden [Goodson et al., 2011]. The World Health Organization (WHO) has recommended that countries take the opportunity of the current goals of control and elimination of measles to introduce rubella vaccines in combination with other vaccines [WHO, 2012]. The WHO recommends that countries like Uganda, without rubella vaccination programs, assess the burden of rubella and CRS before the introduction of a routine vaccination program. Routine rubella vaccination is expected to begin in Uganda by 2015 [GAVI Alliance, 2012]. Uganda is already involved in integrated case-based surveillance including laboratory testing to confirm measles and rubella.

Circulating RV genotypes, which can be critical for monitoring the efficacy of control programs, have so far not been documented in Uganda. Only one previous report [Caidi et al., 2008] has identified RV genotypes from Uganda: a 1G virus collected in Uganda in 2001 and a 1G virus imported into the United States from Uganda in 2007. In order to characterize the baseline of endemic rubella genotypes in Uganda, positive viral samples collected from 12 districts located throughout the country from outbreaks over several years were analyzed to determine the distribution of rubella genotypes in Uganda. This report expands our knowledge of wild-type RVs in Uganda from 2003 through the first quarter of 2012.

MATERIALS AND METHODS

Sample Collection and Serology Testing

Throat swabs and oral fluids obtained using Oracol devices (Malvern Medical, Worcester, UK) were collected from patients who had symptoms of rash and fever. All the samples were collected and transported in boxes containing ice packs during outbreak investigations as part of routine measles-surveillance between March 2003 and April 2012. From 2003 until 2007, anti-rubella IgM ELISA testing was performed on measles-surveillance serum samples that tested negative or indeterminate for measles IgM. Starting in 2007 all measles-surveillance sera were tested in parallel for measles and rubella. Data on rubella cases in Uganda were obtained from summaries produced by the Measles Reference Laboratory at the Uganda Virus Research Institute, covering the time period from January 2003 to April

2012. Laboratory testing for rubella IgM antibody was performed using the Enzygnost Anti-Rubella Virus IgM kit (Siemens, Marburg, Germany).

Identification of Rubella Viruses

Oracol samples were processed as described in Abernathy et al. [2009] for direct RNA extraction. Oracol samples used for inoculation were eluted in the viral growth media used to collect the throat swabs. All throat swabs and the Oracol samples eluted in growth media were inoculated onto an 80–90% confluent layer of Vero/SLAM cells in T_{25} flasks [Ono et al., 2001]. After 5 days, the culture media from the flask was harvested and passed to a second flask with a confluent monolayer of cells. Since RVs from clinical samples generally do not exhibit cytopathic effect, the presence of the RV was confirmed by RNA extraction, followed by reverse-transcription PCR (RT-PCR), for all samples and also by detection of the RV E1 protein in infected cells using an immunocolorimetric assay (ICA) [Chen et al., 2007] in a subset of samples. RNA was extracted from both harvests of cultured materials and directly from the clinical materials using the QIAamp Viral RNA Extraction Mini Kit (QIAGEN, Valencia, CA). The presence of RV RNA was detected using an RT-PCR assay which amplified a 185-nucleotide (nt) fragment of the E1 coding region [Zhu et al., 2007]. The RT-PCR assay described by Zhu et al. was modified by the addition of a second reverse primer (RV12-2: CCACGAGCCGCGAACAGTCG), which enhances detection of clade 2 RVs by changing three of the nucleotides in the primer sequence to match the clade 2 consensus sequence for the region, and by the use of the Qiagen OneStep RT-PCR kit.

Genotyping RT-PCR and Sequencing Assays

RNA extracts of the samples that were positive in the 185-nt RT-PCR assay were used as templates in two genotyping RT-PCRs using the Qiagen OneStep RT-PCR kit and primers which amplify two overlapping fragments that encompass the 739-nt sequence required for genotype assignment [WHO, 2005]. One set of primers (8633F: AGCGACGCGGCCTGCTGGGG and 9112R: GCGCGCCTGAGAGCCTATGAC) amplifies a 480-nt portion of the RV genome and the second set (8945F: TGGGCCTCCCCGGTTTG and 9577R: CGCCCAGGTCTGCCGGGTCTC) amplifies a 633-nt portion of the RV genome. The cycling conditions were 50°C for 30 min, 95°C for 15 min followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and finally a 10-min 72°C incubation. The PCR products were purified using ChargeSwitch PCR Clean-Up Kit (Life Technologies, Grand Island, NY). Sequencing was carried out using the BigDye Terminator version 3.1 cycle sequencing kit (Life Technologies) and a model 3130 ABI Genetic Analyser (Life Technologies). The two sequences for each sample were aligned and edited to generate a 739-nt sequence using MEGA version 4.0.2 [Tamura et al., 2007].

