Seroepidemiological survey of rubella immunity among three populations in Shiraz, Islamic Republic of Iran

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مسح مصلي وبائي لمناعة الحصبة الألمانية بين ثلاثة مجتمعات في شيراز بجمهورية إيران الإسلامية

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خلاصة: استخدمنا مقابسة الإليزا اللامباشرة في فحص 1193 عينة مصل بحثاً عن أضداد الحصبة الألمانية من صنف الأيج ج IgG، والأيج م IgM، وذلك في مسح مصلي وبائي أجريناه على ثلاث عينات سكانية مختلفة بحسب السن والجنس في شيراز. وشملت الدراسة 203 من الأطفال الذين تبلغ أعمارهم 2-7 سنوات، و255 من ثنائيات الأمهات والولدان (عينات من دم الحبل السري)، و400 فتاة وسيدة تبلغ أعمارهن 14-70 سنة. وتبيّن أن إيجابية المصل بين النساء اللواتي تتراوح أعمارهن بين 14 و70 سنة من العمر كانت بنسبة 96.2%. ولم توجد في عينات دم الحبل السري وعددها 255 أي عينة إيجابية للأيج م. وبلغت إيجابية الأمصال بين الأطفال المئتين وثلاثة نسبة 97.0% (أعلى كثيراً مما تضمّنته التقارير من قبل). وربما كان سبب ذلك أوبئة الحصبة الألمانية التي يتواتر حدوثها كل ست إلى عشر سنوات. وتناقش المقالة تأثير تطبيق التطعيم ضد الحصبة الألمانية.

ABSTRACT We used indirect ELISA assay to test 1193 sera for rubella IgG and IgM antibodies in a seroepidemiological survey of three age- and gender-differentiated sample populations in Shiraz: 203 children aged 2–7 years, 255 paired mothers and neonates (cord blood) and 480 women aged 14–70 years. Seropositivity among women aged 14–70 years was 96.2%. No IgM positive case was found among the 255 tested cord blood samples. Seropositivity among the 203 children was 97.0% (much higher than previously reported). This may be due to rubella epidemics, which tend to occur every 6–10 years. The impact of introducing rubella vaccination is discussed.

Enquête séro-épidémiologique sur l'immunité contre la rubéole dans trois populations à Chiraz (République islamique d'Iran)
RESUME Nous avons utilisé la méthode immuno-enzymatique indirecte pour tester 1193 sérums à la recherche d'anticorps IgG et d'IgM antirubéoleux dans une enquête séro-épidémiologique comprenant trois populations de l'échantillon différenciées par l'âge et le genre à Chiraz : deux cent trois (203) enfants de 2 à 7 ans, 255 mères et nouveau-nés appariés (sang du cordon) et 480 femmes âgées de 14 à 70 ans. La séropositivité parmi les femmes âgées de 14 à 70 ans s'élevait à 96,2 %. Aucun cas IgM positif n'a été trouvé dans les 255 échantillons de sang de cordon testés. La séropositivité parmi les 203 enfants s'élevait à 97,0 % (bien supérieure à ce qui avait été signalé précédemment). Ceci peut être dû aux épidémies de rubéole qui ont tendance à survenir tous les 6-10 ans. L'impact de l'introduction de la vaccination contre la rubéole fait l'objet d'un examen.

Introduction

Rubella is an exanthem disease of childhood, presenting with rash, fever and lymphadenopathy. It is a disease with a high risk of damage to the fetus. Since 1970, comprehensive vaccination against rubella has been undertaken in industrialized countries [1]. In the past few years, some developing countries have begun the vaccination programme against rubella [2].

Although rubella is endemic in the Islamic Republic of Iran, there is a lack of adequate information about the immune status of populations to the disease in different parts of the country. Rubella vaccination is currently not considered a health priority in the country. However, the high risk of damage to fetal organs and congenital malformations after intrauterine infection, coupled with the endemicity of the disease in the Islamic Republic of Iran, would suggest the necessity of vaccinating those at risk in the population. Selection of an appropriate, efficacious policy of vaccination is, therefore, needed. Different vaccination strategies are used in different parts of the world [3–5], including: universal vaccination of children and susceptible adults; vaccination of 15–24-month-old children, as well as children entering or ending school; and vaccination of 12–13-year-old girls, and women in the postpartum period. However, in populations with high levels of natural immunity in women of childbearing age, premarital testing and vaccination of seronegative females is recommended [6].

