

Measles in Sudan

Diagnosis, Epidemiology and Humoral Immune Response

Mazelen in Soedan

Diagnostiek, epidemiologie en humorale immuunrespons

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To Mohammed and Nimah,
my dear parents, lovely family
and to Sudan

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Abbreviations

A	adenine
Ab	antibody
AFU	arbitrary fluorescence units
ANOVA	analysis of variance
BAL	broncho-alveolar lavage
BCG	Bacillus Calmette-Guérin
B-LCL	B lymphoblastic cell line
C	cytosine
CCID ₅₀	cell culture infectious dose 50%
CD	cluster of differentiation
CDC	Centre of disease control
CDV	canine distemper virus
CFR	case fatality rates
CNS	central nervous system
CPE	cytopathic effect
CT	cycle threshold
CTL	cytotoxic T lymphocyte
DMSO	dimethyl sulfoxide
DMV	dolphin morbillivirus
DTP	diphtheria-tetanus-pertussis
EBV	Epstein-Barr virus
EDTA	ethylene-diamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPI	expanded programme of immunisation
Erasmus MC	Erasmus Medical Center
F	measles virus fusion protein
FACS	fluorescence activated cell scanner
F(ab')	fragment of antigen binding
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
G	guanine
GMLN	global measles laboratory network
H	measles virus hemagglutinin
HLA	human leukocyte antigen
HIV	human immunodeficiency virus
HRPO	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IH	immunohistochemistry
IL	interleukin
i.m.	intra-muscular
i.n.	intra-nasal
i.t.	intra-tracheal
IU	international units
L	measles virus large protein
LAV	live attenuated vaccine
LLC	lung lavage cells
M	measles virus matrix protein

mAb	monoclonal antibody
MCP	membrane cofactor protein
MIBE	measles inclusion body encephalitis
MMR	measles-mumps-rubella
MOI	multiplicity of infection
MHC	major histocompatibility complex
MSMV	monk seal morbillivirus
MV	measles virus
MV-EDM	measles virus Edmonston
MV-EZ	measles virus Edmonston Zagreb
N	measles virus nucleoprotein
NK	natural killer
nt	nucleotide
OD	optical density
P	measles virus phosphoprotein
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDV	phocine distemper virus
pfu	plaque-forming units
PHA	phytohaemagglutinin
PMV	porpoise morbillivirus
PO	peroxidase
PPRV	peste-des-petits-ruminants virus
RNA	ribonucleic acid
RNP	ribonucleoprotein complex
RPV	rinderpest virus
RT-PCR	reverse transcriptase polymerase chain reaction
SCID	severe combined immunodeficiency
SD	standard deviation
SLAM	signalling lymphocytic activation molecule
SSPE	subacute sclerosing panencephalitis
T	thymine
TCID ₅₀	tissue culture-infectious dose 50%
Th	T helper lymphocyte
Th1	T helper lymphocyte type 1
Th2	T helper lymphocyte type 2
TMB	tetra-methyl benzidine
TNF	tumor necrosis factor
Tween-20	polyoxyethylenesorbitan monolaurate
UNG	uracil-N-glycosylase
UNICEF	United Nations Children's Fund
UV	ultraviolet
VN	virus neutralisation
WBC	white blood cells
WHO	World health Organisation
wt	wild-type
www	world-wide-web

Chapter 1

General Introduction

Measles

Measles is a highly contagious disease, which is transmitted via the respiratory route. After an incubation time of one to two weeks, disease starts with a prodromal phase of fever, cough and coryza. At this stage the typical red “Koplik spots” can be seen on the buccal mucosa. A few days later a generalised maculopapular rash appears, often in combination with conjunctivitis (1). Measles is associated with a transient immunosuppression, which significantly contributes to its morbidity and mortality. Paradoxically, the disease also results in lifelong immunity (2).

Introductions of measles in virgin populations and endemic transmission in populations with inadequate medical care are associated with high mortality (1). Measles remains the most important cause of vaccine-preventable deaths in developing countries. It was estimated that measles caused 777 000 deaths in 2002, 84% of which occurred in only 11 countries in Asia and Africa (3).

Common complications of measles include pneumonia, diarrhoea, laryngo-tracheobronchitis, eye complications, otitis media and stomatitis (1). These complications, which are the direct result of measles-related immunosuppression, are the most important determinants of morbidity and mortality. Pneumonia is the leading complication in this aspect, accounting for more than half of the measles related deaths (2). The only means of case management recommended by the World Health Organisation (WHO) is administration of vitamin A [in developing countries (4)] and treatment of complications with antibiotics [for review see (2;5)]. The outcome of MV infection may also be influenced by concurrent infection with immunosuppressive agents, e.g. human immunodeficiency virus (HIV) (2;6).

Neurological complications of measles are relatively rare. Two rare chronic progressive infections of the CNS exist: measles inclusion body encephalitis (MIBE) in immunocompromised or malnourished individuals

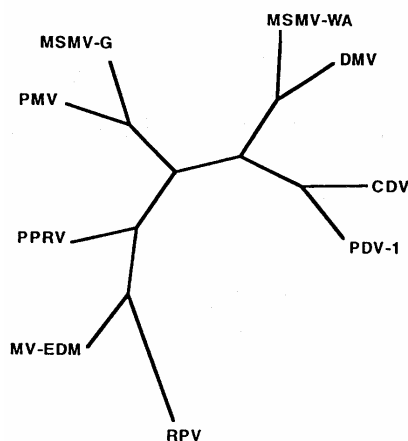


Figure 1

A maximum likelihood tree of N protein fragment sequences (121 nucleotides) of different morbilliviruses. DMV, dolphin morbillivirus; PMV, porpoise morbillivirus; PDV, phocine distemper virus; CDV, canine distemper virus; RPV, rinderpest virus; MV-EDM, measles virus Edmonston strain; PPRV, peste des petits ruminants virus; MSMV-G, monk seal morbillivirus from Greece; MSMV-WA, monk seal morbillivirus from West Africa. Adapted from Osterhaus et al. Vaccine 1998;16:979-981.

and sub-acute sclerosing panencephalitis (SSPE) in individuals with normal immune function (1). The latter occurs years after initial infection and is always fatal (7;8).

Measles Virus

Classification

Measles virus (MV) is a member of the genus *Morbillivirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*. The virus infects humans and has no animal reservoir, although under certain conditions non-human primates can also be infected. Measles is a relatively new disease of humans. It is postulated that MV developed from rinderpest virus (RPV, figure 1) in environments where cattle and humans lived in close proximity (1). Since large populations of susceptible individuals are needed to sustain the continuous presence of the virus, it is thought that the disease established itself in the early centres of civilisation in the Middle East (9).

Morphology

The viral core is a pleomorphic ribonucleoprotein particle (RNP), consisting of the negative sense, non-segmented, single stranded RNA genome contained within a helical nucleocapsid (figure 2a). The genome consists of 15894 nucleotides, encoding six structural proteins. These are the nucleoprotein (N), phosphoprotein (P), large protein (L), matrix protein (M), fusion protein (F) and haemagglutinin (H). In addition, two non-structural regulatory proteins are encoded through alternative reading frames: C and V (figure 2b). The N, P and L proteins, of which L is the RNA polymerase, are bound to the RNA and form the nucleocapsid (figure 2a). This nucleocapsid is surrounded by a lipid membrane, which is composed of the M protein, and the two transmembrane glycoproteins F and H (figure 2a) (1).

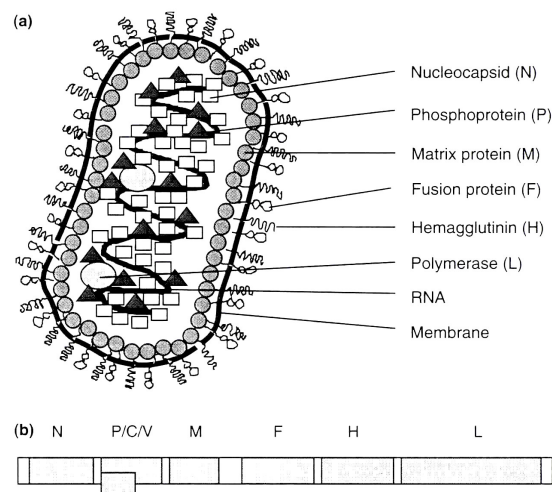


Figure 2

The MV particle and genomic organisation. Taken from Ian C. D. Johnston et al. Trends in Microbiology 1995; 3: 361-365.

Replication

MV infection starts by binding of the H protein (a type II glycoprotein) to its cellular receptor. The F and H proteins facilitate fusion of the virus envelope with the cell membrane (10), resulting in release of the nucleocapsid into the cytoplasm. The process of transcription and replication proceeds by the synthesis of genomic RNA and viral proteins, of which N is the first transcribed followed by P, M, F, H and L (figure 2b). The viral proteins are synthesised either in the cytosol (N and M) or in the endoplasmic reticulum. Finally, new virus particles are released from the cell membrane by budding, which is regulated by the presence of M protein.

Cellular receptors

The first protein identified as cellular receptor for MV was CD46 (membrane cofactor protein, MCP), which is ubiquitously expressed on human nucleated cells (11). However, conflicting results were obtained with regard to the ability of wild-type clinical strains to interact with CD46 (12;13). In addition, the tissue distribution of CD46 was not in accordance with the pathogenesis of measles.

It is now generally accepted that CD46 mainly functions as receptor for laboratory-adapted or vaccine strains of MV and most wild-type MV strains do not bind to CD46. Recently, signalling lymphocytic activation molecule (SLAM, CD150) has been identified as a receptor for both laboratory-adapted and wild-type strains of MV (14-18). SLAM is expressed on activated B and T cells, monocytes and dendritic cells (19-22), and its usage as a receptor can explain the tropism of wild-type MV in these cells (18;23). MV, however, also infects epithelial cells, endothelial cells and neural cells, suggesting the existence of an additional unknown receptor or an alternative uptake mechanism (24).

Genetic variability

MV is a monotypic virus: infection provides immunity from all circulating strains. However, the MV genome replicates by an RNA-dependent RNA polymerase, which lacks proofreading capacity. The resulting variability (1) has been documented, initially as variation in monoclonal antibody reactivity, plaque morphology, fusogenicity, temperature sensitivity, ability to induce interferon, and subsequently as nucleotide sequence variability [for review see (25)]. However, viruses with the maximal sequence variation from vaccine strains are still neutralised by antiserum to vaccine virus (26). In addition, serum of people infected decades ago still neutralises recent wild-type viruses (1). Yet, with the increased mutation rates (25;27), the possible emergence of a strain that can escape vaccine immunity can not be excluded (27).

Genetic variation occurs at varying levels in the whole MV genome, the most variable being the N and H genes which display up to 8% variation (28). This variation is stable upon propagation in tissue culture and thus a suitable tool for the molecular characterisation of wild-type isolates (25),

facilitating molecular epidemiological studies (29;30). Laboratory studies show that the mutation rates of MV are similar to other RNA viruses (31). However, molecular epidemiological studies based on the N and H genes show little variation within a single chain of measles transmission (32;33).

Epidemiology

Measles is among the most infectious of communicable diseases (1;34)). It is estimated that 76% of susceptible household exposures lead to measles, and the average number of secondary cases produced by a measles case is 15-20 cases (1). In temperate climate zones, MV infections used to peak in winter and early spring seasons possibly indicating the increased survival of the virus at lower humidity (1). The main transmission pathway for MV is through direct exposure to infected individuals. However, the virus remains viable and infectious for hours in respiratory droplets. Infected individuals can transmit the disease for a period of one week before, until one week after onset of clinical symptoms. The maintenance of MV in a population is dependent on the presence of susceptible individuals. Since older persons in a population are generally immune due to previous exposures, measles is basically a childhood disease in populations where the virus is endemic. Results of mathematical modelling and observations in island populations suggest that a population size of 250 000 to 500 000 is required to sustain endemic MV circulation (1).

Measles related mortality is highest in very young infants, adults, and patients with low socio-economic status and in the absence of medical care. Although malnutrition did not seem to affect mortality rates, the source of the virus did: mortality is increased in secondary cases in a household as compared to index cases [as reviewed in (1)]. This can probably be explained by a higher virus inoculum in the latter group. Most of the fatal measles cases occur in developing countries, with case fatality rates (CFR) often exceeding 1% (5).

The introduction of measles vaccination has changed the epidemiology of measles. Vaccination reduced the numbers of susceptible individuals, resulting in an increase in the time between epidemics and an increase in the mean age at infection. A substantial number of vaccinated individuals is still susceptible or can become susceptible again to MV infection, due to primary or secondary vaccination failure (35;36). These infections can either result in no detectable clinical signs at all (subclinical infection associated with a strong secondary immune response), mild respiratory symptoms or in classical measles. The possibility of MV transmission from subclinical measles cases to susceptible individuals remains a controversial issue, but it is generally thought that the contribution to the epidemiology of measles is limited.

Molecular characterisation of wild-type MV isolates has become an important tool in measles surveillance. When combined with standard epidemiological information it enables the identification of the sources and

transmission pathways. Molecular surveillance also allows the differentiation between indigenous and imported MV strains (25;37-39).

The increasing number of reported MV sequences from different parts of the world allowed phylogenetic studies, resulting in the identification of 8 clades comprising 22 genotypes (28). The WHO has standardised the nomenclature of wild-type MV strains and the minimum amount of sequence data to genotype a virus strain or nucleotide sequence. For this purpose the 456 nucleotides coding for the carboxyl terminal of the N-gene need to be sequenced. To identify a new genotype, however, it is essential to provide the complete H gene sequence. In addition, it is also recommended to provide H gene sequences from representative isolates from each genotype (38;40). MV genotypes are not geographically restricted, but tend to be endemic in certain areas (25). In countries where measles remains endemic a few genotypes are usually the cause of the transmission, while in areas where measles is eliminated more (import-related) genotypes are responsible for fewer infections (28).

Central and west Africa is dominated by clade B MV strains. This clade consists of three genotypes B1, B2 and B3 (33;41). Genotype B3 was recently divided into two clusters (39). Genotype B3 cluster 2 is confined to western Africa, whilst cluster 1 shows wide distribution in sub-Saharan Africa (39;42). Genotypes D2 and D4 were found in South Africa and recently also in Kenya (27;43). In Morocco genotype C2 was found to circulate, which was restricted to European countries (44).

Pathogenesis

MV is spread by aerosols and enters via the respiratory route. The

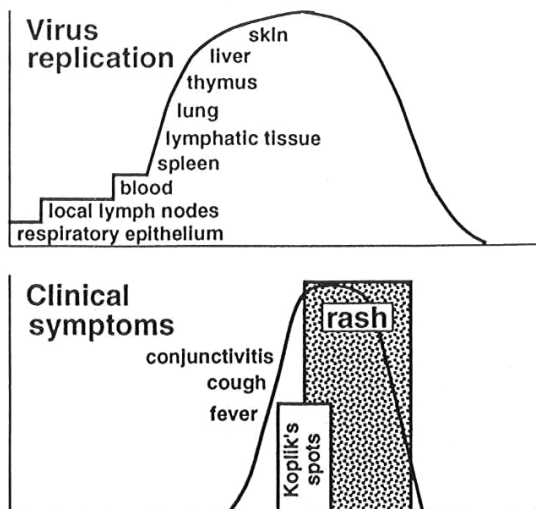


Figure 3

Diagram of the pathogenesis of measles. Virus replication begins in the respiratory epithelium and spreads to monocytes / macrophages, endothelial cells, and epithelial cells in the blood, thymus, spleen, lymph nodes, liver, skin, and lung and to the conjunctivae and the mucosal surfaces of the gastrointestinal, respiratory, and genitourinary tracts. Clearance of virus is approximately coincident with fading of the rash. Taken from Griffin DE, Bellini WJ. Measles virus. In: Fields BN, Knipe DM, Howley PM, eds. Fields Virology. 3 ed. Philadelphia: Lippincott - Raven Publishers, 1996:1267-312.

virus initially infects the respiratory epithelia, and extends to the local lymphoid tissues perhaps through pulmonary macrophages and dendritic cells. Replication of the virus in the lymphoid tissues leads to spread of the virus through a viremic phase, resulting in infection of a variety of organs including skin, conjunctiva, kidney, lung, gastrointestinal tract, respiratory mucosa, genital mucosa and liver (for review see (1) and figure 3).

The appearance of clinical signs coincides with the induction of MV-specific immune responses. The typical measles rash and conjunctivitis are mediated by the activity of MV-specific T cells, i.e. are manifestations of immunopathology. In patients with compromised cellular immunity infection with MV is often fatal, but may not be recognised as measles due to the absence of a rash.

Immunity

Antibody responses

Antibodies are important mediators of protection from infection. This is best illustrated by the protection of infants born to sero-positive mothers through passively acquired maternal antibodies (1). MV-specific virus neutralising (VN) antibodies were also shown to be protective in adoptive transfer experiments and in immune therapy (45;46).

MV-specific antibodies are detectable from the onset of rash, and remain present for life. However, several changes in antibody isotype distribution take place over time. MV-specific IgM antibodies are the first to appear, have a relatively low affinity and disappear within a few months (47-49). Detection of MV-specific IgM is considered the gold standard for the laboratory diagnosis of measles (50).

MV-specific IgG antibodies can also be detected from the onset of rash, but peak one to two weeks later and remain present life-long. They undergo affinity maturation, and are the major mediators of virus neutralisation (51). Protective levels of MV-specific VN antibody responses were found to be between 0.1-0.2 international units (IU)/ml (52;53). The IgG subclass composition during different phases of the immune response is still a matter of discussion (54-57).

The kinetics and possible role of measles virus-specific serum IgA are also unclear. Specific IgA can be detected during the acute and convalescent phase of the infection, but in some individuals also during the memory phase (47;58;59). These antibodies may also be of importance in protection (60).

MV-specific antibodies can be directed to most of the viral proteins, but the most immunogenic proteins are the N, F and H proteins (61). N-specific antibodies are the most abundantly and rapidly produced (61). However, N-specific antibodies are considered to have limited biological function. H-specific antibodies are considered the most important contributors to virus neutralisation (62), although F-specific antibodies may neutralise as well (1;63).

T-cell responses

The role of T-cell immunity in clearance of an existing MV infection is considered to be more important than the role of antibodies (1). This is illustrated by the observation that children with hypogammaglobulinemia recover uneventfully from measles, while children with T-cell deficiencies are at risk of developing fatal complications (64;65). During the acute phase of measles, MV-specific CD8⁺ cytotoxic T lymphocytes (CTLs) were demonstrated in blood and broncho-alveolar lavage cells (66-68). Later in the course of infection MV-specific CD4⁺ helper T lymphocytes (Th cells), with and without cytolytic capacity, were demonstrated (69-72). CD4⁺ and CD8⁺ T-cells were both detectable in the memory phase long after infection (53;73), thus indicating the role of T-cells in the maintenance of lifelong immunity against measles. The balance between CTLs and Th cells on the one hand and between Th cell subsets producing different cytokine patterns on the other have been subject of extensive research in relation to measles immunopathogenesis or vaccine safety [for review see (74)].

Immunosuppression

Measles is associated with a characteristic immunosuppression, which was first documented in 1908 by clinical observations, and then confirmed by sufficient in vivo and in vitro evidence [for review see (1)]. The mechanisms and factors involved in this immunosuppression are still poorly understood. MV infects both lymphocytes and antigen-presenting cells (APCs), resulting in acute damage to the immune system (75). However, other mechanisms mediated by the F and H proteins in the absence of infectious virus have also been described (76). Recently, the N protein was reported to bind specifically to a new cell receptor on thymic epithelial cells inhibiting cell proliferation (77). Measles immunosuppression is therefore most likely a multifactorial process (78).

Animal models

A variety of animal models were used to study the pathogenesis of measles, MV-specific immune responses or alternative vaccination strategies. Small laboratory animals including hamsters (79), mice (80;81), ferrets (82) and rats (81;83) were used in measles research. Most rodents are not susceptible to infection with wild-type MV strains. However, limited wild-type MV replication has been reported in the respiratory tract of cotton rats, and they appear to be the most promising rodent model for measles (80;84-90). Neurotropic rodent-adapted strains of measles virus have been developed, which do not produce acute disease as in humans but can serve as models for neurologic disease like SSPE (82;91-93).

Non-human primates are highly susceptible to MV infection, as illustrated by outbreaks in monkey colonies and by experimental infection (94). The pathogenesis of measles in monkeys resembles that in humans,

although severity varies in the different species (94-98). Monkeys are thus the most relevant animal models for measles research [for review see (94)]. Monkeys used as models in measles research include marmosets (*Sanguinus mystax*), baboons (*Papio hamadrius*), cynomolgus- and rhesus macaques (*Macaca fascicularis* and *Macaca mulatta*), respectively (94). Monkey models allowed the detailed analysis of mechanisms underlying the immunopathogenesis of classical measles, atypical measles, measles in the immunocompromised host and in the immunised host (8;95;96;99-101). In addition, they allowed comparison of the virulence of different wild-type strains and comparisons of novel vaccines and vaccination strategies (97;102;102-104).

Prevention and control

Clinical diagnosis

The WHO clinical case definition for measles states: “any person with a generalised maculopapular rash, fever of 38°C or higher and one of the following: cough, coryza or conjunctivitis; or any person in whom a health professional suspects measles” (105). However, it has been documented that clinical manifestations are very likely to be altered in very young children, immunocompromised patients, or previously vaccinated individuals (106). In addition, other viral and bacterial diseases share many of the features (1). As the incidence of measles decreases due to successful measles control measures, the reliability of clinical diagnosis decreases making laboratory confirmation more important (107).

Laboratory diagnosis

Analogous to the existing polio laboratory network, a Global Measles Laboratory Network (GMLN) has been developed (108). The GMLN has a tiered structure with national and sub-national laboratories, regional reference labs and global specialised labs, and implements standardised testing and reporting procedures. During the early stages of the accelerated measles control campaigns the main aim of the GMLN will be to monitor indigenous measles virus (MV) transmission and to trace small or large outbreaks, as opposed to laboratory diagnosis of individual cases (108). Therefore, collection of clinical samples from representative numbers of patients for laboratory diagnosis and collection of sequences for molecular surveillance will be needed.

Laboratory diagnosis of MV infection can be based on detection of either the virus itself or specific IgM antibodies. Virological diagnosis may be performed by virus isolation, direct detection of the virus or viral antigens, or detection of viral RNA. Serological diagnosis may be performed by demonstration of MV-specific IgM antibodies in a single specimen or a more than fourfold titer rise in IgG antibodies in paired samples [for review see (109)].

Serum based IgM detection by ELISA using a single sample collected during the presence of clinical symptoms is selected by the GMLN as the

standard method for the laboratory confirmation of clinical diagnosis (50;108). To this end several “in house” or commercially developed validated and non-validated ELISAs are used in different laboratories, using either indirect or class capture systems [for review see (108;109)]. Although most systems work well, the GMLN recommends the use of the Dade Behring indirect IgM assay or equivalent assay systems.

The countries with the highest mortality levels often have limited infrastructure, resulting in difficulties with collecting, storing and shipping the conventional clinical samples. Whereas serum has always been the standard clinical specimen for this purpose, alternative samples including oral fluids and filter paper blood spots have been used successfully (108). Both oral fluid samples and filter paper blood spots can be used for IgM detection as well as detection of MV genomic RNA by RT-PCR and collection of sequences (110-113). Several studies compared filter paper samples and serum samples in the detection of MV-specific IgM and IgG antibodies using in house and commercial ELISAs (114;115). The most important current aspect of the alternative sampling methods is the possibility to test them under field conditions.

Vaccination

In the 1960's formalin or tween-ether inactivated whole virus vaccines adjuvanted with alum were developed. These vaccines induced proper seroconversion rates, but specific antibody levels waned over time. More importantly, when vaccinated individuals encountered a wild-type MV, they developed a severe form of disease characterised by high fever and an immune-mediated pneumonia. These immunopathological manifestations were referred to as “atypical measles” (116;117).

During the same period live attenuated vaccine (LAV) strains were also developed, which proved to be safe and effective. In large parts of the world these are now used in combination with live-attenuated mumps and rubella virus strains in a trivalent vaccine (measles-mumps-rubella, MMR). Although these vaccines have a number of important disadvantages (e.g. requirement of a cold chain, interference by maternal antibodies), they have also proven to be effective in eliminating MV from large parts of the world. This has especially been the result of introducing a second opportunity for measles vaccination at a later age, and adopting a strategy of “catch-up, keep-up and follow-up” (118)(www.measlesinitiative.org).

Several experimental measles vaccines and different vaccination strategies to combat the disadvantages of the LAV vaccine have been evaluated, but none of these have yet held sufficient promise to seriously compete with LAV (for review see (119;120). Recently, the development of needle-free alternative methods of LAV administration has received considerable attention, of which especially the aerosol route of vaccination is considered promising (121-123).

Measles in Sudan

Sudan is the largest country in Africa, located in the northeast (figure



Figure 4. Map of Sudan.

4). Measles is an endemic disease in Sudan. It is the third common cause of childhood deaths, preceded by gastro-enteritis and non-specific fever (124). The incidence of the disease is greatly underestimated due to the general instability of the population; influx of immigrants from other countries, and the spread of wrong beliefs of not taking measles patients to hospitals (124). In 2001 the number of reported measles cases in Sudan was 4362 (figure 5). These reported incidence rates are all hospital-based and do not reflect the real incidence in the community (124).

A number of epidemiological studies involving the morbidity rates and age of infection have been carried

out in different parts of Sudan. These showed that most measles cases occur during the first five years of life (124-126). A community-based study was performed in a suburban area in Khartoum, and showed a seasonal pattern in MV infection with incidence rates peaking during winter (124). The risk factors predisposing to severe disease were found to include malnutrition, poverty, overcrowding and poor sanitation (124;126).

In 1989 a randomised, blinded and placebo-controlled clinical trial was performed in Khartoum to study the efficacy of immunisation with LAV of increased titer (127). At that time this strategy was being evaluated to break the barrier of maternal immunity, allowing vaccination at an earlier age (128). Although this strategy was later abandoned because of an observed

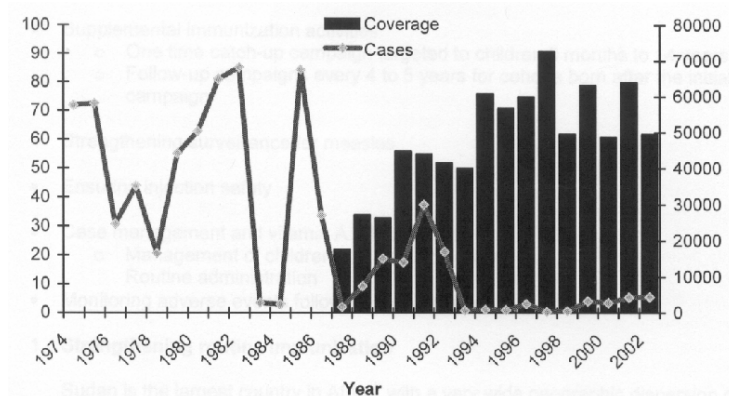


Figure 5

Measles cases and vaccination coverage in Sudan, 1974-2002. Reproduced with permission from "Measles mortality reduction in Sudan", WHO/EMRO Technical Report, 21-29 September 2003.

increased mortality in comparison with infants obtaining LAV of standard titer (129), these problems were not observed in Sudan (127). Recently, it was hypothesised that the observed increased mortality may have been the result of changes in the vaccination schedule (i.e. the order in which different vaccines are administered) rather than the LAV titer (130;131).

The most common long-term measles complications in Sudan are eye lesions, pneumonia and otitis media (124). Measles cases in Sudan are only clinically diagnosed as no serological or virological assays are performed at the community health care units or hospitals (124). In many cases patients with measles have no access to medical treatment (124).

The live attenuated Schwarz vaccine was introduced in Sudan in the late 1970s. In 1985 the Ministry of Health introduced countrywide measles vaccination at 9 months of age, through the expanded programme of immunisation (EPI) services. This resulted in a remarkable reduction in the incidence of measles, as shown by the WHO records (figure 5). Despite these extensive efforts, low vaccination coverage and high incidences of vaccine failure were reported (124;132). Vitamin A supplementation in the early course of infection was found to reduce the frequency of complications and mortality and proved to enhance recovery from complications (124). Vitamin A is thus therapeutically administered to measles cases reporting to hospitals and health centres.

