



Research Paper

Immunolocalization and Distribution of Rubella Antigen in Fatal Congenital Rubella Syndrome



Mihaela Lazar^{a,c,d}, Ludmila Perelygina^a, Roosecelis Martines^b, Patricia Greer^b, Christopher D. Paddock^b, Gheorghe Peltecu^e, Emilia Lupulescu^c, Joseph Icenogle^{a,*}, Sherif R. Zaki^b

^a Measles, Mumps, Rubella and Herpesvirus Laboratory Branch, Division of Viral Diseases, 1600 Clifton Rd, Atlanta, GA, USA

^b Infectious Diseases Pathology Branch, Division of High-Consequence Pathogens and Pathology, Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA, USA

^c National Laboratory for Measles and Rubella, National Institute of Research-Development for Microbiology and Immunology "Cantacuzino", 103 Splaiul Independenței, Bucharest, Romania

^d Department of Biology, University of Bucharest, 4-12 Blvd. Regina Elisabeta, Bucharest, Romania

^e Filantropia Clinical Hospital, 11-13 Blvd. Ion Mihalache, Bucharest, Romania

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ABSTRACT

Background: An estimated 100,000 cases of congenital rubella syndrome (CRS) occur worldwide each year. The reported mortality rate for infants with CRS is up to 33%. The cellular mechanisms responsible for the multiple congenital defects in CRS are presently unknown. Here we identify cell types positive for rubella virus (RV) in CRS infants.

Methods: Cells and organs involved in RV replication were identified in paraffin-embedded autopsy tissues from three fatal case-patients by histopathologic examination and immunohistochemical (IHC) staining using a rabbit polyclonal RV antibody. Normal rabbit antisera and RV antisera preabsorbed with highly purified RV served as negative controls.

Results: RV antigen was found in interstitial fibroblasts in the heart, adventitial fibroblasts of large blood vessels, alveolar macrophages, progenitor cells of the outer granular layer of the brain, and in capillary endothelium and basal plate in the placenta. The antibody specificity was verified by IHC staining of multiple tissue sections from other infectious disease cases. RV infection of each cell type is consistent with abnormalities which have been identified in patients with CRS, in the heart, large blood vessels, and brain. Antigen distribution was consistent with inflammatory response to vascular injury and systemic spread of RV.

Conclusions: The identification of RV positive cell types in CRS is important to better understand the pathology and pathogenesis of CRS.

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1. Introduction

Rubella virus (RV) is an enveloped, positive-sense, single-stranded RNA virus of the genus *Rubivirus*, the family *Togaviridae*. Congenital rubella syndrome (CRS) is a serious disease caused by RV infection in utero (Plotkin et al., 2011). During the period of maternal viremia, RV may infect the placenta, pass the placental barrier and enter the fetal bloodstream, possibly through emboli of necrotic endothelial cells originating from infected chorion (Tondury and Smith, 1966; Driscoll, 1969; Ornoy et al., 1973; Lima et al., 1977). If the fetus is infected during the first trimester of pregnancy, the virus usually persists until term in multiple fetal organs causing a spectrum of birth defects that includes

hearing impairment, and ocular and cardiovascular abnormalities (Rawls, 1968; Singer et al., 1967; Esterly and Oppenheimer, 1973). Birth defects in individual cases can be minor or severe, affecting many organs or only a few (Lindquist et al., 1965; Rosenberg et al., 1981). Gestational age at the time of maternal infection is the most important determinant of fetal damage. The risk of fetal infection with congenital anomalies is highest during the first 11–12 weeks of gestation, but then sharply decreases with increasing gestational age (Alford et al., 1964; Sheridan, 1964; Miller et al., 1982). Fetal infection after 16 weeks of gestation typically results in an infant without clinical signs of CRS and such cases are classified as congenital rubella infection (CRI) if laboratory confirmed by IgM ELISA or virus detection.