Phylogenetic Analysis

Genotype assignments for the 20 Uganda 739-nt sequences were determined following the recommendation of the WHO [2005] as follows: an alignment of the Uganda sequences with the 32 WHO rubella reference sequences, made using the ClustalW program in the Mega5.2 software package [Tamura et al., 2011], was used to construct a tree using the MEGA5.2 neighbor-joining algorithm. Genotypes were determined based on clustering patterns of the

unknown virus sequences with sequences of reference viruses of known genotype. There were approximately 65 sequences 739-nt or longer for genotype 1G viruses in GenBank and 46 of these were used with 19 Ugandan viruses to create a genotype 1G-specific tree. The years of collection were from 1991 to 2012; some identical sequences and very similar sequences from the same country and year were not included. The number of 1E sequences of 739-nt and longer in GenBank is considerably higher, approximately 350, 68% of which were derived from surveillance in China. To construct a genotype-specific 1E tree, 56 representative 1E sequences from 1997 to 2012 and 1 Uganda 1E sequence were used. Highly similar sequences from countries with multiple sequences such as China, Russia, and France were omitted. Phylogenetic analysis for the genotype-specific trees was done using the MEGA5.2 neighbor-joining algorithm with 1,000 bootstrap replicates. Mean genetic distances within and between groups were computed using the MEGA5.2 Distance program.

RESULTS

Rubella Incidence in Uganda

The percent of measles-surveillance serum samples that were positive for rubella is shown in Figure 1. Increases in rubella cases can be seen in the years 2004–2005, 2007–2008, and 2011. The majority of cases identified as rubella during the study period were older than 5 years; however, it is not known how many were of child-bearing age.

Rubella Virus Detection

Before 2010, the focus was primarily on measles and few rubella outbreaks were investigated. A small number of throat swab samples were collected from three outbreaks in 2003, 2008, and 2009. Reductions in measles cases and greater interest in rubella control starting in 2010 led to an increased identification of rubella outbreaks. When at least five cases of suspected measles were reported from the same area, sera were tested in parallel for measles and rubella IgM. If three or more sera tested IgM positive for rubella, a confirmed rubella outbreak was reported and investigators were sent to collect blood and viral samples from persons with rubella clinical symptoms. From 2010 to April 2012 such investigations resulted in collection of a total of 117 study samples comprised of 64 (54.7%) throat swabs and 53 (45.3%) oral fluids from 16 districts in Uganda. The WHO recommends sequencing of representative samples from outbreaks [WHO, 2005]; therefore, in cases where both throat swabs and oral fluids were collected from the same patient, only the throat swab was tested. Samples that were collected later than 7 days from onset were also not tested. Thus, a total of 68 of the 117 samples (58%) from the 16 districts were tested by the 185-nt RT-PCR, resulting in a positivity rate of 50% (34/68). Fourteen of the 34 185-nt RT-PCR positive samples from 2010 to 2012 (from 9/16 districts) and 6 of the isolates from previous years (from 3 districts) gave strong genotyping RT-PCR template production and sequencing results and were used for phylogenetic analysis (Table I). Nineteen of the viruses that were sequenced were isolates from cell culture (confirmed by RT-PCR and/or ICA) and one virus was sequenced directly from an oral fluid sample.

