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Comparison of different serological test-systems to analyse specific IgM and IgA immunresponses against measles virus

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“You see, Momo,” he told her one day, “it’s like this. Sometimes, when you’ve a very long street ahead of you, you think how terribly long it is and feel sure you’ll never get it swept.”

He gazed silently into space before continuing. “And then you start to hurry,” he went on. “You work faster and faster, and every time you look up there seems to be just as much left to sweep as before, and you try even harder, and you panic, and in the end you’re out of breath and have to stop - and still the street stretches away in front of you. That’s not the way to do it.”

He pondered a while. Then he said, “You must never think of the whole street at once, understand? You must only concentrate on the next step, the next breath, the next stroke of the broom, and the next, and the next. Nothing else.”

Again he paused for thought before adding, “That way you enjoy work, which is important, because then you make a good job of it. An that’s how it ought to be.”

Monologue, “Beppo Roadsweeper”

Michael Ende, “Momo”, K. Thienemanns Verlag, Stuttgart, 1973

To my parents

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Table of abbreviations

Aa	amino acid
AFU	arbitrary fluorescence unit
aq. bidi	aqua bidestillata
BSA	bovine serum albumin
CDC	Centers of Disease Control and Prevention
C.U.	Capture Units
EBV	Epstein-Barr Virus
EDTA	ethylene diamin tetraacetic acid
Ef	efficiency
ELISA	enzyme linked immunosorbent assay
ER	endoplasmatic reticulum
FACS	flourescence activated cell scanner
F-FACS	Mel-JuSo/MV-F/wt tested in FACS
FCS	fetal calf serum
F	fusion protein
FITC	fluorescein isothiocyanate
h	hour
H	hemagglutination protein
H-FACS	Mel-JuSo/MV-H/wt tested in FACS
HI	hemagglutination inhibition
HIV	Human Immunodeficiency Virus
IgA	immunoglobulin A
IgG	immunoglobulin G
IgG-Enzygnost	Enzygnost® Anti-Measles-Virus/IgG
IgM	immunoglobulin M
IgM-Enzygnost	Enzygnost® Anti-Measles-Virus/IgM
IU	international units
kDa	kilodalton
L	large protein

mM	milli Mol
M	matrix protein
mAb	monoclonal antibody
MWRST	Mann-Whitney Rank Sum Test
MCV	measles containing vaccine
MHC	major histocompatibility complex
min	minutes
MMR	Measles- Mumps- Rubella
MV	measles virus
MV-F	measles virus fusion protein
MV-H	measles virus H protein
MV-NP	measles virus nucleoprotein protein
N	nucleoprotein
N_2	nitrogen
NPV	negative predictive value
NT	neutralization assay
O.D.	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PO/POD	Peroxidase
PPMC	Pearson Product Moment Correlation
PPV	positive predictive value
PSG	penicillin/streptomycin/glutamin
RF	rheumatoid factor
RNA	ribonucleic acid
rpm	rounds per minute
Se	sensitivity
SFV	Semliki Forest virus
Sp	specificity
SROC	Spearman Rank Order Correlation
SSPE	subacute sclerosing panencephalitis
T_C	T- cytotoxic lymphocyte

T_H	T- helper lymphocyte
WHO	World Health Organisation
wt	wild-type

1 Introduction

More than 70% of children who die under the age of 5 years do so from only 5 illnesses (WHO, 1999 a):

- pneumonia
- diarrhoea
- measles
- malaria
- malnutrition

Measles still ranks as one of the leading causes of childhood mortality in the world (Norrby and Gollmar, 1975; Asaad, 1983; Preblud and Katz, 1988; Sabin, 1991; Weiss, 1992; Tulchinsky et al., 1993). It accounts for the majority of the estimated 1.6 million annual deaths caused by childhood vaccine-preventable diseases (WHO, 2001 a). Before a measles vaccine became available, virtually all individuals contracted measles with an estimated 130 million cases each year. Despite the remarkable progress made in measles control with the introduction of measles vaccination in 1963 and improved monitoring of virus circulation and transmission (Rima et al., 1995; Rota et al., 1996; Kreis et al., 1997; Hanses et al., 1999; Mulders et al., 2001), the disease continues to be a major health problem worldwide, accounting for more than 30 million cases globally each year. Nearly 900.000 of them are fatal (WHO, 2001 a).

1.1 The measles virus

1.1.1 Classification and morphology

The measles virus (MV) is a member of the morbillivirus genus of the family of paramyxoviridae. Members of this genus include canine distemper virus, rinderpest virus, and peste-des-petits-ruminants virus. Other morbilliviruses such as phocine, seal and dolphin distemper virus have been found over the last years among large sea mammals (Visser et al., 1993; Blixenkrone-Moller et al., 1996) and among large predators such as bears and lions (Follmann et al., 1996). Murray et al. (1995) and Selvey et al. (1995) have described a morbillivirus, which affects horses (Table 1.1).

Family Paramyxoviridae
Subfamily Paramyxovirinae
Genus Paramyxovirus
Sendai virus (mouse parainfluenza virus type 1)
Human parainfluenza virus type 1 and type 3
Bovine parainfluenza virus type 3
Genus Rubulavirus
Simian virus 5 (Canine parainfluenza virus type 2)
Mumps virus
Newcastle disease virus (Avian paramyxovirus 1)
Human parainfluenza virus type 2, type 4a and 4b
Genus Morbillivirus
Measles virus
Dolphin morbillivirus
Canine distemper virus
Peste-des-petits-ruminants virus
Phocine distemper virus
Rinderpest virus
Subfamily Pneumovirinae
Genus Pneumovirus
Human respiratory syncytial virus
Bovine respiratory syncytial virus
Pneumonia virus of mice
Turkey rhinotracheitis virus

Table 1.1: Examples of members of the family paramyxoviridae

Morbilliviruses are distinct from other paramyxoviruses in that they do not have neuramidase activity and that they cause formation of intranuclear inclusion bodies.

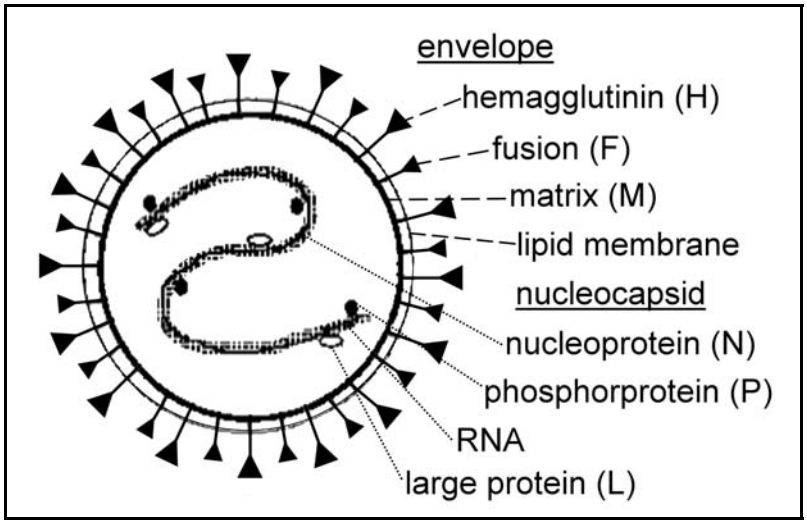


Fig. 1.1: The measles virus

The measles virions are pleomorphic and range in size from 100 to 300 nm. The lipid bilayer envelope originates from the plasma membrane of the host cell. Inserted into the envelope are glycoprotein spikes with a length of approximately 9 to 15 nm that are composed of viral transmembrane hemagglutinin (H) and fusion (F) glycoproteins (Fig. 1.1). On the inner surface of the envelope is the matrix (M) protein. The nucleocapsid has a coiled helical structure with a diameter of 21 nm (Lund et al., 1984). It consists of the nucleocapsid (N) protein, surrounding the genomic RNA, to which the phospho- (P) and large- (L) proteins are bound. The MV genome is composed of a single-stranded non-segmented RNA of negative polarity. It is about 16.000 nucleotides in length and codes for the following six major structural proteins (Bellini et al., 1994, Griffin and Bellini, 1996):

- hemagglutinin protein (MV-H),
- fusion protein (MV-F),
- matrix protein (MV-M),
- nucleocapsid protein (MV-N),

- phospho protein (MV-P),
- large protein (MV-L).

The MV-H protein is a receptor-binding and hemagglutinin type II transmembrane glycoprotein that resides on the surfaces of infected cells and virions as a disulfide-linked homodimer. The monomeric H has 5 potential glycosylation sites grouped within a small region of the extracellular domain.
H is closely associated with the F protein on the cell surface and acts in conjunction with F during the fusion of the virions envelope with the cell membrane and entry of the nucleocapsid into the cytoplasm (Malvoisin et al., 1993). Both hemagglutination and hemadsorption are properties of the MV-H (Varsanyi et al., 1984; Takehara et al., 1992). Interaction between the MV and the target cell appears to be the primary event in the MV infection (Devaux et al., 1996). Responsible for the interaction is the junction between the MV-H protein and the “human membrane cofactor protein” (CD46) (Naniche et al., 1993; Dorig et al., 1993; Tatsuo et al., 2000 a). CD46 is a regulatory glycoprotein of the complement pathway, which is located on the surface of all human hematopoeitic cells, except erythrocytes. It protects cells from destruction by the complement pathway. CD46 is also present on monkey erythrocytes. Interaction between CD46 and MV-H inevitably leads to agglutination (Perles et al., 1962). Recently, a receptor for wt MV-strains was discovered and identified as the human SLAM (signalling lymphocyte-activation molecule (CDw150)) (Tatsuo et al., 2000 b). Many human cells are SLAM negative and CD46 positive but still susceptible to wt MV, although wt MV-strains are not supposed to use CD46. This phenomenon is probably due to the newly identified CD46 binding-site, located at the wt MV-H (Masse et al., 2002).
The MV-F protein is a type I membrane glycoprotein synthesized as an inactive precursor F on the endoplasmatic reticulum (ER) and cleaved enzymatically on the golgi apparatus to yield the mature F1 (42 kDa) and F2 (20 kDa) fragments (Wild and Buckland, 1995). These two subunits are covalently coupled by a disulphide band. MV-F, closely associated with MV-H, is responsible for the fusion of the viral membrane with the membrane of the target cell (Choppin et

al., 1981; Richardson et al., 1986; Buckland et al., 1987; Malvoisin et al., 1993; Wild et al., 1994). Antibodies to either protein can neutralize the MV (Giraudon et al., 1985; Malvoisin et al., 1990).

The MV-M protein is a basic protein holding several conserved hydrophobic regions. It is supposed to interact with the transmembrane proteins, H and F and with the nucleocapsid to play a key role in virion-maturation, stabilization, and organization of the membrane environment in preparation for budding virions (Griffin and Bellini, 1996).

The MV-N protein is the most abundant and major protein both in the virion and in the infected cell. It is synthesized on free ribosomes and folded in the cytoplasm (Gombart et al., 1993). MV-N serves several functions in virus replication, including (i) encapsidation of the genome RNA into an RNAse-resistant nucleocapsid (the template for RNA synthesis), (ii) association with the P-L polymerase during transcription and replication and most likely (iii) interaction with the M protein during virus assembly (Griffin and Bellini, 1996).

The MV-P protein is a modular protein that is named so for its highly phosphorylated nature. It plays a central role in RNA synthesis. Together with the MV-L protein it forms the viral polymerase (P-L).

1.1.2 Isolation of MV

The MV was first isolated in tissue culture in 1954 by Enders and Peebles, which inoculated primary human kidney cells with the blood of David Edmonston, a child with measles (Enders et al., 1954). MV can be adapted to grow in a number of primary human cells (e.g. blood, lung, conjunctiva, kidney, intestine, amnion, skin, muscle, thymic stroma, foreskin, and uterus) and human cell lines (e.g. WI-38, MRC-5, Hep-2 and HeLa) (Milovanovic et al., 1957; de Maeyer et al., 1965; Katz et al., 1965; Numazaki et al., 1989). More recently sensitive and reproducible isolation has been achieved using an Epstein-Barr virus (EBV) transfected marmorset B lymphocyte cell line, B95-8 (Kobune et al., 1990). The virus generally replicates slowly, and 3 to 5 days of culture are needed before plaques / syncytia-formation, the first observable signs of virus growth become visible.

1.1.3 Virus replication

An overview of the life cycle of the virus is shown schematically in Fig. 1.2. Adsorption of the MV-H to the cellular receptor of the host cell (CD46) triggers a conformational change in the MV-F, which leads to the fusion of the viral membrane with the cellular plasma membrane. The consequence is the release of the viral nucleocapsid into the cytoplasm of the host cell. The typical cytopathic effects of syncytia-formation due to cell-cell fusion become visible. Transcription and replication synthesizes new virus-specific molecules, which are then released as progeny virions. Virus entry largely determines the cell tropism between different MV strains (Tatsuo et al., 2000 a). In cell culture, single cycle growth curves are generally of 14 to 20 h duration.

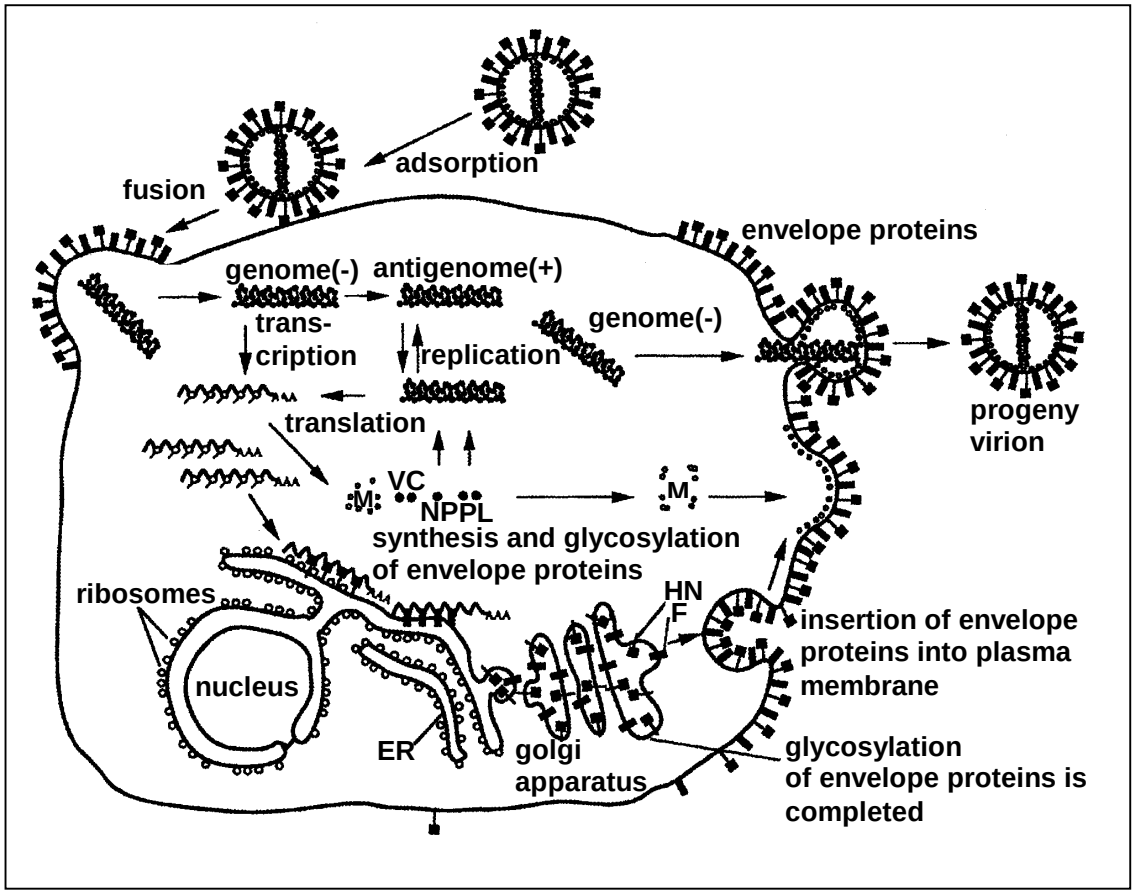


Fig. 1.2: Schematic representation of the life cycle of measles virus.

1.1.4 Molecular epidemiology

With the use of attenuated live virus vaccine and massive global vaccination campaigns, the incidence of measles morbidity and mortality has been dramatically reduced during the last years. Still, the disease remains a serious public health-problem in many parts of the world (Norrby and Gollmar, 1975; Asaad, 1983; Preblud and Katz, 1988; Sabin, 1991; Weiss, 1992; Tulchinsky et al., 1993). Measles infection is contagious with a transmission risk of minimum 95% (Griffin and Bellini, 1996). To further reduce measles, it is of great importance to improve the understanding of virus circulation patterns and to further concentrate on new strategies to interrupt virus transmission. Molecular epidemiology of field isolates has proven to be a powerful tool to monitor

circulation of infectious virus, to trace routes of transmission and to assess the success of measles control strategies (Mulders et al., 2001).

Paramyxo- and rhabdoviridae are the only viruses with a non-segmented RNA of negative polarity, which is the reason for a lack of greater genetical recombinations. The antigenic “shift” or “drift” phenomena known for instance in influenza viruses does not take place on this scale within the paramyxovirus genus. Therefore, MV infection induces immunity against all wild-type (wt) viruses, as only one serotype exists. Although, only one serotype of MV has been identified, recent MV isolates have undergone a significant genetic drift relative to the Edmonston wt strain. It was thought that this may be due to an increased immunological pressure as a result of vaccination campaigns (Klingele et al., 2000). Variability is found in the N, M, and H genes and, to a lesser extent, in the F gene. Genetic differences are few among vaccine strains, whereas wt viruses are more variable. Given the increased mutation rates over time (Taylor et al., 1991; Rota et al., 1994; Rima et al., 1995; Kreis et al., 1997; Hanses et al., 1999), especially in the MV-N, as the most variable of the MV genome, the possibility of emerging escape mutants cannot be excluded (Schrag et al., 1999; Klingele et al., 2000). Molecular analysis of virus isolates in the Americas have demonstrated that the virus circulation had been effectively disrupted and has ceased in large parts of the continent (Rota et al., 1996, Bellini and Rota, 1998).

1.2 Measles disease

1.2.1 Clinical features

Measles is a highly infectious virus that is transmitted by small droplet nuclei (< 5 µm in diameter) via the respiratory route. Measles infection is characterized by a latent period (10 - 14 days), a prodrome of fever and malaise, cough,

coryza, and conjunctivitis (2 - 3 days), and a maculopapular rash (3 - 5 days) (Griffin and Bellini, 1996) (Fig. 1.3).

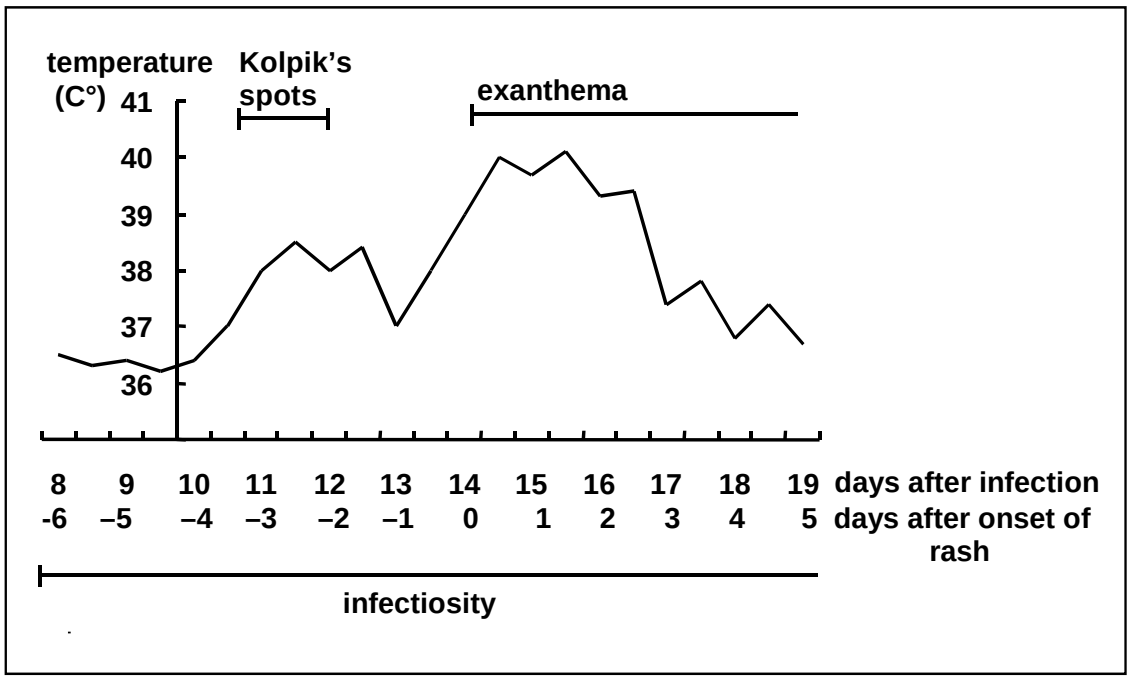


Fig. 1.3.: Clinical course of measles and its infectiosity.

Upon contact, the virus replicates in the upper respiratory tract and in local lymph nodes before spreading systematically (Gresser and Chany, 1963; Peebles, 1967; Osunkoya et al., 1974 a, b; Frazer and Martin, 1978; Hypia et al., 1985). The infection spreads after 2 - 4 days throughout the whole reticulo-endothelial system (van Binnendijk et al., 1994), where virus replication leads to lymphoid hyperplasia and formation of MV-specific multinuclear giant cells (Warthin - Finkeldey giant cells), which arise from cell to cell fusion (Hathaway, 1935; Gordon and Knighton, 1941; Pinkerton et al., 1945; Corbett, 1945; Grist, 1950; Enders et al., 1957; Sherman and Ruckle, 1958; Cascardo and Karzon, 1965; Chiarini and Norrby, 1970; Archibald et al., 1971; Hall et al., 1971; White and Boyd, 1972).

The reticulo-endothelial system is the matrix for the second (5 to 7 days after contact), far stronger viremia that is carried to all tissues mostly by lymphocytes and monocytes (Gresser and Chany, 1963; Peebles, 1967; Osunkoya et al., 1974 a, b; Joseph and Oldstone, 1974; Frazer and Martin, 1978; Hypia et al.,

1985). The following virus replication and the progressing immune response indicate the end of the incubation period and are responsible for early prodromal symptoms. During the latent period leucopenia is the only indicator for an infection. Leukocytes are infected and used by the virus for systematic spread and therefore reduced in their amount (Whittle et al., 1978; Arneborn and Biberfeld, 1983; Hirsch et al., 1984; Griffin et al., 1986).

