Iranian Journal of Virology 2012;6(4): 20-26


**Original Article**

# Evaluation of Cell Mediated Immunity following Rubella Vaccination Using Lymphocyte Proliferation

**Jafari E[1], Mohammadi A[2], Arabzadeh SAM[1], Esna-Ashari F[2*], Sadigh ZA[2], Shokri GhR[2], Shahbazi R[2], Foroughi A[2]**

1. Microbiology&Virology Department, Kerman University of Medical Sciences, Kerman, Iran.
2. Human viral vaccines Department, Razi Vaccine and Serum Research Institute, Karaj, Iran.

## Abstract

**Background and Aims:** *Rubella* is predominantly a childhood disease that is endemic throughout the world and when *rubella* outbreaks occur, they are accompanied by birth defects following congenital rubella syndrome. Immunity to *rubella* virus as a teratogenic agent has an important role for prevention of these serious congenital defects. Lymphocyte proliferation assay is a way for investigation of human cell-immunity and its ability against *rubella* infection.

**Materials and Methods:** The blood samples were obtained in sodium heparin tubes. Ficoll was added to separate lymphocytes. The cells were cultured with RPMI 1640 medium with 15% calf serum in microplates and incubating at 37°C in 3-5% $CO_2$. Mitogens including Phytohemagglutinin and *rubella* hemagglutinin antigen (derived Takahashi strain) were added, separately. Then a fluorescent nucleotide was added. On day $10^{th}$-$11^{th}$ the wells stained and observed.

**Results:** Lymphocytes stimulated with the mitogens were observed directly with an inverted microscope. Their aggregation and growth were detected after two days. Also lymph proliferation was shown using labeled nucleotide comprising a new fluorophore, by fluorescent microscopy. Response to full particle of attenuated virus was better than antigens derived from different parts of the virus.

**Conclusion:** Comparison of the data with previous studies on proliferation of specific lymphocytes in response to *rubella* vaccination confirms our results. Thus cell-immunity to *rubella* infection was activated timely, in individuals who were vaccinated against *rubella* virus approximately 10 years before or exposed to it, but the intensity of responses to different antigens varied in each subject.

**Keywords:** *Rubella*; Vaccination; Cell-Mediated Immunity; Fluorescence Microscopy

## Introduction

*Rubella* virus (RV) is an enveloped positive-single stranded RNA virus of the genus *Rubivirus* in *Togaviridae* family that is transmitted by aerosol via the respiratory tract. The RV virion contains a RNA genome enclosed within an icosahedral capsid composed of multiple copies of a basic protein, C and surrounded with a lipid bilayer in which viral glycoproteins El and E2 are inserted (1).

Rubella is predominantly a childhood disease that is endemic throughout the world. Primary replication occurs in the nasopharynx. The incubation period of *rubella* is 14 to 21 days, with most patients developing a rash 14 to 17 days after exposure. During the first week after

***Corresponding author:** Esna-Ashari F, Ms.C. Human viral vaccines Department, Razi Vaccine and Serum Research Institute, Karaj, Iran.
Email: fesna2002@yahoo.com




[ DOI: 10.21859/isv.6.4.20 ]

exposure, there are no symptoms but in the second week, lymphadenopathy may be noted. Later in the second week, virus appears in the blood and there may be a mild self-limiting fever, malaise and conjunctivitis (1, 2). When *rubella* outbreaks happen, in consequence of maternal infection during the first trimester of pregnancy, congenital *Rubella* syndrome (CRS) occurs. This disease accompanied by birth defects such as cataracts, cardiac abnormalities and mental retardation (3, 4).

Immunity to *rubella* virus as a teratogenic agent has an important role to prevent these serious congenital defects and is conventionally determined by measuring specific immunoglobulin G (IgG). To design better vaccination strategies, it is essential to define the critical immunological mechanisms for effective immunity to *rubella* vaccine. Virus specific T cells play a prominent role in viral immunity particularly in the elimination of infected cells even in the absence of antiviral antibodies. In other words, investigation of rubella cell immunity is a confident way for following up active protection in time past (2, 5).