Phylogenetic Analysis

Phylogenetic analysis of the 20 Uganda sequences with the 32 WHO rubella reference sequences showed that two genotypes, 1G and 1E, were identified in Uganda between 2003 and the first quarter of 2012 (data not shown). Of the 20 sequences obtained, 19 (95%) belonged to genotype 1G and 1 sequence belonged to genotype 1E. In order to further analyze the sequences, 46 additional 1G sequences and 56 additional 1E sequences were obtained from Gen-Bank and used to create genotype-specific trees. Phylogenetic analysis of the genotype 1G sequences yielded a complex branching pattern for the 19 Ugandan viruses (Fig. 2A). Eighteen of the Uganda sequences formed a cluster with 86% bootstrap support. This cluster, however, can be further divided into two main groups, lineages 1 and 2. Lineage 1 contains 14 Uganda viruses from eight districts; other viruses in this group are a virus from a CRS case imported into the United States from Tanzania in 2012, and a virus from Great Britain identified in 2012. Collection years for all viruses in this group range from 2003 to 2012. The mean intragroup genetic distance is 0.5%. The second main group (lineage 2), a deeper branch with 96% bootstrap support, contains two viruses from Oyam from 2009 and two viruses from 2011 (Kabarole and Mityana), as well as a virus imported into the United States from Uganda in 2007 and a virus imported into Great Britain from Africa (country not specified) in 2004. The mean intragroup genetic distance for this cluster is higher at 1.3%. The 19th 1G Ugandan virus (Kiboga.UGA/18.03) grouped separately with a Ugandan virus identified in 2001, which also serves as a reference virus for the 1G genotype; this branch (lineage 3) also had high bootstrap support (84%). Mean intergroup genetic distances between the three lineages range from 1.8% between lineages 1 and 3 to 2.7% between lineages 2 and 3. No Uganda sequences were found on any of the other branches on the tree and the mean genetic distance between a group composed of lineages 1, 2, and 3 and the other sequences on the genotype 1G tree is 3.1%.

Phylogenetic analysis of the 1E genotype (Fig. 2B) showed that the Uganda 1E sequence (Arua.UGA/36.11) was part of a four-sequence cluster with 97% bootstrap support and mean intragroup genetic distance of 1.4%. The mean genetic distance between this cluster and the other 1E viruses is 2.8%. In addition to the Uganda virus, this cluster contains two viruses imported in the United States (one CRS case from Sudan in 2012 and one in 2008 from an unknown source) and one virus from Sudan collected in 2005.

Temporal and Geographic Distributions of Genotype 1G

Temporally, lineage 1 was detected in 2003, 2008, 2010, 2011, and 2012, while lineage 2 was identified in 2007 (one virus imported into the United States from Uganda), 2009, and 2011 (Fig. 3). Lineage 3 was detected in 2001 and 2003. In terms of geographic distribution (Fig. 3), in one district (Kiboga), viruses of two lineages (1 and 3) were identified in the same year (2003). Single lineages were associated with outbreaks in 2008 (lineage 1 in Kasese), 2009 (lineage 2 in Oyam), and 2010 (lineage 1 in Jinja). In 2011, five districts had outbreaks, two caused by lineage 2 virus (Mityana and Kabarole) and three caused by lineage 1 virus (Tororo, Rakai, and Luwero). In the first 4 months of 2012, two outbreaks of lineage 1 viruses occurred in Lwengo and Kiryandongo. Almost all of the districts experiencing outbreaks were relatively rural, with only Arua and Kasese containing urban centers with populations of 100,000 or more.

DISCUSSION

This study is the first report of a detailed molecular examination of RVs in a central African country and increases by almost fourfold the number of RV sequences collected from central African countries that are available in GenBank. The study investigated the genotype distribution of RVs in Uganda during 2003 and 2008–2012 to establish a genetic baseline before routine rubella vaccination is implemented. In addition, this study provides information of interest to the global distribution of RVs as very few studies of RV circulation using molecular epidemiological methods have been performed in countries with low rates of vaccination or no vaccination program [Tran et al., 2012]. Genotype 1G viruses were found to be widely distributed in Uganda, occurring in 11 out of the 12 districts from which virus sequences were obtained. Interestingly, the Uganda 1G viruses segregated into at least three distinct lineages. The high bootstrap support for these lineages (86% for lineages 1 and 2, 96% for lineage 2, and 84% for lineage 3) indicates that these are phylogenetically distinct clusters. The literature is not consistent on the terminology for sub-genotype designations. Zhu et al. [2012] used the term cluster, while Donadio et al. [2003] and Tran et al. [2012] used the term lineage as was done here. One of the viruses from 2003 grouped with the first Uganda RV identified in 2001 [Caidi et al., 2008], but this lineage was not detected in later years. The largest group (lineage 1) contained viruses from 2003 through 2012 from eastern, central, and western districts. In addition, a virus that originated in Tanzania, a neighboring country was located in this lineage. There was remarkably little sequence variability of 0.5% seen in this lineage over a 9-year period. Viruses from lineage 2, which exhibited over twofold higher sequence variability, were collected between 2004 and 2011 and of the four viruses collected in Uganda, two were from an outbreak in a northern district and two others were from western and central districts. These findings suggest that considerable variation exists within genotype 1G in Uganda and that the transmission patterns within the country (and possibly with neighboring countries) are consistent with co-circulation of different lineages. It is interesting to note that lineages 1 and 2 were mostly identified in alternating years from 2007 to 2012, the exception being 2011. Patterns of co-circulation of different RV lineages of one genotype within a country have been reported previously [Donadio et al., 2003; Tran et al., 2012; Zhu et al., 2012]. Other lineages of genotype 1G have been found in Ethiopia, Europe, and western Africa (Cote d'Ivoire and Ghana) (Fig. 2A).