A significant non-CRS manifestation of in utero rubella virus infection is spontaneous abortion which occurs in about 20% of rubella infections acquired during pregnancy. The limited fetal damage after the first trimester is presumably because organogenesis is complete, maternal IgG is transferred to the fetus, and a fetal immune response is present. Late manifestations of CRS have also been recognized. Ocular

* Corresponding author at: Centers for Disease Control and Prevention, 1600 Clifton Rd NE, MS C22, Atlanta, GA 30333, USA.
E-mail address: jci1@cdc.gov (J. Icenogle).

consequences of CRS are observed after the neonatal period and CRS has recently been associated with Fuchs heterochromic iridocyclitis (Winchester et al., 2013; de Groot-Mijnes et al., 2006). A rare disability associated with CRS is progressive rubella panencephalitis (Townsend et al., 1976). Another late onset disability is insulin-dependent diabetes mellitus; in a follow-up study, 40% of CRS patients developed evidence of overt or latent diabetes (Menser et al., 1974).

Children with CRS may be infectious for the entire first year of life, rarely even longer (Cooper and Krugman, 1967; Rawls et al., 1967), and can transmit the virus causing infection in non-immune individuals (Hardy et al., 1965; Greaves et al., 1982; Schiff and Dine, 1965). The infant mortality rate for CRS can be as high as 33% (Cooper et al., 1965; Toizumi et al., 2014). Disability-adjusted life years (DALY) lost per CRS case was estimated to be between 19 and 38 depending on the country income and health care resources (Simons et al., 2014). Rubella and CRS are rare in most developed countries but are still a significant risk for non-immune pregnant women in developing regions and an estimated 100,000 CRS cases occur worldwide each year (Cutts and Vynnycky, 1999). In the absence of vaccination, rubella is an endemic disease with epidemics every 6 to 9 years (Horstmann, 1971).

The pathology of CRS was comprehensively documented following the major rubella pandemic in 1963–1965. Histopathologic investigations of fetal CRS cases revealed tissue damage in multiple fetal organs such as the heart, blood vessels, crystalline lens, ears, brain, teeth, and liver (Tondury and Smith, 1966; Driscoll, 1969; Esterly and Oppenheimer, 1969). Based on the localization of lesions, it was suggested that endothelial cells, myocardial cells, skeletal muscle cells and particular epithelial cells of the developing lens and inner ear were infected with RV (Tondury and Smith, 1966). Only one study demonstrated the presence of RV antigen in tissue sections of the heart and skeletal muscle of a single CRS case; each focus of RV infection involved only a few cells, which were histologically entirely normal (Woods et al., 1966). However, modern techniques and reagents should provide more precise identification of cell types infected by RV. A recent study of three aborted fetuses with congenital rubella infection identified RV antigen in multiple organs, including the lung, kidney, brain, spleen, heart and ciliary body (Nguyen et al., 2015). Epithelial cells and mononuclear progenitor cells in multiple organs and neuronal cells in cerebral cortex were reported to be the predominant cell types, in which RV capsid protein was detected by immunohistochemical (IHC) staining. However, the precise cell types in which RV replicates and persists in infants carried to term or born with CRS have remained largely uncharacterized.

A nationwide rubella outbreak occurred in Romania in 2011–2012 with more than 20,000 rubella cases in unvaccinated individuals, and 22 laboratory confirmed CRS cases, including ten deaths and one stillbirth reported by the end of 2012 (Janta et al., 2012). Rubella virus genotype 2B was associated with this outbreak (unpublished data).

Here, we describe the clinical manifestations, laboratory investigations, and histopathologic changes in formalin-fixed, paraffin-embedded (FFPE) tissue specimens obtained from three children with fatal congenital rubella, and the application of an IHC assay for the detection and localization of rubella virus antigens. We assert implications of the IHC results for CRS pathogenesis. These three cases were all carried to or almost to term and thus this study describes RV antigen positive cell types in CRS, rather than cells that were transiently infected during pre-term pathogenesis.