In the current study, we examined humoral and cellular immune responses to *rubella* vaccination by *rubella*-specific lymphoproliferation and measuring antibody levels.

## Methods

**Blood donors**

Blood was obtained, with informed consent from 12 healthy individuals that some of them were vaccinated from one to ten years ago and the others were exposed to rubella infection (without determined history of vaccination).

**Isolation of peripheral blood mononuclear cells (PBMC)**

Blood sample (6 ml) was collected from each person, in sterile plastic tubes containing sodium heparin(Golden Vac). It was mixed well and diluted with sterile RPMI 1640 medium (Gibco) without serum 1:1. Then the diluted blood was carefully poured onto a 4 ml Ficoll solution (Kronberg/Taunus) and centrifuged at 2500rpm for 30 minute for separating buffy coat layer and then washed twice in sterile RPMI 1640 medium in 2000rpm for 5 min to separate lymphocytes from platelets. The viability of isolated cells was determined by the trypan blue exclusion test (6). The remaining of blood (2ml) was incubated at 4°C overnight and centrifuged for serum separation. Each serum sample was separately stored in deep freeze (20°C).

**Mitogens**

Phytohemagglutinin (PHA, Baharafshan) at concentration of 100 μg / ml, *rubella* hemagglutinin antigen (1:128), In-house standard (IhS) including live particle (Takahashi strain: $10^3$ CCID50/ml) [7] were used.

**Lymphocyte proliferative assay**

The lymphocytes (100μl/well) were cultured in (100 μl/well) RPMI 1640 supplemented with 2% bicarbonate, 15% irradiated calf serum (Razi Institute), 0.3% KN (kanamycin-neomycin) and 1% Tricin at a concentration of $2 \times 10^5$/ml on a 96-well flat-bottom microplate in duplicate at 37°C in 3-5% $CO_2$. At each plate, medium without cells was added to 4 wells as a negative control. After 3 days mitogen and *rubella* antigens were added, separately (50μl/well) in each micro plate at quadruplicate. After 24-48hr incubation fluorescent nucleotide (Alexa Fluor Dyes, Invitrogen) was added to positive wells from each microplate.

**Fluorescent staining**

On day $10^{th}$ -$11^{th}$ following the stimulation, the cultures were halted after 1hrs incubation with colchicine (0.2μg/ml, Baharafshan) at room temperature (RT), KCL (0.075M) was added for 15min. Then the wells were fixed with ethanol-acetate (3:1). After air drying, they were stained with Hoechst 33258 using a modification of the S.A. Latt method (8). The slides were screened after 60-minute incubation (with the dye H33258) in dark condition at RT then examined fluorescence microscopy.

**Serological test**

Hemagglutination Inhibition (HI) and ELISA test were done on the serum samples. The EUROIMMUN Anti-*Rubella* Virus ELISA



[ DOI: 10.21859/isv.6.4.20 ]

(IgG) was used to measure antibody titer against *rubella* virus (9, 10).

## Results

**Proliferative activity of lymphocytes in vitro**

Lymphocytes were observed directly with inverted microscopy. The conventional fluorescence microscopy was used to analyze the in vitro proliferation of lymphocytes labeled with an intracellular fluorophore. Lymphocytes from all donors showed a significant increase of cell proliferation with regard to mitogenic stimulation by PHA(Fig. 1-b) and antigenic stimulation by Rub-IhS (Fig. 1-c) and Ag HA (Fig. 1-d) at day 5-6 upon stimulation and the greatest observed stimulation ratio belonged to PHA and RubIhS. However the responses between individuals were different, the degree of responses had direct correlation with fluorescent intensity (Fig. 2, Table 1).

