PROMOTION OF ORAL FLUID METHODS FOR EVALUATION AND SURVEILLANCE OF THE MEASLES IMMUNIZATION PROGRAMME IN ETHIOPIA

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A thesis submitted in fulfillment of the requirements of The University of Warwick Graduate School for the degree of Doctor of Philosophy

September 2002

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ACKNOWLEDGEMENTS

I acknowledge groups involved in the study:

(i) Dr James Nokes, Warwick University, UK, and KEMRI/Wellcome Trust Collaborative Research Unit, Kilifi, Kenya. I am greatly indebted for his additional excellent supervision of my PhD research programme.

(ii) Dr David Brown, Director, Dr Bernard Cohen, Dr Jin Li, Dr Andrew Vyse, Dr Dahn Samuel, Enteric, Respiratory and Neurological Virus Lab, Central Public Health Lab, London UK.

(iii) Prof Felicity Cutts, Dr Phillippa Cumberland, Tropical and Infectious Disease Unit, London School of Hygiene and Tropical Medicine.

(iv) Dr Eshetu Lema, Head of Dept of Infectious and Other Disease Research, Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia.

(v) Dr Fikre Enquselassie, Department of Community Health, Faculty of Medicine, University of Addis Ababa, Ethiopia.

Many people and organizations in Ethiopia contributed towards making this thesis possible. I would like to thank MOH, WHO/EPI country office, Butajira Rural Health Project, Arsi Zone Health Department, Asela Health Centre, Asela Hospital and Arada, Woreda 25 and Akaki Health Centres in Addis Ababa for permission to use their facilities for this study. I am grateful to the children and their mother or guardians who agreed to participate in the study. I acknowledge all the other participants, survey teams and medical personnel for their support this work.
The study was financially supported by the Wellcome Trust (Ref.No. 047413) and WHO (GPV, DVB Project Grant No.V21/181/133). Supplementary support was obtained from BASICS, USA, and the British Council, Addis Ababa.

I am gratefully acknowledge examiner of this research degree Professor Andrew Easton, Chairman of the Faculty of Science, University of Warwick and Professor Peter Smith of London School of Hygien & Tropical Medicine.

Finally, but not least, I would like to thank my wife, Fikerete, for her full support and taking care of the children during my study absence.
This thesis is dedicated to the world’s children, especially to Ebenezer, Maranata, Hanna, Zion, Jeremiah and Lydia, who need to be free of measles.
DECLARATION

The research work contained in this thesis is my own work notwithstanding that it was contained with two collaborative research projects. Funds received from Wellcome Trust (Ref.No. 047413) and WHO (GPV, DVB Project Grant No.V21/181/133) assisted towards carrying out this research. Related supplementary support was obtained from BASICS, USA, and the British Council, Addis Ababa. The candidate undertook the work with general support from:

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Material clearly indicated in the text as published by the candidate as principal author has been used in the thesis. The thesis has not been submitted for a degree at another university.
Abstract

This work aims to demonstrate the use of oral-fluid methods in evaluating the effectiveness of a vaccination programme at the individual (vaccine conversion) and population levels (herd immunity and virus transmission and origin) within a developing country context. The setting for this work was Ethiopia - a country beset with huge economic, social and logistical difficulties in vaccine programme implementation. The study comprises the following: First, the development and evaluation of highly sensitive measles specific IgG/IgM ELISAs using oral-fluid, second, the application of these assays to evaluate routine and campaign measles vaccination programmes, and third genotyping of measles virus strains circulating in the country, again using oral-fluid samples.

Paired blood and oral fluid samples were obtained from 787 individuals of all ages from rural Ethiopia for evaluation of the measles enhanced IgG antibody capture (GAC) enzyme linked immunosorbent assay (ELISA). Relative to serum, oral fluid assay sensitivity and specificity were: 97.4% and 91.1% for measles IgG. This work is the development and evaluation of a new method that has contributed scientifically to vaccination programme evaluation and refinement.

Pre- and post-vaccine antibody determined in 296 children attending for routine measles immunization in Addis Ababa suggested the average vaccination age at which 92.6% (200/216) seroconversion rate attained was about nine and half months. Oral-fluid based testing show 87.3% (185/212) seroconversion rate for IgM antibody compared to the 92.6% seroconversion rate for serum. This work included the development and use of an oral-fluid enhanced MACELISA as a useful substitute to serum in evaluating vaccine seroconversion.

RT-PCR was performed for oral-fluid and serum samples collected from outbreaks and sporadic measles cases across the country to study the molecular genotype characteristics of the strains. Sequence analysis of outbreaks and sporadic case samples revealed that the viruses of the D4, D8 and B3 genotypes were found in the country. This study also demonstrates the practicality of integrating oral-fluid based genotyping into measles surveillance efforts.

Pre-campaign survey work carried out in Assella town by collecting oral-fluid samples from 1928 children aged 9-59 months visiting vaccination stations, and post-campaign survey work undertaken by cluster-based random sampling of 750 oral fluid samples from eligible individuals aged between 9 months to <20 years clearly show (i) a shortfall in measles 'immunity' in the target age group (9-59 months), and (ii) a significant deficit in 'immunity' in those too old to have received the vaccine. This work demonstrates for the first time the merit of oral-fluid sampling in evaluating a measles vaccine campaign.

The main achievements summarized above, give weight towards the practicality of using oral fluid in evaluating and refining immunization programmes in the developing country setting. It waits to be seen if the non-invasive technology will gain wider support in the measles control activities.
ABBREVIATIONS AND ACRONYMS

A  Adenosine
Ag  Antigen
aa  amino acids
BCG  Bacille-Calmette-Guerin
BRHP  Butajira Rural Health Project
C  Cytidine
CDC  Centers for Diseases Control
cDNA  copy Deoxyribonucleic Acid
CFR  Case Fatality Rate
CFT  Complement Fixation Test
CPHL  Central Public Health Laboratory
dNTPs  deoxynucleoside triphosphates
DPT  Diptheria Pertusis and Tetanus
EHNRI  Ethiopian Health and Nutrition Research Institute
ELISA  Enzyme Linked Immunosorbent Assay
EPI  Expanded Programme on Immunization
FCS  Foetal Calf Serum
FITC  Fluorescein Isothiocyanate
G  Guanosine
GACELISA  IgG Antibody Capture ELISA
GACRIA  IgG Antibody Capture Radioimmunoassay
GPV  Global Programme of Vaccination
<table>
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<tr>
<td>HI</td>
<td>Haemagglutination Inhibition</td>
</tr>
<tr>
<td>HRPO</td>
<td>Horse Raddish Peroxidase</td>
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<tr>
<td>IF</td>
<td>Immuno Fluoresence</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IU/ml</td>
<td>International Units per millilitre</td>
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<tr>
<td>K</td>
<td>Kappa Statistic</td>
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<tr>
<td>kd</td>
<td>kiladalton</td>
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<tr>
<td>MAB</td>
<td>Monoclonal Antibody</td>
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<tr>
<td>MACELISA</td>
<td>IgM Antibody Capture ELISA</td>
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<tr>
<td>MACRIA</td>
<td>IgM Antibody Capture Radioimmunoassay</td>
</tr>
<tr>
<td>mg/L</td>
<td>milligrams per liter</td>
</tr>
<tr>
<td>MMR</td>
<td>Measles, Mumps and Rubella</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>MV</td>
<td>Measles Virus</td>
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<tr>
<td>NGO</td>
<td>Non Governmental Organization</td>
</tr>
<tr>
<td>NHS</td>
<td>Negative Human Sera</td>
</tr>
<tr>
<td>NIDs</td>
<td>National Immunization Days</td>
</tr>
<tr>
<td>NNT</td>
<td>Neonatal Tetanus</td>
</tr>
<tr>
<td>NPA</td>
<td>Nasal Pharyngeal Aspirate</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative Predictive Value</td>
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<td>NRS</td>
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nt  nucleotide
OD 450/620  Optical Density at 450/620
OPV  Oral Polio Vaccine
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
P/N  Positive Control to Negative Control Ratio
PPV  Positive Predictive Value
RH  Radial Haemolysis
RHB  Regional Health Bureau
RNA  Ribonucleic Acid
RNase  Ribonuclease
RIA  Radioimmunoassay
RT-PCR  Reverse Transcription PCR
SNNPR  Southern Nations, Nationalities Peoples Region
T/N  Test Sample to Negative Control Ratio
TMB  Tetra Methyl Benzidine
TT  Tetanus Toxoid
U  Uracil
μl  microlitre
WHO  World Health Organization
INTRODUCTION

1. OVERVIEW

The burden of measles is estimated to be 30 million cases and nearly one million related deaths each year (WHO, 1999). In Ethiopia, the Ministry of Health (MOH) estimated 55,000 children under 5 years of age die from measles and its complications every year (MOH, 1998).

Key strategies for the local elimination/total eradication of measles as a disease are as follows. The spread of measles infection through a population requires that a chain of infectives should be maintained. Protection against this spread of infection can be taken at two points. First, the route from susceptibles to recovereds can be short-circuited by the establishment of immunization (Hinman, 1966; Spink, 1978). Second is to interrupt the mixing of infectives and susceptibles with protective barriers (e.g. isolation) (Curtin, 1985). The greatest potential is with vaccination.

Acquired immunity after measles illness is permanent. Live attenuated measles virus, when administered at recommended ages, produces about 85% immunity after one dose and greater than 90% immunity after two doses (Hull et al., 1983; Strebel, 1998; Cutts et al., 1999). Vaccine-induced immunity is long lasting and protective to all the diverse geographic origin strains. Widespread vaccination has resulted in interruption of measles virus transmission in a number of countries. For instance, the Gambia in 1968-69, the English speaking Caribbean islands, Cuba, Chile, the USA over short periods in 1993, 1995, and 1996 (de Quadros et al., 1988; Bellini and Rota, 1998). Similar achievements
were obtained in England and Wales through 1995-2000 (Ramsay et al., 2003). The success of recent mass vaccination campaigns in these countries has suggested that global eradication of measles may be possible. Reaching this goal will require continued commitment to increase vaccination coverage levels with a co-coordinated global effort.

WHO targeted measles elimination by the year 2000 in the American region, by 2007 in the European region and by 2010 in the eastern Mediterranean region (CDC, 1999). The WHO current policy to the region of Africa is adopting a strategy aiming to raise routine vaccine coverage to at least 80% and using supplemental campaigns in non-polio-reservoir countries by 2003 (WHO-Afro, 2000).

Laboratory investigation will play a critical role in monitoring the success of measles control strategies and as we shall see this role will require the development of more sensitive diagnostic methods suitable for diagnosis and surveillance, genetic analysis of measles strains and a technology which is transferable worldwide.

Measles diagnosis relies increasingly on serological tests (Arista et al., 1995). Serum based diagnosis can be made by virus isolation, by demonstration of a significant increase in specific immunoglobulin G (IgG) titers, or by the detection of anti-measles virus (MV) IgM antibodies by using radio-immunoassays (RIA), enzyme-linked immunosorbent assays (ELISAs) and direct or indirect fluorescence-antibody techniques (Perry et al., 1993; Arista et al., 1995; Samuel et al., 1998; Lee et al., 1999; Nigatu et al., 1999).
The effectiveness of an immunization programme can be evaluated through serological survey methods. The need for techniques that obviate the requirement for blood sampling promotes the application of oral-fluid based methods to the evaluation of the immunization programs. Oral-fluid based testing has an advantage of convenience, avoidance of inadvertent transmission of blood-borne pathogens, ease of use in paediatric and geriatric populations; as well as the potential for blood-free home and work place collection of patient samples.

To facilitate the use of oral fluid in measles diagnosis an IgG and IgM capture ELISA has been developed (Chapter 2, 3). Demonstration of the utility of oral fluid in epidemiological studies and measles vaccination evaluation utilizing these methods is described in this thesis (Chapter 3, 6). The use of molecular epidemiology in the control/elimination of measles infection by monitoring the virus circulation is also undertaken in this research work (Chapter 4, 5).

II. OBJECTIVES OF THE STUDY

Of the “ten elements of surveillance” summarized by WHO (1968) at least in six of them viz a viz, morbidity reporting, epidemic reporting, laboratory investigations, individual case investigations, epidemic field investigations and surveys, the laboratory has a role in providing serological results for measles surveillance. This indicates that the general advantages of measles surveillance data depend in large part on laboratory results. The requirement of blood specimens for laboratory results can limit the yield of data for measles surveillance. In this respect we need another source of human biological material
for measles surveillance, which is inexpensive and simple to collect, acceptable to donor and collector and provides accurate representation of serological status.

Oral fluid has been explored as a source of human biological material for surveillance of viral diseases (Parry et al., 1987). It has clear advantages over venipuncture in surveillance and epidemiology of viral diseases. In the UK, oral-fluid sampling and screening has been used for the surveillance of measles, mumps and rubella (MMR) since 1994 (Ramsay et al., 2003). This has permitted the impact of MMR vaccination programme to be monitored and evaluated in a way which may not have been possible through blood collection alone.

Measles serological surveys could play a role in the evaluation of immunization programmes (Babad et al., 1995; Cox et al., 1998). Immuno-serological cross-sectional measles surveys have particular importance to determine immunization programme strategy in relation to age groups, geographic areas, socioeconomic groups and risk population groups. Follow-up serological measurements in measles immunized persons has importance to determine the proportion developing immune responses, quality and extent of response, duration of response and level of protection against measles infection. Periodic measles serological surveys have advantage to identify groups who are not receiving measles vaccines or who have inadequate responses. The importance of seroepidemiology for such purposes is paramount although the necessity for venipuncture reduces the ease and acceptability of this method. New methods that obviate the requirement for blood sampling could further encourage the application of measles
serological surveys for the evaluation of measles immunization programme. To achieve
the aforementioned roles at better performance this thesis work was undertaken for
measles vaccination programme evaluation and surveillance based on oral-fluid
collection and screening methods. The purpose of this thesis is, therefore, to explore the
development and evaluation of oral fluid as a diagnostic specimen for measles virus with
particular reference to the developing country setting. The technologies developed (see
A2, appendix attached to Chapter 2) have increased the level of sensitivity and specificity
where salivary examination for measles IgG and IgM is practical and convenient. Using
PCR technology we found oral-fluid from measles cases to be useful in the molecular
characterization of measles virus. Success of the measles vaccination programme can be
assessed using oral fluid specimens as markers of sero-conversion.

The thesis specifically aims to achieve the following: -

1. The development of a GACELISA for the detection of measles specific IgG in oral-
   fluid, with performance (sensitivity and specificity) that makes it suitable for
   replacement of serum assays, particularly for estimating population immunity.
2. The development of a MACELISA for the detection of measles specific IgM in oral-
   fluid, suitable in performance to replace serum assays.
3. Demonstration of the use of these assays in the estimation of measles antibody
   (immunity) prevalence in the vaccine-targeted population and in monitoring the
   outcome of a measles vaccination programme (routine and campaign) in a developing
   country setting.
4. Demonstrate the utility of oral fluid to study the molecular epidemiology of measles
   virus in a developing country in a period of accelerated measles control.
Community surveys of measles specific IgG/IgM are useful to guide the design of measles control programmes. For example these help in (a) defining levels of immunity to measles pre- and post-vaccination efforts, i.e. assessing the effectiveness of the vaccination programme, (b) identifying age groups in which a significant susceptible proportion remain, (c) assessing sero-conversion rates following vaccination. Analysis of the genetic characteristics of wild-type measles helps to elucidate the origin and transmission pathways of measles virus (Rota et al., 1996). The genetic data when analysed with other epidemiological data provides a means to assess the efficacy of measles control programmes. For such molecular studies measles RNA can be detected by RT-PCR from isolates, oral fluid, blood, throat-swabs, urine collected from acute cases. There have been no systematic studies made to evaluate the relative sensitivity of these different samples. The primary role of this study is in the demonstration of the use of oral fluid as a clinical specimen for detecting IgG/IgM antibody for evaluating measles control strategies and the virus genome for molecular epidemiological studies.

The thesis has 7 chapters. Chapter 1 reviews the literature on issues of measles virus and immunization strategies. This is followed in Chapter 2 by description of the development of a GACELISA for the detection of measles specific IgG in oral fluid. Chapter 3 presents a study of pre- and post-vaccine status of measles antibody in infants using serum and oral-fluid testing: an evaluation of routine immunization in Addis Ababa, Ethiopia. The basis for this evaluation is in part a novel enhanced MACELISA for the detection of measles specific IgM in oral fluid. An analysis of measles viruses circulating in Ethiopia during 1998-9, in which a new genotype is identified, is presented in Chapter
4. In Chapter 5 the serological and molecular characterization of measles virus outbreaks reported in Ethiopia during 2000 – 2002 is presented. Results of an evaluation of a measles immunization campaign using oral-fluid antibody surveys are presented in Chapter 6. Chapter 7 presents some future developments and applications of measles oral fluid methods. It has two parts (1) future technical developments (2) future wider applications of oral-fluid methods.
CHAPTER 1
LITERATURE REVIEW

1. MEASLES VIRUS

1.1.1 Classification and morphology

The position of the measles virus is presented in the Seventh Report of the International Committee on Taxonomy of Viruses (ICTV) (van Regenmortel et al., 2000). A basic division is made between families whose member viruses have deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) as their genetic material. The Paramyxoviridae is an RNA family that the measles virus belongs. The measles virus is not distinguishable from other members of the paramyxovirus family by electron microscopy (Cliff et al., 1993). Measles virions are spherical, enveloped particles with a helical nuleocapsid (Norrby, 1964). The diameter of the pleomorphic particles varies between 100 and 250 nm. The envelope carries surface projections (peplomers) with a length of 9-15nm. There are two different peplomers, which have distinct morphologies: the haemagglutinin (H) peplomers appear conical, whereas the fusion (F) peplomers are shaped like dumbbells with ends of unequal size. On the inside surface of the envelope is the matrix (M) protein, which is thought to interact with the peplomers and with the nucleocapsid and to play a key role in virus maturation. Purified matrix (M) protein appears as short, tubular structures with a width of 8 nm. The nuleocapsid has a helical structure with a diameter of 17-18 nm. The nucleocapsid is packed within the envelope in the form of a symmetrical coil of undefined nature.
1.1.2 Measles clinical features

Figure 1.1. Time course (as day 0 is assumed the onset date of rash) of clinical events in measles disease (temperature given in °F, where 38 °C = 100.4 °F). (Source: Modified from Cliff et al, 1993)

<table>
<thead>
<tr>
<th>Days of Illness</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measles</td>
<td>104</td>
<td>103</td>
<td>102</td>
<td>101</td>
<td>100</td>
<td>99</td>
<td>98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rash</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koplik's</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coryza</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cough</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The incubation period is usually 10 days (and can range from 7 to 18 days) from exposure to onset of fever. This is the period of maximum respiratory transmission to susceptible individuals. Prodromal fever, conjunctivitis, coryza, cough and Koplik spots on the buccal mucosa characterize the disease. A characteristic red rash (maculo-papular erythematous rash) appears on the third to seventh day, beginning on the face, becoming generalized and lasting four to seven days. The period of communicability continues for 4 to 5 days after rash onset, although the patient is then less infectious. The time course of clinical events in measles disease is shown in Figure 1.1.
I.1.3 Natural infection

Measles virus enters through the respiratory tract and is carried by lymphocytes to the regional lymph nodes. It then spreads from cell to cell and is transported by wandering macrophages or lymphocytes to the reticulo-endothelial system. Activated thymus-dependent lymphocytes form a line of sensitized cells capable of reacting with measles antigen. T-cells then react with the infected cells liberating virus into the extra-cellular fluids where it stimulates B-lymphocytes to produce antibody. Once released from cells virus is disseminated by the blood stream to the skin, mucous membranes and other tissues where it proliferates. Large numbers of thymus-dependent immunocytes follow and give rise to a delayed-hypersensitivity reaction manifesting as the clinical syndrome of measles.

I.1.4 The immune response to measles infection

Cell-mediated immune responses appear to be important in both the pathology and recovery from measles disease. It is required to clear measles virus infection. However, both humoral and cell-mediated immunity appear to be capable of preventing infection in normal individuals exposed to the virus. Measles-specific immune suppression begins with the onset of clinical disease, before the rash, and continues for many weeks after apparent recovery. Antibodies are first detectable when the rash appears. The life-long protection results from natural infection in large, relatively dense populations (Black, 1966). IgM antibodies are produced initially, followed by IgG and IgA in serum and secretions. IgM antibodies peak at 7-10 days after rash onset and fall rapidly, rarely being
detected more than 8 weeks after rash onset (Pederson et al., 1996; Bouche et al., 1998). The presence of IgM is generally accepted as evidence of primary measles infection (by wild or vaccine virus). IgG antibody peaks about 2 weeks following rash onset and subsequently declines, but is detectable for years after infection.

### I.1.5 Growth in cell cultures

This review is based on my present work experience at CPHL (see Chapter 2) with reference to other studies cited in the text. While humans are the only hosts, measles can be propagated in vitro in cell cultures and lines. Virus can be isolated successfully from respiratory secretions and blood during the prodromal phase up to about the first or second day of rash using human kidney cell cultures (Enders and Peebles, 1954). Available reports show that human embryo kidney and monkey kidney were equally sensitive for the primary isolation of measles virus (Matumoto, 1966). The virus can also readily be isolated from blood and respiratory secretions obtained during the catarrhal prodrome and on the first day of the rash if inoculated into primary cultures of human or monkey renal cells (Enders et al., 1957; Enders, 1962). After adaptation to these cells, conversion to growth in continuous cell lines seems to be reasonably easy. It can successfully be propagated in a wide variety of cells which include human amnion, dog kidney, bovine kidney, conjunctival cells, human liver, and chick embryo (Bech, 1958; Mutai, 1959; Gendelman et al., 1984). Measles virus grows in continuous line cells of human origin such as FL, AV-2, HEP-2, KB, and HeLa, and also the simian cell lines BSC-1 and MS (Dekking and McCarthy, 1956; Black et al., 1956; Kohn and Yassky, 1962). Recent works show success of the method of isolation of the virus from clinical
specimens (primary isolation) using an Epstein-Barr virus-transformed marmoset B lymphoblastoid cell line, B95a (Kobune et al., 1990).

Multiplication of MV produces characteristic changes in cell cultures. The main features being development of multi-nucleated cells or syncytia, formation of cytoplasmic and intranuclear inclusion bodies, and vacuolization of cytoplasm with slow destruction of the cell sheet. In HEp-2 or FL cell cultures inoculated with more than one virus particle per cell, intracellular virus is first detected after 12 to 18 hours and increases until 24 to 48 hours (Bellini and Rota, 1995). Virus release into the fluid medium begins at about 30 hours and increases until 36 to 48 hours. After a plateau of several days, both titers decline. With a small inoculums or inoculation at the time of implanting the cells, the phase of growth and decline of infective virus are somewhat prolonged.

1.1.6 Differential diagnosis

The non-specific nature of the prodromal signs and the existence of mild cases make clinical signs unreliable as the sole diagnostic criteria of measles disease. Misdiagnosis of measles is, for example, more common among young infants, and outbreak associated cases are more likely to be laboratory confirmed than sporadic cases (Ramsay and Emond, 1978; Ramsay et al., 2003). Measles may resemble infections with rubella, dengue fever, ECHO, coxsackie, parvovirus B19 and herpes virus 6 viruses, as well as some bacterial and rickettsial diseases (Nur et al., 1999). Moreover, there are other conditions that may present in a similar form, including Kawasaki’s disease, toxic shock and drug reactions. Selection of appropriate testing algorithms will depend upon the
prevalence of these conditions in countries, and the availability of adequate laboratory services. Countries in the elimination phase with successful measles immunization programmes are finding that a high percentage of suspected measles cases are due to rubella (WHO, 1999). As measles and rubella may be coincidentally eradicated with use of MMR vaccine, testing negative measles serum samples for rubella will provide useful information for rubella surveillance.

1.1.7 Immunological detection of measles infections

Measles virus can be detected from various clinical samples by using serological methods, cell cultures techniques or molecular techniques. Samples that can be collected at different stages of the measles infection for virus isolation and serological tests are outlined in Table 1.1.
The following is a summary of identification methods available for laboratory diagnosis of measles infection.

**I.1.7.1 Serological assays**

Measles infection is diagnosed serologically by 1) detecting measles specific IgM antibodies; or 2) quantifying measles specific immunoglobulins in order to demonstrate a significant rise in IgG between paired acute and convalescent sera.
I.1.7.1.1 Measles Specific IgM Antibodies

IgM produced on primary vaccination declines more rapidly than IgM produced in response to the wild virus. Vaccine and wild virus IgM cannot be distinguished by serological tests. A vaccination history is therefore essential for interpretation of test results. The following methods are commonly used to detect measles-specific IgM.

- **IgM capture ELISA**, requires only one blood sample for case confirmation. Assays show 97% sensitivity compared with the plaque reduction neutralization test (PRNT) in detecting infection in vaccinated infants (Erdman et al., 1991). In clinically confirmed cases, the sensitivity and specificity of capture assays were 91.8% and 98.2% respectively, while the positive and negative predictive values were 98.2% and 92.0% respectively (Arista et al., 1995). Capture ELISA assays are considered superior to indirect assays, since they do not require the removal of IgG antibodies. Several capture IgM ELISA kits are commercially available.

- **IgM indirect ELISA**, requires only one blood sample for case confirmation. In clinically confirmed cases, the sensitivity and specificity of indirect assays were 90.3% and 98.2% respectively, while the positive and negative predictive values were 98.2% and 90.5% respectively (Arista et al., 1995). Indirect ELISA assays are the most widely used. However, this type of assay requires a specific step to remove IgG antibodies. Problems with the incomplete removal of the IgG can lead to inaccurate results.

- Serologic tests are effective in identifying cases of SSPE (Table 1.1). Patients with this disease have serum antibody titers, which are 10-100 times higher than those seen
in late convalescent-phase sera. There is pronounced local production of oligoclonal measles virus antibodies in the CNS (Vandvik et al., 1973).

I.1.7.1.2 Measles Specific IgG Antibodies

- Several capture and indirect IgG ELISA kits are commercially available.
- Recently newer capture ELISA methods specific for IgG and IgM of measles virus that have about 97% sensitivity and about 90% specificity have been developed at CPHL, London, UK, and CDC, Atlanta, USA, for detecting measles infection using oral fluid (Helfand et al., 1996; Nigatu et al., 1999).

I.1.7.1.3 Quantification of Measles-specific immunoglobulins

- Virus neutralization, the plaque reduction neutralization test (PRNT), requires two serum samples, acute and convalescent, and shows 100% sensitivity in confirming clinical measles. Single titers of greater than 120 are consistent with 100% protection against clinical measles (Chen et al., 1990; Ratnam et al., 1995; Lee MS et al., 1999).
- Haemagglutination inhibition (HI) requires two serum samples, acute and convalescent, and shows 98% sensitivity in detecting antibody increase in vaccinated students and 100% sensitivity in vaccinated infants (Kalter et al., 1991).

Generally complement fixation methods and radio-immunoassays (RIA) have been developed as the most useful method in the past employed with diagnosis of measles. (Perry et al., 1993).
I.1.7.2 Virus isolation

The growth of measles virus in cell culture is mentioned previously in detail. The virus can be cultured with difficulty from urine, nasopharyngeal or oral-fluid specimens or peripheral blood lymphocytes during the prodrome and rash stages of the disease (Table 1.1). Thereafter virus excretion declines rapidly. Detection and identification of the virus in cell culture may take several weeks. Possession of a measles virus isolates permits genomic analysis and comparison with other strains from different locations and years, providing information on its origin and transmission history.

I.1.7.3 Reverse transcription polymerase chain reaction (RT-PCR)

Amplification of measles RNA after reverse transcription (RT-PCR) is done in specialized laboratories and can be use for measles surveillance programmes.

II. MOLECULAR BIOLOGY OF MEASLES VIRUS

II.1.1. Genomic structure

The measles negative-sense non-segmented (single-stranded) RNA genome consists of six transcription units (Figure 1.2), which generates the mRNAs for six structural and two non-structural proteins (Barrett et al., 1991). Three structural proteins are complexed with viral RNA, and the other three structural proteins participate in the formation of the virus envelope (Table 1.2). The major internal component is the phosphorylated N (nucleocapsid) protein. This has a molecular weight (MW) of approximately 60 kd. The other internal virion components are the L (large protein or polymerase) and P (phosphoprotein) which are present only in limited quantities. The P protein shows a high degree of sensitivity to proteolytic enzymes. The envelope components consist of the M
(matrix) protein and the H (haemagglutinin) and F (fusion) peplomers. The M protein has MW of 36-37 kd, with some size variation in different virus strains (Wechsler, 1982). The glycosylated H protein has MW of 79 kd. The F protein is synthesized in a precursor form as a glycoprotein with MW of 55-60 kd (Graves et al., 1978; Ogura et al., 1988). The mRNA for the P-protein also encodes, in an overlapping reading frame, the non-structural C protein and V protein (Bellini et al., 1985). The functional importance of these structural proteins is mentioned in Table 1.2. Research shows Baculovirus expression of the nucleoprotein genes of measles virus and utility of the recombinant protein in diagnostic enzyme immunoassays (Hummel et al., 1992). The in-house Microimmune measles specific IgG and IgM oral-fluid capture assays developed at CPHL demonstrated the importance of nucleoproteins in immunological assays. The IgG assay was used to undertake the work mentioned in Chapter 6.