Aims and outline of thesis

Measles remains endemic in Sudan. However, epidemiological and virological information about the disease is largely lacking. The aims of this thesis were to study diagnostic and epidemiological aspects of measles in Sudan. In addition the clinical samples were used to study pathogenesis and virus-specific immune responses. These studies were initiated as part of a research project performed in collaboration between the University of Khartoum and the Erasmus MC in Rotterdam, which was funded by the INCO-DC program of the European Commission. The project at large aimed at analysis of diagnostic, epidemiological and immunological aspects of measles in Sudan.

In **chapter 2** diagnostic and epidemiological aspects of measles in Sudan are described. **Section 2.1** describes serological and virological confirmation of clinical measles cases from Sudan. In **section 2.2** a review of diagnostic, and epidemiological studies performed within the collaborative research project described above is presented. **Section 2.3** describes the molecular characterisation of the wild-type MV isolates circulating in Sudan. In **section 2.4** the surveillance of measles in Sudan is continued using filter paper blood samples. **Section 2.5** describes the development of a real-time RT-PCR for detection of MV genome. The value of the assay in assessment of measles diagnosis and pathogenesis is discussed.

In **chapter 3** the MV protein-specific responses during the acute and convalescent phases of the infection are assessed in patient specimens.

In **chapter 4** aspects of the virulence and pathogenesis of the wild-type strain circulating in Khartoum are studied in cynomolgus and rhesus macaques. The data are compared with those obtained in parallel experiments with a European wild-type strain of MV.

Finally, **chapter 5** provides a summarising discussion of the thesis.

Chapter 2

Diagnosis and epidemiology

Section 2.1

Serological and virological characterisation of clinically diagnosed measles cases in suburban Khartoum

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Abstract

Measles continues to be a major childhood disease in terms of global morbidity and mortality. In the main areas of its endemicity the only available means of diagnosis are based on clinical criteria: the presence of a maculopapular rash and fever accompanied by cough, coryza and/or conjunctivitis. We have studied 38 clinically diagnosed measles cases in Khartoum, Sudan, by means of serology, reverse transcriptase PCR (RT-PCR) on throat swabs and virus isolation from lymphocytes. On the basis of serology, 28 patients were diagnosed as having an acute measles virus (MV) infection, while in 10 cases the clinical symptoms proved to have other causes. It was shown that in cases with low serum immunoglobulin M (IgM) levels, an additional measurement of IgG or virus neutralising (VN) antibodies was necessary to discriminate between patients with an acute MV infection sampled during an early stage of the disease and patients who had experienced a MV infection in the more distant past. The serological laboratory diagnosis was validated by a MV-specific RT-PCR: for all confirmed measles cases tested a fragment of the correct size which hybridised with a third MV-specific primer could be amplified, while all serologically negative cases were also RT-PCR negative. MV could be isolated from 17 out of 23 of the serologically confirmed cases, demonstrating that virus isolation is less reliable as a diagnostic tool than serology or RT-PCR. This study stresses the urgent need for a rapid diagnostic field test for measles.

Introduction

Measles is a highly infectious respiratory virus infection, with typical clinical symptoms including maculopapular rash, fever, cough, coryza and conjunctivitis. The causative agent of the disease, measles virus (MV), is a negative strand RNA virus of the genus *Morbillivirus*, family *Paramyxoviridae* (1). Measles continues to be a major cause of childhood morbidity and mortality worldwide, with an estimated one million fatal cases each year (38). Although the introduction of live attenuated MV vaccines has largely abrogated the endemic circulation of wild-type MV in the industrialised world, vaccination has been less successful in large areas of Africa and Asia (133). This is thought to be the combined result of insufficient vaccination coverage due to limited infrastructure and/or political instability and inherent disadvantages of the live attenuated vaccine such as the need for cold chain maintenance and the interference by maternal antibodies (134;135).

Considering the World Health Organisation (WHO) aim to eradicate measles in the beginning of the next century, more insight is required in the epidemiology and immunopathogenesis of measles in areas where the virus remains endemic. During the course of an eradication campaign, the identification of clinical cases becomes increasingly important. At present, measles diagnosis in third world countries is almost exclusively based on the evaluation of clinical symptoms. However, due to the immunopathological nature of at least part of the typical clinical symptoms of measles, not all patients infected with MV present typical symptoms (1;136). Furthermore, not every disease which fulfils the clinical criteria for measles is necessarily caused by an infection with MV (106;137).

The "gold standard" for laboratory diagnosis of an MV infection is the demonstration of specific IgM (50), either by a capture enzyme linked immunosorbent assay (ELISA) (138) or by immunofluorescence (139). A rapid, cheap and accurate test to detect MV-specific IgM antibodies in a field setting is urgently needed but, at present, is not available. Diagnostic measures based on demonstration of the presence of the virus (either by RT-PCR or by virus isolation) are equally valid, but generally less practicable in a routine setting. However, the widespread application of lymphoblastoid cell lines instead of the traditional Vero cell cultures for the isolation of wild-type MV strains (140) has greatly facilitated MV isolation procedures. As a spin-off, sequence analyses of the increasing pool of MV strains isolated in different parts of the world has proven a powerful tool for molecular epidemiological studies, showing the global distribution of different MV genotypes (29;141). These studies will be of crucial importance during the final stages of the MV eradication program.

Here, we present the serological and virological characterisation of a group of 38 clinical measles cases collected in Khartoum, Sudan, by demonstration of MV protein-specific serum IgM, IgG and VN antibody

levels, RT-PCR signals in throat swabs, and MV isolation from peripheral blood mononuclear cells (PBMC).

Materials & Methods

Patients.

Clinical materials were collected from infants who complied with the WHO clinical case definition for measles: "any person with a generalised maculopapular rash (i.e. non-vesicular), and a history of fever of 38°C or more, and at least one of the following: cough, coryza (i.e. runny nose), or conjunctivitis (i.e. red eyes); or: any person in whom a health professional suspects measles" (105). The clinical symptoms were always still present at the moment of sampling. Samples were collected after having obtained informed consent from the parents. The collection of clinical specimens was an integral part of an ongoing prospective measles study in Khartoum (started in April 1997), which was approved by the medical ethical committee of the University of Khartoum. The samples collected during the first 6 months of the integral study period are presented here.

Study area.

Most of the patients (n=30) were sampled through a network, which was set up in the residential area Haj Yousif for finding cases of measles. This area has an estimated number of 500,000 inhabitants, mainly comprised of displaced people from the south and west of Sudan. Health care in the area is provided through volunteer health centres, often by staff members with poor clinical backgrounds. Measles vaccination coverage is low, and endemic MV transmission occurs throughout the year. The cases included in this study did not present as an outbreak, but were spread over the 6 month period. The number of cases observed during this period was not substantially different from any other period of the year, or from any other year between 1995 and the present. The number of reported patients that could not be sampled was less than half of the number of patients included in the study. Eight additional patients were sampled in paediatric hospitals in Khartoum.

Samples.

Clinical specimens collected consisted of a throat swab and a heparinised blood sample (approximately 3 ml). Peripheral blood mononuclear cells were isolated by density gradient centrifugation in Khartoum and were frozen in RPMI supplemented with 40% foetal bovine serum (FBS) and 10% dimethyl sulfoxide in liquid nitrogen. Plasma and throat swabs were frozen at -70°C.

Serology.

Plasma levels of IgM or IgG specific for the two MV transmembrane glycoproteins, the fusion protein (F) and haemagglutinin (H), were determined by an immunofluorescence assay using transfected human

melanoma cell lines as targets, as previously described (139). Briefly, melanoma cells expressing either the F protein (Mel-JuSo/MV-F) or the H protein (Mel-JuSo/MV-H) or the untransfected parental cell line (Mel-JuSo/wt) were incubated with diluted plasma samples. The samples were prediluted 1:10 in PBS supplemented with 2% FBS (for measurement of MV-specific IgG), or in GullSorb reagent (Gull Laboratories, Salt lake City, UT, USA) to precipitate all plasma IgG for measurement of MV-specific IgM. Subsequently, the samples were diluted 1:10 in PBS supplemented with 2% FBS to reach a final dilution of 1:100. After one hour on ice the cells were washed and stained with FITC-labelled rabbit anti-human IgM or IgG (F(ab')₂ fragments, DAKO, Glostrup, Denmark). Results are expressed as the fluorescence signal (histogram peak channel) measured on a FACScan (Becton-Dickinson, Mountainview, CA, USA), in arbitrary fluorescence units (AFU). Fluorescence signals measured on the untransfected cell line were always below ten (data not shown).

Plasma levels of IgM specific to the nucleoprotein (N) were measured in a capture ELISA, using peroxidase labelled purified baculovirus-expressed N (N-PO). Plasma samples were diluted 1:100 in ELISA buffer (Meddens Diagnostics, Vorden, The Netherlands) and incubated on ELISA plates (Greiner, Alphen a/d Rijn, The Netherlands) coated with rabbit anti-human IgM (Meddens Diagnostics). After one hour at 37°C, plates were washed in water containing 0.05% Tween-80 and were subsequently incubated with N-PO. Following another hour incubation at 37°C, the plates were washed again and subsequently coloured using tetramethylbenzidine as a substrate. Extinctions were read in an ELISA reader at 450 nm.

Plasma levels of VN antibodies were measured as previously described (61) with minor modifications. Briefly, serial twofold dilutions of the plasma samples were tested for their ability to neutralise 60 TCID₅₀ of the MV Edmonston strain. Plasma dilutions were prepared in Dulbecco's Modified Eagle Medium (BioWhittaker, Verviers, Belgium) supplemented with 2% FBS, of which 50 µl was incubated with 50 µl of the virus working dilution in 96 wells flat bottom plates (Greiner). After 1 hour at 37°C (neutralisation phase), Vero cells were added (10⁴ cells in 50 µl per well). Cells were microscopically monitored for cytopathic effects (CPE) during the following week. For each plasma sample, eight dilutions (1:32 to 1:4096, dilution during neutralisation phase) were tested in triplicate. The results are shown as the dilution at which 50% of the cultures was neutralised, calculated as previously described (142), standardised to the WHO international standard (0.2 I.U./ml), which was found to have a 50% VN titer of 54.

Virus isolation.

MV was isolated from PBMC by an infectious centre assay as previously described (95). PBMC (3.2x10⁵) were divided over 8 wells of a 96-well round-bottom plate (greiner) and stimulated with phytohaemagglutinin (PHA-L, Boehringer, Mannheim, Germany) for one hour at 37°C, after which twofold serial dilutions were prepared in eight-fold

in RPMI supplemented with 10% FBS. Subsequently, a standard amount (5×10^3 per well) of a human Epstein-Barr virus (EBV)-transformed B lymphoblastic cell line (B-LCL) previously established from a healthy volunteer (GR) was added to each well. In the case of positive virus isolations, cytopathic changes (CPE) were usually observed 2 to 4 days after culture at 37°C . The level of viraemia (i.e. the number of MV-infected cells per 10^6 PBMC) was determined by calculating the number of PBMC per well resulting in 50% of the cultures showing CPE (142), and is presented as the number of MV-infected cells per 10^6 PBMC.

In the case of a positive MV isolation, as determined by the observation of typical MV-related CPE, the supernatant of one to three wells showing CPE at the upper range of the serial dilutions was harvested, and co-cultivated with approximately 5×10^6 B-LCL GR in a 25 cm^2 culture flask. When these cells showed CPE two to three days later, cell-free supernatant was harvested and aliquots were frozen at -70°C .

RT-PCR.

The presence of MV genomic RNA in throat swab samples was determined by RT-PCR by using a forward primer in the N gene and a reverse primer in the region between the N gene and the P-C-V gene. Briefly, RNA was isolated from $200 \mu\text{l}$ of throat swab material by using the High Pure Viral RNA kit (Roche Diagnostics, Almere, The Netherlands), and was analysed by RT-PCR by using random hexanucleotides for first strand synthesis. Primers used for amplification were as follows: forward 5'-TTAGGGCAAGAGATGGTAAGG-3' (MV-N1, position 1090-1110) (41), and reverse 5'-TTATAACAATGATGGAGGG-3' (MV-N2, position 1633-1615). PCR products were separated on 2% agarose gel, and blotted onto Hybond N⁺ membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Hybridisation was performed using a ^{32}P -labelled oligo probe: 5'-GCCATGGCAGGAATCTCGGAA-3' (MV-prN2, position 1498-1518).

Results

Laboratory diagnosis by serology.

Blood samples were collected from 38 clinically diagnosed measles patients (age range 5 months to 14 years) within six days after onset of the rash (table 1). Immunofluorescence using MV F and MV H transfected cell lines demonstrated the absence of MV glycoprotein-specific IgM in the plasma of ten of these patients (figure 1A, open symbols). In one patient, SM32, the result of the assay was indeterminate based on previously established cut-offs (signal <30 AFU), while the other 27 patients all demonstrated the presence of both MV F- and MV H-specific plasma IgM (figure 1A, filled symbols). The MV F- and MV H-specific immunofluorescence signals showed a good correlation (linear regression analysis, $r^2=0.82$). We subsequently compared the mean MV glycoprotein-specific IgM response with the MV N-specific IgM response (figure 1B).

Table 1. Summary of patient baseline data and results of laboratory assays.

ID ^a	Gender ^b	Age (mo)	Rash (days after onset) ^b	IgM ^c (plasma)	RT-PCR ^d (throat)	MV-isolation (PBMC)	No. of infected cells ^e (per 10 ⁶ PBMC)
SM4	F	16	1	+	ND ^f	-	<3
SM5	M	12	1	+	+	ND	ND
SM8	F	42	3	+	+	ND	ND
SM11	M	8	2	+	ND	+	6
SM14	F	24	2	+	ND	-	<3
SM16	M	18	2	+	ND	-	<3
SM23	F	48	5	+	+	+	6
SM24	F	14	5	+	+	+	10
SM25	F	48	3	+	+	+	925
SM26	F	20	3	+	+	+	50
SM27	M	23	3	+	+	+	3
SM28	F	15	2	+	+	+	350
SM29	M	168	4	+	ND	+	150
SM30	M	48	3	+	+	ND	ND
SM31	F	120	4	+	+	+	3
SM32	M	14	2	+/-	+	+	9700
SM33	F	96	2	+	+	ND	ND
SM34	M	132	4	+	+	+	80
SM37	M	7	4	+	+	+	925
SM38	M	11	4	+	ND	-	<3
SM39	M	10	6	+	+	-	<3
SM40	F	24	2	+	+	+	200
SM41	M	36	2	+	+	ND	ND
SM42	F	12	4	+	+	+	525
SM43	F	5	3	+	+	-	<3
SM44	F	8	3	+	ND	+	100
SM45	F	10	3	+	ND	+	300
SM46	F	23	3	+	+	+	3
SM1	F	48	3	-	-	ND	ND
SM2	F	48	2	-	-	ND	ND
SM3	M	54	3	-	-	ND	ND
SM9	M	15	1	-	-	-	<3
SM17	M	42	3	+/-	-	-	<3
SM18	M	18	4	-	-	-	<3
SM19	M	36	2	-	-	-	<3
SM20	M	11	3	-	-	-	<3
SM21	M	20	4	-	-	-	<3
SM22	M	33	4	-	-	ND	ND

a: Laboratory-confirmed MV cases (see results section) are listed in the upper part of the table, serologically confirmed MV-negative cases in the lower part; b: F: female, M: male; c: as shown in figure 1; d: positive if fragment of correct length which hybridized with the specific probe was amplified; e: determined by infectious center test as described in materials and methods; f: ND = not done.

Of the 10 patients who were IgM negative in the glycoprotein-specific assays, one patient (SM17) gave a low positive result in the N-specific IgM capture ELISA, while the other nine were negative in this assay. The 27 patients who were glycoprotein-specific IgM positive and the indeterminate patient (SM32) were also N-specific IgM positive, although SM32 showed an N-specific signal which was intermediate between SM17 and the 27 high positives.

On the basis of these MV IgM assays, 36 out of 38 cases could be diagnosed as being either acute MV infections (n=27) or non-measles rash diseases (n=9). In order to serologically diagnose the two low positive IgM patients, additional IgG and VN assays were carried out. As shown in figure 1C, SM17 had high serum levels of glycoprotein-specific IgG and VN antibodies while SM32 was glycoprotein-specific IgG and VN negative, suggesting that the first was a non-measles rash disease patient while the second was sampled in an early stage of a MV infection. Of the other nine MV IgM negative cases, two (SM9, SM19) contained high levels of MV-specific IgG, three contained low levels of MV-specific IgG and four were completely IgG negative (figure 1C).

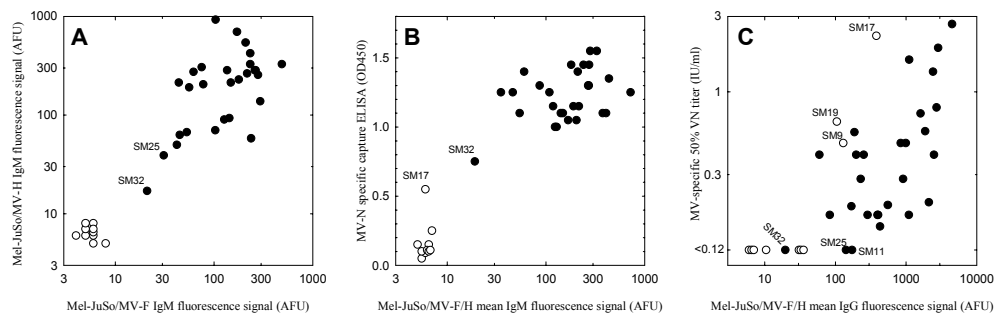


Figure 1

Relationships between MV F- and MV H-specific IgM levels (A), MV glycoprotein-specific IgM and MV-N specific IgM levels (B), and MV glycoprotein-specific IgG and VN antibody levels (C). Laboratory-confirmed MV cases (see results section) are shown as black symbols, while non-measles rash disease cases are shown as open symbols.

Laboratory diagnosis by RT-PCR and virus isolation.

In addition to serology, MV-specific RT-PCR on throat swab material and MV isolation from PBMC were carried out with the clinical specimens available. As shown in table 1, 20 out of 20 serologically confirmed MV cases tested (including SM32) were RT-PCR positive, while all 10 serologically confirmed MV negative cases (including SM17) were RT-PCR negative. MV could be isolated from PBMC of 17 out of 23 serologically confirmed MV cases (including SM32), and from none out of six serologically confirmed MV-negative cases (including SM17, see table 1). Levels of infected cells were as high as about 10,000 infected cells per 10^6

PBMC (see table 1), with the highest level in patient SM32. MV isolation from swab material was far less successful than from PBMC: MV could be isolated from only three of the 20 serologically confirmed cases tested.

Discussion

In the present paper, we have serologically and virologically analysed 38 clinically diagnosed measles cases in Khartoum, Sudan. Measurement of MV-specific IgM, IgG and VN antibodies, as well as RT-PCR on throat swab samples and MV isolation from PBMC, demonstrated that in 28 of the 38 cases (74%) the disease was indeed caused by an acute MV infection, but in 10 patients (26%) the disease had another cause.

Misdiagnosis of measles on clinical grounds has often been reported (106;137;143). The diagnosis of measles can be difficult, even for experienced practitioners, especially in individuals with a pigmented skin. A number of different infectious agents, including Parvovirus B19, human herpesvirus type 6, Dengue virus, Epstein-Barr virus, *Mycoplasma pneumoniae* and *Rickettsia conorii*, are known to cause symptoms that can easily be confused with measles, and are also known to be endemic in this part of Africa (137). In the framework of the envisaged MV eradication program, rapid and reliable assays to diagnose these infections will be crucial.

Measurement of MV-specific IgM antibodies proved to be sufficient to diagnose 36 of the 38 clinically diagnosed measles cases. We measured the IgM antibody response to the three major immunogenic MV proteins: the transmembrane glycoproteins F and H and the internal protein N. For routine diagnostic purposes, a capture ELISA such as the one presented here based on peroxidase labelled N would be the first choice. In case of low positive specific IgM signals, there are three theoretical possibilities: the patient is in an early stage of a MV infection (with a nascent IgM response), the patient had a MV infection some months ago and is now suffering from a non-measles rash disease, or the patient was previously vaccinated and is undergoing a secondary immune response associated with a transient low IgM response. The latter category should be discriminated from the first two on basis of the clinical signs: if the patient has a normal measles rash accompanied with conjunctivitis, the patient is most probably undergoing a primary immune response. Since this was the case for SM17 and SM32, we tried to discriminate between the first two possibilities by measuring MV-specific IgG and VN antibody levels. During the early phase of a MV-specific immune response, specific IgM antibodies appear before or at the same time as specific IgG antibodies. When the levels of IgM antibodies start to decrease a few weeks later, the levels of specific IgG and VN antibodies have reached a plateau value (1;139). SM17 could be identified as a case of non-measles rash disease, since the low MV-specific IgM antibody levels were accompanied by high IgG and VN antibody levels. In contrast, SM32 proved to be a laboratory-confirmed MV infection, since the low levels of

MV-specific IgM antibodies were accompanied by undetectable IgG and VN levels. This serological diagnosis was confirmed by RT-PCR and MV isolation: SM17 was negative while SM32 was positive in both assays. Furthermore, assessment of the level of viraemia further confirmed that patient SM32 was in an early stage of infection, as this patient had the highest level of infected cells.

To our knowledge, the use of an RT-PCR for diagnosis of normal measles cases has not been described previously. In our study, an RT-PCR using throat swab materials proved to completely correlate with the serological diagnosis of MV. The RT-PCR described may be used in serologically doubtful cases. However, it will especially be of value in cases of suspected MV infections in immunocompromised individuals in which clinical and serological diagnosis is often impossible. MV isolation from PBMC proved less reliable as a diagnostic tool, since MV could not be isolated from six out of 23 (26%) serologically confirmed MV cases. We hypothesise that in most of these cases this was due to practical problems related to sub-optimal sampling, handling and/or freeze-thawing procedures. Isolation and storage of PBMC in an African setting is often difficult to organise, as the required freezing facilities (-135°C or lower) are rarely available. The success rate of virus isolation from PBMC was significantly higher than the success rate of virus isolation from swab material: in 85% of the serologically confirmed MV cases no MV could be isolated from swabs. This included some of the cases in which the presence of MV genomic RNA was demonstrated by RT-PCR. Apparently, the capacity of viable PBMC to produce new virus particles, especially following PHA stimulation, strongly favours this method of MV isolation. An alternative and generally easy to obtain clinical source for the isolation of MV would be urine. However, this was not evaluated in the present study.

In conclusion, our study has shown that a substantial percentage (26%) of the measles cases identified on the basis of the symptoms specified in the WHO clinical case definition (105) were misdiagnosed. Serological methods were sufficient for a laboratory diagnosis of MV infection: measurement of MV-specific IgM alone could diagnose 95% of the patients, while the remainder could be diagnosed with an additional IgG or VN assay. RT-PCR on throat swab material proved to be an equally valid diagnostic assay, which is, however, less practicable in a routine setting. In combination with virus isolation it does, however, provide the tools for phylogenetic analyses, allowing molecular epidemiological studies which will be of crucial importance in the end stages of the envisaged MV eradication program.

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Section 2.2

Prevention of measles in Sudan: a prospective study on vaccination, diagnosis and epidemiology

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Abstract

Despite the availability of safe and effective live attenuated vaccines, measles continues to be endemic in many developing countries. Control and elimination of measles will be especially difficult in East Africa, because of its limited infrastructure and political instability. We have studied diagnostic and epidemiological aspects of measles in suburban Khartoum, Sudan. Prospective studies were carried out in a cohort of clinically diagnosed measles cases and in a cohort of new-borns, which were both followed up for two years. The studies intended to provide a rational basis for improvement of measles vaccination strategies, and strengthen measles research infrastructure in Khartoum.

Introduction

Measles continues to be a major childhood disease, resulting in an estimated 1 million fatal cases each year (1). Although measles vaccination has largely abrogated endemic transmission of measles virus (MV) in the industrialised world, it has been less successful in developing countries. This is thought to be the combined result of insufficient vaccination coverage and inherent disadvantages of the live attenuated vaccine such as the need for cold chain maintenance and interference by maternal antibodies (144;145). The World Health Organisation (WHO) aims at global control and eradication of measles during the coming decade (38). It may be anticipated that achievement of this goal will be most difficult in East Africa, due to its limited infrastructure and political instability.

Study design

We have studied diagnostic and epidemiological aspects of measles in suburban Khartoum, Sudan. Prospective studies were carried out in a cohort of clinically diagnosed measles cases and in a cohort of new-borns, which were both followed up for 2 years. For the first cohort, clinical materials were collected from 197 infants who complied with the WHO clinical case definition of measles: "any person with a generalised maculopapular rash (i.e. non-vesicular), and a history of fever of 38°C or more, and at least one of the following: cough, coryza (i.e. runny nose), or conjunctivitis (i.e. red eyes)" (105). Samples were collected within a week after onset of rash, following informed consent of the parents.

Most patients were sampled through a network of active measles case finding, which was set up in the residential area Haj Yousif. This area has an estimated number of 500,000 inhabitants, mainly displaced people from the south and west of Sudan. Health care is provided through health centres most often run by non-governmental organizations, and staffed mainly by nurses, midwives and medical assistants. Measles was found to be endemic in this area. A number of additional patients were sampled in paediatric hospitals in Khartoum. Vaccination status, age, gender, clinical scoring and socio-economical information was recorded for each patient using questionnaires.

The clinical materials collected included a throat swab, a whole blood sample spotted on filter paper, and a heparinised venous blood sample. From this last sample peripheral blood mononuclear cells (PBMC) were isolated and stored in liquid nitrogen, and plasma was stored at -70°C. For the second cohort, filter paper blood samples were collected from a cohort of new-borns in a rural area (146) at birth (cord blood) and at the age of 6, 12 and 24 months (by heel prick). Vaccination status and family background information was recorded for each subject.

Diagnostic studies: serology versus virological parameters.

In a first study, the diagnostic value of detection of MV-specific serum IgM, RT-PCR analysis on throat swabs, and MV isolation from PBMC was compared using clinical specimens of 38 clinically diagnosed measles cases (147). We found that measurement of serum IgM was sufficient when high levels were present. However, in cases with low serum IgM levels measurement of IgG or virus neutralising (VN) antibodies was necessary to discriminate between patients with an acute MV infection sampled during an early stage of the disease and patients with a history of previous MV infection. The serological laboratory diagnosis was confirmed by a MV-specific RT-PCR on throat swab materials. Using a set of MV N-specific primers an amplicon of the correct size which hybridized with a specific probe was obtained for all confirmed measles cases tested, but for none of the non-measles rash disease cases. MV isolation proved to be less sensitive as diagnostic method. We therefore defined the laboratory diagnosis of measles in our cohort by:

- high MV-specific serum IgM levels; OR:
- intermediate to low MV-specific serum IgM levels accompanied with low serum IgG or VN antibody levels and positive RT-PCR signals on throat swab samples.

Using this definition we could diagnose 192 of the suspected measles patients, of whom 145 (76%) proved to be patients with an acute MV infection, while in 45 cases (24%) the clinical symptoms proved to have other causes (see figure 1). In some of the non-measles patients IgM antibodies specific for rubella, human herpesvirus type 6, Epstein-Barr virus, dengue virus, sandfly fever or Rickettsia species could be detected.

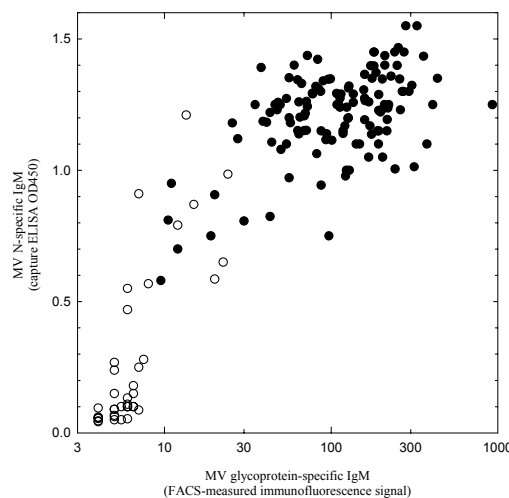


Figure 1

MV-specific serum IgM levels of 168 infants in cohort B, as measured by IgM capture ELISA using recombinant MV-N directly coupled to peroxidase (vertical axis) or an immunofluorescence method using human melanoma cells transfected with the MV F and MV H proteins (means of MV F and MV H signals on horizontal axis). The symbol colour represents the laboratory measles diagnosis based on serology (MV-specific IgM, IgG and VN antibodies) and RT-PCR: black symbols represent acute MV-infected patients, white symbols represent non-measles rash disease patients.