**Antibody response**

he survey on humoral immunity of two group showed in individuals who were exposed to *rubella* virus or were vaccinated 1 year earlier, antibody titers remained seropositive (1:32), but in individuals who were vaccinated 10 years befor, the *rubella* virus antibodies declined (HI titer lower than 1:16) (Table 2).

## Discussion

To design better vaccination strategies, it is essential to define the critical immunological mechanisms for effective immunity to *measles*, *mumps*, and *rubella* vaccines. Recent data demonstrate the importance of cell mediated immunity (CMI) in controlling and protecting against viral diseases (11, 12). For example, *measles* virus specific CD8+ cytotoxic T cells

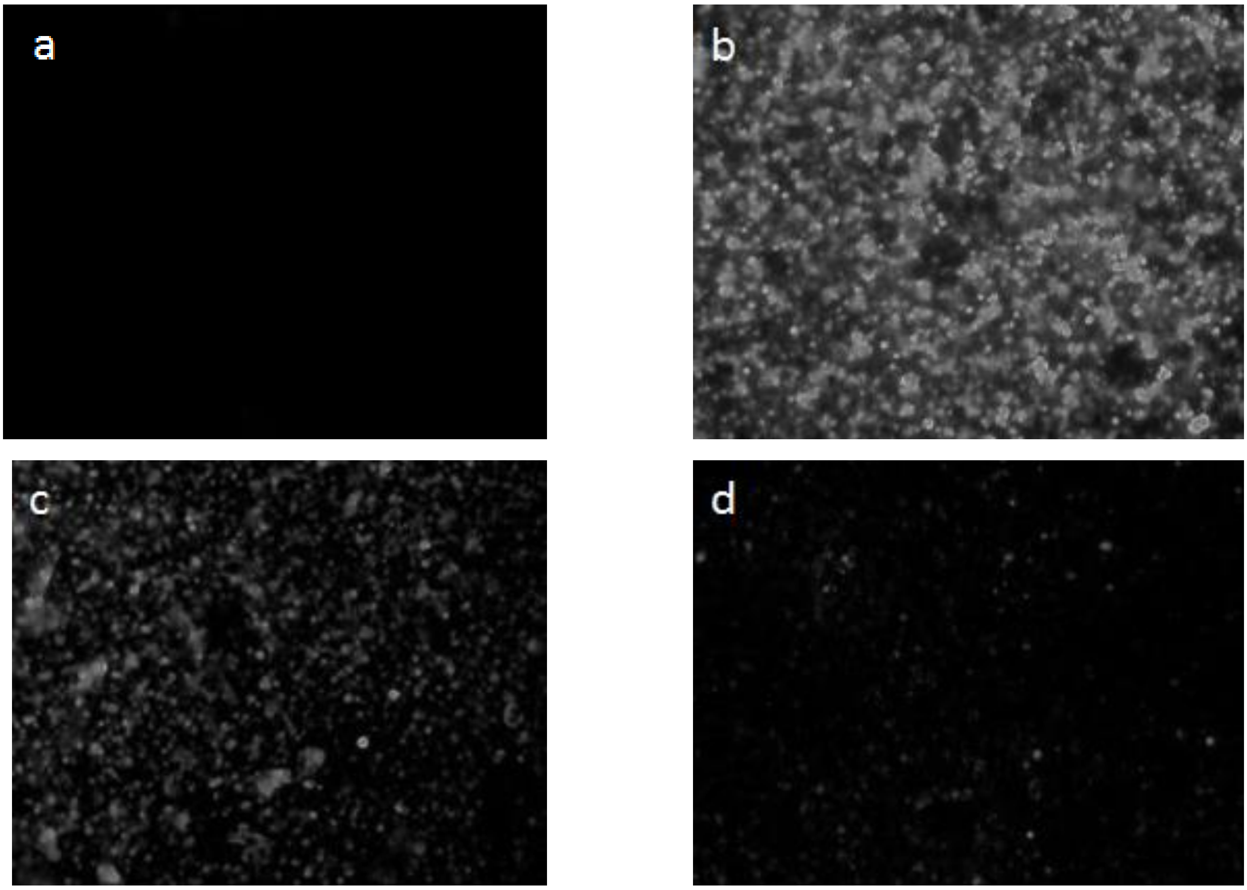


**Fig. 1.** Lymphocyte proliferation within microplate. (a)Negative control, (b)Positive control (PHA), (c) RubIhS and (d) Ag HA.