Kolpik's spots appear as bluish-grey-white specks on red base in the buccal mucosa around the 11th day after infection. With another rise of temperature they pass into the typical measles exanthema, which starts with infection of dermal epithelial cells and spreads from cranial, starting on the face and behind the ears to caudal (fades the same direction). Kolpik's spots are pathologically similar to the epithelial giant cells, but involve submucous glands. Measles is highly contagious 1 - 2 days before prodrome until the rash has reached the lower extremities (Christensen et al., 1953; Bloch et al., 1985) (Fig. 1.3).

In uncomplicated measles, appearance of the rash indicates the onset of the virus-specific immune response, the beginning of virus clearance and clinical recovery. Treatments for measles include interferon, thymic humoral factor, thymostimulin, levaminsole, ribavirin, and immune globulin. In developing countries, administering high doses of vitamin A to children hospitalised with measles has effectively decreased overall mortality and morbidity (Dollimore et al., 1997; Rosales, 2002). Vitamin A is recommended for children with acute measles by the World Health Organization (WHO).

1.2.2 Complications

Although usually a mild or moderately severe illness at childhood, measles can result in many complications, which occur mainly due to the appearing immune suppression. The frequency of complications varies in different parts or the world. In industrialized areas, complications occur in around 10 - 15% (WHO, 1999 a). Interstitial pneumonia, caused by an inflammation of the lower respiratory tract is one of the most common, but rather mild complications (Gremillion and Crawford, 1981). Bacterial or viral superinfections can lead to chronic pulmonary disease, bronchopneumonia, laryngotracheobronchitis and

otitis media and worsen the chances of a recovery without intensive medical treatment drastically (Olson and Hodges, 1975; Gremillion and Crawford, 1981; Kaschula et al., 1983; Beckford et al., 1985; Morton and Mee, 1986). Particularly in young patients, diarrhoea is an additional common complication (Arya et al., 1987; Akramuzzaman et al., 2000) that often compounds the borderline nutritional status of young children in developing countries (Axton, 1979; Feachem and Koblinsky, 1983; Duggan et al., 1986). Especially a low vitamin A status has been associated with a higher rate of complications (Dollimore et al., 1997). Even though there is little evidence that the brain parenchyma is an important target for MV replication (Enders, 1962; Hall et al., 1971; Moench et al., 1988), postinfectious encephalomyelitis complicates 1 in 1.000 cases of measles, mostly in individuals over 2 years of age (ter Meulen and Liebert, 1993). The induction of an autoimmune response to myelin basic proteins leads to the characteristically demyelination, which is frequently accompanied by seizures and multifocal neurological signs. Entering the brain, MV can replicate in neurons and glial cells and might lead to the slowly progressive neurological disease (measles inclusion body encephalitis) 1 - 6 months after transmission or to the more rarely (1 in 1.000.000) subacute sclerosing panencephalitis (SSPE) some years after the infection (WHO, 1998 c; Croxson et al., 2002).

Measles is further considered to be an important cause of childhood blindness (Reddy et al., 1986) because of corneal lesions and keratitis, especially in vitamin A deficiency areas.

In general, vitamin A appears to be a particularly important nutrient influencing the outcome of measles (Barclay et al., 1987; Hussey and Klein, 1993, Ekanem et al., 2000, Villamor and Fawzi, 2000), as well as factors such as:

- exposure at extreme age
- underlying illness
- low socio-economic status
- malnutrition
- lack of access to medical care

(Smythe et al., 1971; Dagan et al., 1987; Diaz et al., 1992; Aaby et al., 1993 Rodgers et al., 1993; Samsi et al., 1994). Recently, a decrease in overall mortality, pneumonia-specific mortality, and severity of measles associated diarrhoea was described by D'Souza and D'Souza (2001) after administration of two doses of 100.000 / 200.000 IU of vitamin A. However, the case fatality rates in developing countries are estimated to be still 3 - 5%, as opposed to 0.1% in many industrialized regions (WHO, 1998 c).

1.3 Immunity against measles virus

1.3.1 Immune response

Immunity has both non-specific and specific components. Innate, or non-specific immunity refers to the basic resistance to the disease that an individual is born with (anatomic, physiologic, endocytic and phagocytic, and inflammatory barriers). Acquired, or specific immunity requires the activity of a functional immune system, involving lymphocytes and their products. Functionally, an immune response involves two interrelated events: recognition of an antigen and response to that antigen (Kuby, 1997). Humoral (B cells) and cell-mediated (T cells) immunity resembles the specific immune response. Both appear to play an important role in the recovery from MV infection. They only recognize an antigen if displayed together with a major histocompatibility complex (MHC class I or II) on the cell surface. Antigen presenting cell's (APC), such as macrophages, dentritic cells, and B cells are able to internalise antigen produced outside of the host cell by endocytosis or phagcytosis. They degrade the ingested exogenous antigen into peptide fragments within the endocytic processing pathway and present those peptide fragments complexed with class II MHC.

Endogenous antigen (e.g. viral and tumour pathogens) are produced within the host cell itself. They are degraded within the ER and displayed with a class I

MHC on the cell surface. Since all nucleated cells express class I MHC molecules, all cells producing endogenous antigen use this route to process antigen. In general, T cytotoxic (T_C) cells display CD8 receptors and recognize antigen associated with class I MHC cells, whereas T helper (T_H) cells express CD4 receptors and recognize antigen associated with class II MHC cells. The generation of both humoral and cell-mediated immune response depends on the activation of T_H cells. The interaction of a T_H cell with an antigen-MHC II molecule complex on the surface of e.g. a dentritic cell generates a signal that leads to activation and proliferation of antigen-specific T_H cells. After the binding of a T_C cell to an antigen-MHC class I complex and further cytokine secretion of T_H cells, the proliferation of T_C cells into memory T_C cells and cytotoxic T lymphocytes starts immediately. T_C cells mediate a membrane damage leading to cell lysis. The B cell, also a potent APC cell, presents antigenic peptides together with MHC class II and attracts herewith T_H cells. As a result of the interaction, the T_H cells activate the B cells, which differentiate into a population of both antibody-secreting plasma cells and memory cells (Kuby, 1997).
Although cell-mediated immune response is important (Beauverger et al., 1996), the humoral response has been found to have positive impact on the clinical course of measles infection (Aaby et al., 1987).
Exact evaluation of the relative importance of these two mechanisms is difficult since MV causes a depression of the normal immune functions.

1.3.2 Specific immune response against MV

Clearance and recovery from infection are associated with production of serum-antibodies as well as establishment of cellular immunity. The measles-specific immune response begins with the onset of the maculopapular rash together with the general appearance of virus-specific B and T cells. The immune response continues for many weeks after apparent recovery. Although already verified by many scientists, the interaction of different components of the immune system and their first detectable antibody appearance during recovery from measles infection still needs further investigation.

Measles-specific IgM is the first antibody-isotype that is found in serum after primary measles infection. It enriches the serum transiently, starting with onset of rash (Forghani et al., 1983; Tuokko and Salmi, 1983; Lievens and Brunell, 1986). It can be detected in most individuals 3 days after onset of rash (Rossier et al., 1991; Perry et al., 1993). IgM peaks on day 7 - 10 and wanes again within weeks (Pederson et al., 1986). A single serum specimen collected at the appropriate time (Helfand et al., 1997; Helfand et al., 1999 a, b) is now accepted to be sufficient to diagnose measles (Vuorimaa et al., 1978; James, 1990; Ozanne and d'Halewyn, 1992). However, the absence of IgM does not exclude infection, as the sensitivity of some of the IgM assays may be low.

Measles-specific IgM switches later to IgG and IgA (Schluederberg, 1965; Ehrnst, 1978; Mathiesen et al., 1990). IgG becomes detectable in serum soon after onset of rash, peaks within about 4 weeks and subsequently declines, but persists for life as IgG memory cells. Measles IgG titers tend to be lower and wane faster after measles vaccination as opposed to wt infection (Christenson and Bottiger, 1994; Davidkin and Valle, 1998). The most rapidly and most abundant antibodies are directed against the N-Protein, followed immediately by antibodies against the hemagglutination and fusion proteins and those antibodies are believed to be mainly responsible for virus neutralization. Erdman et al., (1991) found that the peak IgA response to measles in serum occurs at about 3 - 10 days after the onset of rash. Furthermore, IgA, IgM and IgG antibodies are found in secretions (Dubois-Dalcq et al., 1984; Perry et al., 1993; Brown et al., 1994).

However, several investigations have shown that the cellular immunity is also of great importance for recovery from measles infection (Samb et al., 1995; Ward et al., 1995). Patients with agammaglobulinaemia or the deficiency of a single immunoglobulin, such as the selective IgA-deficiency recover uneventfully from MV infection, whereas patients with deficits in cellular immunity acquire severe and progressive disease (Griffin and Bellini, 1996). Especially, T_H and T_C cells are involved. T_C cells are detectable in blood at the time of the rash. They eliminate infected cells via the MHC class I restricted pathway by inducing proliferation of cytotoxic T lymphocytes, which damage the membrane of the

altered self cell leading to virus clearance. T_H cells rather work as messenger and activator cells and are important for antibody production (Griffin et al., 1994). They become activated themselves by contacting a MHC class II antigen complex and an additional co-stimulatory signal from an APC. Secretion of the different cytokines determines the further function of the two different types of T_H cells:

- type 1 produces interferon-γ and interleukin (IL)-2, which activate macrophages leading to a delayed type hypersensitivity skin test response and promoting T cell proliferation
- type 2 produces IL-4, 5 and 10, which are important for B-cell growth and differentiation as well as macrophage deactivation.

The immune response against natural measles infection leads mainly to the activation of T_C cells, which are important for virus clearance, as well as of type 2 T_H cells needed for antibody production. However, there is also in vivo and in vitro evidence of immune suppression during measles infection. The specific immune suppression begins with the onset of clinical disease, before the rash, and continues for many weeks after apparent recovery. During this period superinfections account for most of the morbidity and mortality associated with measles. In vivo, delayed type hypersensitivity skin test responses to recall antigens, such as tuberculin, are suppressed for several weeks after recovery from infection (Tamashiro et al., 1986). In vitro, lymphoproliferative responses to mitogens are suppressed and lymphokine production is abnormal (Ward et al., 1991; Karp, 1999).

The MV infection leads to a life long immunity and re-exposure is not required (Panum, 1938). There is no evidence for persistence of latent virus, but it is still possible that viral antigens stay in antigen-retaining cells in germinal centres leading to long-term antibody production (Donaldson et al., 1986). It has also been shown that MV can re-infect fully protected individuals resulting in an asymptomatic secondary immune response (Huiss et al., 1997). Furthermore,

the virus could even be isolated from naturally-immune but asymptomatically re-infected individuals (Vardas and Kreis, 1999).

1.3.3 Surveillance of measles immunity

The number of measles cases has declined substantially mainly as a result of mass vaccination campaigns. However, it has also limited the experience of doctors and health care workers, and made it increasingly difficult for them to diagnose measles (Lawrence et al., 2001). Moreover, incomplete protection after vaccination can result in a mild or asymptomatic MV infection (Edmonson et al., 1990). Diseases with similar skin involvement like measles and patients with dark skin add to the difficulties of measles diagnosis (Zenel, 2000). All this led to the conclusion that only clinical diagnosis of measles lacks the precision required for an effective disease control (Adjaye et al., 1983; Norrby and Oxman, 1990; Brown et al., 1994; Ferson et al., 1995; Ratnam et al., 2000). However, as the notification of every measles case should trigger a public health response with minimal delay, accurate and rapid serological diagnosis is of paramount importance to avoid outbreaks that could possibly arise from only one misdiagnosed patient (Paunio et al., 1998).

Currently, many different serological techniques are used to diagnose and monitor measles. The WHO recommended serological criteria for measles diagnosis are isolation of MV, a significant increase in IgG antibody-titer or presence of measles-specific IgM antibodies in serum (WHO, 1999 a). MV isolation is time consuming and expensive, but possibly helpful in cases, such as wt virus contact after recent primary measles vaccination (Helfand et al., 1999 a). The disadvantage of measles-specific IgG detection in assays, such as hemagglutination inhibition (HI), radioimmunoassay (RIA), plaque reduction neutralization (PRN) and neutralization (NT) assay (Hierholzer et al., 1969; Albrecht et al., 1981; Orenstein et al., 1987; Ratnam et al., 1995), is the need of a second serum sample, drawn a few days after the first one, in order to see a significant rise in the IgG antibody-titer. Without a second serum sample accurate interpretation may be difficult. In association with this method, a delay in the start of precautions and of suitable medical treatment is inevitable.

Measles-specific IgM can be detected in test-systems like RIA and enzyme linked immunosorbent assay (ELISA). RIAs are reliable but require adequate facilities to store, use and dispose the radioactive materials needed for performance. IgM ELISAs are available in two formats: (i) Indirect ELISAs (Arista et al., 1995), in which the whole MV is directly coated onto a solid phase are the most widely used. However, specific IgG antibodies may cause false negative results by inhibiting the binding of IgM causing a risk of further of spreading the disease. Also, the IgM rheumatoid factor (RF), which can be frequently detected in serum from patients with rheumatoid arthritis, and occasionally also in serum from normal individuals may be a source of false positive reactions (Kolb and Allner, 1986; Jenkerson et al., 1995). The production of RF may also be activated during measles (Tuokko and Salmi, 1983). (ii) Capture ELISAs (Erdman et al., 1991), in which the solid phase is coated with an anti-human IgM-specific antibody, are considered to be superior to indirect assays, since they are less susceptible to the above mentioned problems (Erdman et al., 1991; Hummel et al., 1992). In order to facilitate assay performance, labelled antigens for detection of virus-specific IgM have been developed (Chiodi et al., 1986; Salonen et al., 1986; Tuokko, 1988). More recently, immunoassays based on recombinant MV-H or -F protein measurements using H or F transfected human melanoma cell lines, either as extracted or cell-surface expressed antigens have been established, such as the H-ELISA (Bouche et al., 1998 a, b, c) or FACS-measured immunofluorescence assay (de Swart et al., 1998).

Reliable clinical and laboratory surveillance and prompt investigation of cases and contacts are essential for the timely implementation of control measures to interrupt virus circulation (Lambert et al., 2000). To achieve this goal, highly sensitive and specific tests are needed. The major requirements of such laboratory tests are accuracy, simplicity of the test itself and of sample collection, stability of materials, affordability and high sensitivity and specificity.

1.3.4 Alternatives

Phlebotomy is uncomfortable for patients, especially for infants or small children and requires trained staff and sterile tools. In 1999, the WHO reported that up to 50% of injections given in third world countries were not sterile, carrying the risk of iatrogenic infections (e.g. hepatitis, HIV and other bloodborn pathogens) (WHO, 1999 b). Proper collection and destruction of used syringes add to the difficulties, especially in developing countries (Cutts and Steinglass, 1998). In this context, oral fluids may represent a more acceptable alternative. Tests based on saliva specimens have already made a substantial contribution to the diagnosis of MV and it has been shown that the whole range of measles-specific antibody-isotypes is represented in oral fluids (Roitt and Lenher, 1983; Brandtzaeg, 1989). Serological MV diagnosis using saliva samples has gained interest due to the simple, less dangerous collection method and the rather large amount of fluid that can be used for testing (Malamud, 1992; George and Fitchen, 1997).

The usefulness of IgA as the principal immunoglobulin of external secretions has been described by several investigators (Friedman et al., 1982; Friedman et al., 1983; Friedman et al., 1989; Garrido et al., 1997; Yan et al., 2002). Previous saliva studies highlight the advantages of oral fluid usage opposed to serum (Malamud, 1992; Thieme et al., 1994; Helfand et al., 1996; George and Fitchen, 1997). New combinations of antibodies, specimens and test-systems allow improvement of serological MV diagnosis in all directions.

1.4 Measles eradication

1.4.1 Measles vaccines and vaccination strategies

The first MV-vaccine that was used in the 1960's was based on MV, inactivated by tween-ether or formaldehyde treatment, modelled on the successful vaccine for polio. It failed to induce antibodies against the F-protein, which was thought

to be the reason for insufficient protection (Norrby et al., 1975; Merz et al., 1980). Furthermore, antibody-titers declined rapidly resulting in new susceptibility of recipients to measles (Rauh and Schmidt, 1965; Fulginiti et al., 1967; Nader et al. 1968; Hall and Hall, 1979; Frey and Krugman, 1981). A number of vaccinees developed a severe disease called “atypical measles syndrome” after re-exposure to wt virus . Several attenuated measles vaccines were developed at this time by adaptation of MV and long term passage on various cell lines (e.g. AIK-C, Schwarz, Edmonston B, Moraten, Edmonston - Zagreb).

The currently used attenuated live virus vaccine (Moraten and Schwartz) triggers a seroconversion of more than 90% (Hayden, 1979; Preblud and Katz, 1988), but is not resistant to maternal antibodies, which are abundant until the 9^{th} – 12^{th} month of age.

In 1983, researchers developed a vaccine that was especially created to overcome this problem. It was the high-titer vaccine Edmonston-Zagreb-Strain, which could be used in infants of 4 - 6 months of age (Whittle et al. 1988). In 1990 the vaccine was recommended by the WHO for use in infants especially in problematic areas, but Weiss (1992) described a measles correlated mortality 80% higher in children, vaccinated with the high-titer Edmonston-Zagreb-Strain, than in those vaccinated with the normal standard vaccine. Therefore the vaccine was recalled in the same year.

Nowadays, measles vaccination is combined with mumps and rubella in a trivalent (MMR) vaccine, which is mostly injected in two doses in order to overcome primary and secondary vaccine failure (Peltola et al., 1994; Slater et al., 1999; Paunio et al., 2000 b).

Since 1989, the WHO/UNICEF Joint Committee on Health Policy declared control as the first step toward measles eradication. Infants should be vaccinated with the first boost at the age of 12 months (infants in developing countries at the age of 9 months) and again at age 5 – 6 years with the second boost (Weiss, 1992). If a child was vaccinated at the age of 6 months already, an additional boost at the age of 15 months is recommended (Vidyashankar, 2002). If

vaccination was delivered according to the recommended time schedule, the incidence of measles could be considerably reduced (Siedler et al., 2002).
Vaccination against measles is the tool of worldwide eradication efforts, but until a herd immunity of at least 95% has not been reached, waning antibody-titers in mothers, consequently followed by lower antibody-titers in newborns, are critical problems, especially in developing countries. The low maternal titer leads to a loss of measles-specific antibodies in infants long before the recommended time of vaccination. In developing countries this gap results in the death of many infants (Krugman et al., 1985; Black, 1989). On the other hand, persisting maternal antibodies can lead to the neutralization of the vaccine virus and inevitably prevents an active immune response. Waning maternal antibodies would leave the child largely unprotected.
New possible strategies to overcome these problems are urgently needed. Some have been recently described by Osterhaus et al. (1998), Butts et al. (1998), Polack et al. (2000) and El Kasmi and Muller (2001).

1.4.2 Side effects of measles vaccination

Mild adverse events following measles vaccination, such as local reactions, moderate fever (up to 5%) or measles rash (2%) have been reported (Peltola and Heinonen, 1986). They occur less frequently after the second dose of measles vaccine (Chen et al., 1991) and tend to occur only in those individuals not protected by the first dose (Davis et al., 1997). However, it has only recently been shown by Shinefield et al. (2002) that MMR was generally well tolerated and immunogenic in healthy children with 12 months to 6 years of age.
Anaphylactic reactions following measles vaccination are extremely low and case reports have shown that individuals experiencing anaphylactic reactions had IgE antibodies to gelatine, a stabilizer used in vaccine production (Sakaguchi et al., 1995; Pool et al., 2002). There is no evidence for increased cases of aseptic meningitis, encephalopathy, SSPE or Guillain-Barré Syndrome caused by measles vaccination (Miller et al., 1997; da Silveira et al., 1997; Duclos and Ward, 1998; Makela et al., 2002). The alleged associations between inflammatory bowel disease, including Crohn’s disease and autism have been

refuted as groundless (Duclos and Ward, 1998; Farrington et al., 2001; Makela et al., 2002; Thjodleifsson et al., 2002).

The combination vaccine (MMR) produces an immunological response equal to that of the single antigen shots (Decker and Edwards, 1999). It appears that MMR vaccination induces a very mild but transient depression of immune responses for maximal 3 weeks (Pabst et al., 1997) and should therefore not be used in HIV infected children with severe immunosuppression (American Academy of Pediatrics, 1999). Scott et al. (1999) has shown that mild illness (e.g. rhinorrhoea, cough, diarrhoea, fever, conjunctivitis or rash) gives no reason to delay measles vaccination, since there were no significant differences in post-vaccination antibody-levels among children (age 3.5 and 6 months) with symptoms compared to those without. The hypothesis that natural measles infection compared to vaccination could offer protection to some extend against atopic disease has been proven wrong by Paunio et al. (2000 a).

The evidence of the safety and efficiency of MMR vaccine is so overwhelmingly conclusive that health professionals should not hesitate about recommending its use (Elliman and Bedford, 2001).

1.4.3 Eradication efforts

Theoretically, measles represents an ideal target for eradication through vaccination for the following reasons:

- measles virus is monotypic
- absence of an animal reservoir
- no healthy carriers
- cheap and effective vaccine.
- long-term immunity (> 20 years)

Even though the incidence of measles morbidity and mortality has been dramatically reduced due to massive global vaccination campaigns over recent years (WHO, 1998 a, b), the disease remains a serious public health problem in

many parts of the world (Fig. 1.4) with most deaths occurring in the developing countries (mainly Africa and Asia). 13 countries reported routine measles vaccine coverage below 50% (WHO, 2001 b).

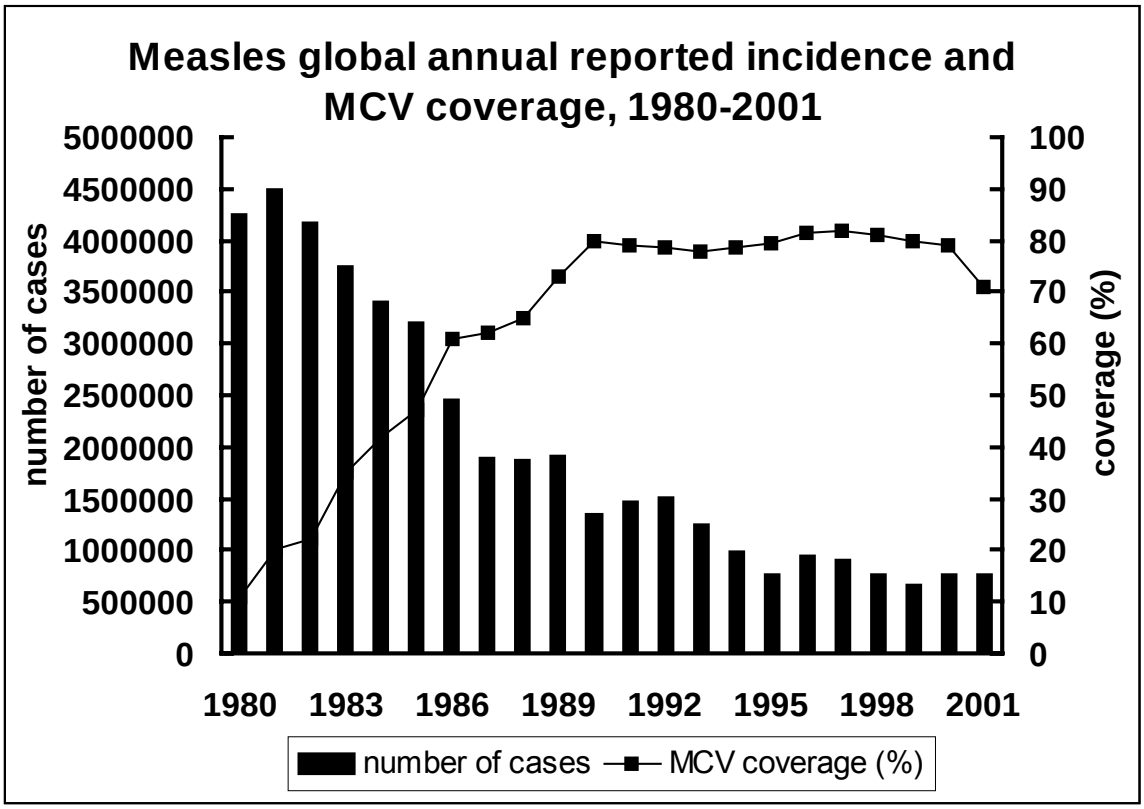


Fig. 1.4: Measles global annual reported incidence and MCV (measles containing vaccine) coverage from 1980 – 2001 (WHO, 2002).