Considerable geographic variations in the seropositivity rate for rubella because of differences in temperature have been reported by other investigators [7]. However, adequate information about immunity to the disease in different parts of the Islamic Republic of Iran is missing. The results of the few investigations undertaken reveal a high seropositivity rate, ranging from 74.8% among women of childbearing age in Ahwaz and Dezful [8] to 97.5% among children by age 15 years in Teheran [9]. However, cases of congenital rubella syndrome have been reported in Ahwaz and Dezful where high temperatures and a low seropositivity rate coexist.

In order to design an efficient and economical vaccination policy, it is necessary to have adequate information about the immune status in different geographical parts of the country, with the different climates in different time intervals. Most of the information about rubella epidemiology in the Islamic Republic of Iran is limited to a few geographical regions in discontinuous time intervals. A major reason for this lack of information is because of the high cost of the commercial rubella kits available for such studies — the availability of homemade assays would clearly be more cost-effective. To achieve this, an indirect enzyme-linked immunosorbent assay (ELISA) was designed and standardized according to World Health Organization standard serum to perform a quantitative test. Since 1991, when a rubella outbreak was reported in Shiraz [10], there has been no official report of seroprevalence of rubella immunoglobulins in the city. Therfore a seroepidemiological survey was performed on 1193 sera from three different populations according to age and sex.

Methods

Blood samples

The study population consisted of three age groups. There were 203 blood samples collected from children (ages 2–7 years) with no major health problem referred to Shahid Rezaiee Polyclinic, Shiraz; 255 paired ma-

ternal and cord blood samples collected from pregnant women (ages 14–40 years); and 480 blood samples collected from women (ages 15–70 years) attending the Zeinabie Gynaecologic Clinic for routine examination. Sera were separated from blood samples on the same day of sampling, aliquoted in 0.5 mL volumes, and stored at –20 °C until use. Demographic data, including age, sex, number of children, previous abortions, abnormalities in the family and address were collected at the time of sampling.

Virus culture

Takahashi strain of rubella virus, which is used for optional vaccination in the Islamic Republic of Iran, was cultured in Vero cell line. Monolayers of Vero cell line were grown in RPMI-1640 containing 10% fetal bovine serum at 37 °C in a 5% carbon dioxide-containing atmosphere. At the time of confluency (2–3-day-old cultures), cultures were washed with Hanks balanced salt solution, and rubella seed virus 10^3 TCID50 in 2 mL was added. After 1 hour, adsorption at room temperature with periodic rolling, followed by 3 hours at 37 °C, maintenance medium containing 3% fetal bovine serum was added to each monolayer. Maintenance medium was replaced, 48 hours after infection, with RPMI-1640 medium without serum. To achieve high titre stocks of virus and to restrict defective interfering particles during serial un-

diluted passage, rubella virus was alternatively passaged on Vero and BHK-21 cell lines [11].

Antigen preparation

Detergent-solubilized whole virus preparations were prepared according to the method described by Mitchell et al. [12,13]. In brief, Takahashi strain of virus was grown on monolayers of Vero cells and harvested from culture fluids 72, 96 and 120 hours after infection. Culture supernatants were pooled and clarified by centrifugation at $1000 \times g$ for 20 minutes at 4 °C. The clarified culture supernatant was concentrated by polyethylene glycol 6000 precipitation by adding 2.2% (w/v) sodium chloride followed by 6% (w/v) polyethylene glycol 6000. After overnight incubation at 4 °C with continual stirring, the fluid was pelleted by centrifugation at $1000 \times g$ for 1 hour at 4 °C. The viral pellet was resuspended in proper volume of phosphate-buffered saline containing 0.5% Triton X-100.

