SERO-EPIDEMIOLOGY OF MEASLES IN CHILDREN FROM DISTRICT FAISALABAD PAKISTAN

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ABSTRACT: A comparative cross sectional study was conducted on blood samples (n=200) collected from children of 1 to 10 years of age, selected by convenient sampling method. Different coupling agents were used to bind measles antigen with erythrocyte suspensions of different concentrations collected from different species for standardization of Indirect Hemagglutination Assay (IHA) which was then used for testing of serum samples for the detection and quantification anti-measles antibodies. Overall prevalence of anti-measles antibodies was 93.5 percent in target population. A statistically significant association was found between vaccinated and non-vaccinated individuals with appreciably high antibody titres in vaccinated individuals ($P < 0.01$). Measles vaccine coverage was approximately 60 percent nationwide a complete mismatch to the global standards, and was lowest among all other vaccines included in the Expanded Program on Immunization (EPI). Moreover, IHA proved to be a simple and convenient tool for serodiagnosis and for monitoring of protective humoral immune response against measles vaccine.

Keywords: Measles, Serodiagnosis, Indirect Hemagglutination Assay, Humoral Immune Response.

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INTRODUCTION

Measles ranks among the most communicable diseases which is caused by measles virus (MV) of genus *Morbilivirus* and *Paramyxoviridae* family (Fazlalipour *et. al;* 2008). Measles is prevalent worldwide but it has become an epidemic particularly in developing countries. Although an effective, safe and economical vaccine has been available since four decades, measles remains the most frequent cause of mortality in children below 5 years age (WHO, 2006). The higher morbidity and mortality rates are linked to poverty, malnutrition, secondary bacterial infections and subdue vaccination coverage (Commey and Dekyem, 1994).

Measles affects more than 95% of exposed individuals in the absence of vaccination and spread by the respiratory route (van den Ent *et. al;* 2011). In Pakistan about 1 million children in the age of five or less than five suffer from measles and about 20,000 children die because of this disease (WHO, 2008). From 1982 to 1991 no outbreaks of measles were observed. However, occurrence of serious outbreaks afterwards had highlighted that either there were time lapses during the vaccine campaigns or vast demographic areas were not properly covered (Murray and Rasmussen, 2000). The Expanded Program on Immunization (EPI) was initiated by World Health Organization (WHO) in 1974 and later in 1978 Pakistan adopted this, and is still ongoing for the control of vaccine preventable diseases in the infants and children. Among the all other EPI vaccines, measles vaccine has been reported to have least coverage (Mehnaz, 2009). The goal set by WHO under the umbrella of EPI was provision of 80-90% immunization coverage by 2010 in Pakistan. The global target of the Program is to immunize over 95% of infants.

Diagnosis of measles is often simple for the physicians in areas where disease is endemic or outbreaks are common, but correct diagnosis based on symptomology is an uphill task if the incidence rate is low and/or other pathogens are largely responsible for illnesses causing rash and fever (Moss and Strebel, 2011). The role of laboratory diagnosis is of great significance in the differential of measles from other diseases with similar clinical presentations, as the disease prevalence falls (Featherstone *et. al;* 2011). Measles is often diagnosed based on serological testing. Acute cases are mostly diagnosed by enzyme immunoassay (EIA) with the detection of MV specific IgM in a single specimen of serum, eluates of dried blood spots and oral fluid. The sensitivity of indirect EIA has been reported to be 77-90% during initial three days after the onset of rash and approximately 100% at the day four for the detection of MV specific IgM antibodies (Helfand *et. al;* 1997; Tipples *et. al;* 2003). Anti-measles IgG antibodies can be detected with the onset of rash and peak within a couple of weeks later, which mostly persists for life long (Grandien *et. al;* 1994).

Since IHA is inexpensive and does not require sophisticated equipments, therefore it was selected for testing serum samples. The protocol for IHA was standardized and optimized with some modifications fit for present scenario and then employed for serological

diagnosis and assessment of humoral immune response against measles.