A virus of genotype 1E was identified in the Arua district, located in northwestern Uganda in 2011 (Fig. 3). Further viral surveillance will be required to determine whether this is an endemic strain as this is the first documentation of the presence of genotype 1E in Uganda. On the genotype 1E tree, the Uganda virus branched with a virus collected in the neighboring country of Sudan in 2005, as well as a virus from Sudan imported into the United States in 2012, suggesting that similar viruses are likely endemic in the region. Other lineages of 1E are distributed widely globally, primarily in Europe and Asia [Abernathy et al., 2011].

Molecular epidemiology and phylogenetic analyses have become important tools in monitoring virus circulation and the progress of elimination efforts, and these baseline data should be useful in the near future for classifying RVs found in Uganda as either indigenous

or imported. In particular, the genetic data can help confirm the sources of virus or suggest a source(s) for unknown source cases as well as to establish links between various outbreaks. The ability to identify phylogenetically distinct lineages within genotypes will likely improve the utility of virologic surveillance for monitoring the presence or absence of endemic lineages, for identifying replacement of one lineage for another, and for providing more specific information about the effectiveness of control programs.

Virologic surveillance has helped to document the interruption of transmission of endemic measles and rubella in some regions [Icenogle et al., 2011]. The molecular characterization of RVs has provided a valuable tool for measuring the effectiveness of control programs and this will likely be true in Uganda where the vaccine has not yet been introduced. Even countries that have rubella vaccination programs experience outbreaks because of the presence of susceptible individuals and viral importations from endemic areas. Elimination and maintenance of elimination of this viral disease requires achieving very high levels of population immunity and good laboratory-based surveillance to rapidly detect and control periodic outbreaks. Although the rubella case data presented are not an accurate measure of rubella incidence in Uganda due to the fact that rubella is not reportable in Uganda and sera were collected from cases that met the measles case definition, it does provide additional data contributing to an understanding of the periodicity of rubella in Africa [Goodson et al., 2011]. The increase in incidence of rubella seen in 2011 in Uganda is interesting in the light of imported CRS cases into the United States from Africa in 2012 [CDC, 2013]. Although none were from Uganda, two were from neighboring countries (Sudan and Tanzania), which may have had concurrent increases in rubella incidence.

There is a need to determine more viral sequences from other districts of Uganda in subsequent years to increase understanding of transmission patterns of 1G lineages within the country and to determine if genotype 1E viruses are more prevalent than the current data suggests, as well as to detect any other genotypes that may be present or introduced into the country.

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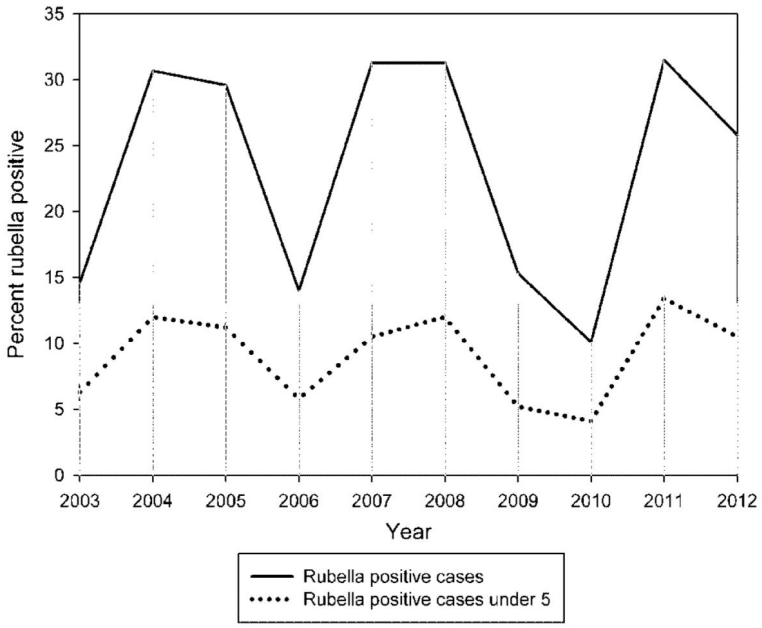

Fig. 1. The percentage of serum samples collected for measles-surveillance purposes in Uganda that tested positive for rubella IgM by year of collection.

