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SHORT COMMUNICATION

Sero-epidemiology and Risk Factor Analysis of Measles Among Children in Pakistan

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Abstract

Comparative cross sectional study was conducted on blood samples (n = 231) collected from children of 1 to 10 years of age in Punjab Pakistan through convenient sampling method. Indirect haemagglutination assay (IHA) was standardized and used for serodiagnosis and evaluation of humoral immunity against measles. Associated risk factors including age, gender, locale, and vaccination status were analyzed. Geometric mean titre (GMT) of vaccinated individuals was significantly higher ($p < 0.001$) than that of non-vaccinated individuals showing that IHA titre of vaccinated individuals was a measure of humoral immune response; whereas, in case of non-vaccinated individuals an indicative of exposure to the measles infection.

Key words: measles, sero-epidemiology, geometric mean titre, IHA

Measles, a disease caused by Measles virus of genus Morbillivirus is a highly contagious disease, which is transmitted through respiratory droplets of infected person (Rasool *et al.*, 2016). Measles virus possess H-protein for it attachment to the target site, which is the main cause of this disease. The genome of Measles virus is composed of non-segmented single stranded RNA, which encodes for different proteins (Griffin *et al.*, 2007). Before the availability of vaccine against measles, about 2.6 million deaths have been reported annually due to this extremely hazardous disease (Perry and Halsey, 2004). Globally, measles was a main reason of early childhood mortality because no efficient and cost-effective vaccine was available against it. The measles prevalence rates are more among children above 12 months of age with high severity in children with vitamin A deficiencies (Merajuddin *et al.*, 2015).

Recently, several disastrous epidemics have been exploded in different areas of the world including Europe and China. About 1.5 million deaths were reported in 2010 only. According to a report of WHO in 2012, about 1.2 million individuals were died of measles and majority of them were young children up to 5 years of age. According to an estimate, the case fatality ratios (CFRs) of measles are about 0.1% in developed countries and up to 30% in immigrant people (Perry and Halsey, 2004). During the time period of 1999–2005, measles mortality and morbidity rate has been reduced up to 60%; however, yet causalities due to measles are still far above the ground in various parts of the world (Cohen *et al.*, 2009).

Measles can lead to many severe complications including pneumonia, encephalitis and even death. After infection, contagious encephalitis may also develop about 1/1,000 registered measles patients and mortality rate is about 2–3 deaths/1,000 measles cases (Gindler *et al.*, 2004). A considerable reduction in prevalence, morbidity and mortality from measles may be achieved by proper immunization coverage. In Pakistan, the usual immunization coverage for measles remains < 60% (Zahoor *et al.*, 2015). The major factors for low vaccination coverage include the lack of education and lack of motivation. In non-vaccinated individuals, the risk of measles complications is very high and these complications can only be reduced by proper vaccination (Mohammad *et al.*, 2011).

Several studies recommend that the routine vaccination program is the only way to achieve high level of immunity in the community (Shakurnia *et al.*, 2013). Generally in Pakistan, the vaccination exposure against vaccine treatable infections varied from 56% to 88% among various populations of different provinces in

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2011 and 2012. A large figure of measles epidemics with high morbidity and mortality rates has been recorded in various regions of Pakistan (Merajuddin *et al.*, 2015). In Pakistan, while considering the measles vaccination approach, measles epidemics, poor vaccination knowledge and lack of vaccination services in distant and countryside regions, the various society dependant studies have been carried out in different regions of Pakistan to assess the measles incidence in infants in spite of immunization (Merajuddin *et al.*, 2015).

The significance of an early and accurate diagnosis cannot be overlooked to adopt the effective control measures against a disease (Moss and Strebel, 2011). Clinicians can perform these EIAs very easily with only single sample of minute quantity and carried out after 4 weeks of manifestation of rash (Ratnam *et al.*, 2003). The diagnosis of measles can be done through different ways now a day. Initially, in early days only clinical diagnosis was done without laboratory confirmation. The laboratory diagnosis was carried out by applying usual techniques such as haemolysin inhibition, haemagglutination inhibition (HI), complement fixation, and plaque-reduction neutralization (PRN) for determination of antibodies against measles while immunefluorescence antibodies technique was used for recognition of measles (Featherstone *et al.*, 2011).