Using mock-infected culture supernatant of Vero cells, a control antigen was prepared using the same procedure as rubella antigen and used in an indirect ELISA assay, including eight positive sera with different but known concentrations of anti-rubella IgG. Only two sera revealed an absorbance of more than 0.6, which was equal to 20 IU/mL in the assay. However,

Table 1 **Comparison of the absorbance of 8 tested sera with control and rubella antigens prepared from mock infected and rubella virus-infected culture supernatant fluid**

Reaction	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6	Serum 7	Serum 8
Reaction with control antigen	0.24	0.22	0.23	0.47	0.43	0.75	0.35	0.87
Reaction with rubella antigen	1.30	1.38	1.28	1.87	1.59	1.90	1.33	1.86

the reactivity of these sera with rubella antigen was much greater (Table 1).

Whole virus ELISA

Different concentrations of Triton X-100-solubilized rubella antigen were titrated against negative and positive control sera of a commercial kit (Alfa Biotech, Italy) containing 0, 6.25, 12.5, 25, 50 and 100 IU/mL anti-rubella IgG to find the best concentration for antigen coating. Rubella antigen was diluted in carbonate–bicarbonate buffer (pH = 9.6) and coated onto the ELISA plate in proper concentration. Unbound antigen was washed three times with phosphate buffered saline containing 0.05% Tween 20, and plates were blocked with phosphate buffered saline containing 1% gelatine for 1 hour at 37 °C. After the next three washes, patient sera (diluted 1:100) were added to wells and incubated at 37 °C for 1 hour. Each plate contained a 6-point dilution series of a pooled serum, which was standardized against WHO RV-IgG reference serum (NIBSC, United Kingdom). A blank well containing phosphate buffered saline Tween 20 instead of serum was also included in each plate. Specific IgG was detected using a horseradish peroxidase-conjugated goat anti-human IgG (Calbiochem, United States of America). Optimally diluted, labelled anti-IgG was added onto ELISA plates and incubated for 45 minutes at 37 °C. After the final three washes with phosphate buffered saline Tween 20, enzyme substrate, orthophenylene diamine dihydrochloride (DAKO, Denmark) and hydrogen peroxide in citrate buffer (pH = 5.5) were added in 100 μL volumes. Colour development was allowed to continue for 10 minutes and then stopped by adding 50 μL of 12.5% sulfuric acid solution. Absorbance was measured at 490 nm using an ELISA spectrophotometer (Titertecplus-MS2 reader, Finland). Concentrations of rubella virus-specific IgG (IU/mL) were determined from standard curves plotted for each plate by the formula:

$$\ln Y = \alpha_o + \alpha_1 \ln X$$

where Y is absorbance value as dependent variable, X is the concentration of anti-rubella IgG in sera, α_o is the Y intercept and α_1 is the slope of the fitted line.

Since levels of 19.0 ± 3.7 IU/mL in the in-house whole-virus ELISA were shown to be equal to 15.3 ± 2.4 IU/mL in the Dia-Medix commercial assay [12], the level of 20 IU/mL was considered as the baseline for detection of negative cases. To detect IgM anti-rubella antibody, coating, blocking, serum dilution and incubation times and temperatures were performed as for the IgG-rubella assay. To avoid the effect of potentially present rheumatoid factor and to remove the unwanted competition of IgG antibodies with IgM antibodies, total serum IgG was absorbed by adding goat anti-human IgG in proper dilution at the time of serum addition. Positive, negative and cut-off sera of a commercial kit (Alfa Biotech, Italy) were included in the assay. Absorbance was measured at 490 nm and positive and negative results were determined according to the cut-off value.

Statistical methods

The student t-test for unpaired and paired samples was used to compare the mean concentration of anti-rubella IgG between the study groups. The chi-squared test was used to evaluate the effect of different factors on seropositivity rate. The Levene test was used to analyse the homogeneity of variances of anti-rubella IgG concentration between groups. Statistical analyses were performed using SPSS, version 6.0.

