



# A novel cell-based high throughput assay to determine neutralizing antibody titers against circulating strains of rubella virus



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## ABSTRACT

A large rubella outbreak occurred in Japan 2013, and 14,344 rubella and 45 congenital rubella syndrome (CRS) cases were reported. At that time, the populational immunity was above the protective threshold assessed by hemagglutination inhibition (HI) titer. The genotype 2B rubella virus (RV) strains were responsible for the outbreak, which are non-indigenous in Japan. In this work, a cell-based high throughput assay was established to measure the neutralizing antibody (NA) titer against circulating RV isolates. RV infection poorly induces cytopathic effects in tissue culture, preventing the casual measurement of NA titer. Our assay system has overcome this hurdle. Using this assay, we re-evaluated the antibody prevalence rate against circulating viral isolates using human sera collected before the outbreak. Individuals with protective IgG titer ( $\geq 10$  IU/ml) represented 88.1% of the population. Consistently, 85.2% of the population had protective neutralizing antibody titers ( $\geq 1:8$ ) against the vaccine strain. In contrast, 50.5% of the population had protective neutralizing antibody titers against circulating genotype 2B RV strains. These data suggest that the herd immunity assessed by HI titer should have been appreciated deliberately.

## 1. Introduction

Rubella is a mild contagious disease that predominantly affects children and is caused by the rubella virus (RV), a togavirus of the genus Rubivirus (Gershon, 2014). When a pregnant woman is infected with RV, congenital rubella syndrome (CRS) can occur. No specific treatment is available for rubella and CRS (Gershon, 2004). However, the live attenuated rubella vaccine is available. Most of the licensed rubella vaccines are based on genotype 1a strains, such as RA27/3 (Rubella vaccines, 2011). Other rubella vaccine strains of the 1a genotype include Matsuura, Takahashi, and TO-336 strains, used primarily in Japan (Shishido and Ohtawara, 1976).

RV is grouped into two clades and further divided into 13 genotypes (clade 1: 1a, 1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I, and 1J; clade 2: 2A, 2B, and 2C) based on a 739 nucleotide sequence within the E1 gene (Rubella virus nomenclature update, 2013). Having 0.8–2.1% of amino acid differences, no serotype has been defined (Frey, 1994). Repeated exposure to circulating RV or vaccinations should therefore provide protection against RV infection of any genotype, except in the case of immunocompromised individuals (Tingle et al., 1985; Anon., 2005).

The genotypes of current epidemic RV strains are 1E, 1G, 1J, and 2B (Shishido and Ohtawara, 1976). A genotype 1E strain was initially detected in France in 1995 (Vauloup-Fellous et al., 2010). In 1997, it spread to North America (Icenogle et al., 2006). Genotype 1E strains replaced the circulating strains (1F and 2B) and became the major epidemic strain in China after 2001 (Zhu et al., 2012). In the late 2000s, genotype 2B strains, first isolated in Israel 1968, have become the dominant RV genotype globally (Rubella virus nomenclature update, 2013; Abernathy et al., 2011). In Japan, the rubella endemic was attributed to five indigenous genotypes 1a, 1C, 1D, 1E, and 1J (Kato, 2004). RV genotype 1E strains have started to spread since 2010, followed by genotype 2B strains replacing 1E strains since 2011. Genotype 2B strains caused the most recent rubella outbreak, which peaked in 2013. The mechanism of the genotypic shift remains largely unclear (Icenogle et al., 2006; Zhu et al., 2010).

Herd immunity plays a critical role in the prevention of rubella in the human population. The protective threshold of herd immunity is 80–85% against rubella (Anderson and May, 1990), which is likely achieved by vaccinating  $\geq 95\%$  of children. Protection against RV infection is commonly assessed by either IgG levels specific to viral

**Abbreviations:** RV, rubella virus; CRS, congenital rubella syndrome; HI, hemagglutination inhibition; NA, neutralization antibody; TCID, tissue culture infectious dose; GMT, geometric mean titer; CPE, cytopathic effect

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antigens  $\geq 10$  IU/ml (Skendzel, 1996) or hemagglutination inhibition (HI) titer  $\geq 8$ , which is the reciprocal of the highest dilution of serum that completely inhibits hemagglutination (Tingle et al., 1985). These laboratory tests utilize antigens of vaccine strains, and these parameters correlate well with neutralizing antibody (NA) titer (Lambert et al., 2014; Brown et al., 1969). The NA titer most directly represents the protective humoral immunity against RV (Charlton et al., 2016). However, the use of NA titer is limited due to the high technical skills required and the time constraints (Lambert et al., 2014; Castellano et al., 1981; Meegan et al., 1982; Truant et al., 1983; Dimech et al., 1992; Dimech et al., 2008; Dimech et al., 2013). This is partly due to the fact that RV does not induce apparent cytopathic effects (CPE) in tissue culture (Bellini and Icenogle, 2015).