MATERIALS AND METHODS

Experimental Design: Children of 1 to 10 years of age were selected as target population, and divided into two groups *i.e.* vaccinated and unvaccinated, each group was further subdivided into two on the basis of gender *i.e.* male and female (n=50 each) from district Faisalabad, Punjab, Pakistan. Sampling was done from urban, peri-urban and rural areas.

Serum Samples: A total of two hundred serum samples were collected. For this purpose about 3ml of blood was collected aseptically by venipuncture and placed into Gel and Clot Activator tubes immediately. The specimens were kept at laboratory temperature for 1-2 hours for clot formation and centrifuged at 3000 rpm for 5 minutes (Hettich® EBA 20, Germany). The separated sera were placed in Eppendorf tubes, properly labeled and stored at -20°C until further use.

Data Collection: A data sheet was maintained for each collected sample bearing person's name, age, gender, vaccination status and locale.

Source of Measles Antigen: Live attenuated (Freeze Dried) Measles Vaccine (Indonesia) procured from Health Department; Government of the Punjab, Pakistan was used as antigen source.

Complement Inactivation: The separated sera were heat treated in water bath at 56°C for half an hour for inactivation of the complement proteins to prevent the non-specific hemolysis caused by complement proteins (Soltis *et. al;* 1979).

Indirect hemagglutination Assay: Different coupling agents (0.1% Gluteraldehyde and 10 mg/dl Tannic acid) were evaluated to bind antigen with erythrocyte suspensions of different concentrations (0.5 and 1.0%) from different species (Sheep, Chicken and Rabbit) for standardization and optimization of the test. The optimized test conditions showing reproducible results were used for testing the serum samples. IHA was carried out using sheep RBC's (1%) fixed with 0.1% Gluteraldehyde, tanned with (10 mg/dl) Tannic acid and adsorbed with ultrasonicated measles antigen. IHA antibody titres of all the serum samples were determined against measles virus antigens by using the microtitration technique (Sakata and Sugiura, 1988). The test was carried out in 96 well U-shaped Titreteck microtitartion plates. Agglutination was read by the pattern method and the highest dilution of a test serum showing partial agglutination was taken as the endpoint. The reciprocal of the end point serum dilution was expressed as antibody titre.

Data Analysis: Geometric Mean Titres (GMTs) of different experimental groups i.e. Unvaccinated (males and females) and Vaccinated (males and females) were calculated. Mean antibody responses among the various groups were analyzed statistically using student t-test (Minitab® version 17.0, Minitab Inc. USA). A *P*-value of ≤ 0.05 was considered significant.

RESULTS

The Indirect Hemagglutination Assay (IHA) was optimized using different coupling agents like Gluteraldehyde (0.1%) and Tannic acid (10 mg/dl) with erythrocytes obtained from venipuncture of sheep, chicken and rabbit respectively. Reproducible results of IHA test were observed using 1% sheep RBC's fixed with Gluteraldehyde (0.1%) tanned with (10 mg/dl Tannic acid) and adsorbed with ultrasonicated measles antigen. The 0.5% suspension of sheep erythrocytes was also treated with same protocol but results were not well reproducible. The pattern of agglutination was found to be consistent in case of 1% sensitized sheep RBC's in contrast to 0.5% sensitized sheep RBC's. Whereas 0.5% and 1% suspension of sensitized RBC's of chicken as well as rabbit failed to produce reproducible results fixed with 0.1% Gluteraldehyde and tanned with (10 mg/dl) Tannic acid and hemolysis was observed during tanning process. In case of sensitized chicken and rabbit RBC's (0.5% and 1%) agglutination pattern was not recorded at all.

IHA titre of 16 or higher was taken as positive and below than 8 as negative. The titre of 8 was also considered as negative because its specificity was questionable and could not be verified by competition. The specificity of the IHA test was verified by at least a fourfold reduction in agglutination titre in the second dilution series to which measles virus antigen was added as a competitor before sensitized erythrocytes.