MV is still endemic in various regions and outbreaks continue to occur even in highly vaccinated populations (Maurer and Muhlemann, 1998; Paunio et al., 1998; Hanses et al., 1999, Kelly et al., 2001; van den Hof et al., 2002). In developing countries, MV patients are usually not older than 5 years, whereas patients in first world countries are a few years greater (CDC, 1996; Singh et al.,1997). Because of the highly reproductive rate of measles infection, herd immunity should be kept at 93 – 95% to eradicate the virus (Hethcote, 1983, Anderson and May, 1990; Peltola et al., 1994). This goal has neither been reached in developing, nor in industrialized countries. In the United States, the number of measles cases decreased dramatically (95%) to less then 1500 cases in 1983 after the development of the first measles vaccine (Katz, 1985). Greater measles epidemics arose again in 1989 - 1991 including up to 26.000

cases. Now the virus no longer circulates in the US and all cases are due to virus importation. Factors, such as

- poor acceptance vaccination
- insufficient vaccine potential
- ineffective surveillance system

contributed to renewed outbreaks.

Although Argentina launched a massive vaccination campaign in 1993, MV has re-emerged causing 15.000 cases reported from July 1997 to December 1998, probably due to an accumulation of susceptible individuals and new unprotected annual birth cohorts; inadequate vaccination in poverty-stricken areas, and migration of children from neighbouring countries with unknown measles vaccination status (Barrero et al., 2000). The vaccination coverage has declined from 99% in 1995 and 1996 to 92% in 1997 (WHO, 2001 b). In Nigeria, for instance, vaccination coverage has remained low. Since the introduction of measles vaccination into the national vaccination program in 1979, two mass immunization campaigns have been carried out in 1983 and 1995. Vaccination coverage has reached 69% in 1997, but faded again to only 30% in 2000 (WHO, 1999 a, 2001 b). This consequently facilitates the virus to spread and cause outbreaks or let measles even stay endemic.

To further reduce measles, it is essential to improve the understanding of virus circulation and to concentrate on new strategies to stanch the spread of infection (Miller, 2000).

The WHO and UNICEF will assist affected countries to:

- provide a first dose of measles vaccine to all infants
- guarantee a “second opportunity” for vaccination to increase the probability that as many children as possible are immunized and to assure that those immunized are responding to the vaccination
- establish an effective system to monitor coverage and conduct measles surveillance

- improve management of complicated measles cases including vitamin A supplementation (Cutts et al., 1999; WHO 2001 a).

The priorities for countries pursuing accelerated measles control include improving routine vaccination coverage levels to at least 80% in all districts of every country, achieving at least a coverage of 90% nationwide. Priorities for countries and regions with a measles elimination goal include improving routine vaccination coverage levels to at least 90% in all districts of every country (resulting in nationwide coverage greater than or equal to 95%). Adherence to these priorities will ensure that the measles morbidity and mortality burden will decrease and that the measles disease reduction targets can be reached. Eradication would avert the current annual deaths due to measles and save about $ 1.5 billion in treatment and prevention costs (Orenstein et al., 2000).

1.5 Goals of this thesis

1.5.1 Comparison of 5 different serological measles IgM assays

Vaccination reduces the number of measles cases but consequently it also reduces the experience of doctors to diagnose clinical measles. Therefore, new accurate serological assays are needed for the timely implementation of standard measles control measures.
A panel of 225 serum and plasma samples was collected in order to compare a set of five different serological assays:

- Indirect whole measles virus ELISA (Enzygnost® Anti-Measles Virus/IgM (IgM-Enzygnost))
- Capture measles-IgM ELISA (Measles-IgM Comfort EIA (IgM-Comfort EIA))

- Recombinant H-ELISA (H-ELISA), performed following a standard protocol and production of recombinant MV-H protein and the control antigen as described by Bouche et al. (1998 a, b, c)
- FACS-measured immunofluorescence assay based on human melanoma permanently transfected cells expressing the MV-H protein (H-FACS)
- FACS-measured immunofluorescence assay based on human melanoma permanently transfected cells expressing the MV-F protein (F-FACS)

The performance of each test severely depends on its cut-off value. Therefore, evaluation was done with the standard characteristics (sensitivity and specificity) and additionally with the “receiver operating characteristic curves” (ROC), which do not depend on a discrimination threshold, but calculate the performance of each assay with all possible cut-off values.

1.5.2 Evaluation of IgA response against MV-H

Samples for serological measles testing are usually collected by vein-puncture, which is uncomfortable and bears certain risks for patients and medical personnel. It further requires trained staff and sterile tools. These conditions are often not fulfilled, especially in developing countries. In this context, oral fluids may represent a more acceptable alternative.
Therefore, a panel of 273 serum and saliva samples, derived from

- vaccinees
- late convalescents
- acute phase measles virus patients

was compiled.

FACS-measured immunofluorescence assay (based on human melanoma permanently transfected cells expressing the MV-H protein) was used to measure MV-specific IgA antibodies against the MV-H protein.

2 Materials and Methods

2.1 Test panels

2.1.1 Panel A for comparison of MV-specific IgM assays

Group	Origin	IgM-Enzygnost	IgM-Comfort EIA	H-FACS	F-FACS	H-ELISA
1	Patients Luxembourg	101	101	101	101	68/101
2	Patients Nigeria	37	37	37	37	-
3	Late convalescent RF positives	37	37	37	37	-
4	Late convalescent parents	22	22	22	22	-
5	Late convalescents Nigeria	10	10	10	10	-
6	Late convalescents and vaccinees Luxembourg	18	18	18	18	14/18
Total		225	225	225	225	82

Table 2.1: Distribution and origin of human serum and plasma samples tested in 5 assays.

A total of 101 sera was collected from 67 patients (median age 8 years, age range 1 to 35 years) during a measles outbreak in Luxembourg in 1996 and from several other isolated cases in Luxembourg (Table 2.1, group 1). Blood was taken from 14 days before up to 59 days after onset of rash. 28 patients were at least bled twice. All patients were confirmed as acute measles cases by detection of specific IgM antibodies in the IgM-Enzygnost. In some patients, measles was also confirmed by an increase of specific antibodies in paired sera

(n = 32) and/or the clinical case definition of the Centre of Disease Control and Prevention (CDC) (Atlanta, USA) (n = 24). The CDC criteria include (i) generalized maculopapular rash for 3 days or more; (ii) fever of at least 38.3°C, if measured; (iii) and at least one of the following symptoms: cough, coryza or conjunctivitis. These sera were previously also analysed by Bouche et al. (1998 c) using the H-ELISA. As stated in the latter study, only the sera drawn until day 19 (68 sera originating from 48 patients) after onset of rash were evaluated here (group 1).

In addition, the following sera were included: (i) 37 acute phase (day 1 - 6 after onset of rash) plasma samples (median age 2 years, age range 7 months to 35 years) collected in Nigeria for MV isolation (group 2), (ii) 87 IgG positive and IgM negative serum samples, including 37 measles IgM negative, but IgG and RF IgM positive samples (group 3), 22 strong IgG positive serum samples (> 0.8 O.D.; tested in IgG-Enzygnost) originated from fully protected, late convalescent parents who had children with measles (group 4), 10 IgG positive and IgM negative sera from Nigeria (group 5) and 18 measles IgG positive and IgM negative serum samples, obtained from late convalescents and from one vaccinated person (median age 49 years, age range 13 to 74 years), of which 14 were additionally analysed in H-ELISA (group 6).

During the experimental period of our study, it was not possible to test the samples from group 2 – 5 in the H-ELISA. For a better understanding, panel A is therefore divided into 6 groups. Group 1 and 6 (n=82) were tested in all 5 assays (IgM-Enzygnost, IgM-Comfort EIA, H-FACS, F-FACS, H-ELISA), whereas these 82 samples plus the samples from group 2 – 5 were only tested in 4 assays (the latter mentioned, except the H-ELISA) (n=225).

2.1.2 Panel B for evaluating IgA response against MV-H

Specimen	Group	Immunological status	Luxembourg	Nigeria	Argentina	Total
Serum	1a	Vaccinees	24	20	-	44
	1a	Late convalescents	30	20	-	50
	1b	Patients	37	-	63	100
Saliva	2a	Vaccinees	14	-	-	14
	2a	Late convalescents	19	-	-	19
	2b	Patients	14	-	32	46
	Total					273

Table 2.2: Distribution and origin of human serum and saliva samples tested in the H-FACS.

94 Serum samples from 24 Luxembourgian (median age 12 years, range 12 to 27 years) and 20 Nigerian vaccinees (median age 11 years, range 4 to 15 years). 30 sera were collected from Luxembourgian late convalescent individuals (median age 34 years, range 26 to 38 years), which included 10 MV IgG low-titer (0.2 – 0.8 O.D.; tested in IgG-Enzygnost; median age 29.5 years) and 10 MV IgG high-titer samples (2.4 – 2.8 O.D.; median age 30 years). Those were age matched with 20 Nigerian (median age 30 years, range 25 to 38 years) late convalescent sera (Table 2.2, group 1a).

37 Serum samples (16 paired with saliva) were collected from MV patients originating from Luxembourg within 14 days before until 46 days after onset of rash (median age 8.5 years, range 1 to 35 years). 63 Patient samples (30 paired with saliva) were collected from Argentina MV patients (median age 1 year, range 2 months - 42 years), taken during the 1997 - 1998 outbreak (group 1b).

33 Saliva samples from 14 Luxembourgian vaccinees (median age 24 years, range 18 – 45 years) and 19 late convalescents (median age 28 years, range 22 – 47 years) (group 2a).

46 Acute phase patients saliva derived from 14 Luxembourgian patients (median age 9 years, range 7 to 35 years), drawn 14 days before until 28 days after onset of rash and 32 Argentine patient samples from day 1 - 4 after onset of MV rash (median age 1 year, range 3 months - 5 years) (group 2b).

2.1.3 Specimen handling

2.1.3.1 Materials

Reagents		
Serum, plasma and saliva samples		Panel A, B
Instruments		
Saliva collection	Saliviette	Sarstedt, Nümbrecht, Germany
Serum collection	Z-Vaccutainer	BD Vacutainer systems, Plymouth, UK
Plasma collection	K3E-Vaccutainer	BD Vacutainer systems, Plymouth, UK
Sterile disposable pipette	Finetip Transfer pipette	SIGMA Chemicals Co., Mo., USA
Waterbath	37°C	Julabo, Seelbach, Germany
Centrifuge	Heraeus Biofuge Stratos	Heraeus, Hanau, Germany
Eppendorf tubes	1.5 ml	Greiner, Frickenhausen, Germany
1-Channel pipette	100-1000 µl	Dunn, Labortechnik GmbH, Asbach, Germany

Table 2.3: Materials for specimen handling

2.1.3.2 Procedure

Blood was drawn into EDTA Vaccutainer and transferred under sterile conditions into 15 ml tubes. If plasma was extracted, sample was centrifuged with 2000 rpm (739 x g) for 20 min at 20°C. Serum samples were drawn into Vaccutainer without EDTA. Samples were centrifuged with 3000 rpm (1663 x g) for 30 min at 20°C. Supernatant was removed without disturbing the pellet. Plasma and serum samples were transferred aseptically to sterile 1.5 ml tubes and with no delay stored at – 20°C.

Saliva samples were collected in the morning (before test persons have brushed their teeth) with a small cylinder of cotton wool. The cotton wool swab was placed under the tongue and kept in the mouth until swallowing could not longer be prevented. This took 30 to 45 s. Cotton wool swab was then returned to the suspended insert, firmly closed with the stopper and kept at 4°C until all

samples have been collected. In Argentina, saliva samples were collected with a disposable, sterile pipette. Subsequently, all saliva samples were treated in our laboratory essentially as described by Friedman (1982):
Samples were thawed and inactivated in the waterbath at 56°C for 1h and thereupon centrifuged for 5 min at 10 000 rpm (1792 x g) to remove debris, gross bacterial contamination and mucin. The supernatants were transferred to fresh tubes and stored at – 20C°. Samples were kept on ice during all procedures.

2.1.4 Assays performed on different panels

Panel	Number of samples	Tests
A	225 serum and plasma samples	Enzygnost® Anti-Measles-Virus/IgM (IgM-Enzygnost),Measles-IgM Comfort EIA (IgM-Comfort EIA), H-ELISA, IgM-Mel-JuSo/MV-H/F-FACS (H-/F-FACS)
B	273 serum and saliva samples	IgA-Mel-JuSo/MV-H-FACS

Table 2.4: Distribution of panels and assays used in this study

2.2 Cell culture

2.2.1 Transfected human melanoma cells (Mel-JuSo)

Mel-JuSo/MV-H and -F transfected and Mel-JuSo/wt melanoma cells were kindly provided by Dr. de Swart (Rotterdam) (de Swart et al., 1998). The melanoma cell line Mel-JuSo was established in the Institute for Immunology (Munich) from a primary tumour (Johnson et al., 1981). Originally, the human Mel-JuSo melanoma cells (Johnson et al., 1981) were transfected with the full length Edmonston F- or H-genes (de Swart et al., 1998). The resulting cell lines, Mel-JuSo/MV-H and Mel-JuSo/MV-F, displayed identical growth characteristics

as the parental Mel-JuSo/wt cell line: fast-growing, adherent cells. Cells were seeded into culturing flasks containing medium supplemented with fetal bovine serum, antibiotics, sodium pyruvate and fungizone. Chilled EDTA (which chelates the calcium required for cell adhesion) was used for harvesting.

2.2.2 Materials

Reagents		
Culture medium	Basic medium RPMI 1640	Gibco, Bruxelles, Belgium
	+ 10% Fetal bovine serum heat inactivated (50 ml/500 ml basic medium)	Gibco, Bruxelles, Belgium
	+ 1% PSG-mixture containing Penicillin (10000 IU/ml), Streptomycin (10000 µg/ml), Glutamine (29.2 mg/ml) (5 ml/500 ml of basic medium)	Gibco, Bruxelles, Belgium
Mel-JuSo cells	Human melanoma cells transfected with MV-H (Mel-JuSo/MV-H) or F (Mel-JuSo/MV-F) and not transfected wild-type cells (Mel-JuSo/wt)	Dr. de Swart, Rotterdam, The Netherlands
PBS-EDTA	Phosphate buffer	Gibco, Bruxelles, Belgium
	+ EDTA 0.1g per 500 ml of phosphate buffer	Sigma, St. Louis, Missouri, USA
Instruments		
Laminar flow unit	Uniflow UVUB	Uni Equip, Martinsried, Germany
Incubator	37°C, 5% CO_2	Heraeus, Hanau, Germany
Centrifuge	CR4-12	Jouan, St. Herblain, France
Centrifuge tubes	15 ml, 50 ml	Nunc, Roskilde, Denmark
Counting chamber	Neubauer improved hemocytometer	Greiner, Frickenhausen, Germany
Counting dye	Trypan blue 0.4 %	Sigma, St. Louis, Missouri, USA
Microscope	ID 03	Zeiss, Frankfurt, Germany
Liquid nitrogen reservoir	Chronos 80	Messer Griesheim, Mudersbach, Germany
Culture flasks	175 cm²	Nunc, Roskilde, Denmark
Sterile pipettes	2 ml, 10 ml, 25 ml	Nunc, Roskilde, Denmark
Pipette aid	pipetus-akku	Hirschmann, Germany

Table 2.5: Materials for cell treatment

2.2.3 Procedure

2.2.3.1 Cell culturing

In order to standardize the H- or F-expression, one stock of each Mel-JuSo/MV-H and Mel-JuSo/MV-F cells, was used. Cells were stored in liquid nitrogen (N_2) in amounts of 5 x 10^6 / vial. 3 days prior to the experiment, cells were seeded in 175 cm² culture flasks and cultivated in culture medium under standard cell culture conditions. Mel-JuSo/wt cells were used from a continuous culture.

2.2.3.2 Thawing the cells

All following steps were performed under sterile conditions. 50 ml Mel-JuSo culture medium was filled in a 50 ml centrifuge tube. Mel-JuSo cells were collected from the N_2 tank and without delay placed in the 37°C waterbath until frozen complex was loose. Subsequently, the content of the vial was added to 50 ml culture medium and distributed over two 175 cm² cell culture flasks containing 25 ml of the Mel-JuSo cell/culture medium mix. After 3 days, the semiconfluent cell monolayer was used for flow cytometry measurements.

2.2.3.3 Harvesting cells

After 3 days, culture medium was removed and 15 ml of PBS-EDTA were added to the flask. Cells were returned to the incubator for 10 min in order to allow detachment from the flask. Detached cells were swirled gently and 14 ml were harvested and added into a 15 ml centrifuge tube. To the 1 remaining ml of cell suspension, additional 24 ml of culture medium was added for further culturing. The harvested cells were centrifuged at 1200 rpm (266 G) for 5 min at 4°C. Supernatant was removed and the pellet was resuspended in 1 ml FACS medium and kept on ice. Cells were counted using trypan blue solution. The number of cells per ml of suspension was determined as follows:

$$n = C \times 10^4 \times 2 \qquad (C = \text{Cell count per large square})$$

2.2.4 Transfected baby hamster kidney cells (BHK-21 cells)

The parent line of BHK-21(C-13) was derived from the kidneys of five unsexed, 1-day-old hamsters (Mesocricetus auratus, Syrian golden) by Macpherson and Stoker (1962). Following 84 days of continuous cultivation, interrupted only by an 8-day preservation by freezing, clone 13 was initiated by single-cell isolation (Macpherson, 1963). This line has been used as a host for transformation with expression vectors containing selectable and amplifiable marker DNA's.

Semliki Forest virus (SVF) vectors allow for the expression of protein-encoding sequences in virtually any animal cell, since the virus has a broad host range. A protein-coding sequence of interest is subcloned into a plasmid vector, which is subsequently used to produce recombinant RNA in vitro (Liljeström and Garoff, 1991). This RNA is transfected into the cells and amplified by virtue of its self-encoded RNA replicase.

BHK-21 cells were transfected with the resulting RNA of three overlapping cDNA fragments of the MV H-protein, which were obtained by reverse transcription-PCR from total RNA of virus-infected Vero cells.

BHK-21 cells transfected with the recombinant SFV1 RNA of the H protein were referred to as BHK-H; control cells transfected with SFV1-β-galactosidase RNA were called BHK-gal. High levels of expression were confirmed by flow cytometry. At 24h posttransfection, the cells were mechanically disrupted in hypotonic solution (10mM Tris-HCl [pH7.6], 0.5 mM $MgCl_2$). The nuclei and the large debris were sedimented at 500 x g (5 min, 4°C). Total membranes were sedimented from the supernatant by centrifugation at 110.000 x g (45 min, 4°C). After resuspension in phosphate buffered saline-0.5% Nonidet P-40, the pellet containing insoluble debris and the total membrane were centrifuged again at 14.000 x g (15 min, 4°C) to remove insoluble particles. The supernatant containing the total membrane fraction was stored at 4°C in 0.05% azide. The total protein concentration was determined by the method of Bradford (Bradford et al., 1976). This work was done by Dr. Fabienne Bouche, Laboratoire National de Santé, Luxembourg.

2.3 Serological methods

2.3.1 Indirect whole measles virus ELISA (IgM-/IgG-Enzygnost®)

2.3.1.1 Principle

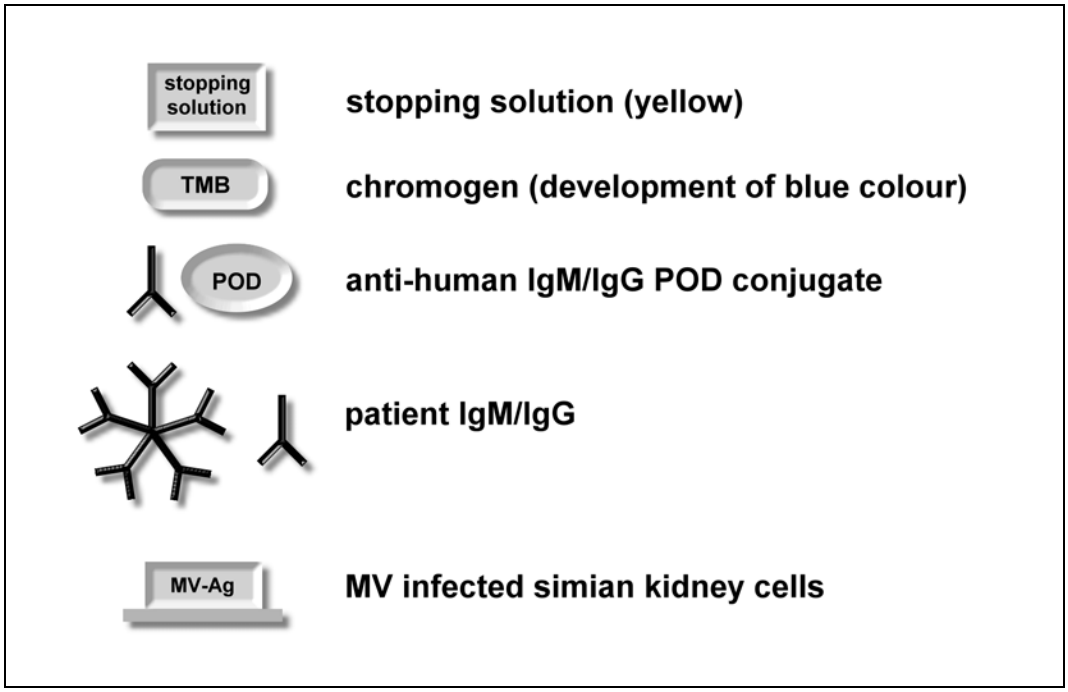


Fig. 2.1.: Schematic representation of IgM-/IgG-Enzygnost (step 1-5, starting from the bottom)

The IgM-/IgG-Enzygnost® (Dade Behring, Marburg, Germany) are commercial ELISA-Kits for detection and quantification of MV-specific IgM or IgG antibodies. The assay is based on directly coated MV-antigen. MV simian kidney infected cells were used as positive antigen and the negative control antigen corresponds to the non infected cells (step 1). For IgM determination the added rheumatoid factor (RF) absorbent binds to IgG and IgG-linked IgM-RF present in the test sample and is thus eliminated. This step is not needed for IgG measurements. MV-specific IgM or IgG from the test sample (added in the second step) binds to the infected cells at the bottom of the well and the µ-chain

or y-chain-specific anti-human / peroxidase (POD) conjugate further binds to this complex (step 3). The enzyme component of the conjugate transforms the substrate into blue chromogen (step 4). The reaction is terminated by the addition of stopping solution (blue changes to yellow) (step 5). The intensity of the yellow colour correlates with the amount of MV-specific IgM or IgG antibodies in the sample and is evaluated with a photometer at a wavelength of 450 nm.