[ DOI: 10.21859/isv.6.4.20 ]

**Table 1**. Proliferative activity of lymphocytes exposed to different mitogens.

| Samples | PHA | | IhS | | HA Ag | |
|---|---|---|---|---|---|---|
| | Pw | FI | Pw | FI | Pw | FI |
| **1** | 8/8 | ++++ | 7/8 | +++ | 6/8 | ++ |
| **2** | 7/8 | ++++ | 6/8 | +++ | 7/8 | ++ |
| **3** | 8/8 | ++++ | 7/8 | +++ | 7/8 | ++ |
| **4** | 8/8 | ++++ | 7/8 | +++ | 6/8 | ++ |
| **5** | 7/8 | ++++ | 7/8 | +++ | 5/8 | ++ |
| **6** | 6/8 | ++++ | 4/8 | ++ | 3/8 | + |
| **7** | 7/8 | ++++ | 6/8 | +++ | 6/8 | ++ |
| **8** | 6/8 | ++++ | 4/8 | ++ | 2/8 | + |
| **9** | 8/8 | ++++ | 7/8 | +++ | 7/8 | ++ |
| **10** | 8/8 | ++++ | 7/8 | +++ | 7/8 | ++ |
| **11** | 8/8 | ++++ | 7/8 | +++ | 7/8 | ++ |
| **12** | 7/8 | ++++ | 6/8 | +++ | 5/8 | ++ |
| **Total %** | 7.3/8 | | 6.2/8 | | 5.6/8 | |

Pw: Positive Wells, FI: Fluorescent Intensity
+: 10-25%, ++: 25-50%, +++: 50-75%, ++++: 75-100%. Samples 1-3 were exposed to *Rubella* infection, samples 4-8 and 9-12 were vaccinated 1 year and 10 years prior to this experiment, respectively. For each sample, eight wells were stimulated with different mitogens and labeled nucleotide were added to positive wells which showed cell growth, to determine proliferative responses.

**Table 2**. Qualitative test results obtained from two serological assays HI & ELISA

| Samples | Anti-Rubella Virus ELISA(IgG) EUROIMMUN | HI Test |
|---|---|---|
| 1 | 40(IU/ml) | 1:32 |
| 2 | 43(IU/ml) | 1:32 |
| 3 | 39(IU/ml) | 1:32 |
| 4 | 51(IU/ml) | 1:32 |
| 5 | 50(IU/ml) | 1:32 |
| 6 | 87(IU/ml) | 1:32 |
| 7 | 33(IU/ml) | 1:16 |
| 8 | 13(IU/ml) | 1:8 |
| 9 | 27(IU/ml) | 1:16 |
| 10 | 16(IU/ml) | 1:8 |
| 11 | 11(IU/ml) | 1:8 |
| 12 | 10(IU/ml) | 1:4 |

In ELISA: Negative range = 8 (IU/ml) , Cut-off =10 (IU/ml) , Positive range ≥11 (IU/ml)
Immune antibody titer of HI test for Rubella is considered to be ≥ 1: 8. Cut off: the upper limit of the reference range of non-infected persons.

proliferate and activate in the peripheral blood with *measles* rash onset, suggesting a central role in viral clearance (13, 14). Further, individuals who are infected with human immunodeficiency virus (HIV) and those with impaired cell-mediated immunity have a higher morbidity and mortality rate with *measles* co-infection compared to those who are immunologically intact (15, 16), demonstrating the importance of CMI in *measles* virus elimination. Similarly, Lovett and colleagues (17) and Hyöty and colleagues (18)