The numbers for 2012 reflect data collected in the first quarter of 2012.

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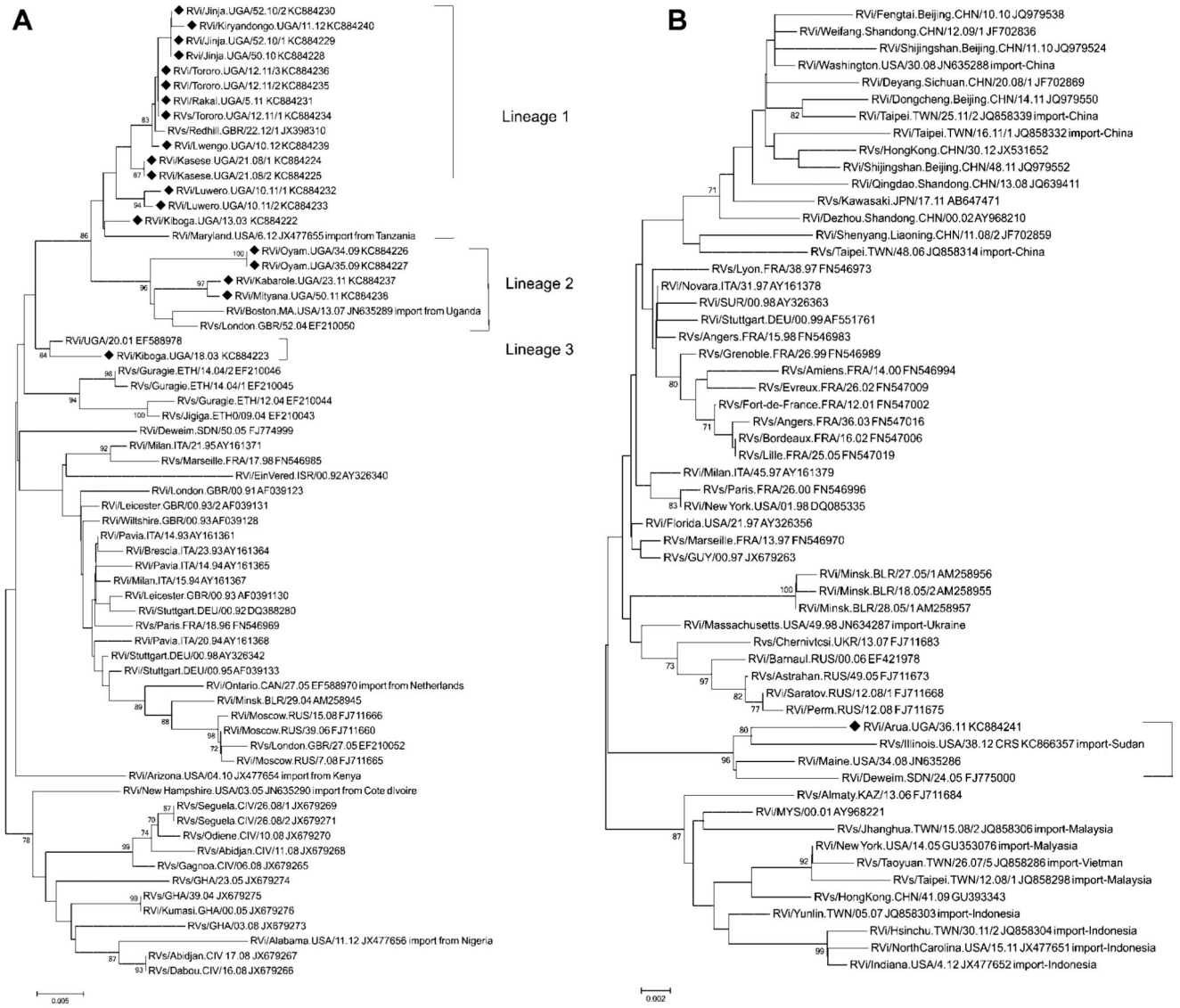

Fig. 2. The genetic relationships of the 739 nt sequences were inferred using the Neighbor-Joining method.