The entire measles virus genome has been sequenced and it contains about 15,900 nucleotides (Rota et al., 1995), but the exact number varies between virus strains and even between viruses of the same strain with different passage histories. Sequence of the nucleotid genome shows genetic hetrogeneity for the N, F, H, M, P and L genes (Taylor et al., 1991; Baczko et al., 1991; Komase et al., 1995; Rima B et al., 1995a; 1995b). The highest degree of variation is found in the C terminus of the N and H genes (Taylor et al., 1991). The sequence of the C-terminal 151 amino acids of the N protein analyzed in 18 MV strains show up to 7.2% divergence in the nucleotide sequence and 10.6% divergence in the amino acid sequence between the most unrelated strains (Taylor et al.,
1991). Compared to the H and N genes, the F, M, and L genes of the wild-type MVs are relatively conserved (Komase et al., 1995; Rota et al., 1992).

Table 1.2. Structural components of the measles virus

<table>
<thead>
<tr>
<th>Component</th>
<th>MW (kd)</th>
<th>Virion location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-nucleoprotein</td>
<td>60</td>
<td>Dominating internal protein</td>
<td>Protects viral RNA</td>
</tr>
<tr>
<td>P-polymerase(phospho) protein</td>
<td>72</td>
<td>Internal; associated with N</td>
<td>Probably part of transcription complex</td>
</tr>
<tr>
<td>L-large protein</td>
<td>180-200</td>
<td>Internal; associated with N</td>
<td>Part of the transcription complex</td>
</tr>
<tr>
<td>M-matrix</td>
<td>36-37</td>
<td>Inside virion envelop</td>
<td>Assembly of virions</td>
</tr>
<tr>
<td>H-haemagglutinin</td>
<td>79</td>
<td>Transmembranous envelope glycoprotein</td>
<td>Adsorption to nucleated cells and erythrocytes</td>
</tr>
<tr>
<td>F-fusion factor</td>
<td>41(F1) and 18-20(F2)</td>
<td>Transmembranous envelope glycoprotein</td>
<td>Cleaved protein; active in fusion of cells, haemolysis, and virus entry</td>
</tr>
</tbody>
</table>

II.1.2. Genetic diversity of MV and geographical distribution of genotypes

MV is serologically monotypic (Rota et al., 1994). However, genetic heterogeneity exists among MVs and provides a basis for the study of their molecular epidemiology. Sequencing of the MV nucleoprotein (N) and haemagglutinin (H) genes is used to assign MVs to one of eight clades, designated A to H. Clades B, C, D and G are further subdivided into genotypes B1, B2 and B3, C1 and C2, D1 to D8, G1 and G2, respectively (Chibo et al., 2000). Clades are used to indicate the relationship between the various genotypes. The genotype designations are the operational taxonomic units. A unified nomenclature guideline for MV strains and a definition of their genotypes has been proposed by WHO (WHO, 1998; 2001).

The virus is distributed worldwide, and molecular genotyping procedures have been able to demonstrate significant differences between isolates from different locations (Bellini
and Rota, 1998). The following table shows the geographical distribution of measles virus genotypes.

Table 1.3. Measles virus genotype distribution worldwide (Source: Rota et al., 1994; Bellini and Rota, 1998; WHO, 1998; 2001; Chibo et al., 2000 etc.)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>vaccines, pre-vaccine and recent wts</td>
</tr>
<tr>
<td>B1</td>
<td>Africa, USA</td>
</tr>
<tr>
<td>B2</td>
<td>Africa, USA</td>
</tr>
<tr>
<td>B3</td>
<td>Nigeria, Ghana, Sudan, Congo, Gambia, Cameroon, USA</td>
</tr>
<tr>
<td>C1</td>
<td>Japan 1984, Germany 1992</td>
</tr>
<tr>
<td>C2</td>
<td>Morocco, Western and Central Europe, USA, Brazil, UK, Australia</td>
</tr>
<tr>
<td>D1</td>
<td>Australia, UK 1974, INACTIVE*</td>
</tr>
<tr>
<td>D2</td>
<td>South Africa, Zambia</td>
</tr>
<tr>
<td>D3</td>
<td>Japan, Philippines</td>
</tr>
<tr>
<td>D4</td>
<td>South Africa, USA, Pakistan, Iran, India, Ethiopia, Australia, Russia</td>
</tr>
<tr>
<td>D5</td>
<td>Malaysia, Australia, Japan, Thailand</td>
</tr>
<tr>
<td>D6</td>
<td>Western and Central Europe, USA, Brazil, UK, Argentina, Chile, Turkey</td>
</tr>
<tr>
<td>D7</td>
<td>Australia (Victoria), Europe, Germany</td>
</tr>
<tr>
<td>D8</td>
<td>South Asia, Middle East, Balkans, Ethiopia</td>
</tr>
<tr>
<td>D9</td>
<td>Venezuela, Indonesia (West Java)</td>
</tr>
<tr>
<td>E</td>
<td>Germany 1971, INACTIVE*</td>
</tr>
<tr>
<td>F</td>
<td>Spain 1965, INACTIVE*</td>
</tr>
<tr>
<td>G1</td>
<td>USA 1983, INACTIVE*</td>
</tr>
<tr>
<td>G2</td>
<td>Indonesia, Malaysia, Thailand</td>
</tr>
<tr>
<td>G3</td>
<td>East Timor, Indonesia</td>
</tr>
<tr>
<td>H1</td>
<td>China, USA, UK, Australia, Korea, Mongolia</td>
</tr>
<tr>
<td>H2</td>
<td>Vietnam</td>
</tr>
</tbody>
</table>

* = Circulation terminated

II.1.3. Molecular amplification techniques used for the detection and quantification of measles virus

The development of molecular amplification procedures has enabled the specific detection of measles RNA and its genomic sequences (Figure 1.2). These techniques have been used to determine infection of a person from samples of different source (see Table 1.1). Brain tissues from patients have been used for PCR in detecting subacute sclerosing panencephalitis (SSPE) associated cases (Godec et al., 1990). Peripheral
blood mononuclear cells have also been used in PCR for looking at infection of monocytes during acute measles cases (Esolen, 1993). Nasopharyngeal aspirates (NPA) were used in PCR for detection of measles virus (Shimizu, 1993). Recently sera and plasma specimens have been used for molecular identification of distinct haemagglutinin measles virus and for detection of virus infection in patients (Nakayama et al., 1995). Clinical samples such as CSF, NPA, throat swab, oral fluid and urine have also been used at CPHL for detecting measles virus RNA by PCR (Jin, 1996). These marked developments have been enables to determine truly infected measles cases, particularly when serological results are indeterminate and to study biological variation of different strains. This enables to gain wider acceptance promises in the future (see Chapter 7).
Figure 1.2. Structure and genomic organization of measles virus and proteins encoded by its genes. Eight gene products are indicated; N (nucleocapsid), P (phosphoprotein), C (nonstructural), V (nonstructural), M (matrix), F (fusion), H (haemagglutinin), and L (large or polymerase). The number of amino acids in different proteins is shown under the boxes. Regions indicated by explosion stars are the virus genome amplified for first and second round PCR. (upper). Cross-sectional part of the measles virion (lower). (Data derived mainly from Alkhatib and Briedis, 1986; Bellini et al, 1985; Bellini et al, 1986; Blumberg et al., 1988; Buckland et al., 1987; Gerald et al., 1986; Richardson et al., 1986; Rozenblatt et al., 1985).
III. MEASLES VACCINES AND IMMUNIZATION

The development of live attenuated measles virus vaccines began soon after the isolation of the virus by Enders and Peebles (1954). By the end of 1950s, Enders and colleagues had developed the Edmonston B strain of live attenuated measles vaccine by subjecting the virus to passage in different cell cultures (Enders, 1962). The Edmonston B vaccine was frequently found associated with rash and fever greater than 39.5 °C (Krugman et al., 1962). To alleviate this problem gamma globulin was often administered simultaneously to reduce the occurrence of high fever and rash. In the early to mid 1960s a formalin-inactivated measles vaccine derived from the Edmonston strain was used. Administration was normally given as three doses of inactivated virus at monthly intervals (Krugman et al., 1965). Use of inactivated vaccine was stopped in 1967, when it was realized that immunity was short-lived, and that recipients were at risk of atypical measles on exposure to live measles virus (ACIP, 1967). By the mid to late 1960s, new strains of measles vaccine had been developed in the USA, Japan, Yugoslavia, the USSR, and China, by further attenuation of Edmonston (AIK-C), Edmonston A (Schwarz), Edmonston B (Moraten, Edmonston Zagreb (EZ)) or separate isolates (Leningrad 16, CAM-70, Shanghai-191). Further attenuated vaccines were associated with lower rates of clinical reactions and were suitable for widespread use without the need for concurrent administration of gamma globulin.

Different factors affect the response to immunization, such as, age and maternal antibody level (Cutts, 1993). Wesley et al., 1978 reported that the response to measles immunization was delayed among malnourished children. The optimal age at delivery of measles vaccine depends upon the relationship between the average age at infection and
the rate of loss (average duration) of maternal antibodies specific to measles (McLean and Anderson, 1988; McLean, Nokes and Anderson, 1991). Maternal antibodies typically provide protection during the first 6 months of life, but often longer (McLean, Nokes and Anderson, 1991; Williams, Cutts and Dye, 1995). Interference with the replication of vaccine virus is frequently still seen at the age of 12 months (Albrecht et al., 1977; Marks et al., 1978; Nader et al., 1968). As a consequence vaccination in the first year of life gives inadequate immunity to measles. The earlier the age the lower the seroconversion rate (McLean, Nokes and Anderson, 1991; Williams, Cutts and Dye, 1995). The requirement for delay until maternally derived antibody has been lost is an impediment for early vaccination. The duration of maternally derived immunity in a child depends on the mother's antibody titer, the efficiency of transfer across the placenta and the rate of catabolism in the child (Lee TL, 1983; Black et al., 1986). A child exposed to many infections makes a large variety of IgGs; in order to keep the total blood IgG level in the normal range, catabolism is accelerated and passively acquired antibodies are swept out at an accelerated pace. In this way, early susceptibility to measles is strongly correlated with low economic status (Black et al., 1986). To meet this challenge age cross-sectional sero-epidemiological surveys and seroconversion studies are important for recommending the proper age for vaccination. An evaluation of the routine immunization programme in Ethiopian children, reported here (Nigatu et al., in press), gives support for the WHO recommended age for measles vaccination at 9 months (WHO, 1979). The ability of a measles vaccine to induce an immune response, particularly in the presence of maternal antibody, varies according to the strain and the dose of vaccine (McLean et al., 1991). Studies compared the effect of standard EZ and Schwarz vaccines found that EZ
vaccine gave superior sero-conversion rates to Schwarz (Whittle et al., 1988; Markowitz et al., 1990c; Job et al., 1991). Another study compared AIK-C vaccine with EZ vaccine and found that this vaccine was as immunogenic as EZ vaccine (Tidjani et al., 1989).

Serological studies in developing countries have shown sero-conversion rates following immunization at age 9 months of 80% to 90% (Black et al., 1984; Ndikuyeze et al., 1988; Li-Min Huang et al., 1990). Field studies of vaccine efficacy have given estimates of 85% protection (Hull et al., 1983).

IV. WHO/EPI PROGRESS TOWARDS GLOBAL MEASLES CONTROL AND ELIMINATION

In 1989, the World Health Assembly resolved to reduce global measles morbidity by 90% and measles mortality by 95% by 1995, compared with the disease burden during the pre-vaccine era (WHO, 1997; 1999). By 1996, the estimated incidence and death rates for measles worldwide were reduced by 78% and 88%, respectively. In 1990, the World Summit for Children adopted a goal of vaccinating 90% of the world’s children against measles by 2000 (WHO, 1993; WHO, 1994a). However, routine measles vaccination coverage has remained relatively stable since 1990, and an estimated 1 million children continue to die from this preventable disease each year (WHO, 1999). During the 1990s, the widespread use of innovative measles-control strategies in the Region of the Americas and in countries such as Mongolia, South Africa, and the United Kingdom demonstrated that high-level measles control and even interruption of transmission is feasible over large geographical areas (Bellini and Rota, 1998; Ramsay et al., 2003).
IV.1.1 Stages of measles control

Based on implementation of a combination of vaccination and surveillance strategies, countries are considered to be in 1 of 3 stages: control, outbreak prevention, or elimination (WHO, 1997; 1999) (see Figure 1.3).

Figure 1.3. Phases for measles control/eradication programmes (source: WHO, 1999)

1. Measles Control

Control is defined as the reduction of disease incidence and/or prevalence to an acceptable level as a result of deliberate efforts, requiring continued interruption measures. In the control stage, the objective is to achieve high routine coverage with 1 dose of measles vaccine among infants to reduce measles morbidity and mortality. To
accelerate measles control in large urban and other high-risk areas with a substantial proportion of unvaccinated children and measles associated deaths, mass vaccination campaigns targeting children aged 9 months to 3-14 years have been recommended (de Quadros et al., 1996; Cutts et al., 1999).

2. Measles Outbreak Prevention (MOP)
MOP aims to maintain low incidence and prevent outbreaks by the administration of supplemental doses of measles vaccine through mass vaccination campaigns. As programmes plan for elimination of measles, a high coverage of single dose vaccine with supplementary immunization is assumed be sufficient to interrupt transmission (WHO, 1994b). The Africa region is adopting to raise routine vaccine coverage to at least 80% and using supplemental campaigns in all non-polio-reservoir countries by 2003 (WHO-Afro, 2000).

3. Measles Elimination
Elimination is defined as the reduction of endemic incidence of a disease to zero as a result of deliberate efforts, requiring continued control measures. WHO region of the America and Europe and Western Pacific aims to meet elimination goals (WHO, 1999). Although there has been tremendous success in the reduction of measles endemic incidence in many countries with measles elimination, the total interruption of measles transmission remains a major challenge due to importation of measles cases to these regions (Bellini and Rota, 1998; Hanratty et al., 2000, Ramsay et al., 2003).
IV. 1.2 Measles control and surveillance in Ethiopia

Ethiopia is implementing the measles outbreak prevention phase of the Accelerated Measles Control Strategy (AMCS). This phase envisages 5 inter-linked interventions i.e. (i) measles vaccination through the routine EPI, (ii) targeted campaigns through the high risk approach, (iii) prevention of measles complications through effective case management, (iv) monitoring of EPI and epidemiological data to predict outbreaks and identify reasons for the outbreaks, and (v) increasing public awareness on measles infection, treatment and confidence in immunization. Targeted campaigns through the high risks approach are necessary to supplement the routine immunization activities and to extend full coverage to the un-reached communities. In response to this the Ministry of Health EPI in collaboration with WHO/EPI and other NGO's launched four measles immunization campaigns (Source: Family Health Department, MOH, 1999; 2002).

The first measles immunization campaign was conducted in 1998 and targeted 9 main cities in Ethiopia i.e. Addis Ababa, Mekele, Bahir Dar, Dire Dawa, Harrar, Jimma, Nazareth, Dessie, and Gonder. 256, 689 children aged between 9-59 months were reached during this campaign. As a continuation of the measles control strategy, in 1999, a second round of a measles immunization campaign was undertaken in 9 ‘high risk’ zones in the three Regions of Amhara, Oromia, Southern Nations (total population of 23 million). During this campaign 2,964,675 children age between 9-59 months were reached with measles vaccination representing 95% coverage of the targeted population. In a further continuation of the measles immunization campaign program under-five children were vaccinated in 45 zones in 2000 and another 18 zones were covered in 2001.
V. ORAL FLUID AS A DIAGNOSTIC FLUID

The concentration of antibody in saliva was found at much lower levels compared to plasma (Table 1.4). This has limited its use as diagnostic specimen for viral immunological assays. However, research demonstrated that salivary antibody has two sources, the parotid and crevicular crevice, with different concentration levels of immunoglobulins (Mortimer and Parry, 1988). The transudate that comes from the gingival crevice, whilst being lower in concentration, closely reflects the immunoglobulin class and specificities of antibody found in plasma (Mortimer and Parry, 1991; Nishanian et al., 1998). The major reason for this is that the majority of the antibody present in the transudate comes from the small capillary bed beneath the margin that separates the teeth and gum. These properties of crevicular fluid lead investigators for measurements of virological markers of immune activation as an alternative to serum.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>IgG (mg/L)</th>
<th>IgM (mg/L)</th>
<th>IgA (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>14730</td>
<td>1280</td>
<td>2860</td>
</tr>
<tr>
<td>Parotid saliva</td>
<td>0.36</td>
<td>0.43</td>
<td>39.5</td>
</tr>
<tr>
<td>Crevicular fluid</td>
<td>3500</td>
<td>250</td>
<td>1110</td>
</tr>
<tr>
<td>Whole saliva</td>
<td>14.4</td>
<td>2.1</td>
<td>19.4</td>
</tr>
</tbody>
</table>

The other problem associated to the use of saliva as a viral diagnostic fluid is the need of immunological assays that have higher sensitivity. The development of antibody capture assays, $^{125}$I labeled (RIA) or ELISA, that are able to generate higher signals by capturing
a higher proportion of the total immunoglobulin (present in the oral fluid) specific for the antigen under test, enabled saliva to be used for successful immunological assays (Duermeyer et al., 1979; Flehmig et al., 1979). Presently the production of purified nucleoprotein through Baculovirus expression (Hummel et al., 1992) increases the utility of saliva in diagnostic enzyme immunoassays.

The value of saliva in screening for HIV infection is now well established with the use of IgG captures radioimmunoassay (Parry, 1993). The methodology has been applied to salivary diagnosis of measles, mumps, rubella, Epstein-Barr virus and hepatitis A and B infection (Parry, 1993; Vyse et al., 1997; 1999; Nigatu et al., 1999). Veterinarians found it useful for detecting feline immunodeficiency virus (FIV), (Poli, 1992) and feline leukemia virus (Lewis, 1987). Using PCR investigators found Hepatitis C virus RNA in saliva of patients with post-transfusion hepatitis C infection (Wang, 1991). Its potential application in bacteria was demonstrated with the measurement of specific IgA antibody to *Bordetella pertussis* antigens in saliva for diagnosis of whooping cough (Granstrom, 1988). Other possibilities were seen in the diagnosis of cysticercoids by measuring specific salivary antibody to *Taenia solium* larvae (Feldman, 1990). Measuring of specific IgA antibodies to gliadin is used as a screening marker for coeliac disease (Hakeem, 1992).

Generally saliva as diagnostic fluid has the following advantages: -

(1) humanitarian- the patients are spared the discomfort of repeated venipunctures;

(2) clinical- with less stress, non risk of anemia, infection or thrombosis;
(3) for children- saliva sampling is the technique of choice;

(4) economic- patients can collect themselves, thereby saving technicians' time, samples may also be mailed, eliminating travel time;

(5) eliminates the issue of protection of privacy and adulteration during sample collection;

(6) the ease and low cost of collection are major benefits in large-scale studies.
CHAPTER 2

DEVELOPMENT OF GACELISA FOR THE DETECTION OF MEASLES SPECIFIC IgG IN ORAL FLUID

Summary

An IgG antibody capture enzyme linked immunosorbent assay (GACELISA) for the detection of measles specific IgG in oral fluid was developed using an FITC/anti-FITC amplification system. The GACELISA was evaluated by testing paired oral fluid and serum samples from 787 subjects in an epidemiological study of measles in rural Ethiopia. Oral fluids were tested by GACELISA and corresponding serum samples by a sensitive indirect ELISA for measles IgG (Behring Enzygnost). By comparison with the serum measles IgG assay, the oral fluid GACELISA had a sensitivity of 97.4% (95% confidence intervals: 95.9, 98.2) and a specificity of 91.1% (81.9, 94.3), with no significant differences observed by age group. Total IgG concentrations were measured on a subset of 160 oral fluids by an in-house ELISA. This showed that false negative GACELISA results tended to occur in samples with low concentrations of total IgG, although the trend was not statistically significant. We conclude that the overall performance of the GACELISA was satisfactory, showing close agreement to the serum ELISA, and has potential to serve as an easily transferable tool for large scale epidemiological studies as required for the World Health Organisation's programme for the global control of measles.
2.1. Introduction

Radio-immunoassays for the detection of virus specific IgG and IgM in oral fluid have been developed for hepatitis A and B (Parry et al., 1989), HIV (Hunt et al., 1993), measles mumps and rubella (Perry et al., 1993) and parvovirus B19 (Rice and Cohen, 1996). These assays are sensitive and specific but are inconvenient because of the biological hazard posed by the use of the radioisotope, I^{125}, the high costs of specialized equipment required and the short shelf life of reagents. These limitations are an impediment to the wider adoption of oral fluid viral-specific antibody assays in laboratories, most particularly, in the developing world. There is a need, therefore, for more simple and robust assays, to facilitate greater use of oral fluid assays in both field epidemiology and surveillance contexts. The enzyme linked immunosorbent assay (ELISA) is presently the most feasible and appropriate assay format for such development and has been employed for the detection of HIV-1 specific IgG in oral fluid (Connell et al., 1993). More recently, an IgG antibody capture ELISA (GACELISA) using the FITC/anti-FITC amplification system has been developed for rubella specific IgG (Vyse et al., 1999).

We now describe the development of an FITC/anti-FITC GACELISA for measles specific IgG in oral fluid. Although capture ELISA has been used previously to detect measles specific IgM in oral fluid (Helfand et al., 1996), we believe this is the first time such a method has been developed for measles specific IgG. The usefulness of an IgG antibody type assay is as a measure of past exposure to infection or vaccination, by which to estimate immune status of the individual or population. Such an assay would, ideally,
perform to a high specification on samples collected under demanding conditions of the field and across a wide range of age groups, but in particular young children. In accord with this we evaluated the measles GACELISA by testing oral fluid and matching serum samples collected from 787 individuals in a rural district of southern Ethiopia in 1997. The present study forms an important stage in a programme of research in Ethiopia investigating the utility of oral fluid as a replacement for large-scale blood specimen collection for epidemiological studies (Nokes et al., 1998a,b; Vyse et al., 1999). It also demonstrates the potential of the methods used for accessing hard to reach groups and children, using non-medical or non-nursing personnel, in countries with only basic laboratory facilities.

2.2. Materials and Methods

2.2.1 Study area and population

The study was undertaken in the densely populated rural district of Butajira, 130 km south of Addis Ababa, Ethiopia. The district is situated at altitudes ranging from 2300 m in the North West to 1500 m in the South West. The 250,000 inhabitants are predominantly subsistence farmers with a main town of Butajira of some 10,000 occupants. Since 1986 this has been the study base for a longitudinal demographic and health research programme called the Butajira Rural Health Project (Shamebo et al., 1992; Shamebo et al., 1993), providing an ideal framework for epidemiological studies of vaccine-preventable viral infections. Routine measles vaccination of infants is offered through the Expanded Programme on Immunization at the recommended age of 9 months, but coverage is low and variable (Anon., 1995). Ethical approval was gained from the Faculty of Medicine Scientific and Ethics Committee, Addis Ababa University,
and Coventry Research Ethics Committee. Agreement to participate in the study was gained through verbal consent of the subject or their guardian.

2.2.2 Specimen collection

Oral fluid was collected using a sponge swab (diameter 1.5cm x height 2 cm) and processed as described previously (Nokes et al., 1998b). Specimens were kept in a cool box with ice packs prior to transportation to the field station each day. Transport buffer (see A2, Appendix attached to this Chapter) made weekly and stored in a refrigerator at 4-10°C, was added to each collection tube prior to storage at 4-10°C for up to one week until transfer to the EHNRI laboratory in Addis Ababa. Venous blood was collected by Vacutainer system (5mL Vacutainers and 21G or 22G needles, Becton Dickinson, Oxford, UK) usually on a certain day of a week and transported that same day to the laboratory in Addis Ababa. Of the 9 peasant associations (PAs) and one urban dwelling association (UDA), 4 PAs and 1 UDA were selected based on conducive conditions for the study. Total sample sizes of 5000 or 1000 for each cluster were determined. Households were identified from the census and 167 households were selected at random from each cluster. Paired blood and oral fluid samples were available from 787 subjects, representing approximately 20% of individuals who provided oral fluid (n=5000) between May and November 1997. Individuals were aged between 0 and 84 years with the following age stratification and sex ratios: 0-4 years, n=89 (% male=47.2); 5-9, 144 (50.7%); 10-19, 238 (50.8%); 20-29, 58 (32.8%), 30-49, 156 (34.6%); 50+, 100 (54.0%) (totaling 785 since 2 individuals had no age recorded).
2.2.3 Total IgG concentration determination

Total IgG concentration in oral fluids was measured by ELISA (Connell et al., 1990; see A2, Appendix attached to this Chapter).

2.2.4 Serum measles antibody assays

Serum samples were screened for measles specific IgG by a commercially available enzyme immunoassay kit (Behring Enzygnost, Dade Behring, Milton Keynes, UK). Antibody status (negative, equivocal or positive) and antibody concentration in milli-international units (mIU/mL) were determined according to the manufacturer's instructions. The relationship between antibody status and units was identified from the results as the following: <150 mIU/mL negative; 150-335 mIU/mL equivocal; >335 mIU/mL positive. Serum samples giving measles specific IgG results discordant to those of corresponding oral fluid specimens were screened for measles antibody by plaque reduction neutralization test (PRNT, Brugha et al., 1996) using the wild type 'Loss' strain of measles (Sinitsyna et al., 1990) as challenge virus.

2.2.5 GACELISA for measles specific IgG

Wells of microtitre plates ("U" well Maxisorb, Life Technologies, Paisley, UK) were coated with 100μl of a 5.7 g/L anti-human IgG solution (Dako, Ely, UK) diluted 1:1000 in 0.05M carbonate/bicarbonate buffer, pH 9.6. After overnight incubation at 4°C, the unoccupied binding sites were blocked by 2 hours incubation at 4°C with 200μl of 5% Sol-u-pro (Dynagel Inc, Calumet City, Ill, USA). The Sol-u-pro solution was aspirated and the plates air dried by inverting and leaving at 37°C overnight. The dried plates were
stored at 4°C in plastic bags containing a sachet of desiccant until required. Plates were incubated with 100µl undiluted oral fluid samples at 37°C for 30 minutes on a plate shaker set at 650 rpm (Dako, Ely, UK). Plates were then washed four times in phosphate-buffered saline (PBS) pH 7.4 (Oxoid Ltd, Basingstoke, UK) containing 0.05% Tween 20 (PBS-T) and incubated at 37°C for 1 hr with shaking with 100 µl/well of measles antigen diluted 1:2000 in GACELISA buffer (PBS with 10% Fetal Calf Serum (FCS) and 0.2 % Tween-20). After washing 4 times with PBST, the plates were incubated at 37°C for 1hr with shaking with 100µl/well of anti-measles haemagglutinin-FITC conjugate (Chemicon Inc., Temecula, California, USA) diluted 1:4000 in PBS containing 10% FCS, 5% Normal Rabbit Serum (NRS), 2% measles antibody Negative Human Serum (NHS) and 0.2% Tween-20. Plates were washed 4 times with PBST and incubated at 37°C for 30 minutes with shaking with 100µl/well of anti-FITC-Horse radish peroxidase (Chemicon, Inc.) diluted 1:4000 in PBS containing 10% FCS, 10% NRS, 2% NHS and 1% Tween-20. Wells were washed 4 times with PBST and incubated at room temperature, in the dark, for 15 minutes with 100µl/well of TMB (3,3',5,5'-tetramethyl benzidine) substrate. The reaction was stopped by adding 50µl 2M H₂SO₄. Optical density (OD) was measured at 450/620 nm (iEMS plate reader 4F, Life Sciences International, Basingstoke, UK) and converted to mOD (mOD = OD X 1000). For each microtitre plate, the mean mOD value plus 3 standard deviations (sd) calculated from the test results for subjects who had negative serum IgG results (mODneg+3sd) was used as the cut-off value, i.e. samples were defined as GACELISA positive if the mOD>(mODneg+3sd). (For standard operating procedure see A2). The number of oral fluid samples with corresponding negative sera used to determine the cut-off mOD in
each GACELISA plate ranged from 11-34. Different cut off value were used for each plate. Data stored on EPINFO V.6 were analysed using STATA V7.0 (Stata Corp, College Station, Texas, USA) and Excel (Microsoft for Windows 95 v7.0a).

2.2.6 Measles antigen preparation

One 50 ml flask of Vero cells was infected with the wild type Loss virus strain (Sinitsyna et al., 1990) and when cythopathic effect (CPE) was positive after 3 days the cells were scraped into the medium and inoculated into eight 600 ml flasks of Vero cells. When CPE was advanced (about 6 days) the cells were scraped into the medium and cell debris pelleted at 500g for 10 minutes. The pellet was resuspended in 8ml of the supernatant (S1), disrupted by sonication for about 2 minutes in a bath sonicator and centrifuged at 500g for 20 minutes. The supernatant (S2) was collected and the pellet of cell debris discarded. S2 was then combined with S1 and centrifuged at 20000g for 1 hour at 5°C. The supernatant was discarded and the pellet resuspended in 4 ml of PBS by sonication. Fetal calf serum added to a final concentration of 10% and the antigen was aliquoted and stored at -70°C. Control antigen from uninfected Vero cells was prepared in the same way.

2.2.7 Stability of measles antigen

Aliquots of measles antigen, both undiluted and diluted 1:2000 in GACELISA buffer, were placed at +8, +22, and +37, and undiluted at -70°C, for up to 30 days. The OD values of measles positive and negative control serum samples diluted 1:100 in GACELISA buffer were determined by GACELISA using aliquots of antigen stored for
0, 3, 7, 14, 21 and 30 days. (Control sera were those in use in the Enteric and Respiratory Virus Laboratory: a measles IgG negative plasma unit from an individual blood donor and a pool of measles IgG positive sera). The haemagglutination activity of the stored aliquots was measured at 37°C using 0.5% rhesus monkey erythrocytes suspended in PBS containing 2% (w/v) dextrose and 0.2% (w/v) bovine serum albumin.

