III. MATERIAL AND METHODS

To recapitulate, the major objectives of the study were -

1. To study the epidemiology of measles in the slums of Vadodara city.
2. To determine the optimum age for measles vaccination in children.

The specific objectives of the study were -

1. To study the various factors which may influence the management of measles in the community.
2. To study the correlation between maternal and cord blood measles antibody levels vis-a-vis the maturity, birth weight and sex of the newborn.
3. To study the fall in passive measles immunity.
4. To determine the seroconversion rates at different ages in order to evaluate the efficacy of further-attenuated Schwarz measles vaccine.

This chapter is described under the following heads -

I. Epidemiological study
   A. Baseline survey
   B. Follow up survey

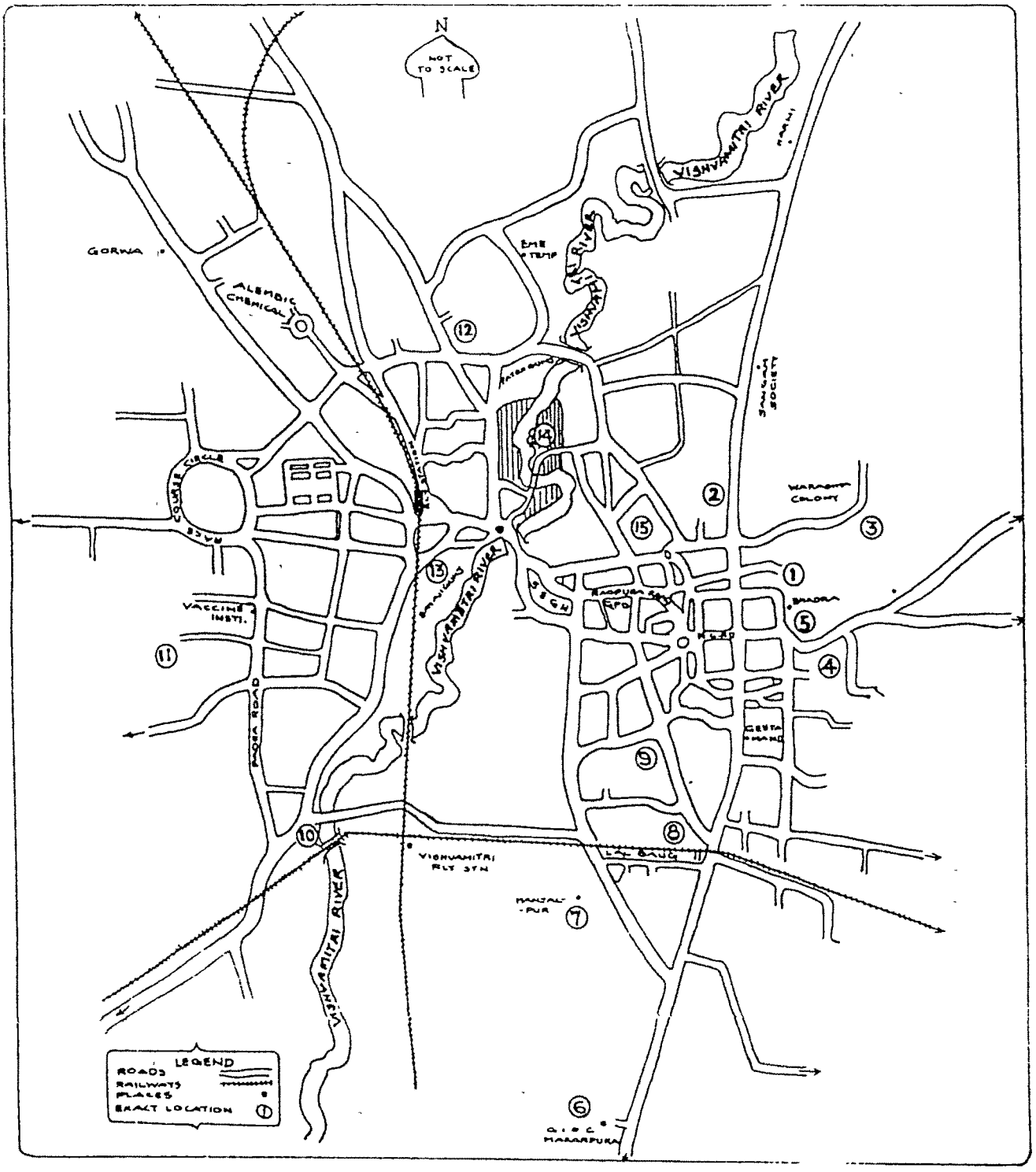
II. Serological study
   A. Maternal vs. cord blood study
   B. Waning of passive antibody study
   C. Seroconversion study
   D. Methodology

