Successful Detection and Genotyping of Rubella Virus From Preserved Umbilical Cord of Patients With Congenital Rubella Syndrome

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Keywords. congenital rubella syndrome; preserved umbilical cord; retrospective investigation.

Congenital rubella syndrome (CRS), first documented in 1941 by Gregg [1], presents with multifarious manifestations involving multiple organ systems, including the ophthalmic, auditory, cardiovascular, and encephalic systems. These manifestations are identified in "transient," "permanent," and "delayed" (late-onset) manners.

Although investigation of CRS can be aided by disease surveillance information, it is challenging to confirm suspected cases in countries where rubella is completely or nearly eradicated. Cases of newborns with CRS born by women reinfected with either symptomatic or asymptomatic form of rubella have been reported in the literature. CRS cases with strictly delayed manifestations are likely to be missed at birth. For such cases, specimens for investigation may be limited by the time the symptoms become salient.

Between 2012 and 2013, Japan suffered from an epidemic of rubella [2], accumulating CRS cases in excess of 40 [3]. Clinical manifestations of CRS differ in each case. Some cases that were completely asymptomatic at birth subsequently developed into hearing difficulty and/or other CRS-associated symptoms.

Received 7 July 2014; accepted 28 October 2014; electronically published 6 November 2014.

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Clinical Infectious Diseases® 2015;60(4):605–7

DOI: 10.1093/cid/ciu882

In a quest for feasibly available potent specimens for retrospective investigation of CRS, we investigated preserved umbilical cords, which are usually treasured lifelong as an embodiment of maternofilial bond in the Japanese culture.

MATERIALS AND METHODS

Specimens

Specimens in this study were collected with informed consent of the patient's guardian and ethical approval from our institute's internal review board. Preserved umbilical cords were voluntarily provided from individuals diagnosed with CRS (recruited from a CRS patient organization; diagnosed elsewhere), as well as from individuals with no signs nor circumstances indicative of CRS, serving as negative controls. Typically, the withered umbilical cord stump and/or a small portion of the severed umbilical cord are wrapped in paper, cotton, or gauze and preserved in a small wooden box (Supplementary Figure 1). A small fragment of approximately 20 mg was chipped off from each preserved umbilical cord for investigation.

The diagnosis of all our subjects, except for those born before April 1999, is based on the criteria under which all CRS cases are mandatorily reported to the national epidemiological surveillance system [2].

RNA Extraction

RNA was extracted as described elsewhere [4]. In brief, RNA was extracted by ISOGEN reagent (Nippon Gene Co, Ltd) in combination with a PureLink mini kit (Thermo Fisher Scientific), and eluted with nuclease-free water in volumes corresponding to 4 times the mass of the starting material. In the instance that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) was negative (employed as a control for RNA extraction), high-salt precipitation was further performed and the RNA reanalyzed.

Real-time Polymerase Chain Reaction

Two primer/probe sets ("A" [5] and "B" [6]) for detecting rubella virus (RV), confirmed to be of comparable detection limits under our settings (approximately 1×10^1 copies/20 μL; data not shown), were adopted from the literature. Another primer/probe set was employed for detecting mRNA for GAPDH [7], as a control of RNA extraction. RNA transcribed in vitro served as positive controls. Four microliters of the eluted RNA was amplified in a 20-μL reaction on a CFX96 detection system (Bio-Rad). The thermal conditions were as follows: 42°C

for 5 minutes and 95°C for 3 minutes, followed by amplification cycles of 95°C for 5 seconds and 60°C for 20 seconds.

Genotyping

Each specimen positive for RV by at least 1 detection method was subjected to genotyping. Expecting efficient transcription, complementary DNA was reverse-transcribed using random 15-mers [8]. To amplify the 739-bp sequencing window used for genotyping [9], amplification of a 1039-bp amplicon using the 2 primers, RV-gt-F and RV-gt-R, was attempted. If this failed, another protocol that amplifies the sequencing window in 2 overlapping short amplicons was adopted from the domestic manual of infectious agent detection for RV [10]. The sequences and orientations of the utilized primers are listed in Supplementary Figure 2. Successfully amplified products by either strategy were sequenced at Eurofins Genomics K.K. (Tokyo, Japan).

The determined sequences were aligned with the sequences of the 32 World Health Organization reference strains [9]. Thirty-nine RV sequences determined in Japan between 2011 and 2013 were also chosen from GenBank to be included in this alignment. Using the 739-bp sequencing window, a dendrogram was constructed using the MEGA6 software [11].

RESULTS

Specimens Subjected to Analysis

A total of 8 umbilical cord specimens were collected and subjected to investigation; 6 were from individuals diagnosed with CRS (specimens 1–6), whereas 2 were from individuals with no signs nor circumstances indicative of CRS, serving as negative controls (specimens 7 and 8). The profiles of the 8 individuals from whom the umbilical cords were derived, and the analysis results mentioned hereafter, are summarized in Table 1.

Real-time Polymerase Chain Reaction

All 6 CRS specimens proved positive for RV by primer/probe set B; among these, 2 were not proven positive by set A. The 2 negative control specimens remained negative for both RV detection methods. All 8 specimens proved positive for GAPDH mRNA, suggesting successful RNA extraction, although 1 negative control (specimen 8) required additional high-salt precipitation.