[ DOI: 10.21859/isv.6.4.20 ]

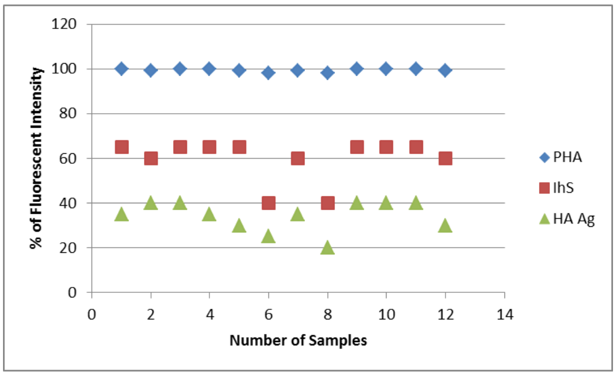


**Fig. 2.** Comparison of fluorescent intensity of each individual in response to different mitogens. After PHA which is a nonspesific stimulator, greatest ratio of observed stimulation in the samples belonged to RubIhS.

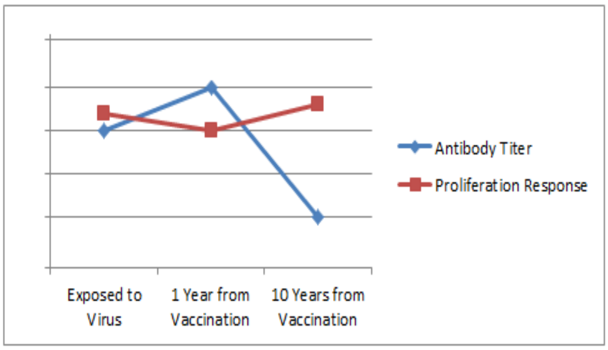


**Fig. 3.** Comparison of antibody titer and proliferation responses in different subjects. Individuals who regularly were exposed to *Rubella* virus showed high titers of antibody compared to those which 10 years passed from their vaccination but had almost similar lymphoproliferative responses.

demonstrated that induction of specific cellular immunity in response to *rubella* and *mumps* is necessary for recovery from disease and long-term protection.

The lymphocyte proliferation assay (LP) and the chromium-release assay have been widely used in the past for investigation of CMI. Since estimation of CMI not only is critical for effective immunity and long term protection against viral infections but also is the best for evaluation of vaccine efficacy and post marketing surveillance, we evaluated a surrogate measure of CMI, lymphoproliferative response, across the *Rubella* components of MMR-Razi through 12 blood sample in appropriate vaccinated individuals or exposed to the particle. Further an evaluation of the correlation within CMI responses and ELISA to this component of MMR-RAZI was done 1&10 years after vaccination.

In vitro lymphocyte proliferation assays can be used to analyze immune reactions and to reach a better understanding of cell-cell interactions during the course of immunological events. In general, radioactive tracers such as [3 H] thymidine are added to lymphocyte preparations and incorporated into newly synthesized DNA during cell division and proliferation (19). The radioactive uptake can be measured using a beta counter and correlates with the amount of cell proliferation. However working with radioactive substances and expensive counting equipment in the method, has motivated a quest for easier alternatives.

In this study two different measurement systems for analyzing immunity against *rubella* vaccine Takahashi strain have been done and compared. For evaluating CMI, Fluor chrome based method using Alexa flour dye was installed in human viral vaccine department of RAZI institute. The results of the various experiments were compared and analyzed in order to investigate whether non-radioactive Fluor chrome assays could be substituted as the previous radioactive [3 H] thymidine labeling method or not. In parallel HI and ELISA was done on same samples for judgment of *rubella* humoral immunity.