I. EPIDEMIOLOGICAL STUDY

The city of Vadodara has 8 wards. All slums under each ward were listed. A total of 15 slums, each having a population of less than 1000, two from each ward, were randomly selected (except ward number 1, where the only slum was included in the study) (Fig.7).

FIGURE-7

CITY MAP OF VADODARA SHOWING THE SLUM AREAS IN THE EPIDEMIOLOGICAL STUDY



1 - JUNIGADHI HARIJANWAS 2 - VYARAWADI 3 - BHATHUJI CHAWK
4 - BAMANPURA 5 - BAUCHAWAD 6 - MAKARPURA G.I.D.C.
7 - MANJALPUR VILLAGE 8 - BABAJIPURA 9 - KUMBHARWADA
10 - MUNJMAHUDA 11 - SAYED VASANA 12 - PENSIONPURA
13 - PARASHURAM BHATTHA 14 - JALARAMNAGAR 15 - NAVIDHARATI

A. Baseline Survey

A baseline survey was conducted in 15 slum areas of Vadodara city during the month of January 1983, a peak season for measles epidemic.

Demographic data

A total of 1754 families were surveyed and recorded by a house-to-house survey (Annexure:1). In the study area, there were 1581 children in the age group 0-6 years. The birth date, weight and vaccination status of each child were recorded. The age of the child and vaccination status were confirmed from the birth records given by the Corporation Health Authority or from the ICDS Anganwadi Cards or from the parents. The children were weighed using a Salter spring balance which was checked frequently for accuracy.

The nutritional grading was done as per the recommendations of the Nutrition Subcommittee of the Indian Academy of Pediatrics (IAP Classification) (249) (Annexure : 2).

Sample selection

During house-to-house survey, the following procedure was adopted for selection of sample. A history of measles disease during the preceding 10 days was elicited. The specific symptom complex of the disease and the nature and mode of the spread of the exanthematous lesion were inquired into in great detail to confirm the diagnosis of measles.

A 10-day-recall method was used as measles has a set of distinct symptoms and signs lasting for 7-10 days which may be adequate for the diagnosis (29). The relative seriousness of the disease makes it an experience that is not easily forgotten by the respondent.

In a study by John et al (141), children with a past history of measles did not subsequently develop the disease.

A close correlation between positive serological findings and the history of measles given by the parents has been observed in various studies (26,162, 197,228,239,283,296). In a study by Nanavaty et al (239), a 97.80% positive correlation was found between serological findings and history of measles given by the parents. In a study by Shah et al (296), in a rural

population, 27 out of 28 children who had history of measles disease had also positive serological findings. Khare et al (162) from Delhi have also reported positive serological evidence in 91.60% of children reported to have measles.

Thus, in case of measles, a retrospective study can be relied upon especially if the disease has occurred in the recent past.

Although prospective studies are better, due to constraints of finance, manpower and time, this study was conducted retrospectively.

A total of 200 cases of measles were identified and recorded separately (Annexure·3).

The beliefs regarding causation, rituals observed and treatment taken, if any, for measles were inquired into and recorded.

B. Follow Up Survey

Within 4-6 weeks of the first visit, all the recorded cases of measles were followed up. Of the 200 initial cases, 185 children could be contacted and recorded in the follow up study. During the second visit, the children were weighed and measles-related complications, if any, were inquired into and recorded. The diagnosis of the complications was made on the basis of detailed information about symptoms, signs and treatment taken, if any, for the same.