Results

In total, 735 serum samples of women aged 14–70 years and 203 serum samples from children aged 2–7 years were collected from December 8, 1998 to February 20, 1999. Of 735 female subjects, the sera of 255 pregnant mothers were also grouped with the paired cord blood sam-
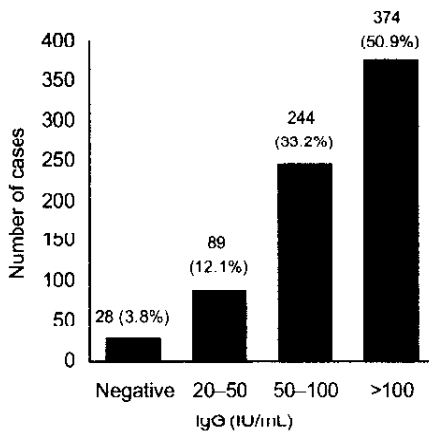

Figure 1 **Anti-rubella IgG concentration in women aged 14–70 years.**

ples collected at the time of delivery. Of 643 samples with known address, 262 (40.7%) were collected from women who lived in areas surrounding Shiraz city (rural) and 381 (59.3%) from women who lived in Shiraz city itself (urban). Of 666 serum samples for which the number of children in the family was known, 513 (77.0%) belonged to women with fewer than 3 children, 99 (14.9%) belonged to women with 3–5 children and 54 (8.1%) belonged to women who had 6 or more children. Among women of childbearing age, 606 had no history of abortion and 129 had at least one case of abortion (86 had had one, 30 had had two and 13 had had more than two). Of the 735 women, 46 reported abnormality among their children or a close relative.

Seropositivity rate in women aged 14–70 years

The rate of seropositivity among women was found to be 96.2% (707 out of 735). The average age ± standard deviation of the test group was 26 ± 8 years and the mean IgG concentration was 111.13 ± 60.79 IU/mL. Of the 735 sera tested in this group,

Table 2 Anti-rubella IgG concentration in 643 women by age, Shiraz, Islamic Republic of Iran

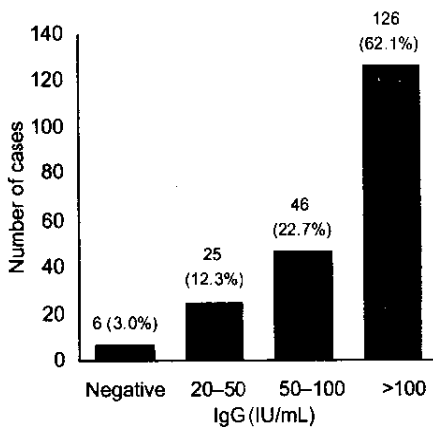| Age group (years)	No.	%	No. negative	No. positive	IgG concentration Mean ± s (IU/mL)
< 20	106	16.5	3	103	112.19 ± 58.38
20–24	229	35.6	7	222	106.76 ± 55.37
25–29	146	22.7	4	142	104.72 ± 57.32
30–34	71	11.0	4	67	114.75 ± 71.65
35–39	45	7.0	2	43	128.41 ± 68.40
40–44	28	4.4	0	28	116.43 ± 61.09
45–49	10	1.6	0	10	155.46 ± 51.02
≥ 50	8	1.2	0	8	118.62 ± 90.36
Total	643	100	20	623	111.31 ± 60.21

s = *standard deviation.*

Figure 2 **Anti-rubella IgG concentration in children aged 2–7 years.**

28 sera (3.8%) had less than 20 IU/mL anti-rubella IgG and were therefore considered to be negative. Anti-rubella IgG concentrations among the 735 women are shown in Figure 1. Mean rubella-specific IgG concentration with regard to age distribution, and the frequency of positive and negative cases in each age group are shown in Table 2. No negative case was found among women aged > 40 years. No significant difference was found between the eight age groups with regard to specific IgG concentration (Kruskal–Wallis test, $P = 0.09$).

Of the 262 rural and 381 urban women, 9 (3.4%) and 11 (2.9%) respectively were negative. The difference was not statistically significant. However the mean concentration of rubella-specific IgG in rural cases (mean ± standard deviation − 103.64 ± 61.58 IU/mL) was significantly lower than in urban cases (117.59 ± 59.59 IU/mL) (Student t-test, $P < 0.05$).

A higher percentage of seronegativity was found in women with fewer than 3 children compared to women with 3 or more children (3.9% versus 1.3%), although this was not significant. The mean concentration of rubella-specific IgG in the former group was significantly lower than in the latter (108.44 ± 59.04 IU/mL versus 119.88 ± 63.20 IU/ml) ($P < 0.05$).