This study showed that a proliferation of lymphocyte in response to *rubella* antigen appears both in 1&10 year after vaccination (Table 1, Fig. 2). This result reveal that in group study after PHA which is a nonspecific stimulator, highest observed stimulation ratio in all of the samples belonged to Rub IhS. It's confirmed the presence of *rubella* CMI in all of cases against TAKAHASHI strain of RAZI vaccine, although the serologic evidence revealed that there is seropositivity in >91.6% of samples (Table 2). It means 8.3% of sample showed sero negativity meanwhile was CMI positive.





[ DOI: 10.21859/isv.6.4.20 ]

All cases that vaccinated last year were still seropositive (100%). The other vaccinated people to 85% were still seropositive, after10 years but their mean HI titers had dropped to 1: 8 (Table 2). In a sensitive population, *rubella* outbreaks may be explosive. But a history of having been exposed to a *rubella* outbreak does not necessarily indicate immunity to *rubella* as showed in. In the other hand there are immune persons (CMI positive) that in humoral assay showed negative results (Table 2, Fig 3).

Approximately 5% of RV-vaccinated people do not seroconvert and are therefore regarded as non-responders (2). However, with regard to vaccination against other viral diseases it is reported that persistent sero negative people develop CMI and can therefore be classified as immune (2). There are only few reports about CMI in RV. Toyoda and colleagues (20) found a generally good correlation between antibody levels and the expression of interleukin-2 receptor alpha on T lymphocytes cultured with RV antigen. However, in several individuals the correlation was poor. Ovsyannikova and co-workers (21, 22) showed that it is genetically determined whether a vaccinated person develops humoral immunity or CMI to RV. A correlation between the proliferation index of antigen-stimulated lymphocytes and antibody response to RV was not detected (2).

In *rubella* virus diagnostics, no routine test for the assessment of CMI exists. Conventionally the correlate of immunity to RV is a hemagglutination inhibition titer ≥1:32 or an antibody level ≥15 IU/ml (2). In future, we aim to determine if a correlative and predictive intra class relationship exists between CMI responses to individual virus, the *measles*, *mumps*, and *rubella* components of the MMR – RAZI vaccine by lymphoproliferation assay in compare of a relatively new method, the interferon-gamma (IFNγ)-ELISpot (23).


## Acknowledgements

The authors would like to thank Razi Vaccine and Serum Research Institute for providing facilities and financial support and the staff of Human viral vaccines department for their help in progression of this research.


## References


1. Hobman TC, Chantler J. Rubella Virus. *In* Knipe DM, Howley PM (editor), Fields Virology, 5th Edition. Lippincott /Williams & Wilkins Co, Philadelphia, 2007; P: 1069–1100.
2. Reef SE, Plotkin SA. Rubella Vaccine. Vaccines, 6th Edition. Saunders, Philadelphia**,** 2012;P: 688 – 717 .
3. Webster WS. Teratogen update: congenital rubella. Teratology. 1998; 58(1):13-23.
4. Cutts FT, Robertson SE, Diaz-Ortega J, Samuel R. Control of rubella and congenital rubella syndrome (CRS) in developing countries, Part 1: Burden of disease from CRS. Bulletin of the World Health Organization. 1997;75(1):55.
5. Robertson S, Cutts F, Samuel R, Diaz-Ortega J. Control of rubella and congenital rubella syndrome (CRS) in developing countries, Part 2: Vaccination against rubella. Bulletin of the World Health Organization. 1997;75(1):69.
6. Strober W. Trypan blue exclusion test of cell viability. Current Protocols in Immunology. 2001:A. 3B. 1-A. 3B. 2.
7. Esna-Ashari F, Shafyi A, Taqavian M, Mohammadi A, Sadigh Z, Sabiri G, et al. Microtitration of Rubella Virus in Monovalent Vaccinal Products. Iranian journal of public health. 2011;40(1).
8. Latt SA. Microfluorometric analysis of deoxyribonucleic acid replication kinetics and sister chromatid exchanges in human chromosomes. Journal of Histochemistry & Cytochemistry. 1974;22(7):478-91.
9. Leland DS. Clinical virology: WB Saunders Philadelphia; 1996.
10. Hay FC, Westwood OM. Practical immunology: 4th Edition, Blackwell Science Ltd, Oxford, UK; 2002.
11. Mitchell LA, Tingle AJ, Décarie D, Shukin R. Identification of rubella virus T-cell epitopes recognized in anamnestic response to RA27/3 vaccine: associations with boost in neutralizing antibody titer. Vaccine. 1999;17(19):2356-65.
12. Pukhalsky AL, Shmarina GV, Bliacher MS, Fedorova IM, Toptygina AP, Fisenko JJ, et al. Cytokine profile after rubella vaccine inoculation: evidence of the immune-suppressive effect of vaccination. Mediators of inflammation. 2003;12(4):203-7.