2.3. Results

2.3.1 Titration and stability of measles antigen

From the titration illustrated in Figure 2.1, the optimum dilution of measles antigen for GACELISA was chosen as 1:2000 in GACELISA buffer since after this dilution factor the OD reading start to decline. The same preparation of antigen had a haemagglutination titer of 1:640.

Figure 2.1. Titration of measles antigen (filled circles) and control antigen (open circles) in GACELISA using the measles IgG positive control serum.
The effect of storage of antigen, diluted or undiluted, at different temperatures (+8, +23, +37 or -70°C) on its activity in GACELISA is shown in Table 2.1. After 30 days, no decline in antigen activity was detected at any of the storage temperatures. In addition, no decline of antigen activity was detectable by haemagglutination assay (results not shown).

Table 2.1. Stability of measles antigen at various temperatures and storage periods. Measurements are of mean OD values with positive (+) and negative (-) control serum samples.

<table>
<thead>
<tr>
<th>Storage T°</th>
<th>Storage period (days)</th>
<th>0</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>8°C diluted</td>
<td>+</td>
<td>2.658</td>
<td>2.654</td>
<td>2.494</td>
<td>3.299</td>
<td>2.813</td>
<td>2.828</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.062</td>
<td>0.105</td>
<td>0.093</td>
<td>0.122</td>
<td>0.700</td>
<td>0.125</td>
</tr>
<tr>
<td>8°C undiluted</td>
<td>+</td>
<td>2.783</td>
<td>2.509</td>
<td>2.142</td>
<td>2.798</td>
<td>2.611</td>
<td>2.298</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.073</td>
<td>0.109</td>
<td>0.054</td>
<td>0.069</td>
<td>0.059</td>
<td>0.056</td>
</tr>
<tr>
<td>23°C diluted</td>
<td>+</td>
<td>2.786</td>
<td>2.918</td>
<td>2.670</td>
<td>3.106</td>
<td>3.012</td>
<td>2.962</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.070</td>
<td>0.147</td>
<td>0.132</td>
<td>0.109</td>
<td>0.073</td>
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<td>23°C undiluted</td>
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<td>2.531</td>
<td>2.567</td>
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<td></td>
<td>-</td>
<td>0.078</td>
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<td>0.054</td>
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<td>37°C diluted</td>
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<td>-</td>
<td>0.069</td>
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<td>0.127</td>
<td>0.146</td>
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<td>0.107</td>
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<tr>
<td>37°C undiluted</td>
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<td>2.706</td>
<td>2.707</td>
<td>2.578</td>
<td>3.098</td>
<td>2.916</td>
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</tr>
<tr>
<td></td>
<td>-</td>
<td>0.066</td>
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<td>0.083</td>
<td>0.075</td>
<td>0.071</td>
</tr>
<tr>
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<tr>
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<td>-</td>
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<td>0.072</td>
<td>0.067</td>
<td>0.073</td>
<td>0.062</td>
<td>0.069</td>
</tr>
</tbody>
</table>
2.3.2 Comparison of measles specific IgG measurements in oral fluid and corresponding sera

Paired oral fluid and serum measurements were obtained for 787 individuals. Results were plotted (log scale, Figure 2.2) to show the quantitative relationship between measles specific IgG in oral fluid measured by GACELISA and in corresponding serum samples measured by Behring ELISA. This showed a direct relationship between measles IgG levels in oral fluid and in serum (Figure 2.2). Fourteen sera giving results falling within the two vertical lines in Fig 2 were equivocal by ELISA (i.e. >150 but <335 mIU/mL). Of these, 10 had paired oral fluid samples, which were GACELISA positive. A correlation coefficient of r=0.581 for serum positives (n=683) was calculated for serum antibody concentration and oral fluid mOD (using log-transformed data). The horizontal line in Figure 2.2 represents the mean of the cut-off mOD values estimated for the GACELISA (mean mOD 114, range 96.4-143, n=9).
Figure 2.2. Relationship between measles-specific IgG concentration measured in serum (Behring ELISA; mIU/mL) and in oral-fluid GACELISA; mOD for 787 sample pairs from southern Ethiopia, 1997. Vertical lines mark the equivocal range in serum ELISA (antibody levels 150-335 mIU/mL). The horizontal line identifies the average cut-off level for oral-fluid GACELISA (114 mOD). Filled markers indicate 27 samples with discordant results for further analysis (see Table 2.3).

In qualitative analysis, and excluding 14 pairs with equivocal serum results, the GACELISA results agreed with serum results in 746 of 773 (96.5%) of samples tested, with a kappa statistic of 84% (i.e., the percentage of the difference between random and perfect agreement, \( \kappa=0.837 \ z=23.3, \ P<0.0001 \)). Overall sensitivity and specificity of the oral fluid GACELISA in comparison to the serum Behring ELISA were 97.4% and 91.1%, respectively (Table 2.2). The considerably larger sample size of serum positives
(n= 683) compared with negatives (n=90) yields greater confidence in the sensitivity estimates (Table 2.2). No change in sensitivity or specificity is discernible with age (note overlapping confidence intervals in Table 2.2).

Table 2.2. Evaluation of oral fluid GACELISA against serum Behring ELISA

<table>
<thead>
<tr>
<th>Age class (years)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a/(a + b)(^a)</td>
<td>proportion</td>
</tr>
<tr>
<td>0 - 4</td>
<td>43/43</td>
<td>1</td>
</tr>
<tr>
<td>5 - 9</td>
<td>101/105</td>
<td>0.962</td>
</tr>
<tr>
<td>10 - 19</td>
<td>220/226</td>
<td>0.973</td>
</tr>
<tr>
<td>20 - 29</td>
<td>54/56</td>
<td>0.964</td>
</tr>
<tr>
<td>30 - 49</td>
<td>150/152</td>
<td>0.987</td>
</tr>
<tr>
<td>50+</td>
<td>95/99</td>
<td>0.960</td>
</tr>
<tr>
<td>Total (^c)</td>
<td>663/681</td>
<td>0.974</td>
</tr>
</tbody>
</table>

\(^a\) Where a = Behring ELISA+/GACELISA+, b = Behring ELISA+/GACELISA-, c = Behring ELISA-/GACELISA+, d = Behring ELISA-/GACELISA-.  
\(^b\) Exact limits based on the F distribution (Armitage and Berry, 1987).  
\(^c\) Total does not includes samples from 2 individuals for whom no age was available.

Specimens from 23 of 27 oral fluid/serum pairs showing discordant results were retested (Table 2.3). In general, this showed the results of both GACELISA and Behring ELISA were reproducible. However, 5 serum samples giving reactions close to the "cut off" OD re-tested equivocal, 3 oral fluids initially negative retested GACELISA positive and 1 serum initially Behring ELISA positive re-tested negative. All 23 of the sera from this panel were also examined by PRNT that had a detection limit of 150 to 320 mIU/ml. The 17 sera from the set where Behring ELISA was initially positive were PRNT positive.
whereas only one of six sera where Behring ELISA was initially negative was PRNT positive.

Table 2.3. Further results for 23 paired oral fluid and serum samples showing discordant results: repeat GACELISA and Behring ELISA, and PRNT

<table>
<thead>
<tr>
<th>Initial results</th>
<th>No.</th>
<th>Repeat results</th>
<th>Serum PRNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral fluid GACELISA - Serum Behring ELISA +</td>
<td>17</td>
<td>3 14</td>
<td>17 0</td>
</tr>
<tr>
<td>Oral fluid GACELISA + Serum Behring ELISA -</td>
<td>6</td>
<td>6 0</td>
<td>1 5</td>
</tr>
</tbody>
</table>

2.3.3 Total IgG content of oral fluid

In order to investigate the quality of oral fluid samples, total IgG concentrations were measured in a subset of 160 samples selected based on corresponding sera with different concentration of total IgG. The concentration of total IgG ranged from 1 to more than 60 ng/mL. The effect of total IgG concentration in oral fluid on performance of measles GACELISA was analysed (Table 2.4). A trend was apparent for sensitivity of the GACELISA to increase with increasing total IgG concentration. However, sample sizes were small and there was no statistical evidence for an association between GACELISA sensitivity and total IgG in oral fluid (Fisher's Exact P=0.42). Of 160 oral fluids tested, 136 (85%) gave the same results as the Behring ELISA on corresponding sera. Matching results, either GACELISA+/Behring+ or GACELISA-/Behring-, were obtained with oral fluids with total IgG concentrations as low as 1.1 to 5.3ng/mL and as high as >60ng/mL.
Discordant results, which were obtained with 23 paired oral fluid/serum samples, varied, however, according to total IgG concentration of oral fluid. Most of the discordant results were in the category GACELISA-/Behring+ and were more frequent with oral fluids with a low total IgG concentration (Table 2.4). Only 2 discordant results were in the category GACELISA+/Behring- and both were with oral fluids with high total IgG concentrations.

Table 2.4. Performances of measles GACELISA in 160 oral fluid samples compared to Behring ELISA in corresponding sera with different concentration of total IgG

<table>
<thead>
<tr>
<th>Total IgG conc. In oral fluid (ng/mL)</th>
<th>Number of paired oral fluid / serum samples</th>
<th>Sensitivity of GACELISA vs Behring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GACELISA+ Behring +</td>
<td>GACELISA- Behring -</td>
</tr>
<tr>
<td>1.1 - 5.3</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>5.4 - 10</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>10.1 - 20</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>20.1 - 40</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>40.1 - 60</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>91 (56.9)</td>
<td>45 (28.1)</td>
</tr>
</tbody>
</table>

2.4. Discussion

Of importance to any measles vaccination control programme is the capability to assess the effectiveness of immunizations, to identify individual and population susceptibility, and verify disease diagnosis. In this context the need for simple robust assays for the determination of immune status is recognised, and is particularly acute in the developing world (Cutts & Brown, 1995). Ideally, assessment of immune status should be made on the basis of specimens collected by painless non-invasive means (especially relevant to
measles as an infection and disease predominantly of the young), using assays based on standard technology suited to most basic laboratories around the world. Previous work has clearly shown the relative ease and acceptability of collecting oral fluid samples compared to collecting blood samples, from individuals of all ages, either within communities (Nokes et al., 1998b; Azevedo Neto et al., 1995; Cutts et al., 1995) or in the medical environment (Brown et al., 1994; Ramsay et al., 1998). The adequacy of oral fluid for specific IgG determination for a range of viruses has been demonstrated (Vyse et al., 1997; 1999; Nokes, 1998a, b; Parry et al., 1993; Perry et al., 1993; Rice and Cohen, 1996; Ramsay et al., 1998). The present work now describes an assay for measles specific IgG using oral fluid which, since it has an ELISA format, is more simple and robust than the radio-immunoassay previously used to measure measles IgG in oral fluid (Perry et al., 1993). Moreover, as the method does not require a high degree of laboratory sophistication, it could be performed in most laboratories in both developed and developing countries. The assay has the limitation of reproducibility since the components of the assay are not provided as ready to use reagents.

The measles GACELISA method described here is similar to that recently developed to measure rubella specific IgG in oral fluid (Vyse et al., 1998). It used the same FITC/anti-FITC amplification system (Samuel et al., 1998) to enhance the sensitivity of the GACELISA to a level comparable to that of corresponding radio-immunoassays. The GACELISA also uses a monoclonal antibody to measles haemagglutinin and Vero cell culture-grown measles antigen. Other than the measles antigen, all reagents are commercially available; thus minimising problems of availability of reagents to any
laboratory wishing to establish the assay. One practical issue investigated was the
stability of the measles antigen at a range of storage temperatures. In the presence of
10% fetal calf serum, in either a concentrated or diluted suspension, no loss of
antigenicity in GACELISA or haemagglutination assay was observed after 30 days
storage at temperatures ranging from -70 to +37°C. (We did not measure the infectivity
of measles virus stored under these conditions). The results indicate that low temperature
storage of measles antigen for GACELISA is therefore not essential.

The performance of the GACELISA was assessed by testing a large panel of paired oral
fluid and serum samples collected during a community-based health project in rural
Ethiopia. It is clear that oral fluid collected in this way using a simple sponge swab
device provides an adequate sample for measles IgG determination. Corresponding serum
samples were tested by a commercial indirect ELISA (Behring) which can detect as little
as 80 mIU/ml of measles specific IgG (Hesketh et al., 1997) although, following the
manufacturers guidelines, a cut-off level of 150mIU/ml was used in this study.
Compared to this sensitive serum ELISA, the oral fluid GACELISA had a sensitivity of
97%. This was higher than the 82% sensitivity of the similar oral fluid rubella FITC/anti-
FITC GACELISA (Vyse et al., 1998). The sensitivity of the rubella GACELISA was
shown to decrease markedly with increased age of the subject (Vyse et al., 1998). By
comparison, no age-specific sensitivity of the measles GACELISA described in this study
was observed. Greater precision of the estimate of specificity of 91% (95%CI: 82%-94%)
for the oral fluid GACELISA, compared to Behring ELISA on serum, would be
desirable. In particular, where the identification of true negatives for selective
vaccination, or the proportion seronegative for triggering campaign intervention, are the objectives, then a high specificity assay would be critical (Cutts & Brown, 1995).

Discordant results from 23 of 27 oral fluid/serum sample pairs were investigated by testing serum samples by PRNT. This confirmed a positive Behring result in 17 sera and a negative Behring result was confirmed in five of six sera (Table 2.3). These PRNT results support the conclusion that the oral fluid GACELISA was less sensitive than the serum Behring ELISA and that a small number of oral fluid samples gave falsely positive GACELISA results. The reason for false positive reactions is not known but two oral fluids giving false positive reactions contained high concentrations of total IgG (Table 2.4).

The concentration of total IgG in oral fluid is variable and is considered to be an important factor in determining the quality of an oral fluid for virus specific antibody assay (Parry, 1993). For the measurement of antibody to hepatitis A and B and HIV, a minimum total IgG of 0.5 ng/mL in oral fluid was deemed to be required (Parry, 1993). For oral fluid EBV antibody, a minimum of 2 ng/mL was required (Vyse et al., 1997). In our study of measles IgG, a minimum level of total IgG was not determined but many oral fluids with as little as 1.1 to 5.3 ng/mL gave results matching those of corresponding sera (Table 2.4). However, the sensitivity of the oral fluid GACELISA tended to increase with increased total IgG concentration (Table 2.4), though the association was not significant and requires further investigation. By contrast, it was previously observed in a study of measles IgG in oral fluid samples collected in Bolivia that the sensitivity of the
virus specific antibody assay used (radio-immunoassay) decreased with increased total IgG concentration, a phenomenon ascribed to competition from local production in the oral cavity of IgG of other specificities (Cutts et al., 1995). In a more recent study (Nokes et al., 1998b) it was shown that the relationship between rubella-specific and total IgG in oral fluid was variable, differing between type of collection device used; for the sponge swab device used in this study, the association was positive. We did not find false negative measles specific IgG results in oral fluids with high levels of total IgG in the study reported here but we did find a few false positive results. Our understanding of the composition and properties of oral fluid remains at an early stage and future investigation in this direction would be useful. The relationship between the data presented in Table 2.2 and Table 2.4 were not comparable as samples tested in Table 2.4 are not representative samples selected randomly. All the discordant samples were also included in this test panel.

We conclude that, in spite of minor deficiencies, the specificity (91.1%) and certainly the sensitivity (97.4%) of the oral fluid measles GACELISA can be considered adequate for sero-epidemiological studies. Coupled to the numerous practical advantages of both oral fluid sampling and ELISA methodology, this new measles assay should be useful for population screening, surveillance and assessing the efficacy of vaccination programmes as required for the World Health Organisation's objective of the global control of measles (World Health Organisation, 1997).
I. MEASUREMENT OF TOTAL IgG CONCENTRATION IN ORAL FLUID

1. IgG standard Curve

1.1. A calibration curve ranging from 2.5 mg/L to 0.039 mg/L is prepared in PBST as described below. Each standard is tested in duplicate in each assay or each plate.

1.2. Check the concentration of the standard in use. In a separate microtitre plate prepare a 2.5 mg/L. For example dilute a 45 mg/L calibrant 1 in 18 (10ul to 170ul PBST).

1.3. Prepare the remaining standards by doubling dilutions, from the 2.5 mg/L-diluted standard, in PBST (50ul standard + 50ul PBST). A new pipette tip must be used for the preparation of each dilution.

2. Test Procedure

2.1. This assay is only accurate in the approximate range of 0.1 mg/L to 10 mg/L. Therefore, if a sample is likely to contain a concentration of IgG outside this range it must be tested at a different dilution.

2.2. Add 40ul of PBST to the wells. Add 10ul of oral-fluid to its allocated microtitre well.

2.3. After the addition all the samples add a further 10ul of PBST to wells A1 and A2 to act as blanks for the assay. Add 10ul of each of the standards, in duplicate, to their allocated wells in columns 1 and 2.

2.4. Cover the plate with a plate sealer and incubate at 37 °C for 2 hours.

2.5. Wash the microtitre plate 4 times with a plate Washer.

2.6. Prepare a 1 in 10,000 dilution of rabbit HRPO conjugated anti-human IgG (Dako) [1ul + 10ml, 10mls per plate], and add 100ul to each well.

2.7. Cover the plate with a plate sealer and incubate at 37 °C for 30 minutes.
2.8. Wash as described previously but include an additional wash, giving a total of 5 washes.

2.9. Add 100μl TMB substrate to each well, cover the plate with a plate sealer and incubate in the dark at room temperature for 25 minutes.

2.10. Stop the reaction with 0.25M H2SO4 and read in a plate reader at 450nm (reference 620nm) using wells A1 and B1 as blanks. It is recommended that a reader capable of reading OD values > 3.0 is employed in order to extend the dynamic range.

2.11. Appropriate software (e.g. Mikrotek-Laborsysteme) should be used to analyse the data and to produce the standard curve against which oral fluid sample concentrations are determined.

Useful notes:

- The critical part of this assay is the preparation of the calibration curves. Great care is needed when preparing the dilution series.
- When calculating the actual IgG concentration do not forget to compensate for the sample dilution.

II. MEASLES IgG & IgM DIAGNOSIS IN ORAL-FLUID USING SIMPLE CAPTURE ELISA

1. Processing of Oral-fluid Samples

1.1. Pink Sponge Swabs (Malvern Medical)

Add 1ml. Transport medium (recipe below) to swab in tube. Mix the swab thoroughly in the medium, creating a froth. Invert the sponge stick in the tube and replace the cap. Centrifuge the tube at 2000 rpm for 5 minutes. Discard the sponge stick immediately and pipette the oral fluid into a storage vial.
1. 2. Omni-sal (SDS)

Detach the flat sponge swab from the stick using a disposable pipette or pipette tip, into the tube. Take an Omni-sal filter plunger from the laboratory stock and push it into the tube. Push it down onto the sponge using sufficient pressure to filter the fluid. Pipette the fluid, which is blue colored, into a storage vial.

1. 3. Orasure

Break off the small plastic tip at the bottom of the orasure tube. Place the tube within a centrifuge tube (blue-capped, graduated plastic tubes). Centrifuge at 2000rpm for 5 minutes. Pipette the fluid from the centrifuge tube into a storage vial.

Transport medium for use with pink sponge swabs

PBS with

- 10% Fecal Calf Serum (FCS)
- 0.2% Tween 20
- 0.5% Gentamicin (50mg/ml stock)
- 0.2% Fungizone

2. Blocking of Plates with SOL-U-PRO: Preparation of Plates for Long Term Dry Storage

2. 1. Coat plates with 100ul DAKO anti human IgG or IgM at the appropriate dilution in carbonate / bicarbonate buffer (recipe below) and incubate over night at 37 °C.

2. 2. Aspirate (i.e. remove but do not wash) the anti-human IgG / IgM solution and add 200 ul of 5% Sol-u-pro (Sol-u-pro is provided as a 25% stock solution; to make a 5% solution dilute the stock 1:5 in distilled water).

2. 3. Leave at room temperature for a minimum of 1 hour and a maximum of 4 hours.
2.4. Aspirate the solution, invert the plates and leave at 37 °C over night.

2.5. Package the dried wells into plastic bags containing a sachet of desiccant previously heated sufficiently to remove any moisture. Ensure the bags are properly sealed.

2.6. Store the plates at 4 °C (plates will last ~3 months).

Coating Buffer

0.05M Carbonate-bicarbonate pH 9.6 preparation

Solution A: 0.2M anhydrous sodium carbonate

\[21.2g \text{Na}_2\text{CO}_3 / 1000ml \text{H}_2\text{O}\]

Solution B: 0.2M sodium bicarbonate

\[16.8g \text{NaHCO}_3 / 1000ml \text{H}_2\text{O}\]

Add

\[16ml \text{Solution A}\]
\[34ml \text{solution B}\]
\[150ml \text{H}_2\text{O}\]
\[2ml 8\% \text{sodium azide}\]

3. Preparation of Measles Antigen for Solid Phase Assays

3.1. Infect one 50 ml flask of Vero cells with Loss virus. When cytopathetic effect (CPE) is just +++ (about 3 days) scrape the cells into the medium and inoculate in to eight 600 ml flasks of Vero cells.

3.2. When CPE is advanced (about 6 days) scrape the cells into the medium, and pellet cell debris at 2000 rpm for 10 minutes.
3.3. Remove supernatant (S1). Resuspend pellet in 8 mls of supernatant and disrupt cells by sonication for about 2 minutes. Spin at 2000 rpm for 20 minutes, remove supernatant (S2) and discard pellet.

3.4. Add S2 to S1, and spin at 40,000 rpm for 1 hour at 5 °C.

3.5. Discard supernatant and resuspend pellet in 4 mls of PBSA. Sonicate to break up clumps, add FCS to a final concentration of 10%, aliquot and store at -70 °C.

[Initial harvest is spun in 16 universals; pooled supernatant is spun in 10 1” x 3 1/2” Beckman tubes].

Final antigen concentration is about 1 in 80.

4. Fluorescin Isothio Cyanate (FITC) Labeling of Antibody

4.1. Labeling reaction

4.1.1. To 1 mg of antibody in 0.5 ml of 0.1M carbonate / bicarbonate buffer, pH 9.2-9.3 containing 0.15M NaCl.

4.1.2. Add 9.2 μl FITC (5 mg /ml in absolute alcohol kept dry with anhydrous NaSO₄ or MgSO₄) in 3 equal parts with stirring over a 5 minute period.

4.1.3. Leave to react for a further 45 minutes at room temperature in the dark.

4.2. Separate unbound FITC

4.2.1. Pre-block PD 10 column (Pharmacia) with 1ml 1% bovine serum albumin (BSA).

4.2.2. Wash column with 3x column volume of PBS with NaN₃

4.2.3. Add conjugate to column and elute with PBS with NaN₃, collecting 0.5ml fractions.

4.2.4. Measure OD280 and OD495 of fractions.
4.2.5. Pool fractions with peak OD (usually fractions 6-8).

4.2.6. Add glycerol to final concentration of 20-25% and store in dark at 4 °C.

5. Preparation of TMB Substrate

5.1. TMB: 100mg in 10 ml DMSO
final concentration 42 mM

5.2. Citrate / Acetate

Buffer pH 6.0 : 0.1M citric acid 21.014 g / L
0.2M sodium acetate 16.40 g / L

2ml 0.1M citric acid
48ml 0.2M sodium acetate
50 ml distilled water

5.3. For 10 ml of substrate : 10ml of citrate / acetate buffer

7.5ul H2O2 6% 20 volumes
100ul TMB solution - add slowly

5.4. Stop solution: 2M H2SO4

5.5. Read OD at 450 / 620nm

6. Procedure for Measles FITC/Anti-FITC GACELISA

6.1. Materials:
- Anti-human IgG (Fc) specific antibody coated microtitre wells (In-house preparation)
- Positive and negative control serum (tested in other serum assays) diluted 1/100 before use in GACELISA buffer (10%FCS, 0.2% Tween-20 in PBS)
- Measles Loss virus antigen (cell lysate) diluted 1/2000 in GACELISA buffer
- Anti-measles haemagglutinin-FITC conjugate (1/4000) in PBS containing 10% FCS, 5% Normal rabbit serum (NRS), 2% Measles-negative human serum (NHS) and 0.2% Tw-20
- Anti-FITC-HRP conjugate diluted 1/4000 in PBS containing 10% FCS, 10% NRS, 2% NHS and 1% Tw-20
- Wash buffer (PBS containing 0.05% Tw-20)
- TMB substrate-freshly prepared from stock TMB in DMSO added to citrate/acetate buffer, 0.1M, (pH 6.0) containing hydrogen peroxide
- 2M H2SO4

6.2. Procedure:

6.2.1. Add 100ul of diluted POS and NEG control serum samples in duplicate to assigned wells.

6.2.2. Add 100ul of each of the saliva samples to assigned wells.

6.2.3. Incubate at 37°C for 30 minutes in plate shaker (~500 rpm).

6.2.4. Wash wells 4 X in wash buffer.

6.2.5. Add 100 u/ well of the diluted measles antigen at 37 °C for 60 minutes with shaking as in step 6.2.3.

6.2.6. Wash wells 4 X in wash buffer.

6.2.7. Add 100 ul / well of the diluted anti-measles haemagglutinin-FITC conjugate and incubate at 37 °C for 60 minutes with shaking as in step 3.

6.2.8. Wash 4 X in wash buffer.
6.2.9. Add 100 ul / well of the diluted ant-FITC-HRP conjugate and incubate at 37 °C for 30 minutes with shaking.

6.2.10. Wash wells 4 X with wash buffer.

6.2.11. Add 100 ul/well of freshly prepared TMB substrate and incubate at room temperature, in the dark, for 15 minutes.


6.2.13. Read optical density at 450/620.
# WORKSHEET FOR MEASLES IN GACELISA

**DATE_____________**

**OPERATOR_____________**

<table>
<thead>
<tr>
<th>Preparation of Anti-MH-FITC conj.</th>
<th>Total volume = 6000ul (6strips)</th>
<th>Total volume = 8000ul (9strips)</th>
<th>Total volume = 12000ul (12strips)</th>
<th>Incubation °C</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>4968 µl</td>
<td>6624 µl</td>
<td>9936 µl</td>
<td>37°C</td>
<td>1hr</td>
</tr>
<tr>
<td>10% FCS</td>
<td>600 µl</td>
<td>800 µl</td>
<td>1200 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% NRH</td>
<td>300 µl</td>
<td>400 µl</td>
<td>600 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% NHS</td>
<td>120 µl</td>
<td>160 µl</td>
<td>240 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% Tw-20</td>
<td>12 µl</td>
<td>16 µl</td>
<td>24 µl</td>
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<td></td>
</tr>
<tr>
<td>Anti-MH-FITC 1/10 stock</td>
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<td>20 µl</td>
<td>30 µl</td>
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</tbody>
</table>

<table>
<thead>
<tr>
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<th>Total volume = 4620 µl (6strips)</th>
<th>Total volume = 6160 µl (9strips)</th>
<th>Total volume = 9240 µl (12strips)</th>
<th>Incubation °C</th>
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<tr>
<td>PBS</td>
<td>4620 µl</td>
<td>6160 µl</td>
<td>9240 µl</td>
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<td>30 min.</td>
</tr>
<tr>
<td>10% FCS</td>
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<td>800 µl</td>
<td>1200 µl</td>
<td></td>
<td></td>
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<td>800 µl</td>
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</tr>
<tr>
<td>2% NHS</td>
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<td>160 µl</td>
<td>240 µl</td>
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<table>
<thead>
<tr>
<th>TMB substrate preparation</th>
<th>Citrate/acetate buffer 6 ml</th>
<th>8 ml</th>
<th>12 ml</th>
<th>Dark (RT) 15 min.</th>
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</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>4.5 µl</td>
<td>6 µl</td>
<td>9 µl</td>
<td></td>
</tr>
<tr>
<td>TMB</td>
<td>60 µl</td>
<td>80 µl</td>
<td>120 µl</td>
<td></td>
</tr>
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</table>
CHAPTER 3

PRE- AND POST-VACCINE MEASLES ANTIBODY STATUS IN INFANTS USING SERUM AND ORAL-FLUID TESTING: AN EVALUATION OF ROUTINE IMMUNIZATION IN ADDIS ABABA, ETHIOPIA

Summary

This study evaluated pre- and post-vaccine antibodies in children attending for routine measles immunization in Addis Ababa. Infants who presented to 3 health centers between September-November, 1998 for routine measles vaccination were enrolled in the study. In total 296 infants (median age 9 months) provided blood and oral-fluid samples, of which 230 (77%) returned to provide post vaccine samples (median interval of 15 days). Screening of sera was undertaken using commercial indirect ELISA kits, and of oral fluids using an in-house IgM-capture ELISA. Pre-vaccination serology showed 1.4% IgM positive, 2.0% IgG positive, and 97.0% seronegative. Post-vaccination seroprevalence of IgM and IgG was 91.3% and 85.0%, respectively, and 92.9% overall. The seroconversion rate was 92.6% (95%CI 88.2-95.7). Based on oral fluid results, 87.3% (95% CI 82.0-91.4) of children showed specific IgM antibody conversion. These results are in support of the recommended age for measles vaccination in Addis Ababa, and show the merit of oral-fluid IgM screening as a non-invasive alternative to blood for assessing vaccine immunogenicity.
3.1. Introduction

Worldwide it is estimated that measles kills some 880,000 children annually, a toll more than any other vaccine-preventable disease. The global plan, established by the World Health Organization (WHO) and UNICEF, is to cut this burden by two-thirds between 2000 and 2005, and thereafter to prevent 600,000 measles fatalities annually (Brown, 2000). Half of the total deaths are concentrated in three African countries (Congo, Ethiopia and Nigeria) and one Asian country (India). Progress towards the control of measles requires that countries develop national capacity to measure programme effectiveness by which to assess and refine immunization policy. This includes evaluation of vaccine effectiveness in infants attending routine immunization clinics.