Seropositivity rate in paired mothers and cord blood samples

The mean concentration of anti-rubella IgG in mothers' sera (109.98 ± 54.18 IU/mL) was significantly higher than the mean concentration of anti-rubella IgG in cord blood samples (91.39 ± 54.03 IU/mL) (Student t-test for paired samples, $P < 0.001$). Of 255 mothers, 2 had < 20 IU/mL anti-rubella IgG and were thus considered negative. However, the number of negative cases was higher (10 out of 255) among neonates. Of the seronegative neonates, two were born of mothers who were seronegative, and

Table 3 **Comparison of anti-rubella IgG in mothers' sera and the corresponding cord sera (n = 255)**

Source	Anti-rubella IgG (IU/mL)							
	< 20		20–50		50–100		100	
	No.	%	No.	%	No.	%	No.	%
Mothers' blood	2	0.78	25	9.80	97	38.04	131	51.37
Cord blood	10	3.92	58	22.74	84	32.94	103	40.93

were in the youngest age group (i.e. < 20 years old). The eight others were born of mothers with anti-rubella IgG concentration of < 50 IU/mL. A comparison of the representative data for mothers and neonates is shown in Table 3.

Seropositivity rate among children aged 2–7 years

The mean age ± standard deviation was 4.5 ± 1 years. The seropositivity rate in the children was 97% and the mean concentration of specific IgG was 131.21 ± 71.24 IU/mL. Of 203 serum samples in this age group, 6 (3.0%) had an anti-rubella IgG concentration of < 20 IU/mL (Figure 2). Of these, 5 of 102 (4.9%) were males and 1 of 101 (1.0%) was female. However, the observed difference was not significant. The mean concentration of anti-rubella IgG for males was 136.06 ± 73.95 IU/mL, compared to 127.61 ± 67.72 IU/mL for females.

Comparison of the rubella-specific IgG in women (group 1) and children (group 3)

The mean concentration of anti-rubella IgG in women's sera was significantly lower than in children's sera (111.13 IU/mL compared to 131.21 IU/mL). In addition, the distribution of IgG concentrations around the mean was significantly different between these groups (Levene test of homogeneity, $P < 0.05$). In this regard, the variation in anti-rubella IgG concentration in children was significantly higher than in women.

Anti-rubella IgM determination in cord blood samples

To assess possible rubella infection of the neonates, 255 cord blood samples were tested in an IgM-ELISA assay. No IgM-positive case was detected.

Discussion

The observed 96.2% seropositivity rate among women of childbearing age is comparable to the reported prevalences of 93% [14] and 94.9% [15] of the same age group in the Islamic Republic of Iran. Since the study population was from a low socioeconomic population, the high seropositivity rate is not unexpected. Saidi et al. reported the seropositivity rate in a low socioeconomic group to be as high as 99%, compared to 92% in the high socioeconomic group in their study [14]. In a study between 1993 and 1995, Modarres et al. reported seropositivity to be 96.5% in a low socioeconomic group compared to 93% in the high socioeconomic group [15]. The rate of seropositivity in our study is also comparable to Saudi Arabia [16,17], Kuwait [18], Nigeria [19], Bahrain [20], Gambia [21], Turkey [22] and India [23]. However, it is much higher than the reported prevalence in Taiwan [24,25], Angola and Ghana [26].

The frequency of seropositivity in urban participants was slightly higher than in rural participants (97.1% versus 96.6%), but this was not statistically significant. This might be due to the fact that the rural participants were from villages very close to Shiraz, and the same factors involved in the spread of the virus in the city would also have influenced the epidemiology of the disease in these areas. In addition, the mean anti-rubella IgG concentration was significantly lower in rural participants compared to urban participants. Similarly, Saidi reported a lower haemagglutination-inhibition titre in rural cases compared to urban cases (1:138 versus 1:165) [14].

In previous studies, family density had been reported to influence the level of rubella antibody [5]. In the present study, the mean IgG concentration in women with

fewer than 3 children was lower than those with 3 or more children.