2. Materials and Methods

FFPE autopsy tissues of three CRS case-patients with serology or RT-PCR confirmed RV infection were submitted to the CDC for histopathologic and immunohistopathologic evaluation. No other potential causes of defects in these infants (e.g. environmental exposures or genetic mutations) were noted.

2.1. Clinical History and Tissue Samples

Case 1. A premature girl (33 gestation weeks) was born on November 13, 2012. The course of pregnancy was not medically supervised. The APGAR (Appearance, Pulse, Grimace, Activity, Respiration) score was 7 at 1 min and 9 at 5 min after birth. Scores of 7 and above indicate that a newborn is healthy and does not require immediate medical interventions. The girl weighed 2050 g and had pulmonary hypertension. CRS diagnosis was laboratory confirmed by a positive test for rubella immunoglobulin M (IgM ELISA) from serum collected on the 24th day of life. She died after 96 days. Immunological tests were negative for other teratogenic pathogens (cytomegalovirus (CMV), mycoplasma pneumonia, and herpes simplex virus (HSV) types 1 and 2). Examination and autopsy revealed bilateral congenital cataracts, liver dystrophy, cardiomegaly and multiple cardiac anomalies including atrial septal defect, patent ductus arteriosus, and patent foramen ovale. FFPE tissue samples were available from the cerebral hemisphere, cerebellum, myocardium, aorta, lung, liver, pancreas, intestine, kidney, spleen and thymus.

Case 2. A girl (35 gestation weeks) was stillborn on November 23, 2012. The mother was serologically confirmed (IgM ELISA) to have had rubella in the first trimester of pregnancy (week 13). Autopsy revealed a globular heart with a single atrium, primary pulmonary atelectasis, and cerebral edema. FFPE blocks were prepared from the placenta, myocardium, liver, lung, spleen, kidney, and umbilical cord. Note that CRS is by definition a syndrome which must be found in a live infant. We have considered this term stillbirth a CRS case for the purposes of this study.

Case 3. A boy was born on December 12, 2012 after 39 weeks gestation period having birth weight of 1160 g, consistent with small for gestational age. APGAR score was 4 at 5 min. The course of pregnancy for this case was not medically supervised. The clinical signs included respiratory distress, congenital pneumonia, cataract, glaucoma, hearing loss, congenital heart disease, patent ductus arteriosus, splenomegaly, microcephaly, developmental delay, radiolucent bone disease, and jaundice within 24 h. Serum and nasopharyngeal swab were collected 5 days after the birth. The serum was IgM-positive and rubella virus was detected in nasopharyngeal swab by real-time RT-PCR. The baby died when 21 days old after cardiorespiratory failure. FFPE blocks from the liver, lung, kidney, spleen, pulmonary artery, brain, and skeletal muscle were available.

2.2. ELISA

The detection of rubella IgM was performed using Enzygnost Anti-Rubella Virus/IgM kit (Siemens Healthcare Diagnostics Products GmbH) and Rubella virus IgM micro-capture kit (IBL International).

2.3. PCR

Fresh tissue samples from the lung, kidney, spleen, lens, liver, brain, thymus, and heart were collected only for **Case 3**. All tested samples were positive by real-time RT-PCR assay, which was previously described in detail (Abernathy et al., 2009) using the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, United States). Skeletal muscle and myocardium samples were negative. Subsequent genotyping based on the standard genotyping window (nt 8731–9469) was performed by conventional RT-PCR reactions (QIAGEN OneStep RT PCR Kit (QIAGEN, Hilden, Germany), using the protocol recommended by the Global Measles and Rubella Laboratory Network with minor modifications (Standardization of the nomenclature for genetic characteristics of wild-type rubella viruses, 2005; Namuwulya et al., 2014). For sequencing PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit was used (Applied Biosystems, Foster City, California) and sequences were determined on a PRISM 3100-Avant Genetic

Analyzer (Applied Biosystems, Foster City, California). The genotype of rubella virus was identified as 2B (GenBank Acc. # KR021370).