The bootstrap consensus tree inferred from 1,000 replicates is shown with bootstrap values of 70% or greater shown at the appropriate nodes. The genetic distances were computed using the Maximum Composite Likelihood method and are in units of the number of base substitutions per site. All taxa are labeled with WHO names and GenBank accession numbers. **A**: The tree is composed of 65 rubella virus genotype 1G sequences; the 19 Uganda sequences in this report are marked with a black diamond. Lineages are denoted by brackets. **B**: The tree is composed of 57 rubella virus genotype 1E sequences; the group containing the one Uganda sequence (marked with a black diamond) is denoted with brackets.

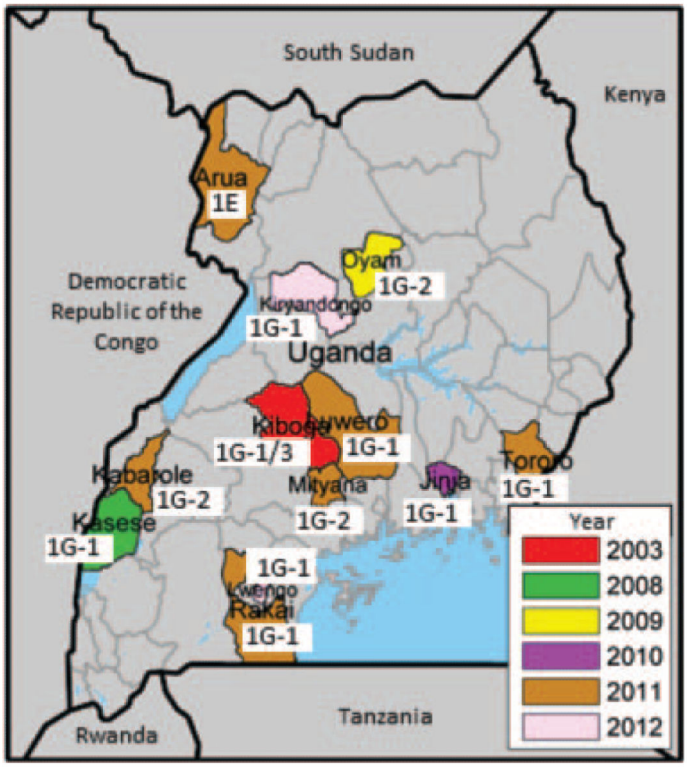

Fig. 3. A map of Uganda in which the 12 districts where the 20 Uganda viruses were collected are color coded by year.

The distribution of genotypes and lineages of 1G in the 12 districts are also shown. The shape files used to construct the maps were obtained from http://www.gadm.org/.

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TABLE I

Rubella Viruses Identified in This Study

WHO Name[a]	Accession number	Year	Genotype
RVi/Kiboga.UGA/13.03	KC884222	2003	1G
RVi/Kiboga.UGA/18.03	KC884223	2003	1G
RVi/Kasese.UGA/21.08/1	KC884224	2008	1G
RVi/Kasese.UGA/21.08/2	KC884225	2008	1G
RVi/Oyam.UGA/34.09	KC884226	2009	1G
RVi/Oyam.UGA/35.09	KC884227	2009	1G
RVi/Jinja.UGA/50.10	KC884228	2010	1G
RVi/Jinja.UGA/52.10/1	KC884229	2010	1G
RVi/Jinja.UGA/52.10/2	KC884230	2010	1G
RVi/Rakai.UGA/05.11	KC884231	2011	1G
RVi/Luwero.UGA/10.11/1	KC884232	2011	1G
RVi/Luwero.UGA/10.11/2	KC884233	2011	1G
RVs/Tororo.UGA/12.11/1	KC884234	2011	1G
RVi/Tororo.UGA/12.11/2	KC884235	2011	1G
RVi/Tororo.UGA/12.11/3	KC884236	2011	1G
RVi/Kabarole.UGA/23.11	KC884237	2011	1G
RVi/Mityana.UGA/50.11	KC884238	2011	1G
RVi/Lwengo.UGA/10.12	KC884239	2012	1G
RVi/Kiryandongo.UGA/11.12	KC884240	2012	1G
RVi/Arua.UGA/36.11	KC884241	2011	1E

[a]WHO names are composed of district, country, epidemic week, and year of collection. RVi and RVs indicate that sequences were determined from isolates or directly from the clinical sample, respectively.