Diagnostic studies: whole blood spotted on filter paper

In many tropical countries collection and storage of serum and/or throat swabs for diagnostic purposes is logistically complicated. We were able to show that blood samples spotted on filter paper are suitable for the laboratory diagnosis of measles using a combination of RT-PCR analysis and IgM detection (111). First it was shown that *in vitro* MV-infected cells diluted in human blood and spotted on filter paper can be detected by RT-PCR. Low numbers of infected cells remained detectable after 25 weeks storage of the filter paper at room temperature, 4 weeks at 37°C, or 2 weeks at 45°C. Subsequently, this RT-PCR was applied to filter paper blood samples collected from 117 clinically diagnosed measles patients in Sudan in 1997 and 1998. Laboratory diagnosis carried out as described above had confirmed 90 cases as acute MV infections, while 27 proved to be non-measles rash disease cases. Positive RT-PCR signals were detected in filter paper blood samples of 43 of the 90 (48%) confirmed cases, but in none of the 27 non-measles cases. In addition, MV-specific IgM levels measured in reconstituted filter paper samples correlated well with those measured in serum samples. Measles diagnosis based on the combination of filter paper RT-PCR and IgM detection had a sensitivity of 99% and specificity of 96%. Another advantage of this diagnostic approach is that sequencing of the RT-PCR products allows phylogenetic analysis of the measles virus strain involved.

Measles in khartoum: vaccination coverage and epidemiology

Measles continues to be the most common immunizable disease in febrile children who present at the Emergency Hospitals in Khartoum. The most recent measles vaccination coverage data reported for Sudan by the Expanded Programme for Immunization (EPI) was a national coverage of 63% (148). Vaccination status of the children enrolled in our clinical measles cohort study in Khartoum, as evidenced by inspection of a vaccination card, demonstrated high coverage of DTP, polio and BCG vaccines, but rather low measles vaccine coverage (table 1). The vaccination coverage in the non-measles rash disease patients, which may be expected to represent a random sample of the population, was found to be 70%. Measles vaccination coverage was expected to be significantly lower in the group of confirmed measles cases, since measles vaccination should of course protect against measles. It was therefore worrying that still 59% of this group proved to have been vaccinated against measles.

The median age of the laboratory-confirmed measles cases in the cohort was 24 months (range 5 - 168). Of 91 confirmed measles cases that were followed up for more than three months, 34 (37%) recovered without complications, 48 (53%) developed complications and 9 (10%) died during the first month after measles. Frequently observed complications included gastroenteritis and pneumonia.

Table 1: Vaccination status in clinically diagnosed measles patients in suburban Khartoum, based on inspection of a vaccination card.

	Confirmed measles (n=65)	non-measles rash disease (n=34)
DTP1	65 (100%)	33 (97%)
DTP2	63 (97%)	32 (94%)
DTP3	62 (95%)	30 (88%)
Polio1	64 (99%)	33 (97%)
Polio2	63 (97%)	33 (97%)
Polio3	62 (95%)	31 (91%)
BCG	63 (97%)	32 (94%)
Measles	38 (59%)	23 (70%)

Laboratory diagnosis confirmed the clinical diagnosis in 65 cases as acute measles virus infections, but demonstrated that in 34 patients the clinical symptoms had other causes (non-measles rash disease patients).

Newborn cohort study: sero-epidemiology of measles

In order to assess levels of both maternally derived and acquired (either through vaccination or infection) MV-specific antibodies, blood samples were collected on filter paper from new-borns in a rural area outside Khartoum (146) at birth and at the age of 6, 12 and 24 months. Initially, 196 cord blood samples were collected. 182 infants could be sampled at the age of six months, 172 infants at the age of 12 months and 168 at the age of 24 months. The most important reason for dropping out of the cohort was death of the child (n=20), indicating that infant mortality in this area is high (10%). Other reasons for dropping out included refusal (n=11), and moving out of the area (n=9). Filter paper blood samples were reconstituted and first analysed for total IgG1: samples with levels outside the normal range of infants of the respective age were considered invalid. MV-specific IgG serology was carried out by ELISA and FACS-measured immunofluorescence. At the age of six months 60-80% of the infants had less than 0.2 I.U./ml MV-specific IgG. At the age of twelve months 35-50% of the cohort still had MV-specific IgG antibody concentrations below this protective level, while at 24 months this percentage had dropped to 10-20% (161).

Discussion and conclusion

In conclusion, the study carried out in Khartoum provided valuable data. The diagnostic studies in the clinical measles cohort clearly showed that implementation of proper laboratory diagnostics will be essential in the framework of the anticipated global eradication campaign for measles. It was

also shown that finger prick blood samples collected on filter paper are suitable clinical specimens for this purpose. Measles vaccination coverage was found to be relatively low. However, it was worrying that many confirmed measles cases had a history of measles vaccination, which raises doubts about vaccination efficacy. The maternal antibody measurements, as carried out in the cohort of newborns, suggested that the age of nine months, at which EPI in Khartoum presently vaccinates for measles, is properly chosen to avoid interference by maternally transferred MV-specific antibodies. We are presently preparing a measles vaccination efficacy study in Khartoum, which will be carried out in 2001. It is expected that vaccination coverage and -efficacy will both have to be improved before measles can be controlled in and eventually eliminated from suburban Khartoum.

Acknowledgements

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Section 2.3

Genetic characterisation of wild-type measles viruses circulating in suburban Khartoum, 1997-2000

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Abstract

Measles remains endemic in many East African countries, where it is often associated with high morbidity and mortality. We have collected clinical specimens from Sudanese measles patients between July 1997 and July 2000. Sequencing of the 3' 456 nucleotides of the nucleoprotein gene from 33 measles virus (MV) isolates and 8 RNA samples extracted from clinical specimens demonstrated the presence of a single endemic MV strain with little sequence variation over time (overall nucleotide divergence of 0 to 1.3%). This was confirmed by sequencing of the complete H gene of two isolates from 1997 and two from 2000, in which the overall divergence ranged between 0 to 0.5%. Comparison with MV reference strains demonstrated that the viruses belonged to clade B, genotype B3, and were most closely related to a set of viruses recently isolated in Nigeria. Our study demonstrates a remarkable genetic stability of an endemically circulating MV strain.

Measles virus (MV) is considered a monotypic and antigenically stable virus, which has long masked the presence of nucleotide variation (149). Sequence analysis of vaccine and wild-type MV isolates revealed the presence of nucleotide variation over the entire genome, the most variable genes being the nucleoprotein (N) and the haemagglutinin (H) genes with about 7% nucleotide variability between the most distantly related sequences (40;41;141). The 456 nucleotides encoding the carboxyl-terminus of the nucleoprotein are the most variable region of the MV genome, as the nucleotide variability in this region can exceed 12% between different genotypes (40;41). This variability was found to be unaffected by propagating the virus in tissue culture, making it a suitable tool for the molecular characterisation of wild-type MV isolates (25). This region has now been assigned by the WHO as the minimum sequence required to genotype a wild-type MV isolate (141). Complete H gene sequences should be obtained from viruses that may represent new genotypes, or from representative viruses of a larger set of viruses (40). The generation of a database of sequences of different wild-type MV isolates allowed phylogenetic studies, resulting in the identification of 8 clades and a still increasing number of genotypes (40;141). Some of these are considered inactive, but the majority is still actively circulating, and to some extent geographically restricted (27;29). Genetic characterisation of wild-type isolates, when combined with the standard epidemiological methods, may enable the identification of source and transmission pathways of wild-type viruses and the differentiation between indigenous and imported viruses (25;37;39;141). Characterisation of globally circulating MV strains, and particularly from countries where measles remains endemic, will therefore contribute to these studies.

One of the regions of the world where measles remains endemic is East Africa. However, no MV isolates from this region have been sequenced, except for the NY 94 and NY 96 isolates which were isolated in New York and epidemiologically linked to Kenya (37;150). The sequence database of the available African MV isolates shows the complexity of the distribution of virus genotypes. In southern Africa viruses of clades A and D were found to predominate (27;141;150). Clade B viruses were shown to predominate in western and central Africa. This clade comprises three genotypes, B1, B2 and B3 (33;37;40;150;151).

We studied the phylogenetic characteristics of 41 wild-type MV sequences obtained in suburban Khartoum between July 1997 and July 2000. The majority of the patient samples (n=18) had been collected in 1997, and the rest in 1998 (n=10), 1999 (n= 8) and 2000 (n=6) (see table 1). Heparinised blood samples, throat swabs and blood samples spotted on filter paper were collected from clinically diagnosed measles patients in suburban Khartoum in the framework of a prospective measles study in suburban Khartoum, as previously described (147;152). Samples were collected within seven days after onset of rash, upon having obtained informed consent of the parents or guardians.

Table 1: Summary of sequences determined

Patient ID	Sequence available (WHO-nomenclature)	GenBank accession no.		Material ¹	Year
		N gene	H gene		
SM11	MVi/Khartoum.SUD/23.97	AF149065		Vi	1997
SM23	MVi/Khartoum.SUD/26.97	AF311785		Vi	1997
SM24	MVi/Khartoum.SUD/28.97	AF311786		Vi	1997
SM25	MVi/Khartoum.SUD/30.97/1	AF311787		Vi	1997
SM26	MVi/Khartoum.SUD/30.97/2	AF149064		Vi	1997
SM27	MVi/Khartoum.SUD/31.97/1	AF149066		Vi	1997
SM29	MVi/Khartoum.SUD/33.97	AF311788	AF453430	Vi	1997
SM31	MVi/Khartoum.SUD/34.97/1	AF149061		Vi	1997
SM32	MVi/Khartoum.SUD/34.97/2	AF149063		Vi	1997
SM34	MVi/Khartoum.SUD/34.97/3	AF311789		Vi	1997
SM37	MVi/Khartoum.SUD/35.97	AF149062		Vi	1997
SM40	MVi/Khartoum.SUD/36.97/1	AF311790		Vi	1997
SM42	MVi/Khartoum.SUD/36.97/2	AF311791	AF453431	Vi	1997
SM44	MVi/Khartoum.SUD/37.97	AF311792		Vi	1997
SM45	MVi/Khartoum.SUD/38.97/1	AF311793		Vi	1997
SM46	MVi/Khartoum.SUD/38.97/2	AF311794		Vi	1997
SM54*	MVs/Khartoum.SUD/40.97	AF311795		Fp	1997
SM64*	MVs/Khartoum.SUD/44.97	AF311796		Ts	1997
SM111*	MVs/Khartoum.SUD/6.98	AF311797		Fp	1998
SM120	MVs/Khartoum.SUD/21.98	AF311798		Ts	1998
SM125	MVi/Khartoum.SUD/22.98	AF311799		Vi	1998
SM133	MVi/Khartoum.SUD/26.98	AF311800		Vi	1998
SM135	MVi/Khartoum.SUD/27.98/1	AF311801		Vi	1998
SM136	MVi/Khartoum.SUD/27.98/2	AF311802		Vi	1998
SM149*	MVs/Khartoum.SUD/33.98	AF311803		Fp	1998
SM154*	MVs/Khartoum.SUD/34.98	AF311804		fp and ts	1998
SM161*	MVs/Khartoum.SUD/38.98	AF311805		Fp	1998
SM170	MVi/Khartoum.SUD/1.99/1	AF311806		Vi	1999
SM172	MVi/Khartoum.SUD/5.99	AF311807		Vi	1999
SM176	MVi/Khartoum.SUD/8.99	AF311808		Vi	1999
SM177	MVi/Khartoum.SUD/11.99	AF311809		Vi	1999
SM180	MVi/Khartoum.SUD/13.99	AF311810		Vi	1999
SM184	MVi/Khartoum.SUD/22.99	AF311811		Vi	1999
SM192*	MVs/Khartoum.SUD/23.99	AF311812		Ts	1999
SM194	MVi/Khartoum.SUD/24.99	AF311813		Vi	1999
SM00-1	MVi/Khartoum.SUD/28.00/1	AF311814		Vi	2000
SM00-2	MVi/Khartoum.SUD/28.00/2	AF311815		Vi	2000
SM00-3	MVi/Khartoum.SUD/28.00/3	AF311816		Vi	2000
SM00-4	MVi/Khartoum.SUD/28.00/4	AF311817		Vi	2000
SM00-5	MVi/Khartoum.SUD/28.00/5	AF311818	AF453432	Vi	2000
SM00-6	MVi/Khartoum.SUD/28.00/6	AF311819	AF453433	Vi	2000

¹: vi = virus isolate, fp = blood sample spotted on filter paper, ts = throat swab

*: Samples cloned into PCR 2.1 vector system prior to sequencing.

MV isolates (n=33) were obtained by co-cultivation of phytohaemagglutinin-activated peripheral blood mononuclear cells of the

Sudanese patients with a human Epstein-Barr virus-transformed B-lymphoblastic cell line of a healthy donor as previously described (147). MV isolates were frozen after two or maximally three passages in the same cell line.

Total viral RNA was isolated from MV isolates or throat swab samples using the high pure viral RNA isolation kit (Roche Diagnostics, Almere, The Netherlands). RNA from filter paper blood samples was extracted by a modification of the High pure viral nucleic acid isolation kit (Roche), as previously described (111). The first strand cDNA synthesis was primed by random hexanucleotides (Promega, Leiden, The Netherlands) as previously described (147). Primers used for amplification of the 456 nucleotides coding for the carboxyl-terminus of the N protein were 5'-TTAGGGCAAGAGATGGTAAGG-3' (MV-N1, positions 1090 to 1110) and 5'-TTATAACAATGATGGAGGG-3' (MV-N2, positions 1633 to 1615). The PCR products from the virus isolates were then directly sequenced using the Dye Terminator cycle sequencing kit (PerkinElmer, Oosterhout, The Netherlands), using the same primer set and following the manufacturer's protocol. Sequencing was performed on a 373A automated sequencer (Applied Biosystems, Nieuwekerk aan de IJssel, The Netherlands). The RT-PCR products obtained from throat swabs or blood spotted on filter paper, were in some cases not sufficient for direct sequencing. These samples, indicated in table 1 by an asterisk, were cloned into the PCR 2.1 vector system (Invitrogen, Breda, The Netherlands) prior to sequencing. Mini-preparations of plasmid DNA were isolated by Alkali method (153) and sequenced with the M13 forward and reverse primers. The complete coding region of the H gene was amplified in three overlapping PCR fragments using a slight modification of the primers used by Truong et al. (150) and Chibo et al. (154). The primer combinations were as follows: 5'-TTAGGGTGCAAGATCATCCACA-3' (MV-H1, positions 7249-7270) and 5'-GACCATTACTGACTGGTTGC-3' (MV-H6, positions 8120-8100); 5'-CACCTCAGAGATTCAGTACC-3' (MV-H3, positions 7590-7610) and 5'-GAGCCGTGTGTGATCAATGG-3' (MV-H8, positions 8586-8567); 5'-GTACCGAGTGTGTTGAAGTAGG-3' (MV-H5, positions 8023-8043) and 5'-GGGTGACATCATGTGATTGG-3' (MV-H2, positions 9157-9138). The PCR products amplified from the virus isolates were then directly sequenced as described for the N gene using the above primers and the following additional primers: 5'-GATCTCTGAAGTCGTACTCC-3' (MV-H4, positions 7676-7657) and 5'-GATCTGAGTCTGACAGTTGAGC-3' (MV-H7, positions 8516-8537). The sequences determined are available from the GenBank database (accession numbers are shown in table 1).

The sequences obtained were aligned with the reference sequences representing the different genotypes described by the WHO (40) using the Clustal-W function of the BioEdit program (T. Hall, Department of Microbiology, North Carolina State University). Distance matrices were calculated using Maximum Likelihood function of the PHYLIP-package (155), the phylogenetic relation was then inferred using the neighbour joining

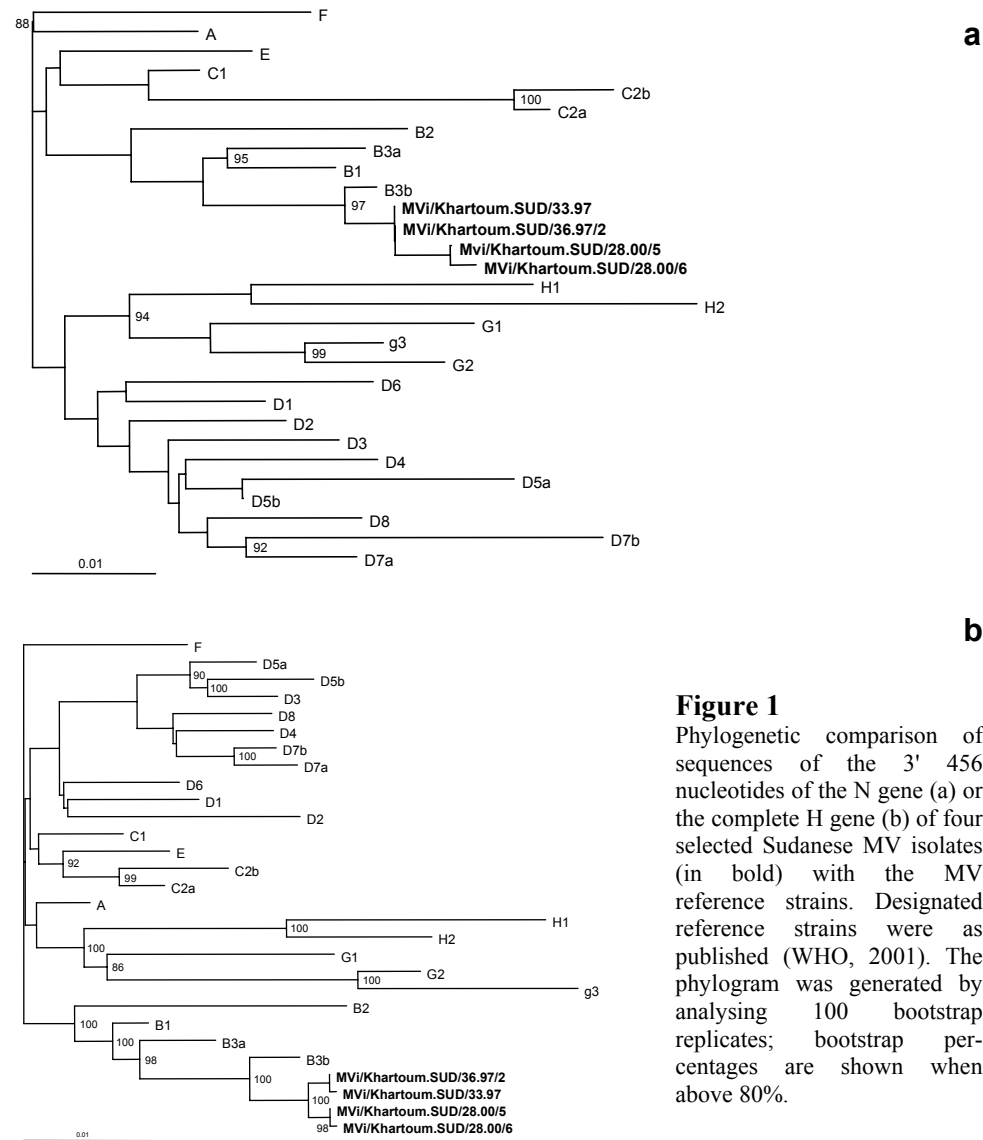
method of the PHYLIP-package (155) in combination with bootstrap analysis (100 replications).

The 41 partial N gene sequences studied were closely related, as over the 3 year period a divergence of only 0 to 1.3% was found in this hypervariable region of the MV genome. Half of the point mutations were silent. One nucleotide mutation ¹³⁷⁰A→G [numbering according to Mori *et al.* (156)] was found in five out of nine 1998 sequences, seven out of eight 1999 sequences and five out of six 2000 sequences, but in none of the sequences obtained in 1997. This mutation was not found in any MV strain described in GenBank (blast search). Since the data set was found to be homogeneous, we randomly selected two sequences from 1997 and two from 2000 of which the nucleotide sequence of the H gene was determined. Analysis of these sequences confirmed the observed high homology, as the 1997 isolates differed from the 2000 isolates by only 4 nucleotide mutations (0.2%), all of which were silent. As shown in figure 1, the Sudanese sequences clustered with the reference strain of genotype B3.

For a more comprehensive analysis of the relationship between the Sudanese viruses and other clade B virus strains, a dendrogram was made of a larger group of the Sudanese N-sequences and a large number of previously described clade B N- sequences (figure 2). From this analysis, it became apparent that the Sudanese sequences were most closely related to a group of sequences which was recently described in Nigeria, and assigned to genotype B3, cluster 1 (151). Although our study area was rather restricted, the highly conserved sequences among the isolates over a three-year period strongly suggest that our isolates represent an indigenous and probably dominant strain circulating in greater Khartoum and probably elsewhere in Sudan. The continuous arrival of new refugees from the south and west of Sudan in the main study area suggests that at least in these areas the same strain predominates.

The observed high conservation over the 3-year period suggests a relatively high genetic stability of wild-type MV isolates. These results are in agreement with previously observed low mutation rates in H gene sequences of MV isolates collected in Madrid from 1993 to 1996 (32). Similarly, during the last three years of endemic MV circulation in the USA, H and N gene sequences differed by less than 0.5% (37). However, in a previous study in Nigeria and Ghana a much higher variability was found between different virus isolates analysed (150;151). This difference may be explained by two factors. Firstly, travel in and out of Sudan is more restricted than travel in and out of Nigeria, which reduces the chances of importation of new MV strains into Sudan. Secondly, the level of sequence variation in a situation of endemic virus circulation will be directly related to the absolute number of simultaneously infected patients (i.e. chains of transmission), since each infection may potentially result in the occurrence of new mutations. The numbers of MV-infected patients in Nigeria may indeed be higher than those in Sudan, since reported MV vaccination coverage in Nigeria is much lower than that in Sudan, 26% and 63% respectively (111;148). Other phylogenetic

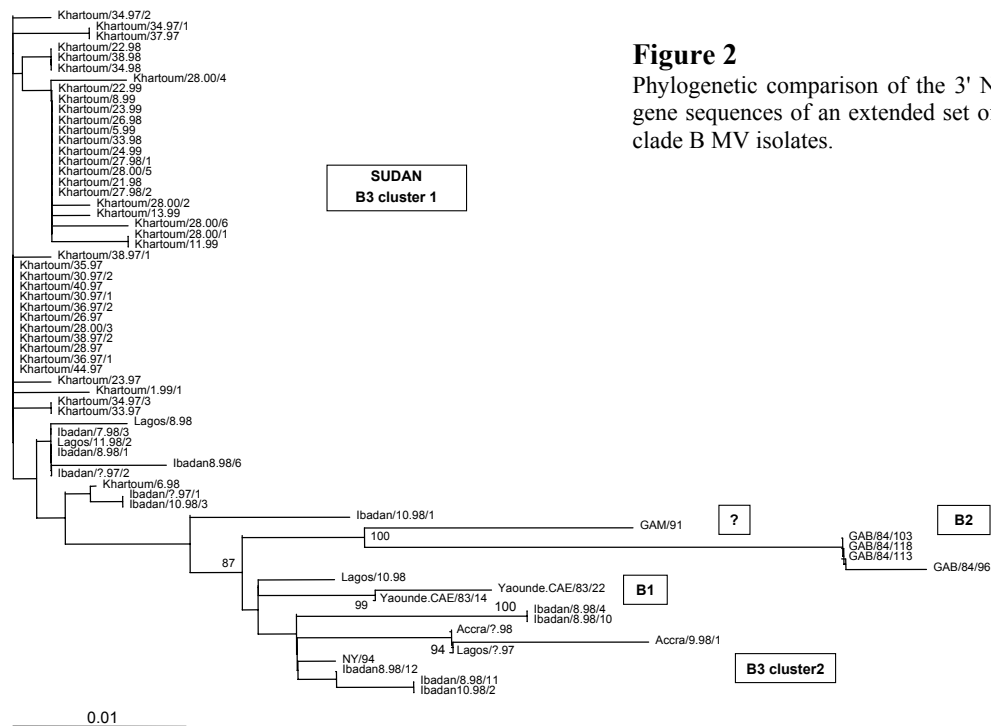
studies in areas with high levels of measles transmission (e.g. China, Vietnam) also found a relatively high variability within the locally circulating genotype (26;157).



Comparison of the Sudanese isolates with the reference sequences of the known wild-type MV clades and genotypes allowed the allocation of the isolates in clade B, to which isolates from Central and Western Africa had

been placed (141). The Sudanese isolates were clearly distinct from the reference sequences of the known genotypes of clade B. Analysis of all the isolates of clade B of which N sequences were available, allowed the allocation of the Sudanese isolates with those from Nigeria within the genotype B3, cluster 1.

Until recently no standard criteria for the definition of clades and genotypes were available. However, two approaches were proposed by Kreis *et al.* (27) and Hanses *et al.* (151) based on the percentage divergence, and common characteristic mutations between the isolates, respectively. The second approach provides more information about the common evolutionary background of viruses, but it requires the presence of larger numbers of isolates (151). The WHO now proposes a minimum nucleotide divergence of 2.5% for the partial N sequence and 2.0% for the complete H sequence from the most closely related strain as standard criteria for defining new genotypes (40). According to the above criteria, our isolates together with the Nigerian B3, cluster 1 isolates would form a new genotype, with a divergence of 3.4% and a total of 5 set-specific mutations in the COOH-N and a divergence of 2.0% in the H gene. Sequences of the proposed genotype B3 cluster 2 and genotype B1 are closely associated. They form, however, a heterogeneous group with a nucleotide divergence of up to 2.7% in the N gene, which may comprise more than one group or subgroup. More sequences from different African countries will thus be required for a better definition of clade B genotypes.



Globally, different MV clades show a certain degree of geographical restriction. The movement of infected people within and between geographical areas will determine viral epidemiology. The close similarity between our MV isolates and those isolated in Nigeria may therefore be explained by the historical and continuing links between Sudan and Northern Nigeria.

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Section 2.4

Surveillance of measles in Sudan using filter paper blood samples

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submitted

Abstract

Dried blood spots collected on filter paper are considered potential clinical specimens for measles surveillance because of their ease of collection, storage and transport. We have evaluated the usefulness of these samples for surveillance of measles in a field setting. Blood spots were collected by finger prick from 316 clinically diagnosed measles patients in suburban Khartoum, mostly within a week after onset of the rash. Samples were collected between October 2000 and April 2003, and stored at 4°C. Measles virus (MV)-specific IgM antibodies were detected in 200 (63%) of the samples using an in-house IgM capture ELISA. For 201 samples reconstitution and IgM measurement was repeated one year after initial testing with essentially the same results, showing the stability of IgM in the filter paper under these conditions. In a limited number of samples (n=38) MV-specific IgM was also tested with a commercial indirect IgM ELISA. Although the results of the two assays correlated well, the in-house IgM capture ELISA proved slightly more sensitive. MV-specific RT-PCR amplicons were obtained from 16 of 57 (28%) samples tested. Sequencing of the 3' 456 nucleotides of the nucleoprotein gene showed the continued endemic circulation of the previously identified genotype B3 viruses in this region. Although problems related to limited sample quantities were encountered, the present study confirms the usefulness of dried blood spots for measles surveillance. The results also demonstrate that measles continues to be endemic in Sudan.

Introduction

Measles continues to be a major cause of vaccine-preventable deaths in developing countries. In 2002 the disease caused an estimated 777,000 deaths, 84% of which occurred in only 11 countries (Afghanistan, Burkina Faso, Democratic Republic of the Congo, Ethiopia, India, Indonesia, Niger, Nigeria, Pakistan, Somalia, Uganda) in Africa and Asia (3). As the control and elimination campaigns of measles progress, measles surveillance will become increasingly important. Surveillance requires both laboratory confirmation of clinically diagnosed measles cases (109) and genetic characterisation of selected wild-type viruses for molecular epidemiology (28). However, the countries with the highest mortality levels often have limited infrastructure, resulting in difficulties with collecting, storing and shipping the necessary clinical samples.

Analogous to the existing polio laboratory network, a Global Measles Laboratory Network (GMLN) has been developed (108). The GMLN has a tiered structure with regional laboratories, national laboratories, regional reference labs and global specialised labs, and implements standardised testing and reporting procedures. During the early stages of the accelerated measles control campaigns the main aim of the GMLN will be the monitoring of indigenous measles virus (MV) transmission and the tracing of measles outbreaks, as opposed to laboratory diagnosis of individual cases (108). Therefore, clinical samples will need to be collected from representative numbers of patients for laboratory diagnosis and/or identification of circulating MV strains by sequence analysis.