The Expanded Programme on Immunization (EPI) in Ethiopia was launched in 1980 (MOH, 1981a; 1981b), and offers a single dose of measles vaccine at 9 months of age. In 1999 the national measles coverage was 53% with uptake ranging from 7-88% (the highest 88% recorded for Tigray region the lowest 7% recorded for Somali region) in the different administrative regions (Source: Department of Family Health, MOH, 2000).

Research has demonstrated oral fluid to yield detectable levels of immunoglobulins (IgG and IgM antibodies) against a wide variety of infections (Parry et al., 1989; 1993; Hunt et al., 1993; Perry et al., 1993; Rice and Cohen, 1996; George and Fitchen, 1997; Nokes et al., 1998a; 1998b; Vyse et al., 1999; Helfand et al., 1996; Nigatu et al., 1999) using sensitive and specific antibody capture assays. The detection of measles specific IgM antibodies in oral fluid by antibody capture ELISA (MACELISA) has previously been
reported in a study of children who received measles vaccine (Helfand et al., 1996). The position of non-invasive antibody testing for measles grows stronger as emphasis on vaccine programme surveillance increases, accompanied by technical developments in oral-fluid assays (Helfand et al., 1996; Nigatu et al., 1999) and favorable evaluations under a variety of settings (Helfand et al., 1996; Nokes et al., 2001; Vyse et al., 2001; Ramsay et al., 2003).

The aim of this study is to evaluate pre- and post-vaccine antibody in children attending for routine measles immunization in Addis Ababa. Furthermore, we aim to evaluate the use of oral-fluid testing for measles specific IgM as an alternative to serum assays in estimating vaccine response and effectiveness. For this an enhanced measles MACELISA that incorporates an amplification stage is developed.

3.2. Materials and methods

3.2.1. Study population

Addis Ababa, the capital of Ethiopia, has a population of 2,570,004 (density of 4,847.8/km² (CSA, 2000) settled at 2000-2800m altitude above sea level. Administratively there are 6 ‘Zones’ (each divided 4-7 Weredas), and 28 ‘Weredas’ (=district) each with a population of 45,277-153,688. In 1998/99 measles vaccination was given in 7 hospitals, 17 health centres, 9 health clinics, 20 health posts, 104 out reach sites under the Region 14 Health Bureau. The estimated measles vaccination coverage in 1998/99 (1991 E.C.-Ethiopian Julian Calendar) was 86% with variation in the different ‘Zones’ and ‘Weredas’(63-100%). Measles vaccine consumption for the year was 10,250
vials given to 32,548 children aged under one year (Source: Addis Ababa City Government Health Bureau).

Three of 19 Government health centers were selected for the study: one from the center of the city (Arada, Wereda 1), one in the outer city (Akaki, Woreda 26 and 27), and the third from western part of the city (Wereda 25). All infants who presented to these health centers between September-November, 1998 for routine measles vaccination were eligible for enrolment in the study. All the children had received vaccinations of BCG and OPV at birth, and 3 doses of DPT and OPV according to the recommended schedule of the EPI Global Advisory Group (6, 10 and 14 weeks) (WHO, 1985). Oral informed consent was obtained from guardians. Permission for this work was obtained from Addis Ababa City Government Health Bureau (No.1370/172) in 29th August 1998.

3.2.2. Sample collection

Blood samples were collected by finger prick using Safety Flow Lancet into Microtainers (Becton Dickinson, Oxford, England). Oral-fluid specimens were collected and processed as described previously (Nokes et al., 1998b) by sponge swab collection devices (Oracol: Malvern Medical Developments, Worcester, England) from infants before they received live attenuated measles vaccine (Schwartz strain, SmithKline Beecham Biologicals, Rixensart, Belgium). Mothers were requested to return with their infants two weeks after vaccination for a second oral-fluid and blood sample. Samples collected were processed at the virology laboratory of the Ethiopian Health and Nutrition Research Institute.
(EHNRI). Of specimens were transported on dry ice to the UK for advanced laboratory analysis.

3.2.3. Serum measles IgM/IgG determination

Serum samples were screened for measles virus (MV) specific IgM and IgG by using a commercial ELISA kit (Enzygnost for IgM, and IgG; Behring Diagnostics, Marburg, Germany). In the IgG ELISA optical density (OD) readings of <0.100, >0.200 and between 0.100-0.200 obtained from 1:231 IgG serum dilution were considered as negative, positive and equivocal respectively. The limit of the IgG detection of the test is 150 mIU/ml, equivalent to an OD of 0.100. Similarly, in the IgM ELISA OD readings of <0.100, >0.200 and between 0.100-0.200 obtained from 1:42 IgM dilution were considered as negative, positive and equivocal respectively.

3.2.4. Oral-fluid MV IgM detection

MV specific IgM in oral fluid was determined by MACELISA, which was a modification of the FITC/anti-FITC IgG capture ELISA (GACELISA) described previously (Nigatu et al., 1999). For detailed laboratory operating procedure see A2 (Appendix for Chapter 2). A description of these modifications follows. Wells of microtitre plates (Maxisorb “U” wells, Life Technologies, Paisley, UK) were coated with 100μl of a 11.2 g/L solution of rabbit anti-human IgM serum (Dako, Ely, UK) diluted 1:3000 in 0.05M carbonate/bicarbonate buffer, pH 9.6. After addition of measles antigen, anti-measles haemagglutinin monoclonal antibody-FITC conjugate (Chemicon Inc., Temicula, CA., USA) diluted 1:3000 (instead of 1:4000 for GACELISA). Anti-FITC horseradish
peroxidase conjugate and TMB were then added and the reaction stopped by adding 100 ul of 0.5M HCl.

OD results from the MACELISA were expressed as T/N ratios (test sample OD/negative control OD). The receiver operating characteristics (ROC) curve was generated (Choi, 1998) to determine the appropriate cut off T/N value (set to the maximum sensitivity and specificity relative to serum results). Relative to serum IgM results (excluding equivocal results), the MACELISA was 93.6% (190/203) sensitive and 93.4% (284/304) specific, using a T/N ratio of 1.14. T/N ratios less than the cutoff value (1.14) were considered as negative in the analyses.

3.2.5. Statistical analysis

Analysis was conducted using STATA V7.0 (Stata Corp, College Station, Texas, USA). Comparison between median ages uses the non-parametric Kruskall-Wallace test (with ties). Estimates of the proportions seropositive for either IgM or IgG exclude samples with equivocal results. Overall proportions based on serum specific IgM or IgG results exclude only samples equivocal by both tests. Estimates of seroconversion are derived from the proportion of individuals specific-antibody negative pre-vaccine whose status changes to specific-antibody positive post-vaccination. Proportions seropositive are compared using Fisher's Exact test. Exact binomial confidence intervals are calculated for prevalence estimates.
3.3. Results

A total of 296 infants were recruited with median age 9 months (range 5-40 m) all providing a blood and an oral-fluid sample. The number and median age of individuals attending Akaki, Arada and Woreda 25 clinics were, respectively, 86, 8m, 41, 9m and 169, 10m (median ages did not differ between clinics: $\chi^2(2) = 5.235, P=0.073$). Of the 296 recruits, 230 (77%) returned to provide post-vaccine samples (median interval of 15 days, range 14-46 days) of oral-fluid and blood. Results of the measles-specific IgG and IgM tests on serum samples from pre- and post-vaccinated children are shown in Table 3.1. Seroprevalence estimates were made excluding equivocal results. Pre-vaccination, 1.4% of children were IgM seropositive and 2.0% IgG positive. Post-vaccination seroprevalence of IgM and IgG was 91.3% and 85.0%.

Table 3.1. Seroprevalence of measles antibody status (IgM and IgG) in the pre- and post-vaccinated children in Addis Ababa, Ethiopia 1998.

<table>
<thead>
<tr>
<th>Results</th>
<th>IgM</th>
<th></th>
<th>IgG</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>4</td>
<td>199</td>
<td>6</td>
<td>170</td>
</tr>
<tr>
<td>Negative</td>
<td>285</td>
<td>19</td>
<td>290</td>
<td>30</td>
</tr>
<tr>
<td>Equivocal</td>
<td>6</td>
<td>9</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>NTa</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>296</td>
<td>230</td>
<td>296</td>
<td>230</td>
</tr>
<tr>
<td>Proportionb</td>
<td>1.38 %</td>
<td>91.3 %</td>
<td>2.03 %</td>
<td>85.0 %</td>
</tr>
</tbody>
</table>

*NT = Sample collected but not tested because of insufficient serum
b Proportion = Number Positive / Number (Positive + Negative)
Of the 4 pre-vaccination IgM positive samples, 3 were IgG negative (2 aged 9m and 1 age 10m) and one was high titer IgG positive (age 9m). All the 4 samples were repeated by Behring serum IgM ELISA and found to remain positive. Of 6 pre-vaccination IgM equivocal results all were IgG negative. Of 6 pre-vaccine sera that were measles-specific IgG positive, 5 had high titres (>900mIU/ml), three of which were aged 9m, one 10m and one 11m. One of the 5 was IgM positive, and one (age 10m) had low level antibody (296mIU/ml) for which the paired post-vaccine sample showed a greater than fourfold rise in titer (i.e. suggesting this individual had residual maternal antibody).

Of 19 children specific-IgM negative post-vaccination, in pre-vaccination samples 5 were IgG positive and one other was IgM positive. Of 9 post-vaccination IgM equivocal samples, all were IgM and IgG negative pre-vaccine and, post-vaccine, 2 were IgG equivocal and 2 were IgG positive. The time between the pre- and post-sample collection for the 2 IgG positive specimens was 15 and 39 days. Post-vaccine, there were 30 samples negative and 28 equivocal for measles specific-IgG; results which should be interpreted in relation to the short interval (median of 15 days) between vaccination and second sample collection. Of the 30 samples IgG negative post-vaccination, pre-vaccination, none were IgG positive, 1 was IgM positive and 2 had equivocal IgM status.

Overall prevalence and antibody conversion rate for the sera (based on IgG and IgM results) and oral fluid are presented in Table 3.2.
Table 3.2. Overall antibody prevalence and conversion rate in vaccine recipients in Addis Ababa, Ethiopia 1998.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Pre-vaccine % (positive/total) 95% CI</th>
<th>Post-vaccine % (positive/total) 95% CI</th>
<th>Antibody conversion % (converters/total) 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>3.04% (9/296)</td>
<td>92.9% (208/224)</td>
<td>92.6% (200/216)</td>
</tr>
<tr>
<td></td>
<td>1.40%-5.69%</td>
<td>88.7%-95.9%</td>
<td>88.2%-95.7%</td>
</tr>
<tr>
<td>Oral fluid</td>
<td>6.76% (20/296)</td>
<td>86.9% (199/229)</td>
<td>87.3% (185/212)</td>
</tr>
<tr>
<td></td>
<td>4.18%-10.2%</td>
<td>81.8%-91.0%</td>
<td>82.0%-91.4%</td>
</tr>
</tbody>
</table>

Exact binomial 95% confidence intervals (CI) are shown. There was no significant difference between the three clinics in seroprevalence pre-vaccination (Fisher's Exact P=0.066) or post-vaccination (P=0.752). Compared with serum estimates, oral-fluid prevalence was nearly 4% higher pre-vaccine, and 6% lower post-vaccine, although these differences were not significant (95% confidence intervals on prevalence estimates overlap). The overall antibody conversion rate for serum was 92.6% (200/216), which was higher (but not significantly higher) than for oral-fluid, 87.3% (185/212).
3.4. Discussion

3.4.1. Pre- and post-vaccination status of children

Assessment of vaccine recipient children (median age 9 months) at three representative vaccine clinics in the city of Addis Ababa, showed an absence of measles-specific IgM and IgG antibody in 99% and 98% of serum samples, respectively. Considering either antibody class, the data suggest that 97% (95%CI 94-98%) of the children attending for routine (9 months of age) measles vaccination would respond to vaccine. Following measles vaccination the seroconversion rate (based on either antibody class) was 93% (95% CI 88-96). Children attending for routine measles vaccination had a median age of 9m; only 5% were less than 9m and 10% were 1 year or over. Together these results indicate a highly successful routine immunization programme. The present seroconversion rate study confirms vaccinating children at age 9 months in the Ethiopia setting is in accordance to the WHO recommendation (WHO/MOH-Kenya, 1977; WHO, 1979) and previous mathematical model studies made for developing countries (MacLean and Anderson, 1988; Nokes et al., 1990).

A full assessment of whether or not the present age at immunization is optimal should consider the prevalence of residual maternal antibodies and proportion with evidence of recent infection in samples from infants attending for routine vaccination. In this study, of 15 samples that were positive or equivocal for serum measles-specific antibody prior to vaccination, only 1 appeared to indicate the presence of residual maternal antibody (low specific-IgG titer with subsequent four fold rise post-vaccination). In contrast there were 8 pre-vaccine serum samples specific-IgM positive and/or high specific-IgG titer
positive, indicative of recent infection (6 aged 9m, and 1 each aged 10 and 11m). These results suggest that at the routine target vaccination age a negligible proportion of infants have residual maternal antibody, but a small but significant proportion (8/296=2.7%) have been exposed to measles virus. This is, at least, an indication of the need to discourage any delay in bringing children for measles vaccination beyond the appointed age.

With an average interval of 15 days between receiving vaccination and the collection of second samples, the study was primarily designed to investigate the development of measles virus specific IgM antibodies. Consequently the results yield a high proportion of serologically negative and equivocal IgG results post-vaccination, compared to IgM data. Taking IgM serological data alone yields a serconversion estimate of 90%, only marginally lower than that based on IgG and IgM results.

3.4.2. Serum/oral fluid comparison

The detection of oral fluid specific IgM for measles virus by antibody capture ELISA in pre- and post-vaccinated infants has been previously described (Helfand et al, 1996). In the present work a new MACELISA test is described that employs an FITC/anti-FITC amplification system (Samuel, Patt and Abuknesha, 1998) to enhance the sensitivity of the oral fluid assay to a level comparable to that of the corresponding 'gold standard' serum testing using a commercial kit. Using this amplification system, adequately sensitive and specific measles and rubella virus specific IgG oral fluid assay methods have been developed (Vyse et al., 1999; Nigatu et al., 1999). The IgM assay developed
in this study demonstrated 93.6% sensitivity and 93.4% specificity, which is comparable to the results of 91% sensitivity and 95% specificity found in a previous oral-fluid testing study (Helfand et al., 1996). Based on oral-fluid samples screened using the MACELISA, an estimate of specific antibody conversion of 87% (82-91) was obtained. Although lower than the overall serconversion rate obtained by Behring ELISA, the result is not significantly lower. With such performance the oral-fluid testing method (MACELISA) would be of use for measuring measles antibody prevalence in pre- and post-vaccine infants.
CHAPTER 4
MEASLES VIRUS CIRCULATING IN ETHIOPIA DURING 1998-9: IDENTIFICATION OF A NEW GENOTYPE

Summary

Molecular characterization of measles virus (MV) strains contributes to measles control programmes by enabling the source and transmission pathways of the virus to be investigated. An outbreak of measles in 1998/99 in the Bedelle district in western Ethiopia, where routine measles vaccine coverage was <50%, provided an opportunity for a molecular epidemiological study. Sporadic cases of measles in the city of Addis Ababa, at the same time, where vaccine coverage was 88%, were also examined. To identify the genotype and source of MV strains causing the outbreak in Bedelle paired serum and oral-fluid samples were collected from 55 cases from the outbreak and from 2 sporadic cases in the city. Serum and oral-fluid samples were tested for MV-specific IgM. Oral-fluid samples were tested for MV RNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Nucleotide and deduced amino acid sequences were analyzed and compared to those of reference MV genotypes. 53 of 55 cases from the Bedelle outbreak and both sporadic cases from Addis Ababa were positive for serum MV-specific IgM. Oral-fluid MV-specific IgM was positive in 40 (75%) of the outbreak cases and in both of the sporadic cases. RT-PCR using oral fluid was positive in all 55 serologically confirmed cases. Sequence analysis was performed on 28 strains from the outbreak; all were identical and closely related to the reference genotype D4 strain. One of the strains from Addis Ababa was also similar to genotype D4 but the strain from the other sporadic case was distinct. It was assigned to clade D but was not closely related to any other
The MV strain in the Bedelle outbreak was identified as genotype D4. A similar D4 strain was identified in sporadic cases in Addis Ababa. The Bedelle outbreak may therefore have been caused by the introduction of a case from an urban area where measles is endemic into a population with low vaccine coverage. One other sporadic case in Addis Ababa was due to a distinct genotype and might be representative of a previously undescribed genotype within clade D. The study clearly demonstrates the use of oral fluid for viral genetic analysis, but is less convincing for a role in IgM-based diagnosis.

4.1. Introduction

Analysis of sequence data of different measles genotypes has been used as an epidemiological tool to investigate transmissions of measles in endemic infections and in outbreaks. Genetic analysis of strains associated with the 1994 and 1995 measles outbreaks in the United States confirmed that infections were the result of international importation of the virus (Rota et al., 1995). Recent studies demonstrated the endemic co-circulation of two distinct measles genotypes of clade B in western and central Africa (Hanse et al., 1999) while in countries from southern Africa genotypes of Clade A and clade D predominate (Truong et al., 1999). Apart from one report of genotype D4 from Kenya (Truong et al., 1999) there is no information available on measles strains circulating in countries of eastern Africa such as Ethiopia. Molecular identification of the geographical origin of measles virus strains is a key strategy in achieving the aims of the World Health Organization measles elimination/eradication programme (WHO, 2001).
In November 1998 a measles outbreak was reported in the regional state of Oromia in western Ethiopia, affecting 3 districts. An outbreak investigation was mounted in one district, Bedelle, during which paired oral fluid and serum samples were collected for MV-specific IgM serology to confirm recent measles infection. Oral fluids have also been used as a source of viral RNA for genetic analysis of measles virus strains (Jin et al., 1996; 1997) and it was decided to use these samples to characterise the virus strain causing the outbreak. Measles virus strains detected in oral fluids from sporadic cases from Addis Ababa in 1998 were also analysed. The phylogenetic relationships of these Ethiopian strains and of others from different geographical origins were studied in order to investigate the source of the outbreak and to identify epidemiologically related strains.

4.2. Materials and Methods

4.2.1. Study areas and populations

i). The measles outbreak occurred from 25/12/98 to 30/1/99 in Illubabor, one of 12 administrative zones of the Oromia regional state in western Ethiopia. The population of Illubabor zone is 954,352 (Population and Housing Census Commission, 1995) and consists of 12 districts with one hospital, five health centers and 78 health stations. All health facilities undertake integrated maternal and child health activities including immunization services for children less than one year of age and women of childbearing age. There are 57 static sites and 105 outreach teams conducting immunizations. Measles immunization coverage in the zone was 36% in 1998.

Of the 12 districts in the Illubabor zone, three were affected by the 1998/9 measles outbreak. These were Chora, Bedelle and Gachi districts, which lie adjacent to each other.
between longitude 36°20' and latitude 8°20' and an altitude of 1500-2000 meters above sea level (Figure 4.1). Chora district was the first to report the outbreak (25/12/98) followed by Bedelle and Gachi. A total of 30 villages and two towns were affected by the epidemic

ii). Addis Ababa (population 2.4 million in 1998) has six administrative zones with different vaccination coverage. For the target population (children of 9 months) of 32,548 measles vaccination coverage was 88.5% in 1998 (Source: statistical record of region 14 Health Bureau).

4.2.2. Study subjects and specimens examined

The study subjects were 55 patients visiting the Bedelle Health Center and the Health Clinic in Abdella; a town located 15km south west of Bedelle town (Figure 4.1). Acute measles patients reported by Health Post attendants (non-medical personnel trained for notifying infectious epidemic diseases) were also traced from house to house in the different villages of Bedelle district. The clinical characteristics of the measles infection observed by the Health Center doctor or nurse were recorded. Paired oral fluid and serum samples were collected from patients 2 to 8 (mean 4.4) days after onset of symptoms. Oral fluid specimens collected using the sponge swab collection devices were processed as previously described (Nokes et al., 1998b; Nigatu et al., 1999) in the Health Center and transported in liquid nitrogen to EHNRI, Addis Ababa for storage at −20°C. Specimens packed in polystyrene boxes with dry ice were then transported to CPHL, UK for measles IgM testing and genotyping. In addition to the outbreak samples, 2 paired
oral fluid/serum specimens were collected from Addis Ababa. One was collected from a patient diagnosed at a private clinic in 'Zone 5' and the other from a patient at Akaki Health Center in 'Zone 6'.

4.2.3. Serum MV IgM

MV-specific serum IgM was detected by antibody capture ELISA according to the manufacturer’s instructions (Light Diagnostics, Chemicon International, Inc., Temecula, CA, USA). The Light Diagnostics measles capture ELISA utilises a recombinant MV nucleoprotein antigen.

4.2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Nucleic acid was extracted from oral fluid specimens using the silica-guanidinium thiocyanate method and MV cDNA produced by RT-PCR as described previously (Jin et al., 1996). The primers for amplifying and sequencing a 550 and a 450-nucleotide (nt) fragment of the haemagglutinin (H) gene, the entire H gene and the entire nucleoprotein (N) gene have been described (Jin et al., 1997; 1996; 1998). In parallel with negative controls (deionised H2O), the laboratory MV strain, Loss (Sinitsyna et al., 1990), was used as positive control. The full laboratory protocol is attached as A4 (Appendix for this Chapter).

4.2.5. Nucleotide and amino acid sequence determination

PCR amplicons were excised and purified from agarose gels prior to sequencing (Jin et al., 1996). Sequencing was performed using the Taq DyeDeoxy-terminator cycle
sequencing kit (PE Applied Biosystems, Warrington, UK) with specific primers (Jin. et al., 1997; 1996; 1998) in an ABI373A automatic DNA sequencer. Nucleotide and deduced amino acid (aa) sequences were analysed with SeqEd (version 1.0.3. programme, PE Applied Biosystems). Phylogenetic trees were constructed either by the Clustal of Megalign program (DNASTAR, Madison, W1, USA) or bootstrap analysis (1,000 times) using neighbour joining of the PAUP 4.0 Beta 2 packages (Sinauer Associate, Inc., Sunderland, MA, USA).

4.3. Results

4.3.1. Epidemiological findings

A total of 1,603 cases fulfilling the WHO case definition of measles infection (Strebel, 1998) were reported in the three districts. The outbreak was first reported in Chora district on 25/12/98 and within 20 days spread to all 20 villages in this district. After this time the number of reported cases declined. The incidence of measles was higher in the Chora district (1,087 per 100,000) than in Bedelle (373 cases) and Gachi (221 cases) districts to which it subsequently spread (Figure 4.1)
Figure 4.1. Districts affected by the 1998-99 measles outbreak in Ethiopia. The area in which measles infection occurred is highlighted.

The age group most affected was children between 1 and 4 years who comprised 47.7% of clinically reported cases. Most of the remaining cases were in children between 5 and 14 (39.5%) or in infants less than one year (9.8%). Only 3.1% of the clinically reported cases were in those aged 15 years or more. The age distribution of the subset of the
laboratory confirmed cases from Bedelle district was similar (Table 4.1), though fewer of the laboratory confirmed cases were in children less than 5 years.

Table 4.1. Age distribution of reported and laboratory confirmed measles cases

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Reported cases ( % )</th>
<th>Confirmed cases ( % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1</td>
<td>156 (9.8)</td>
<td>2 (3.8)</td>
</tr>
<tr>
<td>1 - 4</td>
<td>762 (47.7)</td>
<td>14 (26.4)</td>
</tr>
<tr>
<td>5 - 9</td>
<td>393 (24.6)</td>
<td>19 (35.8)</td>
</tr>
<tr>
<td>10 - 14</td>
<td>238 (14.9)</td>
<td>15 (28.3)</td>
</tr>
<tr>
<td>15+</td>
<td>50 (3.1)</td>
<td>3 (5.7)</td>
</tr>
<tr>
<td>Total</td>
<td>1599</td>
<td>53</td>
</tr>
</tbody>
</table>

Reported coverage for measles vaccine was less than 50% in the 3 districts. Of the 1603 reported cases, 1440 were in non-vaccinated people and only 163 in those who were vaccinated. Management of the epidemic consisted of supportive therapy (antibiotics, oral re-hydration salt (ORS) administration and vitamin A supplement) and health education. Schools in affected area were closed for approximately one month and 7823 children under 5 years in adjacent villages were given measles vaccine.

4.3.2. Laboratory confirmation

Of 55 cases from Bedelle district, 53 were confirmed as recent measles by both IgM serology and RT-PCR with the primer set for amplifying a 450 nt region of the H gene. The two cases that were not confirmed were an infant of 7 months and an 18-year-old female with a clinical diagnosis of varicella. Of the 53 confirmed, 40 (75%) were measles IgM positive in oral-fluid samples. The oral fluid measles IgM detection rate increased
from 63-67% at days 2 and 3 to 100% at days 6 and 7 (Table 4.2). The two sporadic cases from Addis Ababa were both IgM and RT-PCR positive on oral fluids.

Table 4.2. Oral fluid measles-IgM detection and date of sample collection in days after onset of symptoms in laboratory confirmed cases from Bedelle outbreak

<table>
<thead>
<tr>
<th>Oral fluid measles IgM</th>
<th>Days after onset of symptoms</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Pos</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Neg</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>%</td>
<td>67</td>
<td>63</td>
</tr>
</tbody>
</table>

4.3.3. Genetic characterization of MV strains

Of the 53 PCR positive specimens, 28 chosen at random from the Bedelle outbreak and two from the sporadic cases in Addis Ababa were sequenced, initially over the 450-nt region of the H gene. Identical sequences were obtained in all 28 specimens from the Bedelle outbreak, and two distinct strains were identified from the sporadic cases. Subsequently, the entire H and N genes of these three representative strains were amplified by RT-PCR and the amplicons were directly sequenced. The three strains were named as MVs/Bedelle.ETH/5.99 (ETH10/99) from a 5-year-old child from the Bedelle outbreak and MVs/Addis Ababa.ETH/50.98 (ETH54/98) and MVs/Addis Ababa ETH/2.99 (ETH55/99) from 10 and 2-year-old sporadic cases from Addis Ababa respectively.
Figure 4.2 shows the phylogenetic relationships between the three Ethiopian and 33 other MV strains including 18 WHO reference strains (WHO, 1998), 10 previously reported African strains (Truong et al., 1999), one suspected Indonesian strain, assigned recently to genotype G2 (Swart et al., 1999) and three strains (UK140/94 (D8), UK160/94 and UK226/94) identified in the UK in 1994 (Jin et al., 1998) which are closely related to ETH54/98. The phylogenetic tree in Figure 4.2 was constructed by analysing the entire H gene sequence (1854-nt). The three Ethiopian (ETH) strains were clustered in the D but located on two different branches. Strains ETH10/99 and ETH55/99 were closest to genotype D4 and there were respectively, 32 (1.7%) and 29 (1.6%) nucleotide differences and 7 (1.1%) and 3 (0.5%) amino acid differences between these two ETH strains and the D4 reference strain (Monteral.CAN/89). There was at least 2.5% divergence between these two ETH strains and other genotypes of clade D. Strain ETH54/98 showed at least 2.4% divergence to all reference genotypes but over 98% similarity to the three strains (UK140/94 (D8), UK160/94 and UK226/94) identified in the UK in 1994 (Jin et al., 1998) and formed a new branch together with these three strains. The degree of divergence would suggest that this group should be considered an additional genotype of clade D.

A similar phylogenetic tree based on the C-terminus of the N gene was constructed by the Neighbour-joining method (PAUP version 4.0) and subjected to bootstrap analysis (1,000 times, Figure 4.3). Strains ETH10/99 and ETH55/99 were close to genotype D4 but strain ETH54/98 appeared on a new branch with the strains detected previously in the UK. This confirmed phylogenetic relationships based on analysis of the H gene. On the basis the
entire N gene sequence, there were 34 (2.2%) and 32 (2.0%) nt differences and 8 (1.5%) and 5 (0.9%) aa differences between the ETH strains 10/99 and 55/99 and D4 reference stain (Montreal.CAN/89), while there was at least 3% divergence from other reference genotypes. There were 17 (1.1%) nt differences between strain ETH54/98 and the UK strains and at least 2.7% divergence from other genotypes.