Genotyping

Genotyping could be achieved from 2 of the 6 RV-positive specimens. According to the dendrogram, these sequences clustered most closely with clade 2B, close to sequences from Japan (Supplementary Figure 3).

DISCUSSION

In this report, RV was successfully detected from preserved umbilical cords of all 6 CRS cases by real-time polymerase chain reaction (PCR). Despite the potential degradation of RNA during preservation and further specimen processing procedures, we managed to genotype the viral sequence in 2 of 6 RV-positive specimens. Accuracy and reliability of these sequences were suggested, as they clustered closely to other sequences determined in Japan between 2011 and 2013. To the best of our knowledge, there are no reports of utilizing preserved umbilical cord for detection or genotyping of RV in the literature.

In most retrospective studies on infections, the availability of specimens of interest is scarce, especially in the case of apparently healthy individuals. Nonetheless, there have been several reports of successful retrospective investigation of prenatal viral infections in developed countries, such as the utilization of Guthrie cards (dried blood spots) and umbilical cord blood. However, these specimens are not always readily accessible; Guthrie cards are conserved at public health agencies, and

Table 1. Background of Study Subjects and Results

Specimen No.	Status	Year/Week of Birth	CRS Triads			Detection/ΔCt		Accession No.[a]
			CA	HL	CCD	Set A [5]	Set B [6]	
1	CRS	2013/28	–	+	–	−2.6	−4.0	AB920562
2	CRS	1982/46	+	+	+	ND	2.5	
3	CRS	2012/47	–	+	+	ND	6.0	
4	CRS	2013/30	–	+	+	2.4	0.9	
5	CRS	2013/31	–	+	+	1.8	−0.5	AB920563
6	CRS	2002/48	–	+	–	1.2	0.5	
7	NC	2009/46	–	–	–	ND	ND	
8	NC	2011/42	–	–	–	ND	ND	

A negative ΔCt value indicates that the threshold cycle of the specimen was smaller than the control RNA (ie, the specimen proved positive faster than the control).
Abbreviations: ΔCt, difference of threshold cycles of the specimen and control RNA of 4×10^2 copies/reaction; CA, cataract; CCD, congenital cardiovascular disease; CRS, congenital rubella syndrome; HL, hearing loss/impairment; NC, negative control; ND, not detected.

[a] From DNA Data Bank of Japan (DDBJ).

umbilical cord blood is usually cryopreserved at appointed facilities. In contrast, preserved umbilical cords kept in the patient's home are easily accessible. In addition to our present investigation, there are reports on the successful utilization of preserved umbilical cords for retrospective investigation of prenatal infections by both DNA and RNA viruses.

The availability of preserved umbilical cord might be misunderstood as a limitation to our study, as some may believe that this is limited to the Japanese cultural background. To the contrary, similar customs of preserving the umbilical cord are documented among many countries and cultures (Supplementary Data 4). Thus, by paying attention to the cultural background of the patients, similar investigations might be possible worldwide.

There are several limitations to our study. First, the number of evaluated specimens was not substantial enough to discuss sensitivity/specificity. Nonetheless, we have demonstrated the suitability of preserved umbilical cord as a specimen for retrospective molecular detection of RV in suspected CRS cases. Second, the accuracy of the diagnosis of the oldest patient, born in 1982, was uncertain. However, this patient presented with the classic triad of CRS (ophthalmic, auditory, and cardiovascular involvements; Table 1); combined with the fact that Japan experienced a rubella epidemic in 1982 [12], evidence supporting the diagnosis of this case seems sufficient. Third, genotyping was not possible for 4 of the 6 CRS specimens. In addition to the potential degradation of RNA during preservation, the continuous force applied during the extraction procedure may have increased the chance of RNA shearing into fragments too short to serve as templates for sequence determination (739 nucleotides), but still permissible for real-time PCR detection (129 nucleotides). Meanwhile, RV had smaller threshold cycles in successfully genotyped specimens (Table 1), suggesting that higher RNA concentration favors genotyping. Hence, further optimization of the extraction procedure to overcome these factors may improve the suitability of the extracted RNA as a template for genotyping, as well as for real-time PCR-based detection, boosting the value of preserved umbilical cord as a specimen for retrospective molecular investigation.

Despite being a congenital condition, late-onset manifestations of CRS can take years before it is noticed. A definitive diagnosis can allow adequate patient care to be initiated earlier, and substantial proof of prenatal infection can also provide answers to patients who wish to know the cause of their conditions. Furthermore, diagnosing otherwise missed CRS cases may help to unveil unknown aspects and/or manifestations of the syndrome. However, current laboratory confirmation of CRS becomes very difficult after 1 year; in such situations, preserved umbilical cord can serve as an alternative specimen that extends this 1-year limit.

In conclusion, this is the first report of the successful detection and genotyping of RV from preserved umbilical cord specimens of CRS patients. Notably, a specimen that has been preserved for 31 years was still of value. Our findings add to the evidence that preserved umbilical cord is a useful archive of prenatal information and can be utilized for retrospective investigations of other prenatal infections.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. This study was approved by the Internal Review Board at the National Center for Child Health and Development, Japan (NCCHD). The authors thank Dr Julian Tang from the Department of Education for Clinical Research, NCCHD, for reviewing this manuscript. The authors also thank all the volunteers for providing their preserved umbilical cords.

Financial support. This work was supported by NCCHD, Japan (grant number 24-11).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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