The detection of MV-specific IgM antibodies remains the gold standard for measles laboratory diagnosis (50). Whereas serum has always been the standard clinical specimen for this purpose, alternative samples including oral fluids and filter paper blood spots have been used successfully (108). Oral fluid has two major advantages: the sampling method is non-invasive, and avoids handling of blood with the associated safety and waste disposal problems. Filter paper blood spots have as advantage the ease of sample collection by heel- or finger prick, which requires less medical training and is usually more acceptable for both parents and infants than venepuncture. However, the most important advantage of filter paper blood samples is the ease of storage and transport: whereas oral fluids require a cold chain, filter paper blood spots can be stored at ambient temperature and shipped by regular mail (111).

Both oral fluid samples and filter paper blood spots can be used for IgM detection as well as MV strain identification by sequence analysis (110;111;113). Several studies compared filter paper samples and serum samples in the detection of MV-specific IgM and IgG antibodies using in house enzyme immunoassays. Recently, two studies showed good correlation between MV-specific IgM and IgG measured in filter paper samples and serum using a commercial EIA and have adapted the commercial indirect EIA to the use of filter paper blood specimens (114;115). We and others have

previously reported the adaptation of in-house RT-PCR assays to the amplification of MV genomic sequences from filter paper blood samples (111;112). In addition, we have used filter paper blood samples in the identification of the MV strain circulating in Sudan (158).

The most important current aspect of the alternative sampling methods is their evaluation under field conditions. The purpose of the present study was to evaluate the use of filter paper blood samples for the surveillance of measles in Sudan.

Materials & Methods

Patients and samples

As part of an ongoing measles surveillance program, whole blood samples spotted on filter paper (sample collection paper 903, Schleicher & Schuell, Dassel, Germany) were collected from clinically diagnosed measles patients in Khartoum, Sudan. All patients fulfilled the WHO clinical case definition for measles (105). This study was approved by the medical ethical committee of the Institute of Endemic Diseases, and samples were obtained with informed consent of the parents. Filter paper blood samples were obtained by heel- or finger-prick from 252 patients between October 2000 and January 2002 (set 1, shipped to Rotterdam in February 2002), and from 70 patients between February 2002 and April 2003 (set 2, shipped to Rotterdam in April 2003). All samples were stored dry at 4°C.

Study area

The patients included in this study were sampled through a measles surveillance network that was set up in a suburban residential area (Haj Yousif) in Khartoum as previously described (147;152). From the end of the previous study surveillance was continued using filter paper samples only.

Sample reconstitution for serology

Filter paper blood spot disks of 6 mm diameter were cut out using a paper puncher. For the in-house capture ELISAs one disk was eluted in 100µl PBS supplemented with 2% FBS overnight at 4°C. This reconstituted sample was considered as a 1:20 serum dilution. For the commercial measles IgM ELISA two 6mm disks were eluted in 220µl PBS supplemented with 0.5% tween and 5% dry milk powder overnight at 4°C as previously described (114).

In-house total IgM ELISA

Total (non virus-specific) IgM was measured (qualitatively) in a sandwich ELISA. Briefly, plates pre-coated with polyclonal anti-human IgM antibodies (Meddens Diagnostics, Vorden, The Netherlands) were incubated with the reconstituted samples diluted 1:5 in ELISA buffer (Meddens), i.e. at an estimated final dilution of 1:100. After washing, the plates were incubated with a peroxidase-labelled anti-human F(ab)₂ IgM conjugate (Biosource,

Nivelles, Belgium). Plates were coloured using TMB as a substrate, and extinctions were measured at 450nm. Samples with extinctions below 1 were excluded from further analysis, the majority of samples resulted in maximum extinctions (3.5).

In-house MV-specific IgM ELISA

MV nucleoprotein (N)-specific IgM was measured in a capture ELISA. Briefly, plates pre-coated with polyclonal anti-human IgM antibodies (Meddens) were incubated with the reconstituted samples diluted 1:5 in ELISA buffer (Meddens). After washing, the plate was incubated with a baculovirus-produced recombinant MV-N protein (kind gift of Dr. T.F. Wild, Lyon, France) labelled with peroxidase (Meddens). Plates were coloured using TMB as a substrate, and extinctions were measured at 450nm. An arbitrary cut-off for positivity was defined at an OD₄₅₀ of 0.3 (111). A pooled IgM-negative human serum sample was included as control on each plate, and consistently gave extinctions between 0.1. An IgM-positive human serum was also included as control on each plate, and consistently gave extinctions above 1.

Commercial MV-specific IgM ELISA

For the MV-specific indirect ELISA (Dade Behring Enzygnost, Marburg, Germany, kits kindly provided by Dr. D. Featherstone, WHO, Geneva), samples were reconstituted and processed as described previously (114). Since in preliminary assays extinctions of the positive controls were below the lower limit as provided by the manufacturer, the substrate incubation time of the ELISA was extended from 30 to 60 minutes.

RT-PCR, sequencing and phylogenetic analysis

For reverse transcriptase polymerase chain reaction (RT-PCR) two 6mm disks diameter were eluted in 250µl cold RNase-free water. After brief mechanical manipulation with a sterile pipette to achieve complete elution, samples were centrifuged at 10 000 g for 15 seconds and 200 µl was mixed with the lysis buffer provided by the “High pure viral nucleic acid kit” (Roche Diagnostic, Almere, The Netherlands). The RNA was eluted in 40µl water, of which 10µl was used in the RT-PCR assay. RT-PCR was carried out as described previously (147), using the forward primer 5'-TTAGGG CAAGAGATGGTAAGG-3' (position 1090-1110), and reverse primer 5'-TTATAACAATGATGGAGGG-3' (position 1633-1615). The amplified products were then visualised in ethidium bromide staining. When limited in quantity the PCR products were re-amplified using the same primer combination to allow sequencing. The products were then sequenced using the Big dye[®] Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster city, USA) on a 3100 Sequencer (Applied Biosystems).

Results

MV-specific IgM detection: assay validation and effects of storage

For laboratory confirmation of the clinically diagnosed measles cases, MV N-specific IgM antibodies were measured in reconstituted filter paper blood samples. For the samples of set 1 (n=252), these assays were first carried out in March 2002, i.e. after 2 to 17 months storage of the samples at 4°C. In this assay, 28 (11%) samples had to be excluded from analysis

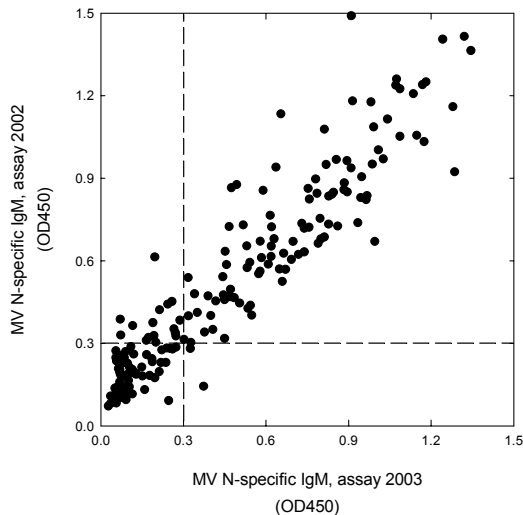
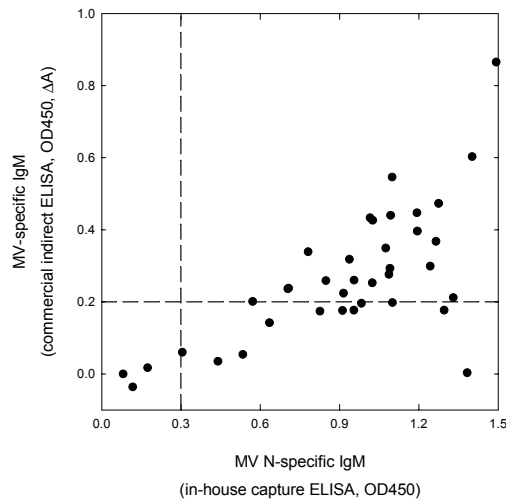


Figure 1

MV N-specific IgM antibody levels in filter paper blood samples of 201 clinically diagnosed measles patients, as measured by the in-house IgM capture ELISA. The vertical axis shows results of the assay performed in 2002 (i.e. after storage for 2-17 months at 4°C), while the horizontal axis shows the results of the assay performed in 2003 (i.e. after storage for 18-33 months at 4°C).

because the reconstituted samples contained insufficient quantities of total IgM. In July 2003 (18-33 months after collection) 228 samples of this set were reconstituted again for measurement of N-specific IgM. This time five (2%) of the reconstituted samples contained insufficient amounts of total IgM. As shown in figure 1, the results obtained with the samples that were tested in both assays (n=201) showed a good correlation ($r^2=0.88$), demonstrating stability of IgM antibodies in the filter paper samples under these conditions. On basis of the combined results of both assays, 147 of 247 (60%) samples of set 1 were found IgM positive. The second set of samples (n=69) was tested in July 2003, i.e. 3 to 17 months after sample collection. The reconstituted samples all contained sufficient quantities of total IgM, and 53 (77%) samples were IgM positive.

From a limited number of samples (n=38) the material available after completion of serological and virological analyses was sufficient to be tested in the WHO-advised commercial Dade Behring assay in parallel with our in house assay. The results of the two assays correlated well, although the in house capture ELISA was slightly more sensitive than the commercial indirect ELISA (figure 2).

**Figure 2**

MV-specific IgM antibody levels in filter paper blood samples of 38 clinically diagnosed measles patients. The vertical axis shows the results of the commercial indirect ELISA, while the horizontal axis shows the N-specific IgM antibodies measured by the in-house IgM capture ELISA.

MV-specific IgM detection: laboratory diagnosis

The IgM results obtained using the in house assay were used for laboratory confirmation of the clinically diagnosed measles cases. As shown in table 1, the number of IgM-positive patients in the complete cohort was 200/316 (63%). IgM results were affected by the time of sample collection, as in samples collected within three days after onset of rash 67/132 (51%) were IgM positive while in those collected four days or later after onset of rash 133/184 (72%) were IgM positive. The median age of the patients included was 42 months, this was 48 and 36 months for the IgM positives and negatives, respectively. Almost half of the patients had been vaccinated against measles, which was similar for the IgM positives and negatives (43 and 48%, respectively).

Table 1. Patient baseline data and MV-specific IgM results.

	Total	IgM positive	IgM negative
Number of patients	316	200	116
Median age (months)	42	48	36
Gender (F/M)	152/164	99/101	53/63
Measles vaccination status			
Vaccinated	141 (45%)	85	56
Not vaccinated	171 (54%)	111	60
Unknown	4	4	0
Days after onset of rash			
0-3	132	67 (51%)	65 (49%)
4-7	167	120 (72%)	47 (28%)
>7	17	13 (76%)	4 (24%)

RT-PCR, sequencing and phylogenetic analysis

A subset of 57 samples (of which 47 and 10 were MV IgM positive and negative, respectively) collected in different years was tested by RT-PCR. Sixteen samples (28%) were found RT-PCR positive, of which 2 were MV IgM negative. From 15 of the resulting amplicons partial (3' 456 nucleotides) N gene sequences were obtained (table 2). Phylogenetic analysis using the WHO-assigned reference strains showed that the sequences belonged to genotype B3, cluster 1 (figure 3). The sequences were also closely related to those obtained from Sudan during the period 1997-2000, with a maximum divergence of 2% between the most distant viruses during seven years. In addition, the results revealed the fixation of the previously observed nucleotide mutation ¹³⁷⁰A→G in all sequences collected in Khartoum since 2001. Another 12 set-specific mutations occurred in the set of sequences. In the most a specific pattern for the occurrence of the mutations was followed, as a ¹⁵⁷²G→A was found in sequences obtained from patients M243, M272 and M273 (2001) and were never found again.

Table 2. Summary of sequences determined.

Patient ID	Sequence available (WHO nomenclature)	GenBank Accession no.	Year
M227	MVs/Khartoum.SUD/34.01	AY456396	2001
M238	MVs/Khartoum.SUD/35.01	AY456397	2001
M243	MVs/Khartoum.SUD/36.01/1	AY456398	2001
M253	MVs/Khartoum.SUD/36.01/2	AY456399	2001
M272	MVs/Khartoum.SUD/36.01/3	AY456400	2001
M273	MVs/Khartoum.SUD/36.01/4	AY456401	2001
M347	MVs/Khartoum.SUD/14.02	AY456402	2002
M349	MVs/Khartoum.SUD/16.02	AY456403	2002
M358	MVs/Khartoum.SUD/17.02	AY456404	2002
M375	MVs/Khartoum.SUD/32.02	AY456405	2002
M376	MVs/Khartoum.SUD/34.02/1	AY456406	2002
M379	MVs/Khartoum.SUD/34.02/2	AY456407	2002
M408	MVs/Khartoum.SUD/8.03	AY456408	2003
M409	MVs/Khartoum.SUD/11.03/1	AY456409	2003
M410	MVs/Khartoum.SUD/11.03/2	AY456410	2003

The ¹²⁵⁰T→C mutation was also found in sequences from patients M349 and M376 (2002) and M410 (2003), this mutation occurred previously in one of the isolates from 1999 and one from 2000. Furthermore, three point mutations ¹²⁷⁴T→C, ¹⁶⁴³T→C and ¹⁶⁵⁵T→C occurred in combination with each other, and were found in none of the 2001 cases, 2/6 of the 2002 sequences and 2/3 of the 2003 sequences. The ¹⁶⁴³T→C and ¹⁶⁵⁵T→C were also found independently of the ¹²⁷⁴T→C in only one sequence from 2001

sequences, all three mutations were found in combination with a ¹⁵³⁰G→A mutation in two of the 2003 sequences.

Discussion

In the present study we have evaluated surveillance of measles using dried blood spots collected on filter paper in a field setting in Sudan. MV-specific IgM was detected in 63% of the samples and fifteen partial N gene sequences were collected from clinically diagnosed measles patients sampled between 2001 and 2003. Collectively these data demonstrate that measles continues to be endemic in Khartoum.

MV-specific IgM was detected in 200 of 316 (63%) samples using an in house-developed IgM capture ELISA based on peroxidase-labelled N protein. In a previous study performed in the same area, laboratory diagnosis based on the combination of serology and RT-PCR confirmed 145 of 192 (76%) clinically diagnosed patients as measles cases (152). The percentage IgM positives was lower in samples collected in the first three days after onset of rash than in those collected afterwards, confirming previous observations (159). This suggests that the total percentage of laboratory-confirmed cases is an under-representation of the true number of measles cases.

The in house capture IgM ELISA was shown to be more sensitive than the Dade Behring indirect IgM ELISA. This observation is in accordance with a recent evaluation of different commercial measles IgM ELISAs, in which the capture assays tested, proved more sensitive than the indirect assays (160). If the Dade Behring assay had been used in the present study, the percentage of IgM positives would have been lower than the percentage we now detected. However, the Dade Behring assay proved to be more specific than the IgM capture assays, resulting in higher positive predictive values. Therefore, the WHO continues to advise use of the Dade Behring IgM assay. Since the emphasis is on outbreak diagnosis rather than diagnosis of individual cases in measles control countries, the use of either type of assay would actually result in similar conclusions.

The repeated reconstitution and IgM testing of 201 samples confirmed that IgM is fairly stable during storage of filter paper blood samples at 4°C. Despite the 16 months between the first and second measurement, the results of the two assays were almost identical. Riddell et al. reported that testing within six months after sample collection still gave reliable results (114), but the present study suggests that the period during which the sample remains stable may actually even be longer. A possible explanation for the observed prolonged stability in our study may again be the use of an IgM capture ELISA: degradation of half the total IgM in the sample would reduce the specific signal in an indirect ELISA, but if the amount of IgM was still sufficient to saturate the capturing plate the resulting extinction in the capture ELISA would remain unaffected.

A substantial number (43%) of the laboratory-confirmed measles cases in the present cohort had been vaccinated against measles. In our previous cohort study in the same area this was 59% (161). We recently performed a measles vaccination efficacy study in Khartoum, during which relatively low seroconversion levels were detected by virus neutralisation in sera collected four to six weeks after vaccination (manuscript in preparation). These observations raise serious concerns about the effectiveness of measles

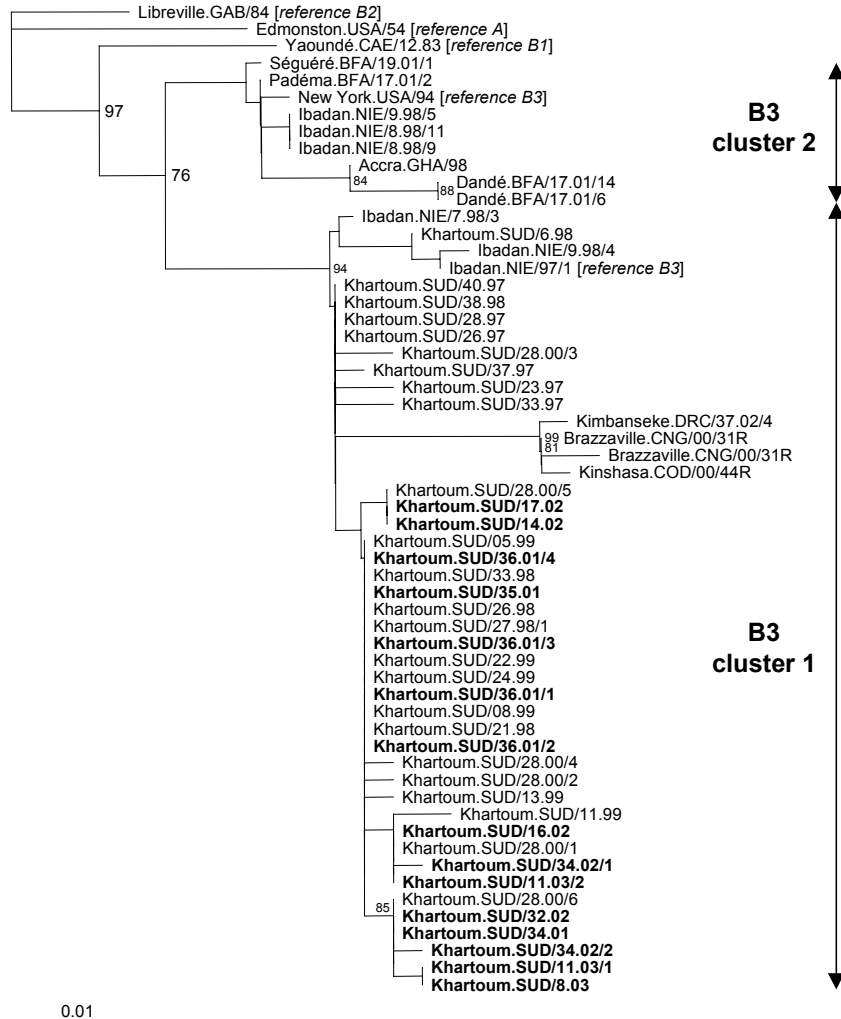


Figure 3

Phylogenetic comparison of the 3' N gene sequences determined in the present study (shown in bold), a selection of 24 other Sudanese sequences (158), an extended set of genotype B3 sequences and the reference sequences of clade A and genotypes B1 and B2. This phylogram was generated by analysing 100 bootstrap replicates, bootstrap values are shown when above 75%. Sequence identification is according to the WHO-nomenclature.

vaccination in the area.

The 15 partial N gene sequences studied confirmed the continued circulation of genotype B3 cluster 1 in Khartoum. The results also demonstrate a high genetic stability of wild-type viruses circulating in Khartoum, as over a 7 year study period the maximum nucleotide divergence was 2% (158 and present study). This observation is in agreement with previously described low mutation rates within single chains of transmission (32;37).

The Sudanese sequences contained 12 set-specific mutations, five of which were shared with other Sudanese sequences (158). One of these mutations ¹³⁷⁰A→G was fixed in all recent sequences. This mutation was first found in 1998, was found in the majority of the sequences obtained in 1999 and 2000, and was now found in all sequences obtained from 2001 until 2003. Interestingly, the set-specific mutations occurred in a specific pattern, as some mutations are apparently fixed over time whilst others do not occur again. This localisation of point mutations in specific patterns may suggest the existence of separate transmission pathways.

Although dried blood spots collected on filter paper proved to be applicable for measles surveillance, problems were encountered with limited sample quantity. The samples were collected by finger prick, and in many cases spots were not completely saturated with blood. The Dade Behring assay uses two 6mm disks, so if a technical problem occurs in the assay rendering the result invalid (which happened in the present study), repetition of the assay may be impossible due to limited sample quantity.

In conclusion, the present study confirmed the value of filter paper blood samples for measles surveillance in a field study in Khartoum. These samples have as advantages the ease of transportation, the lack of cold chain issues, the ability to be used in combination with standard commercial assays, and the perception of being less invasive than venepuncture. Application of single-use lancets should be advised for safety reasons. Although in areas with sufficient infrastructure collection of serum and throat swab or oral fluids is probably preferable, filter paper blood samples should be a valuable addition to the GMLN for use in remote areas. The present study also demonstrated that measles continues to be endemic in Khartoum, associated with the circulation of genotype B3 viruses.

Acknowledgements

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Section 2.5

Development of a semi-quantitative real-time RT-PCR for the detection of measles virus

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submitted

Abstract

Real-time detection of polymerase chain reactions allows convenient detection and quantification of virus-derived nucleic acids in clinical specimens. We have developed a real-time RT-PCR assay for the detection of measles virus (MV) genomic RNA, and compared it to a well-established conventional RT-PCR assay. Based on a serial dilution of the live-attenuated MV Edmonston-Zagreb vaccine, the detection limits were approximately 0.1 and 0.02 cell culture infectious dose 50% units (CCID₅₀) per test for the conventional and TaqMan RT-PCR assays, respectively. Furthermore, tissue materials spiked with known quantities of MV were equally well detected in both assays. The TaqMan assay was linear within a range of $10^{4.4}$ to $10^{-0.6}$ CCID₅₀/ml, with an intra-assay variability lower than 3% and an inter-assay variability ranging from 1.5% at $10^{4.4}$ CCID₅₀/ml to 8.7% at $10^{-0.6}$ CCID₅₀/ml. The TaqMan assay could detect representative wild-type viruses from the currently active MV clades, and could detect MV genome in clinical specimens obtained from measles patients. Finally, quantification of MV RNA in peripheral blood mononuclear cells or broncho-alveolar lavage cells from cynomolgus macaques collected at different time points after experimental infection showed a good correlation with virus isolation data. In conclusion, the TaqMan assay developed is specific, sensitive, rapid and reproducible, and can be of use for diagnostic purposes or for studies on the pathogenesis of measles.

Introduction

There are a number of laboratory methods for the diagnosis of measles, of which detection of measles virus (MV)-specific serum IgM antibodies remains the gold standard (50). Virus detection is an equally valid method, but MV isolation has limited sensitivity (147). Reverse transcription polymerase chain (RT-PCR)-based methods have been described for the detection of MV genomic RNA, which are more sensitive, reproducible and robust (162). Different clinical specimens can be used in these assays, including throat swabs, urine, oral fluids and filter paper blood samples (111;113;147;152;163). As an added value, sequences can be obtained for molecular epidemiological studies (28).

The development of fluorescence-based real-time detected RT-PCR methods allowed the quantitative detection of RNA sequences (164;165;165). In combination with automated RNA isolation technologies these methods allow processing of large amounts of samples in a short period of time with a minimal risk of cross-contamination.

The quantitative aspect of the assay is especially important in the diagnosis of chronic infections (165-167), and has limited value for the diagnosis of measles (167). However, in pathogenesis studies the ability to quantify MV genomes may be of interest. In some cases MV-specific real-time RT-PCR assays have been used without indication of their specificity and sensitivity in comparison with conventional RT-PCR assays (168-170). However, in a recent study evaluating different diagnostic assays a real-time RT-PCR assay using the Light Cycler instrument was used which proved to be equally sensitive as a nested RT-PCR assay (113;169;170).

Here we describe the development of a MV-specific quantitative real-time PCR assay, and compare it to an established conventional RT-PCR. Different RNA isolation methods were tested, and the new assay was validated using different virus strains and clinical samples.

Materials & Methods

Test samples

Live attenuated MV vaccine strain Edmonston Zagreb (MV-EZ, kindly provided by the Serum Institute of India, batch MVA-750, potency $10^{4.083}$ cell culture infectious dose 50% units [CCID₅₀] per 0.5ml) was used for evaluation of assay sensitivity and as a standard in further experiments. In addition, specimens used in a previous study comparing different conventional RT-PCR assays (162) were also used for evaluation of assay sensitivity (samples kindly provided by Dr. M.A. Afzal, National Institute for Biological Standards and Control, United Kingdom).

To test the ability of our primer / probe combination in detecting wild-type strains, virus isolates from different currently active MV clades (171) were tested: MVi/Khartoum.SUD/34.97/2 [B3] (158), MVi/Bilthoven.NET/47.91 [C2] (37), MVi/Amsterdam.NET/3.98 [D4] (172), MVi/Alblasserdam.

NET/27.99 [D6] (173), MVi/Amsterdam.NET/49.97 [G2] (174), and MVi/Amsterdam.NET/27.97 [H1] (172). All viruses were grown in human Epstein-Barr virus transformed B-lymphoblastic cell lines to titers of approximately 10^4 CCID₅₀/ml.

Clinical materials consisted of throat swabs and filter paper blood spots collected from clinically diagnosed measles patients in Sudan, after obtaining informed consent (147;158;158).

Peripheral blood mononuclear cells (PBMC) and broncho-alveolar lavage (BAL) cells were collected from a cynomolgus macaque (*Macaca fascicularis*) at different time points after experimental infection with wild-type MV strain BIL (intra-tracheal challenge dose 10^5 CCID₅₀). This animal experiment had been performed as part of a different study, which was approved by the Animal Ethics Committee and performed in accordance with animal experimentation guidelines. Immediately after collection PBMC and BAL cells were counted, and 5×10^5 cells were frozen as dry pellet at -70°C . Virus loads had been determined in these same specimens by virus isolation using an infectious centre assay as previously described (103). For RNA isolation pellets were thawed and resuspended in 400 μ l PBS.

RNA isolation

For the conventional RT-PCR, RNA was isolated using the High Pure viral RNA kit (Roche Diagnostics, Almere, The Netherlands), with some modifications. Briefly, 200 μ l of sample was mixed with 400 μ l of lysis buffer, put on the column and centrifuged for 15s at 8000g. For filter paper blood samples the blood spots were eluted in 200 μ l of distilled water before addition of the lysis buffer. The filters were then washed twice with wash buffer 2 as described in the kits protocol. Finally, RNA was eluted in 50 μ l of elution buffer, aliquotted and stored at -70°C .

For the TaqMan RT-PCR assay, RNA was isolated from 200 μ l sample using the MagNA Pure LC Total nucleic acid isolation kit (Roche Diagnostics). RNA was eluted in 50 μ l. RNA isolations from the vaccine titrations were also performed using the conventional RNA isolation kits in parallel with the MagNa Pure protocol.

Conventional RT-PCR

RT-PCR was carried out as previously described (147), using the forward primer MV-N1 5'-TTAGGGCAAGAGATGGTAAGG-3' (positions 1090-1110) and reverse primer MV-N2 5'-TTATAACAATGATGGAGGG-3' (positions 1633-1615) (Isogen BV, Maarssen, the Netherlands). Amplified products were visualised on agarose gel with ethidium bromide staining, followed by southern blotting using a biotin-labelled oligonucleotide probe MV-prN2 5'-GCCATGGCAGGAATCTCGGAA-3' (positions 1498-1518) (Eurogentec s.a., Seraing, Belgium). A positive RT-PCR signal was defined as an amplified product of the right size (544 nucleotides), which hybridised with the specific probe.

Real-time quantitative RT-PCR

Primers and probe were designed using the primer express software (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) based on sequences of the MV nucleoprotein gene (figure 1).