II. SEROLOGICAL STUDY

The study group consisted of newborns, their mothers and children attending the outdoor patient departments or admitted in the Paediatric Wards of S.S.G.Hospital and B.A.G.Hospital, Vadodara.

A. Maternal vs. Cord Blood Study

Sample selection

A total of 187 paired blood samples (2 ml each) were collected from the mother and cord at the time of delivery. The samples were designated

to four subgroups, as per the maturity and birth weight of the newborn (79) as described below -

(i) Full term adequate for gestational age (FTAGA)
(ii) Full term small for gestational age (FTSGA)
(iii) Preterm adequate for gestational age (PTAGA)
(iv) Preterm small for gestational age (PTSGA)

The weights were taken on the Detecto horizontal bar infant weighing scale with a minimum calibration of 25 gm. The maturity was determined after 24 hours of birth by the method described by Dubowitz (79). The observations were recorded in a proforma (Annexure:4).

B. Waning of Passive Antibody Study

Sample selection

A total of 623 blood samples (2 ml each) were collected randomly from children (0-6 years) to determine the passive measles haemagglutination inhibition (HAI) antibody seropositivity at different ages. Children with any acute illness or fever at the time of observation, a prior history of measles or measles vaccination, or a contact with a patient suffering from measles in the preceding two weeks, were excluded from the study. The age, sex and address of each child were recorded. The age was recorded in number of completed months. Each child was weighed and the nutritional status was assessed according to the IAP Classification(249) (Annexure:2).

The children were divided into nine subgroups according to age (months) at the time of vaccination as described below -

(1) 0
(2) 3-5
(3) 6-8
(4) 9-11
(5) 12-14
(6) 15-17
(7) 18-20
(8) 21-23
(9) 24-72

C. Seroconversion Study

Sample selection

A total of 327 children (6-72 months) who were eligible for measles vaccination, were included in the study. The weight, sex, address, nutritional status, time of vaccination and dates of blood collection (pre- and postvaccination) were recorded (Annexure-6). They were stratified for their age (months) into seven subgroups as shown below -

(1) 6-8
(2) 9-11
(3) 12-14
(4) 15-17
(5) 18-20
(6) 21-23
(7) 24-72

The blood samples (2 ml each) were collected from each child twice - once on the day of vaccination and a repeat $\geqslant$ 4 weeks after vaccination.

The primary HAI antibody response to measles virus is a mixture of IgM and IgG immunoglobulins with a rapid disappearance of IgM by 3 weeks, but indefinite persistence of IgG after reaching peak levels within 4-6 weeks (227). Therefore, the postvaccination blood sample was collected after $\geqslant$ 4 weeks.

The Schwarz strain of further attenuated, lyophilised freeze-dried vaccine (Rouvax-Merieux, France or Rimevax-Smith Kline RIT, Belgium) was used in the study. The cold chain was maintained scrupulously. A dose of 0.5 ml of reconstituted vaccine was administered subcutaneously on the anterolateral aspect of the left thigh. The potency of the vaccine used was tested randomly at the Central Research Institute (CRI), Kasauli, Himachal Pradesh and was reported to be in the range of 2000 to 3000 $TCID_{50}$ per dose.

D. Methodology

The HAI test, as described by Saha, CRI, Kasauli (18, 281) was used. The researcher underwent training for two weeks at the CRI, Kasauli, for the same.

The choice of a serological test for measles can be made purely on the basis of sensitivity and convenience, because the several manifestations of the immune reactions move in synchrony, and all are measles virus specific (165).

The detection and quantitation of measles antibody is usually performed by one of the three methods: virus neutralisation (N), complement fixation (CF) or haemagglutination inhibition (HAI).

Following vaccination or natural infection, measles HAI and neutralising antibodies appear earlier and persist longer than CF antibodies (171).

The currently practised test using tween 80-ether purified antigen recommended by Norrby is 5-10 times more sensitive than the HAI test using the classical antigen (246,349) and is considered one of the most sensitive tests for measuring HAI antibody titres as low as 1:2 (338,356). Non-specific positive reactions, when using HAI test, are not a common problem (165).