2.4. Antibodies

Rabbit polyclonal anti-rubella antibody was raised against purified rubella virions (strain HPV77, Meridian Life Science) by the CDC core facility. The specificity of the antibody was documented with Western blotting and immunofluorescence assays using RV infected and mock infected cell cultures (data not shown) and verified immunohistochemically by testing the antibody against cases or cell controls positive for other infections associated with congenital abnormalities (*Toxoplasma gondii*, varicella zoster virus, parvovirus B19, CMV, HSV-1) and measles virus.

2.5. Histopathology and Immunohistochemistry

Hematoxylin–eosin (H&E) staining was performed for histopathological evaluation. The IHC assay for RV antigen was performed on 3- μ m sections of FFPE tissues. Deparaffinized and rehydrated tissue sections were placed in a LAB Vision autostainer and digested in 0.1 mg/ml proteinase K (Roche Diagnostics GmbH Mannheim, Germany). The staining process was done with a LabVision System on IntelliPATH Autostainer 2 \times Secondary using UltraVision LPValue Large Volume Detection System AP Polymer kit (Thermo Scientific, UK) according to the kit instructions. Tissue sections were incubated with a rabbit anti-RV polyclonal antibody for 30 min followed by sequential incubations with enhancer, polymer and fast red substrate (Dako Corporation). Sections were then counterstained in Meyer's hematoxylin (Fisher Scientific) using the Sakura Automatic Slide Stainer and mounted with aqueous mounting medium (Lerner Laboratories, Pittsburg, PA). The optimal dilution of the anti-rubella antibody for the IHC assay was determined (1:500) by using a series of titrations applied to sections from FFPE blocks with RV-infected A549 human lung carcinoma cells (ATCC#CCL-185) mixed with normal human tissues, such sections also served as a positive control in each IHC assay. Negative controls were run in parallel and consisted of sequential tissue sections of case patients each incubated with either normal rabbit serum or the rubella antibody pre-absorbed with purified rubella. Interpretation of IHC assay results for rubella virus included determination of the location of the

positive reaction in a cell (nuclear or cytoplasmic staining), the type of cells infected and the intensity of staining.

3. Results

Histopathologic changes and IHC evidence for the presence of RV antigens were documented in some organs from all cases.

3.1. Lung

Microscopic examination of all lungs from cases showed congestion, intra-alveolar edema, and interstitial inflammation (Fig. 1a–c). The inflammation was mild and consisted predominantly of mononuclear cells. Histopathological changes of diffuse alveolar damage (DAD) including edema, hyaline membranes, and inflammation were seen in only one case (Case 1). Mild medial arterial hypertrophy was seen in some vessels in Cases 1 and 2, however luminal vascular occlusion or arterial thrombi were not identified. Immunohistochemical staining exhibited viral antigens in alveolar macrophages. Globular aggregates of rubella antigens were seen (Fig. 1d). In Cases 2 and 3, no significant histopathologic findings were observed.

3.2. Heart

Morphologic myocardial damage in the CRS autopsy cases was not significant. In some areas the heart showed reactive myocytes with plump nuclei and interstitial inflammation (Fig. 2a). No evidence of myocarditis was observed in Case 2. Heart tissue was not available for Case 3. Viral immunostaining was seen in fibroblasts in Case 1 (Fig. 2b) and Case 2 (not shown).

3.3. Aorta and Pulmonary Artery

Multiple sections of the aorta (Fig. 3a) and pulmonary artery (not shown) were examined and no morphological features were seen. However, numerous fibroblasts strongly positive for RV antigens were detected in adventitia of aorta (Case 1) and in the pulmonary artery (Case 3) (Fig. 3b).