RNA was amplified using the EZ RT-PCR amplification kit on an ABI 7700 sequence detection system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Briefly, 20µl of RNA put into 30µl amplification mix containing 1µl (20 pmol) of each of the primers MV-fpr6 5'-GGCAAGAGATGGTAAGGAGGT-3' (positions 1094-1114) and MV-rpr6 5'-CGGCAGTGATACCGAGTTC-3' (positions 1147-1165), and 1µl (10 pmol) of probe 5'-FAM-ATGCCAATGTGGAAGTACCTTTCCA-

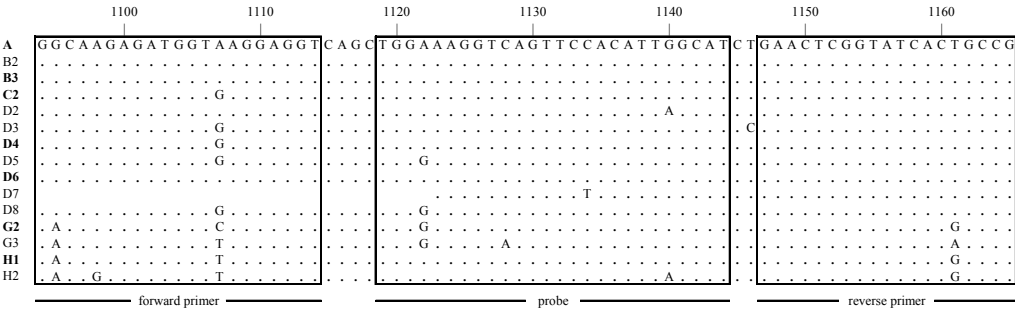


Figure 1
N gene sequence alignment of currently active measles virus genotypes (reference strains as indicated in WHO, 2003), showing the region containing the primers and probe used in the TaqMan assay. For genotype D7, the sequence of MVi/DenHaag.NET/11.03/1 was kindly provided by Dr. R.S. van Binnendijk. Dots indicate similarity to the genotype A reference strain, which has 100% similarity to the TaqMan primers and probe. Genotypes from which representative viruses were tested in the present study are shown in bold

TAMRA-3' (positions 1119-1144). Following uracil-N-glycosylase (UNG) amplicon degradation and reverse transcription (2 min at 50°C, 30 min at 60°C), an UNG inactivation step of 5 min at 95°C was performed. The cDNA was then amplified for 50 cycles (15 s at 95°C and 1 min at 62°C). The cycle threshold (CT) value was calculated automatically when the FAM-specific MV signal was above the background, and used to get a semi-quantitative impression of the MV viral load.

Results and Discussion

Detection limits, linearity and reproducibility

For the determination of detection limits, RNA was isolated from a serial tenfold dilution of live-attenuated MV-EZ vaccine. The RNA was isolated from four individual titrations, and all quadruplicates were tested in TaqMan and conventional RT-PCR assays. In the TaqMan assay 0.24 CCID₅₀/ml was detectable, while in the conventional RT-PCR assay 2.4

CCID₅₀/ml still resulted in a positive signal (figure 2). Since the TaqMan assay was based on a larger RNA input than the conventional RT-PCR (in both cases RNA was isolated from 200µl and eluted in 50µl, of which 20 and 10µl were used for the TaqMan and conventional RT-PCR, respectively), the detection limits were 0.02 and 0.1 CCID₅₀ per test, respectively. At a concentration of 0.024 CCID₅₀/ml (0.002 CCID₅₀/test) only one out of the four samples tested positive by TaqMan. The sensitivity of the assay was 90% at 0.24 CCID₅₀/ml and 100% at all higher concentrations tested (figure 2). The TaqMan assay was linear within a range of 10^{4.4} to 0.24 CCID₅₀/ml (figure 2).

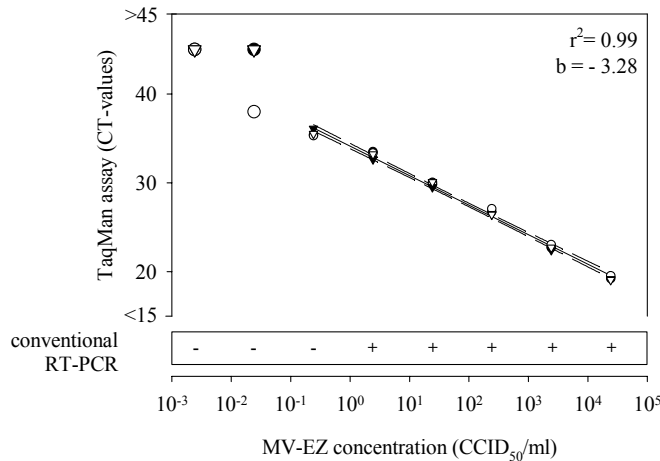


Figure 2

Linear regression curve based on four independently processed tenfold serial dilutions of the MV-EZ vaccine. The results of the regression analysis (correlation coefficient r^2 and slope b) are shown in the upper right corner of the graph.

To determine the inter and intra assay variation of the TaqMan assay 16 samples of the MV-EZ vaccine at a concentration of 10^{4.4} and 0.24 CCID₅₀/ml were tested in three independent assays. The inter-assay variability ranged from 1.5% of CT value of 23.1 at 10^{4.4} CCID₅₀/ml to 8.7% of a CT value of 36.5 at 0.24 CCID₅₀/ml and the intra-assay variability ranged from 1.1% of CT value of 23.1 at 10^{4.4} CCID₅₀/ml and 2.3% of CT value 36.5 at 0.24 CCID₅₀/ml. For the TaqMan assay RNA isolated using either the automated MagNA Pure technique or the conventional RNA isolation kits gave similar results for the vaccine titration, throat swabs and filter paper blood samples (data not shown), and the MagNA Pure was selected as the standard method of RNA isolation for this assay.

Due to the absence of an internationally validated standard for the quantification of MV RNA, and to correlate the performance of our assay to other MV-specific RT-PCR assays described, clinical samples spiked with MV as previously described (175) were tested in our TaqMan assay. The qualitative results were identical to those obtained using our conventional RT-PCR (table 1). The assay sensitivity based on a serial dilution of sample

R was 1.34×10^{-4} and 0.67×10^{-4} plaque forming units for the conventional and TaqMan RT-PCR assay, respectively.

Table 1. Comparison of conventional and TaqMan RT-PCR in detecting MV RNA in spiked clinical specimens obtained through an international collaborative study (162).

Sample code	Sample description	Conventional RT-PCR	TaqMan RT-PCR
A	CD ¹	-	-
B	CD	-	-
C	CD	-	-
D	CD	-	-
E	CD + SSPE ²	+ ⁶	+ ⁷
F	CD	-	-
G	CD + MV ³	+	+
H	CD	-	-
J	LYM ⁴ + SSPE	+	+
K	LYM	-	-
L	LYM + MV	+	+
M	LYM	-	-
R	MV RNA ⁵	+	+

¹ CD = resected gut of a Crohn's Disease patient

² + SSPE = material spiked with 10 μ l of brain suspension of an SSPE patient

³ + MV = material spiked with 210 plaque forming units of wild-type MV strain 94/31825

⁴ LYM = lymphocytes of a Crohn's Disease patient

⁵ RNA isolated from wild-type MV strain 94/31825

⁶ designated positive if amplicon of right size hybridized with specific probe

⁷ designated positive if CT-value was below 40 in association with a good amplification curve

3.2 Virus strains and clinical specimens

The TaqMan RT-PCR assay could detect RNA samples isolated from virus strains belonging to different active wild-type MV clades (genotypes A, B3, C2, D4, D6, G2 and H1, data not shown).

The detection of MV RNA in throat swab samples (n=41) by both the conventional and TaqMan RT-PCR also resulted in similar data (table 2A). The TaqMan assay was however more sensitive, since in four of the throat swab samples a positive signal was detected, which was undetectable by the conventional RT-PCR. Similar results were obtained from filter paper blood samples (n=11) since in four samples a positive signal could be detected by TaqMan and not by the RT-PCR assay (table 2B).

Table 2. Examination of clinical specimens by conventional and TaqMan RT-PCR

a. Throat swabs

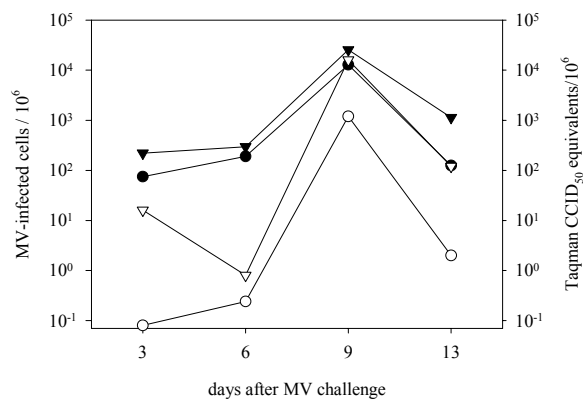
Assay		TaqMan RT-PCR	
		Positive	Negative
Conventional RT-PCR	positive	22	0
	negative	4	15

b. Filter paper blood samples

Assay		TaqMan RT-PCR	
		Positive	Negative
Conventional RT-PCR	positive	6	0
	negative	4	5

3.3 Viral RNA quantification during experimental infection

Quantitative detection of MV genome in PBMC and BAL cells obtained from experimentally infected macaques correlated well with virus isolation data (figure 3). However, the TaqMan assay underestimated the actual infectious dose. Whether this is due to the fact that in this animal the cell associated virus load was determined while the TaqMan assay uses a cell-free virus stock as quantification reference, remains to be determined.

**Figure 3**

Virus loads in PBMC (circles) and BAL-cells (triangles) of a cynomolgus macaque at different time points after experimental infection with MV strain Bil [C2], as determined by virus isolation (black symbols) or TaqMan (white symbols). Virus isolation results are expressed as infected cells per 10⁶ total cells on the left y-axis, while TaqMan results are expressed as MV-EZ CCID₅₀ equivalents on the right y-axis.

In conclusion, the TaqMan assay developed is specific, sensitive, rapid and reproducible, and can be of use for diagnostic purposes or for studies on the pathogenesis of measles.

Chapter 3

Measles virus-specific antibody responses

Chapter 3

Measles virus protein-specific IgM, IgA and IgG subclass responses during the acute and convalescent phase of infection

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Abstract

The availability of new generation serological assays allowed re-evaluation of the antibody response to measles virus. Here, IgM, IgA, total IgG and IgG subclass responses were studied to the three major immunogenic measles virus proteins: the fusion protein (F), haemagglutinin (H) and nucleoprotein (N). Plasma samples were obtained from clinically diagnosed measles cases (n=146) in Khartoum (Sudan) within a week after onset of the rash. Convalescent phase samples were collected from 32 of 117 laboratory-confirmed measles cases at different time points after onset of rash. Glycoprotein-specific IgM, IgG and IgA antibody levels correlated well to the N-specific response. For IgG and IgA, responses to F were higher than to H. IgA antibody levels were undetectable in about one third of the laboratory-confirmed cases during the acute phase, but positive in all patients tested one to four weeks after infection. IgM levels declined rapidly and were lost three to six months after infection. IgA levels declined slowly during the first year but did not return to background levels during the subsequent two years. IgG avidity maturation was detected during a three to six month period after infection. The predominant IgG subclasses during the acute phase were IgG1 and IgG3. The latter was lost in the convalescent phase, while the IgG4 isotype showed a slight rise afterwards. Interestingly, acute phase IgG3 and IgA responses were associated, and were only detected in samples with high IgG. This study provides a comprehensive perspective on the antibody response to wild-type measles virus infection.

Introduction

Measles virus is a member of the family *Paramyxoviridae*, genus *Morbillivirus*. Despite being associated with immunosuppression, measles virus infection induces strong specific cellular and humoral immune responses (1;70). Whereas measles virus-specific T cells are considered essential in virus clearance, virus neutralising antibodies are capable of conferring protection from infection, as illustrated by maternal antibody-mediated protection of young infants (1). Measles virus-specific antibodies are detectable from the onset of rash, and remain present for life. However, several changes in antibody isotype distribution take place over time. IgM antibody levels peak several days after onset of rash, and decrease during the following months. Detection of measles virus-specific IgM is considered the gold standard for the laboratory diagnosis of measles (50). Measles virus-specific IgG antibodies can also be detected from the onset of rash, but peak one to two weeks later and remain present life-long. They undergo affinity maturation, and are the major mediators of virus neutralisation (51). The IgG subclass composition during different phases of the immune response is a matter of discussion (54-57). The kinetics and possible role of measles virus-specific serum IgA are also unclear. Specific IgA can be detected during the acute and convalescent phase of the infection, but in some individuals also during the memory phase (58;59).

Measles virus protein-specific antibody responses to the N, H, F and matrix protein (M) have been shown (176). The N protein is the most abundantly present during acute infection, which could account for its immunodominance (177). However, N-specific antibodies are considered to have only limited biological function. Virus neutralising antibodies are directed towards the H and F transmembrane glycoproteins (51). The introduction of recombinant technology has enabled the detection of protein-specific responses at the isotype level (139;178-180). For the glycoprotein-specific responses, immunofluorescence assays using intact transfected target cells may be preferred, since this results in detection of antibodies that recognise the extra-cellular domain of the protein in its natural conformation. We have previously shown that these responses correlate well with virus neutralising antibody responses (139). N-specific antibody responses can be detected easily by ELISA systems, using purified recombinant baculovirus-produced N (181;182).

In the present study, these new generation serological techniques were used to re-evaluate the measles virus protein-specific antibody response at the isotype level, using samples collected in the framework of a cohort study in suburban Khartoum, Sudan (152;161).

Materials & Methods

Patients and samples

Plasma samples (n=169) were collected from Sudanese infants who complied with the WHO clinical case definition for measles, as previously described (147;152;161). Informed consent was obtained from the parents, and the study was approved by the medical ethical committee of the University of Khartoum. As described previously, detection of measles virus-specific IgM in these plasma samples resulted in the identification of three groups: IgM high-positive laboratory confirmed cases (n=117), IgM-negative "non-measles rash disease" cases (n=29), and samples with low or borderline IgM levels (n=23) (147;152). The latter group consisted of both laboratory-confirmed and non-measles rash disease cases as defined by additional IgG serology and RT-PCR analysis on throat swab and filter paper blood samples (147;152), but was excluded from the present study to obtain a homogeneous set of acute phase samples. The 29 IgM-negative samples were included in the serological analysis as clinical controls. Convalescent phase samples were collected from 32 of the 117 laboratory-confirmed measles patients during a 3-year follow-up period, at the following time intervals after onset of rash: 1-6 days (n=30), 7-13 days (n=5), 14-27 days (n=5), 1-3 months (n=5), 4 to 6 months (n=5), 7 to 12 months (n=8), 1 to 2 years (n=13) and more than 2 years (n=10) after initial sampling.

Measles virus glycoprotein-specific serology

Plasma antibody levels specific for F and H were determined by an immunofluorescence assay using transfected human melanoma cell lines as targets, as described previously (139). Briefly, melanoma cells expressing either the F protein (Mel-JuSo / measles virus F) or the H protein (Mel-JuSo / measles virus H), or the untransfected parental cell line (Mel-JuSo/wt), were incubated with plasma samples diluted 1:100 in phosphate-buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS). For measurement of IgM or IgA plasma samples were pre-diluted in GullSorb reagent (Gull Laboratories, Salt lake City, UT, USA) to precipitate all plasma IgG. After one hour on ice the cells were washed and stained with FITC-labelled rabbit anti-human IgM, IgA or IgG (F(ab')₂ fragments, DAKO, Glostrup, Denmark).

For measurement of measles virus glycoprotein-specific IgG subclass levels, the plasma incubation was followed by incubation with monoclonal antibodies specific for human IgG1 (HP6188, CLB, Amsterdam, The Netherlands), IgG2 (HP6014, CLB), IgG3 (HP6050, Cappel, Organon Teknika, The Netherlands) or IgG4 (HP6196, CLB) at a concentration of 2 µg/ml. After one hour incubation on ice the cells were washed again, and stained with RPE-labelled goat-anti-mouse Ig antibodies (DAKO). Results are expressed as the fluorescence signal (histogram peak channel) measured on a FACScan (Becton-Dickinson, Mountain View, CA, USA), in arbitrary fluorescence units (AFU). This fluorescence intensity was previously shown

to correlate with the quantity of specific antibodies (139). Fluorescence signals measured on the untransfected cell line were less than ten AFU (data not shown). For each sample the mean of the fluorescence signals to F and H was calculated after log10-transformation, and plotted against the N-specific signal as "glycoprotein-specific" response. As arbitrary cut-off a signal of 30 AFU was used as defined previously (139).

Measles virus nucleoprotein-specific serology

Plasma levels of nucleoprotein (N)-specific IgM, IgA, IgG2, IgG3 or IgG4 were measured by capture ELISA systems using purified recombinant baculovirus-expressed N (produced as described by Ravel et al., 1997). Plasma samples were diluted 1:100 in ELISA buffer (Meddens Diagnostics, Vorden, The Netherlands) and incubated in ELISA plates (Greiner, Alphen a/d Rijn, The Netherlands) coated with rabbit anti-human IgM or IgA (Meddens) or on plates coated with monoclonal antibodies specific for human IgG2, IgG3, or IgG4 (CLB). After one hour at 37°C plates were washed in PBS containing 0.05% Tween-20, and were subsequently incubated with peroxidase labelled N (N-PO, protein labelled by Meddens). Following another hour incubation at 37°C, the plates were washed again and subsequently coloured using tetramethylbenzidine as a substrate. Extinctions were read in an ELISA reader at 450nm using a reference filter of 620nm. Arbitrary cut-off values were designated for each assay at approximately three times the signal obtained with negative serum samples.

N-specific total IgG and IgG1 titres were measured in an indirect ELISA: plates were coated with recombinant N (50 ng/well) in PBS for 2 hours at 37°C or overnight at room temperature. After washing the plates in PBS containing 0.05% Tween-20, serial two-fold plasma dilutions (1:40 to 1:81920) were prepared in ELISA buffer (Meddens) supplemented with 5% normal goat serum, and incubated at 37°C for 1 hour. Subsequently, the plates were washed again and incubated with a peroxidase labelled rabbit-anti-human IgG (DAKO) or with a human IgG1-specific monoclonal antibody (HP6188, CLB). Finally, the IgG1 plates were incubated with peroxidase-labelled goat-anti-mouse Ig, (DAKO) and subsequently coloured using tetramethylbenzidine as a substrate. Extinctions were read in an ELISA reader at 450 nm and a reference filter of 620nm. Endpoint titres were defined as the highest serum dilution that still resulted in an extinction of more than three times the background. Cut-off titers were defined as titers more than three times higher than titers obtained with IgG-negative samples.

IgG avidity

The avidity of measles virus N-specific antibodies was measured in an indirect ELISA using an elution technique. Plates were coated with recombinant N as described above, washed and incubated with serial two-fold plasma dilutions (1:40 to 1:81920) at 37°C for 1 hour. Subsequently, the plates were washed again and incubated with 6M urea (in PBS) or PBS only for 10 minutes at room temperature. Finally, the plates were washed again

and incubated with peroxidase-labelled goat-anti-human IgG, and subsequently coloured. Avidity indexes were expressed as the ratio of the two end point titres: titre (urea wash) / titre (PBS wash) x 100, as described previously (183).

Results

Protein-specific IgM, IgG and IgA responses during the acute phase

Plasma IgM responses to the F and H proteins were strongly correlated (figure 1a, $r^2=0.85$). The mean glycoprotein-specific response also showed a good correlation to the N-specific IgM response (figure 1b, $r^2=0.83$). Two clearly distinct clusters of IgM-negative and IgM high-positive samples could be discerned in both plots.

Plasma IgG responses to F and H showed a good correlation ($r^2=0.77$), but contrary to the IgM responses the IgG responses to the F protein were usually higher than those to the H protein (figure 1c). In the non-measles rash disease cases (white symbols) the difference between the F- and H-specific IgG response was not as evident. The correlation between the mean glycoprotein-specific IgG response and the N-specific response was substantially lower ($r^2=0.46$).

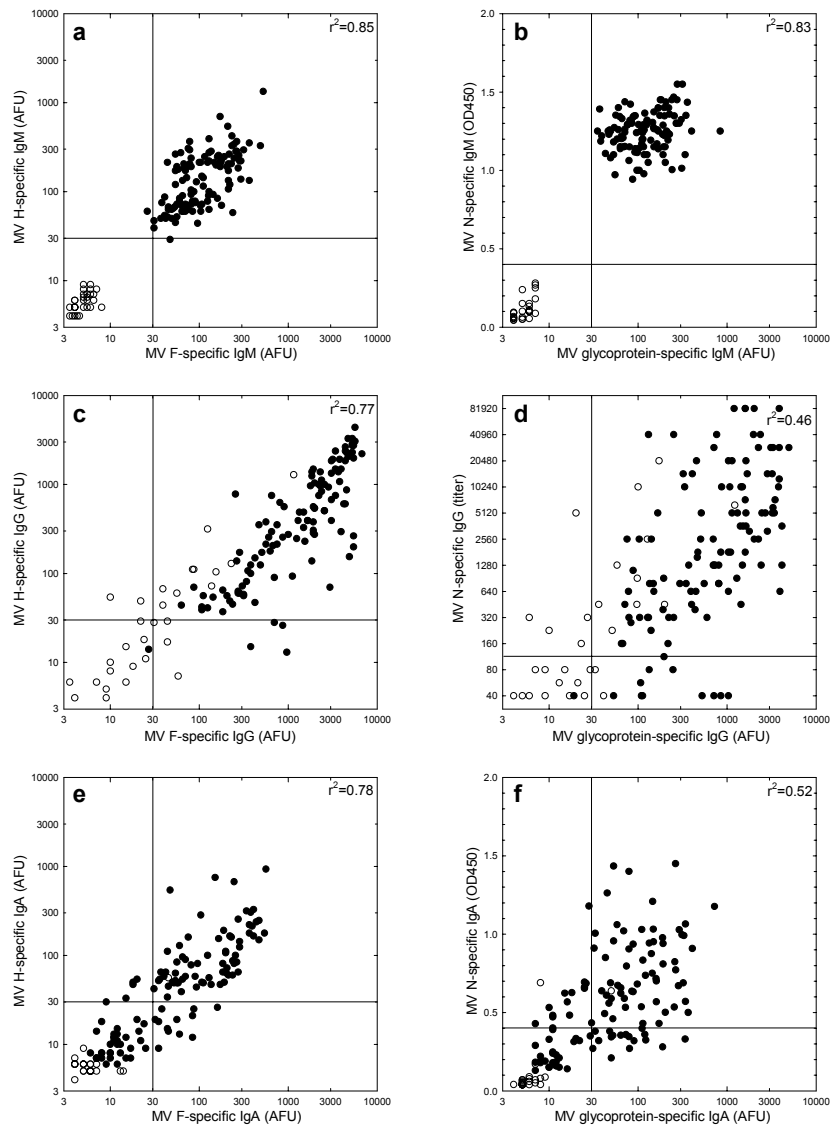
Plasma IgA responses to the F and H proteins again showed a good correlation ($r^2=0.78$). A substantial number of laboratory-confirmed measles cases showed a low or undetectable measles virus-specific IgA response during the acute phase of the infection. In the non-measles rash disease cases (white symbols) only one patient had glycoprotein-specific IgA and two patients had N-specific IgA responses. The same pattern was evident from the correlation between the glycoprotein-specific and the N-specific IgA response (figure 1f, $r^2=0.52$).

Protein-specific IgM, IgG and IgA responses during the convalescent phase

As shown in figure 2a, measles virus protein-specific IgM antibodies were readily detectable in convalescent phase samples collected from laboratory-confirmed measles until a month after onset of rash. In some patients, low-positive glycoprotein- and N-specific IgM levels remained detectable until a year after onset of rash.

Measles virus protein-specific IgG levels showed a highly variable pattern during the acute phase. However, from one week after onset of rash onwards high IgG levels to all three proteins were detected in all samples (figure 2b). Responses showed a decline between month 1 and 3 after infection and remained stable thereafter.

Measles virus protein-specific IgA was detectable in all samples collected between one to four weeks after onset of rash. Afterwards levels dropped gradually, but contrary to specific IgM levels did not return to negative values in all patients and even tended to be slightly higher again in samples collected more than six months after measles (figure 2c). Again

**Figure 1**

Relationships between measles virus F- and H-specific IgM (a), IgG (c), and IgA (e) antibody responses, and between measles virus glycoprotein- and N-specific IgM (b), IgG (d) and IgA (f) antibody responses in plasma samples collected from clinically diagnosed measles patients. Black symbols represent laboratory confirmed measles cases. Quadrant lines represent arbitrary assay cut-off values. Linear regression correlation coefficients are shown in the upper right corner of each plot.

similar to the acute phase, IgA responses to the F protein were higher than those to the H protein (figure 2c).

Measles virus N-specific IgG avidity indexes were highly variable in the samples collected during the first few weeks after onset of rash (figure 2d). The levels in the samples collected during the later stages of the disease suggest that affinity maturation is complete by three months after infection.

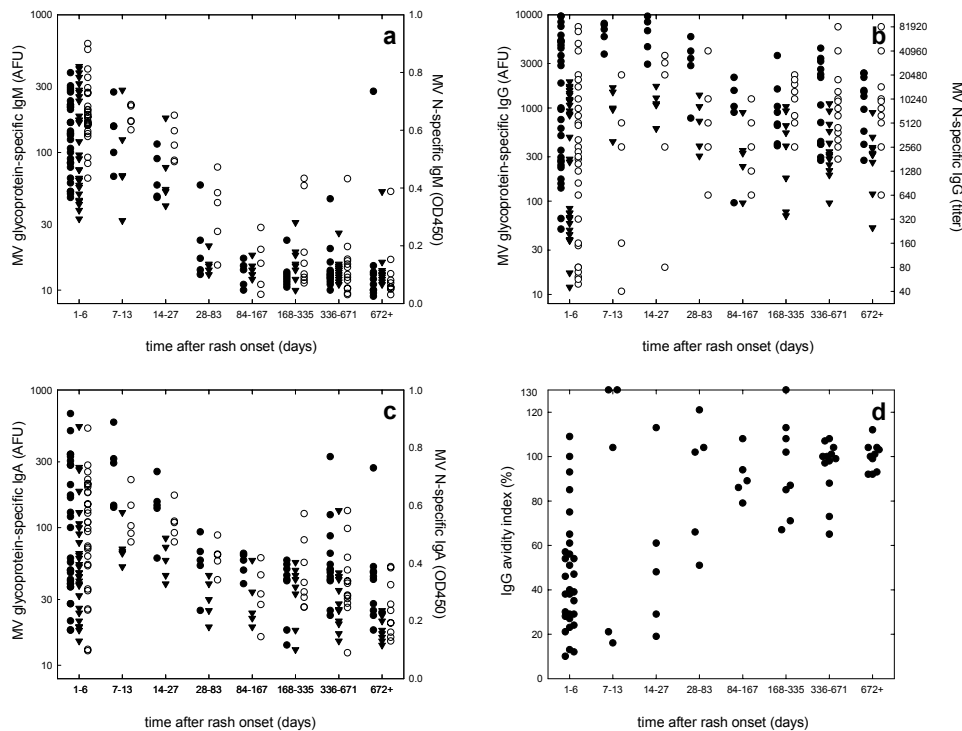
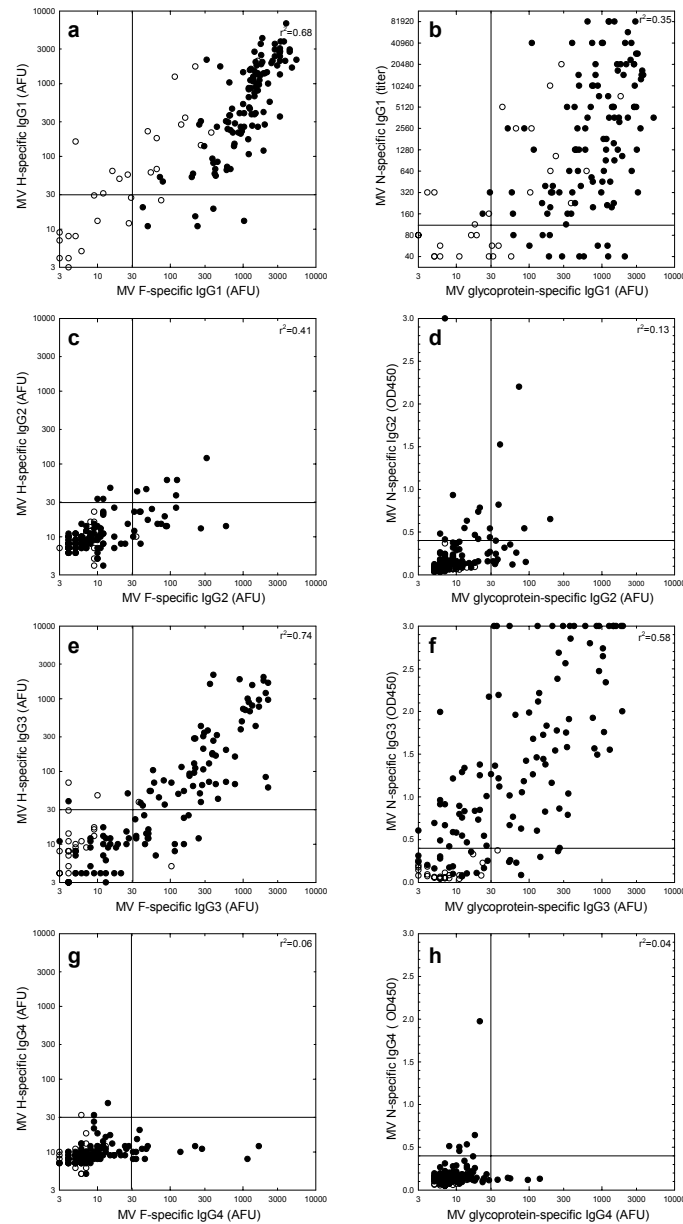


Figure 2

Measles virus F- (black circles), H- (black triangles) and N- (white circles) specific IgM (a), IgG (b) and IgA (c) antibody responses and measles virus-N-specific IgG avidity (d) profiles of laboratory-confirmed measles patients for whom follow-up samples were available.