The HAI test is a most convenient and reproducible technique (150), is equally sensitive to and has a good correlation with neutralisation test (25, 171).

The HAI test is more sensitive than the CF test (25,171) and has currently gained acceptance as a method of choice (338). A positive CF test is a reliable index of recent infection or of resistance but its absence is not conclusive evidence of susceptibility. A negative HAI or N test can serve as an index of susceptibility (171,219,311).

However, in all HAI test, there is a requirement for pretreatment of the serum to remove non-specific inhibitors and agglutinins which, if not removed, affect the sensitivity and reliability of the test, adding to cost, complexity and time needed to conduct the test proper (34).

MEASELISA, an enzyme-linked immunosorbent assay for the detection of measles IgG antibody now available, is found to be comparable to the HAI test, when comparing sensitivity, specificity and accuracy (34,338).

1. Collection of Blood Samples

About 2 ml of cord or venous blood was collected in autoclaved plain bulbs. The cord blood was collected directly under sterile conditions while venous blood was drawn by venepuncture using a sterile disposable needle and syringe. The samples were allowed to clot at room temperature, centrifuged, and the sera thus separated were transferred to sterile bulbs which were then stored in a deep freeze until tested.

2. Haemagglutination Inhibition (HAI) Test

a) Principle

Measles virus has been shown to possess the capacity of adsorbing to Rhesus monkey red blood cells and causing their agglutination. This haemagglutination is readily inhibited by the specific antibody to measles. The HAI test furnishes a sensitive method for detecting measles HAI antibodies. This method can detect an antibody titre as low as 1:2 (356).

b) Equipment : (Photograph:1)

(i) Graduated centrifuge tubes (capacity 15 ml)
(ii) Plastic plates with 'U' shaped wells
(iii) Microdiluters (0.025 ml)
(iv) Microdroppers (0.025 ml and 0.05 ml)
(v) Mirror for reading the plates
(vi) Aluminium foils
(vii) Micropipettes (2 ml)

c) Reagents

(i) Measles haemagglutination antigen
(ii) Rhesus monkey red blood cells (RMR)
(iii) Physiological saline, pH 7.2
(iv) Alsever's solution
(v) Known positive and negative measles antisera.

d) Preparation of solutions

(i) Physiological saline

To 1000 ml of distilled water, 9 gm of sodium chloride was added. The solution was autoclaved at 15 lb pressure for 15 minutes. The pH was adjusted to 7.2 by adding a few crystals of Na_2HPO_4.

(ii) Alsever's solution

Dextrose	-	20.5 gms
Sodium chloride	-	4.2 gms
Sodium citrate	-	8.0 gms
Citric acid	-	0.55 gms

The above ingredients were dissolved in 1000 ml of distilled water and the solution autoclaved at 15 lb for 15 minutes and pH adjusted to 7.2.

e) Washing of glassware

Washing of glassware was carried out in the following steps -

(i) Put in boiling detergent solution for one hour.
(ii) Rinsed and brushed with tap water.
(iii) Kept overnight in 10% hydrochloric acid.
(iv) Washed with tap water and kept overnight in distilled water.
(v) Washed 3 times in distilled water.
(vi) Dried at 70°C in a hot air oven.
(vii) Plugged with cotton gauze.
(viii) Sterilised in a hot air oven at 160°C for 2 hours.

Used glassware was filled with water and decontaminated in an autoclave at 121°C for 20 minutes, put in detergent solution, rinsed and brushed with tap water and then steps (iv) to (viii) above were carried out.

f) Procedure

Day 1

(a) Blood of Rhesus monkey was collected under sterile conditions in Alsever's solution in a ratio of 1:4 and was distributed in small

aliquots and stored at 4°C. This blood can be used upto 10 days, and 2 ml of blood is enough for 50 tests.

(b) Washing of RBCs was carried out as follows -

(i) The required quantity of preserved blood was centrifuged in graduated centrifuge tubes at 2000 rpm for 10 minutes at room temperature.