2.3.1.2 Materials

Reagents for IgM-Enzygnost		
Anti-measles virus/IgM test plate for 48 test samples (LOT No. 429136)	6 strips of 8 wells in the left row coated with MV- antigen and 8 wells in the right row coated with control-antigen	
Anti-MV reference P/P (positive control) (LOT No. 429233)	anti-measles virus/IgG with RF in TBS (20 mmol/l)	
Anti-MV reference P/N (negative control) (LOT No. 429561)	human monoclonal IgG-specific for MV in TBS (20 mmol/l)	
Sample buffer POD	TBS 0.3 mol/l (for diluting the test samples and the anti-MV references)	
Anti-human IgM/POD conjugate (LOT No. 424021)	µ-chain-specific, goat antibody conjugated with POD in 0.05 mol/l TBS	
Conjugate buffer	EDTA, 37 mg/l in 0.01 mol/l PBS	
RF absorbent	sheep antibodies against human IgG Fc-fragment in 10 mmol/l PBS (8.2 g/l)	
Reagents for IgG-Enzygnost		
Anti-measles virus/IgG test plate for 48 test samples (LOT No. 429436)	6 strips containing 8 wells in the left row coated with MV-antigen and 8 wells in the right row coated with control-antigen	
Anti-MV reference P/N (positive control); (LOT No. 429565)	human IgG-specific for MV in TBS, 20 mmol/l	
Sample buffer POD	TBS, 0.3 mol/l (for diluting the test samples and the anti-MV references)	
Anti-human IgG/POD conjugate (LOT No. 423925)	y-chain-specific, Fab' component of a rabbit antibody conjugated with POD in 0.05 mol/l TBS	
Conjugate buffer	EDTA, 37 mg/l in 0.01 mol/l PBS	
Supplementary reagents for IgM/IgG		
Washing solution POD for IgM/IgG	Dade Behring, Marburg, Germany	
Chromogen TMB for IgM/IgG	Dade Behring, Marburg, Germany	
Substrate buffer TMB for IgM/IgG	Dade Behring, Marburg, Germany	
Stopping solution for IgM/IgG	Dade Behring, Marburg, Germany	
Test sera, plasma and control samples	Panel A	
Instruments		
Incubator	37 ° C	Heraeus, Osterode, Germany

Photometer for microtiter plates	Titertek Multiskan Plus MK II, 450 nm	Flow Laboratories, Asse-Relegem, Belgium
1-Channel pipette	0.5-10 µl, 10-100 µl, 50-200 µl, 100-1000 µl	Dunn, Labortechnik GmbH, Asbach, Germany
8- and 12-Channel pipettes	50-200 µl	Dunn Labortechnik GmbH, Asbach, Germany

Table 2.6: Materials for IgM/IgG-Enzygnost (all reagents are included in the kit)

♦ **Procedure (IgM-Enzygnost®)**

Reagents were equilibrated at room temperature before the assay was started. Sera, plasma samples or anti-MV references (positive and negative controls) were pre-diluted 1:21 in sample buffer (20 µl + 400 µl). RF absorbent was reconstituted in 5 ml H_2O and 200 µl of the diluted sample were added to an equal volume of RF absorbent (1:42) to precipitate IgG and IgG-linked IgM-RF, which could interfere with IgM detection. Pre-diluted anti-MV reference sera were not treated with RF absorbent. After an incubation time of 15 min at room temperature (18° - 25°C), 150 µl of each sample were placed into a well coated with MV-antigen and one well coated with control-antigen. The reference P/N (negative control) was added to one pair of wells at the start of the series (wells A1/A2), whereas the reference P/P (positive control) was loaded into the second (wells B1/B2) and last pair of wells of each plate. For the transfer of the pre-diluted sera and plasma samples an 8-channel pipette was used and samples were mixed thoroughly after dispensing. Plates were covered with an adhesive foil and incubated for 60 min at 37°C. Samples were then washed with no delay. Washing solution was not introduced too quickly into the wells since excessive foam formations had to be avoided. Washing solution was thoroughly aspirated. 180 µl washing buffer was added to each well and aspirated after allowing the buffer to react for 1 – 2 min. Washing was repeated 4 times. Then, 100 µl conjugate buffer (250 µl of anti-human IgM/POD conjugate in 12.5 ml conjugate buffer) was added to each well. The test plate was again covered with an adhesive foil and incubated for another 60 min at 37°C. Washing was repeated as described above. Thereafter, substrate buffer (1 ml chromogen in 10 ml substrate buffer) was distributed to each well. The test plate was

incubated for further 30 min at room temperature and after adding 100 µl stop solution to each well, absorbance was measured in the photometer at the reference wavelength of 450 nm. 6 IgM positive and negative serum samples were taken along on all test plates as an additional internal laboratory controls for reproducibility.

For each test and reference sample the difference in absorbance (∆A) in O.D. of the value obtained for MV-antigen and control-antigen ($\Delta A = A_{antigen} - A_{control\ antigen}$) was calculated. Results were only used for further evaluation if the following validation criteria were met: The ∆A-values for each pair of reference P/P wells must be within the range defined by the lower and upper margins given by the manufacturer. In addition, the ∆A-values of the individual readings of the reference P/P at the start and the end of the series must not deviate from the mean of these two readings by more than ± 20% and each pair of reference P/P wells must reach or exceed a value of 0.2 O.D. The ∆A for the reference P/N should always be less than 0.1 O.D. The reference P/P is not only used for validation of the test, but is also necessary for the calculation of a correction factor. The nominal value given in the enclosed table of values for the reference P/P was divided by the mean value of its ∆A values obtained in the test. The ∆A-values of the samples, determined in the same series, were corrected by multiplying with this factor and the cut-offs for negativity (< 0.1 O.D.) and positivity (> 0.2 O.D.) were applied as recommended by the manufacturer.

- **Procedure (IgG-Enzygnost®)**

Sera, plasma samples or the anti-MV reference (positive control) were pre-diluted 1:21 in sample buffer (5 µl + 100 µl). A volume of 200 µl sample buffer was distributed into each well of the test plate. 20 µl of the pre-diluted reference P/N were added into the first pair of MV-antigen and control-antigen wells (A1/A2). Afterwards, 20 µl of each test sample were placed into the following pairs of MV-antigen / control-antigen wells, however, the last pair on the plate was filled again with the pre-diluted reference P/N. Further steps were performed as described for IgM-Enzygnost except for the preparation of

conjugate buffer. In this case, 250 µl of anti-human IgG/POD were added to 12.5 ml conjugate buffer.

Calculation and validation was performed as described for the IgM-Enzygnost with the following exception: each pair of reference P/N wells must reach or exceed a value of 0.5 O.D. Correction factor was calculated with the reference P/N as described above. The corrected ΔA-value was used for the calculation of the antibody-activity of the sample using the following formula (α-method): $\log_{10}$ mIU/ml = $\alpha * \Delta A^{\beta}$. The values for the lot-dependent constants α and β are also given in the manufacturer's table of values. The cut-offs for negativity (< 0.1 O.D.) and positivity (> 0.2 O.D.) were taken as recommended.

2.3.2 Capture measles-IgM ELISA (IgM-Comfort EIA)

2.3.2.1 Principle

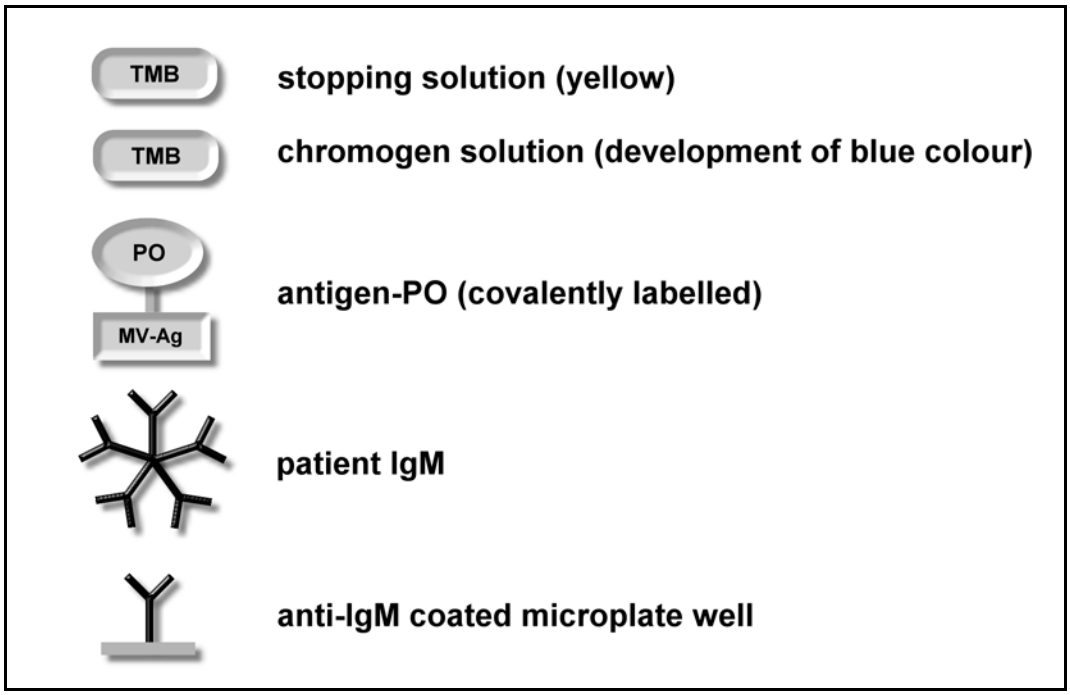


Fig. 2.2: Schematic representation of the capture measles IgM-Comfort EIA (step 1-5, starting from the bottom)

The measles IgM-Comfort EIA (Meddens Diagnostics, The Netherlands) is a commercial antibody-capture ELISA kit for the detection of measles-specific IgM

antibodies. Antibodies specific for the µ-chain of human antibodies are coated to the wells (step 1). The diluted patient sample is incubated in the wells and IgM antibodies bind to the immobilised anti-human IgM antibodies (step 2). The measles antigen is prepared by purification of the whole virus. Peroxidase (PO) is then conjugated to this antigen. For the reduction of aspecific reactivity, control-antigen is added to this conjugate. After removing unbound components, measles-PO conjugate is dispensed into the wells (step 3). This antigen-conjugate will then complex to the captured measles-specific IgM. Due to the added chromogen, colour will develop proportionally to the amount of measles-specific IgM bound in the wells (step 4). The reaction is stopped (step 5) and measured at the absorbance at 450 nm.

2.3.2.2 Materials

Reagents	
Anti-human IgM coated microtiter plate for 88 test samples; (LOT No. 9902.1)	12 strips (8-well breakable) in a special holder, each strip contains 8 wells
Positive control, RED; (LOT No. 9901.01)	human MV-IgM positive sera
Negative control, YELLOW; (LOT No. 9801.02)	human MV-IgM negative sera
Cut-off control, GREEN; (LOT No. 9901.01)	human MV-IgM low positive sera, diluted in a special buffer such that it gives the appropriate signal strength to distinguish between positive and negative samples.
Control-antigen (100 x concentrated); (LOT No. 9901.01)	human cellular components
PO-labelled Measles conjugate (100 x concentrated); (LOT No. 9901.01)	Purified whole MV conjugated with POD
Dilution buffer, BLUE; (LOT No. 9902.02)	phosphate buffered saline enriched with proteins and bacteriostatic substances
TMB (tetramethylbenzidine) substrate (10 x concentrated); (LOT No. 9902.04)	Meddens, Brummen, The Netherlands
Rinsing buffer (10 x concentrated); (LOT No. 9901.01)	Meddens, Brummen, The Netherlands
TMB diluent; (LOT No. 9812.04)	Meddens, Brummen, The Netherlands
Stop solution; (LOT No. 9902.01)	Meddens, Brummen, The Netherlands
Instruments	
	2.3.1.2

Table 2.7: Materials for IgM-Comfort EIA (all reagents are included in the kit)

2.3.2.3 Procedure

Sera and plasma samples were pre-diluted 1:21 in dilution buffer (10 µl + 200 µl) and mixed thoroughly. Control sera were ready to use. 100 µl of positive and negative control serum were dispensed in duplicate in A1/B1 and C1/D1, respectively. The cut-off control serum was loaded into the wells E-H1. After an incubation time of 30 min in a resealable bag at 37°C, plates were rinsed 5 times by filling all wells to the rims (300 µl) with rinsing buffer. After 5 washing cycles, plates were placed on absorbent paper to remove residual washing solution. 100 µl diluted measles-PO conjugate (12.0 ml dilution buffer + 120 µl measles-PO conjugate + 120 µl control antigen / 12 stripes = 1 complete plate) was dispensed per well. Plates were incubated for 30 min at 37°C and washed again. Subsequently, 100 µl of diluted TMB (10.8 ml TMB diluent + 1200 µl TMB substrate / 12 stripes) were added to each well. The plate was incubated in the dark for 15 min at room temperature. The reaction was stopped by adding 100 µl stop solution leading to a colour shift from blue to yellow. Within 10 min the absorbance was read with the photometer at the reference wavelength of 450 nm. 6 IgM positive and IgM negative serum samples were tested in all assays as an additional internal laboratory control.
A test was only validated when the following criteria were met: The absorbance of the cut-off sample should be between 0.150 O.D. and 0.500 O.D. The abundance of measles-IgM was expressed in “Capture Units” (C.U.) and calculated as follows:

$$\text{Measles-IgM “abundance”} = \frac{\text{(mean) absorbance of control and test sample}}{\text{mean absorbance of cut-off serum}}$$

The measles IgM-specific “abundance” of the negative control should be > 0.7 C.U., whereas the positive control should lead to an abundance > 2.0 C.U.
After calculation of the abundance of each serum, positivity for measles-IgM could easily be interpreted. As recommended by the manufacturer, a serum was considered positive for measles-specific IgM antibodies when the

abundance was ≥ 1.1 C.U. and negative when < 0.9 C.U. A serum was considered equivocal, if abundance was measured between 0.9 and 1.1 C.U.

2.3.3 Recombinant H-ELISA (H-ELISA)

2.3.3.1 Principle

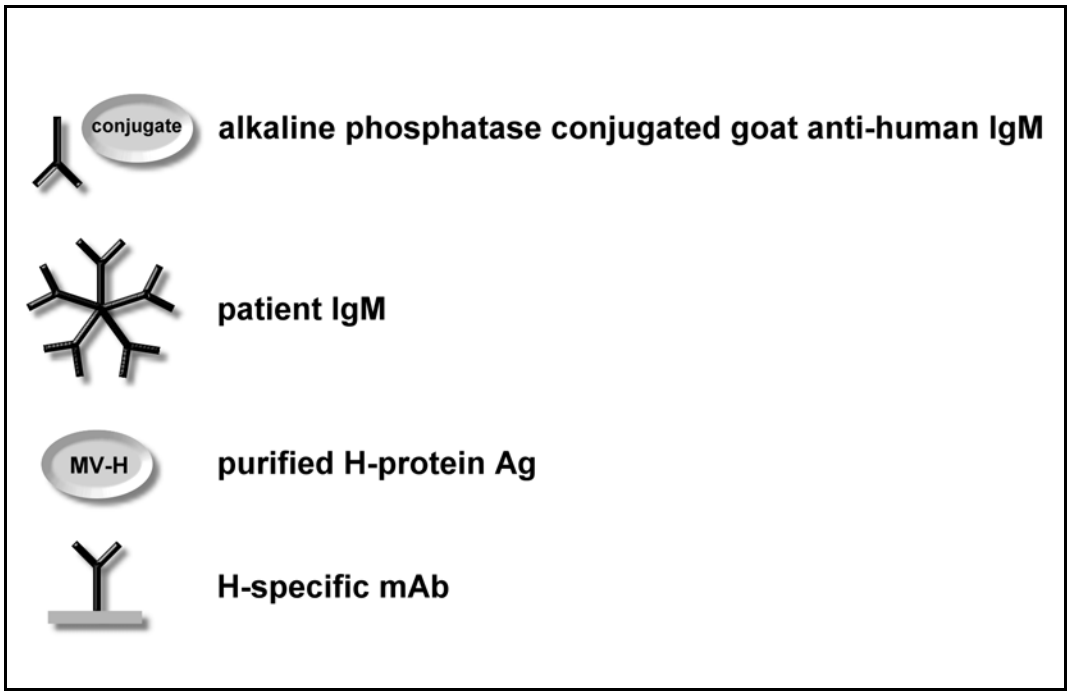


Fig. 2.3: Schematic representation of MV H-ELISA (step 1-4, starting from the bottom)

The recombinant H-ELISA was performed following a standard protocol and production of recombinant H-protein and the control-antigen as described by Bouche et al. (1998 a, b, c).

A mixture of three H-specific monoclonal antibodies (mAb) was coated onto microtiter plates (step 1). After a washing step, H-antigen or negative control was added to the wells (step 2). Sera were diluted 1:10 in GullSORB to eliminate interference with IgG. They were further diluted to a final concentration of 1:25 in a modified commercial dilution buffer (Enzymum-test) and dispensed into the wells (step 3).

Specific IgM antibodies were detected by using alkaline phosphatase-conjugated goat anti-human IgM and p-nitrophenylphosphate (step 4). After a 2h incubation time at 37°C, reactivity was measured at 405 nm.

2.3.3.2 Materials

Reagents		
Antigen	BHK-H	2.2.4
Control-antigen	BHK-gal	2.2.4
ELISA-TBS	(15 mM Tris(hydroxymethyl)-aminomethanacetate, 136 mM NaCl, 2 mM KCl)	Sigma, München, Germany
Coating buffer	1 mM sodiumbicarbonate ($NaCO_3$), pH 9.6	Sigma, München, Germany
Blocking buffer in aq.bidi pH 7.4	TBS, 1% BSA	Sigma, München, Germany
Dilution buffer in aq.bidi pH 7.4	TBS with 0.1% Tween 20 (polyoxyethylen- sorbitan-monolaurate), 1% BSA, 5% goat serum	Boehringer, Mannheim, Germany
IgG-Absorptionreagenz	GullSORB	Gull Laboratories GmbH, Bad Homburg, Germany
Washing buffer in aq.bidi pH 8.0	TBS, 0.1% Tween 20	
Substrate buffer	1mM AMP (2-amino-2-methyl-1-propanol), 0.1 mM $MgCl_2$ $6H_2O$	Sigma, München, Germany
anti-human-IgM-AP (1:1000)	Goat anti-human IgM, µ-chain-specific, conjugated with alkaline phosphatase,	Southern Biotechnology Associates, Birmingham, USA
ExtraAvidin®-AP	Streptavidin conjugated with alkaline phosphatase	Sigma, St. Louis, Missouri, USA
Sustrate	p-nitrophenylphosphate disodium salt (Sigma 104) as 5mg tablets	Sigma, München, Germany
microtiter plates	96 wells, 12 columns/8 rows, U-shaped	Nunc, Roskilde, Denmark
Instruments		
		2.3.1.2

Table 2.8: Materials for recombinant H-ELISA

2.3.3.3 Procedure

Microtiter plates were coated with 50 µl of a mixture of three conformation-dependent H-specific mAbs (5 µg/ml) in 0.1 M sodium bicarbonate buffer (pH 9.6). The mAbs were derived from mice immunized with the Edmonston MV strain. The plates were washed three times with 1% Tween 20 in Tris-buffered saline (15 mM; pH 8.0) and incubated for 75 min at room temperature with 50 µl of the above H-antigen/well (10 µg of protein/ml) or negative control antigen. The plates were blocked with 1% bovine serum albumin in Tris-buffered saline (15 mM; pH 7.4). Test sera were diluted 1:10 in GullSORB to eliminate interference by IgG. The sera were further diluted to a final concentration of 1:25 in a modified commercial dilution buffer and added for 75 min at room temperature to the antigen-coated microtiter plates. The plates were washed three times with the above-mentioned wash buffer. Alkaline phosphatase-conjugated goat anti-human IgM (1:1.000) and *p*-nitrophenylphosphate (0.5 mg/ml; 100 µl/well) were used to develop the assay. Optical density (OD) was measured at 405 nm following a 2 h incubation period at 37°C. Data are expressed as milli-OD (mOD). The threshold for positivity was defined as the mean mOD (+ 2 standard deviations [SD]) of the IgM-negative sera, measured after 2 h. This assay was carried out by Dr. Fabienne Bouche, Laboratoire National de Santé, Luxembourg. Results have been published by Bouche et al. (1998 c).