Protein-specific IgG subclass responses during the acute phase

The acute phase F-, H- and N-specific IgG subclass response was dominated by IgG1 and IgG3, although IgG2 and IgG4 responses were detectable in a minority of the samples (figure 3). IgG3 was, similar to IgA, not detectable in all laboratory-confirmed cases, and both isotype responses were strongly correlated, as shown for F in figure 4a ($r^2=0.68$). Both IgA and IgG3 were only detected in samples with high IgG levels (figures 4b and c).

**Figure 3**

Relationships between measles virus F- and H-specific IgG1 (a), IgG2 (c), IgG3 (e) and IgG4 (g), and between measles virus glycoprotein- and N- specific IgG1 (b), IgG2 (d), IgG3 (f) and IgG4 (h) subclass responses in plasma samples collected from clinically diagnosed measles patients. Black symbols represent laboratory confirmed measles cases. Quadrant lines represent arbitrary assay cut-off values. Linear regression correlation coefficients are shown in the upper right corner of each plot.

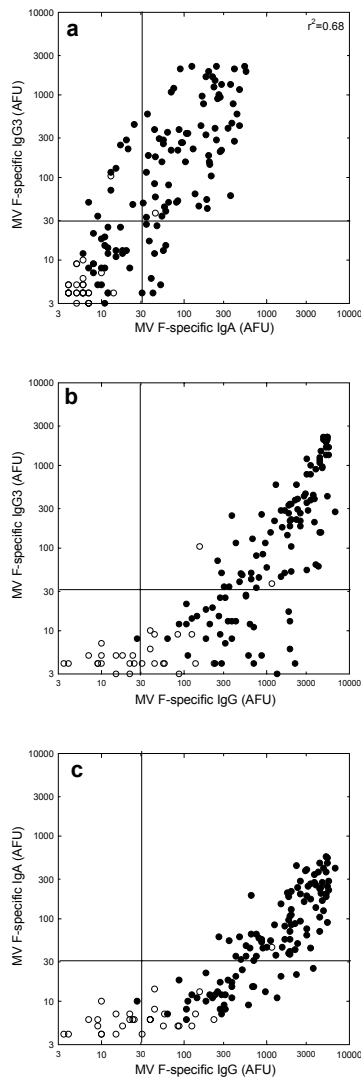


Figure 4
Correlation between measles virus F-specific IgA and IgG3 (a), IgG and IgG3 (b), and IgG and IgA (c) antibody responses in plasma samples collected from clinically diagnosed measles patients. Black symbols represent laboratory confirmed measles cases. Quadrant lines represent arbitrary assay cut-off values. Linear regression correlation coefficients are shown in the upper right corner of each plot.

Protein-specific IgG subclass responses during the convalescent phase

The IgG1 responses during the convalescent phase remained high from a week after onset of rash (figure 5a). The IgG3 responses to all three proteins remained detectable for three months after onset of rash, showed a rapid decline from six months onwards, and was rarely detectable more than one year after infection (figure 5c). Measles virus-specific IgG2 was hardly detected (figure 5b), whereas specific IgG4 showed a slight tendency towards higher levels in samples collected during the late convalescent phase (figure 5d).

Discussion

In the present study, F-, H- and N-specific antibodies of different isotypes were measured during the acute and convalescent phase of measles virus infection. The isotype and kinetics were similar for the antibody response to all three proteins.

Historical records show that N-specific antibodies are both the first to appear and the most abundant (177;184). This was confirmed in the IgM assays, where low-positive signals were more readily detected to N than to F or H, both in the acute (152) and the convalescent phase. However, it should be noted that direct comparison between these fundamentally different assays in a quantitative sense is impossible. Comparison between the F- and H- specific responses, which were measured in identical assays, showed similar responses to both proteins for IgM, but higher responses to F than to H for total IgG, the IgG subclasses and IgA. The F- and H-genes used to obtain the transfected cell lines were derived from the Edmonston measles virus strain (genotype A) (139), while the infants of the present cohort study were infected with wild-type measles virus belonging to genotype B3 (158). Since the H

gene is more variable than the F gene (1), some B cell epitopes may have changed in the B3 H protein, precluding detection of antibodies to these epitopes in the assay used. This difference could perhaps be measured more easily for IgG than for IgM antibodies because of their higher avidity.

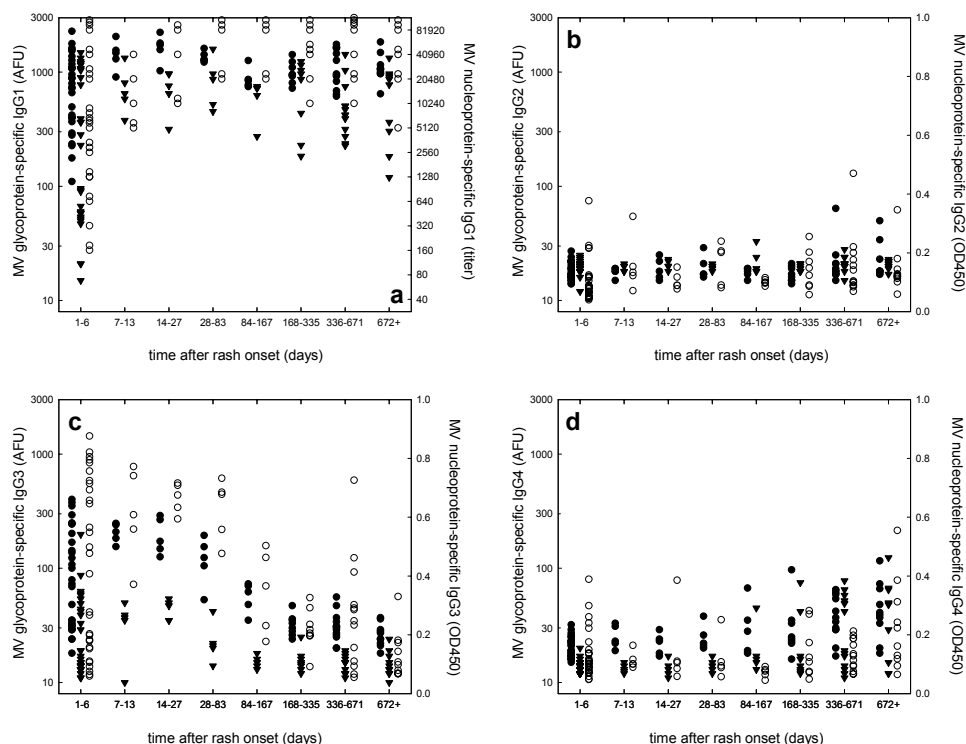


Figure 5

Measles virus F- (black circles), H- (black triangles) and N- (white circles) specific IgG1 (a), IgG2 (b), IgG3 (c) and IgG4 (d) responses in plasma samples collected from laboratory-confirmed measles patients for whom follow-up samples were available.

The kinetics of measles virus-specific IgM responses was largely as expected: specific IgM was detectable in all samples for four weeks after onset of rash (48;51). However, in some patients N-specific IgM could be detected until more than six months after onset of rash. This demonstrates the risk of using highly sensitive IgM detection assays for laboratory diagnosis in areas where measles remains endemic (147;152). One subject had an unexplained measles virus-specific IgM antibody response to all three proteins in samples obtained one and two years after laboratory-confirmed measles.

Measles virus-specific IgG antibodies were detected in all acute phase samples and peaked two to three weeks after infection. Measles virus N-

specific IgG antibody avidity increased during the first three months after infection, associated with a slight decline in IgG levels. This could be due to the loss of the less avid IgG antibodies. Some patients had high avidity antibodies even during the acute or early convalescent phase. It is tempting to speculate that this may have been the result of a previous measles vaccination, resulting in a secondary immune response associated with a transient IgM response and a rapid IgG response of high avidity.

Measles virus-specific IgA antibodies were not detectable in all acute phase samples, which could be related to the kinetics of this response. Specific IgA was only detectable in samples with high IgG levels, suggesting that these antibodies appear slightly later than specific IgM and IgG. The samples collected one to four weeks after onset of rash were all IgA positive. Afterwards IgA levels declined, but not to background levels such as IgM. In some late convalescent samples the IgA levels seemed slightly higher again. This confirms the limited diagnostic value of measurement of serum IgA antibodies (58).

Similar to the majority of respiratory virus infections, the predominant measles virus-specific IgG subclasses during the acute phase were found to be IgG1 and IgG3. IgG1 peaked 1-2 weeks after onset of rash and remained high thereafter, while IgG3 levels declined during the first year post infection. Interestingly, IgG3 and IgA showed a strong correlation, and both could be detected only in samples with high IgG levels. This does not support the hypothesis that IgG3 antibodies would appear earlier than IgG1 (57). We found only few samples with measles virus-specific IgG4 antibodies. During the acute phase there was a small subset of samples in which some F- or N-specific IgG4 could be detected. Interestingly, IgG4 levels seemed to be higher in late convalescent phase samples. It has been suggested that IgG4 is related to prolonged or repeated exposure to antigen (185), suggesting that IgG4 could be a marker of either persistent measles virus infection or a secondary immune response to subclinical re-infection. Prolonged measles virus-specific IgG4 responses were also shown in subacute sclerosing panencephalitis (SSPE) patients (56). A similar pattern was shown for Puumala virus, where specific IgG4 antibodies were detected two years post infection and remained present for ten years (186). Almost no measles virus protein-specific IgG2 antibodies were detected in the acute or memory phases. IgG2 antibodies are almost exclusively polysaccharide-specific, and thus less important in antiviral responses (187). However, RSV-specific IgG2 antibodies were described against the G protein and not the F protein, which was explained by the heavy glycosylation of the G protein (188). A more recent study showed a transient measles virus-specific IgG2 antibody response during the acute phase of measles virus infection (189), which could not be confirmed in the present study.

In conclusion, the present study provides a comprehensive perspective on the specific antibody response to natural measles virus infection. The fact that data obtained using fundamentally different assays (e.g. immunofluorescence versus capture ELISA) showed a good correlation,

strengthens the results obtained. The possible activation of measles virus-specific IgG4 antibodies in some of the late convalescent samples could be indicative of persistence of virus or viral antigens or recurrent subclinical measles virus infections, and deserves further attention. Since selection of antibody isotype seems mostly influenced by the geography of antigen encounter (190), these aberrant IgG subclass responses could perhaps provide new clues concerning the pathogenesis of measles.

Acknowledgements

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Chapter 4

Measles virus infection in macaques

Chapter 4

Infection of cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) with different wild-type measles viruses

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Manuscript in preparation

Abstract

Experimental measles virus (MV) infections in macaques have been used as animal models for vaccination and immunopathogenesis studies. However, a number of studies suggested that rhesus monkeys (*Macaca mulatta*) displayed more characteristic clinical features of measles than cynomolgus monkeys (*Macaca fascicularis*). In the present study we have infected both animal species with two different wild-type MV strains, and compared clinical, virological and immunological parameters. The viruses used were a genotype C2 virus isolated in the Netherlands in 1991 (MV-Bil) and a genotype B3 virus isolated from a severe measles case in Sudan in 1997 (MV-Sudan). All the rhesus monkeys developed skin rash and conjunctivitis upon infection, which was less obvious in cynomolgus monkeys. Measurement of body temperature using transponders showed no or very mild fever in either species. In both animal species similar MV-specific IgM and IgG responses were detected. Virus re-isolation profiles from peripheral blood mononuclear cells and broncho-alveolar lavage cells suggested that viraemia appeared earlier and lasted longer in animals infected with MV-Sudan than in animals infected with MV-Bil. This was also reflected by the earlier appearance of MV-specific IgM antibodies in monkeys infected with MV-Sudan than in animals infected with MV-BIL. Collectively, these data show that MV-Sudan is more pathogenic for non-human primates than MV-Bil, which may render it more suitable for use in future pathogenesis studies.

Introduction

Measles remains one of the most important causes of vaccine-preventable deaths in developing countries (3). A safe and effective live-attenuated measles virus (MV) vaccine is available, which has successfully been used to achieve elimination of endemic MV transmission in large parts of the world (191).

To improve vaccination coverage and efficacy in tropical countries with limited infrastructure, mass vaccination campaigns can be highly effective. However, injection safety and safe waste disposal in these countries where infections with HIV and hepatitis B virus are often highly prevalent is difficult to achieve. Therefore, the World Health Organisation (WHO) is currently evaluating alternative non-parenteral routes of administration of the existing live-attenuated MV vaccine (121;122).

Measles is associated with a transient immunosuppression, which accounts for a large part of the associated morbidity and mortality. Paradoxically, the disease also results in lifelong immunity, resulting in an epidemiological pattern of childhood infections in areas of MV endemicity. The pathogenesis of measles and the associated immunosuppression are still poorly understood (2), although the recent identification of signalling lymphocyte activation marker (SLAM, CD150) as a cellular receptor for MV infection has provided new insights (16;18). Besides SLAM and CD46, which was previously identified as receptor for laboratory-adapted and vaccine strains of MV (11), it is suspected that other modes of entry and/or other receptors may exist (24;192).

For measles pathogenesis studies or evaluation of alternative MV vaccination strategies, animal models are required. Rodents are usually not susceptible to infection with wild-type MV strains, with the exception of cotton rats (87;88) and SCID mice xeno-grafted with human cells (193). The only animals with similar susceptibility to MV infection as humans are non-human primates (94). In recent years, macaque models have been developed and used for vaccination and pathogenesis studies (95;102;120;194). Although both rhesus and cynomolgus macaques were used, clinical symptoms like rash and conjunctivitis were especially reported in rhesus macaques (102).

Due to differences in preparation of stocks of challenge virus, origin of animals and experimental procedures, it is often difficult to compare results obtained by different research groups. We therefore decided to study MV infection "back to back" in both animal species. A second objective of the study was to compare the differences in pathogenesis of infection with two wild-type MV isolates. The first wild-type MV strain used was MV-Bil, a genotype C2 virus isolated in the Netherlands in 1991 and used as challenge virus in both cynomolgus and rhesus macaques by us and others (95;100;102). The second MV strain was a genotype B3 virus isolated from a severe measles patient in Khartoum in 1997 (MV-Sudan). In the present

study, clinical parameters, MV replication kinetics and antibody responses were monitored over time.

Materials & Methods

Macaques

The study was performed in seven rhesus monkeys (*Macaca mulatta*) and eight cynomolgus monkeys (*Macaca fascicularis*). The animals were juvenile (2-4 years) and seronegative for measles as determined by virus neutralisation. Body temperatures were measured by telemetry (195), the resulting data were first averaged per animal per hour and subsequently per group per hour. The study was approved by the animal ethics committee, and performed according to guidelines for animal experimentation.

Viruses

Two wild-type MV-strains were used for experimental infection of macaques: three rhesus and four cynomolgus macaques were infected with MV-Bil (MVi/Bilthoven.NET/91, genotype C2), while four rhesus and four cynomolgus macaques were infected with and MV-Sudan (MVi/Khartoum.SUD/34.97/2, genotype B3, see section 2.3 table 1). Both viruses were isolated from peripheral blood mononuclear cells of a measles patient in human B-lymphoblastoid cell lines (BLCL). A third passage in BLCL was used for infection. The macaques were intra-tracheally infected with 10^3 CCID₅₀ in a volume of 5ml phosphate-buffered saline (PBS).

Samples

EDTA blood samples were collected at days -6, 3, 6, 9, 13, 17, 24 and 30 after infection. Plasma was separated by centrifugation, heat inactivated (30 minutes 56°C) and stored at -20°C. PBMC were isolated by density gradient centrifugation, resuspended in RPMI-1640 supplemented with antibiotics and heat-inactivated foetal bovine serum (R10F), counted and used fresh for virus isolation (see below). Broncho-alveolar lavages (BAL) were collected on days -6, 3, 6, 9, 13 and 17 after infection, by intra-tracheal infusion of 10ml PBS through a flexible catheter. Recovered BAL fluid was centrifuged, and BAL-cells were resuspended in R10F, counted and used fresh for virus isolation.

MV isolation

MV was isolated in BLCL using an infectious centre test as previously described (103). Briefly, 3.2×10^5 PBMC (first stimulated with phytohaemagglutinin-L for one hour at 37°C) or BAL cells were transferred to eight wells in the first column of a 96-wells round bottom plate (each well containing 4×10^4 cells). Subsequently, two-fold dilutions were prepared to obtain a cell density gradient of 2×10^4 to 10 cells. Subsequently, BLCL were added (1×10^4 cells/well) and plates were incubated at 37°C. Cytopathic changes were monitored by light microscopy after co-cultivation for 3 to 6

days. Numbers of cells resulting in 50% of the cultures becoming infected were calculated using the formula of Reed and Muench (142).

MV-specific antibody responses

MV F- and H-specific IgM and IgG antibodies were determined in plasma using an immunofluorescence assay using transfected human melanoma cell lines as targets, as described previously (139). As conjugates FITC-labelled rabbit anti-human IgM or IgG (F(ab')₂ fragments, DAKO, Glostrup, Denmark) were used which were shown to cross-react with macaque antibodies (139).

MV nucleoprotein (N)-specific IgM was measured in a capture ELISA as previously described (196). Capturing plates were coated with a polyclonal anti-human IgM serum (Meddens Diagnostics, Vorden, The Netherlands), which was shown to cross-react with macaque IgM. Specific signals were detected with a recombinant baculovirus-produced purified N preparation (kind gift of Dr. T.F. Wild, Lyon, France) which was peroxidase labelled by Meddens Diagnostics.

N-specific IgG responses were measured in an indirect ELISA using baculovirus-produced purified N, as previously described (196). As conjugate peroxidase-labelled rabbit-anti-human IgG (DAKO) was used, which cross-reacts with macaque IgG.

Haematology

White blood cell counts were measured using an automated haematology analyzer (Sysmex, Renton, Washington). Thin blood films were prepared from EDTA blood, and coloured with Giemsa stain (Merck). Differential cell counts were obtained by counting 500 cells per slide, and numbers of lymphocytes, neutrophils, eosinophils and monocytes were calculated by multiplying these percentages with the white blood cell counts obtained for the same sample.

Statistical analysis

Virus loads were summarised by the area under the curve (AUC, above the detection limit 3) between day 0 and 17, using the trapesoidal method of numerical integration after natural logarithmic transformation. The effects of animal species and virus strain were estimated using multiple linear regression analysis. The hematologic parameters and antibody responses were analysed using mixed model analysis of variance (ANOVA) with explanatory factors time, species and virus strain, and with the baseline measurement of each variable as continuous covariate.

Results

Clinical features

All rhesus monkeys developed a skin rash and conjunctivitis between one and two weeks after infection. Levels and kinetics differed between

animals, but did not seem to be related to the virus strain used for infection. Monitoring of these symptoms was difficult without capturing and sedating the animals, which was only done on the days of sampling. A similar skin rash was seen in a minority of the cynomolgus macaques, although less profound and again not related to one particular virus strain.

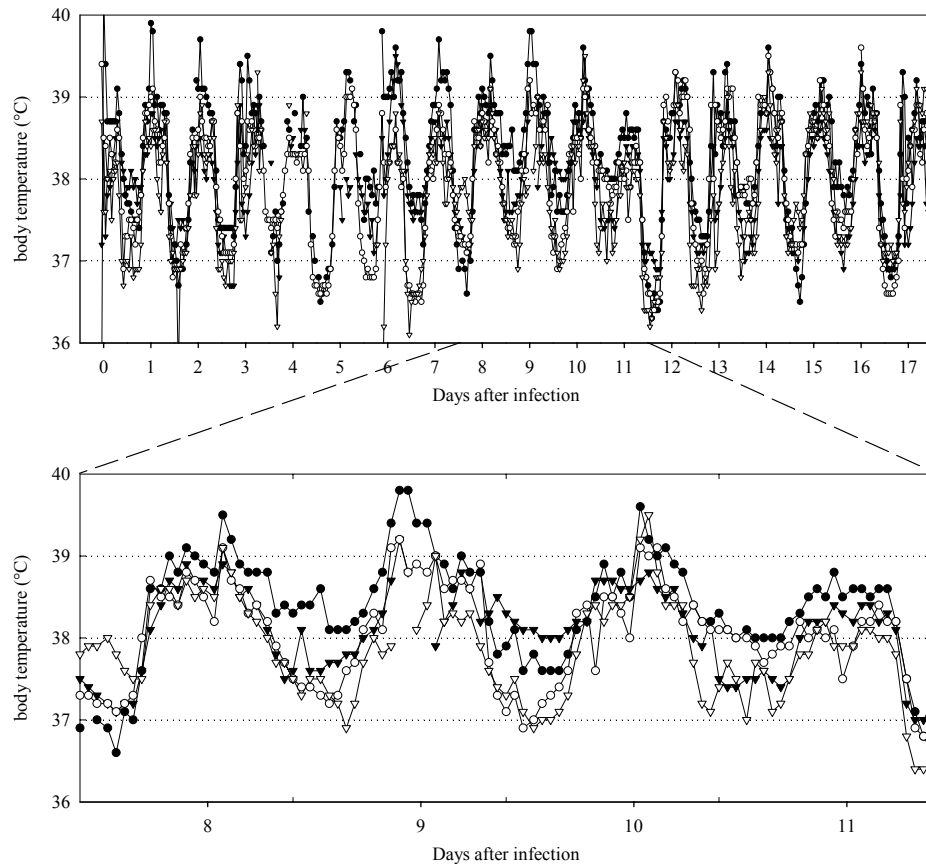


Figure 1

Body temperature as measured by telemetry. Black symbols represent the animals infected with MV-Sudan, white symbols represent animals infected with MV-Bil. Circles represent rhesus macaques and triangles represent cynomolgus macaques. The lower panel represents an enlarged part of the upper panel.

Measurement of body temperatures demonstrated a rhythmic pattern, with mean temperatures fluctuating between 36.5°C at night and 39.5°C during the day (figure 1, upper panel). Between 8 to 11 days after infection a slight (1°C) increase in body temperatures was recorded during the night

time, especially in the animals infected with MV-Sudan (figure 1, lower panel).

Peripheral lymphopenia and neutropenia were observed in all animals between days 6 and 13 (figure 2, upper panels). Numbers of neutrophils measured at day 13 were significantly higher ($p=0.042$) in the rhesus macaques as compared to the cynomolgus macaques. Interestingly, an

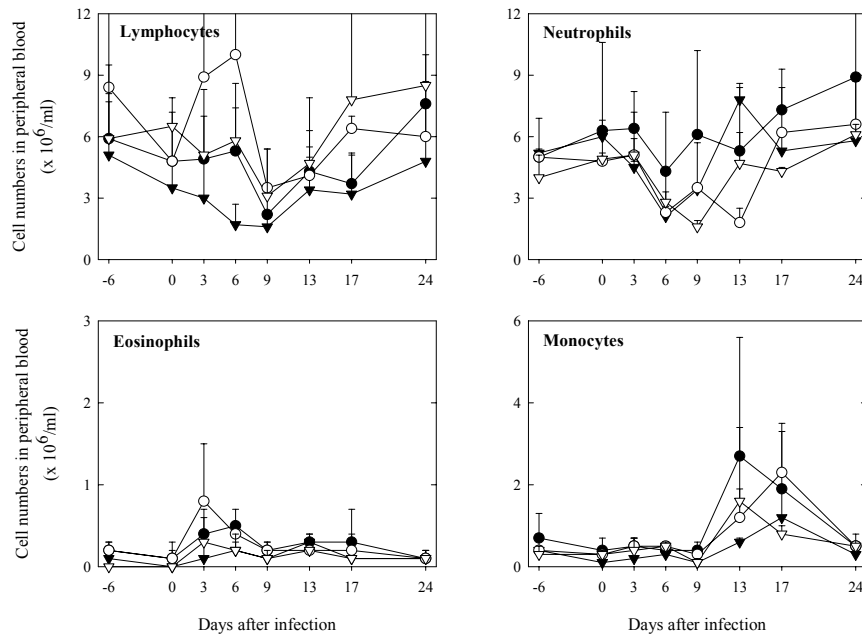


Figure 2

Numbers of lymphocytes, neutrophils, eosinophils and monocytes in peripheral blood at different time points after infection. Symbols and fills are identical to figure 1.

increase in numbers of peripheral monocytes was observed in all animals on days 13 and/or 17 (figure 2, lower right panel), and no significant variation between the different animal species or virus strains was found. No eosinophilia was seen in any of the animals (figure 2, lower left panel).

Virus isolation

Quantification of MV-infected cells in BAL cells and PBMC showed that the peak of virus replication was usually on day 6 after infection for MV-Sudan and on day 9 for MV-Bil. In addition, a trend was detected of higher numbers of infected cells both early (day 3) and late (day 13) after infection with MV-Sudan when compared to MV-Bil (figure 3). This was also reflected by calculations of the areas under the curve between days 0 and 17 (table 1). Virus loads in BAL cells of rhesus macaques infected with MV-

Sudan were significantly higher than in rhesus macaques infected with MV-Bil ($p<0.001$), this effect was not significant in cynomolgus macaques ($p=0.058$). Virus levels in BAL cells were higher in rhesus than in

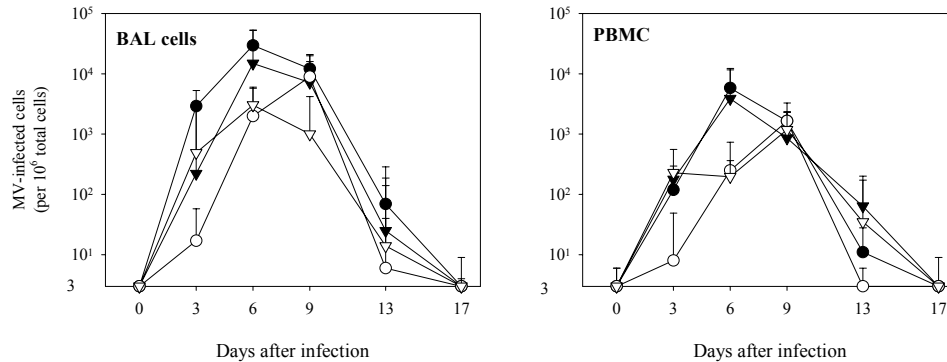


Figure 3

Numbers of MV-infected BAL cells or PBMC per 10⁶ cells. Symbols and fills are identical to figure 1.

cynomolgus macaques infected with MV-Sudan ($p=0.019$), this effect was not found after infection with MV-Bil ($p=0.32$). No significant differences were found in the PBMC compartment between the virus strains or macaque species.

Table 1. MV loads in BAL cells and PBMC, as determined from virus isolation data shown in figure 2 (AUC₀₋₁₇).

Macaque	Virus	BAL cells	PBMC
Rhesus	MV-Sudan	90.5 ± 6.1	61.7 ± 9.0
Cynomolgus	MV-Sudan	76.0 ± 8.4	66.5 ± 16.2
Rhesus	MV-Bil	57.8 ± 5.1	40.3 ± 8.1
Cynomolgus	MV-Bil	64.0 ± 9.0	57.7 ± 15.6

Antibody responses

MV protein-specific IgM antibodies were detected in all animals from days 13 until day 30 after infection (figure 4). N-specific IgM antibodies peaked earlier in the animals infected with MV-Sudan as compared to those infected with MV-Bil (days 9 and 13, respectively), but showed similar kinetics in both macaque species (figure 4, upper left panel). The same pattern could be observed for IgM responses to F and H, which on day 9 were also higher in the animals infected with MV-Sudan than in animals infected with MV-Bil. However, individual variation was substantially higher in these responses.

The kinetics of MV-protein specific IgG antibodies were largely similar in all animals, with responses being detectable from day 13 onward and reaching maximum levels on days 24 (figure 5). Earlier onset of N- and F-specific IgG responses was observed in cynomolgus macaques infected with MV-Sudan, mainly caused by one outlier animal.