The minimum IHA antibody titre was 32 while the highest was 1024 in case of unvaccinated males. Whereas minimum IHA antibody titre was 32 and the highest was 512 in case of unvaccinated females. Out of 50 unvaccinated males, 7 (14%) individuals were declared negative while remaining 43 (86%) were declared positive indicative of exposure to measles. Out of 50 unvaccinated females, 6 (12%) were declared negative and remaining 44 (88%) as positive which indicated the possible exposure to natural infection. The calculated GMT was 91.83 and 102.57 of unvaccinated males and females respectively. Upon comparison of Geometric Mean Titres of unvaccinated males and females, *P*-value was > 0.05 so results were not statistically significant.

It was found that all the individuals developed humoral immunity in response to vaccination but variation in IHA titres was recorded. The lowest IHA

antibody titre was 16 while the peak titre was 1024 in case of vaccinated males. Whereas the lowest IHA antibody titre was 32 and peak titre was 1024 in case of vaccinated females. The calculated GMT was 202.3 and 222.84 of vaccinated males and females respectively. Geometric Mean Titres of vaccinated males and females were compared and recorded as statistically unrelated ($P > 0.05$).

GMTs of 100 vaccinated (212.32) and 100 unvaccinated (64.05) individuals were also compared and results were highly significant which depicted that antibody titre was appreciably high in vaccinated than unvaccinated individuals ($P < 0.01$). The GMTs of male (136.14) and female (151.01) individuals were compared without consideration of vaccinated or unvaccinated. The results showed that GMTs were statistically non-significant irrespective of gender ($P > 0.05$). However, statistical analysis displayed highly significant results of comparison between Geometric Mean Titres of vaccinated (202.30) and unvaccinated (91.83) males ($P < 0.01$). GMTs of vaccinated females (222.84) were significantly higher ($P < 0.01$) than unvaccinated (102.57) females (Table 1, Figure 1 and 2).

Table 1. Geometric Mean Antibody Titres against Measles virus by Indirect Haemagglutination assay in Children of District Faisalabad, Pakistan.

Titres	Male		Female		Total	
	Unvaccinated (%)	Vaccinated (%)	Unvaccinated (%)	Vaccinated (%)	Unvaccinated (%)	Vaccinated (%)
≤1:16	7(14)	1(2)	6(12)	0(0)	(13)	(1)
≤32	6(12)	3(6)	1(2)	3(6)	(7)	(6)
64	10(20)	5(10)	11(22)	5(10)	(21)	(10)
128	9(18)	10(20)	14(28)	11(22)	(23)	(21)
256	9(18)	16(32)	12(24)	14(28)	(21)	(30)
512	7(14)	14(28)	6(12)	14(28)	(13)	(28)
1024	2(4)	1(2)	0(0)	3(6)	(2)	(4)
Negative	7(14)	0(0)	6(12)	0(0)	(13)	(0)
Low + ≤1:64	16(32)	9(18)	12(24)	8(16)	(28)	(16)
High + >1:64	27(64)	41(82)	32(64)	42(84)	(59)	(83)
Total	50	50	50	50	100	100
GMT	91.83	202.3	102.57	222.84	64.05	212.32