2.3.4 FACS-measured immunofluorescence assay (FACScan)

2.3.4.1 Principle

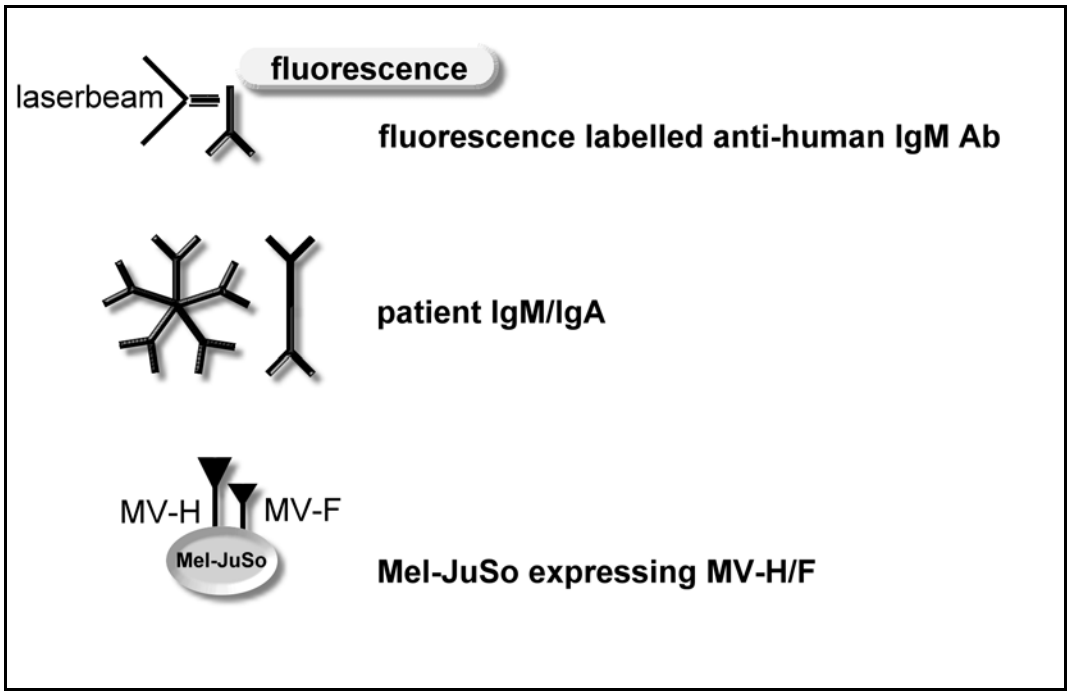


Fig. 2.4: Schematic representation of Mel-JuSo/MV-H/F IgM/IgA FACScan (step 1-5, starting from the bottom)

Human melanoma cells, stably transfected with MV-H and -F protein (de Swart et al., 1998), presented in their native membrane-inserted conformation were used as target cells. Fluorescence labelled antibodies directed against H and F were generated and detected by flow cytometry. The sample passes the laser beam in a stream, which allows all cells to be scanned for fluorescence intensity. The amount of signal detected is then converted in linear correlation into arbitrary fluorescence units (AFU) geometrical mean values.

2.3.4.2 Materials

Reagents		
FACS medium	KH_2PO_4 2.19 g/l, NaCl 90 g/l KH_2PO_4 7.26 g/l + 0.5 % sodium azide (0.25 ml/50 ml) + 5 %	Sigma, St. Louis, Missouri, USA

	BSA (2.5 ml/50 ml)	
FACS tubes	Round bottom tubes 1ml	Greiner, Frickenhausen, Germany
Anti-human IgA conjugate	Rabbit anti-human IgA FITC ($F(ab')_2$ fragments)	DAKO, Glostrup, Denmark
Anti-human IgG conjugate	Goat anti-mouse IgG FITC ($F(ab')_2$ fragments)	Sigma, St. Louis, Missouri, USA
Anti-human IgM conjugate	Goat anti-human IgM RPE	Southern Biotechnology Association, Birmingham, USA
IgG-Absorptionreagenz	GullSORB	Gull Laboratories GmbH, Bad Homburg, Germany
Dead cell excluder	Propidium Iodide (PI)	Sigma, St. Louis, Missouri, USA
Instruments		
Flow cytometry	Fluorescence-activated cell scanner (FACS); Epics Elite ESP	Beckman Coulter, Miami, Florida, USA
		2.3.1.2

Table 2.9: Materials for H- and F-FACScan

2.3.4.3 Procedure

FACScan was performed with minor modifications as described by de Swart et al., 1998. Final concentration for best results in the FACScan was 2 million cells per 1 ml FACS medium. For IgG depletion sera and plasma samples were diluted 1:10 in GullSORB and without delay further diluted to the standard laboratory concentration of 1:100 in FACS medium. Saliva samples were diluted 1:1 with FACS medium.

50 µl of the diluted serum/plasma/saliva sample or mAb were added to 50 µl of Mel-JuSo/MV-H (H-FACS), Mel-JuSo/MV-F (F-FACS) and Mel-JuSo/wt cell suspension. Dilutions were transferred into two 96-well plates. After 30 min incubation time on ice, 150 µl of FACS medium were added to all tubes and plates were centrifuged at 1200 rpm (266 x g) for 5 min at 4°C. Supernatant was removed without disturbing the pellet. The second wash was carried out with 200 µl FACS medium.

Negative controls were wt Mel-JuSo/MV-H/-F cells (well A1), Mel-JuSo cells plus anti-human IgA/IgM conjugate (B1) or an irrelevant mAb BNP146 (C1). Positive controls for H expression were BH67 (D1) and BH216 (E1) in the H-FACS (Ziegler et al., 1996). F expression was controlled with F186 (D1) and OST3 (E1) in the F-FACS (mAb were kindly provided by Dr. F. Wild, Lyon). 50

µl of FACS medium were added to well A1. 50 µl of IgG/FITC (1:200 in FACS medium) were added to each well containing the control mAb. To well B2 and the wells with serum and saliva were either added 50 µl IgA/FITC (1:25) or IgM/RPE (1:200), depending on the test performed. Cells were then incubated for 15 min. Subsequently, samples were washed again and resuspended in 150 µl FACS medium. 20 µl of PI were added before the analysis on the flow cytometer in order to exclude dead cells. Fluorescence intensity was expressed in arbitrary fluorescence units (AFU) geometrical mean values. Threshold for IgM H/F-FACS was calculated as follows:

The mean values +1 and +2 standard deviation(s) of 58 IgM negative samples were used to define the threshold for negativity and positivity, respectively. Values between these thresholds ($1.9 \leq$ H-FACS < 2.9 AFU, $2.3 \leq$ F-FACS < 3.5 AFU) were considered to be undefined. Sera with a background signal on the wt-FACS > 1.9 AFU for the H-FACS and > 2.3 AFU for the F-FACS were considered undefined as well. Threshold calculation for IgA H-FACS was calculated as follows:

An arbitrary serum cut-off of ≤ 1.5 AFU mean value for negative IgA titers was determined by calculating the average of 20 cord blood samples multiplied by 3. Values of all cord blood samples were uniformly negative. AFU mean values > 1.5 were regarded positive. Serum samples with Mel-JuSo/wt IgA intensity fluorescence > 1.5 were excluded.

All saliva samples with AFU values ≤ 1.2 were considered negative. The cut-off for saliva samples was based on the average x 3 of one MV negative saliva sample and one saliva sample from a patient with IgA deficiency, tested at least 4 times. AFU values > 1.2 were considered positive. Saliva samples with a background signal of > 1.2 on the wt cell line (Mel-JuSo/wt) were excluded.

2.4 Statistical evaluation

The statistical evaluation was done in collaboration with Prof. Dr. Dietz, Department of Medical Biometry, University of Tübingen, Germany.

Data were analysed according to the definitions of Bland (1995) using standard statistical characteristics such as sensitivity (Se), specificity (Sp), efficiency (Ef), positive predictive value (PPV) and negative predictive value (NPV).

The Se is the proportion of disease positives who are tested positive, while the Sp is the proportion of disease negatives who are tested negative. The Ef of a test describes how many sera were positive with respect to all sera measured. The PPV resembles the probability that a subject, which is test positive will be a true positive (i.e., has the disease and is correctly classified), whereas the NPV describes the probability that a subject, which is test negative will be a true negative (i.e., does not have the disease and is correctly classified). They depend on the prevalence of the condition (i.e. the prevalence of measles), as well as on the Se and the Sp.

For standard assay evaluation, most authors use the characteristics mentioned above. To compare different tests using these standard methods, a set cut-off is necessary, which immediately makes test performance depend on its discrimination threshold and leads to results susceptible to selection bias. In contrast, receiver operating curve (ROC) characteristics evade such problems by calculating the sensitivity and the specificity for every observed data value. The ROC curve is a graph of true positive rates (Se) on the vertical axis against the false positive rates (Sp) on the horizontal axis. The area under the curve (AUC; values range from 0 - 1) represents a general judgement of test performance considering all possible cut-off values (van der Schouw et al., 1994; Greiner 1995 a; Greiner et al., 1995 b; Laheij et al., 1998; Hartter et al., 2000 a).

The program GraphROC for Windows (Version 2.0) used for ROC and AUC calculation was developed by Veli Kairisto and Allan Poola in 1995. It was created for estimating clinical characteristics of quantitative laboratory tests.

Perfect discrimination between presence or absence of measles-specific IgM antibodies would emerge as a ROC creating the biggest possible AUC (=1) defined as

$$f_{(x)} = \begin{cases} 0\ ,\ x = 0 \\ 1\ ,\ x\ > 0 \leq 1 \end{cases}$$

whereas worst discrimination ability would lead to the ROC

$$f_{(x)} = x$$

and the arising diagnostic performance would not be better than chance.

The correlation coefficient (R) for the IgM assay controls (2.3) was calculated with the Pearson Product Moment Correlation (PPMC), which is used when measuring the strength of association between pairs of variables ignoring, which variable is dependent or independent. The relationship, if any, between the variables is a straight line, and the residuals (distances of the data points from the regression line) are normally distributed with constant variance. The normality distribution may be regarded as the fundamental probability distribution of statistics. It has a mean of 0, a standard deviation of 1 and a “bell-shaped” graph. A normal distribution of values was requested for calculation of R. If values failed the normality test (i.e., for the calculation of internal controls of the IgM-Comfort EIA) the data was log10 transformed, before running the PPMC in order to obtain a normal distribution.

Normality distribution of the IgM assays failed due to a larger amount of negative tested samples. Therefore the Spearman Rank Order Correlation (SROC) was applied. This correlation is used when measuring the strength of association between pairs of variables without specifying, which variable is dependent or independent and the population is not normally distributed.

For calculation of the P-value of the vaccinees and late convalescents cohort (panel B, group1a/2a, 2.1.2), the Mann-Whitney Rank Sum Test (MWRST) was used. This nonparametric test is the analogue of the two sample t-test. Nonparametric tests do not require that the data is normally distributed. They perform a comparison on ranks of the observations. The MWRST tests the null hypothesis (hypothesis of no difference). If the data are not consistent with the

null hypothesis, the difference is said to be statistically significant. Disregarding the error of first and second kind, the conventional compromise is to say that differences are significant if the probability (p) is less than 0.05.
Statistical tests were performed using the Sigmastat software (Jandel Scientific, Erkrath, Germany).

3 Results

3.1 Comparison of different serological measles IgM assays

3.1.1 Panel used

A panel of 225 serum and plasma samples from Luxembourg and Nigeria was compiled to evaluate the overall performance of 4 different MV IgM-specific assays in comparison with the IgM-Enzygnost, chosen to serve the gold standard (panel A, 2.1.1).

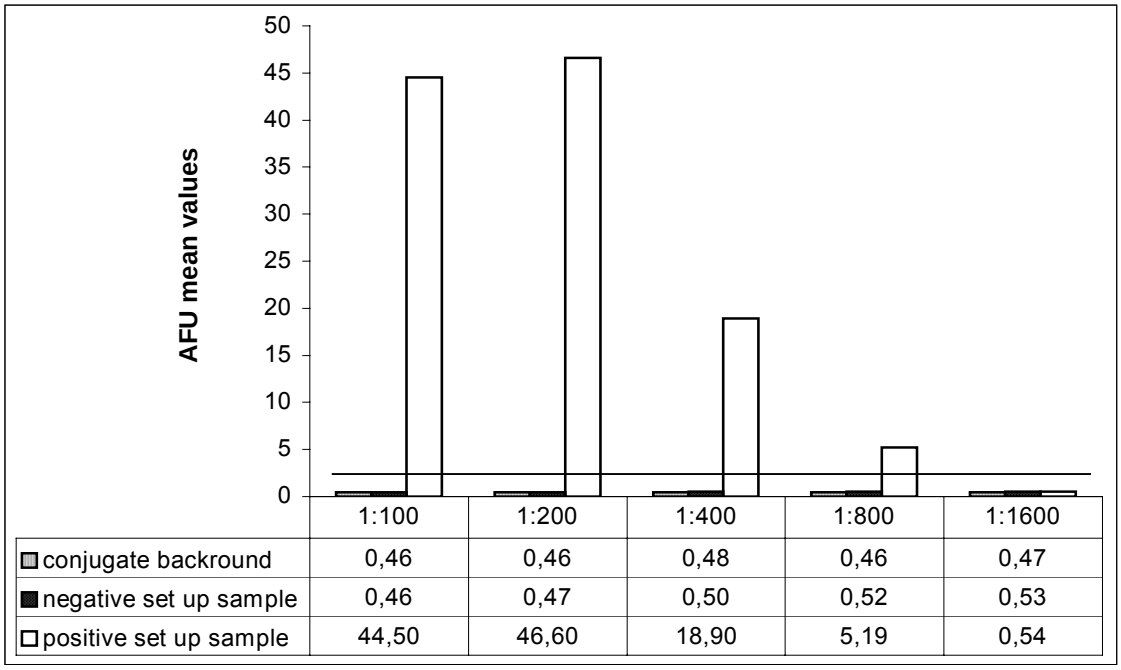
3.1.2 Test standardization

In addition to the controls provided by the manufacturer, 6 IgM positive and negative serum samples were taken along as a laboratory-internal reproducibility control in each IgM-Enzygnost and IgM-Comfort EIA test plate (R = 0.917 – 0.999, R = 0.977 – 0.998, respectively). Controls for IgM-FACScan are listed in Table 3.1. Values were only taken into account when the assay complied with the validation criteria (see 2.3).

	Reagents	Antigen	Dilution	Conjugate	Dilution	AFU
Negative	Mel-JuSo	-	-	-	-	≤ 0,4
Controls	Mel-JuSo	-	-	anti-human IgM	1:200	≤ 0,6
	Mel-JuSo	BNP146	1:1000	anti-mouse IgG	1:200	≤ 1,3
Positive	Mel-JuSo	BH67	1:1600	anti-mouse IgG	1:200	≥ 24
Controls	Mel-JuSo	BH216	1:1000	anti-mouse IgG	1:200	≥ 17
	Mel-JuSo	F186	1:250	anti-mouse IgG	1:200	≥ 56
	Mel-JuSo	OST3	1:1250	anti-mouse IgG	1:200	≥ 73

Table 3.1: Controls of Mel-JuSo/MV-H/-F FACScan

For the IgM-FACScan the dilution of 1:200 anti-human IgM conjugate (goat anti-human IgM RPE) was found to be optimal and used in all assays (Fig. 3.1).



	1:100	1:200	1:400	1:800	1:1600
conjugate backround	0,46	0,46	0,48	0,46	0,47
negative set up sample	0,46	0,47	0,50	0,52	0,53
positive set up sample	44,50	46,60	18,90	5,19	0,54

Fig. 3.1: Titration of RPE-conjugated F(ab')2 fragment of goat anti-human IgM (µ-chains) using one negative sample (white dotted bar) and one high positive sample (white) for IgM FACS analysis. The horizontal line shows the cut-off for positivity. All controls met the conditions mentioned above.

3.1.3 Gold standard

Serum or plasma samples were classified for each test as positive, negative or undefined using the recommended thresholds (see section 2.3). The IgM-Enzygnost was used as the gold standard as it has been shown by several authors to be a highly reliable test system for measles-specific IgM and it is also the one recommended by the WHO (Ozanne and d'Halewyn, 1992, Arista et al., 1995, Salmaso et al., 2000).

3.1.4 Characteristics of the different assays

The sensitivity (Se), specificity (Sp), efficiency (Ef), positive predictive value (PPV) and negative predictive value (NPV) were calculated for each test using the IgM-Enzygnost as the gold standard. Group 1 – 6 of panel A were tested by

IgM-Enzygnost, IgM-Comfort EIA, H-FACS and F-FACS (n=225). Group 1 and 6 from panel A were tested in addition by H-ELISA (n=82). Samples, which were undefined in the IgM-Enzygnost (3/225 and 1/82), were excluded in the calculation of characteristics and receiver operating characteristic curves. The actual size of panel A was therefore 222 and 81, respectively. The IgM-Enzygnost determined 135 (of 222) and 63 (of 81) samples, respectively as being positive. 10 Samples, tested in the F-FACS, were undefined because of high background-signals on Mel-JuSo/wt. These samples were left in the test panel. No serum or plasma samples were tested undefined in the Mel-JuSo/wt H-FACS.

A valid comparison of the assays was complicated by the fact that some assays displayed a more or less important grey zone (IgM-Enzygnost, IgM-Comfort EIA, H-FACS and F-FACS), whereas the H-ELISA did not. When the undefined or grey zone serum or plasma samples were excluded from the analysis (method A) and the cut-off value for negativity was applied, the values for sensitivity and specificity ranged from 87.0% (F-FACS) to 99.3% (IgM-Comfort EIA) and from 97.5% (F-FACS) to 100% (IgM-Comfort EIA), respectively (Table 3.2). The number of false negative samples was significantly lower in the IgM-Comfort EIA (1/222) compared to H- and F-FACS (6/222 and 16/222, respectively).

Since detection of IgM-positive individuals is the major purpose of these measles assays, the characteristics of each test were recalculated by considering the undefined sera as being positive (method B). According to this evaluation, the values for NPV did not change compared to method A, but there was an increase in sensitivity, a decrease in specificity, PPV and efficiency for all assays except IgM-Comfort EIA, which did not further improve. Compared to the other assays, the performance of the F-FACS deteriorated mainly because of its high percentage of false negative and undefined sera (20/222). There were samples that fell into the grey zone on Mel-JuSo/MV-F as well as sera that gave too high background-signals on the Mel-JuSo/wt strain.

	Results of tests	Number of sera IgM-Enzygnost positive	negative	Sp (%)	Se (%)	PPV (%)	NPV (%)	Ef (%)	Undefined results (%)	False negative and undefined (%)*	False positive and undefined (%)**	False negative (%)	False positive (%)
Method A													
IgM-Comf. EIA	positive	133	0	100	99,3	100	98,9	99,6	0	0,5	0	0,5	0
	negative	1	87										
H-FACS	positive	124	1	98,8	95,4	99,2	93,2	96,7	0	2,7	0,5	2,7	0,5
	negative	6	82										
F-FACS	positive	107	2	97,5	87,0	98,2	82,8	91,1	0	7,2	0,9	7,2	0,9
	negative	16	77										
Method B													
IgM-Comf. EIA	positive	133	0	100	99,3	100	98,9	99,6	0,5	0,9	0	0,5	0
	negative	1	87										
	undefined	1	0										
H-FACS	positive	124	1	94,3	95,6	96,3	93,2	95,1	4,1	5,0	2,3	2,7	0,5
	negative	6	82										
	undefined	5	4										
F-FACS	positive	107	2	88,5	88,2	92,3	82,8	88,3	9,0	12,6	4,5	7,2	0,9
	negative	16	77										
	undefined	12	8										

* Percentage of individuals who would possibly be disregarded in standard measles precautions due to false negative and undefined results.
**Percentage of individuals who would possibly be treated for measles without necessity due to false positive and undefined results.

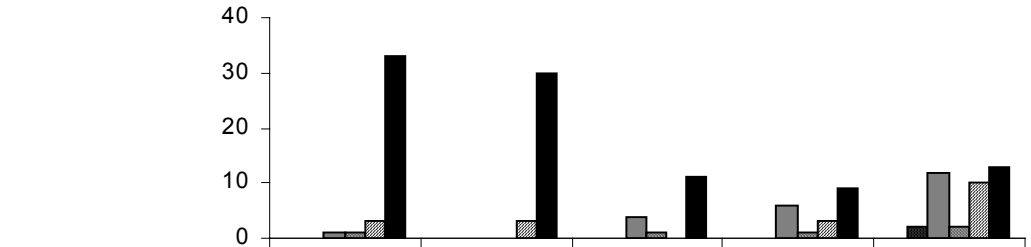
Table 3.2.: Evaluation of 222 serum and plasma samples in IgM-Comfort EIA, H-FACS and F-FACS against the gold standard (IgM-Enzygnost). Method A: undefined results were excluded; method B: undefined results were counted as positive.

Characteristics of all 4 assays (n=81 samples), including the H-ELISA, compared to the IgM-Enzygnost (gold standard), are presented in Table 3.3. Independent of the calculation method (method A or B), IgM-Comfort EIA and H-FACS gave the best correlation in all characteristics compared to the gold standard. However, the H-FACS determined one sample as undefined. Since this sample was positive in the gold standard, it did not decrease the results of characteristics in method B. F-FACS showed only small deviations in its performance. Because of the sample, tested undefined in F-FACS, which was negative in the IgM-Enzygnost, the F-FACS performed better if method A was used for calculation of characteristics.

	Results of tests	Number of samples IgM-Enzygnost positive	negative	Sp (%)	Se (%)	PPV (%)	NPV (%)	Ef (%)
Method A								
IgM-Comfort EIA	positive	63	0	100	100	100	100	100
	negative	0	18					
H-ELISA	positive	62	1	94.4	98.4	98.4	94.4	97.5
	negative	1	17					
H-FACS	positive	62	0	100	100	100	100	100
	negative	0	18					
F-FACS	positive	57	0	100	98.3	100	94.1	98.7
	negative	1	17					
Method B								
IgM-Comfort EIA	positive	63	0	100	100	100	100	100
	negative	0	18					
	undefined	0	0					
H-ELISA	positive	62	1	94.4	98.4	98.4	94.4	97.5
	negative	1	17					
H-FACS	positive	62	0	100	100	100	100	100
	negative	0	18					
	undefined	1	0					
F-FACS	positive	57	0	94.4	98.4	98.4	94.4	97.5
	negative	1	17					
	undefined	5	1					

Table 3.3: Evaluation of 81 sera in all 4 assays against the gold standard (IgM-Enzygnost). Method A: undefined results were excluded; method B: undefined results were counted positive.

Another important purpose of a competent test-system is the early determination of MV-specific IgM antibodies during measles. Therefore, false negative or undefined tested samples of the Luxembourgian patients cohort (2.1.1) were plotted against the time of onset of rash starting with day one after onset of rash (n=96) (Fig 3.2).



	1-9 days after onset of rash	10-19 days after onset of rash	20-29 days after onset of rash	30-39 days after onset of rash	40-59 days after onset of rash
IgM-Enzygnost	0	0	0	0	0
IgM-Comfort EIA	0	0	0	0	2
H-ELISA	1	0	4	6	12
H-FACS	1	0	1	1	2
F-FACS	3	3	0	3	10
total amount of sera	33	30	11	9	13

Fig. 3.2: Patients, that would have been left untreated due to false negative and undefined results, using the IgM-Enzygnost (black dotted bar) as gold standard. White dotted bar = IgM-Comfort EIA, grey bar = H-ELISA, bar with thin lines = H-FACS, bar with thick lines = F-FACS.

