AUS DEM LEHRSTUHL
FÜR MEDIZINISCHE MIKROBIOLOGIE UND HYGIENE
PROF. DR. DR. ANDRÉ GESSNER
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

Measles virus neutralization assay with endpoint determination using RT-qPCR

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

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Tag der mündlichen Prüfung:	31.03.2025

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Zusammenfassung

Das Masernvirus ist ein humanpathogenes Paramyxovirus, welches vermutlich vom Rinderpestvirus der frühen Antike abstammt. Es ist hochkontagiös und löst bei Menschen die Masern aus, eine Erkrankung, welche mit schweren Komplikationen und Todesfällen einhergehen kann. Seit 1963, also 60 Jahren, ist ein wirksamer, sicherer und kostengünstiger Impfstoff verfügbar, welcher attenuierte, lebende Masernviren des Stammes Edmonston enthält. Seit den 90er Jahren wird die regionale Elimination der endemischen Masern weltweit als Ziel der Staaten aller WHO-Regionen angestrebt. Durch die Einführung der Routineimpfung und ergänzende Impfaktivitäten wurden weltweit wesentliche Fortschritte zur Bekämpfung der Masern erreicht. Laut Schätzung der World Health Organization (WHO) konnten durch diese Impfungen im Zeitraum 2000-2021 mehr als 56 Millionen Todesfälle verhindert werden. Dennoch kommt es weltweit immer noch zu großen Masernausbrüchen, auch in Deutschland. Daher ist ein Schutz gegen Masern wichtig und es bestehen weiterhin verschiedenste Fragestellungen im klinischen Alltag.

Hierbei müssen auch Patienten und Patientinnen beachtet werden, die keinen Impfschutz aufbauen oder wegen vorliegender Kontraindikationen nicht geimpft werden können. Gerade wenn die Impfung mit Lebendviren ein gesundheitliches Risiko darstellt, sollten nach Kontakt zu Masernviren bei fehlendem Impfschutz Therapieoptionen sorgfältig abgewogen werden. Neben einer Impfpasskontrolle kann der Frage nach einer Immunität gegen Masern mittels ELISA-Verfahren nachgegangen werden. Hierbei werden IgG-Antikörper gegen das Masernvirus gemessen. Bei negativem Ergebnis aber, also im ELISA-Verfahren nicht nachweisbaren IgG-Antikörpern, kann ein Neutralisationstest, der FRNT, durchgeführt werden. Hierbei werden neutralisierende Antikörper gegen das Masernvirus im Blut gemessen. Selbst bei negativem ELISA kann ein positiver FRNT-Test Immunität und somit Schutz vor Erkrankung nachweisen. Die Messung neutralisierender Antikörper ist somit für Fragestellungen in Diagnostik und Forschung von großem Interesse. Der als Goldstandard verwendete FRNT wurde durch die WHO standardisiert und wird über fünf bis sechs Tage durchgeführt, eine Zeitspanne, welche im klinischen Alltag entscheidend sein kann. Gemessen wird der Titer neutralisierender Antikörper durch die Reduktion einer auf dem Zellrasen getesteten Virusinfektion mit Masernviren.

Mit einem alternativem Masernvirus-Neutralisationstest, genannt NT-qPCR, welcher durch Endpunktbestimmung mittels Polymerase Kettenreaktion (PCR) ausgewertet wird, würde sich die Laufzeit des Testes verkürzen.

Zu diesem Zweck wurde eine RT-qPCR etabliert. Die relative Quantifizierung wurde mit Hilfe von Masernvirus-RNA oder spezifisch hergestellter DNA-Fragmente als Standard durchgeführt. Der FRNT wurde mit den Masernvirus-Genotypen Edmonston wild-type (Genotyp A) und MVi/Offenburg.DEU/10.19 (Genotyp D8) eingestellt. Weiterhin wurde zur Endpunktbestimmung des Testes die Extraktion der Masernvirus-RNA oder die Lyse mit direkter Weiterverwendung des Lysates in der RT-qPCR verglichen. Die Neutralisationstiter wurden mittels linearer Regression, sowie mittels nicht-linearer Regression und gewählter dose-response-inhibition-curve berechnet. Alle NT-qPCRs wurden zunächst mit einer im ELISA IgG-positiv getesteten (20-00848) und einer IgG-negativ getesteten (20-00762) Probe durchgeführt, um Vergleichbarkeit über alle getesteten Varianten hinweg herzustellen. Als Referenz wurde in den NT-qPCRs mit beiden Erntevarianten, Extraktion und Lyse, der für den FRNT entwickelte 3. Internationale Standard der WHO, sowie käufliches Immunglobulin (KEDRION) mit bekanntem FRNT-Titer eingesetzt.