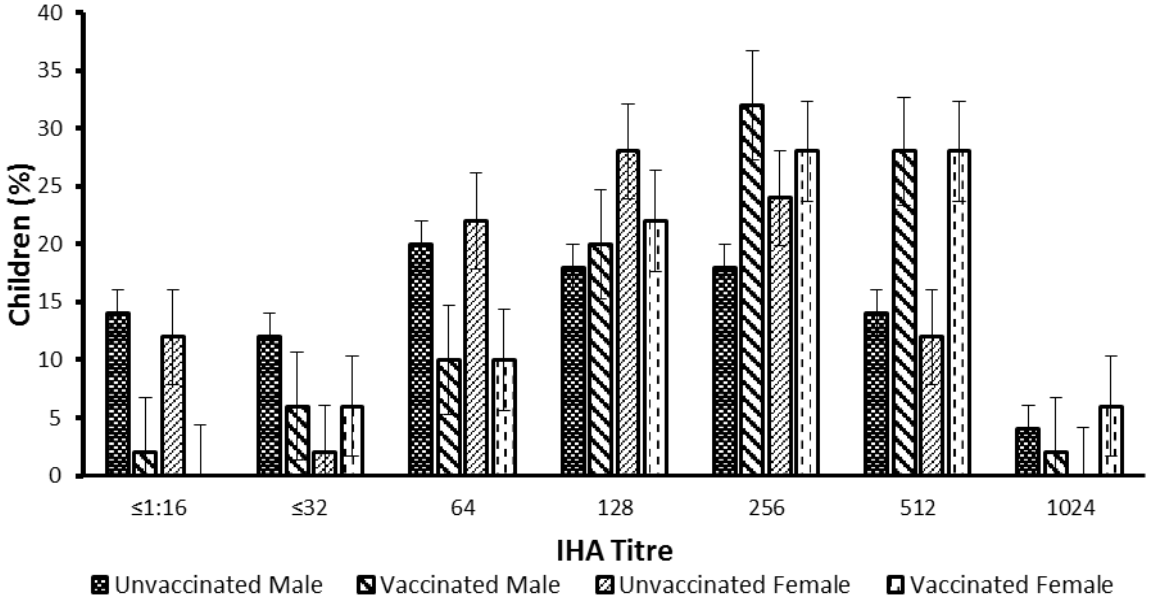
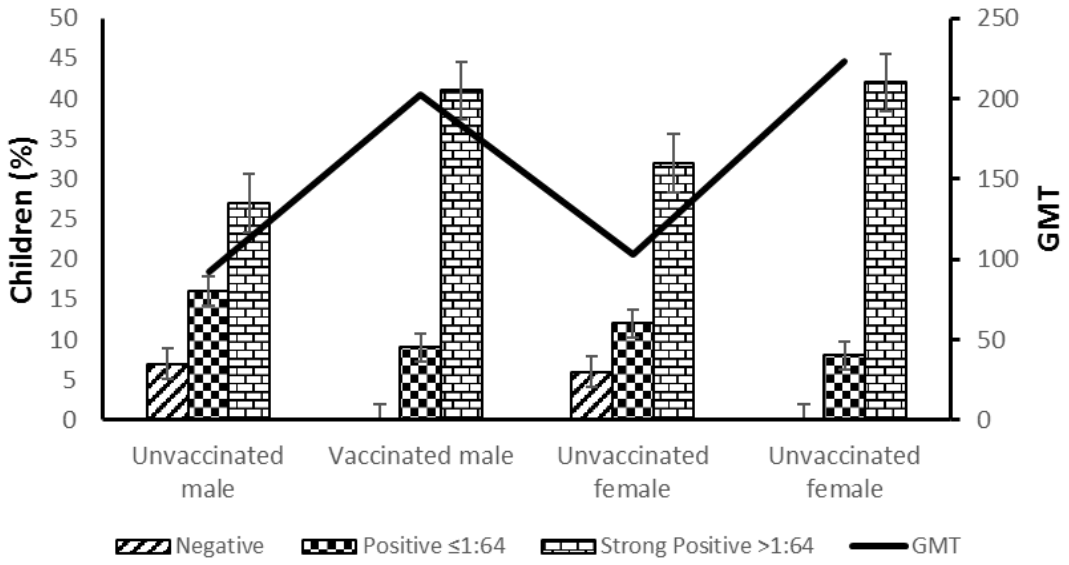


Figure 1: The IHA antibody titers in un-vaccinated and vaccinated children of either sex



Fiigure 2: The GMTs in male and female children showing negative, positive and strong positive results

DISCUSSION

In the present study, antibody levels were measured against measles using standardized IHA with 1% sheep RBC's fixed with 0.1% Gluteraldehyde tanned with (10 mg/dl) Tannic acid and adsorbed with ultrasonicated measles antigen among unvaccinated and vaccinated individuals from district Faisalabad, Punjab, Pakistan, for serodiagnosis and assessment of immunity. Serological tests with high sensitivity forms the gold standard for confirmatory diagnosis of suspected cases of measles (WHO, 2007). Several tests have been used for serodiagnosis as well as for assessment of humoral immune response. The IHA test with monkey erythrocytes sensitized with measles virus hemagglutinin (HA) and fixed with glutaraldehyde was reported to be specific and sensitive for anti-measles antibodies (Ghyka *et. al*; 1973).