Antibodies against Measles can also be detected using indirect haemagglutination assay (IHA). IHA is found to be simple, specific and cost effective therefore, it was chosen for the assessment of serum samples in present study. After optimization of test conditions, IHA was used for the evaluation of humoral immune response against measles and for assessment of certain risk factors associated with the disease in District Sargodha and Khushab of Punjab Pakistan.

Children of 1 to 10 years of age from different areas of district Sargodha and Khushab, Punjab Pakistan were selected as target population. They were further divided and assessed on the basis of different parameters *i.e.* gender (male and female), area (urban, peri-urban and rural), age (1–3 years, 4–6 years and 7–10 years) and vaccination status (vaccinated or non-vaccinated). A total of 231 serum samples (134 from Sargodha and 97 from Khushab) were randomly collected from children ranging from 1 to 10 years of age. 138 serum samples from male and 93 samples from female children were collected. Similarly, 139 serum samples were from vaccinated children of both sexes and 92 samples from non-vaccinated ones. Complete record of collected samples was maintained on data sheet containing information like individual's name, age, locale, gender and vaccination status. The prior permission for blood sampling was obtained from Ethical Review Committee of Government College University Faisalabad. After collection, blood samples were properly labelled and stored in Gel & Clot Activator Vacutainers®. These Samples were then, centrifuged at 3000 rpm for 5 minutes for collection of serum. The collected sera were labelled and kept at –20°C till further processing. Serum samples were heat inactivated at 56°C for about 30 minutes in water bath to inactivate the complement proteins for preventing non-specific hemolysis (Soltis *et al.*, 1979). Blood samples were collected from sheep, chicken, and rabbit and centrifugation of these blood samples was carried out at 1500 rpm for 5 minutes to get RBCs. Pelleted RBCs were washed using phosphate buffer saline (PBS) and 10% stock suspension of erythrocytes was prepared from washed RBCs of sheep, chicken and rabbit using PBS for optimization of test conditions. The erythrocytes were fixed with 0.1% gluteraldehyde (Gluteraldehyde 25% Applichem™) and tanned with tannic acid (10 mg/dl) for about 30 minutes at 37°C (Iwasa *et al.*, 1977). Live attenuated (freeze dried) Measles vaccine (Indonesia) was obtained from Health Department, Government of the Punjab and used as antigen. After ultrasonication (Donald *et al.*, 1967) of antigen, sensitization of RBCs was done by treatment with measles antigen for a 1 hour at 37°C. 1% and 2% suspension of sensitized RBCs of sheep, chicken and rabbit were prepared. The optimized test conditions showing best and reproducible results were used for testing of serum samples.

Indirect haemagglutination antibodies (IHA) titers of all the serum samples were measured against measles virus antigens by applying the technique explained by Sakata and Sugiura (1988). The test was performed using micro-titration plates each comprising of 96 U-shaped wells. The highest dilution of each serum sample showing a clear haemagglutination pattern was considered as end point and taken as positive whereas button formation as negative. The IHA antibodies titre was exhibited as the reciprocal of its end point dilution. The IHA titers of all the serum samples thus obtained were recorded and Geometric mean titers (GMTs) were calculated and analyzed on the basis of gender, locale, age groups and vaccination status. The data was analyzed statistically by independent t-test and a one way Analysis of Variance (ANOVA). A value of $p \leq 0.05$ was considered as significant (Ruzauskas, 2005).

Indirect Haemagglutination Assay was optimized using gluteraldehyde (0.1%) and tannic acid (10 mg/dl) with RBCs obtained from different species including sheep, chicken and rabbit. Following optimization of test conditions, it was used for the sero-diagnosis and evaluation of humoral immune response against measles in Sargodha and Khushab districts of Punjab, Pakistan. 2% sheep erythrocyte suspension fixed with 0.1% gluteraldehyde and tanned with tannic acid (10 mg/dl), and adsorbed with ultra-sonicated measles antigen showed the clear and reproducible results in

terms of agglutination and button formation as compared to 1% sheep RBCs.