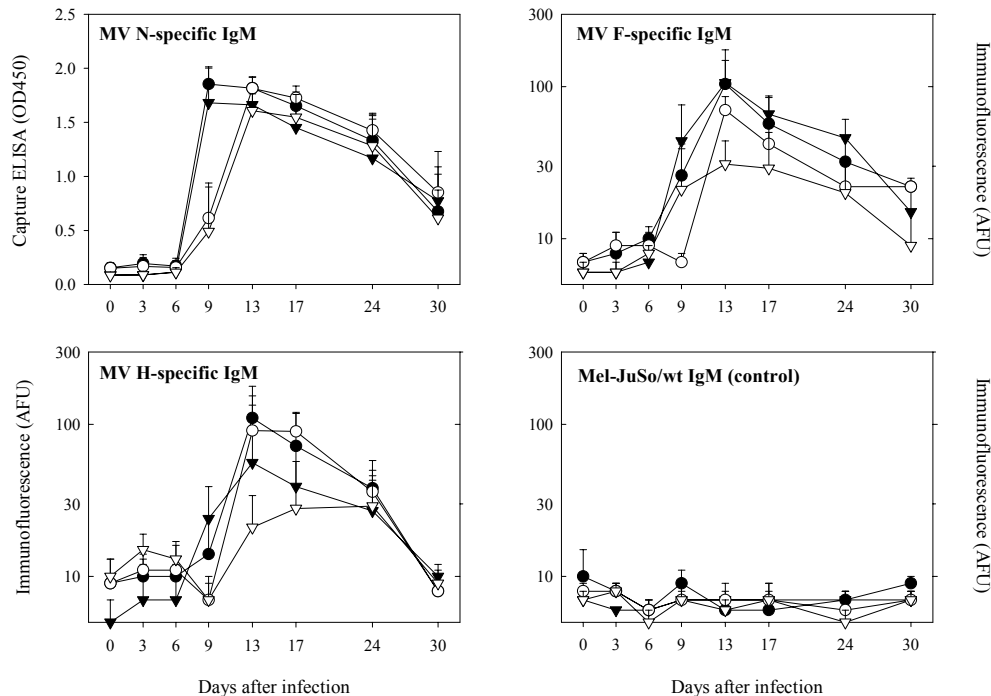


Figure 4

Plasma IgM responses to the MV N protein (upper left), F protein (upper right), H protein (lower left) or untransfected Mel-JuSo/wt cells (lower right, control for F- and H-responses). Symbols and fills are identical to figure 1.

Discussion

In the present study we have demonstrated that rhesus and cynomolgus macaques are equally susceptible to infection with wild-type MV. Previous reports of more distinct skin rash and conjunctivitis in rhesus than in cynomolgus macaques (102) were confirmed, but virological and immunological post-infection parameters were largely similar between the two species. However, MV-Sudan was found more pathogenic than MV-Bil.

Measurement of body temperature in non-human primates using standard methods can be unreliable due to changes in body temperature as a result of the stress associated with capturing the animal. We have previously successfully used telemetry to demonstrate the development of fever after infection of macaques with influenza virus strain H5N1. Indeed, the present study showed changes in body temperature after MV infection, although to different levels in the individual animals. Animals infected with MV-Sudan showed the most significant (approximately 1°C) increase in night temperature during the days immediately after the peak in virus replication.

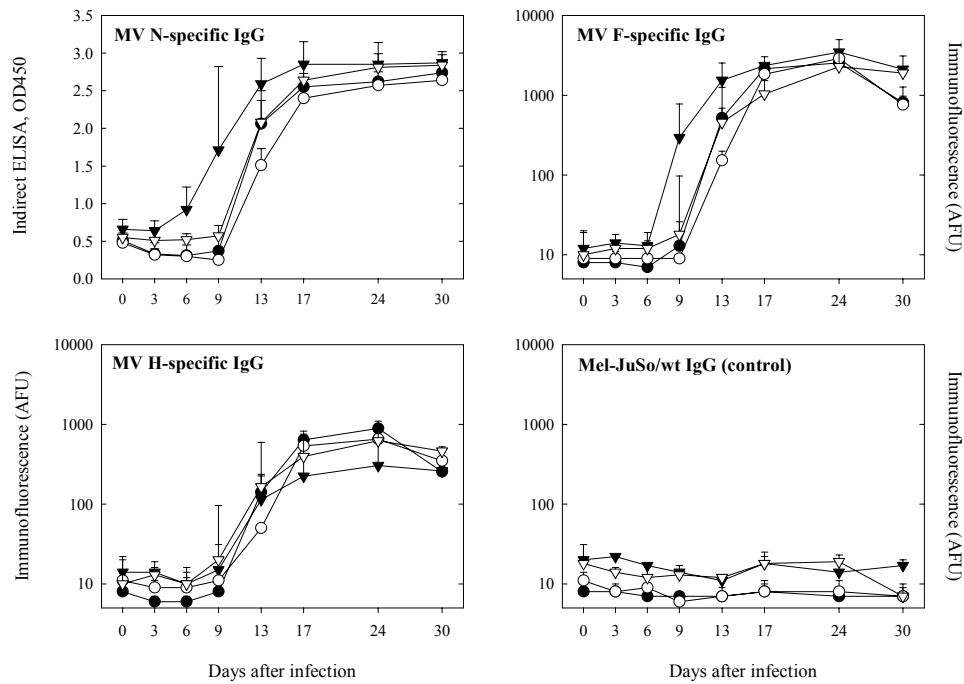


Figure 5

Plasma IgG responses to the MV N protein (upper left), F protein (upper right), H protein (lower left) or untransfected Mel-JuSo/wt cells (lower right, control for F- and H-responses). Symbols and fills are identical to figure 1.

Skin rash and conjunctivitis were more evident in rhesus macaques than in cynomolgus macaques, confirming previous observations (100;102). This was not accompanied by differences in MV replication kinetics or specific antibody responses, suggesting that both animal species were equally susceptible to MV. In addition, haematological parameters measured showed a lymphopenia and neutropenia during the peak of virus replication in all animals, followed by monocytosis a few days later, essentially confirming previous observations (102). The observed clinical differences could either

result from specific cells (e.g. endothelial cells) being susceptible to MV infection in rhesus macaques but not or to a lesser extent in cynomolgus macaques. However, it has been speculated that measles rash and conjunctivitis have an immune-mediated origin, since they are often absent in immunocompromised patients infected with MV (1;174). Therefore, an alternative explanation is that there may be qualitative or quantitative differences in the MV-specific cellular immune response between rhesus and cynomolgus macaques. This hypothesis is currently under investigation.

MV-Sudan proved to be more pathogenic in macaques than MV-Bil, as demonstrated by increased levels of virus replication and more rapid onset of specific IgM responses. This virus was isolated from a severe measles patient in Khartoum in 1997. Measles-associated morbidity and mortality in Sudan is relatively high, with case-fatality rates between 1 and 10% (161). Although MV is a monotypic virus genetic differences exist, and little is known about the biological differences between members of the different clades (141;171).

The present study suggests that the virus strain circulating in Khartoum is more pathogenic than the european strain MV-Bil, which may at least in part account for the observed clinical severity of measles in Sudan.

Acknowledgements

We thank Dr. R.S. van Binnendijk for critical comments to the manuscript, and R. Dias d'Ullois for zootechnical assistance.

Chapter 5

Summarising discussion

Despite the availability of safe and effective live attenuated vaccines, measles remains endemic in many developing countries. Little is known about the pathogenesis of measles virus (MV) infections in the areas of its endemicity, largely due to the limited infrastructure and political instability. Whereas measles in industrialised countries is often considered an “innocent” childhood disease, measles case-fatality rates are often well above 1% in developing countries, and can be even higher in outbreak situations (5). One explanation for this observation is that measles is associated with a transient immunosuppression resulting in an increased susceptibility to other infectious agents: the infectious pressure of many human pathogens is substantially higher in developing countries than in the industrialised world. In combination with vitamin deficiencies (e.g. vitamin A) and inadequate case management this increases the risk of MV infection developing into a serious life-threatening disease (3). However, prior to or co-infections with other pathogens could also play a role in modifying the specific immune response to MV infection. In particular the high prevalence of parasitic infections in many developing countries could affect the development of the cellular immune system towards a dominance of T-helper (Th) cells producing type 2 cytokines such as IL-4, IL-5 IL-10 and IL-13. Such responses have been associated with immunosuppression (197), and counterbalance Th responses producing type 1 cytokines (such as IL-12 and interferon- γ) which sustain cytotoxic T cell (CTL) response and have been associated with clearance of MV infection (198).

Towards the control and eventual eradication of measles more insight will be required in the pathogenesis of measles in areas of its endemicity. However, another reason to study measles in these regions is that it allows the development and validation of alternative surveillance methods. Surveillance will become of crucial importance during the next decade(s), since several organisations including the World Health Organisation (WHO), United Nation Children’s funds (UNICEF), the American Red Cross and the Centres for Disease Control (CDC) have recently joined forces to proceed to a phase of accelerated measles control which should result in a substantial reduction in measles mortality (118), (www.measlesinitiative.org). It has been shown that in areas with decrease MV circulation the clinical diagnosis of measles becomes increasingly unreliable, and other rash-causing diseases such as those caused by infection with rubella virus, parvovirus B19, or human herpesvirus-6 are often mistaken for measles (199).

We have studied diagnostic, virological and immunological aspects of measles in suburban Khartoum, Sudan. Prospective studies were carried out in two cohorts of clinically diagnosed measles cases. In **chapter 2** the diagnostic value of different laboratory methods was evaluated and compared to clinical diagnosis. Sequence information was obtained from wild-type MV strains circulating in Sudan, allowing molecular epidemiological studies. Furthermore, surveillance of measles in Sudan was continued using filter paper blood samples, adding to the field evaluation of this clinical specimen for use in the global measles laboratory network (GMLN). Finally, a real-

time RT-PCR assay was developed for the semi-quantitative detection of MV genomic RNA in clinical samples. In **chapter 3**, MV-specific antibody responses during the acute and convalescent phases of the infection were assessed using clinical specimens. In **chapter 4**, aspects of the immunopathogenesis of a representative wild-type MV isolate from Sudan were studied in a macaque model.

Study design

The patients included in our studies were enrolled through two cohorts of clinically diagnosed measles cases. In the first cohort, 197 infants complying with the WHO clinical case definition of measles (105) were included. Samples were collected within a week after the onset of rash, following informed consent by the parents. Most patients were sampled through a network of active measles case finding, which was set up in the residential area Haj Yousif. This area has an estimated number of 500.000 inhabitants, mainly displaced people from the south and west of Sudan.

University of Khartoum - Institute of Endemic Diseases COHORT B == MEASLES DIAGNOSIS FORM

1. Date of visit (ddmmyy)	_____
2. Patient's number	_____
3. Patient's name	_____
4. Patient's age (months)	_____
5. Patient's sex	1 = Male 2 = Female
6. Village name / number	_____
7. Interviewer's name / number	_____
8. Mother's name	_____
9. Mother's education (number of school years)	_____
10. Father's education (number of school years)	_____
11. Father occupation	_____
12. Family income (dinars)	_____
13. Number of siblings	_____
14. Number of rooms	_____
15. Water source	1 = Piped indoors 2 = Piped outside 3 = Deep wells 4 = Nile 5 = Canals 8 = Other _____
16. Toilet facility	1 = Open space 2 = Communal latrine 3 = Private latrine 4 = Siphon 8 = Other _____
17. Electricity present	0 = No 1 = Yes
18. Household amenities	0 = None 1 = Radio 2 = Refrigerator 3 = TV 4 = Combination 8 = Other _____
19. Transport to health centre	1 = On foot 2 = Cart 3 = Bus 8 = Other _____
20. Distance of house from health centre by usual transport (minutes)	_____
21. Duration of breast feeding (months)	_____
22. History of childhood diseases	0 = None 1 = TB 2 = Whooping cough 3 = Polio 4 = Measles 5 = Diphtheria 8 = Other _____ 9 = Unknown
23. Contact with measles case in:-	0 = None 1 = Household 2 = Neighbours 3 = School 8 = Other _____ 9 = Unknown
24. Measles contact case	1 = Not vaccinated 2 = Vaccinated 9 = Unknown

25. Vaccination status	0 = No 1 = Yes 9 = Unknown
DPT1	_____ Polio1 _____
DPT2	_____ Polio2 _____
DPT3	_____ Polio3 _____
Measles	_____ BCG _____
26. How long ago (in months) was the child vaccinated for measles	_____
27. Vaccination card present	0 = No 1 = Yes
28. Clinical diagnosis of measles: (a) + (b) + one of (c)	
a. generalized rash of 3 or more days duration	1 = Yes 0 = No
b. fever $\geq 38^{\circ}\text{C}$	1 = Yes 0 = No
c. one of the following:	
cough	1 = Yes 0 = No
running nose (coryza)	1 = Yes 0 = No
red eyes (conjunctivitis)	1 = Yes 0 = No
29. If not measles (i.e. # 0), give probable diagnosis	_____
30. Weight (grams)	_____
31. Height (mm)	_____
32. Skin fold thickness (mm)	_____
33. Head / chest ratio	_____
34. State of dehydration	0 = None 1 = Some 2 = Severe
35. Infection present	0 = None 1 = Otitis media 3 = Pneumonia 3 = Gastroenteritis 4 = Convulsions 5 = Eye lesion 6 = Combinations 8 = Other _____
36. Vitamin A given	0 = No 1 = 1-4 days after the rash 2 = Greater than 4 days
37. Laboratory confirmation of measles	
Rapid field test	0 = Negative 1 = Positive
Standard IgM ELISA	0 = Negative 1 = Positive
Virus culture (Blood)	0 = Negative 1 = Positive
Virus culture (Throat swab)	0 = Negative 1 = Positive
38. Managed at	1 = Home 2 = Hospital
39. Outcome at two weeks	1 = Complete recovery 2 = Died 3 = Complications specify _____ 9 = Unknown
40. Comments	_____

Figure 1. Questionnaire used for collection of baseline information

Health care is provided through non-governmental health centres, staffed by nurses, midwives and medical assistants. A number of additional patients were sampled in paediatric hospitals in Khartoum.

The collection of clinical materials continued from April 1997 until June 1999 and 6 additional samples were collected in July 2000. The clinical materials collected included a throat swab, a whole blood sample spotted on filter paper, and a heparinised venous blood sample. From this last sample peripheral blood mononuclear cells (PBMC) and plasma were separated. A subset of the patients were followed up for two years as described in section 3.1.

The second cohort consisted of 316 infants, who were recruited in the same study area following the same inclusion criteria. The only clinical specimens collected were filter paper blood samples, which were collected between October 2000 and April 2003 and stored at 4°C. Baseline information including age, gender, vaccination status, nutritional status, clinical complications, and family background information was recorded for each subject using a questionnaire (figure 1).

Measles in Sudan: diagnosis, vaccination coverage and epidemiology

The gold standard for laboratory diagnosis of measles is detection of MV-specific IgM antibodies (50). Although specific IgM usually appears at the same time as the clinical symptoms, samples collected during the first days after onset of rash may occasionally be IgM-negative (48). In **section 2.1**, the diagnostic value of detection of MV-specific serum IgM, RT-PCR analysis on throat swabs, and MV isolation from PBMC was compared using clinical specimens of 38 clinically diagnosed measles cases. Measurement of serum IgM identified 27/38 (71%) of the cases as acute measles cases with high levels of MV-specific IgM in plasma. In 9/38 (24%) no MV-specific IgM antibodies were detected, demonstrating that in these patients MV was not the cause of the clinical symptoms. In two patients low MV-specific IgM levels were detected, which did not allow conclusive diagnosis. In these patients an extra measurement of IgG or virus neutralising (VN) antibodies in the plasma was necessary to discriminate between patients with an acute MV infection sampled during an early stage of the disease and patients with a history of previous MV infection (figure 2).

The serological laboratory diagnosis was compared with virological diagnosis by virus isolation from peripheral blood mononuclear cells (PBMC) or detection of MV genomic RNA in throat swab materials by RT-PCR. MV-specific RT-PCR analysis correlated 100% with serological diagnosis, as amplicons of the correct size hybridising with a specific probe were obtained from all confirmed cases that were tested by RT-PCR and from none of the non-measles cases. MV isolation proved to be less sensitive as diagnostic method as in 6/23 (26%) of the confirmed cases no MV could be isolated. The laboratory confirmation of clinical measles in our cohort was thus defined by:

- high MV-specific IgM levels in plasma; OR:
- intermediate to low MV-specific IgM levels in plasma accompanied with low serum IgG or VN antibody levels and positive RT-PCR signals in throat swab samples.

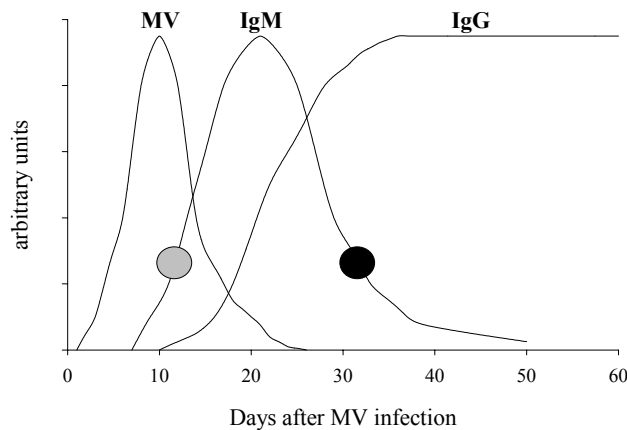


Figure 2

Schematic representation of the kinetics of MV replication and MV-specific IgM and IgG responses. The grey dot indicates low IgM levels during the acute phase of infection, coinciding with detectable MV and low IgG levels. The black dot represents a IgM levels during the convalescent phase of infection, coinciding with high IgG levels and undetectable MV.

In **section 2.2**, the same criteria were used to diagnose the rest of the cohort (n=192) of clinical measles patients, of whom 145 (76%) were confirmed to be patients with an acute MV infection. In 45 cases (24%) the clinical symptoms proved to have other causes. In some of the non-measles patients IgM antibodies specific for rubella virus, human herpesvirus type 6, Epstein-Barr virus, dengue virus, sandfly fever virus or *Rickettsia* species could be detected.

Furthermore, the results of a study assessing the value of dried blood spots collected on filter paper as alternative samples for measles diagnosis were reviewed (111). In this study (not part of the present thesis) it was shown that blood samples spotted on filter paper are suitable for the laboratory diagnosis of measles using a combination of IgM detection and RT-PCR analysis. In addition, sequencing of the RT-PCR amplicons also allows phylogenetic analysis of the causative MV strain.

The longevity of the RT-PCR signals from filter paper was assessed using different storage conditions, and it was then applied to filter paper blood samples collected from clinically diagnosed measles patients in Sudan. Positive RT-PCR signals were detected in 48% of the laboratory confirmed cases, and in none of the 27 non-measles cases.

MV-specific IgM levels measured in reconstituted filter paper samples correlated well with those measured in serum samples. Measles diagnosis based on the combination of filter paper RT-PCR and IgM detection had a sensitivity of 99% and specificity of 96%. The adaptation of in-house RT-

PCR assays to the amplification of MV genome from filter paper blood samples was also described by others (112). The use of FP samples for detection of MV-specific IgM and/ or IgG using commercial validated assays is also well described (114;115). However, the evaluation of filter paper blood samples in a field setting remained essential.

The vaccination status, and basic epidemiological parameters of the clinical measles cohort study in Khartoum was summarised in section 2.2. As evidenced by inspection of a vaccination card, high coverage of DTP, polio and BCG vaccines was demonstrated, but rather low measles vaccine coverage (see table 1, section 2.2). The vaccination coverage in the non-measles rash disease patients, which could represent a random sample of the population, was found to be 70%. This correlated reasonably well with the most recent measles vaccination coverage data reported for Sudan by the Expanded Programme for Immunisation (EPI) and WHO, which was a national coverage less than 70% (148), (132). Measles vaccination coverage was surprisingly high (59%) in the confirmed measles cases.

The median age of the laboratory-confirmed measles cases in the cohort was 24 months (range 5 - 168). Of 91 confirmed measles cases that were followed up for more than three months, 34 (37%) recovered without complications, 48 (53%) developed complications and 9 (10%) died during the first month after measles (161).

Frequently observed complications included gastro-enteritis and pneumonia. It should be acknowledged that in this cohort a number of patients were included from different pediatric hospitals in Khartoum, resulting in an increased frequency of severe measles cases and thus an over-estimation of the case-fatality rate. The Sudanese Ministry of Health recently reported hospital based measles case-fatality ratios of 1-2.8% (132).

In **section 2.3** partial nucleoprotein (N) gene sequences were determined of 41 wild-type MV sequences: 33 from virus isolates and 8 directly from clinical specimens obtained in Khartoum between July 1997 and July 2000. The majority of the sequences (n=18) were from 1997, the others from 1998 (n=10), 1999 (n=8) and 2000 (n=6). The sequences obtained showed high homology with a 0-1.3% divergence throughout the 3 year study period. Half of the point mutations were silent. One mutation (¹³⁷⁰A→G) was found to be more present in the later sequences: 5/9 sequences from 1998, 7/8 sequences from 1999, 5/6 sequences from 2000 and none of the 1997 sequences. The observed high homology was also confirmed by sequencing of the haemagglutinin (H) gene of two representative sequences from 1997 and two from 2000, showing an overall divergence of 0-0.3%. Collectively, these results demonstrated the endemic circulation of a single strain in Khartoum.

Alignment of the obtained sequences with reference sequences of the MV genotypes showed that the Sudanese sequences belonged to genotype B3. Comprehensive comparison of the obtained sequences with all published

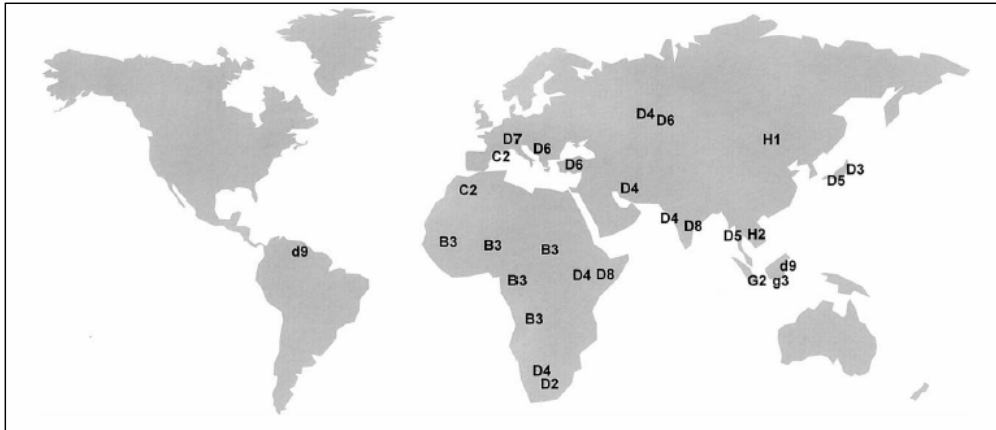


Figure 3

Geographic distribution of MV genotypes associated with endemic transmission based on information available in 2002. Taken from Rota and Bellini, *J Infect Dis* 2003; 187:S270-S276.

clade B sequences showed that they clustered with isolates from genotype B3 cluster 1 which originated from Nigeria (151). Genotype B3 was originally assigned to central and western Africa (42;141;150), but our studies demonstrate the endemic circulation of genotype B3 in eastern Africa as well (figure 3). Although the reference strain of the genotype was isolated in New York and epidemiologically linked to Kenya (141), recent field studies from Kenya demonstrate the endemic circulation of genotype D4 (43).

In **section 2.4**, dried blood spots collected on filter paper were evaluated for surveillance of measles in suburban Khartoum. The GMLN has identified MV- specific IgM detection as the preferred method for measles laboratory diagnosis (108;109). In addition, during the initial phase of the measles control campaign the emphasis will be on outbreak diagnosis rather than diagnosing every single measles patient (108). The laboratory confirmation in our cohort study was therefore conducted by detection of MV-specific IgM antibodies only, and not following the criteria described in section 2.1. Using an in-house MV-specific IgM assay 63% of the patients were confirmed as measles patients. The percentage IgM positives was lower in samples collected in the first three days after onset of rash than in those collected afterwards (see table 1, section 2.4). This confirms previous observations, which show that detection of MV specific IgM has highest sensitivity in the second week of infection (200). In addition, it also suggests that the total percentage of true measles cases in the cohort should be higher than 63%.

IgM detection as performed by the in-house capture ELISA (stated above) was compared to and shown to be more sensitive than the Dade Behring indirect IgM ELISA (advised by the WHO). A recent evaluation of different commercial measles IgM ELISAs also showed that the capture assays tested were more sensitive than the indirect assays. However, the indirect assays proved to be more specific, resulting in higher positive predictive values (160). Since the emphasis is on outbreak diagnosis, the use of either type of assay would actually result in similar conclusions.

The longevity of IgM signals obtained from filter paper blood samples was assessed in a subset of the cohort, and showed that the signal remained detectable for at least 16 months at 4°C. Previous studies using the indirect ELISA approach reported stability of IgM signals for a period of six months at 4°C (114). A possible explanation for the observed prolonged stability in our study may be the use of a capture ELISA, in which some degradation of the total IgM would not affect test results. In contrast, in an indirect assay a slight degradation of the total IgM may be expected to immediately result in a decrease of the ELISA signal.

The percentage of vaccinees in the laboratory confirmed cases in the filter paper cohort was 43%, thus lower than the previously reported 59% (section 2.2), possibly indicating an increase in vaccination efficacy. However, it still raises serious concerns about the efficacy of measles vaccination in the area.

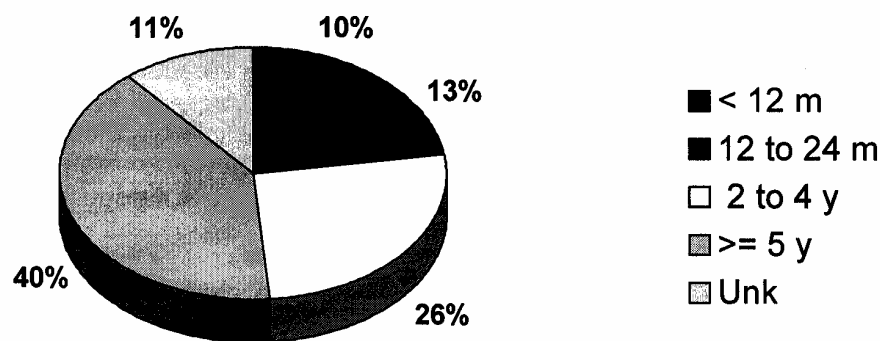


Figure 4

Age distribution of measles patients in Sudan, 2002. Taken from "Measles mortality reduction in Sudan", WHO/EMRO Technical Report, 21-29 September 2003.

The median age of laboratory confirmed cases in this cohort was found to be 48 months as compared to 24 months in the previous cohort (section 2.2). This increase in median age may indicate the effect of vaccination, in accordance with a recent WHO report showing an increase in measles cases among patients ≥ 5 years after year 1989 (figure 4). This indicates that a

single vaccination targeting children under 5 years of age will still leave a gap of susceptible individuals in children < 15 years of age, and stresses the need of a second vaccination (132).

In figure 5, the measles patients sampled in Khartoum are plotted by month of sample collection. Black bars represent laboratory-confirmed cases: before 2000 by measurement of MV-specific IgM in plasma and/or RT-PCR as described in section 2.2, after 2000 by measurement of MV-specific IgM in filter paper blood samples as described above. Grey bars represent the

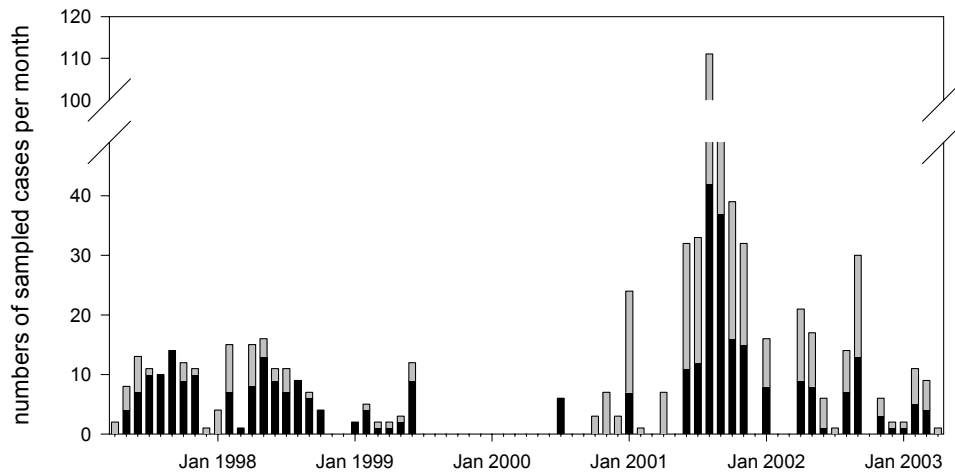


Figure 5

Measles patients sampled in Khartoum by month of sampling. Black bars indicate laboratory-confirmed measles cases.

remaining clinical measles cases. Active surveillance was not equally intensive during all periods, and virtually absent from June 1999 until September 2000. However, the picture indicates that MV was circulating throughout the year. Incidence peaks could be detected, mostly during the late summer or early fall, coinciding with the rainy season.