Herd immunity in the Japanese population was assessed by HI titer in 2012. Individuals with protective HI titer of  $\geq 8$  represent 90.2% of the population: 85.6% among males and 93.7% among females (Anon., 2013a). This was above the protective threshold of rubella. However, rubella outbreak in Japan in 2013 (Anon., 2013b; Nishiura et al., 2015) was the first rubella outbreak with the genotype 2B virus. Rubella patients were mostly males in their late 30s and 40s. This was due to the previous vaccination program in Japan. From 1977–1994, only females at junior high school were targeted for rubella vaccination. Males were left unvaccinated. This unvaccinated population accounted for the adult male dominance of the rubella outbreak in 2013. After 1994, the national vaccination program has changed, and all the children are targeted for rubella vaccination. Since 2006, the current two-dose vaccination program has started. On the other hand, we suspected that the herd immunity scored by HI titer might not accurately represent the protective populational immunity against rubella. Therefore, we re-evaluated herd immunity using the NA titer against the circulating genotype 2B isolates.

## 2. Material and methods

### 2.1. Subjects

Blood specimens were collected from healthy donors chosen randomly at hospitals, clinics, schools, and public health centers under the Preventive Vaccination Law, National Epidemiological Surveillance of Vaccine-Preventable Disease (NESVPD) in Japan 2012. Individuals showing apparent clinical signs suggesting infectious diseases were excluded. Among these specimens, we analyzed 101 specimens with which the comprehensive consent was obtained (Table 1). Among the subjects with vaccination histories, 71.2% (37/52 subjects) confirmed their vaccination histories with their documented records, such as maternity health record book. Specimens MR1 ~ 3 were obtained from healthy individuals at one month post-vaccination of measles and rubella (MR). This study was approved by the Ethical Review Board of

**Table 1**  
Summary of the subjects studied in this work.

		Total	Male	Female	Vaccine history (%) <sup>a</sup>			
					None	Once	Twice	Unknown
No. of subjects		101	47	54	12	38	14	37
Age	Median	18	23	18	–	–	–	–
	Range	0–68	1–68	0–39	–	–	–	–
	Group	0	0	1	0	0	0	1
	1–4	10	7	3	0	7	0	3
	5–9	12	6	6	0	7	5	0
	10–19	32	7	25	3	20	7	2
	20–29	22	13	9	4	4	2	12
	30–39	13	3	10	2	0	0	11
40–49	2	2	0	0	0	0	2	
> 50	9	9	0	3	0	0	6	

<sup>a</sup> MR (measles-rubella) or MMR (measles-mumps-rubella) vaccine.

Osaka Prefectural Institute of Public Health (No. 1302-06 and 1602-09). Written informed consent was obtained from study participants or the guardian of all participating children.

### 2.2. Mammalian cells and transfection

A rabbit cell line RK13 was provided by the National Institute of Infectious Diseases, Tokyo, and was maintained in RPMI1640 (Wako Pure Chemical, Tokyo, Japan) supplemented with 10% FBS (Nichirei Biosciences, Tokyo, Japan), penicillin, and streptomycin (Invitrogen, Tokyo, Japan). Cells were incubated at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Co-transfection of reporter plasmid and a plasmid carrying the puromycin resistance gene pPUR (Clontech, Palo Alto, CA) was carried out with Lipofectamine 2000 (Invitrogen). Cell clones were selected using 1 µg/mL puromycin (Sigma, Tokyo, Japan). Plasmids carrying ISRE-Luciferase reporter units were purchased from Qiagen (Tokyo, Japan).

### 2.3. Virus

The genotype 1a strain, Matsuura, was isolated from freeze-dried live attenuated rubella vaccine BIKEN (Osaka, Japan) (Shishido and Ohtawara, 1976). Endemic genotype 1E and 2B RVi/Osaka JPN strains were isolated from the throat swab of rubella patients in Osaka, Japan from 2012 to 2013. These included 1E strains 52.12 (CRS) [NCBI accession number LC076751], 15.13 [LC076753], 16.13 [LC076754], and 2B strains 14.13 [LC063348], 10.13 [LC076752], and 15.13 [LC076755]. Viral isolation was performed using VeroE6 cells (Early and Johnson, 1988).

### 2.4. Viral titration by RT-PCR

RV was serially diluted in a 10-fold series from 1:10 to 1:10<sup>9</sup> (Vauloup-Fellous et al., 2010). The cells were cultured in a 96 well plate, and were inoculated with virus preparation diluted in 100 µL tissue culture medium. The coculture was allowed to incubate for 4 days. RNA was then extracted from 80 µL of culture supernatant using MagDEA<sup>®</sup> Viral DNA/RNA 200 (GC, Precision System Science, Chiba, Japan). RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen). The NS region was amplified using NSL primers: F3 5'-TCC TTG CGC CGA AGA CT-3' and B3-6 5'-AGA GGG GGT CCA CTT GAG-3' (Early and Johnson, 1988; Okamoto et al., 2016). Each dilution point was examined in 8 replicates. TCID<sub>50</sub> was calculated using the Reed-Muench method (Reed and Muench, 1938).

### 2.5. Determination of the E1 sequence

RNA was extracted from culture supernatant containing a RV

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