13. Jaye A, Magnusen AF, Sadiq AD, Corrah T, Whittle HC. Ex vivo analysis of cytotoxic T lymphocytes to measles antigens during infection and after vaccination in Gambian children. Journal of Clinical Investigation. 1998;102(11):1969.
14. Permar SR, Klumpp SA, Mansfield KG, Kim WK, Gorgone DA, Lifton MA, et al. Role of CD8+ lymphocytes in control and clearance of measles virus infection of rhesus monkeys, Journal of virology. 2003;77: 4396–4400.
15. Good RA, Zak SJ. Disturbancee in Gamma Globulin Synthesis as" Experiments of Nature" E. Mead Johnson Award. Pediatrics. 1956;18(1):109-49.
16. Al-Attar I, Reisman J, Muehlmann M, McIntosh K. Decline of measles antibody titers after immunization in human immunodeficiency virus-infected children. Journal of Pediatric Infectious Diseases. 1995;14: 149– 51.
17. Lovett A, Hahn C, Rice C, Frey T, Wolinsky J. Rubella virus-specific cytotoxic T-lymphocyte responses: identification of the capsid as a target of major histocompatibility complex class I-restricted lysis and definition of two epitopes. Journal of virology. 1993;67(10):5849-58.
18. Hyöty H, Räsänen L, Lehto M, Tanhuanpää P, Eerola A, Surcel HM, et al. Cell-Mediated and Humoral Immunity to Mumps Virus Antigen. Acta Pathologica Microbiologica Scandinavica Series C: Immunology. 1986;94(1-6):201-6.
19. Dhiman N, Ovsyannikova IG, Jacobson RM, Vierkant RA, Pankratz VS, Jacobsen SJ, et al. Correlates of lymphoproliferative responses to measles, mumps, and rubella (MMR) virus vaccines following MMR-II vaccination in healthy children. Clinical Immunology. 2005;115(2):154-61.
20. Toyoda M, Ihara T, Nakano T, Ito M, and Kamiya H. Expression of interleukin-2 receptor alpha and CD45RO antigen on T lymphocytes cultured with rubella virus antigen, compared with humoral immunity in rubella vaccinees. Vaccine 1999;17: 2051–2058.
21. Ovsyannikova IG, Pankratz VS, Vierkant RA, Jacobson RM, Poland GA. Human leukocyte antigen haplotypes in the genetic control of immune response to measles-mumps-rubella vaccine. Journal of Infectious Diseases. 2006;193(5):655-63.
22. Ovsyannikova IG, Jacobson RM, Vierkant RA, Jacobsen SJ, Pankratz VS, Poland GA. Human leukocyte antigen class II alleles and rubella-specific humoral and cell-mediated immunity following measles-mumps-rubella-II vaccination. Journal of Infectious Diseases . 2005;191(4):515-9.
Allmendinger J, Paradies F, Kamprad M, Richter T, Pustowoit B, Liebert U. Determination of rubella virus-specific cell-mediated immunity using IFNγ-ELISpot. Journal of Medical Virology. 2010;82(2):335-40.



[ DOI: 10.21859/isv.6.4.20 ]