Partial N gene sequences were obtained from 15 samples over the 3 year study period (6 from 2001, 6 from 2002 and 3 from 2003). Phylogenetic analysis of these sequences confirmed the continued circulation of MV belonging to genotype B3 cluster 1 in Khartoum. The results also confirmed the homology in Sudanese wild-type MV sequences within a single transmission chain as described in section 2.3. When combining the sequences described in both sections, a maximum divergence of 2% over a seven year study period was found. The sequences contained 12 set-specific mutations, five of which were shared with the previous Sudanese sequences.

The ¹³⁷⁰A→G mutation described above (section 2.4) was fixed in all sequences collected after 2000.

Interestingly, the set-specific mutations occurred in a specific pattern, as some mutations were linked and were apparently fixed over time whilst others were not observed again (figure 6), possibly indicating the presence of multiple transmission lines. Emergence of multiple transmission lines within a genotype in situations of endemic transmission was previously reported (157). However, the driving forces behind these phenomena are still unknown.

Development of a real time RT-PCR for the detection of MV

In **section 2.5** the development of a MV-specific semi-quantitative real-time RT-PCR assay is described. The assay was compared to the previously established conventional RT-PCR as described in section 2.1. Based on a serial dilution of the live-attenuated MV Edmonston-Zagreb vaccine, the detection limits were approximately 0.1 and 0.02 cell culture infectious dose 50% units (CCID₅₀) per test for the conventional and TaqMan RT-PCR assays, respectively. The TaqMan assay was linear within a range of 10^{4.4} to 10^{-0.6} CCID₅₀/ml, with an intra-assay variability lower than 3% and an inter-assay variability ranging from 1.5% at 10^{4.4} CCID₅₀/ml to 8.7% at 10^{-0.6} CCID₅₀/ml.

The TaqMan assay could detect representative wild-type viruses from the currently active MV clades, and could detect MV genome in clinical specimens obtained from measles patients.

Quantification of MV RNA in peripheral blood mononuclear cells or broncho-alveolar lavage cells from cynomolgus macaques collected at different time points after experimental infection showed a good correlation with virus isolation data. The TaqMan assay developed was, thus, specific, sensitive, rapid and reproducible, and can be of use for diagnostic purposes or for studies on the pathogenesis of measles. Real-time detected RT-PCR methods allowed the quantitative detection of RNA sequences (164;165). In combination with automated RNA isolation technologies these methods allow processing of large amounts of samples in a short period of time with a minimal risk of cross-contamination. The quantitative aspect of the assay is less important in the diagnosis of measles than in the case of chronic virus infections (165-167). However, in pathogenesis studies the ability to quantify MV genomes may be of interest.

MV-specific antibody responses

In **chapter 3**, MV protein-specific antibodies of different isotypes were measured during the acute and convalescent phase of MV infection. According to the detection of MV-specific IgM in plasma the samples were divided into three groups: IgM high-positive samples, IgM-negative "non-measles rash disease" samples and samples with low or borderline IgM levels

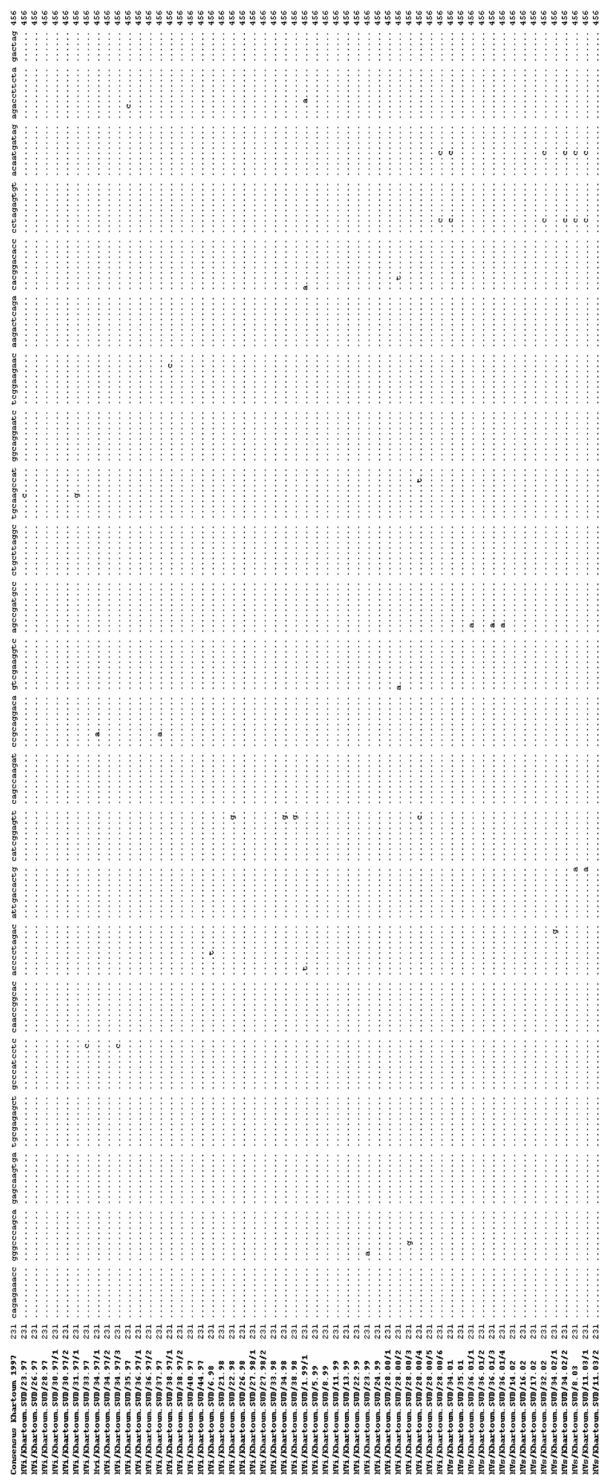


Figure 6
Alignment of 3' partial MV N gene sequences (456nt)
from suburban Khartoum, 1997-2003

as described in sections 2.1 and 2.2. A number of MV IgM high positive patients were followed up for two years, during which samples were collected at different time intervals after onset of disease. The IgM-negative samples were also included as clinical controls for the acute phase measurements. However, the patients with low or borderline IgM values were excluded from analysis to obtain a homogeneous set of acute phase samples.

Isotype kinetics were studied for antibodies specific to the N protein, the H protein and the fusion (F) protein, and proved highly similar. However, in samples with low IgM levels N-specific IgM antibodies were usually more readily detectable than F- or H-specific IgM (see section 2.2, figure 1). This was also reflected in the longevity of the N-specific IgM response in comparison to the F and H responses, and is in accordance with historical records which show that N-specific antibodies are both the first to appear and the most abundant (177;184).

Comparison between the F- and H-specific responses, which were measured in identical assays, showed similar responses to both proteins for IgM, but higher responses to F than to H for IgG and to a lesser extent IgA. The F- and H-genes used to transfect the cell lines used in these immunofluorescence assays were derived from the Edmonston measles virus strain (genotype A) (139), while the infants of the present cohort study were infected with wild-type MV belonging to genotype B3 (sections 2.3 and 2.4). Since the H gene is more variable than the F gene (1), some B cell epitopes may have changed in the B3 H protein, precluding detection of antibodies to these epitopes in the assay used. This difference could perhaps be measured more easily for IgG than for IgM antibodies because of their higher avidity.

The kinetics of MV-specific IgM responses was as expected: specific IgM was detectable in all samples for four weeks after onset of rash (48;179). In some patients the N-specific IgM could be detected until almost a year after onset of rash, demonstrating the risk of using highly sensitive IgM detection assays for laboratory diagnosis in areas where measles remains endemic.

Measles virus-specific IgG antibodies were detected in all acute phase samples and peaked two to three weeks after infection. Measles virus N-specific IgG antibody avidity increased during the first three months after infection, associated with a slight decline in total IgG levels. This could be due to the loss of the less avid IgG antibodies. However, in a substantial number of patients antibodies of high avidity were detected during the acute and early convalescent phase. In some cases this could be explained by previous MV vaccination, resulting in a secondary immune response associated with a transient IgM response and a rapid IgG response of high avidity. In other cases it may however also be the result of very low initial IgG levels, precluding the accurate measurement of antibody affinity.

Measles virus-specific IgA antibodies were not detectable in all acute phase samples, which could in part be related to the kinetics of this response. Specific IgA was only detectable in samples with high IgG levels, suggesting that these antibodies appear slightly later than specific IgM and IgG. The

samples collected one to four weeks after onset of rash were all IgA positive. Afterwards IgA levels declined, but not to background levels such as IgM. In some late convalescent samples the IgA levels seemed slightly higher again. This confirms the limited diagnostic value of measurement of serum IgA antibodies (58), and may suggest the importance of IgA in the memory phase.

The predominant MV-specific IgG subclasses during the acute phase were found to be IgG1 and IgG3. IgG1 peaked 1-2 weeks after onset of rash and remained high thereafter, while IgG3 levels declined during the first year post infection. Interestingly, IgG3 and IgA showed a strong correlation, and both could be detected only in samples with high IgG levels. This does not support the hypothesis that IgG3 antibodies would appear earlier than IgG1 antibodies (57). MV-specific IgG4 antibodies were rarely found in the acute phase, in contrast to observations in historical records on measles (1). However, the levels increased in the late convalescent phase samples. It has been suggested that IgG4 is related to prolonged or repeated exposure to antigen (185), suggesting that IgG4 could be a marker of either persistent measles virus infection or a secondary immune response to subclinical re-infection. Prolonged measles virus-specific IgG4 responses were also shown in subacute sclerosing panencephalitis (SSPE) patients (56). Almost no measles virus protein-specific IgG2 antibodies were detected in the acute or memory phases. IgG2 antibodies are almost exclusively polysaccharide-specific, and probably less important in antiviral responses (187). A more recent study showed a transient MV-specific IgG2 antibody response during the acute phase of MV infection (189), which could not be confirmed in the present study.

MV infection in macaques

Experimental MV infections in macaques have been used as animal models for vaccination and immunopathogenesis studies. Some studies suggested that rhesus monkeys displayed more characteristic clinical features of measles than cynomolgus monkeys (102). In **chapter 4**, rhesus and cynomolgus macaques were infected with wild-type MV strains from Europe (MV-Bil, genotype C2) or Sudan (MV-Sudan, MVi/Khartoum.SUD/34.97/2 genotype B3, see section 2.3, table 1). Clinical, virological and immunological responses were compared between the animal species and between the viruses.

Most rhesus monkeys developed skin rash and conjunctivitis upon infection, which was less evident in cynomolgus monkeys. Measurement of body temperature using transponders did not show substantial fever in either species.

In both animal species similar virus-specific MV-specific IgM and IgG responses were detected. Virus re-isolation from peripheral blood mononuclear cells and broncho-alveolar lavage cells did not differ significantly between the two animal species, but suggested that viraemia appeared earlier and was more prolonged in the animals infected with MV-

Sudan than in animals infected with MV-Bil. This was also reflected in the earlier appearance of MV-specific IgM antibodies in monkeys infected with MV-Sudan than in animals infected with MV-BIL.

Collectively, these data suggest that MV-Sudan may be more pathogenic for primate species than MV-Bil, and this difference might have played a role in the observed high measles case-fatality rates in Sudan.

Conclusions

The studies presented in this thesis have provided more insight in the value of laboratory methods for the diagnosis of measles, epidemiological aspects of the disease in Khartoum and the virus-specific humoral immune response in humans and in experimentally infected non-human primates.

The diagnostic studies have emphasised the importance of laboratory confirmation of the clinical diagnosis of measles. Several laboratory assays for the diagnosis of measles were evaluated and used in a surveillance study in Khartoum. It was shown that finger prick blood spots dried on filter paper provide a good substrate for serological and molecular diagnosis of measles in outbreak situations. The surveillance study also showed that both vaccination coverage and effectiveness should be improved in the study area. The MV strain that circulated in the Khartoum area was shown to belong to genotype B3 and displayed little genetic variation over time.

The studies described in chapter 3 provided a comprehensive insight in the MV protein-specific antibody response. The fact that data obtained with fundamentally different assays (e.g. immunofluorescence versus capture ELISA) showed a good correlation strengthened the results obtained.

The studies in two different macaque species, infected with either a European or a Sudanese MV isolate, showed that the latter is more pathogenic for both species, which may render it more suitable for use in future vaccination and pathogenesis studies.

Finally, these studies have resulted in a strengthening of the laboratory infrastructure for measles research, diagnosis and surveillance in Sudan. This may provide an additional stimulus and opportunity for the ongoing efforts in Sudan to combat measles.

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Nederlandse Samenvatting

Ondanks de beschikbaarheid van een veilig en effectief vaccin, blijft mazelen één van de belangrijkste oorzaken van kindersterfte in ontwikkelingslanden. De Wereld Gezondheids Organisatie (WHO) schat dat er in 2002 777,000 kinderen zijn overleden aan mazelen, waarvan 84% leefden in slechts 11 landen in Afrika en Azië (Afghanistan, Burkina Faso, Democratische Republiek Congo, Ethiopië, India, Indonesië, Niger, Nigeria, Pakistan, Somalië en Uganda). Ook in een groot aantal andere landen in deze regio is de vaccinatiegraad niet hoog genoeg om te voorkomen dat het mazelen virus (MV) in de gemeenschap blijft circuleren.

Mazelen gaat gepaard met een verzwakking van het afweersysteem, die enkele weken tot maanden na het doormaken van de ziekte kan aanhouden. Deze immuunsuppressie verklaart grotendeels het veelvuldig voorkomen van complicaties als longontsteking, middenoorinfectie en maag-darminfecties. In de geïndustrialiseerde wereld wordt mazelen vaak beschouwd als een relatief onschuldige kinderziekte, hoewel ziekenhuisopnames veelvuldig voorkomen en de sterfte wordt geschat tussen 1:1000 en 1:10000. In ontwikkelingslanden hebben mazelenpatiënten vaak minder toegang tot adequate medische zorg, en is de infectiedruk van andere pathogene micro-organismen hoger. Als gevolg hiervan is de sterfte aan mazelen veel hoger: in Soedan wordt deze geschat tussen 1:10 en 1:100.

Dit verschil in ernst van de ziekte illustreert dat mazelen zich in ontwikkelingslanden anders manifesteert dan in geïndustrialiseerde landen. De WHO streeft naar een reductie in mazelengerelateerde mortaliteit (www.measlesinitiative.org), en op lange termijn mogelijk naar wereldwijde controle van het virus. Om dit te kunnen bereiken is meer inzicht nodig in de epidemiologie en pathogenese van mazelen in gebieden waar de infectie gepaard gaat met hoge mortaliteit. Een tweede reden om mazelenonderzoek in deze regio's te doen is dat het de mogelijkheid biedt om nieuwe surveillance methoden te ontwikkelen, te valideren en te implementeren.

Diagnostiek van mazelen in ontwikkelingslanden is vaak alleen gebaseerd op herkenning van klinische symptomen. De definitie van klinisch mazelen is "iedere persoon met een gegeneraliseerde grofvlekkige rode huiduitslag, een geschiedenis van koorts (meer dan 38°C) en ten minste één van de volgende symptomen: hoesten, loopneus of conjunctivitis (rode ogen); of: iedere persoon waarbij een medisch gekwalificeerd persoon mazelen vermoedt". In het verleden is echter aangetoond dat andere ziekten gemakkelijk verward kunnen worden met mazelen, zoals rode hond (rubella virus), de "vijfde ziekte" (veroorzaakt door parvovirus B19) of de "zesde ziekte" (veroorzaakt door humaan herpesvirus type 6). Daar staat tegenover dat een MV infectie in mensen met een verzwakt afweersysteem zeer ernstige klinische symptomen kan veroorzaken in afwezigheid van de karakteristieke huiduitslag, waardoor de ziekte vaak niet herkend wordt.

In dit proefschrift zijn een aantal studies naar diagnostische, virologische en immunologische aspecten van mazelen in Soedan beschreven. Deze studies zijn uitgevoerd in het kader van een samenwerking tussen de Universiteit van Khartoum en het Erasmus MC in Rotterdam, en voor een deel gefinancierd door een subsidie van het INCO-DC programma van de Europese Gemeenschap. In Khartoum werden in twee prospectieve cohort-studies bloedmonsters en keelwabs van klinisch gediagnosticeerde mazelenpatiënten verzameld. Deze cohort-studies werden uitgevoerd in de wijk Haj Yousif, een gebied waarin naar schatting 500,000 vluchtelingen uit het zuiden en westen van Soedan wonen. In aanvulling hierop werden ook klinische monsters verzameld van mazelenpatiënten in kinderziekenhuizen in Khartoum. Alle materialen werden verzameld in de eerste week na het verschijnen van de huiduitslag. Deze klinische materialen vormden de basis voor de in dit proefschrift beschreven studies.

Na **hoofdstuk 1**, waarin een algemene inleiding wordt gegeven op het onderwerp van dit proefschrift, volgt in **hoofdstuk 2** een aantal studies waarin diagnostische en epidemiologische aspecten van mazelen in Soedan zijn bestudeerd.

In **sectie 2.1** wordt een studie beschreven naar de vergelijking tussen de klinische en de laboratorium diagnose van mazelen. Van 38 klinisch gediagnosticeerde mazelenpatiënten kon in 28 gevallen bevestigd worden dat het om een acute MV infectie ging, terwijl in 10 patiënten de klinische symptomen een andere oorzaak bleken te hebben. Alle patiënten konden worden gediagnosticeerd op basis van alleen het meten van MV-specifieke antistoffen in plasma. In de meeste gevallen was meting van alleen MV-specifiek IgM voldoende. In twee gevallen werden laag positieve specifieke IgM responsen gemeten, en was een aanvullende bepaling van MV-specifieke IgG antistoffen of van MV neutraliserende (VN) antistoffen nodig om de diagnose te bepalen. Tijdens de eerste dagen na het verschijnen van de huiduitslag kan de concentratie MV-specifieke IgM antistoffen nog laag zijn, maar in dat geval zijn ook de concentratie specifieke IgG en VN antistoffen nog laag. Indien de patiënt in het recente verleden mazelen heeft gehad (bijvoorbeeld 1 tot 3 maanden voorafgaand aan de huidige ziekte), kunnen ook nog lage concentraties MV-specifiek IgM worden gemeten, maar dan in aanwezigheid van hoge concentraties MV-specifieke IgG en VN antistoffen. Naast serologie werden ook virusisolatie uit lymfocyten en amplificatie van MV genomische sequenties met behulp van RT-PCR op keelwabs monsters getest als mogelijke diagnostische methoden. Virusisolatie bleek wel specifiek maar niet erg gevoelig, maar diagnostiek gebaseerd op RT-PCR bleek in alle gevallen overeen te komen met de serologische diagnose. RT-PCR bleek daarom een interessant alternatief voor monsters waarin diagnostiek op basis van serologie niet meteen duidelijkheid biedt.

In **sectie 2.2** zijn de in sectie 2.1 ontwikkelde criteria gebruikt voor het diagnosticeren van 192 klinisch mazelen patiënten, waarvan 145 (76%) een acute MV infectie bleken te hebben terwijl in 45 gevallen (24%) de symptomen een andere oorzaak hadden. In aantal sera van deze laatste groep

konden IgM antistoffen worden aangetoond tegen rubellavirus, humaan herpesvirus type 6, Epstein-Barr virus, derdedaagse koorts (sandfly fever) virus en *Rickettsia* species. Daarnaast werd een overzicht gegeven van resultaten verkregen in andere deelprojecten van het samenwerkingsverband tussen de Universiteit van Khartoum en het Erasmus MC. Eén van deze studies beschreef de diagnostische waarde van ingedroogde bloeddruppels verzameld op filterpapier. In het verleden was al beschreven dat deze monsters gereconstitueerd kunnen worden, waarna virus-specifieke IgM antistoffen kunnen worden aangetoond. In aanvulling hierop kon worden aangetoond dat deze monsters ook gebruikt konden worden voor het amplificeren van MV genomische sequenties met behulp van RT-PCR. In vitro studies toonden aan dat MV genomisch RNA afhankelijk van de omgevingstemperatuur voor enkele weken tot enkele maanden stabiel blijft in deze monsters. Ook in filterpapier bloedmonsters verzameld in Khartoum konden zowel MV-specifieke IgM antistoffen als RT-PCR signalen worden aangetoond.

In **sectie 2.3** werden gedeeltelijke sequenties van het gen coderend voor het RNA-bindend eiwit (het nucleoproteïne of N eiwit) bepaald voor 41 MV patiënten uit Khartoum uit de periode 1997-2000. Hoewel MV een monotypisch virus is, levert de sequentievariatie in dit deel van het virale genoom een "vingerafdruk" van het virus. Door deze sequenties te vergelijken met sequenties van MV uit andere delen van de wereld, kan de phylogenetische verwantschap worden bepaald. Op deze manier zijn acht genetische hoofdgroepen of "clades" gedefinieerd, en meer dan twintig subgroepen ("genotypes"). Deze groepen zijn tot op zekere hoogte geografisch gerestricteerd, waardoor het plaatsen van een virus in één van deze groepen inzicht kan geven in de herkomst. Het gebruik van dit type informatie wordt ook wel "moleculaire epidemiologie" genoemd. In de studie in sectie 2.3 kon worden aangetoond dat alle 41 sequenties zeer sterk verwant waren, en behoorden tot het genotype B3. Na vergelijking met andere sequenties bleken de Soedanese virussen het sterkst verwant aan virussen geïsoleerd in Nigeria. Deze resultaten werden bevestigd door het bepalen van de volledige sequentie van het gen coderend voor het haemagglutinine eiwit voor een beperkt aantal virussen. Deze resultaten toonden aan dat MV inderdaad endemisch is in Khartoum.

In **sectie 2.4** is de surveillance van mazelen in Khartoum beschreven op basis van alleen filter papier bloedmonsters voor de periode 2001-2003. Van 316 patiënten konden in 200 gevallen (63%) MV-specifieke IgM antistoffen worden aangetoond. Door deze test voor een subset van de monsters een jaar later te herhalen kon worden aangetoond dat IgM antistoffen in filterpapier bloedmonsters opgeslagen bij +4°C meer dan een jaar stabiel blijven. Van 16 monsters verdeeld over 2001, 2002 en 2003 werden N gen sequenties bepaald zoals in sectie 2.3, waarmee kon worden aangetoond dat het genotype B3 MV nog steeds endemisch aanwezig was in Khartoum.

In **sectie 2.5** is de ontwikkeling van een kwantitatieve RT-PCR test beschreven voor mazelen. Op grote laboratoria wordt de moleculaire

diagnostiek van virusinfecties zo veel mogelijk geautomatiseerd uitgevoerd. Voor mazelen is een test ontwikkeld op basis van geautomatiseerde RNA isolatie met behulp van de MagNa Pure in combinatie met "real-time" detectie van MV in een ABI 7700 TaqMan. De assay kon gebruikt worden om MV van de verschillende MV clades aan te tonen, met lage inter-assay en intra-assay variatie.

In **hoofdstuk 3** is de MV eiwit-specifieke antistof respons gedurende de acute en convalescente fase van de infectie bestudeerd. IgM, IgA, IgG en IgG subklasse antistoffen tegen het fusie-eiwit (F), haemagglutinine (H) en nucleoproteïne (N) werden eerst voor alle patiënten gemeten tijdens de acute fase, en daarna voor een geselecteerd aantal laboratorium-bevestigde MV patiënten op verschillende tijdstippen na het verschijnen van de klinische symptomen (van enkele weken tot twee jaar later). Deze studie biedt nieuwe inzichten in de serologische respons na een MV infectie, welke kennis gebruikt kan worden voor zowel diagnostische doeleinden als studies naar de pathogenese van mazelen.

In **hoofdstuk 4** zijn experimentele MV infecties uitgevoerd in apen. Hiervoor zijn twee MV isolaten gebruikt: MV-Bil is een virus dat in 1991 werd geïsoleerd tijdens een mazelen uitbraak in Bilthoven, en MV-Sudan is een virus dat in 1997 werd geïsoleerd uit een ernstig zieke mazelenpatiënt in Khartoum (beschreven in sectie 2.1). Met deze twee virussen werden twee verschillende diersoorten geïnfecteerd: de Java aap en de rhesus aap. Infecties met MV-Bil in Java apen worden op het Erasmus MC gebruikt als diemodel voor mazelen pathogenese- en vaccinatie studies. De rhesus aap werd hiermee vergeleken aangezien in de recente wetenschappelijke literatuur is beschreven dat MV infectie in deze diersoort meer typische klinische verschijnselen van mazelen zou kunnen veroorzaken. De eerste doelstelling van de studie was dan ook om gevoeligheid van de twee diersoorten te vergelijken. Naast MV-Bil werd MV-Sudan gebruikt voor infectie om verschillen in pathogeniteit tussen de twee virussen te bestuderen. MV-Bil is een genotype C2 virus, dat begin jaren '90 circuleerde in grote delen van Europa. Dit virus was dus geassocieerd met relatief lage morbiditeit en mortaliteit, in tegenstelling tot het genotype B3 virus uit Khartoum. De tweede doelstelling van deze studie was daarom om na te gaan of deze verschillen konden worden teruggevonden in de pathogeniciteit van deze virussen voor apen. Rhesus apen vertoonden na MV infectie inderdaad meer zichtbare klinische symptomen dan Java apen: zowel huiduitslag en conjunctivitis werden gezien na infectie met beide virussen in rhesus apen maar in slechts zeer beperkte mate in Java apen. In beide diersoorten kon slechts in geringe mate koorts worden gemeten met behulp van radiotransponders: de meeste temperatuursverhoging (ca 1°C) werd 's nachts gemeten in rhesus en Java apen geïnfecteerd met MV-Sudan. In virusisolatie uit lymfocyten en long lavage cellen bleken weinig verschillen tussen de twee diersoorten, maar werden wel verschillen gevonden tussen de twee virussen. Zowel in Java als in rhesus apen werd meer en langduriger virus geïsoleerd na infectie met MV-Sudan dan na infectie met MV-Bil. MV-

specifieke IgM en IgG antistof responsen lieten weinig verschil zien. MV N-specifieke IgM antistoffen iets eerder aantoonbaar in dieren geïnfecteerd met MV-Sudan, hetgeen waarschijnlijk kan worden als een bevestiging voor de meer uitgebreidere replicatie van dit virus in de apen.

Hoofdstuk 5 is een samenvattende discussie. De studies beschreven in dit proefschrift verschaffen meer inzicht in de laboratoriumdiagnostiek van mazelen, epidemiologische aspecten van mazelen in Khartoum, en de MV-specifieke antistof respons in mensen en experimenteel geïnfecteerde apen. Daarnaast heeft het uitvoeren van deze studies geleid tot een versterking van de laboratorium infrastructuur in Khartoum ten behoeve van mazelen onderzoek en surveillance, hetgeen kan bijdragen aan de bestrijding van mazelen in Sudan.

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Curriculum Vitae

The author of this thesis was born on August 27, 1975, in Maryland (U.S.A). After finishing her B.Sc. honours (first class) in Zoology at the University of Khartoum (Sudan) in November 1996, she was appointed as a teaching assistant at the department of Zoology of the same university. In April 1997 she joined the measles project "Contribution to the elimination of measles from east Africa". In October 1999 she defended her M.Sc. thesis, during the period of which she conducted part of her laboratory work at the department of Virology, Erasmus University Rotterdam. In February 2000, she started her Ph.D. research at the department of Virology of the Erasmus University. This work was conducted under the supervision of Prof. Dr. A. D. M. E. Osterhaus and Dr. Rik L. de Swart, and resulted in the present thesis.

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