The IHA test was similar in principle to the hemagglutination inhibition (HI) test, as both tests exclusively determined the level of antibody directed to the HA spike of MV. The HA spike present on the envelope of virus is transmembrane glycoprotein, immunogenic in nature and helps in attachment of the virus with the cells thus playing significant role in immunity and viral pathogenesis (Griffin, 2007). Hemagglutination inhibition (HI) was a highly economical and convenient test for the detection and quantification of antibodies against HA glycoprotein. HI was time-tested tool for sero-epidemiological studies for viruses having H peplomer e.g., rubella, influenza, Newcastle disease virus. Antibodies directed against HA protein had strong correlation to the immunity against wild type viruses and hence related to virus neutralization potential (Norrby and Gollmar, 1975). However, Hemagglutination inhibition test was not sensitive enough to assess immune status against measles long after infection or vaccination (Neumann *et. al*; 1985; Weigle *et. al*; 1984). The virus Neutralization test (NT) was far more sensitive in comparison to HI test (Orenstein *et. al*; 1987; Kruginan, 1983) but it was very laborious and time grueling for a routine serological testing. The antihemolysin test and ELISA were reported to be as sensitive as the NT test and reliable indicators of immune status (Neumann *et. al*; 1985; Weigle *et. al*; 1984), while another author had concluded that enzyme immunoassay (EIA) was much more sensitive in comparison to HI test, but since the former may detect antibodies irrelevant for protection, it was not necessarily suitable for evaluation of immunity status (Cremer *et. al*; 1985).

In our study, a total of 200 children were screened for the presence of anti-measles antibodies. Of them, 187 (93.5%) children had measles antibody titres ranging from 1:16 to 1:1024 and only 13 (6.5%) children were declared negative. Among unvaccinated children (n=100), 28% had low un-protective antibody titres (≤1:64), 59% of the children had high protective titres (>1:64 to 1024) and 13% children were recorded as negative (<1:16). Hence it can be inferred that 87% of the unvaccinated children had exposure to natural infection at any stage. According to some surveys, nationwide vaccination coverage was poor and on an average one out of every five children was unvaccinated, whereas the situation was more grave in rural areas where two out of every three children were not vaccinated (USAID, 2012).

The vaccination program failure in Pakistan, especially to control measles epidemics had been

associated with multiple factors like; corruption, constant inflow of uninoculated refugees from neighboring country (Afghanistan), parental rejection to get their children vaccinated, war on terror in Khyber Pakhtunkhwa (KPK) province, political turmoil, dwindling security, unawareness of health care personnel about vaccination schedule, lack of skilled vaccinators, and above all the dilemma of vaccine failure itself (Niazi and Sadaf, 2014).

While in case of vaccinated children (n=100), 17% had low un-protective antibody titres (≤1:64) and 83% of the children had high protective titres (>1:64 to 1024). There was no statistically significant association between the IHA antibody titres of the children and their gender (P>0.05) and this was in accordance with the findings of Ogundiji *et al*. (2013). There is huge degree of variation with reference to minimum protective antibody titres in different parts of the world, this sort of variation is attributed to differences in epidemiologic pattern of measles in different geographical regions (WHO, 2007). Antibodies produced in response to measles vaccination had been known to decline with passage of time (Kremer *et. al*; 2006). Therefore in a country like Pakistan, where measles was endemic and immunization coverage was poor, the observed high antibody levels may correspond to higher degree of subclinical infections to which children suffer following early childhood immunization.

It was concluded from the study that the majority of the children had high titres that may possibly confer them with lifelong immunity against measles. However, 17% of the children had low antibody titre which indicated susceptibility of these groups to infection with the wild type virus. This warrants an urgent need for mass scale vaccination in line with the global health standards to protect our future generations from deadly measles. Furthermore all the stake holdres should play their role in achieving the millienium development goals for health and properity set by United Nations Organization (UNO).

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