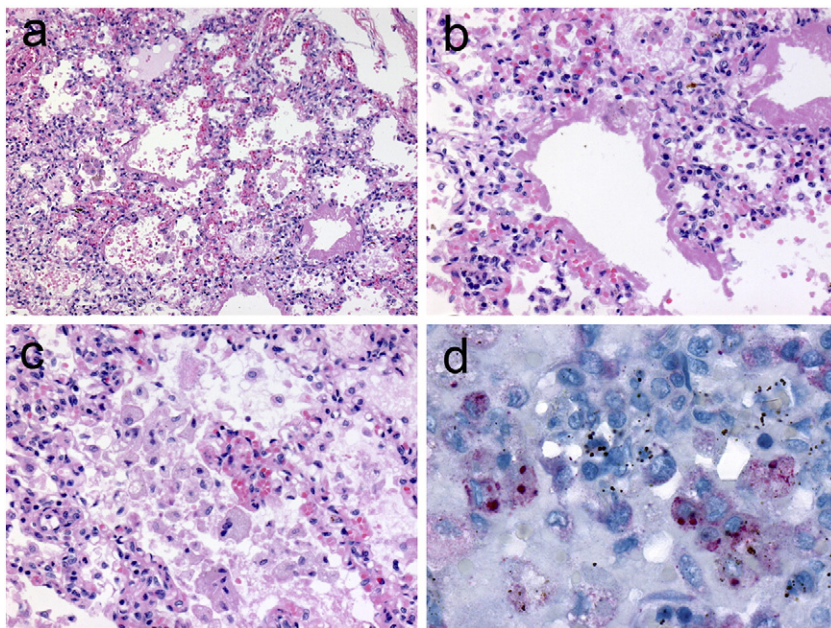


Fig. 1. Pulmonary histopathologic and immunohistochemical features in a fatal CRS case (Case 1). Low-power image of lung (a) shows congestion, edema, mild interstitial inflammation and hyaline membrane formation. Diffuse alveolar damage and interstitial pneumonitis (b). High-power image (c) shows intra-alveolar macrophages. Rubella virus antigens in intra-alveolar macrophages (d). Note immunostaining of globular inclusions.

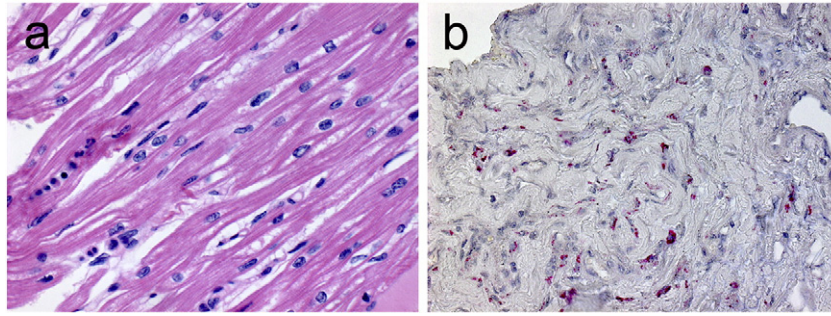


Fig. 2. Cardiac histopathologic and immunohistochemical features in fatal cases of CRS. Higher-power magnification of the heart (a) showing reactive myocytes with plump nuclei and focal inflammatory cells, but no clinically significant inflammation (H&E staining). Abundant immunostaining of interstitial fibroblasts (b).

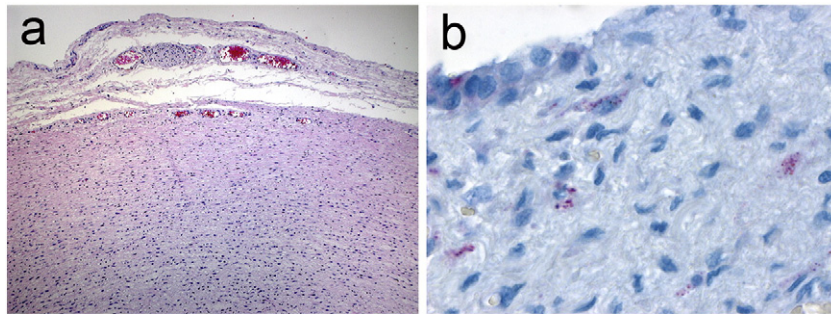


Fig. 3. Aortic histopathologic and immunohistochemical features in a fatal case of CRS (Case 1). Low-power magnification of the aorta (a) shows the lack of pathologic changes (H&E staining). Immunostaining of mesenchymal cells (between myocytes) and numerous fibroblasts in a section of the aorta (b).