The IgM-Enzygnost (gold standard) gave positive results as early as day one after onset of rash with a continuity of 100% until day 59 after onset of rash. The IgM-Comfort EIA tested the same sera positive as the gold standard, except 2 samples. One of the latter was tested negative (day 40) and one sample on day 45 after onset of rash was undefined. The H-ELISA has no grey zone. This test system displayed 23 sera of the panel as being negative (in this case false negative). The H-ELISA has been shown by Bouche et al. (1998 a, b, c) to be a valid test-system for detection of IgM only until day 19 after onset of rash. When only these sera were included there was only one false negative serum on day one after onset of rash, which has also been defined as negative by the F-FACS and undefined by the H-FACS. It is of note that the H-ELISA detected

IgM in a patient serum on the day of onset of rash, whereas all other assays, including the gold standard, detected seroconversion only one day later. The H-FACS tested 4 sera as undefined (1, 27, 39, 43 days after rash onset) and one sample as negative (day 40). The latter sample was also negative in the IgM-Comfort EIA. There were no undefined samples because of high background-signals on Mel-JuSo/wt, whereas 7 of 10 undefined samples, evaluated in the F-FACS, were in the grey zone because of high background-signals on the Mel-JuSo/wt. 9 samples (day 1, 37, 40, 43, 46, 57 after onset of rash) were negative in the latter test. Therefore, IgM-Comfort EIA and H-FACS would have both excluded one patient at day 40 after onset of rash from measles control measures and the H-ELISA one patient at day 1 after onset of rash. F-FACS performed worst with 9 patients, which would have been send home as healthy. It is important for the timely implementation of measles control measures that the detection of IgM is reliable especially during the period of high infectiosity. The risk of virus-spread decreases after around 4 days after onset of rash. According to this, results of the IgM-Comfort EIA and H-FASCS would not miss any contagious patient, whereas the H-ELISA would refrain from precautions for one patient at day one after onset of rash. Nevertheless, only the H-ELISA would indicate an infection risk from a patient at the day of onset of rash. Undefined results delay intervention. H-ELISA would therefore not lead to a slow down in precautions, whereas IgM-Comfort EIA, H-FACS and F-FACS would delay measures in 1, 4 and 10 cases, respectively.

Separately evaluated was the ability of each assay to differentiate between RF IgM-IgG immune complexes and measles-specific IgM antibodies. All 37 samples (2.1.1) were tested negative in the gold standard and IgM-Comfort EIA. H- and F-FACS displayed one false positive, one undefined and 2 false positive values, respectively. Since RF is detected in approximately 5% of healthy individuals, the gold standard and IgM-Comfort EIA were not limited in their performance because of RF.

3.1.5 Receiver operating characteristic curves (ROC)

Most authors use the above characteristics to evaluate and compare tests. However, this method requires the predetermination of a certain cut-off, from which the performance severely depends. Therefore, a better approach is to calculate the performance of the assay for a floating threshold. This allows a comparison of assays independently of a certain cut-off value. Receiver operating characteristic curves (ROC) do not depend on discrimination thresholds. The area under the curves (AUC, values range from 0-1) (van der Schouw et al., 1994; Hartter et al., 2000 a) represents an attractive indicator for the diagnostic performance of a laboratory test that considers all possible cut-off values (Greiner 1995 a; Greiner et al., 1995 b; Laheij et al., 1998). The higher the area under the ROC curve is calculated, the better is the test's performance. The AUC was calculated for the 3 assays (n=222) (excluding the H-ELISA) against IgM-Enzygnost as the gold standard (Fig. 3.3). For method A, the AUC ranged from 0.9986 (IgM-Comfort EIA) to 0.9772 (F-FACS).

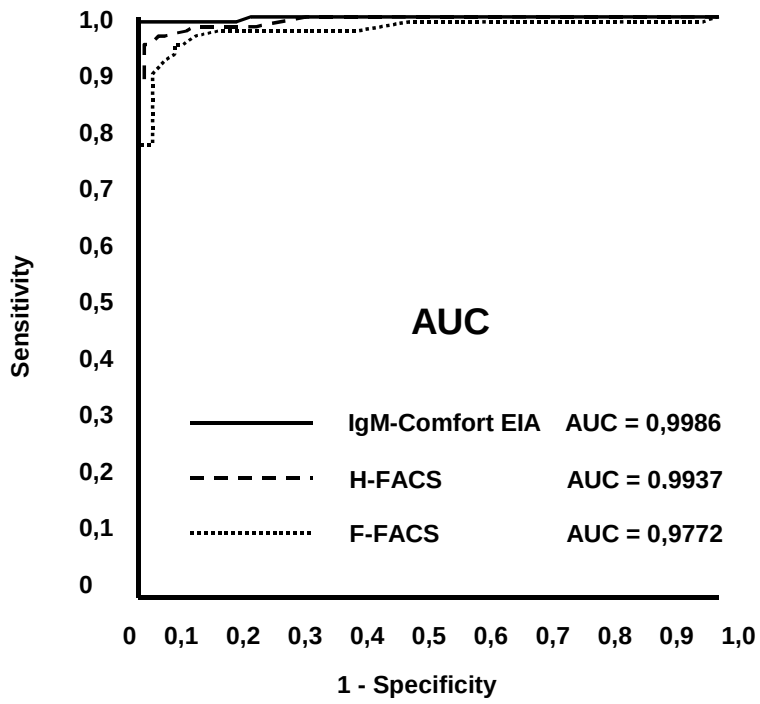


Fig. 3.3: ROC plot of the different assays (method A: excluding the undefined samples in all assays), using the IgM Enzygnost as gold standard (n=222). The AUC indicates the overall performance of each test.

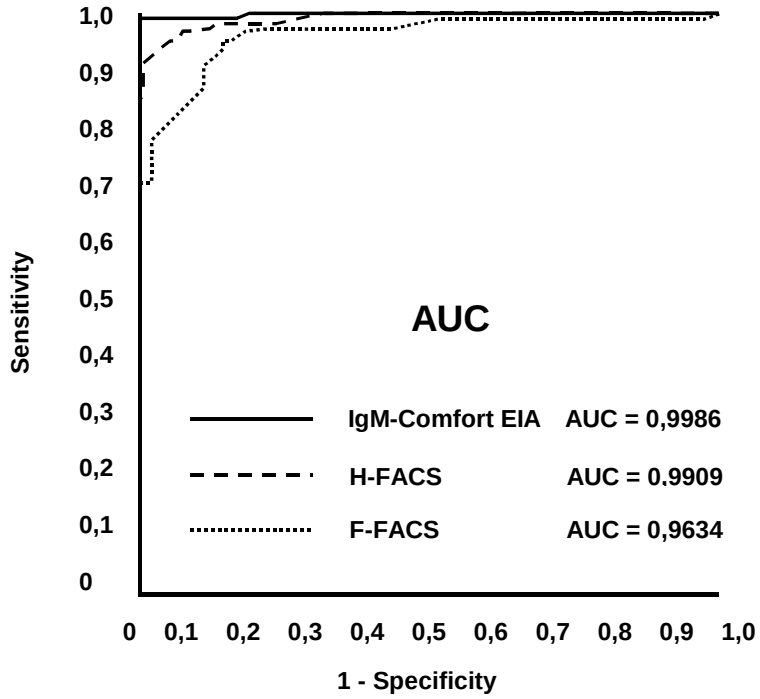


Fig. 3.4: ROC plot of the different assays (method B: undefined results were considered positive), using IgM-Enzygnost as gold standard (n=222). The AUC indicates the overall performance of each test.

For method B, the highest AUC (0.9986) was also found for IgM-Comfort EIA. The AUC for H- and F-FACS was 0.9937 and 0.9634, respectively (Fig. 3.4). To determine the performance independently of thresholds, the sensitivity, specificity and efficiency were plotted against the cut-off (Fig. 3.5). The dotted vertical line represents the location where sensitivity = specificity = efficiency. Since it is of great importance to identify all possible measles-specific IgM positive individuals, thresholds should be located in general to the left of the dotted vertical line in order to minimize false negative results.

The analysis shows that the thresholds recommended by the manufacturer and the authors for IgM-Comfort EIA (0.9) and H-ELISA (0.147), respectively were the optimal choice and cannot be improved by moving the cut-off. The sensitivity for H- and F-FACS could be increased without too much loss of specificity if the threshold was decreased from 1.9 (Se 95.6%) to 1.4 (97%) and 2.3 (88.2%) to 1.7 (91.1%), respectively. However, sera with a background signal on Mel-JuSo/wt > 1.9 AFU for the H-FACS and > 2.3 AFU for the F-

FACS were considered undefined. With a change of cut-off, more samples would then have to be re-tested (H-FACS n = 5; F-FACS n = 47).

The 3 samples, which were undefined in the IgM-Enzygnost and therefore rejected from calculation were mostly tested negative by the other assays. 2 Samples belong to the group of Nigerian plasma samples (group 2, 2.1.1) and have been both tested negative by H- and F-FACS. The IgM-Comfort EIA classified one samples positive and one sample negative. The 3rd serum, which was determined undefined by IgM-Enzygnost is a late convalescent from Luxembourg (group 6, 2.1.1). It was also tested undefined by H- and F-FACS, but negative in IgM-Comfort EIA and H-ELISA. Whereas the IgM-Comfort EIA, as the only assay, would indicate the need of measles control measures in one case, the other assays would either re-test (H- and F-FACS both one time) or send the person home as healthy. Still, for the latter 3 samples we were not able to make a statement about the immunological status, since there was no additional information next to the results from the gold standard.

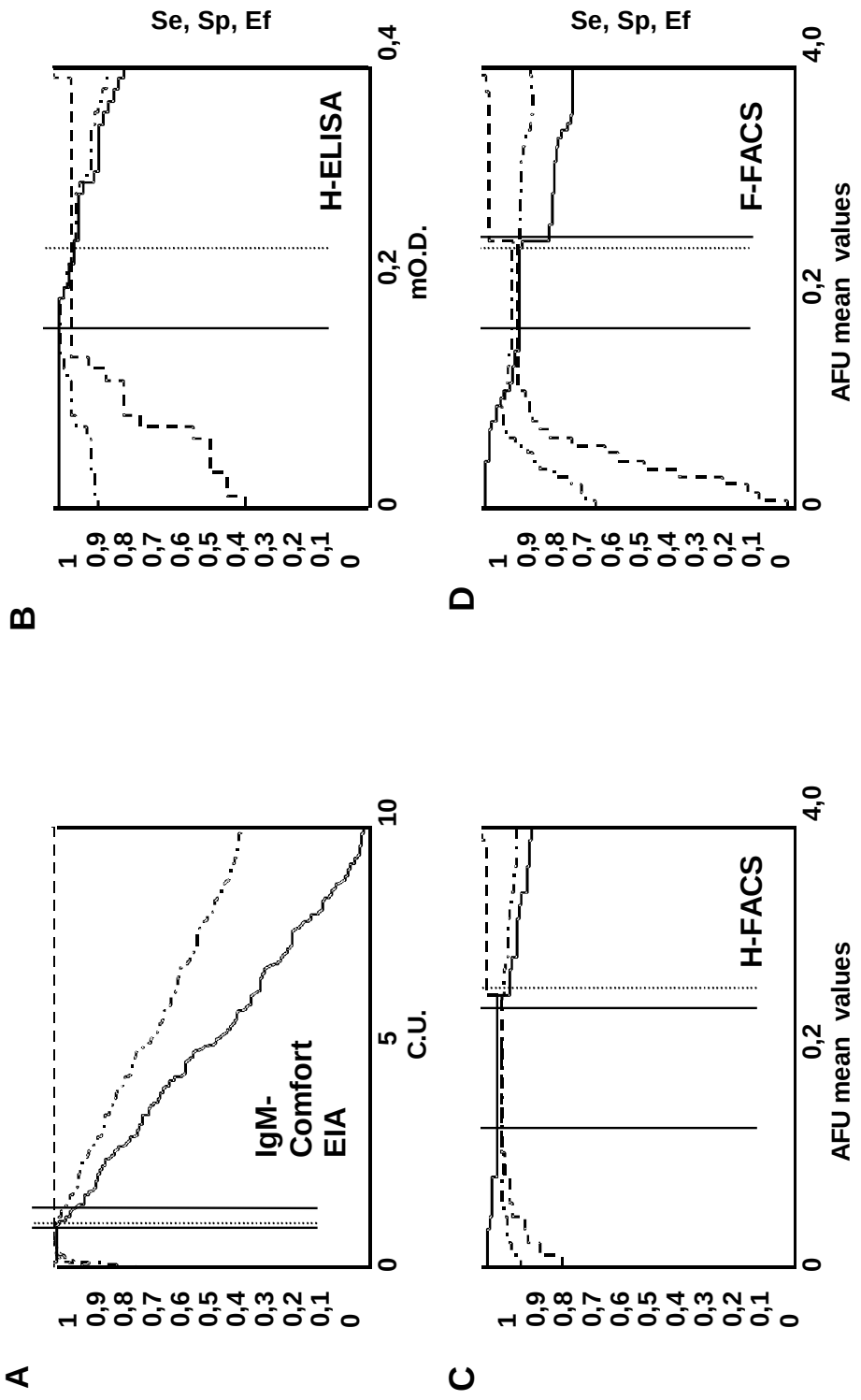


Fig. 3.5: Se (straight line), Sp (dashed line), and Ef (dotted line) of the different assays plotted against cut-off values. Threshold for negativity/positivity (straight vertical line). Vertical dashed line corresponds to a cut-off value where Se = Sp = Ef

3.2 Evaluation of IgA response against MV-H

3.2.1 Panel used

Panel B was chosen to compare the immunological status in serum (n=194) against saliva (n=79) of MV-specific IgA from vaccinees, late convalescents and acute measles patients from Luxembourg, Nigeria and Argentina (see 2.1.2). For MV-IgA detection we have used the Mel-JuSo/H-FACS.

3.2.2 Test standardization

All results obtained with the latter panel using the IgA H-FACS met the validation criteria for IgA H-FACS (see 2.3.4). Data values were declared to be positive or negative as described in section 2.3.4 .
The controls for the Mel-JuSo IgA H-FACS were tested in all assays as listed in Table 3.4.

	Reagents	Antigen	Dilution	Conjugate	Dilution	AFU
Negative controls	Mel-JuSo	-	-	-	-	≤ 0,4
	Mel-JuSo	-	-	anti-human IgA	1:25	≤ 0,5
	Mel-JuSo	BNP146	1:1000	anti-mouse IgG	1:200	≤ 0,5
Positive controls	Mel-JuSo	BH67	1:1600	anti-mouse IgG	1:200	≥ 85
	Mel-JuSo	BH216	1:1000	anti-mouse IgG	1:200	≥ 29

Table 3.4: Controls of Mel-JuSo/MV-H FACScan

High cell surface expression of MV-H was demonstrated with the mAb BH67 and BH216 on the transfected cell line Mel-JuSo/MV-H, whereas low signals were measured on the negative controls (i.e., Mel-JuSo/wt). Flow cytometry histograms with examples of the standard control samples are shown in Fig. 3.6.

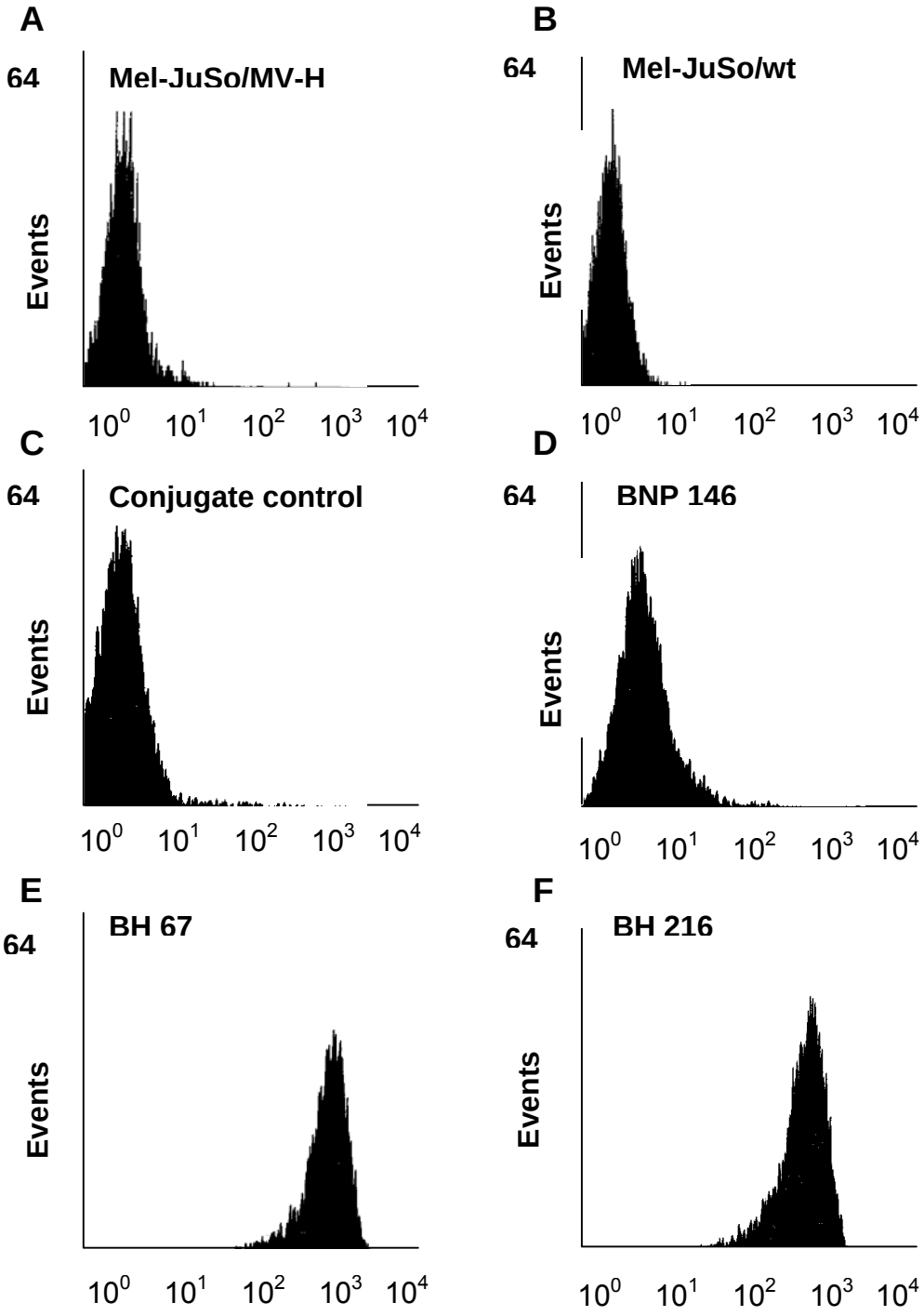
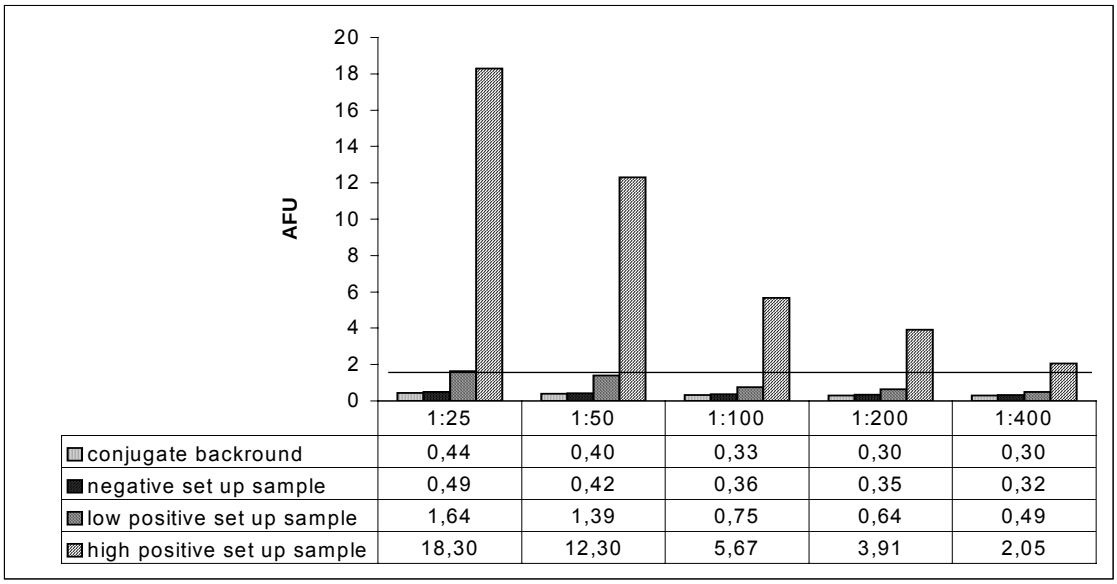


Fig. 3.6: Flow cytometry histograms of negative (A-D) and positive (E-F) controls tested in IgA H-FACS. The amount of events (y-axis) is plotted against the stains measured (x-axis). If stains are measured left or right on the x-axis, fluorescence intensity is low or high, respectively.



	1:25	1:50	1:100	1:200	1:400
conjugate backround	0,44	0,40	0,33	0,30	0,30
negative set up sample	0,49	0,42	0,36	0,35	0,32
low positive set up sample	1,64	1,39	0,75	0,64	0,49
high positive set up sample	18,30	12,30	5,67	3,91	2,05

Fig. 3.7: Titration of FITC-conjugated F(ab')2 fragment of rabbit anti-human IgA (α-chains) using one negative set up sample (white dotted bar), one low positive sample (bar with thin lines) and one high positive sample (bar with thick lines) for IgA FACS analysis. The horizontal line shows the cut-off for positivity.

For the IgA-FACScan the dilution of 1:25 anti-human IgA conjugate (FITC-conjugated F(ab')2 fragment of rabbit anti-human IgA) was found to be optimal and used in all assays (Fig. 3.7).

3.2.3 Serum IgA titers

MV-specific IgA was measured in H-FACS in serum of vaccinees (n=44), of late convalescents (n=50) and of acute phase patients (n=100). Among the vaccinees, 24 sera were from Luxembourg and 20 from Nigeria. Among the late convalescents, 30 sera were from Luxembourg and 20 from Nigeria. All 94 sera were negative for IgM in the IgM-Enzygnost. 2 of the 44 (4.5%) sera were IgA positive in the vaccinated group, whereas 13 were IgA positive (26%) among the late convalescents ($p = 0.007$).