The mean concentration of anti-rubella IgG in cord sera was significantly lower ($P < 0.001$) than that of mothers' sera. Also, the frequency of seronegative cases was found to be significantly higher in cord blood samples. There were eight low positive and two seronegative mothers who gave birth to neonates who were seronegative. This lack of transmission of specific antibodies has been previously reported for measles and tetanus [27]. In a study of transplacental transfer of toxoplasmosis antibodies among European and African cord–mother pairs, a lack of transfer was found among African, but not European pairs [28]. Shortridge and Osmund [29] reported the incidence of maternally acquired rubella antibody to be 84% for the Chinese population and 90% for Caucasians in Hong Kong. They also reported a decrease in this incidence to 54% in Chinese, but not Caucasians, by 2 months. In this regard, the higher concentrations of rubella and measles antibodies in North American or European mother–neonate pairs have been reported in cord blood samples [30]. Ethnic differences and differences in level of specific antibody have been considered possible explanations for these inconsistencies [27,31]. In African mothers who had high levels of antibody and who lived in countries with a high endemicity rate of infectious diseases, higher levels of tetanus antibody revealed a positive correlation with lack of antibody transfer to the fetus [28]. However, in our study we found the opposite to be the case for the transfer of rubella antibody.

The seropositivity rate among children aged 2–7 years was found to be 97%, much higher than previous reports. Saidi reported a rate of 58% for the same age group [14]. In 1970, Naficy et al. reported a rate of 46.5% [9] and in 1996, Modarres et al. reported a rate of 50% for the age group of 1–10 years [15]. The rate of seropositivity among children < 10 years of age in Shiraz had been reported as 46.5% during 1986–90 [10]. However, the authors reported evidence of an outbreak in 1991 in Shiraz, through which the rate of seropositivity increased to 94.5%. There is no official report on the duration of rubella outbreaks in the Islamic Republic of Iran. However, it can be seen from reports from the United States of America and United Kingdom in the pre-vaccination era, that rubella outbreaks seem to appear in 6–9-year cycles. Since the present study was undertaken 8 years after the abovementioned report, it is likely that we faced another outbreak in Shiraz during the sampling period. It is noteworthy that greater variation in the concentration of anti-rubella IgG was found among children than among the women's group. This variation might be due to an active immunological response among children who faced rubella virus for the first time.

In the few reports of the seroepidemiology of rubella from different parts of the country, prevalence rates of 70% to more than 95% are reported among women of childbearing age [8,9,10,14,15]. It seems that in the highly populated urban areas with temperate climates, most of the girls develop anti-rubella IgG by the age of 15 years [9,14,15]. In our study, the seropositivity rate (96.2%) among women in an urban setting with a temperate climate was much higher than the rate reported in Ahwaz and Dezfal (74.8%), a hot area of the country [8]. However, our results are comparable with rates reported from other areas of the country with a temperate climate [15].

To date, rubella vaccination has not been considered a health priority in the Islamic Republic of Iran. However, due to highly variable climate in different parts of the country, deciding on a "yes" or "no" vaccination policy might be reconsidered. It should be noted that most of the studies from which information about Iranian seropositivity rates are drawn are from urban areas with temperate climate. However, in some parts of the country, lifestyles are completely different from urban and even rural life. Some migrating tribes are not settled in a defined place. No data exist about rubella immunity in such tribes.

Previous investigations have shown that the age incidence of rubella infection in the Islamic Republic of Iran is low [14,15]. The introduction of vaccination in other parts of the world has been accompanied by a shift in the age incidence of rubella infection to older age groups [1]. The age of women at marriage in many parts of the Islamic Republic of Iran, especially in rural areas, is low (12–17 years of age). Unless vaccination of all at-risk populations is undertaken, any immunization programme would change the pattern of rubella epidemiology such that the risk of infection for older groups may increase, and the risk of reinfection with wild strains after vaccination may arise.

Acknowledgement

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Control of rubella and congenital rubella syndrome (CRS) in developing countries

This recent WHO publication includes information on the burden of disease from CRS and vaccination against rubella. It also contains selected bibliographies on rubella and CRS by WHO Region, details of selected rubella serosurveys in developing countries and the routine national schedules for rubella immunization by WHO Region. The document is available from: WHO, Department of Vaccines and Biologicals, CH-1211 Geneva 27, Switzerland. Fax: +22 7914193, E-mail: vaccines@who.ch. It is also available on the Internet at:

www.who.int/gpv-documents/