Amino acid differences between the three ETH strains and closely related MV strains are summarised in Table 4.3. Amino acids coded by consensus nt sequences (con.) were based on the sequences of strains selected for phylogenetic analysis that covered all M genotypes defined by the WHO (1998). Compared with the aa sequence of the Mont89/D4 strain, there were 7 (ETH10) and 3 (ETH55) aa differences in the H gene and 8 and 5 aa differences in the N gene. Compared with the aa sequence of the UK140 (D8) strain, there were 13 aa differences in the H gene of strain ETH54 but no aa differences in the N gene because, although there were 17 nt substitutions, all were synonymous.
Table 4.3. Amino acid differences between ETH and closely related MV strains

<table>
<thead>
<tr>
<th>Gene</th>
<th>aa</th>
<th>Con.*</th>
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* amino acids coded by consensus nucleotide sequence
Notes: only differences are indicated and important functional sites are highlighted.
Figure 4.2. Genetic relationships between measles strains detected in Ethiopia and previously reported genotypes. An unrooted tree diagram was constructed on the basis of the entire H gene (1854 nt) sequence using the Clustal method (Megalig programme, DNASTAR). Strains reported in this study are highlighted. WHO reference strains are in boxes and genotypes assigned previously are indicated at the end of the strain designation.
Figure 4.3. Genetic relationships between measles strains detected in Ethiopia and previously reported genotypes on the basis of the C-terminus of the N gene (456 nt). The phylogenetic tree was drawn by bootstrap analysis (1,000 times) using the Neighbor-joining method (PAUP 4.0 package). MV strains reported in this study are highlighted and the reference strains (in boxes) with the designated WHO MV genotype are indicated at the end of each strain designation.
4.4. Discussion

Factors contributing to the spread of measles infection included the low vaccination coverage of less than 50% and the gathering of people in schools and market places. The age groups affected were principally children between 1 and 14, as observed in England and Wales prior to the introduction of measles vaccine (Benjamin, 1968).

Although infections with other agents such as rubella, parvovirus B19, human herpes virus-6 (HHV-6) and Group A streptococcus may resemble measles clinically (Nur et al., 1999), the diagnosis of measles cases in this study by staff at Health Centers was mostly confirmed where laboratory investigations were done. Of the 55 clinically diagnosed cases investigated in the Bedelle outbreak, 53 were confirmed as recent measles by serum IgM testing and PCR. Two cases were not confirmed. One was an adult in whom varicella was suspected. The other was a 7-month infant. A frequent cause of measles misdiagnosis in this age group is roseola infantum, primary infection with HHV-6 (Tait et al., 1996).

The performance of oral fluid IgM assay was poor in detecting early onset natural measles infections. This shows that with greater time from rash onset, there was high predictive value of positive for oral fluid. This suggests a sensitivity problem of the assay, requiring longer developing antibody to be detected. This indicated that taking oral fluid for IgM diagnosis should be excluded, as delays patient diagnosis should not occurs.
Sequence analysis of measles RNA amplified by RT-PCR identified three virus strains. All 28 strains from the Bedelle outbreak were identical and closely related to genotype D4. One of the strains from sporadic cases in Addis Ababa was also similar to genotype D4 but the ETH54 strain from Addis Ababa was distinct. It was closely related to strains identified in the UK in 1994 (Figure 4.2, 4.3). While ETH 54 could be assigned to clade D, it was not identical to any previously described genotype within this clade and might be representative of a previously undescribed genotype. The occurrence of this genotype, in addition to the genotypes A, B1, B2, B3, D2 and D4 previously identified in Africa, suggests that antigenically and genetically distinct MV lineages are co-circulating in Africa. The results in this study indicate the need for continued molecular and antigenic surveillance of wild-type MV strains.

Analysis of aa sequence variation in the entire H and N genes of the three ETH strains revealed that there were significant changes (Table 4.3) affecting sites currently recognised as having important biological and immunological functions. These sites include the predicted B-cell epitope at aa309-318 of the H gene (Partidos et al., 1991; Makela et al., 1989) and three antigenic sites at aa122-150, aa457-476 and aa519-525 of the N gene (Buckland et al., 1989; Komase et al., 1990). Amino acid 416 of the H gene, which is postulated to affect hemagglutination (Saito et al., 1995), was found changed in all three Ethiopia strains compared to the aa coded by the consensus nt sequence (Table 4.3). The nt substitution G to A, which results in the change at aa416, produces an additional potential N-linked glycosylation site which has been found in isolates from Japan (Saito et al., 1995) and the USA (Chil/89, D3; WHO reference strain). Analysis of
the nt sequences of strains ETH10 and ETH55 (detected in 1998/9), the reference
genotype D4 strain (isolated in 1989), strain ETH54 (detected in 1998/9) and the closely
related UK strains (identified in 1994) showed significant genetic relationship.
Furthermore, in previous studies, antigenic reactivity demonstrated by different
monoclonal antibodies indicated that current wild-type MV genotypes contain strain
specific epitopes (Komase et al., 1998). Further studies to attempt isolation of the ETH
MV strains in cell culture and to map their antigenic epitopes and genetic evolution are
currently in progress.
1. Equipment

- Micropipettes capable of dispensing volume range of 1 → 1000 ul range and compatible sterile plugged tips.
- Thermocycler and compatible thin walled micro-reaction tube.
- Sterile 1.5 ml micro-tubes
- Latex gloves and laboratory coat dedicated for use in each lab.
- Glax pyrex conical 500 ml flask.
- Electrophoresis equipment; gel pouring tray, combs, tank unit and power pack.
- Assorted sterile racks
- Dispo jars
- Autoclave bins

2. Reagents

- Polymerase Taq
- 10 X Taq buffer
- Primer ‘h1’ 5’ACTACAAATCAGAGGTCAATTC 3’
- Primer ‘h2R’ 5’AGCATGTCTCCATTGCAACT 3’
- Primer ‘h3’ 5’CAGAGGTCAATTCTCAAACA 3’
- Primer ‘h4R’ 5’CATTCGCAACTTGTCATCTG 3’
- Primer ‘nl’ 5’TGCATACTACTGAGAACAA 3’
- Primer ‘n2R’ 5’TCTCGCACCTAGTCTAGAA 3’
• Primer ‘n3’ 5’ATCAGTAGAGCGGTTGGA 3’
• Primer ‘n4R’ 5’GTCTGAGCCTTGTTCTTC 3’
• MgCl₂ (50mM)
• dNTPs (10mM)
• Sterile distilled high quality water (Sigma)
• Sterile mineral oil
• Seakem Agarose
• TBE buffer
• 0.25% orange G, 10% Ficol in TBE buffer, filtered (+4°C)
• Ethidium Bromide (10mg/ml)

3. Operating Procedure

3.1. Extraction of Measles

For one reaction mix combine

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6</td>
<td>860ul</td>
</tr>
<tr>
<td>Silica</td>
<td>40ul</td>
</tr>
<tr>
<td>Specimen</td>
<td>100ul</td>
</tr>
</tbody>
</table>

Total 1000ul

• Vortex 10 seconds, keep at room temperature for 10 minutes (mix or vortex every 2-3 minutes).
• Centrifuge 15 seconds, discard supernatant by suction
• Wash the silica-NA pellet twice with L2, twice with 70% ethanol, once with acetone (1ml wash solution, vortex briefly, centrifuge for 15 seconds and discard the supernatant).

• Dry the silica-NA pellet at 56°C with an open lid for 10 minutes.

• Add 59ul RNASE free dH2O and 1ul RNAsin, Vortex and incubate for 10 minutes at 56°C, overnight at 4°C.

• Centrifuge for 2 minutes and use 40ul of the supernatant for the cDNA synthesis.

3.2. cDNA Synthesis of Measles Virus RNA

For one reaction mix combine

- Extracted RNA 40ul
- 10 x PCR buffer 10ul
- 50 mM MgCl2 3ul
- 10 mM dNTPs 2ul
- 400u R.T. 2ul (M-MLV Rase)
- RNAsin (40u) 1ul
- pd(N6) 1ul (0.02u)
- H2O 41ul

Total 100ul

The reaction mixture was incubated at room temperature for 10 minutes, at 37°C for 60 minutes, at 95°C for 5 minutes and cooled on ice for 3 minutes. The synthesized cDNA was stored -20°C or used for PCR.
3.3. First round amplification

3.3.1. For 1 reaction mix combine

- 5ul of 10 x Taq buffer
- 1.5ul of 50mM MgCl₂
- 1ul of primer ‘h1’ at 10pmol/ul
- 1ul of primer ‘h2R’ at 10 pmol/ul
- 1ul of primer ‘n1’ at 10 pmol/ul
- 1ul of primer ‘n2R’ at 10 pmol/ul
- 1ul of 10mM dNTP mixture
- 0.4ul of Taq
- 20.175ul H₂O

Final volume of 30ul

3.3.2. Multiply by the above volumes the number of tests you have (plus two to allow for pipetting errors) to make up the bulk reaction.

3.3.3. Pipette 30ul of reaction mix to labeled thin-walled tubes. Add 1 drop of oil to each tube and close the caps.

3.3.4. Take tubes to the thermal cycler room, open caps and short spin the sample to add 20ul of the sample (cDNA) to each tube.

3.3.5. Start the thermal cycling with the following conditions.

- 95°C 2 minutes
- 95°C 1minute (denaturation)
- 50°C 2 minutes (annealing) X 25
- 65°C 3 minutes (extension)
Note: Single gene target PCR can be carried out with either pair of primers and the following thermal cycling conditions:

- 95°C 2 minutes
- 95°C 1 minute (denaturation)
- 50°C 1 minute (annealing) X 25
- 72°C 1 minute (extension)

For PTC-200 Thermal cycler
- Switch on
- Press proceed
- Proceed again
- Press arrow to MPLEX-1
- Press proceed
- "Enabled" - proceed
- Run has started

3.4. Second round amplification
3.4.1. For 1 reaction mix combine

- 5μl of 10 X buffer
- 1.5μl of 50 mM MgCl₂
- 1μl of primer ‘n3’ at 25pmol/μl
- 1μl of primer ‘n4R’ at 25pmol/μl
- 1μl of primer ‘h3’ at 25pmol/μl
1μl of primer ‘h4R’ at 25 pmol/μl

1μl of 10 mM dNTP mixture

30.4 μl H2O

0.4 μl Taq

Final volume of 40μl

3.4.2. Multiply by the above volumes the number of tests you have (plus two to allow for pipetting errors) to make up the bulk reaction.

3.4.3. Pipette 40μl to labeled thin-walled tubes add 1 drop of oil to each tube and close the caps.

3.4.4. Take tubes to the thermal cycler room, open caps and add 10μl of 1st round product to each tube.

3.4.5. Start the cycling with the following conditions

95°C 2 minutes

95°C 1 minute (denaturation)

50°C 2 minutes (annealing) X 35

65°C 3 minutes (extension)

Note: - Single gene target PCR can be carried out with either pair of primers and the following thermal cycling conditions:

95°C 2 minutes

95°C 1 minute (denaturation)

55°C 1 minute (annealing) X 25

72°C 1 minute (extension)
For PTC-200 Thermal cycler proceed as in 3.3.5. above except at stage 4 press arrow to MPLEX-2.

3.5. Agarose gel analysis of PCR products

3.5.1. Make up a 2% agarose gel in TBE buffer by dissolving 1.6g Seaken agarose in 80mls TBE buffer in 500 ml flask in microwave. Pour molten gel on to gel plate with gel comb. When gel has set remove comb.

3.5.2. Combine 10ul of the sample with 5ul of orange G/Ficol with 15ul of TBE buffer. Use a 96 well microtiter plate to prepare the samples for electrophoresis.

3.5.3. Add TBE to the electrophoresis tank so that the buffer just reaches the top surface of the gel, but does not submerge it. Load samples into the dry wells. Electrophoresis samples for 5 minutes until the gel enter. Add TBE to submerge the gel and continue electrophoresis at approximately 120V for 1 hour. Stain with Ethidium Bromide and photograph the gel.

3.6. Gene clean

3.6.1. Fragments were cut from agarose gels, using modified gene clean method.

3.6.2. Put it in tube, add 900ul (9ml 6M NAI + 1ml of 10xTBE) mixture, add 10 ul of silica, keep at 56°C for 10 minutes (vortexing every 2-3 minutes), and then centrifuge for 6 seconds.

3.6.3. Wash the pellet with 1-ml new wash (70% cold ethanol) for 3 times.

3.6.4. Dry the pellet at 56°C for 5 minutes; add 22ul of water, vortex, and keep at 56°C for 3 minutes.
3.6.5. Centrifuge for 30 seconds, and then at 56°C for 3 minutes, centrifuge for 30 seconds.

3.6.6. Store the supernatant at -20°C for sequencing.

3.7. Sequencing

3.7.1. For one reaction mix combine

<table>
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<tr>
<td>DNA</td>
<td>2-9</td>
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<tr>
<td>H2O</td>
<td>9-2</td>
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</tbody>
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Total 20 µl

3.7.2. Spin and 25X cycles (96°C for 30 minutes, 50°C for 15 minutes, 60°C for 4 minutes).

3.7.3. For one reaction mix combine

<table>
<thead>
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<th>Volume (µl)</th>
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<td>The reaction</td>
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<tr>
<td>3M NA AC</td>
<td>2</td>
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<tr>
<td>100% Ethanol</td>
<td>50 (cold)</td>
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3.7.4. Vortex on ice for 10 minutes, centrifuge for minutes.

3.7.5. Wash the pellet with 50µl 70% cold ethanol (no vortex), centrifuge for 5 minutes.

3.7.6. Discard supernatant by suction and dry the pellet at room temperature for sequencing.
Summary

For the past five years, from 1998 to 2002, Ethiopia implemented a measles supplemental immunization (MSI) programme targeted at 9m-59m old children in addition to the routine vaccination given at 9 months. Despite this initiative measles outbreaks occurred throughout the country. In this study serological and molecular diagnosis of measles outbreak samples demonstrated the need for refining of the measles immunization programme. During 2000 – 2002 through the MOH/EPI network suspected measles cases were identified across the country. As a result 150 serum and/or oral-fluid samples were collected from 17 suspected measles outbreaks, which occurred throughout the country. Of the total, 119 and 140 of them were characterized serologically and molecularly, respectively. Based on the serological investigation about 93% of the confirmed cases were children under 15 years of age and 6.5% were 15+ years old. Of the total unvaccinated suspected measles cases (n = 108), 83.3% (90) had confirmed measles infection. Of the total vaccinated measles cases (n =11), 27% (3) had confirmed measles infection. Of 140 samples analysed by RT-PCR measles virus specific cDNA was detected in 39% (54). A total of 31 wild-type measles viruses were characterized by nucleic acid sequence analysis of the nucleoprotein (N) and haemagglutinin (H) genes. Two recognized genotypes (D4 and B3) were identified. Each outbreak comprised of only a single genotype and outbreaks of each genotype tended to occur in distinct geographical locations. The study appeared to clarify the epidemiological links of the
outbreaks of B3 genotype, which occurred in Khartoum, Sudan, near to the Ethiopian border. D4 genotype was previously observed in an outbreak in 1999 and occurs in more diverse locations throughout the country than B3. B3 was first observed in 2002, has now been the cause of 3 outbreaks. It is concluded that both genotypes have contributed to the maintenance of measles in the country. Furthermore, it is concluded that control measures have failed to interrupt transmission. These serological and molecular results provide important information for refining the measles immunization activities. If measles is to be controlled, routine immunization should be strengthened and the MSI should involve older children (5-14 age groups).

5.1. Introduction

In Ethiopia, measles is the commonest single (aetiologically) cause of mortality and morbidity in children. Measles together with malaria, acute respiratory infection, diarrhoea and malnutrition are responsible for 70% of under-five morbidity and mortality (MOH, 1998).

In 1990 (when the EPI began monitoring the routine measles uptake), the national measles immunization coverage was 9%. In subsequent years the coverage increased every year to a maximum of 54% in 1996. This level was maintained for the following three years after which it dramatically falls to 37% in 2000. As shown in Figure 5.1 measles reported cases declined as the EPI coverage increased after 1990 at least for five years. Although the routine measles EPI coverage reached 54% in 1996 and remained relatively stable for the following three years the number of measles cases reported
actually increased (Source: Disease Prevention and Control Department, Family Health Department; MOH 2002).

Figure 5.1. Measles immunization coverage and number of reported measles cases in Ethiopia, 1990-2000*.

From these data it is clear that the measles coverage has remained low over the past decade, and that this level is inadequate for the control of transmission, for which >93% coverage is required (Anderson and May, 1991) During severe outbreaks the case fatality rate is in the range of 15 to 20% (Lindtjorn, 1986), where as in times of low incidence it is 3 to 5% (MOH, 1998).

Since 1998, Ethiopia has implemented the Measles Outbreak Prevention Phase of the Accelerated Measles Control Strategy (MOH, 2001). The term outbreak is used when the
observed number of cases is greater than that normally expected from the same geographical area over the same time period. During this phase, an outbreak is to be defined as more than 10 measles cases per Zone (= District) per week.

As measles control accelerates and cases diminished, the clinical diagnosis becomes less efficient (WHO, 1999). Therefore diagnosis of measles should rely on laboratory confirmation. In this way it is possible to confirm the extent of each outbreak and assist in measuring the effectiveness of the vaccination programme. Molecular characterization of samples from outbreaks can assist in the detection of the different genotypes of the measles virus; identify the transmission pattern and the geographical (transmission) links of the measles outbreaks (Rota et al., 1996). Sequence variability among the strains and comparison with the consensus sequence of clade D genotype was presented in Chapter 4. This present chapter focuses on the differentiation of outbreaks resulting from either case importation or transmission of indigenous MV lineages. The distinction was made through the interpretation of temporal and spatial genotypic pattern. The occurrence of indigenous MV suggests failure of measles control measures to interrupt transmission. There are global implications of studies of this nature in relation to measles elimination nationally and internationally (Bellini and Rota, 1998; Chibo et al., 2000). The purpose of this paper is therefore to present the studies of 17 measles outbreaks reported in the country to identify through serological and molecular epidemiologic analysis, reasons for the outbreaks, which will assist in monitoring and control of the disease in the future. This study was partly linked to The National Measles Reference Laboratory (NMRL) activities at EHNRI. NMRL was established by the support of MOH and WHO country
office. It has a role in monitoring the measles control and surveillance programme by providing results of measles laboratory diagnosis to RHB, MOH/EPI and WHO/EPI.

5.2. Materials and methods

5.2.1 Study area and population

The population for 2002 derived from projecting the 1994 census shows that 86.4% (58,103,000) of the Ethiopian population settled in Tigray (3,901,000), Amhara (17,205,000), Oromia (23,704,000) and SNNP (13,293,000) in high measles risk regions (CSA, 2001). The national routine EPI coverage for measles has declined from 53% in 1999 to 37% in 2000 (see Figure 5.1). The routine measles coverage for Tigray, Amhara, Oromia and SNNPR during 2000 was 72%, 61%, 28% and 24% respectively (MOH, 2001). Because of this low coverage repeated measles outbreaks were reported from various parts of the aforementioned regions for the last three years. In these studies laboratory investigations were performed for 17 suspected measles outbreaks, which occurred in Tigray (5 outbreaks), Oromia (2 outbreaks), Amhara (6 outbreaks) and SNNPR (3 outbreaks) regions (see Table 5.3). One other suspected measles outbreak occurred in Diredawa town and was also investigated. The study areas (districts or towns) where the investigations were carried out showed in Figure 5.2.
Figure 5.2. Districts affected by the 1998-02 measles outbreaks in Ethiopia. The area in which measles infection occurred is highlighted.
5.2.2 Clinical diagnosis

Any person with fever and/or maculo-papular rash and/or cough and/or conjunctivitis was considered as clinically diagnosed for measles (WHO, 1999). The data reported in this study fulfill the above definition confirmed at hospital or health center level by health practitioners.

5.2.3 Outbreak investigation

When an outbreak occurred in a district the case was notified to the Regional Health Bureau (RHB) and Ministry of Health (MOH). Through the networks of the RHB, MOH/EPI and WHO/EPI, District laboratory personnel collected laboratory specimens with the assistance of district polio focal persons from measles outbreaks accompanied by case investigation forms for measles notification. In the case of early notification a team from EHNRI made a further investigations in districts of Gindeberete and Nono, and Tigray region. Usually serum samples reached processed otherwise samples (under cool condition in ice boxes) and forms were sent to EHNRI measles reference laboratory, Addis Ababa, Ethiopia. Specimens were then processed and stored at -20°C until tested. Serological results were reported to respective RHB, MOH/EPI and WHO/EPI country office.

5.2.4 Laboratory confirmation of measles cases and genotyping

Serum or whole blood/oral fluid specimens were collected from 5 or more of the first 10 cases in each Zone (= District) for laboratory investigation. Serum samples were tested for IgM at the National Measles Reference Laboratory, EHNRI using the Behring Kits (Enzygnost for IgM, Behring Diagnostics, Marburg, Germany) and following the
manufacturer instructions. Detection of measles IgM in serum was considered a diagnosed case of measles, which has been confirmed by laboratory. WHO supplied Kits through the WHO/EPI country office.

Polymerase Chain Reaction (PCR) was used to amplify viral genome in oral fluid, whole blood or serum samples collected from suspected measles patients. The full PCR standard operating procedure used for this study was attached in Chapter 4 as A4 (Appendix for Chapter 4). Sequencing of PCR products was carried out as previously described (Jin et al., 1996; Jin et al., 1997; Nigatu et al., 2001). Measles strains characterized were classified according to WHO nomenclature for wild-virus genotypes (WHO, 2001). A phylogenetic tree was constructed by analyzing either the 511 nucleotides (nt) of the H gene sequence or the 456 nt of the N gene sequence (Jin et al., 1998) using the Clustal method (Megaalign Programme, DNASTAR).

5.3. Results

Over the period of 2000 - 2002, the MOH/EPI network was utilized to identify cases of measles-like infection across the country. Through the link, samples were collected from suspected measles outbreaks. The age distribution of laboratory confirmed measles cases are presented in Table5.1. Based on 119 suspected measles cases tested serologically the age group 0-1 contributed to 4.3% of the confirmed cases, 1-5 to 28%, 5-10 to 30.1%, 10-15 to 31.2% and the rest 6.5% was attributed to the age group 15 years and above. About 93% of the cases were under 15 years of age.
Table 5.1. Age distribution of laboratory confirmed measles cases

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>No. tested</th>
<th>No. positive (%)</th>
<th>Confirmed cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>7</td>
<td>4 (57.1)</td>
<td>4 (4.3)</td>
</tr>
<tr>
<td>1 - 4</td>
<td>31</td>
<td>26 (83.9)</td>
<td>26 (28.0)</td>
</tr>
<tr>
<td>5 - 9</td>
<td>43</td>
<td>28 (65.1)</td>
<td>28 (30.1)</td>
</tr>
<tr>
<td>10 - 14</td>
<td>32</td>
<td>29 (90.6)</td>
<td>29 (31.2)</td>
</tr>
<tr>
<td>15+</td>
<td>6</td>
<td>6 (100)</td>
<td>6 (6.5)</td>
</tr>
<tr>
<td>Total</td>
<td>119</td>
<td>93 (78.2)</td>
<td>93</td>
</tr>
</tbody>
</table>

Table 5.2 below shows vaccination status of laboratory confirmed measles cases. In the 108 unvaccinated children 90 cases (83.3%) were confirmed as measles IgM positive. Of the total vaccinated (11) children 3 cases (27.3%) had confirmed measles disease.

Table 5.2. Vaccination status of laboratory confirmed measles cases.

<table>
<thead>
<tr>
<th>Result</th>
<th>Vaccinated (%)</th>
<th>Unvaccinated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>8 (72.7)</td>
<td>18 (16.7)</td>
</tr>
<tr>
<td>Positive</td>
<td>3 (27.3)</td>
<td>90 (83.3)</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>108</td>
</tr>
</tbody>
</table>

The investigation of 17 suspected measles outbreaks is summarized in Table 5.3. The table shows a total of 150 serum/saliva specimens were collected from measles suspected patients. Of the total, 40 serum/oral fluid samples were collected from two measles outbreaks of western Shoa of the Oromia region. A further 52 were serum/oral-fluid samples collected from 5 measles outbreaks of Tigray region in northern Ethiopia. The
remaining 38, 15 and 5 were serum samples collected from 10 suspected measles outbreaks of Amahara region, SNNPR and Diredawa town respectively. Samples of 14 out of the 17 outbreaks were tested serologically, of which 13 (92.9%) outbreaks could be confirmed. A further 3 outbreaks for which there were no serum samples available for serological testing, outbreak samples (whole blood or oral fluid) were tested molecularly by PCR, and 2 were confirmed as measles outbreaks. All the 5 serum samples collected from Bahirdar town were serologically negative. Of these tested by RT-PCR one sample found positive. Of the total 16 laboratory confirmed (serologically and molecularly) outbreaks, 15 (94%) took place between January –August in the dry season. Of the 15, 3 occurred in the month of April and 5 in June. The remaining 1 occurred in November in the rainy season.

The Behring ELISA detected measles IgM in 93 (78.2%) specimens. The RT-PCR amplification of the N nucleocapsid and H haemagglutinin genes were employed on 140 of the 150 specimens (only 10 samples from the target outbreak investigation were excluded). The samples used for PCR were of mixed type (serum, oral fluid, and whole blood). Out of the total, measles virus specific DNA was detected in 54 (38.6%) specimens. Representative samples of 31 PCR positive specimens were selected from 14 outbreaks for sequencing. Sequence analysis of 11 outbreaks revealed that the viruses were D4 genotype. Genotype B3 was identified from the 3 outbreaks of Wolkayet, Woreta and Bahirdar in North West part of the country (see Figure 5.2)
Table 5.3. Serological, PCR and genotype results for specimens collected from measles suspected patients during 2000 – 2002, stratified by geographical location.

<table>
<thead>
<tr>
<th>Region/ Zone/ Town</th>
<th>District (Reported month)</th>
<th>Specimen type (No collected)</th>
<th>No. serologically tested</th>
<th>No. PCR tested</th>
<th>No. genotyped</th>
<th>Prop. (%)</th>
<th>Prop. (%)</th>
<th>No sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oromia</td>
<td>Gendebret (January, 00)</td>
<td>Paired serum &amp; oral fluid (24)</td>
<td>24</td>
<td>20</td>
<td>14</td>
<td>20</td>
<td>83.3</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Nono (November, 01)</td>
<td>Paired serum &amp; oral fluid (16)</td>
<td>16</td>
<td>13</td>
<td>16</td>
<td>8</td>
<td>81.3</td>
<td>8</td>
</tr>
<tr>
<td>Tigray</td>
<td>Atisbi (June, 01)</td>
<td>Serum (7)</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>71.4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ganta Afeshume (February, 01)</td>
<td>Whole blood (15)</td>
<td>ND*</td>
<td>-</td>
<td>15</td>
<td>4</td>
<td>26.7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ofla (August, 01)</td>
<td>Serum (14)</td>
<td>14</td>
<td>12</td>
<td>14</td>
<td>3</td>
<td>85.7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Gulomakada (March, 02)</td>
<td>Oral fluid (4)</td>
<td>ND*</td>
<td>-</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Wolayet (March, 02)</td>
<td>Oral fluid (12)</td>
<td>ND*</td>
<td>-</td>
<td>12</td>
<td>12</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>Amahara</td>
<td>Woreta (April, 02)</td>
<td>Serum (9)</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Gondar town (April, 02)</td>
<td>Serum (6)</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ebinat (July, 02)</td>
<td>Serum (6)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>100</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>Bahirdar town (June, 02)</td>
<td>Serum (5)</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Worellu (April, 02)</td>
<td>Serum (5)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>100</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>Ankesha (Awj) (July, 02)</td>
<td>Serum (7)</td>
<td>7</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SNNPR</td>
<td>Sheko (May, 02)</td>
<td>Serum (5)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>100</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>Lomma (June, 02)</td>
<td>Serum (5)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>100</td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>Damot Gale (June, 02)</td>
<td>Serum (5)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>100</td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>Diredaw town (June, 02)</td>
<td>Serum (5)</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>80.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>150</td>
<td>119</td>
<td>93</td>
<td>140</td>
<td>54</td>
<td>38.6</td>
</tr>
</tbody>
</table>

* = Not done - no serum sample available for testing
** = Sequenced either for h haemaglutinin or n nucleocapsid gene
Figure 5.3 and 5.4 show genetic relationships between measles strains detected in Ethiopia during 2000-2002 and previously reported genotypes (in Ethiopia and worldwide) on the basis of the N gene (456 nt) (Figure 5.3) or H gene (511 nt) (Figure 5.4). MV strains reported in this study or that of Chapter 4 are indicated in circles and boxes. Both Figures show that D4 genotype is the most widely distributed in North, West and Southern parts of Ethiopia. B3 is distributed in specific areas of Amahra and Tigray regions.

Figure 5.3. Genetic relationships between measles strains detected in Ethiopia during 2000-2002 and previously reported genotypes. Unrooted tree diagram was constructed on the basis of the N gene (456 nt).
Figure 5.4. Genetic relationship between measles strains detected in Ethiopia during 2000-2002 and previously reported genotypes. The unrooted tree diagram was constructed on the basis of the H gene (511nt) sequence.