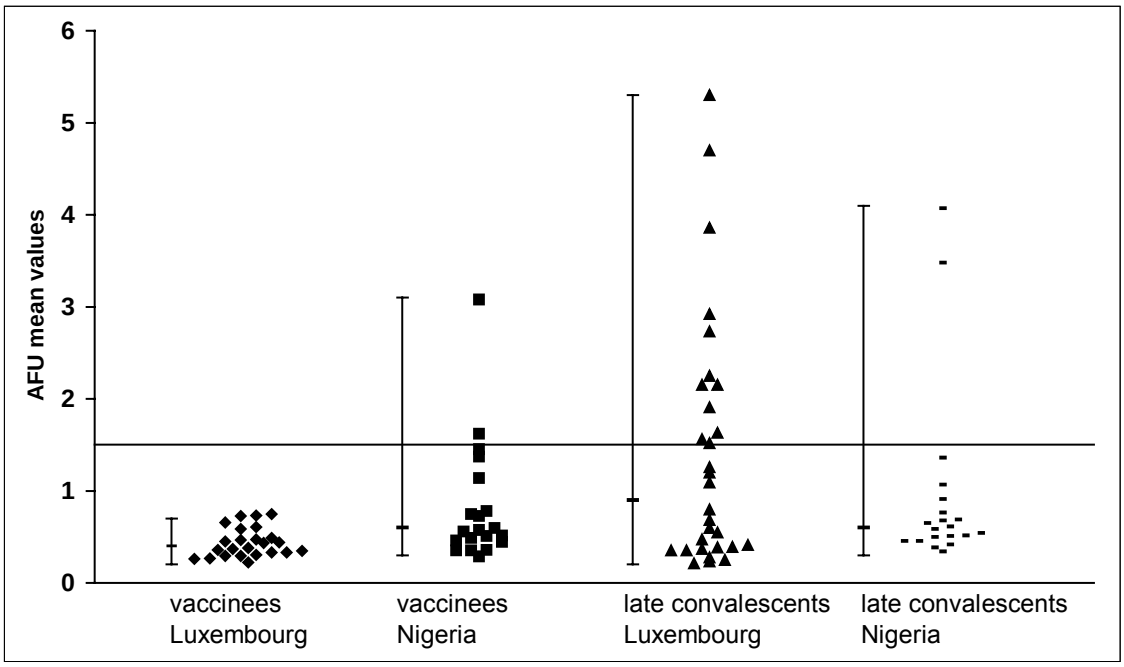


Fig. 3.8: IgA response against MV-H in sera from 44 vaccinees versus 50 late convalescents divided into cohorts of 24 vaccinees from Luxembourg, 20 vaccinees from Nigeria, 30 late convalescents from Luxembourg and 20 late convalescents from Nigeria. Vertical lines indicate the lower and upper range value and the median value calculated for each cohort. Horizontal line indicates the cut-off for positivity.

The Luxembourgian sera displayed a large difference in MV-specific IgA between late convalescents (n=30) and vaccinees (n=24) (Fig. 3.8). The median value of IgA of the Luxembourgian late convalescents was more than twice the median of Luxembourgian vaccinees (0.9 AFU and 0.4 AFU, respectively; p=0.003). Luxembourgian vaccinees (n=24) were all negative in the IgA H-FACS, whereas 11 of 30 (36.6%) Luxembourgian late convalescents gave a positive signal. The MV-IgA median values of the Nigerian cohorts (vaccines versus late convalescents) did not show a significant difference (both 0.6 AFU; p=0.839). In the Nigerian cohort, 2 of either of the 20 vaccinees and late convalescents gave positive IgA antibody-responses. This means that IgA titers in Luxembourgian sera are lower when vaccinated compared to wild-type infection, whereas Nigerian vaccinees compared to late convalescents displayed almost equal results.

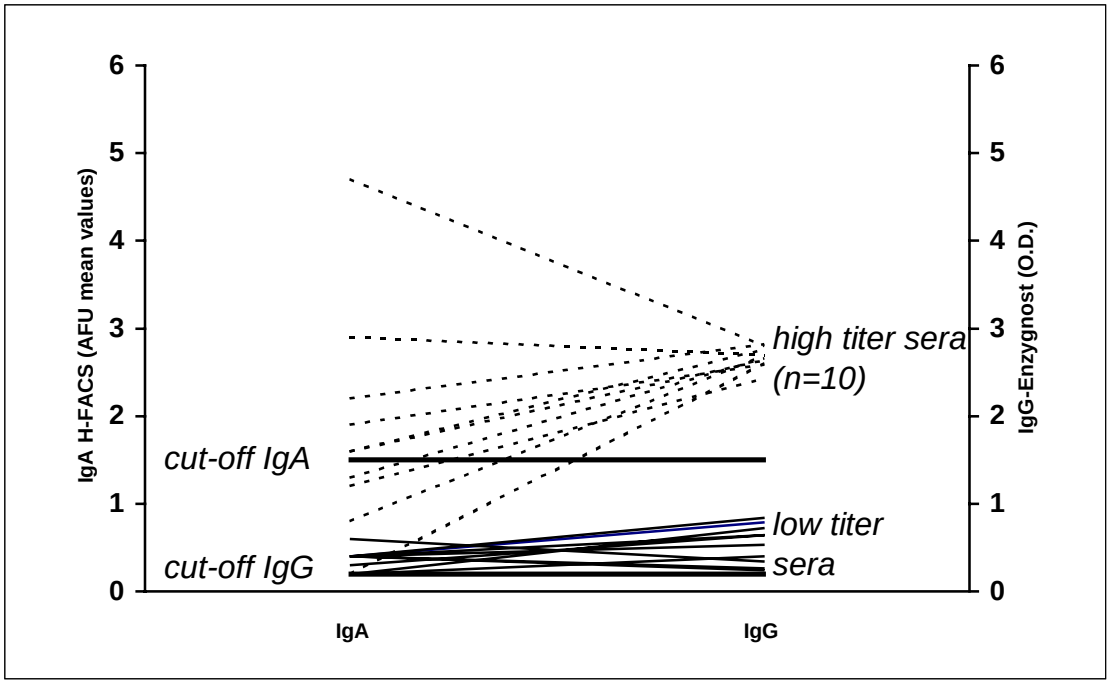


Fig. 3.9: Distribution of 10 MV-IgG low-titer (normal lines) and 10 MV-IgG high-titer (dotted lines) sera from late convalescent measles patients from Luxembourg. Left end of each line indicates the IgA value measured with the IgA H-FACS. Right end of each line shows the IgG value measured with the IgG-Enzygnost. Upper thick horizontal line indicates the threshold of the IgA H-FACS. Lower thick horizontal line shows the threshold of the IgG-Enzygnost.

In order to investigate the influence of IgG on the IgA serum-titer, 10 sera with high IgG and 10 with low IgG were compared using the IgA H-FACS (Fig. 3.9). All sera were collected from Luxembourgian late convalescents. 6 out of 10 (60%) IgG high-titer sera were tested positive for IgA, whereas none of the IgG low-titer samples showed a positive IgA H-FACS signal. The high IgG sera showed a large range of IgA values (0.2 – 4.7 AFU) whereas the low titer sera displayed a small range (0.2 – 0.4 AFU). This means that high titer of IgG in serum is rather related to positive IgA values than low titer of IgG.

The results of IgA responses in serum against MV-H of patients from Luxembourg (n = 37) and Argentina (n = 63) tested in H-FACS are shown in Fig. 3.10. Most of the samples (n = 84) were drawn during the time span when IgA normally appears (14 days before until 5 days after onset of rash). 47 sera from Argentina were drawn on day 1 or 2 after onset of rash. Therefore, the additional group of “1-2 days” was introduced. During some days, more than 1 serum sample was collected and tested in the IgA H-FACS (day 1, 1-2, 2, 3, 4, 5, 6, 11). Median values were presented with upper and lower ranges. On the

days when only one serum was drawn per day, one absolute data value (each without upper or lower ranges) is shown. Serum IgA was negative in only 4 samples. They were collected 14, 8 and 5 days before and on the day of onset of rash (day 0). From day 1 after onset of rash, a significant increase of serum IgA was detected. Even though the median values were positive from day 1 after onset of rash on, there were still 35.8% IgA negatives between day 1 and day 2 after onset of rash. Except one patient, who was IgA negative on day 30 (1.5 AFU), all samples were IgA positive starting on day 2 after onset of rash. This indicates that the serum IgA H-FACS detected measles patients reliably from 2 days after onset of rash, whereas about 64.2 % of the patients were already detected at day 1-2 after onset of rash.

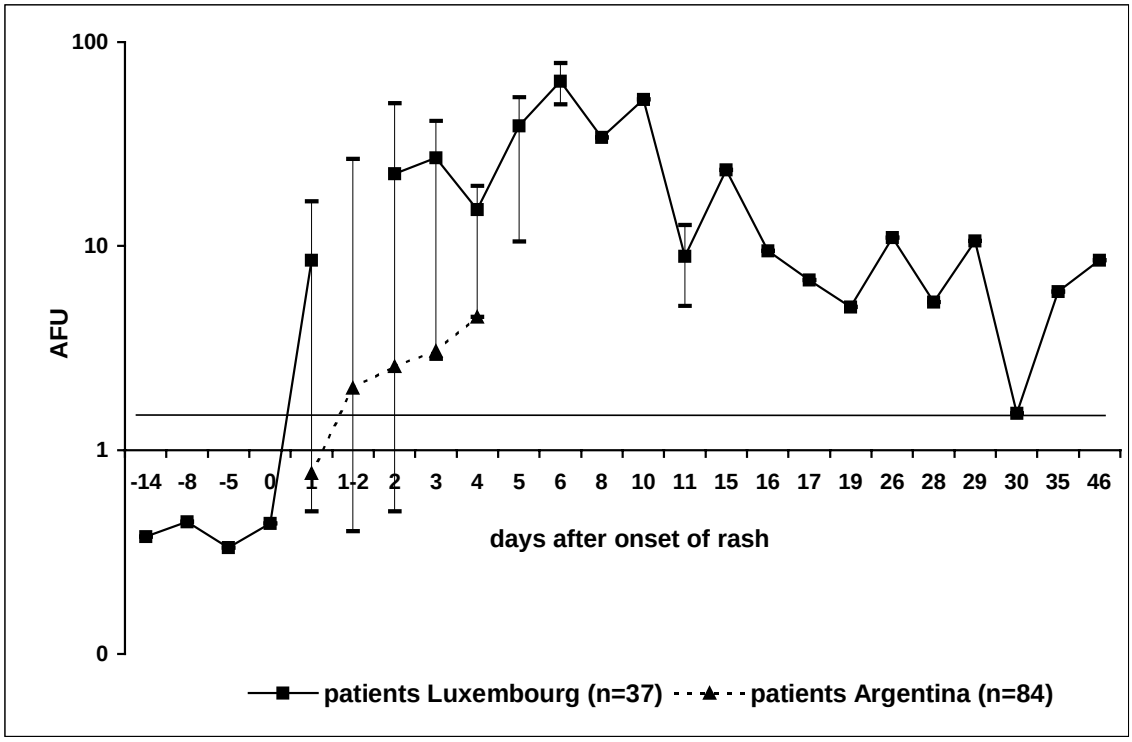


Fig. 3.10: Measles-specific serum IgA response of 100 measles patients from Luxembourg (n=37, normal line with squares) and Argentina (n=84, dotted line with triangles). When more than one sample per day was available, results are expressed in median values with upper and lower ranges. When only one sample was tested per day, absolute values are presented. Horizontal line indicates the cut-off for positivity.

3.2.4 Measles IgA in saliva

MV-specific IgA was measured in saliva of vaccinees (n=14), late convalescents (n=19) and acute phase patients (n=46) by H-FACS. MV-IgA titers in saliva of vaccinees were compared to late convalescents from Luxembourg (Fig. 3.11). Among the 14 vaccinees there was only 1 saliva sample (7.1%) positive for IgA, whereas 8 of 19 (42.1%) late convalescent saliva samples were positive. The difference between both groups was not statistically significant (p=0.061).

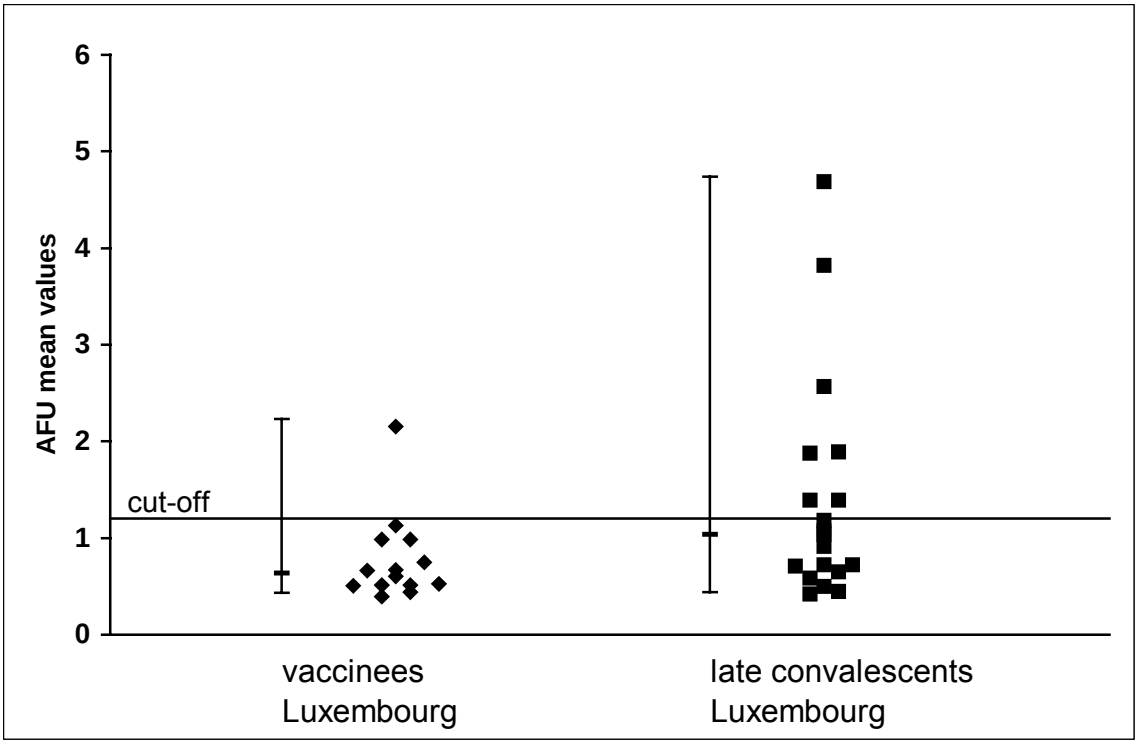


Fig. 3.11: Saliva IgA against MV-H in saliva from 14 vaccinees and 19 late convalescents from Luxembourg. Vertical lines indicate the lower and upper range value and the median value calculated for each cohort. Horizontal line indicates the cut-off for positivity.

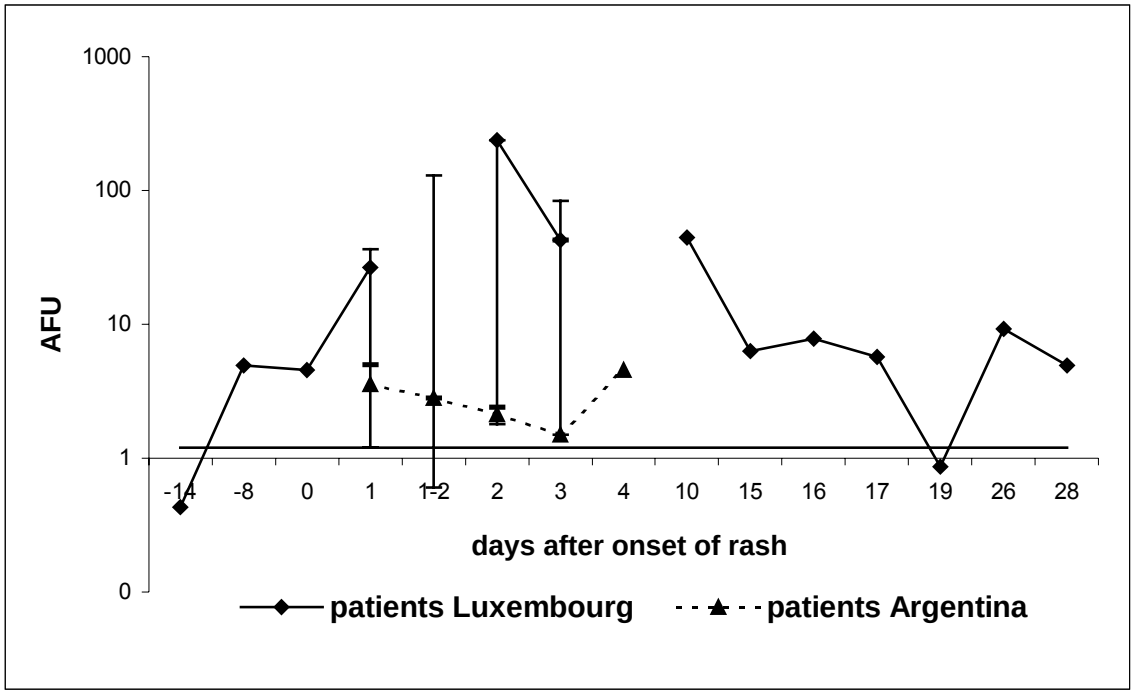


Fig. 3.12: MV-specific saliva IgA of 46 measles patients from Luxembourg (n=14, normal line with squares) and Argentina (n=32, dotted line with triangles). When more than one sample per day was available, results are expressed in median values with upper and lower ranges. When only one sample was tested per day, absolute values are presented. Horizontal line indicates the cut-off for positivity.

IgA was also measured in saliva of 32 Argentine and 14 Luxembourgian MV patients (Fig. 3.12). Positive IgA titers (4 times higher than cut-off) were detected as early as 8 days before onset of rash. Except of 4 saliva samples, all saliva samples were positive for IgA beginning 8 days before onset of rash. 2 of the 4 IgA negative saliva samples, obtained 14 days before and 1-2 days after onset of rash were also negative in the paired sera. The other 2 out of these 4 IgA negative saliva samples had positive IgA values in the paired sera (day 1-2, day 19). This means that MV-specific IgA was detected in saliva by H-FACS from 8 days before until 28 days after onset of rash in 93.3%.

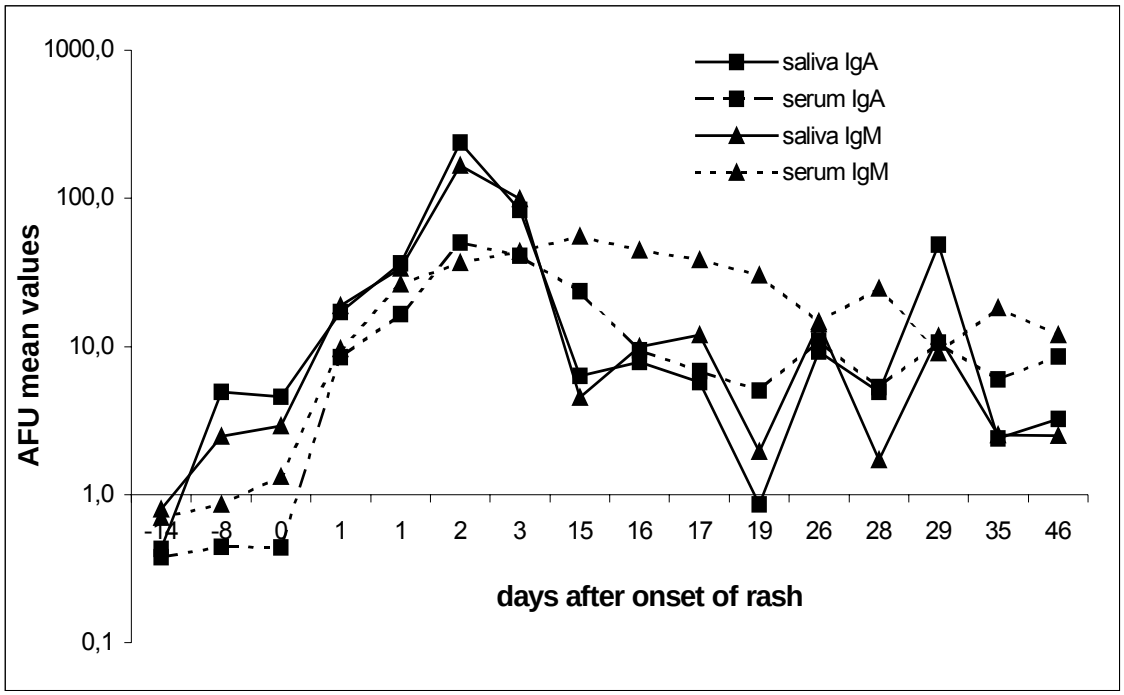


Fig. 3.13: IgA and IgM response of 16 paired serum and saliva samples tested in IgA/H-and IgM/H-FACS. Cut-off values used as defined above (2.3). 2 paired samples were obtained at day 1 after onset of rash.

When serum IgA and serum IgM (by H-FACS) were plotted against IgA and IgM of the paired saliva samples, antibodies in saliva seem to be detected earlier than in serum (8 days before onset of rash, 1 day after onset of rash, respectively) (Fig. 3.13). This would mean that saliva IgA and saliva IgM testing reacted earlier in 2 measles cases compared to serum IgM and in 3 measles cases compared to serum IgA, leading to a quicker start of intervention of measles control measures.

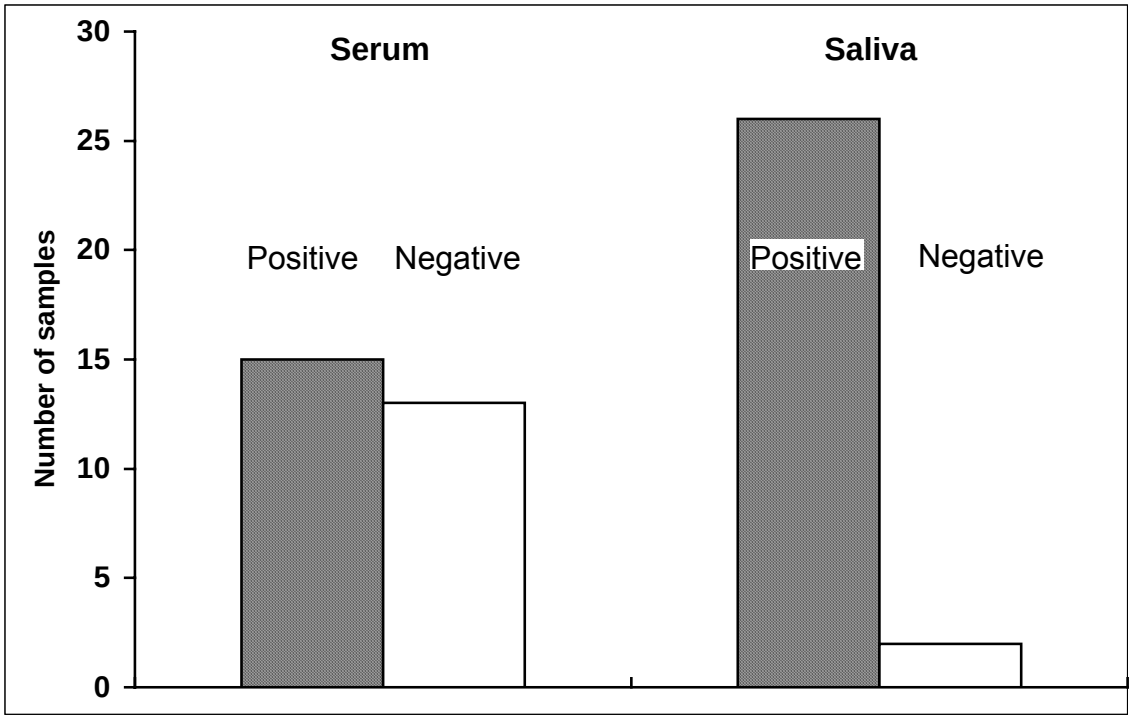


Fig. 3.14: IgA in serum versus paired saliva samples from Argentina (total n=28). Black lined bar = positive, white bar = negative.