3.4. Central Nervous System

No histopathological evidence of encephalitis was observed in any of case-patients with brain samples (Fig. 4a, c). Small areas of calcification were observed. Focal staining of progenitor cells in the external granular cell layer was seen in the cerebellum of Case 1, but not in Case 3 (Fig. 4b, d).

3.5. Placenta and Umbilical Cord

A placenta FFPE block was available only for Case 2. The chorionic villi of the placenta were mature and with abundant blood vessels. The decidua showed focal area of necrosis. No evidence of inflammation was observed. Viral antigens were seen primarily in endothelial cells (Fig. 5a, b) and the basal plate of the placenta (Fig. 5c).

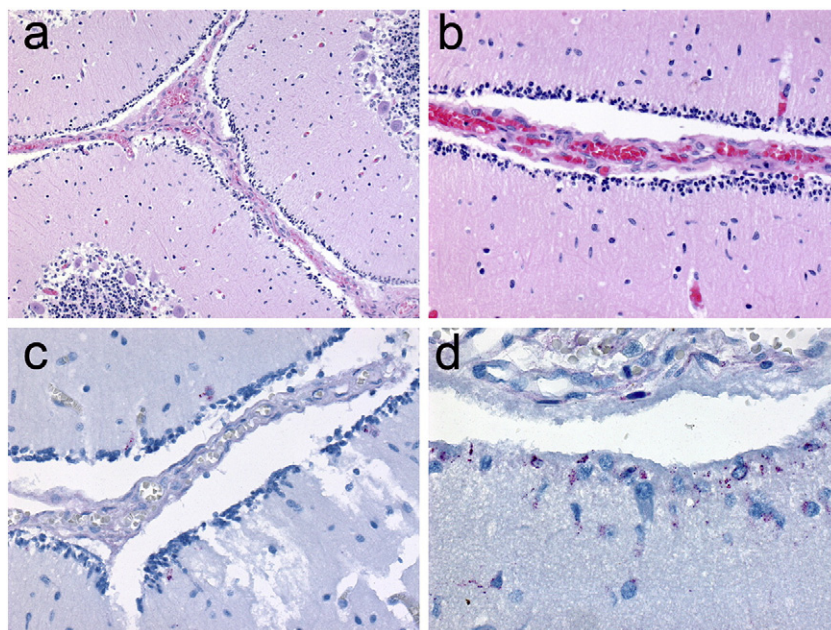


Fig. 4. Central nervous system histopathologic and immunohistochemical features in a fatal case of CRS (Case 1). Low-power (a) and high-power (b) images of the cerebellum show meninges with no evidence of inflammation. Immunostaining of neuronal cells (c and d) in external granular cell layer of the cerebellum.