5.4. Discussion
Measles Outbreak Prevention is the strategy recommended for most of the Eastern African block countries where measles EPI coverage is 50 – 75% (WHO, 1997; WHO-Afro, 2000). Since the routine measles immunization coverage is low (<54%) in Ethiopia the strategy recommended for the country is the Measles outbreak Phase of the accelerated measles immunization control programme. The activities here include
improved surveillance to understand the changing epidemiology of the measles disease and identification of populations at higher risk. The data presented in Table 1 shows that the higher risk populations are less than 15 year children settled in Oromia, Amahara, SNNP and Tigray regions. This is consistent with the national measles cases data reported in the years 1997/98 – 2001/02 that indicates about 95% of cases were less than 15 years of age (MOH, 2002). Table 5.1 also shows an increasing proportion (61.3%) of measles confirmed cases among older age groups, 5 – 14 years. This result is consistent with epidemiological data collected for Tigray region that indicates an increasing proportion of measles cases among older children, 5 – 14 years, over consecutive 7 years from 1994 compared to the children less than five years (Table 5.4). The national data collected on measles cases reported during 1997 – 2002 showed that the age group 5-14 years contributed 40.8% of the cases (MOH, 2002). Both the national data and the data from Tigray region showed the shift of age in occurrence of measles cases from under five to above five years old. An increase in the average age at infection is an expected outcome of the reduced rate of measles transmission in a vaccinated population (Anderson and May, 1991) and is well documented (Bahri et al., 2003). The surveillance data and the present laboratory based findings suggest that the measles supplemental immunization should be delivered to all children 9m-14 years, ie an increased upper age from the current 59 months.
Table 5.4. Distribution of reported measles cases by age groups in Tigray region, 1991-2001*

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>12,000</td>
<td>2,011</td>
<td>3,991</td>
<td>158</td>
<td>350</td>
<td>157</td>
<td>40</td>
<td>47</td>
<td>8</td>
<td>37</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>5-14</td>
<td>5,625</td>
<td>897</td>
<td>1,703</td>
<td>28</td>
<td>467</td>
<td>137</td>
<td>100</td>
<td>214</td>
<td>166</td>
<td>326</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>15+</td>
<td>2,375</td>
<td>160</td>
<td>287</td>
<td>1</td>
<td>29</td>
<td>23</td>
<td>0</td>
<td>20</td>
<td>4</td>
<td>61</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20,000</td>
<td>3,068</td>
<td>5,981</td>
<td>187</td>
<td>846</td>
<td>317</td>
<td>140</td>
<td>281</td>
<td>178</td>
<td>424</td>
<td>164</td>
<td></td>
</tr>
</tbody>
</table>

* = Source: Epidemiology Division, Tigray Regional Health Bureau
** = First six months report only.

Based on Table 5.2 our study showed that the measles outbreaks in Ethiopia mainly involved unvaccinated children. Suspected vaccine failure contributed to 3.3% (3/93) of the total confirmed measles cases only. All 3 were vaccinated confirmed by recall. Thus, there is a clear indication that continued circulation of measles is due to low coverage and not vaccine failure.

The distribution of measles outbreaks reported in Table 5.3 showed that the peak transmission season for measles is in the dry season (months of March-August) with an abrupt fall when the rainy season begins (months of late September-December). Only one outbreak occurred during the four months rainy season. Ten years data on the seasonal distribution of reported measles cases for Tigray region also showed that measles cases were occurred more frequently during the dry season of January, February and March (Table 5.5).
Table 5.5. Seasonal distribution of measles cases by months in Tigray region, 1991-2000*

<table>
<thead>
<tr>
<th>Year</th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>Ma</th>
<th>June</th>
<th>July</th>
<th>Aug</th>
<th>Sept</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1991</td>
<td>5420</td>
<td>9451</td>
<td>2879</td>
<td>423</td>
<td>147</td>
<td>10</td>
<td>154</td>
<td>60</td>
<td>12</td>
<td>15</td>
<td>511</td>
<td>918</td>
<td>20,000</td>
</tr>
<tr>
<td>1992</td>
<td>256</td>
<td>1102</td>
<td>1248</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>51</td>
<td>49</td>
<td>80</td>
<td>70</td>
<td>152</td>
<td>3,068</td>
</tr>
<tr>
<td>1993</td>
<td>1203</td>
<td>1686</td>
<td>2641</td>
<td>72</td>
<td>0</td>
<td>0</td>
<td>44</td>
<td>5</td>
<td>2</td>
<td>17</td>
<td>112</td>
<td>199</td>
<td>5,981</td>
</tr>
<tr>
<td>1994</td>
<td>44</td>
<td>40</td>
<td>27</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>8</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>180</td>
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<tr>
<td>1995</td>
<td>27</td>
<td>56</td>
<td>94</td>
<td>35</td>
<td>0</td>
<td>307</td>
<td>0</td>
<td>26</td>
<td>0</td>
<td>154</td>
<td>0</td>
<td>147</td>
<td>846</td>
</tr>
<tr>
<td>1996</td>
<td>20</td>
<td>39</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>105</td>
<td>72</td>
<td>59</td>
<td>317</td>
</tr>
<tr>
<td>1997</td>
<td>72</td>
<td>20</td>
<td>8</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<td>2</td>
<td>37</td>
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<tr>
<td>1998</td>
<td>38</td>
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<td>0</td>
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<td>185</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>1999</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>78</td>
<td>61</td>
<td>0</td>
<td>39</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>178</td>
</tr>
<tr>
<td>2000</td>
<td>0</td>
<td>0</td>
<td>183</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>128</td>
<td>0</td>
<td>0</td>
<td>424</td>
</tr>
<tr>
<td>Total</td>
<td>7080</td>
<td>12396</td>
<td>7090</td>
<td>543</td>
<td>179</td>
<td>600</td>
<td>395</td>
<td>150</td>
<td>118</td>
<td>499</td>
<td>767</td>
<td>1598</td>
<td>31,415</td>
</tr>
</tbody>
</table>

* = Source: Data collected from Epidemiology Division, Tigray Regional Health Bureau

Associated information collected from this region indicates social activities such as weddings, church ceremonies, and soil and forest protection works undertaken at higher frequency during January-April of the dry season that may markedly influence the opportunities for transmission of the measles virus in crowded or congested conditions. An outbreak investigation study carried out at Nono district indicated that the measles outbreak occurred when the school opened during January. A study undertaken in 1998/9 in Bedelle district (Nigatu et al., 2001) suggested the crowding of students into school, gathering of peoples in market places accompanied by greater movement of peoples promoted the exchange and spread of the measles virus to the adjacent communities and
villages. The link to school opening following vacation has been long documented (Fine and Clarkson, 1982).

The national data shows an increase in number of measles cases as the immunization coverage increases through 1990-2000 (Figure 5.1). This may be attributed to the increased activities of the surveillance programme. As can be seen in Figure 5.2 and Table 5.3 most of the outbreaks occurred in North, North Western and South Western parts of the country. This may be accord with differences in surveillance, vaccine coverage and population density/isolation across the country.

Measles is a disease well known to Ethiopian people especially in rural areas. Lindtjorn (1986) summarized the cultural beliefs or attitudes of Southwest Ethiopian about measles. These cultural factors were observed in areas where outbreaks were observed (Figure 5.2). These attitudes are thought to have influenced the measles immunization control programmes. Cultural belief related to injections administered to the 'measles sick child' was found to affect the collection of blood samples using needles for laboratory tests and drugs given by injection to control measles complications. The belief is “The sick child should not receive injection. If received an injection the disease is thought to aggravate resulting in serious illness and even death”. Because of this cultural factor some of the outbreak communities in Tigray region declined to give blood samples for laboratory analysis. We found that the oral fluid collection devices were the better alternative for collecting specimen for measles testing.
An essential part of the accelerated measles control and surveillance program in Ethiopia is the diagnostic service given by the measles laboratory. Collecting blood specimens from measles patients has a cultural problem which impacts on the effective measles control in the country. The Central Public Health Laboratory (CPHL) is routinely using oral-fluid specimens for measles diagnostic tests in the UK measles surveillance program (Perry et al., 1993; Brown et al., 1994; Ramsay et al., 1997; Vyse et al., 2001; Ramsay et al., 2003). From this study it can be suggested that the establishment of this technique in Ethiopian measles laboratory and its integration into the surveillance system of the national EPI program would play a similarly important role.

In the UK, USA and elsewhere molecular characterization of MV strains circulating in the country is part of the surveillance programme that assists to determine the pattern of measles outbreak transmission (Jin et al., 1997; Bellin and Rota, 1998; Chibo et al., 2000; Ramsay et al., 2003). The strain responsible for the 2000-2002 measles outbreaks reported from most of Tigray, Oromia, SNNP and Amhara regions was genotype D4. The measles strains from sporadic cases in Addis Ababa prior to 2000 (Nigatu et al., 2001), were identified as genotype D4 and D8. No further occurrence of D8 has been documented and it may presume that this was an imported case. Genotype D4 was reported from UK throughout 1995-2000, USA and Canada (Ramsay et al., 2003; Bellini and Rota, 1998). The genotype D8 was reported in 1999-2000 from UK, South Asia, the Middle East and the Balkans (Ramsay et al., 2003). The wide distribution of D4 genotype in the country and the presence of this genotype over a prolonged period through 1998-
2002 suggest this genotype to be the most important for sustainable indigenous transmission of measles in Ethiopia.

Sequence analysis of two outbreaks, those which occurred in Gendebert and Nono of Western Shoa, revealed that the D4 genotype showed a transmission link between the two outbreaks, although each outbreak strains differ each other by 3 nt (nucleotide)/2 aa (amino acids) over time within one year period. A detailed analysis of the relationship between the different outbreaks strains is required to see if there is link or association of transmission.

Clade B viruses are predominantly found in Western and Central Africa. The clade comprises B1, B2 and B3 genotypes where representatives of the clade have been isolated in The Gambia, Cameroon, Gabon, Nigeria, Ghana, Zambia, Kenya and Sudan (Hanses et al., 1999; Troung et al., 1999; WHO, 2001; Mubarak et al., 2002; Kouomou et al., 2002). An importation of clade B virus into the United States from Kenya was identified during 1995-1997 (Bellini and Rota, 1998). Analysis of isolates of clade B showed the Sudanese isolates to be placed within the genotype B3, circulating indigenously in Khartoum over a 3 years period through 1997-2000 (Mubarak et al., 2002). Our investigation showed the presence of B3 genotype in North Western part of Ethiopia bordering Sudan. The similarity between our MV strains with those isolated in Sudan could be explained by the movements of infected peoples across the border of the two countries. However, a more comprehensive analysis of the relationship between the Sudan and the Ethiopia viruses is needed. As the B3 identified in Sudan linked to the
Northern Nigeria strain (Mubarak et al., 2002), the present studies show that this genotype has widened its distribution and now has become a cause of indigenous transmission of measles in Ethiopia.
CHAPTER 6
EVALUATION OF A MEASLES VACCINE CAMPAIGN IN ETHIOPIA USING ORAL-FLUID ANTIBODY SURVEYS

Summary

Campaign vaccination is widely adopted in the accelerated control of measles. Measurements by which to evaluate and refine interventions are essential. The potential contribution of oral-fluid antibody prevalence data in this process has not been demonstrated. An evaluation was undertaken of a measles vaccine campaign in Ethiopia in 1999. In the town of Asela, southern Ethiopia, oral-fluid specimens were collected from 1928 children aged 9m - 5 years attending for measles campaign vaccination. In June 2000, oral fluids were taken from 750 individuals aged 9m-19 years, in the same location, from households selected by cluster surveys methods. Data on vaccine history in the households was recorded. Measles antibody status was determined by using MicroImmune EIA adjusted for known sensitivity and specificity relative to serum assays. Records from the pre-campaign data indicate that 73.6% of children in the target age received measles vaccine. Antibody prevalence in children attending for vaccination (pre-campaign) was 44% (26% in 9-11 month olds rising to 60% in 60-71 month olds). Post-campaign antibody prevalence was 80-90% in the target age group. The proportion negative in older children not targeted by vaccination was 39% in those 7-9 years of age, and 19% in those 10-14 years old. Comparison of antibody prevalence in the pre- and post-campaign children in the targeted age group suggests the campaign raised the immunity by 38-94% (average 77.5%) in proportion to pre-campaign levels (12-51%, average 36%, in absolute percentages). Following the campaign, significant levels of
susceptibility remain in the target ages, and, especially, in older children who were not targeted. These results highlight a need to identify prior susceptibility levels to define target age ranges, and suggest the upper age targeted was too young. The study made clear the problem of reliance on measles surveillance, which was not good enough to inform the decision on which age group to vaccinate in the campaign. The results raise concerns for continued circulation of measles and provide valuable information to health authorities for strategy refinement.

6.1. Introduction

Traditional methods of evaluating the effectiveness of an immunization programme have major limitations, particularly in the context of campaigns (Dietz and Cutts, 1997). These methods are based on estimating vaccine coverage, and case surveillance: reporting of clinical cases and analysis of trends in cases (epidemic/endemic behaviour). Reliability in resource poor countries may be undermined by inadequate communications, infrastructure and surveillance capacity. The percentage of the target population who receive vaccine, based on doses delivered and estimated denominator, is an indicator fraught with problems. Those receiving vaccine may be biased towards those accessible to services, and the denominator may exclude hard-to-reach groups (migrant, unregistered, and living in informal housing). Vaccine coverage surveys are likely to be less reliable in campaigns than for routine vaccination since vaccine certificates may not be issued or are less valued compared to 'vaccination certificates' (eg. Road-in-Health Cards) issued by Health Centers, and will be based more on verbal histories with unknown reliability. Interpretation of case reports following campaigns may suffer from
inadequate reporting infrastructure, misdiagnosis where incidence is low and serological confirmation is absent, and premature interpretation of trends in case reports, i.e. insufficient time may have elapsed for a rebound epidemic to occur (Nokes and Swinton, 1997).

Inadequacy of traditional surveillance techniques in evaluating and for refining vaccination campaigns, suggest that serological surveys could play a larger role. The need for serological evaluation of immunization programs has long been recognized (Horstmann, 1973; Evans, 1980).

Measles cross-sectional serological surveys could provide information as to whether the targeted, hard to reach and isolated populations are reached by vaccination, and identify what age groups should be included in immunization programmes (Babad et al., 1995; Gay et al., 1997a; Miller et al., 1977; Cox et al., 1998). Follow-up serological measurements in measles immunized persons has importance to determine the proportion developing immune responses, quality and extent of response, duration of response and level of protection against measles infection. From cross-sectional and follow-up serological surveys the risk of measles infection outbreak could be assessed.

Experience in the UK shows the use of oral-fluid sampling and screening for the surveillance of measles, mumps and rubella (MMR) since 1994 (Ramsay et al., 1997; Vyse et al., 2001). Using oral-fluid collection in the UK surveillance system contributed and enhanced the MMR disease notification system. Data derived from such a system have been used to refine the MMR vaccination policy (Ramsay et al., 1997). Our recent
studies in rural Ethiopia clearly demonstrated the utility of oral-fluid surveys in determining measles specific IgG in a large community based study (Nokes et al., 2001). This non-invasive technology, which has low risk to operators and donors, was well accepted and we obtained in excess of 90% compliance with oral fluid collection, compared to only 60% in serological surveys in Ethiopia (Nokes et al., 1998b; Fontanet et al., 1998). The study utilised an enhanced GACELISA assay described earlier in Chapter 2. In another study we evaluated pre- and post-vaccine antibody in children attending for routine measles immunization in Addis Ababa. This utilised an oral-fluid MACELISA described in Chapter 3. This work clearly showed the promise of oral-fluid testing as an alternative to serum in estimating vaccine response and effectiveness.

The present study assesses the effectiveness of a measles vaccination campaign in Asela town of Ethiopia using oral-fluid methods. The principal aim of the study is to demonstrate the application of oral-fluid sampling to evaluate a measles campaign. This was addressed through the following two process:

- Assess ‘immune’ status of children receiving vaccine in 1999 campaign in Ethiopia, i.e. identify what proportion of children attending for vaccination were already immune to measles. This would yield important information about the potential impact of the campaign, albeit within a selective sample.

- Estimate post-campaign prevalence of measles IgG in the target age group (9m-59m) and older ages post-campaign (up to 20 yrs). This would generate information on the immunity level in the targeted age group and level of susceptibility in those outside the target group. Ideally a representative cross-sectional oral-fluid survey prior to the
campaign should have been conducted. However there was insufficient time available to carry this out.

Information thus obtained would feed back to health authorities regarding campaign effectiveness and possible refinement to target age groups. During the survey, information was collected on knowledge and attitude of the study groups in relation to routine and campaign vaccinations. However, it was not relevant to this thesis and is not considered further here.

This study adopts a refined measles specific GACELISA (Microimmune EIA) developed as a standardized kit form by the CPHL, UK. This kit has undergone extensive evaluation using set of 717 paired blood and oral-fluid specimens previously reported in this thesis (Chapter 2 Section 2.3.2). The samples derive from a population in the edge of the rift valley in Ethiopia. Compared to sensitive serum assay results the Microimmune EIA had a sensitivity and specificity of 92%.

Ethical clearance was obtained from the National Ethical Committee at Ethiopian Science and Technology Commission (Ref. No. RDHE/84-31/2000) and Coventry Research Ethics Committee, Coventry, UK. Support letter (Ref. No.QEFAIO/2-7/1745) was obtained from The Council of Regional State of Oromiya Health Bureau, Arsi Zonal Health Department that involved with polio NID and measles campaign.
6.2. Materials and Methods

6.2.1. Study setting and population

The study site selected was Asela town with a population size of 50,000 (1994 census) and situated 180 km south of Addis Ababa on edge of rift valley in Arssi Zone, Oromia Region. All other Zones targeted for the 1999 measles campaign through the National Immunization Days (NID) of polio were over 300km from Addis Ababa. The NID utilised static immunization sites; these included regular static (a hospital and a health center) and outreach immunization sites, 14 ‘kebele’ offices. The population size of 9-59m was 6,375 for Asela during the campaign. Pre- and post-campaign selected ‘kebeles’ for the study are shown in Figure 6.1. The selection criteria are presented in the A6 (Appendix to Chapter 6). Measles routine vaccination in Asela was started in 1987. Since 1987 up to 1995 vaccination coverage of the town was reported together with Tiyo district where Asela is located and serving as the capital of the district. During 1999, the time when the campaign was undertaken, routine measles vaccination coverage of the town was 64.3%. No measles cases had been reported in Asela town since 1994 except a month old child was detected as measles suspected case in the hospital in 2000.

6.2.2. Survey methods

Full details of the survey methods including questionnaires used are given in the A6. In summary the survey methods were as follows:

- Collect oral fluid (OF) from children (9m to 5 years) at vaccination posts in Asela town at the time they present for measles campaign vaccination (during Polio NID).
Figure 6.1. Pre- and post-campaign selected 'kebeles' for Asela town measles immunization evaluation surveys conducted in 1999-2000.

• Conduct a cluster sample survey (i.e. representative of the population) of 9m-19yr olds in Asela Town post-campaign (see A6 for how the study design was implemented and the sample size determined).
• Screen using Microimmune OF assay (characteristics: 92% sensitive and 92% specific relative to serum assay)

6.2.3. Pre-campaign survey

The NID measles campaign was carried out between December 3-5th 1999. Vaccine was delivered through different stations over the three days period. From the total 14 ‘kebele’ office stations, 7 were randomly selected for the study. During the measles campaign, children aged 9-59 months visiting the 7 vaccination stations for measles vaccine provided OF samples for the study. Each site was supported by two study staff who enrolled children (parents/guardian), and collected and labeled specimens. In order not to hinder the throughput of children receiving vaccine, minimal questionnaire details were obtained (see questionnaire 1 A6). Data was available that was routinely collected at the time of measles/polio campaign, including household location, child age in months, sex, together with prior history of EPI vaccinations.

6.2.4. Post-campaign survey

The post-campaign community cluster survey was conducted six months after the pre-campaign NIDs between June 9th and July 10th 2000, involving 29 staff. These comprised 7 teams of 3 members to visit households, 3 supervisors, 2 laboratory assistants from Asela Health Centre to process samples for transport, and 3 support staff from the Ethiopian Health Nutrition Research Institute (EHNRI). Staff received training in sample collection, questionnaire delivery, and sample processing prior to the survey. Supervisors monitored work and checked questionnaire completion daily.
Seven clusters or Kebeles were selected out of the total of 14 in the town by probability proportional to population size (PPS) (See A6, appendix attached to this Chapter). The 1994 Government census (CSA, 1998) figures were used for the selection of Kebeles. 70 households were selected from each cluster by simple random sampling using the household registers held at each Kebele (see A6). All individuals from a household aged 9 months to <20 years of age were eligible. 750 individuals were recruited into this study providing questionnaire details and saliva samples. For each eligible individual details were collected on measles vaccination history, history of measles disease, EPI vaccine history, reasons for not receiving vaccines, and knowledge of vaccine preventable diseases (see questionnaire 3 and 4 in A6). For children under 15 years of age an appropriate adult respondent completed the questionnaire. Verbal consent was obtained before samples were taken.

6.2.5. Sample size estimation

6.2.5.1. Pre-campaign

The primary aim is to determine, with a set level of precision, the proportion, p, of measles antibody negative individuals attending vaccination centers who are susceptible at the time of the campaign. Based on measles surveys in urban and rural Ethiopia (Enquselassie et al., in prep; Nokes et al., 2001) it was estimated that 50% of individuals 9m-2 years, and 30% aged 3-5 years would be seronegative. Based on standard sampling theory for precision of prevalence estimates the required sample sizes for each groups were 340 and 560 individuals, respectively, to yield 95% CI for the proportion
seronegative in age class 9m-2 years and 3-5 years of 0.5 +/-0.054 and 0.3 +/-0.038, respectively.

6.2.5.2. Post-campaign
It was assumed that 10% of individuals 9m-<5 years, 15% aged 5-<10 years and 5% aged 10-<20 years would be seronegative in the post-campaign, respectively, with defined 95% CI of +/-0.04, 0.05, and 0.03. The required sample sizes for each groups were, therefore, 196, 195, 203 people, respectively. Based upon the population age-distribution of 10% <5, 12% 5-9, and 32% 10-19 years (1994 census), and an average household size of 5 (i.e. at least one child in each of the two younger age groups should be observed in every other HH), then a sample of 400 HHs should be adequate, or 440 HHs assuming 10% refusal.

6.2.6. Sample collection and processing
Each eligible individual was requested to provide an oral-fluid specimen, using the ORACOL device. Samples were labeled with a unique identifier and transported in stoppered tubes in cooled icebox to the local Health Center laboratory for processing. All samples were processed the same day of collection as described previously (Nokes et al, 1998b) and stored frozen at −10°C. At the end of the field surveys samples were returned to EHNRI and stored at −20°C.

6.2.7. Laboratory methods
Survey samples were transferred to CPHL packed in polystyrene cool boxes with dry ice. Samples were screened using the Microimmune in-house kit developed at CPHL. This
IgG antibody capture ELISA has been developed for commercial application. The kit has been evaluated using 717 paired serum/oral-fluid samples collected from Ethiopia; the paired serum of which had previously been tested by the Behring Enzygnost rubeolla IgG test (Behring Diagnostics, Marburg, Germany) (see Chapter 1 of this thesis). Sensitivity and specificity relative to serological results for this series was found to be 91.7 and 91.9% respectively, results as yet unpublished (Dr D. Samuel, CPHL, personal communication). The principles of the test in brief are as follows. Anti-human IgG coated microwells binds IgG in the test sample. After washing the wells, recombinant measles virus nucleoprotein (rMVN) antigen is added and will bind to wells when there is captured measles specific IgG antibodies. A monoclonal anti-measles antibody linked to HRP is then added to detect the immobilised recombinant measles antigen. After washing, the binding of anti-measles-HRP is visualised by adding TMB substrate. The presence of specific IgG results in HRP oxidising the colorless TMB substrate to an intense blue oxidised product, the signal of which is enhanced by adding a stopping solution, which changes the color to yellow. The optical density of the yellow chromogen is measured at 450/620nm. All components of the assay, apart from the 10 x wash buffer are provided as ready-to-use reagents. The assay incorporates color-coded reagents for monitoring sample addition steps and can be completed within 2h.

Specimens were identified as measles specific IgG positive, negative and equivocal results based on optical density (OD) readings as OD > NC (negative control) x 1.25, OD < NC x 1.1, and OD < NC x 1.25 and > NC x 1.1, respectively.
6.2.8. Data management and statistical analysis - clusters, conversion from oral-fluid prevalence to 'true' prevalence

Data from the questionnaires was double entered and validated using Epi-Info 6.04. Suitable entry checks were coded and data entered into 4 data entry programs (see A6). The laboratory results were entered into an Excel spreadsheet. Both Epi-Info files and Excel files were converted into Stata file format. Cleaning and statistical analysis were carried out using STATA V7.

Prevalence estimates were calculated using 'svy' commands in STATA to adjust the confidence intervals for the clustering effect. The oral-fluid prevalence of immunity estimates and confidence intervals were scaled, using an Excel spreadsheet, to adjust for the sensitivity (92%) and specificity (92%) of the oral-fluid assay compared to the serum assay as described previously (Nokes et al., 1998b). The observed age-prevalence profile from oral fluid can be transformed to the 'true' profile given the following relationship,

\[ P_o = P_s + \alpha (1 - P_s) - \beta P_s, \]

where, \( P_o \) is the proportion oral-fluid ('test') positive, \( P_s \) the proportion serum ('true') positive, \( \beta \) the probability of a false negative (with sensitivity 1 - \( \beta \)), and \( \alpha \) the probability of a false positive (with specificity 1 - \( \alpha \)). Thus, given \( \alpha \) and \( \beta \), and community estimates of prevalence obtained through an oral-fluid survey only, then it is possible to calculate the 'true' antibody prevalence from the above equation by rearranging as follows:

\[ P_s = \frac{P_o - \alpha}{1 - (\alpha + \beta)}. \]
6.3. Results

6.3.1. Sampling and population studies

The following two Tables show information on routine vaccination coverage, and the proportions of vaccine eligible and vaccine recipient children sampled in the pre- and post-campaign measles surveys of Asela town. The latter informs on how well the under 5 years children were covered in the campaign, and also on the sampling intensity of the pre- and post-campaign surveys. Table 6.3 shows the proportion of eligible children sampled stratified by age groups, in the pre- and post-campaign.

Table 6.1. Routine measles vaccination coverage in Asela town, 1996-2001

<table>
<thead>
<tr>
<th>Year</th>
<th>Estimated children</th>
<th>&lt; 1 year</th>
<th>Vaccinated</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996</td>
<td>1234</td>
<td></td>
<td>437</td>
<td>35.4</td>
</tr>
<tr>
<td>1997</td>
<td>1267</td>
<td></td>
<td>730</td>
<td>57.6</td>
</tr>
<tr>
<td>1998</td>
<td>1300</td>
<td></td>
<td>844</td>
<td>64.9</td>
</tr>
<tr>
<td>1999</td>
<td>1333</td>
<td></td>
<td>857</td>
<td>64.3</td>
</tr>
<tr>
<td>2000</td>
<td>1367</td>
<td></td>
<td>805</td>
<td>58.9</td>
</tr>
<tr>
<td>2001</td>
<td>1401</td>
<td></td>
<td>883</td>
<td>63.0</td>
</tr>
</tbody>
</table>

* = Routine vaccination was started in February 1987. Since 1979 up to 1995 vaccination coverage of Asela town was reported together with Tiyo district.

b = Computation of coverage based on the assumption that all the <1year children born within that year are supposed to be vaccinated for measles in the routine EPI activities.

Table 6.1 shows the reported vaccination coverage by year in Asela town, 1996-2001. Over this period coverage increased from 35.4% in 1996 to 63% in 2001. The trend shows that after 1997 the coverage does not seem to markedly increase in the following consecutive years. This proportion may be an underestimate if significant numbers of children receive vaccine in the second year of life.
Table 6.2. Proportion of vaccine eligible and vaccine recipient children sampled in the pre- and post-campaign measles surveys, Asela town, 1999-2000, stratified by kebele

| Vaccine site | Total popn* | <5years Eligible for vacc. (9-59m) | Eligible & vacc. recipient | % Pre-sampled (9-59m) | % Post-sampled (9-59m) | % Vacc. received (60-71m)  

<table>
<thead>
<tr>
<th>Kebele</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E=D/C</th>
<th>F</th>
<th>G=F/D</th>
<th>H</th>
<th>I=H/C</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>1481</td>
<td>230</td>
<td>205</td>
<td>147</td>
<td>71.7</td>
<td>-</td>
<td>-</td>
<td>22</td>
<td>10.7</td>
<td>0</td>
</tr>
<tr>
<td>02</td>
<td>3472</td>
<td>538</td>
<td>463</td>
<td>280</td>
<td>60.5</td>
<td>229</td>
<td>81.8</td>
<td>-</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>03</td>
<td>4255</td>
<td>659</td>
<td>568</td>
<td>511</td>
<td>90.0</td>
<td>-</td>
<td>-</td>
<td>24</td>
<td>4.2</td>
<td>17</td>
</tr>
<tr>
<td>02</td>
<td>5713</td>
<td>801</td>
<td>690</td>
<td>383</td>
<td>55.5</td>
<td>-</td>
<td>-</td>
<td>26</td>
<td>3.8</td>
<td>15</td>
</tr>
<tr>
<td>05</td>
<td>4345</td>
<td>673</td>
<td>566</td>
<td>325</td>
<td>57.4</td>
<td>216</td>
<td>66.5</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>06</td>
<td>4079</td>
<td>632</td>
<td>551</td>
<td>339</td>
<td>61.5</td>
<td>315</td>
<td>92.9</td>
<td>21</td>
<td>3.8</td>
<td>69</td>
</tr>
<tr>
<td>07</td>
<td>5636</td>
<td>874</td>
<td>747</td>
<td>598</td>
<td>80.1</td>
<td>396</td>
<td>66.2</td>
<td>34</td>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td>08</td>
<td>2507</td>
<td>389</td>
<td>333</td>
<td>185</td>
<td>55.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>09</td>
<td>5632</td>
<td>873</td>
<td>780</td>
<td>882</td>
<td>113</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>3.8</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>3137</td>
<td>486</td>
<td>429</td>
<td>316</td>
<td>73.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>1684</td>
<td>261</td>
<td>224</td>
<td>170</td>
<td>75.9</td>
<td>132</td>
<td>77.6</td>
<td>30</td>
<td>13.4</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>2207</td>
<td>342</td>
<td>297</td>
<td>187</td>
<td>63.0</td>
<td>30</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>1055</td>
<td>164</td>
<td>152</td>
<td>118</td>
<td>77.6</td>
<td>93</td>
<td>78.8</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>2728</td>
<td>423</td>
<td>372</td>
<td>254</td>
<td>68.3</td>
<td>186</td>
<td>73.2</td>
<td>-</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Grand total</td>
<td>47391</td>
<td>7345</td>
<td>6377</td>
<td>4695</td>
<td>73.6</td>
<td>1567</td>
<td>75.2</td>
<td>187</td>
<td>5.0</td>
<td>181</td>
</tr>
</tbody>
</table>

* = Based on the population for 1999 derived by projecting the 1994 government census. Projection provided by Arisi Zone EPI Office.