The 30 paired serum and saliva samples from Argentine patients (see 2.1.2) were mostly from day 1 and day 2 after onset of rash (n = 26). 2 of the 30 samples were rejected from evaluation because of high background on Mel-JuSo/wt.

The distribution of IgA positive serum and saliva samples is shown in Fig. 3.14. Except 2 saliva samples taken between day 1 and 2 after onset of rash, all saliva samples were positive by IgA H-FACS (26/28), whereas only 15 of the 28 paired serum samples were IgA positive during the first 4 days after onset of rash. 1 of the 2 saliva samples, which were tested negative for IgA was also IgA negative in serum. The other negative IgA saliva sample was positive for IgA in serum. In this case, the H-FACS was able to detect salivary MV-specific IgA antibodies in 11 measles cases earlier compared to serum.

4 Discussion

Measles elimination is defined as the worldwide interruption of transmission of the virus, and represents the sum of successful elimination efforts in all countries. Following licensure of measles vaccine in 1963, the incidence of the disease and its associated morbidity and mortality has decreased by more than 98% (CDC, 2002). Nevertheless, MV infection remains a serious global health-problem. For each region, the WHO has proposed a measles control program, which includes three sequential steps towards eradication (WHO, 1999 a):

- measles control phase
- measles outbreak prevention phase
- measles elimination phase

Measles control is defined as a significant reduction in measles incidence and mortality. As high levels of vaccine coverage are maintained, the intervals between outbreaks would be lengthened and an increasing proportion of cases would occur among individuals in older age groups. Once measles have been drastically and persistently reduced, improved surveillance is needed to maintain low incidence and prevent outbreaks by separation and timely immunization of susceptible individuals (outbreak prevention phase). Measles elimination could be reached by maintaining the number of susceptible individuals in the population below the critical number (< 5%). During each phase, the number of measles patients will decrease. In areas with a low incidence of measles, the clinical diagnosis in sporadic cases is often complicated because of the widespread of other rash-illnesses (Lawrence et al., 2001). In addition, measles in previously vaccinated or immunosuppressed individuals very often does not meet the clinical case definition (Adjaye et al., 1983; Brown et al., 1994; Ferson et al., 1995). Even though Kolpik's spots and the typical measles exanthema are pathognomonic, they only occur at the end of the prodromal phase, during which the virus has infected other susceptible

individuals. Because of the high communicability of measles, this will normally lead to new cases. Only the prompt detection of patients will allow protective measures such as vaccination or isolation of individuals (e.g. pregnant women, immunodeficient persons, individuals suffering from thrombocytopenia, etc.). Towards the goal of measles eradication, new tests for measles diagnosis and surveillance are urgently needed.

4.1 Comparison of different serological measles IgM assays

The surveillance for cases in areas where measles activity has been eliminated or greatly reduced relies on both, thorough epidemiological investigations and laboratory testing. Antibody detection is the simplest and commonly used method for measles diagnosis. In acute, uncomplicated measles, a significant rise in measles-specific IgG antibodies between paired sera is generally considered diagnostic. A positive test-result for specific IgG antibodies in a single serum specimen indicates past infection with measles virus or measles vaccination. Detection of specific IgM antibodies within the first few days after onset of rash provides a reliable diagnosis in a single specimen of measles. Therefore, the IgM assay is the test of choice for rapid diagnosis of measles.

4.1.1 Demand for new serological assays for measles diagnosis

The main purpose of a measles assay is to identify rapidly and reliably seropositive individuals in order to isolate possible transmitters, vaccinate or re-vaccinate susceptible persons. Measles-specific IgM immune response is short-lived and therefore indicative of recent infection. Correct timing of specimen collection with respect to clinical signs is important for interpreting results (Helfand et al., 1997). To allow rapid intervention, samples must be collected as early as possible. Therefore, the usefulness of an assay will mainly depend on its ability to detect measles-specific IgM antibodies at an early stage after measles infection. Measles IgM can be detected by a number of serological

techniques (Boteler et al., 1983; Weigle et al., 1984; Erdman et al., 1991; Arista et al., 1995; Bouche et al., 1998 a, b, c). Plaque reduction neutralisation (PRN) has long been considered as the gold standard of measles immunity and disease when paired sera were available (Albrecht et al., 1981; Griffin and Bellini, 1996). However, this test is very time consuming (7 days until results are available) and paired sera are needed for confirmation. IgM ELISAs require only one single blood sample for case confirmation and can be performed at most laboratories. Performance of the latter assays, such as sensitivity and specificity have been shown to be equivalent to those of PRN (WHO, 1998 c). Currently, indirect ELISAs, such as the Enzygnost are routinely performed most often. As already presented by several authors and recommended by the WHO, the IgM-Enzygnost was chosen as the gold standard in our study (Ozanne and d'Halewyn, 1992, Arista et al., 1995; Bouche et al., 1998 a, b, c). This type of assay requires a specific step to remove IgG antibodies, which may otherwise either compete with IgM for binding to the antigen or mimic IgM by forming immune complexes with RF. Incomplete adsorption of immune IgG can lead to false negative results, a problem which makes capture ELISAs increasingly interesting (Arista et al., 1995). Immunoassays, such as H-ELISA and FACS analysis, based on recombinant proteins detecting only a defined fraction of the total MV-antibodies have been established (Bouche et al., 1998 a, b, c; de Swart et al., 1998). FACS analysis benefits from the high expression of surface glycoproteins, presented in their native membrane-inserted conformation, the possibility to differentiate between IgM and other immunoglobulins, high specific signals versus low backgrounds and the linear correlation between fluorescence intensity and quantity of specific immunoglobulins in the test sample. H-ELISA has been shown by Bouche et al. (1998 a, b, c) to be a reliable, low cost and rapid diagnostic alternative with enhanced stability. In contrast to previous reports, a larger set of serum and plasma samples (n=225, 2.1.1), highly demanding for the performance of each of the 4 IgM assays, was compiled and used for evaluation (Ozanne and d'Halewyn, 1992, Arista et al., 1995).

4.1.2 Evaluation of different assays

4 high standard MV IgM assays were compared to the IgM-Enzygnost using a for each test highly demanding panel of 225 serum and plasma samples derived from patients, late convalescents, vaccinees and RF positive samples from Luxembourg and Nigeria. The difference of the assays was the principle structure of each test and the subset of antibodies detected. The best correlation was found between assays measuring antibodies directed against the whole MV, such as IgM-Enzygnost and IgM-Comfort EIA (R=0.91) or H-FACS and F-FACS (R=0.92), detecting predominantly antibodies against certain MV-specific surface proteins. In order to evaluate the rank of the H-ELISA in comparison to the other assays, the correlation was recalculated with respect to the validity (only sera until day 19 after onset of rash were included) of the H-ELISA. R-values were then generally lower, since the relative proportion of the positive samples included was higher and the range of the values obtained with the positive samples was larger compared to the range of negative results. The H-ELISA showed the best correlation with the H-FACS and F-FACS (R=0.73 and 0.69 respectively), whereas lower R values were found with respect to whole MV ELISAs (R=0.59, R=0.60). This may be explained by the different subsets of antibodies measured by both types of assays.

Sensitivity was high in all assays when samples measured as undefined were excluded from analysis (method A). However, a higher percentage of false negative results (0.5-7.2%) than false positive results (0-0.9%) (e.g. in the F-FACS) would mean that more individuals would be overlooked by measles control measures compared to those considered unjustifiably infectious. In order to reduce the number of overlooked patients, individuals with undefined immune status should be considered positive and potentially infectious (method B). Under this assumption, sensitivity of all assays increased because of the higher percentage of undefined samples, which are now considered true positive. The performance of H-ELISA did not change under these conditions since this assay does not have a grey zone. Calculation of method B, compared to method A

consequently led to a strong decrease in specificity for all assays, except for the IgM-Comfort EIA.

ROC curve analysis (2.4) evaluated the assays independently of their threshold values (Fig. 3.3, Fig. 3.4). IgM-Comfort EIA and H-FACS performed best in discriminating between patients and healthy individuals when the cut-off value was moved across the complete measurement range and when IgM-Enzygnost was used as the gold standard. H-ELISA was excluded from ROC analysis because of the small number of samples.

GraphROC also calculates sensitivity, specificity and efficiency for each possible cut-off value (Fig. 3.5). The cut-off at the intersection of the lines calculated for sensitivity, specificity and efficiency corresponds to the best performance for the three characteristics. In the case of measles diagnosis, it is better to accept a lower specificity and gain in sensitivity. According to method B, the thresholds recommended by the manufacturer or authors for IgM-Comfort EIA and H-ELISA correspond to the best performance. Both cut-off values for H- and F-FACS could be moved further left of the dotted line in order to increase sensitivity without deteriorating the specificity of 92% and 88%, respectively (Fig. 3.5).

In the cohort of sera from Luxembourgian patients (2.1.1), the IgM-Comfort EIA complied best with the gold standard (Fig. 3.2). Both the IgM-Enzygnost and the IgM-Comfort EIA tested all patients positive from day 1 until day 39 after onset of rash. The 2 false tested samples by IgM-Comfort EIA on day 40 (false negative) and 45 (undefined) should not be taken too seriously, since IgM detection after 5 weeks will remain without consequences both for the patient and his surrounding. The interest of IgM detection as an indicator of current or recent measles infection is essentially lost within one week after onset of rash, because infectiosity terminates after 4 days after onset of rash (CDC, 2002). During this period proper differentiation of true positive and true negative results is critical. The H-ELISA detected a measles patient already at the day of onset of rash (3.1.4). However, the gold standard has tested this sample negative. The patient met the clinical criteria for measles. It is conspicuous that the one sample tested negative by H-ELISA (day 1 after onset of rash) was undefined in

H-FACS and negative in F-FACS, whereas assays based on whole MV-antigen displayed positive results. Previous findings have shown that antibodies against MV-N around day 0 - 2 are detected with a chance of approximately 100%, opposed to antibodies against the MV-H or MV-F, which are positive in only 80% (Griffin and Bellini, 1996).

When H-FACS was compared to F-FACS using the Luxembourgian patient cohort, the F-FACS was more susceptible to interference of background on Mel-JuSo/wt. 7 of 10 sera that were undefined by F-FACS were undefined because of background on Mel-JuSo/wt. As shown by Griffin and Bellini (1996), the relative amount of antibodies to MV-H compared to MV-F is nearly equal until day 6 after onset of rash. Nevertheless, the values obtained with F-FACS are very high also after 6 days after onset of rash (median value from day 1 – 35 was 35.0 AFU, range 3.78 – 228.1 AFU). In summary, the IgM-Comfort EIA has proved to be the most accurate test by its standard characteristics as well as by ROC analysis. The assay is comparable to the IgM-Enzygnost and could be used instead.

4.1.3 User-friendliness of tests

The IgM-Enzygnost has been available for several years in an user-friendly kit, including all necessary reagents required. The assay can be performed within approximately 35 min with another 165 min of incubation time, bringing the total time to 200 min. The fastest test was the IgM-Comfort EIA, mainly because of shorter incubation times (75 min). Another advantage of the IgM-Comfort EIA is the small grey zone and the recommended way of calculation, which interprets the data in relation to the cut-off serum, tested on each plate. Time for performing (65 min) and incubation (55 min) was the same for H- and F-FACS. Material expenditure for the plate assays meets the conditions found in most standard measles surveillance laboratories, but the flow-cytometer required for FACS analysis, is not a standard equipment in every laboratory. If only reagents needed are taken into account, prices differ only slightly for the plate tests. Although reagents for performing the FACS assays are cheap, the costs for cell culturing must also be taken into account.

The overall impression of the IgM-Comfort EIA was best compared to the gold standard in the ease of testing, time and material consumption.

4.2 Evaluation of IgA response against MV-H

Although serum is not convenient to collect and prepare, it is the only body fluid regularly used in tests for viral antibody-detection. Existing laboratory methods largely rely on the detection of a significant rise in measles antibodies or the detection of measles-specific IgM and may be impractical for use in settings where blood samples are difficult to obtain. The use of saliva specimens to detect MV-specific immunoglobulins has some promises for the diagnoses of measles and has been shown to be an increasingly accepted tool in MV surveillance (Friedman, 1982; Friedman et al., 1983; Friedman et al., 1989; Friedman et al., 1996; Malamud, 1992; Thieme et al., 1994; Helfand et al., 1996; Garrido et al., 1997; George and Fitchen, 1997; Nigatu et al., 1999; Madar at al., 2002; Ramsay et al., 2002). Unlike vein-puncture, saliva collection is painless, non-invasive, inexpensive, simple, rapid and save (George and Fitchen, 1997). These and other reasons have inspired researchers to improve saliva based test-systems towards higher sensitivity and specificity and to develop new methods in order to obtain a reliable, inexpensive and rapid test for measles (Pederson et al., 1986; Parry et al., 1987; Erdman et al., 1991; Perry et al., 1993; Helfand et al., 1996; George and Fitchen, 1997, Hartter et al., 2000 a).

4.2.1 Observations on the panel, reagents and the system

To evaluate the potential of saliva as a sample source, a panel of 273 sera and saliva samples (2.1.2), derived from vaccinees, late convalescents and acute phase MV patients was compared with respect to IgA-H responses against MV. Another aim of the study was to establish whether IgA was an acceptable agent to determine the immunological status of measles in serum and saliva. The

objective of analysing MV-IgA was the fact that mucosal fluids, such as saliva, contain predominantly IgA, which seems to be able to bind to pathogens in the lamina propria and the eliminate MV by the secretory pathway (Friedman, 1982; Friedman et al., 1983; Brandtzaeg, 1989; Friedman et al., 1989; Malamud, 1992; Mazanec et al., 1992; Thieme et al., 1994; Etchart et al., 1996; Helfand et al., 1996; Oliveira et al., 1998). Etchart et al. (1996) showed that secretory IgA can be boosted with VV-HA (vaccina virus recombinant expressing MV hemagglutinin) in order to induce MV-specific IgA in the respiratory system, which is the primary site of MV replication. Also Liashenko et al. (1999) published positive results of IgA in nasal washings after application of aerosolised measles vaccine with nasal spray. Findings of Yan et al. (2002) presented anti-H IgA as being the most effective antibody in preventing infection via the apical surface. However, whole saliva is a complex mixture of parotid and submandibular salivary gland secretions, mucin, bacteria, leukocytes, sloughed epithelial cells, gingival crevicular transudate, food particles, and other debris. As a result, the concentration of substances to be detected can vary widely, which complicates the detection and quantification of specific antibodies (Malamud, 1992; George and Fitchen, 1997). The FACScan has previously been shown to produce highly specific signals with low background-values. Therefore, this assay was used in our study for analysis of IgA responses in saliva and serum (de Swart et al., 1997; Hartter et al., 2000 a).

4.2.2 Evaluation of MV-IgA in different specimens

The comparison of IgA titers in vaccinees and late convalescents sera from Luxembourg and Nigeria revealed significantly lower values of IgA in the vaccinated cohort relative to the late convalescents (median values 0.5 AFU and 0.7 AFU, respectively) as tested by H-FACS. This is similar to what has been established for IgG (Christenson and Bottiger, 1994). Only about a quarter (26%) of the late convalescents were IgA positive. If vaccinees and late convalescents were compared for both countries separately, it turned out that significant differences found between Luxembourgian vaccinees and late convalescents (median values 0.4 AFU and 0.9 AFU, respectively) could not be

detected in the Nigerian group. This might be due to the higher likelyhood for Nigerian vaccinees to be re-boosted by regular contacts to MV-patients. It has been shown by Pederson et al. (1986) that vaccinees with low levels of IgA were more susceptible to re-infection leading to a rise of MV-specific IgG and IgA. Suggesting that MV-specific serum IgA antibodies may be of some importance for measles immunity. The trend of increased IgA antibodies associated with increased IgG antibodies was also found in the 20 late convalescent sera that were chosen to reflect 10 individuals which were well protected and 10 individuals which had lower IgG titers. We showed that sera from those late convalescents with low titers of IgG have also low titers of IgA, whereas high titers of IgG do not always associate with high titers of IgA. Following the results of Pederson et al. (1986), which suggested that 2 years after measles vaccination IgA antibodies drop faster than IgG antibodies, it would be interesting to know how long before blood collection, the individuals with high IgG titers (same age group than IgG low titers) had measles, assuming that the IgG high titers with low IgA suffered from measles before the ones with high IgA.

In agreement with Erdman et al. (1991), we also found that the peak IgA response to measles in serum from 100 acute phase patients from Luxembourg and Argentina occurred at about 3 to 5 days after onset of rash. Our study concentrated on the time of appearance of IgA after infection. A significant rise (2 - 41.5 times) of IgA was observed in serum of 100 measles patients from Luxembourg and Argentina on day 1 after onset of rash. Still, the percentage of IgA positives from day 1 until day 46 after onset of rash was only 64.2. As of day 2 after onset of rash all patients were IgA positive, meaning that 35.8% of the sera were negative between day 1 and 2 after onset of rash. The exception on day 30 after onset of rash was possibly due to early waning IgA antibodies. When Argentine and Luxembourgian patient sera were considered separately, it was obvious that the general IgA titer in the 63 sera from Argentina were lower compared to the 37 sera from Luxembourg. This would be due to wrong storage during transport or the phenomena, which has already been described for IgG in Nigerians from Hartter et al. (2000 b). These Nigerian mothers had more total

IgG antibodies, but lower titers of measles-specific IgG compared to German mothers.

In saliva from Luxembourg and Nigeria, almost half (42.1%) of the 19 late convalescents displayed positive IgA values, whereas only one sample was tested positive in the cohort of the 14 vaccinated individuals. This may indicate that IgA antibodies either decrease only in some patients within a few months after infection or the immune response of this vaccinated person was boosted before blood was drawn (Peterson et al., 1986).

Among the 46 saliva samples collected from patients from Luxembourg and Argentina, IgA was already detected in 1 sample 8 days before onset of rash with a 12.25 times increase of titer. Starting on day 8 before onset of rash, 93.5% of the saliva samples were IgA positive. This is the earliest time-point, at which presence of anti-MV antibodies has been detected. In this saliva sample IgA was detected 9 days earlier than serum IgA. The easily detection of saliva IgA would be useful to identify the contracted persons during the prodromal stage. 3 saliva samples were tested negative on day 1-2 (n=2) and day 19 (n=1) after onset of rash. 2 of the samples were positive for serum IgA (day 1-2, day 19), indicating that saliva testing gave 2 false negative results. The sample taken on day 19 after onset of rash was also positive for IgM in serum and saliva. 1 sample on day 1-2 after onset of rash was also serum IgA negative. It was not clear whether this Argentine patient still developed immunoglobulins later or whether he suffered from IgA-deficiency (3.2.4).

The time of appearance and the course of IgA and IgM was compared in 16 paired serum and saliva samples (Fig. 3.13). Both isotypes were detected in saliva as early as 9 days prior to the detection of both isotypes in serum (8 days before and 1 day after onset of rash). No advantage of measuring IgA compared to IgM was found in this set up. The sample tested negative by saliva IgA was also low, but still positive for saliva IgM (Fig. 3.13).

In the group of 18 paired Argentine serum and saliva samples (Table 3.5, Fig. 3.14), it was apparent that 4 saliva samples, taken on day 1-2 after onset of rash, resulted in immensely high IgA values (43.6 – 130.0 AFU) in the IgA H-FACS. The paired sera were compared with the IgA H-FACS and the IgM-

Enzygnost. The single serum, taken on day 1 after onset of rash, was negative in IgM-Enzygnost and IgA H-FACS. The other 3 paired sera, collected on day 1-2 after onset of rash tested positive in IgM-Enzygnost and 2 of the 3 were positive in the IgA H-FACS. The overall positivity of IgA in the 18 Argentine patients was higher in saliva than in sera, which was mostly because of the lack of antibody-detection in serum between day 1 and day 2 after onset of rash in serum.

It is of great importance to have a quick and easy measure of the status of immunity when measles appears. Paunio et al. (1998) has described a “super-spreader”, who infected 22 other students, including 9 vaccinated students. Similar situations could perhaps be avoided if early screening would be possible. The perception that measles is a relatively mild disease, at least in the majority of cases makes parents and medical practitioners often reluctant to draw blood to confirm the diagnosis. A non-invasive test-system could circumvent the reluctance to draw blood and improve MV control.

The results presented in this study suggest that saliva is suitable for laboratory confirmation (H-FACS) of naturally occurring measles infections. Results in our set-up compared well with results obtained by conventional serological assays. Such a test would support early patient isolation and epidemiological surveillance. Salivary antibody-testing may provide better access to epidemic outbreaks, children, large populations, hard-to-reach risk groups. To improve saliva testing, high costs of the test must come down. A flow-cytometer would normally not be available in developing countries. Until now, the MV H-FACS can only be used in well-equipped laboratories for MV surveillances. Even though IgA seemed to be a valuable agent to detect measles patients in serum and saliva at an early stage after infection, the test cannot distinguish between patients, vaccinees, late convalescents and susceptibles.

5 Summary

MV is the most contagious human pathogen. Especially in developing countries, it causes a frequent and fatal disease of young children because of background malnutrition and the risk of opportunistic secondary infections as a result of a transient cellular immunosuppression following acute measles. In industrialized areas outbreaks continue to occur, and rapid diagnosis for the timely implementation of control measures are required. Due to decreasing numbers of cases seen by medical practitioners, diagnosis relies increasingly on serological testing. Since IgM is indicative for acute measles, it is used by most laboratories for detection of measles patients. Four high quality IgM-assays have been evaluated. The IgM-Enzygnost was chosen as a gold standard. Compared to the IgM-Enzygnost (indirect format), the IgM-Comfort EIA (capture format) gave the best performance including ease of test-handling. H-FACS and H-ELISA, based on recombinant H-protein were slightly less accurate, but can be used efficiently during the important period close to onset of rash. The F-FACS was a weaker assay in this study and would require further improvements. An interesting alternative to serum would be saliva-testing. Saliva is more convenient and, to some extend, a safer specimen. Most patients, especially children, would prefer to give saliva, than blood. IgA-H, supposedly the most effective antibody at the mucosal surface, was determined in a panel of paired sera and saliva samples (n=273) using the H-FACS. A correct distribution between vaccinees and late convalescents in serum and saliva cannot be observed in this way. An interesting observation was, that patients could be detected using saliva as specimen already long before IgA was found in serum (8 days before compared to 1 day after onset of rash), strengthen the idea that the reaction of the body during the very early time of virus-entry through the mucosal barrier can be determined with saliva IgA-testing. Nevertheless, IgA was also found in serum and saliva of late convalescents and vaccinees. It is therefore not the isotype to be used for rapid measles diagnostic or for distinction between late convalescents and vaccinees.

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