IHA antibodies titre of 16 or more was considered as positive and < 8 as negative. Similarly, GMT value of 8 was also taken as negative. A total of 231 serum samples from district Sargodha (n = 134) and Khushab (n = 97) were collected from urban, peri-urban and rural areas and processed through IHA. The GMT of all 231 samples of district Sargodha and Khushab showed non-significant ($p > 0.05$) difference among males and females. The mean GMT of male was 77.51; while that of female was 66.15.

Comparison of GMTs of vaccinated and non-vaccinated children of both districts revealed a highly significant ($p < 0.01$) difference. The GMT of vaccinated individuals was higher than that of non-vaccinated individuals. Similarly, a highly significant ($p < 0.01$) difference was found in antibody titre among individuals of urban, peri-urban and rural areas. The mean antibody titre of individuals of urban area was highest when compared to individuals from peri-urban and rural areas. There was also a significant difference in the antibody titre of individuals of district Sargodha and Khushab. The mean antibody titre of individuals of district Khushab was high than that of district Sargodha. The samples obtained from both districts were divided into 3 age groups *i.e.*, 1–3 years, 4–6 years and 7–10 years. A significant difference in the antibody titre of individuals in these age groups was observed. The mean antibody titre of individuals of age 7–10 years was higher than that of other two age groups (Table I).

The GMT of 231 samples of district Sargodha was compared and there was non-significant difference in the results of GMT of both males and females of district Sargodha. The GMT of male was 56.54; whereas that of female was 52.00. GMT of individuals of district Sargodha was compared on the basis of different area like urban, peri-urban and rural. The results revealed that there was a highly significant difference in the antibody titre of individuals in urban, peri-urban and rural areas. The GMT of individuals of urban area was higher than that of peri-urban and rural areas (Table II).

The GMTs of 97 samples of district Khushab was compared and results revealed that there was non-significant difference among males and females of district Khushab. The mean GMT of male was 104.77; while that of female was 87.57. GMTs of vaccinated and non-vaccinated individuals of district Khushab revealed a highly significant difference. The GMT of vaccinated individuals was higher than that of non-vaccinated individuals. In district Khushab, GMT of individuals of urban area was higher than that of peri-urban and rural areas. Similarly, GMT in individuals of age 7–10 years was higher than that of other two age groups (Table III).

Table I
Overall comparison of GMTs of both districts on the basis of different parameters.

Geometric Mean Titre (GMT) and P-values											
Gender		Vaccination status		Age groups (Years)			Locale			District	
Male	Female	Yes	No	1–3	4–6	7–10	Urban	Peri-urban	Rural	Sargodha	Khushab
77.51	66.15	103.65	26.52	21.90	61.96	105.03	109.42	66.56	43.56	54.64	98.21
0.660^{NS}	0.704^{NS}	4.675	5.673	0.001**			0.007**			–2.580	–2.399

Table II
Comparison of GMTs on the basis of different parameters in district Sargodha.

Geometric Mean Titre (GMT) and P-values									
Gender		Vaccination status		Age groups (Years)			Locale		
Male	Female	Yes	No	1–3	4–6	7–10	Urban	Peri-urban	Rural
56.54	52.00	71.72	24.04	15.86	54.27	72.72	100.00	49.09	21.95
0.265^{NS}	0.276^{NS}	2.783	3.628	0.037*			0.001**		

Table III
Comparison of GMTs on the basis of different parameters in district Khushab.