= Total vaccinated population of 7 sampled kebeles was 2084

= Total population of 7 sampled kebeles was 3764

= Data from Arisi Zone EPI Office

Table 6.2 show the sample size of the vaccinated population enrolled in the pre-campaign survey. It also shows the proportion of vaccine eligible children that were sampled in the post-campaign survey. Of the total 6375 measles vaccine eligible children (9-59m) 4695 (73.6%) were vaccinated in the campaign. Of the vaccinated children in the seven selected 'kebeles' (total population= 2084) 1567 (75.2%) were sampled in the pre-
campaign. Of the total vaccine eligible in the seven selected ‘kebeles’ (total population=3764) 187 (5%) of them were selected in the post-campaign.

Table 6.3. Proportion of eligible children sampled by age groups, Asela town pre- and post-campaign measles surveys, 1999-2000.

<table>
<thead>
<tr>
<th>Age group (month)(^a)</th>
<th>Population(^b)</th>
<th>Pre-vaccine sample</th>
<th>Proportion (%)</th>
<th>Post-vaccine sample</th>
<th>Proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>B/A</td>
<td>C</td>
<td>C/A</td>
</tr>
<tr>
<td>9-11</td>
<td>272</td>
<td>94</td>
<td>34.6</td>
<td>6</td>
<td>2.2</td>
</tr>
<tr>
<td>12-23</td>
<td>1220</td>
<td>304</td>
<td>24.9</td>
<td>31</td>
<td>2.5</td>
</tr>
<tr>
<td>24-35</td>
<td>1423</td>
<td>367</td>
<td>25.8</td>
<td>41</td>
<td>2.9</td>
</tr>
<tr>
<td>36-47</td>
<td>1482</td>
<td>386</td>
<td>26.0</td>
<td>46</td>
<td>3.1</td>
</tr>
<tr>
<td>48-59</td>
<td>1980</td>
<td>416</td>
<td>21.0</td>
<td>63</td>
<td>3.2</td>
</tr>
<tr>
<td>Sub total</td>
<td>6377</td>
<td>1567</td>
<td>24.6</td>
<td>187</td>
<td>2.9</td>
</tr>
<tr>
<td>60-71</td>
<td>1569</td>
<td>361</td>
<td>23.0</td>
<td>29</td>
<td>1.8</td>
</tr>
<tr>
<td>Total</td>
<td>7946</td>
<td>1928</td>
<td>24.3</td>
<td>216</td>
<td>2.7</td>
</tr>
</tbody>
</table>

\(^a\) = Although the ‘eligible’ age was 9-59m a significant proportion of children age 5 years i.e. 6 -71 months did come for vaccination, and therefore were sampled in our study. Note that at vaccination posts, the age category of children receiving vaccine was not recorded accurately.

\(^b\) = Based on the population for 1999 derived by projecting the 1994 government census. Projection provided by Arsi Zone EPI office.

Table 6.3 shows ranges of 21-35% (average of 24.6%) of the eligible children were sampled in the age groups between 9-59 months in the pre-campaign survey. Ranges of 2-3% (average of 2.9%) of the eligible children were sampled in the age groups between 9-59 months in the post-campaign survey.
### 6.3.2. Measles Immunity in pre- and post-campaign surveys

Table 6.4. Measles immunity prevalence (observed /‘true’) in a target age group of pre-campaign survey of Asela town, 1999.

<table>
<thead>
<tr>
<th>Age group (months)</th>
<th>Positive / Total</th>
<th>Observed prevalence</th>
<th>95% CI [unadjusted adjusted]</th>
<th>‘True’ prevalence</th>
<th>95% CI [unadjusted adjusted]</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-11</td>
<td>28/94</td>
<td>0.298</td>
<td>0.214-0.398 / 0.216-0.395</td>
<td>0.259</td>
<td>0.160-0.379 / 0.162-0.375</td>
</tr>
<tr>
<td>12-23</td>
<td>108/304</td>
<td>0.355</td>
<td>0.303-0.414 / 0.291-0.425</td>
<td>0.328</td>
<td>0.265-0.394 / 0.251-0.411</td>
</tr>
<tr>
<td>24-35</td>
<td>161/367</td>
<td>0.439</td>
<td>0.389-0.490 / 0.368-0.512</td>
<td>0.427</td>
<td>0.368-0.488 / 0.343-0.514</td>
</tr>
<tr>
<td>36-47</td>
<td>180/386</td>
<td>0.466</td>
<td>0.417-0.516 / 0.381-0.553</td>
<td>0.460</td>
<td>0.401-0.519 / 0.358-0.563</td>
</tr>
<tr>
<td>48-59</td>
<td>235/416</td>
<td>0.565</td>
<td>0.517-0.612 / 0.520-0.609</td>
<td>0.577</td>
<td>0.520-0.633 / 0.524-0.630</td>
</tr>
<tr>
<td>60-71</td>
<td>210/361</td>
<td>0.582</td>
<td>0.530-0.632 / 0.501-0.658</td>
<td>0.597</td>
<td>0.536-0.657 / 0.501-0.688</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>922/1928</strong></td>
<td><strong>0.478</strong></td>
<td></td>
<td><strong>0.474</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.4 shows measles IgG prevalence of 1928 samples in age groups 9-11 months to 60-71 months (ie inclusive of 5 years olds). The Table provides an estimate of the prevalence of measles immunity in children prior to receiving vaccine. 95% CIs are presented as unadjusted and adjusted for clustering effects. The results suggest that 26% (9-11m) to 60% (5 years olds) of children (average of 47%) already had measles antibody prior to the campaign (‘true’ prevalence).
Table 6.5. Measles immunity prevalence (observed /‘true’) by age group in a post-campaign survey of Asela town, 2000.

<table>
<thead>
<tr>
<th>Age group (months)</th>
<th>Positive / Total</th>
<th>Observed prevalence</th>
<th>95% CI [unadjusted / adjusted]</th>
<th>‘True’ prevalence</th>
<th>95% CI [unadjusted / adjusted]</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-11</td>
<td>2/6</td>
<td>0.333</td>
<td>0.084-0.733 / 0.118-0.652</td>
<td>0.301</td>
<td>0.005-0.777 / 0.045-0.681</td>
</tr>
<tr>
<td>12-23</td>
<td>19/31</td>
<td>0.613</td>
<td>0.434-0.766 / 0.454-0.751</td>
<td>0.635</td>
<td>0.421-0.817 / 0.445-0.799</td>
</tr>
<tr>
<td>24-35</td>
<td>32/41</td>
<td>0.780</td>
<td>0.629-0.882 / 0.627-0.883</td>
<td>0.834</td>
<td>0.654-0.955 / 0.651-0.956</td>
</tr>
<tr>
<td>36-47</td>
<td>39/46</td>
<td>0.848</td>
<td>0.713-0.926 / 0.632-0.948</td>
<td>0.914</td>
<td>0.754-1.007 / 0.657-1.033</td>
</tr>
<tr>
<td>48-59</td>
<td>50/63</td>
<td>0.794</td>
<td>0.676-0.876 / 0.653-0.887</td>
<td>0.850</td>
<td>0.710-0.948 / 0.682-0.961</td>
</tr>
<tr>
<td>60-71</td>
<td>24/29</td>
<td>0.828</td>
<td>0.646-0.927 / 0.732-0.894</td>
<td>0.890</td>
<td>0.674-1.008 / 0.776-0.969</td>
</tr>
<tr>
<td>Total</td>
<td>166/216</td>
<td>0.769</td>
<td></td>
<td>0.820</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.5 shows measles IgG prevalence of 216 samples in age groups 9-11 months to 60-71 months (i.e. inclusive of 5 years olds). The Table provides an estimate of the prevalence of measles immunity in children 6 months after the measles campaign of December 1999. The results suggest that 30% - 63%, in age classes 9-11m and 12-23m, respectively, and 83% - 91% in age classes 24-35m to 60-71m (average of 82%) had measles antibody at this time. Note that by July 2000, all children 9-11 months and a significant proportion of those aged 1 year would have been too young to be offered vaccine in the 1999 campaign. Similarly a significant proportion of children aged 6 years (outside the vaccination target age range) would have been aged 5 years at time of the campaign (i.e. within the target age range). Hence the relatively low proportion positive in the 0-1 yr olds and the relatively high proportion positive in the 6 years olds.
Figure 6.2 shows the prevalence of measles specific IgG in individuals from 0-19 years collected in early July 2000. Black bars indicate OF prevalence and open bars 'true' prevalence. The proportion negative in older children was 39% in those 7-9 years of age, and 19% in those 10-14 years old.

Table 6.6 suggests no marked sex difference in measles immunity prevalence (79.5% for male vs 79.2% for female) in the post-campaign survey based on 750 individuals of < 20 year olds. Aggregation into 5 years age groups masks the underlying pattern seen in Figure 6.2. The 15-19 years age group appears to have higher prevalence than other age bands.

Table 6.7. Impact of the measles immunization campaign on measles ‘immunity’ of target age groups of Asela town, 1999-2000

<table>
<thead>
<tr>
<th>Age group (months)</th>
<th>'True' prevalence A</th>
<th>Age group (months)</th>
<th>'True' prevalence B</th>
<th>Difference Prevalence B/(1 - A) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-11</td>
<td>0.259</td>
<td>15-17</td>
<td>0.381</td>
<td>0.122</td>
</tr>
<tr>
<td>12-23</td>
<td>0.328</td>
<td>18-29</td>
<td>0.836</td>
<td>0.508</td>
</tr>
<tr>
<td>24-35</td>
<td>0.427</td>
<td>30-41</td>
<td>0.935</td>
<td>0.508</td>
</tr>
<tr>
<td>36-47</td>
<td>0.460</td>
<td>42-53</td>
<td>0.852</td>
<td>0.392</td>
</tr>
<tr>
<td>48-65</td>
<td>0.593</td>
<td>54-71</td>
<td>0.872</td>
<td>0.279</td>
</tr>
<tr>
<td>Overall</td>
<td>0.481</td>
<td></td>
<td>0.863</td>
<td>0.382</td>
</tr>
</tbody>
</table>

**Note:**
- 'True' prevalence A and B.
- Difference Prevalence B/(1 - A) %.

The 'true' prevalence increased from 25.9% to 38.1%, a difference of 12.2% (16.5% reduction). However, the overall susceptible prevalence reduction was 73.6%.

Table 6.7 compares the estimated true prevalence of measles 'immunity' in 9m-65m olds in the pre-campaign with that of 15-71m old children in the post-campaign. This provides a rough estimate of the effectiveness of the campaign at reducing susceptibility to measles in the target population. Overall, it is estimated that within the target age range 9m-65m, there was a rise in 'true' prevalence of 48% to 86%, or a susceptible prevalence reduction of 74% (Table 6.7). An absolute prevalence increment of between 12% (9-11 month group) to 51% (12-35 month age class) were observed. Reduction in the proportion susceptible was least (16.5%) in the 9-11 month group, and between 67% and
89% in all other group. In the target age range 12m-65m (18-71m post-campaign) measles antibody prevalence was estimated to be between 84-94%.


<table>
<thead>
<tr>
<th>'Kebeles'</th>
<th>Pre-campaign (9m-71m)</th>
<th>Post-campaign (9m to &lt;20 years)</th>
<th>Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos/Tot</td>
<td>%</td>
<td>Pos/Tot</td>
</tr>
<tr>
<td>01</td>
<td>-</td>
<td>-</td>
<td>79/98</td>
</tr>
<tr>
<td>02</td>
<td>120/273</td>
<td>44.0</td>
<td>-</td>
</tr>
<tr>
<td>03</td>
<td>-</td>
<td>-</td>
<td>73/88</td>
</tr>
<tr>
<td>04</td>
<td>-</td>
<td>-</td>
<td>102/123</td>
</tr>
<tr>
<td>05</td>
<td>131/254</td>
<td>51.6</td>
<td>-</td>
</tr>
<tr>
<td>06</td>
<td>176/394</td>
<td>44.7</td>
<td>87/113</td>
</tr>
<tr>
<td>07</td>
<td>250/484</td>
<td>51.7</td>
<td>76/102</td>
</tr>
<tr>
<td>08</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>09</td>
<td>-</td>
<td>-</td>
<td>96/125</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>85/166</td>
<td>51.2</td>
<td>82/101</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>55/115</td>
<td>47.8</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>105/242</td>
<td>43.4</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>922/1928</td>
<td>47.8</td>
<td>595/750</td>
</tr>
</tbody>
</table>

The prevalence of pre- and post-campaign measles immunity by selected 'kebeles' is given in Table 6.8. The highest prevalence of measles immunity (about 51%) was observed in 'kebeles' 05, 07 and 11 in the pre-campaign and in 'kebeles' 01, 03, 04 and 11(about 80%) in the post-campaign. The lowest prevalence observed in 'kebele' 14 (43%) in the pre-campaign and 'kebele' 07 (75%) in the post-campaign. Comparison of measles immunity between the pre- and post-simultaneously selected 'kebeles' shows measles immunity raised from 45% (06 ‘kebele’), 52% (07 ‘kebele’) and 51% (11
'kebele') in pre-campaign to 77%, 75% and 81% in the post-campaign respectively. The resultant reduction in proportion susceptible in each 'kebele' was 52-62% (average 60%).

6.3.3. Measles vaccination status

Data from the pre-campaign survey (Table 6.9) shows that 78% of children had a history of routine measles vaccination (card or recall). Of these children 49-62% were measles antibody positive, which compared with 21% in those without history of vaccination.

Table 6.10 presents descriptive statistics of 606 children under 15 years of age enrolled in the post-campaign survey. Around 86% of these children had a history of routine measles vaccination. In these children who would have been of eligible age for vaccination during the campaign, 88% had a history of routine measles vaccination. In these groups, between 83% and 88% were measles positive. Children without a history of routine vaccination had marginally lower seroprevalence (77-80%). Of children aged 12-23 months 74% had a history of routine measles vaccination in whom measles seroprevalence was between 66-80%. This compared with only 35% seropositivity in children of this group with no routine vaccination.

In Table 6.10 comparison is made of routine vaccination history and seroprevalence in children of eligible age who did or did not attend for campaign measles vaccination. The proportion without history of measles vaccination is slightly lower in those who did not attended for campaign vaccination (16%). Measles antibody prevalence in children vaccinated during the campaign was 89-100%. In children of eligible age who did not receive measles vaccination in the campaign, measles seroprevalence was between 60%
(no history of routine measles vaccination) and 72-85% (history of routine measles vaccination).

Table 6.9. Descriptive statistics for the pre-campaign measles survey

<table>
<thead>
<tr>
<th>Measles vaccination</th>
<th>n</th>
<th>%</th>
<th>Positive</th>
<th>'OF' %</th>
<th>'True' %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes card</td>
<td>25</td>
<td>1.3</td>
<td>15</td>
<td>60.0</td>
<td>61.9</td>
</tr>
<tr>
<td>Yes recall</td>
<td>1482</td>
<td>76.9</td>
<td>729</td>
<td>49.2</td>
<td>49.0</td>
</tr>
<tr>
<td>No</td>
<td>147</td>
<td>7.6</td>
<td>38*</td>
<td>25.4</td>
<td>20.7</td>
</tr>
<tr>
<td>Unknown</td>
<td>194</td>
<td>10.1</td>
<td>91</td>
<td>46.9</td>
<td>46.3</td>
</tr>
<tr>
<td>Missing</td>
<td>80**</td>
<td>4.2</td>
<td>49</td>
<td>61.3</td>
<td>63.5</td>
</tr>
</tbody>
</table>

* = Of the 38 children (7.9%) of them reported to have history of measles infection
** = 97.5% of these children were above 3 old years (36m-71m)
Table 6.10. Descriptive statistics on vaccine uptake from the post-campaign measles survey (for children <15 years)

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>%</th>
<th>Positive</th>
<th>'OF' %</th>
<th>'True' %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Measles vaccination status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Card</td>
<td>84</td>
<td>13.9</td>
<td>67</td>
<td>79.8</td>
<td>85.5</td>
</tr>
<tr>
<td>Recall</td>
<td>434</td>
<td>71.6</td>
<td>336</td>
<td>77.4</td>
<td>82.6</td>
</tr>
<tr>
<td>None</td>
<td>88</td>
<td>14.5</td>
<td>64</td>
<td>72.7</td>
<td>77.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>606</td>
<td>100</td>
<td>467</td>
<td>77.1</td>
<td>82.3</td>
</tr>
<tr>
<td><strong>Routine measles vaccination in eligible children</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Card</td>
<td>46</td>
<td>23.0</td>
<td>37</td>
<td>80.4</td>
<td>86.2</td>
</tr>
<tr>
<td>Recall</td>
<td>130</td>
<td>65.0</td>
<td>106</td>
<td>81.5</td>
<td>87.5</td>
</tr>
<tr>
<td>None</td>
<td>24</td>
<td>12.0</td>
<td>18</td>
<td>75.0</td>
<td>79.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>200</td>
<td>100</td>
<td>161</td>
<td>80.5</td>
<td>86.3</td>
</tr>
<tr>
<td><strong>Routine measles vaccination in children 12-23 months of age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Card</td>
<td>11</td>
<td>35.5</td>
<td>7</td>
<td>63.6</td>
<td>66.2</td>
</tr>
<tr>
<td>Recall</td>
<td>12</td>
<td>38.7</td>
<td>9</td>
<td>75.0</td>
<td>79.8</td>
</tr>
<tr>
<td>None</td>
<td>8</td>
<td>25.8</td>
<td>3</td>
<td>37.5</td>
<td>35.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>31</td>
<td>100</td>
<td>19</td>
<td>61.3</td>
<td>63.5</td>
</tr>
<tr>
<td><strong>Routine measles vaccination in eligible children who reported for measles vaccination in the campaign.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Card</td>
<td>30</td>
<td>24.4</td>
<td>26</td>
<td>86.7</td>
<td>93.7</td>
</tr>
<tr>
<td>Recall</td>
<td>81</td>
<td>65.9</td>
<td>67</td>
<td>82.7</td>
<td>88.9</td>
</tr>
<tr>
<td>None</td>
<td>12</td>
<td>9.8</td>
<td>11</td>
<td>91.7</td>
<td>99.6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>123</td>
<td>100</td>
<td>104</td>
<td>84.6</td>
<td>91.2</td>
</tr>
<tr>
<td><strong>Routine measles vaccination in eligible children who did not report for measles vaccination in the campaign.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Card</td>
<td>16</td>
<td>20.8</td>
<td>11</td>
<td>68.8</td>
<td>72.4</td>
</tr>
<tr>
<td>Recall</td>
<td>49</td>
<td>63.6</td>
<td>39</td>
<td>79.6</td>
<td>85.2</td>
</tr>
<tr>
<td>None</td>
<td>12</td>
<td>15.6</td>
<td>7</td>
<td>58.3</td>
<td>59.9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>77</td>
<td>100</td>
<td>57</td>
<td>74.0</td>
<td>78.6</td>
</tr>
</tbody>
</table>

* = Eligible children are those between 9-59m age group described in Table 6.2.

6.4. Discussion

The objective of this study was to evaluate a measles vaccination campaign in an urban developing country population and demonstrate a transferable oral-fluid methodology for this purpose. Thus the present work highlights the potential of oral-fluid methods to evaluate the effect of the vaccination campaign on population immunity to measles.
The pre-survey is a biased sample in so far as children attending for vaccination may differ in some important characteristics from those who do not attend. For example, we don't know what was the proportion measles positive in children who did not turn up for vaccination. However, nearly three quarters of all children of eligible age did attend for vaccination, and of these vaccinated children within a random sample of 7 vaccination centers three quarters were sampled and immunity tested. Therefore the pre-campaign survey provides a precise estimate of immunity in those receiving vaccine, which in turns constitutes the vast majority of the eligible population.

The post-campaign provided an opportunity to compare routine vaccine uptake in those who attend for campaign vaccination with those who did not. The survey revealed (Table 6.10) little difference between the two groups; children of eligible age attending the campaign 24% had card evidence and 66% recall of routine measles vaccination, as compared with 21% and 64%, respectively. This supports the assertion that the pre-campaign survey provides a reasonably accurate estimate of measles immunity in the community as a whole against which to compare post-vaccination survey data.

Furthermore, the post-vaccine survey comprised a simple random sample of households within clusters selected with probability proportion to size. Prevalence estimates in the pre-campaign survey were weighted for different target population sizes covered by each vaccination center ('kebele') and confidence intervals for both pre- and post-surveys were adjusted for clustering of observations within 'kebeles', (i.e. to take account of the
possibility of reduced variability in immune status of individuals within clusters). We consider estimates of prevalence for such survey to be representative of the community. It should be noted however, that selection of vaccination centers (‘kebele’ offices) should ideally have been made using PPS sampling. However, household registration data at that time was not available.

An indication of the impact of the campaign is thus provided by comparison of the pre- and post-campaign surveys. This suggests campaign vaccination to have elevated immunity in the target age groups by as much as 50% in some age groups, and an average of 36% in the target age group as a whole. A more useful measure of impact is the estimate of the fraction of susceptibles that become immune through the campaign (Table 6.7). This suggests that in age groups 12-23m to 48-65m 67-89% of susceptibles acquired immunity, or an average in the target age group of 74%. If we assume 90% vaccine efficacy, then we might estimate the coverage in the target group was approximately 70%. These estimates of the reduction in susceptible proportion compare well with the estimated proportion of 74% of Asela town eligible children receiving vaccine. No measles infection was reported in the six months period elapsed between the pre- and post-campaign survey as the data from the Health Center and Hospital indicated, and other than one case in 2000, no measles cases were seen in Asela since 1994 (Source: Arsi Zonal Health Bureau, 2001). However, these data may not accurately reflect the rate of natural infection in this period since the community in Southern Ethiopia do not have positive beliefs and attitudes towards seeking health services for the ‘measles sick child’ (Lindtjorn, 1986).
One of the important factors that influence the success of vaccination campaigns is the age range vaccinated (Nokes and Swinton, 1997; Cutts and Steinglass, 1998). The post-campaign survey suggests that there remains relatively high prevalence of susceptibility in older age groups, e.g. 39% of 7-9 years olds, and 19% of 10-14 years olds. The lower immunity levels in the older age groups may be due to historically low routine immunization coverage when these groups were eligible and reduced transmission of measles virus in the community due to moderate immunization coverage. Records of measles coverage of Asela town were reported together with Tiyo district between 1987-1995 and cannot be disaggregated. However, we know that in 1990 the national measles immunization coverage was 9%. Some of the children aged between 12-14 years were not reached by the routine immunization as the Asela routine measles immunization was started in 1987. In future, the effectiveness of campaigns might be increased by an increase in the age range targeted for supplemental immunization (e.g. up to age 9 or 14 years). The overall measles immunity in the under 5 years was found to be 77.5% after the post-campaign survey. The routine and supplemental immunization activities need to be strengthened to increase population immunity in order to establish the protective herd immunity $R_0 \rightarrow > 93\%$ (Anderson and May, 1991).

Pre-campaign immunity was 26% in the 9-11 month olds, rising to 59% in 48-65m age group. This low level of immunity (average $\sim 48\%$) in the pre-vaccine age group appears not to be due to mothers with recent memory of their child attending for routine measles vaccination preferentially deciding not to get them vaccinated in the campaign. In fact
routine measles vaccination history was slightly higher in children attending for campaign vaccination than those not (Table 6.10). Alternatively, the estimated low-level immunity might arise from problems associated with the sensitivity of the assay, for example, in vaccinees with lower levels of measles antibody. Finally, it may be that vaccine coverage data is unreliable or vaccine is not effectively administered. However, our surveys pre- and post-immunization provided estimates of routine vaccination coverage of 78% and 74-88%, respectively; somewhat higher than official coverage statistics (60-65%). While vaccine failure cannot be ruled out, it would appear than in the campaign at least vaccine efficacy was high (~ 90%). Further studies to investigate the performance of the Microimmune assay in highly vaccinated populations; persons of lower vaccine history and in different age groups are required.

The post-campaign survey shows a lower level of immunity in those who did not attend the campaign (79%) compared to those who attended the campaign, which is 91% (Table 6.10). The pre-vaccine survey shows that 78% of the children did have a history of routine vaccination by card and recall (Table 6.9). Similarly in the post-campaign survey 85.5% of the children had a history of routine vaccination by card and recall (Table 6.10). However, in the pre-campaign, of those who said they had been vaccinated (78%), only ~55% were found to have measles immunity. Official coverage data (Table 6.1) shows 60-65% through 1997-2001. It is interesting that the post-campaign prevalence of immunity in campaign non-attenders (79%) was considerably higher than those of the pre-campaign children who attended for campaign vaccination (48%). The explanation
for the lower level of immunity observed in the pre-campaign could be due to vaccine
delivery and efficacy or the assay, as mentioned above.

An issue to be addressed in evaluating the effectiveness of mass immunization campaigns
is to assess whether the hard to reach and isolated populations are reached by vaccination
(Dietz and Cutts, 1997). In this study these groups are not addressed due to the absence of
community census information.

Measles, mumps and rubella surveillance based on oral fluid has been routinely
undertaken in the UK (Ramsay, Brugha and Brown, 1997). The primary role, however,
remains the confirmation of measles diagnosis by detecting measles specific IgM. This
study presents a new model to evaluate a measles vaccination campaign using oral-fluid
antibody surveys. There is only one previous example of a study undertaken to assess
immunity prevalence using OF following a vaccination campaign, which was a study of
rubella in Brazil (Azevedo Neto et al., 1995). However, that study used GACRIA, which
is not easily transferred to developing country laboratories. The present study was
designed and performed in such a way as to be applicable to the developing country
context. In essence it uses a simple transferable oral-fluid method (Microimmune OF kit)
for determining measles specific IgG antibody that opens the way for widespread use in a
surveillance capacity. An obstacle to the widespread use of OF samples for surveillance
has been the lack of commercial tests optimised in a ready-to-use kit form. The
Microimmune IgG capture EIA has been optimised for such purpose. The details of the
measles survey work activities (see appendix A6) presented in this thesis provide a basis
for guidelines for field application of oral-fluid methods. With this regard a generic protocol designed to be prepared for the uses of oral fluid in measles vaccine programme evaluation would be timely by which to accelerate future application of these methods.
MEASLES SURVEYS WORK ACTIVITIES

1. General

The survey teams played an important role in the success of the study. Each survey team comprised 3 members to visit households who received training in sample collection and questionnaire delivery. Survey teams who were visiting the household should first introduce themselves and obtain informed consent from the head of the household by explaining:

- Title of the research project
- Basic information about the disease and objective of the study
- Information about coordinators of the project-they need to describe that although the study was coordinated by EHNRI and University of Warwick it was not possibly realized without the support of health authorities at regional, zonal and local levels.
- Information about how the work was carried out

The interviewers were instructed to pay the maximum effort to obtain the correct answers for each question.

2. Pre-campaign survey and questionnaire

Prior to the surveys of the campaign visits were made to Asela town to present the study plan to local health officials and to administrative officials of the 'kebeles' (urban dwellers associations). During these visits effort was made to identify the vaccine delivery sites. Local ‘kebele’ offices were used as vaccine delivery stations over the three days period. Of the total 14 stations 7 were selected by simple random sampling (i.e. each had equal probability of selection) for the study purpose. At each site we based 2
study staff, that were local nurses, who enrolled children (parents/guardian), filled questionnaires and collected and labeled specimens.

In order not to hinder the throughput of children receiving measles vaccine, brief questionnaire details were obtained. The one page questionnaire was administered to each child prior taking the measles vaccine. Information such as address, name and number of vaccination site, number and code of the child, date and lab number were available to the survey teams prior administering the questionnaire for each child (includes information routinely collected by measles vaccine staff at site). The additional information collected on each child including age, sex, parental education level, history of measles infection and measles vaccination (see questionnaire 1).

3. Post-campaign survey and questionnaire

The sampling frame comprised the register of households at each ‘kebele’. In Asela town there were 14 ‘kebeles’ each with different population size. Seven clusters or ‘kebeles’ were selected out of the total 14 by probability proportional to population size (PPS). Any individuals from a household aged 9 months to <20 years were eligible for inclusion.