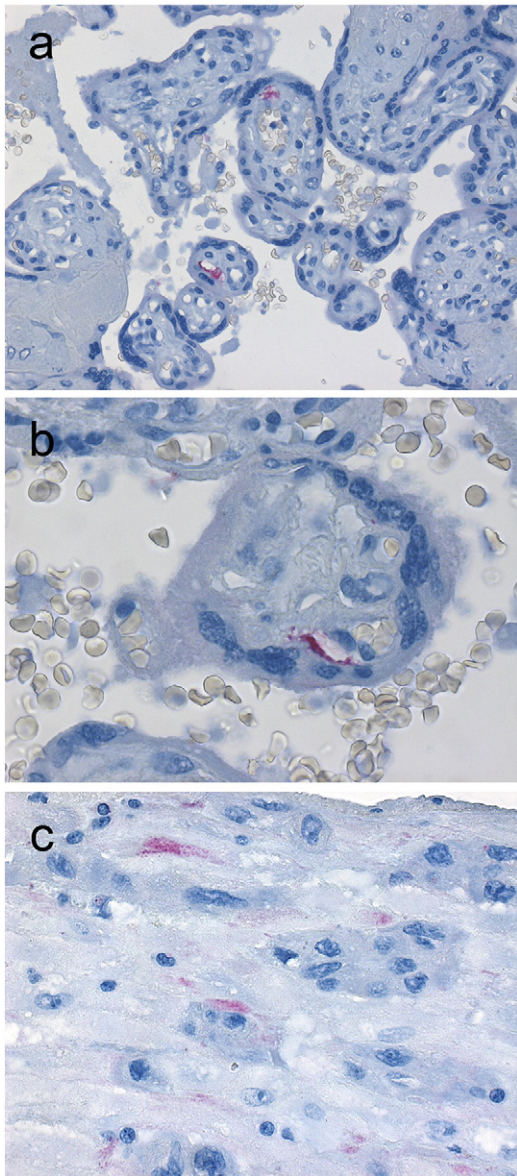


Fig. 5. Immunohistopathologic features in the placenta of Case 2. Low-power (a) and high-power (b) images of sections of chorionic villi show immunostaining of endothelial cells. (c) Section of the placenta (maternal plate) shows extracellular immunostaining throughout the basal plate.

3.6. Other Organs

Histopathological findings in other organs were nonspecific. The liver in **Case 1** showed diffuse small droplet macrovesicular steatosis without significant inflammation, while in **Case 2** there was marked hepatocyte swelling, cholestasis, congestion, and extramedullary hematopoiesis. **Case 3** showed mid-zonal bridging necrosis, marked cholestasis and scattered dystrophic calcifications. No immunohistochemical RV staining was identified in other organs.

4. Discussion

This report presents histopathological and immunohistochemical studies on autopsy samples from three fatal CRS patients that occurred in 2012 in Romania. This study identified new target cells for rubella virus in the tissues of these infants.

One of the important findings was localization of RV antigen in cardiac fibroblasts in the myocardium of two patients and adventitia fibroblasts of large blood vessels (aorta and pulmonary artery). Consistent with descriptions of focal localization of lesions in cardiovascular tissues (Tondury and Smith, 1966), RV antigen staining was found to be focal. The one previous IHC study of an infant with CRS identified very compact scattered foci of rubella infected cells in cardiac muscle fibers (Woods et al., 1966). In cases presented here we did not observe IHC staining in cardiomyocytes. However, areas of histopathology involving only a few cardiomyocytes were often seen near IHC positive fibroblasts. Perhaps the cases we studied had healed lesions after rubella virus was cleared from cardiomyocytes. Cardiac fibroblasts play a critical role in development of the cardiovascular system and maintaining normal cardiac function, while adventitia fibroblasts are important players in inflammatory response to vascular injury (Deb and Ubil, 2014). Infection of these cell types could have significant involvement in the congenital cardiovascular malformations seen in CRS patients.

Another novel finding was the identification of RV antigen in progenitor cells of outer granular layer of the brain. This cell type in the brain of CRS patients has not been previously known to be infected with rubella virus. However, the presence of unresolved outer granular layer and underdeveloped internal granular layer in a CRS case has been reported (Kemper et al., 1973). Mental retardation and microcephaly are frequently seen in infants with congenital rubella syndrome. In vitro studies suggested that the main cell type permissive to RV infection in developing brain tissue is the astrocyte (Chantler et al., 1995). Pathology studies of the brain of infants with congenital rubella syndrome revealed extensive degenerative changes in leptomeningeal and intrinsic arteries and veins of the cerebrum. Vascular damage was associated with foci of necrosis localized in the deep white matter and gray nuclei, but no developmental malformations or significant inflammation of the nervous system was found (Rorke and Spiro, 1967; Rorke, 1973). During CNS development, multipotent neural stem cells give rise first to various kinds of specified precursor cells, which proliferate extensively before terminally differentiating into either neurons or glial cells. These progenitor cells migrate to different areas of the brain and are involved in normal development of the CNS. Thus, RV infection of progenitor cells could lead to a variety of malformations of the CNS, such as those found in CRS patients.