Geometric Mean Titre (GMT) and P-values									
Gender		Vaccination status		Age groups (Years)			Locale		
Male	Female	Yes	No	1–3	4–6	7–10	Urban	Peri-urban	Rural
104.77	87.57	155.47	29.23	34.92	69.17	159.78	146.00	96.52	42.60
0.517^{NS}	0.562^{NS}	4.239	4.634	0.009**			0.029*		

In present study, standardization and optimization of IHA was carried out to assess humoral immune response to measles among children because measles is a highly infectious disease and one of the major reasons of mortality and morbidity among children in the whole world, mainly in developing countries. In urbanized countries, especially Europe and US, measles infection has been controlled through immunization. However, still developing countries are being affected by measles due to inadequate vaccine exposure and inappropriate management of vaccines (Merajuddin *et al.*, 2015). Although vaccine is available for the measles yet it is a major disease in developing and third world countries because of high cost of available vaccine and mishandling of vaccine due to inappropriate storage facilities (Mehnaz, 2011). In Pakistan, vaccination has constantly been under achieved. The WHO reported data reveals that in 2010, usually planned vaccination coverage was 68% with Punjab (86%), Khyber Pakhtunkhwa (74%), Sindh (68%) and a very less proportion was recorded from Baluchistan (43%) (Khan and Qazi, 2014). According to a cross sectional study in 2015, the overall incidence of antibodies against measles was about 93.5% in the population of Faisalabad, Punjab (Rasool *et al.*, 2016).

Measles epidemics were and are still incident in various regions of the world with large ratio of reported cases and mortalities in a short duration of time. This huge gap is due to different reasons including duplicity in healthcare system, poor healthcare facilities, lower immunization coverage, undermined usual immunization, negligence among parents, and lack in quantity of vaccinators (Niazi and Sadaf, 2014). The efficiency of measles vaccine and improvement of immunity against measles among individuals of early age was not most favourable according to WHO strategies and un-vaccinated individuals were at high risk of measles infection (Zahoor *et al.*, 2015). For positive estimation of antibodies level of alleged cases of measles, highly sensitive serological tests are necessary (WHO, 2008).

Considering the significance of this infection particularly in the circumstances of present epidemics, this study was carried out with the aim of optimization of Indirect Hemagglutination Assay for the evaluation of humoral immune response against measles in district Sargodha and Khushab. Erythrocytes of various species including sheep, chicken and rabbit were used to perform IHA (Rasool *et al.*, 2016). In the past, excellent results were obtained when gluteraldehyde treated and sensitized RBCs of monkey were used in IHA (Gykha *et al.*, 1973). Because monkeys are not easily accessible so IHA was carried out with RBCs of sheep, rabbit and chicken in present study. 2% concentration of sheep RBC's revealed clear and reproducible results in this study however 0.5% and 1% erythrocytes concentrations have also been used in the past (Sakata and Suguira, 1988).

In this study, after optimization and standardization of IHA with 2% sheep RBC's fixed with 0.1% gluteraldehyde and adsorbed with measles antigen, IHA titres were calculated from the sera samples (n = 231) collected from children of district Sargodha and Khushab to assess the immunity against measles. The results of this study suggested that there was greater variation in the GMTs of vaccinated and non-vaccinated children of both districts *i.e.* Sargodha and Khushab. Non-significant difference in the GMTs values of males and females of both districts was observed demonstrating that measles infection is not dependant on gender (Ogundiji *et al.*, 2013). Statistical analysis of GMTs of different areas (urban, peri-urban and rural) of both districts showed significant differences in values indicating that less developed areas have more chances of measles infection due to low vaccination coverage, poor sanitary conditions, and lack of awareness in parents about the disease. The GMTs of individuals of age group 7–10 years were higher than other two groups indicating that immunity has been developed in elder children with the passage of time, while early age individuals have more chances of developing the disease.

In conclusion, it was accomplished that IHA with 2% sensitized sheep erythrocytes provided the most clear, consistent and reproducible results as compared to 1% sensitized sheep RBCs. Furthermore, it was found to be an inexpensive and valuable sero-diagnostic tool and hence can be used for the evaluation of humoral immune response against measles among different populations. Furthermore low antibody titers in non-vaccinated children indicate their susceptibility to measles infection with wild type of virus. So there is an urgent need for mass scale vaccination keeping in view the international heath standards to save our future generations against this devastating disease.

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