4. Methods of sample collection

The last Government census was carried out in 1994 (CSA, 1998). A more recent population estimate from 1999 was available, collated by the health center, during active surveillance of households before the NIDs. Unfortunately, this more recent surveillance was thought to be an underestimate on the basis that it was not performed in a way planned for population census. It was therefore thought necessary to use the official 1994 statistics. The census was then projected for 1999 for the selection of ‘kebeles’ (clusters)
with PPS. The process of selection of clusters is described in Table 1 below. The population for each ‘kebele’ (column 2) was accumulated (Column 3). The sampling interval was calculated from the cumulative total divided by the number of clusters required (i.e. 47391/7 = 6770). The starting individual number within the first sampling interval was selected by random number table (i.e. random number between 1 and 6770). The random number selected for the starting point was 639, i.e. selection of ‘kebele’ 1. A further 6 ‘kebeles’ were selected by sequentially adding the sampling interval to the starting number (column 4), i.e. ‘kebeles’ 3, 4, 6, 7, 9, 11 (column 5).

For the household selection within chosen ‘kebeles’, the household registers for each kebele were used (Table 1, column 6). Each register lists the number of households with a named head of household, number of household members, by sex, number in household <5yrs, by sex, and house number. Since there was no community census information about the hard to reach population (such as migrants, those living in informal housing, etc) it was not possible to include this important group in our study.
Table A6.1. Process of selection of 7 clusters from 14 ‘kebeles’ of Asela using PPS.

<table>
<thead>
<tr>
<th>Kebele</th>
<th>Population</th>
<th>Cumulative population</th>
<th>Numbers for selecting clusters</th>
<th>Cluster</th>
<th>Registered number of household s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1481</td>
<td>1481</td>
<td>639</td>
<td>1</td>
<td>280</td>
</tr>
<tr>
<td>2</td>
<td>3472</td>
<td>4953</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4255</td>
<td>9208</td>
<td>7409</td>
<td>2</td>
<td>926</td>
</tr>
<tr>
<td>4</td>
<td>5173</td>
<td>14381</td>
<td>14179</td>
<td>3</td>
<td>829</td>
</tr>
<tr>
<td>5</td>
<td>4345</td>
<td>18726</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4079</td>
<td>22805</td>
<td>20949</td>
<td>4</td>
<td>652</td>
</tr>
<tr>
<td>7</td>
<td>5636</td>
<td>28441</td>
<td>27719</td>
<td>5</td>
<td>988</td>
</tr>
<tr>
<td>8</td>
<td>2507</td>
<td>30948</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>5632</td>
<td>36580</td>
<td>34489</td>
<td>6</td>
<td>684</td>
</tr>
<tr>
<td>10</td>
<td>3137</td>
<td>39717</td>
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<td>11</td>
<td>1684</td>
<td>41401</td>
<td>41259</td>
<td>7</td>
<td>319</td>
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<td>12</td>
<td>2207</td>
<td>43608</td>
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<td></td>
<td></td>
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<tr>
<td>13</td>
<td>1055</td>
<td>44663</td>
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<td>14</td>
<td>2728</td>
<td>47391</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47391</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. Selection of households

Seventy households were to be selected from each ‘kebele’ using the ‘kebele’ registers. The registers were not listed in sequential order as different health workers had completed different sections. Each household in each register from a selected ‘kebele’ was given a new number starting at 1. This way the number of households per ‘kebele’ was checked and the households listed suitably for a random selection. Random number lists of 70 households were generated using Stata after calculating the proportion of households in each ‘kebele’ to be selected.

6. Household information form

This form (attached in this appendix) was designed to help organise the field work and provide a method of monitoring numbers of samples and questionnaires collected and number of households visited. From the ‘kebele’ registers the name of the household and
house number were identified and entered by supervisors onto the form created for each team of field workers for data collection on households selected. When visiting the household it was thought necessary to obtain information on those eligible/ineligible, if consent is given, where necessary, and reasons if unavailable. For practical purposes it was also useful to note whether samples were collected and questionnaires completed for those eligible and if they were not available at the time to note when they would be available for revisit.

7. Questionnaire 3 and 4 for children who participated in the survey

Questionnaire 3 was for <15 years old children for whom the parents/guardian responded. Questionnaire 4 was for children >=15 years, who responded for themselves with or without parents. The two questionnaires are similar except the <15 questionnaire asked some more information about the history and vaccination status of the children (where it was though there would be less recall bias). For some children who were <15 at the time of the measles campaign but outside the target age range, some of the questions specific to participating in the campaign were not relevant.

8. Discussion of questionnaires

The following questions were discussed with the survey teams.

- Misreporting of age is a common problem in Ethiopia. Very few parents keep record of date of birth of their children or issuance of birth certificate is uncommon as most births take place at home. In view of these it was discussed to take the necessary considerations to avoid misreporting age. Age was therefore imputed either by the respondents and/or enumerators on the basis of linking the probable age with some presumably known events.
• BCG scar: It was decided that it would be useful to check for BCG scar where there was only verbal response to whether or not an individual had received the vaccine. The interviewers were familiar with what a BCG scar was as they all work in the Health Centers. This was the case when the interviewers were asked at the training meeting.

9. Staffing for the survey

Three health staffs supervised the survey. Two laboratory personnel from EHNRI, Addis Ababa, processed samples. Another 2 laboratory technicians from the Health Center gave assistance in the sample processing. Seven teams of 3 (1 for each ‘kebele’) carried out the post-campaign survey. Each team had 2 staff from the Health Center and one person from the ‘kebele’ office to show the interviewers to the selected houses. Many staffs from the Health Center were involved with previous pre-campaign survey so have experience both in interviewing and with collecting saliva samples. The health workers were known in the community; thus the compliance was likely to be high as families tend to be more co-operative with people they know working at health center.

10. Training session with interviewers from the Health Center

Distributing consent form, household form and questionnaires to the trainees supported training. General explanation was made on each questionnaire and detail on each question in the questionnaires was also discussed. The Health Center staffs had a briefing before starting the survey that they covered:

• Establishing those in the household eligible/ineligible (household member defined as excluding visitors/live-in helpers of <6 months, as given in Qu.15 of the <15yrs questionnaire)
• Verbal consent to be obtained before samples are taken

• Completion of household information on form

• Indicating on the form that all is completed for a household or when it may be possible to return and finish

• Collection and labeling of samples. A demonstration of taking a sample was given and each interviewer was given a swab to use. Careful labeling was discussed. The ID on the sample will be 'kebele' number/house number/individual number within household. This ID number was entered on the questionnaire, later a separate laboratory number will be given to the samples and put on the questionnaires.

• Each question on the <15 yrs questionnaire was explained and questions answered.

11. Field work

7 teams (1 per ‘kebele’) were sent out with questionnaires, household lists, swabs and icebox. Transport was co-coordinated to deliver and collect interviewers from the field.

Supervisors assigned were visited the teams in the field.

On return

1. Household forms checked

2. Samples placed in racks

3. Questionnaire laboratory specimen number checked with the specimens. Samples and questionnaires matched (household members together) and samples put in rack in order of questionnaires.

4. Samples given laboratory number (001 onwards) and the laboratory number was written on the front of the corresponding questionnaire. The laboratory number and individual household number will be entered with the questionnaire data.
5. The questionnaires are then checked by supervisor and the samples processed.

6. Labels for the sample vials prepared with lab specimen ID (‘kebele’ number/house number/individual number) laboratory number (001 onwards) and date of collection.

7. Specimens processed and kept at $-10^\circ\text{C}$ ready for transport to Addis.

Initially, on return from the field the supervisors discussed both the household form and all questionnaires with the interviewers to check for errors and sort out any problems. There were some problems with a few questions initially but by the third round of fieldwork most problems were resolved.

12. Monitoring of fieldwork

Each day the supervisors visited the survey teams in the field. On return the survey teams had the opportunity to discuss problems with the supervisors.

13. Checking of questionnaire data

During the survey the questionnaires were not systematically checked. However, once the bulk of the fieldwork was completed the supervisors check each questionnaire thoroughly and raise any problems with the relevant fieldworkers. It was thought essential to check as much as possible in Asela. All questionnaires were checked with particular attention given to the first set of questionnaires completed where there were some problems with interpretation of the questions.

14. Completion of survey

Overall the survey was well organised and well co-coordinated. The interviewers were experienced health workers, with knowledge of the community, who worked together well. The laboratory staffs were well organised and there were no problems with the processing of samples.
15. Data Entry

Epi-Info (Epi-Info version 6.04) was used for data entry. Data was then double entered. Two data clerks entered the data from the pre-campaign survey (questionnaire 1) and the post-campaign (questionnaires 3 and 4, and household data form. See attached in this appendix). Four data entry programs, with check files to assist data entry, have been written:

preform.qes and preform.chk - pre-campaign survey questionnaire
form1.qes and form1.chk - post-campaign survey questionnaire - <15 years
form2.qes and form2.chk - post-campaign survey questionnaire - >=15 years
hhform.qes and hhform.chk - post-campaign survey - household information form

16. Management of data entry

Separate pathways were set up for each data entry clerk containing the necessary files for data entry. There were two disks with identical data entry files. One set have 'a' in front of the file names and the other set 'b'. This should avoid any confusion of files between data entry clerks.

Reliable systems of backup were used. Each data entry clerk, in rotation, used at least 2 backup disks, so that at worst only work from one entry session would be lost.

17. Laboratory results

The laboratory results were entered into an Excel spreadsheet.

18. Data Analysis

Epi-Info duplicate files were compared using the 'merge' procedure, and all inconsistencies checked. Epi-Info 6 files and Excel files were converted into Stata file format and the files were merged for analysis.
LAB. number ________________

1. Name of static vaccination site ________________ Date (GC) ________________

Number of static vaccination site (IDI) ________________

2. Child number ________________

3. Child code (IDI child) ________________

4. First name of child ________________

   Family name of child ________________

5. Address:
   
   Woreda ________________
   
   Kebele ________________
   
   House no. ________________

6. Sex __________

7. Age in years and months ________________

8. Parental education ________________

9. Measles vaccination ________________

10. If yes to Qu.9, age at routine measles vaccination (mths) ________________

11. History of measles infection ________________

12. Age when measles infection occurred in years and months ________________

13. Number of children less than 5 years old ________________

14. How many of these children are receiving vaccine in the campaign ________________

15. Child received measles vaccine in this campaign 1. Yes 2. No

Comment ________________
QUESTIONNAIRE 2

HOUSEHOLD INFORMATION FORM

1. Kebele __________
2. Cluster no. __________
3. Name of interviewer __________
4. Serial no. __________
5. Name of household __________
6. House no. __________
7. Number non-eligible __________
8. Number eligible __________
9. Number of eligible consented __________
10. Reason if not available ________________
11. Remarks ________________

Comment ________________
QUESTIONNAIRE 3
QUESTIONNAIRE FOR MEASLES CAMPAIGN EVALUATION
CHILDREN LESS THAN 15 YEARS (PARENT RESPOND)

1. Date of interview (GC) / / Name of Interviewer

2. Zone Woreda Kebele/PA H/No.

3. Lab. Specimen No

4. Age of in Months if less than 5 years Age in years if 5 or over

5. Place of birth:
   1. In Assela town
   2. Elsewhere (specify)

6. Born at:
   1. Home
   2. Hospital
   3. Other (specify)

7. Gender:
   1. Male
   2. Female

8. Details of immunization card:
   1. Original
   2. Replacement
   3. Lost
   4. Never had

9. Vaccination status:

<table>
<thead>
<tr>
<th>Card: write date</th>
<th>Verbal History: Y/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Routine</td>
<td>Campaign</td>
</tr>
<tr>
<td>BCG</td>
<td></td>
</tr>
<tr>
<td>OPV1</td>
<td></td>
</tr>
<tr>
<td>DPT1</td>
<td></td>
</tr>
<tr>
<td>OPV2</td>
<td>Booster polio doses</td>
</tr>
<tr>
<td>DPT2</td>
<td></td>
</tr>
<tr>
<td>OPV3</td>
<td></td>
</tr>
<tr>
<td>DPT3</td>
<td></td>
</tr>
<tr>
<td>Measles 1</td>
<td></td>
</tr>
<tr>
<td>Measles 2</td>
<td></td>
</tr>
<tr>
<td>Other (specify)</td>
<td></td>
</tr>
</tbody>
</table>

10. What was the age at vaccination (months)

11. If yes (vaccinated) to above (9) was the vaccine given in Assella town?
   1. Yes
   2. No

12. How long lived in Assela town (yrs)
   (0= <1 year, 88= all life)

13. Is there History of measles disease?
1. Yes 2. No 3. Unknown

14. If Yes to above (13), What was the age at time of measles infection in months?

15. Family size (exclude visitors < 6 months and helpers (e.g. maid))

16. Number sleep in same bed (inclusive, i.e. answer must be 1 or more)

17. Mothers’ education level (care taker):
   1. Unable to read and write
   2. Able to read and write
   3. From 1-6 grade
   4. From 7-12
   5. Above high School

18. Father’s education level:
   1. Unable to read and write
   2. Able to read and write
   3. From 1-6 grade
   4. From 7-12
   5. Above high School

19. What do you think is the most dangerous disease of children in this area?
   1. ___________  2. ___________  3. ___________

20. EPI disease mentioned in first 3:
   1. Yes  2. No

21. What diseases are prevented by vaccines?

Circle number of EPI diseases mentioned

0 1 2 3 4 5 6

22. What were the vaccines given in the campaign?
   Polio Y/N
   DPT Y/N
   Anti-TB Y/N
   Measles Y/N
   Other (specify) Y/N

23. Do you know of any children who had an abscess after vaccination?
   1. Own child  2. Neighbour’s child  3. Relative’s child  4. Other  5. None

24. If yes to above (23), was this after vaccination in the routine program or vaccination in the campaign?
   1. In routine vaccination  2. Campaign vaccination
25. If a child has been vaccinated against measles, do you think he/she will later catch this disease?
   1. Yes       2. No       3. Don't know

26. If yes to above (25), will the illness be equal to, more severe or less severe than in unvaccinated child?
   1. Equal     2. Worse      3. Mild

27. Has it ever happened that you took your child to be vaccinated but for some reason the health staff did not vaccinate him?
   1. Yes       2. No

28. Did you know about the last national immunization day(s) for polio/measles?
   1. Yes       2. No

29. If yes to above (28), how did you receive the information?
   7. Health staff 8. Other       9. More than one of the above

30. Did you receive prior advice about where to take your child for vaccination during last NIDs?
   1. Yes       2. No

31. How many times did you attend the NIDs last year (circle one)?
   0       1       2       3       >3

32. If you didn't take child for vaccination on NIDs, why not?
   1. Away from area     2. No information     3. Inconvenient time
   4. Mother (self) ill  5. Child ill

Comment ________________________________
QUESTIONNAIRE 4
QUESTIONNAIRE FOR MEASLES CAMPAIGN EVALUATION
CHILDREN OVER 15 YEARS OF AGE

1. Date of interview (G.C) / / Name of Interviewer

2. Zone Woreda Kebele/PA H/No.

3. Lab.Specimen No

4. Age in years if 15 years or over

5. Place of birth:
   1. In Assela town
   2. Elsewhere (specify)

6. Born at:
   1. Home
   2. Hospital
   3. Other (specify)

7. Gender
   1. Male
   2. Female

8. Received measles vaccine during last campaign?
   1. Yes
   2. No
   3. Unknown

9. Received measles vaccination in the routine EPI Program?
   1. Vaccinated(card)
   2. Vaccinated (remember)
   3. Not vaccinated
   4. Unknown

10. What was the age at vaccination (months)

11. If yes (vaccinated) to above (9) was the vaccine given in Assella town ?
   1. Yes
   2. No

12. How long lived in Assela town (yrs)
(0= <1 year, 88= all life)

13. Is there History of measles disease?
   1. Yes
   2. No
   3. Unknown

14. If Yes to above (13), What was the age at time of measles infection in months?

15. Family size (exclude visitors < 6 months and helpers (e.g. maid)

16. Number sleep in same bed (inclusive, i.e. answer must be 1 or more)

17. What do you think is the most dangerous disease of children in this area?
   1. 
   2. 
   3. 

18. EPI disease mentioned in first 3:
   1. Yes
   2. No
19. What diseases are prevented by vaccines?

Circle number of EPI diseases mentioned
0 1 2 3 4 5 6

20. What were the vaccines given in the campaign?

- Polio Y/N
- DPT Y/N
- Anti-TB Y/N
- Measles Y/N
- Other (specify) ________________

21. Do you know of any children who had an abscess after vaccination?


22. If yes to above (21), was this after vaccination in the routine program or vaccination in the campaign?

1. In routine vaccination 2. Campaign vaccination

23. If a child has been vaccinated against measles, do you think he/she will later catch this disease?

1. Yes 2. No 3. Don't know

24. If yes to above (23), will the illness be equal to, more severe or less severe than in unvaccinated child?

1. Equal 2. Worse 3. Mild

25. Has it ever happened that you go to be vaccinated but for some reason the health staff did not vaccinate you?

1. Yes 2. No

26. Did you know about the last national immunization day(s) for polio/measles?

1. Yes 2. No

27. If yes to above (26), how did you receive the information?

1. Neighbour 2. School child 3. Public meeting
7. Health staff 8. Other 9. More than one of the above
CHAPTER 7

FUTURE DEVELOPMENTS AND APPLICATIONS IN MEASLES ORAL-FLUID METHODS

7.1 Future technical developments

The low concentration of IgG/IgM antibodies in oral fluid relative to other diagnostic specimens such as plasma (Mortimer and Parry, 1988) demanded the development of an enhanced immuno assays and of diagnostic techniques based on nucleic acid amplification.

Promotion of the use of oral fluid as viral diagnostic fluid requires that immunological assays have higher sensitivity. The development of antibody capture assays, either $^{125}$I labeled (RIA) or ELISA, that are able to generate higher signals by capturing the proportion of specific to the total immunoglobulin (present in the oral fluid), enabled saliva to be used for successful immunological assays (Duermeyer et al., 1979; Flehmig et al., 1979). Capture ELISA is better for wide-scale use in many laboratories but less sensitive than radioimmuno assays. Hence sensitivity enhancement is required to make best advantage of ELISA. In this study we developed FITC/anti-FITC enhanced capture ELISA that can be used for population and vaccine surveys.

The production of measles antigen for measles diagnosis, such as the one we used for GAC- and MAC-ELISA (see in Chapter 2 and 3), benefited from tissue culture. However, production of purified measles antigens in tissue culture can be difficult. The capture format has been revolutionised by the raising of purified antigen and monoclonal
antibodies for use in oral-fluid measles diagnostics. Cloning and expression of measles genes provides a relatively straightforward alternative approach (Hummel et al., 1992; Bouche et al., 1998), simplifying purification and enabling large-scale production for improvement in measles oral-fluid diagnostic assays.

Kits based on the use of recombinant antigens such as the Light Diagnostic kit (Chemicon Temecula, CA, USA) benefited from the cloning and expression approach. More recently IgG and IgM kits specific to measles have been developed based on such an alternative approach by Microimmune Ltd (Brentford, Middlesex, UK) for both oral-fluid and serum samples. However, there are problems associated with the use of recombinant antigens associated with the production of ‘incorrectly’ processed antigens by most expression systems (Hummel et al., 1992; Bouche et al., 1998) and the problem of using a single cloned antigen to detect a measles antigen that may vary between isolates. This may be resolved by cloning and expressing the most conserved region of the measles gene identified from sequence data of different isolates. Notwithstanding this problem measles antibody assays that are increasingly based on the use of cloned proteins will continue to play a prominent role in oral-fluid diagnostic development. Such immuno-assays may be useful in the future when they become better suited to use with automated systems that are capable of handling all stages of testing from specimen preparation to issuing of diagnostic results.

Microimmune assays are observed to be easy to use, but have not yet been evaluated under a wide range of conditions such as in highly vaccinated populations. Studies of rubella revealed problems of sensitivity in enhanced GACELISA in older age groups.
This appears to be due to decay in the level of specific antibody in serum and in oral fluid (Vyse et al., 1999; Nokes et al., 1998; 2001). Age-related variation in sensitivity was not seen as a big problem in measles assays here and previously (Nigatu et al., 1999; Nokes et al., 2001). However, low-level measles antibodies resulting from vaccine-induced immunity is a feature of many communities, particularly those with high-level routine immunizations coverage. Future work is required to evaluate the performance of newly developed kit assays in such settings.

Assays of measles nucleic acid are fundamentally different from those of measles antibodies, since they detect a component of the measles virus itself, rather than serological evidence of its past presence. Among the several techniques used to detect viral nucleic acids the Polymerase Chain Reaction (PCR) is the one widely used for detection of measles nucleic acid (Shimizu et al., 1993; Nakagomi et al., 1995; Jin et al., 1996). In contrast to direct hybridisation, whose application is restricted to where high concentration of the virus is present, PCR amplifies the probe signal by means of a sequential series of secondary, tertiary, etc. stages. The signal amplification thus increases the sensitivity of detection to a range where it can detect viruses at low concentration in various specimens (Jin et al., 1996). PCR is suitable for the detection of the low concentration of measles virus present in oral fluid. Actually oral fluid is better for nucleic acid extraction than serum or blood because of the absence of PCR inhibitors, such as haem or porphyrin, in the former (Jin et al., 1996). In addition, oral fluid specimens do not need pre-treatment for nucleic acid extraction. In future developments
of measles oral-fluid diagnosis based on the nucleic acid amplification systems are likely to play an increasing part.

The new tool developed by Roche Molecular Biochemicals, MagNA Pure LC DNA isolation kit, for the isolation of nucleic acid from various types of specimen including oral fluid, is a breakthrough that has shortened the tedious manual RNA extraction process in measles nucleic acid detection. This is now practised in many laboratories of industrialized countries but may be restricted to laboratories that have specialised requirements and too costly for most developing countries. The developments of kit-based measles oral-fluid molecular diagnostic assays, like the ARMS-EIA (Amplification Refractory Mutation System-Enzyme-immunoassay), which is under development at CPHL, may place oral-fluid testing within the reach of most diagnostic laboratories. ARMS-EIA is a non-sequencing genotyping method for discriminating between closely related measles strains. It is as easy as PCR and EIA, and compared to PCR it is time saving and does not need interpretation of bands on gels (Dr. Dhan Samuel personal communication).

IgG can be measured in terms of its functional binding avidity. The binding strength between the IgG and the virus antigen is supposed to be low in primary infection and changes to high in past infection. This avidity can be measured by disrupting the interaction using protein denaturants such as urea or diethylamine (Thomas and Morgan-Capner, 1991). Diagnosis of primary infection by IgG avidity assay using serum samples has got relevance for the diagnosis of viral infection such as rubella (Hedman and Seppala, 1988; Thomas and Morgan-Capner, 1988). The detection of antibody with low
or high avidity enables a more accurate diagnosis in differentiating primary infection from past infection.

IgG avidity is useful when the IgM assay result is indeterminate. It may also help in distinguishing primary and secondary ('boosting') response to measles vaccine. Future development of IgG avidity in oral-fluid measured by GACELISA may allow specific, sensitive and accurate diagnosis of primary infection. Our studies (see Chapter 3) show the problem of MACELISA in detecting IgM in oral-fluid samples collected at early onset of measles rash. The future development of IgG avidity that can determine IgM in early-collected oral fluid samples makes MACELISA better use.

Another interesting area to look at in the future is the differentiation between antibodies resulting from vaccine strain and wild type measles virus. This assists in defining vaccine uptake and estimating continued measles transmission. It may be difficult to explain the technical development at this stage. However, it is an area for future research.

Evaluation of a new diagnostic test has the potential sources of bias introduced by the study design. The test’s discriminatory ability, sensitivity, and specificity depend upon the composition of the study population. The study design we used for evaluation of the present measles oral- fluid diagnostic assays (see Chapter 2 and 3) is an area that can be followed in the future for other viral diagnostic test evaluation.
### 7.2 Future wider applications of oral-fluid methods

#### Table 7.1. Setting and details of specific role for non-invasive methods

<table>
<thead>
<tr>
<th>Applications of oral-fluid methods</th>
<th>Setting for country/district</th>
<th>Low /Med uptake routine</th>
<th>High uptake routine</th>
<th>Campaign</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population survey</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Methods: community surveys of IgG across wide age range. Including hard-to-reach groups, informal settlements. Purpose: immunity profiles. Identifies susceptibility gaps and age range for campaigns. Implications: increase in coverage, need for and age range for campaigns.</td>
<td>As previous</td>
<td>As previous plus. Methods: Post-campaign surveys of IgG and perhaps IgM. Purpose: IgG - Identify immunity levels post-campaign. Susceptibility in target age group and outside target group. IgM - indicator of impact ie proportion responding to vaccine. Implications: Age-range for future campaigns; locate problems of vaccine efficacy</td>
<td></td>
<td></td>
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<tr>
<td>Vaccinee surveys</td>
<td></td>
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<tr>
<td>Methods: Vaccine clinic samples pre- and post vaccination. IgM and/or IgG testing. Purpose: Assess efficacy of routine vaccination. Implications: Identify cause of low efficacy.</td>
<td>As previous</td>
<td>As previous plus. Methods: IgG survey of individuals attending vaccine clinics. Purpose: Identify proportion able to respond to vaccine. Implications: Assess potential effectiveness, and suggest alternative method for delivery eg hard-to-reach groups.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td>Not indicated while measles incidence remains high</td>
<td>Method: IgM testing on demand. Purpose: Confirmation of clinical diagnosis</td>
<td>As previous</td>
</tr>
<tr>
<td>Case surveillance: serological and genetic</td>
<td></td>
<td>Not indicated while measles transmission remains high</td>
<td>Method: System of reporting and OF sampling from sporadic cases and outbreaks. IgM and Genotyping Purpose: verify cases, and monitor distribution of virus and endemicity Implications: need for additional control measures</td>
<td>As previous</td>
</tr>
</tbody>
</table>
The application of oral-fluid methods to population surveys, vaccine surveys, diagnosis of clinical cases, and case surveillance for different vaccine uptake settings of a country/district are illustrated in Table 7.1.

The following is a description of some of the applications of oral-fluid methods.

A. Population surveys
Measles antibody population surveys can be used to define the proportions of susceptible and immune in the population. Population immunity may result from natural measles infection or/and measles routine and campaign vaccination. Current methods cannot distinguish between the two. Population immunity surveys can identify in which age groups large pockets of susceptibles remain in unvaccinated populations, in a population with routine immunization, and before and after a vaccine campaign. This would provide valuable information on the age groups to target for vaccination and effectiveness of the routine or campaign vaccination, and clues to where future outbreaks might arise. Similarly, through such surveys hard-to-reach groups in rural/urban under different geographical settings can be reached.

Population surveys may be appropriate at all stages of vaccination programmes, in country/settings of vaccine uptake for low to high, with or without campaigns/accelerated measures. Predominantly, such surveys could assess specific antibody status. However, post-campaigns there might be a role for IgM testing in community survey to establish what proportion of the population actually responded to vaccine. Based on surveys
cluster sampling techniques, as for EPI vaccine cluster sampling, and using of the-shelf EIA kits, the surveys would be rapid and simple to effect.

B. Vaccine surveys

Vaccine surveys assess the level of population immunity attending vaccine clinic to a measles routine vaccination. It can identify the responses to routine vaccine in pre- and post-vaccinated children. The widespread use of serological determinants of vaccine responsiveness is limited by the need to carry out follow up of vaccinees at 2 (IgM) < 4 (IgG) weeks. OF sampling will not improve greatly up on this situation, except that compliance for second samples is likely to be greater than if blood samples are required. However, the future development of oral-fluid IgG avidity measurement cannot be ruled out that may improve this situation.

C. Diagnosis

Measurement of measles antibody present in oral-fluid samples provides information on the status of current and past infection by use of tests for IgG and IgM antibody. Laboratory diagnosis of suspected measles clinical cases can assist in (i) confirmation of the occurrence of measles clinical illness (ii) capability of physicians to diagnose illness and (iii) reporting of the infection to health department. The usefulness of oral fluid in this capacity is at present hindered by the relatively low sensitivity of IgM assays in samples taken early after onset of rash. Delay in collecting a sample may be impractical. Improved sensitivity of assays remains a need.
D. Case surveillance

The recognition and identification of measles outbreaks and sporadic cases using a system of reporting and oral-fluid sampling is established in the UK (Perry et al., 1993; Brown et al.; 1994; Ramsay et al., 2003). For measles epidemic investigation in Ethiopia, where infrastructure is poor and locations of the remote, oral-fluid sampling was found to be appropriate. Especially in the situations where community beliefs or attitudes like “measles sick should not get injection” are present, in which communities declined to give blood specimens, oral-fluid specimens are preferable. Provided reasonable storage conditions while in transit or awaiting transit to the laboratory are made, oral-fluid is a robust sample for IgG testing and IgM testing (UK surveillance and in these studies in Ethiopia) (Nigatu et al., 1999; Morris M et al., 2002). However, further stability studies of OF at different temperature in field conditions are required in the future.

Another area of increasing importance is the application of sequence data obtained from oral-fluid nucleic acid amplification techniques. Genetic information is valuable, in combination with other traditional epidemiological data, to enhance the ability to determine measles transmission pathways and to assess the success of measles control strategies (WHO, 2001).

The practical utility of oral-fluid methods (antibody and genetic) in evaluating and refining measles immunization programmes would, additionally, provide support for a national surveillance initiative. The utility of OF in a population survey is demonstrated
in Chapter 6. The use of OF in a vaccine sero-conversion study is shown in Chapter 3. OF application in molecular epidemiological use is presented in Chapter 4 and 5. In summary, in this thesis work it has been possible to demonstrate the utility of oral fluid in sero-epidemiological and molecular investigations for measles immunization control programs in Ethiopia. It is to be hoped that this work will assist in the wider uptake and acceptance of OF methodology, most particularly in the developing country situation, within the Africa region and elsewhere. There is a strong case and imperative for the promotion of OF methods by WHO in its global programme of control/eradication of measles over the coming decade.
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