(ii) The supernatant fluid and buffy layer of WBCs was aspirated off with a pipette.

(iii) Two ml of physiological saline was added to the RBCs.

(iv) The RBCs were mixed gently with the saline to resuspend the cells and additional quantity of saline was added to bring it to the original volume.

(v) The mixture was centrifuged at 2000 rpm for 10 minutes.

(vi) Steps (ii) to (v) were repeated three times.

(vii) Centrifugation was done again at 2000 rpm for 10 minutes and the supernatant was aspirated and discarded.

(viii) Physiological saline was added to make 50% suspension of RBCs (RMR) (i.e. 1:1 dilution was made).

(ix) For use in the test, a 0.50% suspension of RBCs was prepared by adding physiological saline (i.e. 1:200 dilution was made). The rest was preserved at 4°C.

(c) The treatment of sera was done as follows -

(i) Thawing: To prevent rapid changes in the temperature from the deep freeze to room temperature, the sera kept in the deep freeze were first kept in the lower compartment of the refrigerator before bringing them to room temperature.

(ii) Inactivation of sera: Sera were inactivated by keeping in a water bath at 56°C for 30 minutes.

(iii) Adsorption: Adsorption is done to prevent the antibody other than of measles, reacting with the RBCs. A measured amount, 0.2 ml, of serum was adsorbed on 0.05 ml of 50% RMR by keeping overnight at 4°C.

Day 2

Before performing the HAI test, standardisation tests were carried out. The results were interpreted as follows -

Haemagglutination: Complete agglutination consists of a layer of uniformly agglutinated cells covering the bottom of the well (Photograph:2).

Haemagglutination Inhibition. Absence of agglutination consists of a compact sharply demarcated button of sedimented cells in the centre of the bottom of the well (Photograph:2).

Test 1 · Titration of antigen

(i) With a wax pencil, a 'U' microtitre plate was marked as shown below -

Antigen dilution: 1:2, 1:4, 1.8, 1:16 and so on.

Control was designated 'C'.

(ii) With a 0.025 ml dropper, one drop of diluent (physiological saline) was put in all the wells.

(iii) In the first well, 0.025 ml of measles antigen was added and serial doubling dilutions were made with the help of microdiluters. In the control well, 0.025 ml of normal saline was used instead.

(iv) With 0.025 ml dropper, one more drop of saline was added into each well.

(v) With the help of a microdropper, 0.05 ml of 0.50% RMR suspension was added to each well.

(vi) The plate was shaken gently from side to side on all the sides for thorough mixing.

(vii) The plate was covered with an aluminium foil and incubated at 37°C for 1-2 hours.

(viii) The titrations were read and recorded.

Button formation in the controls indicated the time to take out the plates.

The antigen titre level was given by the end point which was the demarcating line between button formation and haemagglutination.

RESULT

Samples	Wells							
	A	B	C	D	E	F	G	H
1	+	+	+	+	*	*	*	*
2	+	+	+	+	*	*	*	*
C	*	*	*	*	*	*	*	*
Dilution	1:2	1:4	1·8	1:16	1:32	1·64	1:128	1:256

+ = Haemagglutination
* = Button formation
C = Control

Here, the antigen titre is 1:16 which contains 1 HA unit. Therefore, antigen has to be diluted 1:3 with physiological saline so that 0.025 ml antigen contains 4 HA units of antigen.

Test 2 : Immune control

This test is to know the specificity of supplied antigen as measles antigen by using known measles antibody of rabbit's serum available from CRI, Kasauli.

(i) With a wax pencil, the 'U' microtitre plate was marked A,B and so on.

(ii) 0.025 ml of physiological saline was put in all the wells 'B' onwards.

(iii) 0.025 ml of measles serum was put in wells A and B.

(iv) In well B, 0.025 ml of saline was added to double the dilution.

(v) From well 'B' onwards, microdilutions were made with microdiluters.

(vi) 0.025 ml of diluted antigen (4 HA units) was added in all the wells.

(vii) The plate was incubated at 37°C for 45 minutes.

(viii) 0.05 ml of 0.50% RMR was added in all the wells.