Reports of congenital infection with other pathogens support the hypothesis that immature cells are apt to be target cells for infection in the developing brain (Feuer et al., 2003; Dietrich et al., 2004; Tsutsui et al., 2008). HSV, CMV, toxoplasmosis, and other infections are potent disrupters of fetal neurodevelopment leading to abnormalities in brain structure (e.g., hypoplasia of brain regions) and behavior disorders, including mental retardation and learning disabilities (Brown and Derkits, 2010). In a mouse model of fetal CMV infection neural stem progenitor cells in developing brains are the cell type that is most susceptible to CMV infection. The resulting disturbance of cellular events such as proliferation, differentiation and migration was suggested to be the main cause of CMV brain disorders (Tsutsui et al., 2008). Further studies are necessary to determine whether RV infection of neural progenitor cells has any effects on their proliferation, regeneration and differentiation.

The histopathologic changes of DAD were observed only in **Case 1**. This infant had interstitial pneumonia and pulmonary hypertension, which frequently occur in CRS cases (Singer et al., 1967; Boner et al., 1983; Franklin and Kelley, 2001). Pulmonary arterial hypertension has been recently identified as the major contributor to mortality in CRS cases (Toizumi et al., 2014). We detected intense IHC staining in alveolar macrophages. We did not observe staining of alveolar epithelial cells, which were shown to be IHC positive in the rubella infected fetuses examined by Nguyen et al. (2015). Since RV infection in tissues is focal, and RV antigen is typically detected in a limited number of cells, the lack of immunostaining of alveolar epithelial cells in the present study might simply be due to the absence of infected cells in those lung sections that we were able to analyze. Another possibility is RV infection

in alveolar epithelial cells or other cell types in the lung have been cleared, but RV antigen was still present in alveolar macrophages. It is currently unclear whether rubella virus replicates in macrophages.

This study also identified capillary endothelial cells and the basal plate as the primary targets in the placenta. These data are in agreement with the results of one large study of placentas from CRS cases, in which rubella-specific immunofluorescence was detected in epithelial cells of chorionic blood vessels and amniotic epithelium; intracytoplasmic inclusion bodies (virions) were described in decidual cells in the basal plate and cytotrophoblasts (Garcia et al., 1985). Infection of endothelial cells can result in capillary necrosis and blockage of capillaries impacting placenta functions and leading to growth retardation of the fetus. Infection of endothelial cells may account for some of the vascular anomalies and viral spread, whereas infection of cells in the basal plate may lead to placental pathologies and thus account for the multitude of congenital malformations in CRS patients.

One of the overall purposes of this work was to catalogue the cell types with evidence of rubella infection in CRS cases. This study extends the description of the pathology of CRS by IHC beyond the single previous report of RV infected cells in specimens from a single CRS case almost 50 years ago. A complimentary study to catalogue the cell types with evidence of rubella infection from fetuses addressed which RV infected cell types were found during the development of CRS (Nguyen et al., 2015). The only RV positive cell type that was found in the study of fetuses and the current study was neuronal cells. This is not unexpected because the positive cells during progression to CRS and the positive cells in CRS (term babies) may well be different due to transiently infected cells during the progression to CRS. We expect that additional studies of RV positive cell types in RV infected fetuses and in fatal CRS cases will be necessary to fully describe and to understand the molecular pathogenesis and the pathology of CRS. However, as global elimination targets are met, opportunities to conduct such additional studies in the future will diminish (Andrus et al., 2011).

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Potential Conflicts of Interest

The authors do not have a commercial or other association that might pose a conflict of interest. The findings and conclusions in this report are those of the authors and do not necessarily represent the view of the United States Centers for Disease Control and Prevention.

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