(ix) The plate was shaken gently, covered and incubated at 37°C for one hour.

Button formation demarcated the end point.

RESULT

Samples	Wells							
	A	B	C	D	E	F	G	H
1	*	*	*	*	*	*	+	+
2	*	*	*	*	*	*	+	+
Dilution	<1:2	1:2	1:4	1:8	1:16	1:32	1:64	1:128

* = Button formation

+ = Haemagglutination

Button formation is upto F i.e. antibody dilution is 1:32. This test confirms that the supplied solution is measles antigen and reacts properly even after dilution.

Test 3 : Back titration (antigen control)

This test is done to confirm the strength of antigen after dilution.

(i) In the wells from 'B' onwards, 0.025 ml of physiological saline was added.

(ii) In wells A and B, 0.025 ml of diluted antigen (4 HA units) was added and microdilutions carried out from 'B' onwards.

(iii) In all the wells, 0.025 ml of physiological saline was added.

(iv) The plate was incubated at 37°C for 45 minutes.

(v) 0.05 ml of 0.50% RMR was added in all the wells.

(vi) The plate was shaken gently and covered.

(vii) The plate was incubated at 37°C for one hour till button formation occurred.

RESULT

Samples	Wells							
	A	B	C	D	E	F	G	H
1	+	+	+	*	*	*	*	*
2	+	+	+	*	*	*	*	*
Dilution	< 1:2	1.2	1.4	1:8	1:16	1:32	1:64	1:128

+ = Haemagglutination

* = Button formation

Button formation here was from 'D' onwards. So, the antigenic strength after dilution is 4 HA units per 0.025 ml of solution.

Procedure for HAI Test

(i) The titrated antigen used above was used for the sera samples.

(ii) 0.025 ml of physiological saline was put in all wells from 'B' onwards including the last two for control.

(iii) 0.025 ml of adsorbed test serum was added in wells A, B and G (control).

(iv) Microdilution was carried out from 'B' to 'F' and 'G' to 'H'.

(v) 0.025 ml of diluted antigen (4 HA units) was added in all the test wells. To the control wells, 0.025 ml of physiological saline was added.

(vi) The plate was shaken, covered and incubated for 45 minutes at 37°C.

(vii) 0.05 ml of 0.50% RMR was added in all the wells, mixed and the plate incubated at 37°C till control wells showed button formation.

RESULT

Samples	Wells							
	A	B	C	D	E	F	G	H
1	*	*	*	*	+	+	*	*
2	*	*	*	+	+	+	*	*
3	*	*	+	+	+	+	*	*
4	*	*	*	*	*	+	*	*
Dilution	< 1:2	1:2	1:4	1:8	1:16	1:32	Control	Control

* = Button formation

+ = Haemagglutination

Sample 1 has a titre of 1:8
Sample 2 has a titre of 1:4
Sample 3 has a titre of 1:2
Sample 4 has a titre of 1:16.

With each test, positive serum control, antigen control and cell control were put to check the efficacy of the test.

Positive serum control should give known HI titre; antigen control should contain 4 HA units and RBC control should give button formation.

3. Computerisation

The data and results obtained were entered into IBM-PC-XT compatible computer system using d-base 3+ and analysed with the help of a Statistical Package for Social Scientist (SPSS) programme.

4. Statistical Analysis of Data

1. Mean, Standard Deviation (S.D.) and Standard Error (S.E.) were calculated for all the quantitative parameters.

2. Geometric Mean Titre (GMT) was considered at log base 2.

3. Percent prevalence was calculated for all qualitative parameters.

4. Chi-square test was employed to determine the significant differences amongst various age groups for the qualitative parameters.

5. For quantitative analysis -

   - Oneway Anova test was applied to analyse multiple variables.
   - Paired 't' test was used to compare differences between maternal and cord blood measles HAI antibody titres in various subgroups.
   - Independent 't' test was used to compare quantitative parameters between subgroups.

6. Levels of significance selected -

   * = Significant at $P \leq 0.05$
   ** = Significant at $P \leq 0.01$
   *** = Significant at $P \leq 0.001$.