Diese Arbeit soll durch die Etablierung eines Neutralisationstestes mit Endpunktbestimmung mittels RT-qPCR wesentlich zum Übergang hin zu einer zeitnahen, quantitativen Bestimmung sero-protektiver Titer Masernvirus-neutralisierender Antikörper beitragen. Methodische Fragen und Details, welche durch weiterführende Experimente innerhalb eines Anschlussprojektes noch zu bearbeiten sind, werden aufgezeigt. Der NT-qPCR könnte im klinischen Alltag die Klärung eines Immunitäts-Status gegen Masernviren für gefährdete Patientengruppen erheblich beschleunigen und ein hilfreiches Instrument für Forschungsfragen sein.

1 Introduction

1.1 Measles virus infection

Measles, caused by the infection with the measles virus (MeV), is a contagious disease causing thousands of deaths worldwide each year (1). Even though a MeV infection is vaccine-preventable (2,3), measles remains a significant risk for serious complications and death (1), especially for unvaccinated young children (4) and immunodeficient patients (5). During and after measles, patients additionally suffer from immunosuppression and so-called immune amnesia (6). This immune deficiency is described lasting for weeks up to three to five years after infection (7,8), making patients vulnerable to other infections and severe complications (9). A live attenuated measles vaccine has been used for 60 years (10); it is safe, effective, and inexpensive (2,3). The World Health Organization (WHO) defines measles elimination as '[t]he absence of endemic measles transmission in a defined geographical area (e.g. region or country) for ≥12 months in the presence of a well-performing surveillance system [...]' (11, p.91) and aims to eliminate endemic measles transmission regionally and eventually eradicate measles through routine immunization and supplemental immunization with the attenuated vaccine (2,3,12).

1.1.1 Pathogenesis

Measles is transmitted by airborne droplets through the respiratory tract (13) and can infect humans within an incubation period of nine to 19 days (13,14). Early symptoms, called the prodromal state, are cough, coryza, conjunctivitis, and the characteristic white Koplik´s spots seen on the buccal mucosa (13,15). The prodromal state is accompanied by a high fever for four to six days (4,14,15). The prodromal state is followed by a maculopapular skin rash starting behind the ears and spreading over the face throughout the body within two to three days (13–15). After two to three days, accompanied by fever, the skin rash disappears (13,14). The characteristic measles rash is associated with an immune response and may be absent in immunocompromised patients (13,16).

Most severe complications affect infants under five years of age or adults over 30 years of age (4). The most common complications are pneumonia (5,14,15,17), otitis media (5,14,15,17), laryngotracheobronchitis (5,15,17), blindness (4,17), and severe

diarrhea (5,14,17). In addition, due to the ability of MeV to infect neuronal cells, measles can cause different types of encephalitis (5,14,15,17), which are described in detail in Table 1.

Table 1. *Measles related encephalitis. Table is modified after (5,13,14,17,18). SSPE is a notifiable disease since the 1st of march 2020 (19).*

	ADEM	MIBE	SSPE
	= Acute para-/postinfectious or autoimmune encephalitis	= Acute progressive infectious encephalitis/ acute inclusion body encephalitis	= Subacute sclerosing panencephalitis
Incidence	1:1000-2000	1:1500-2000	1:10 000-25 000
Patients' characteristics	children > 2 years	patients with immunodeficiency	more males than females; MeV infection of the patients happened < 2 years
Appearance	simultaneously with or one week after the rash	6-10 months after measles disease	6-15 years after measles disease
Pathogenesis	autoimmune reactions leading to demyelination in the brain	MeV as pathogen spreads in cell-containing regions of the brain	infection of endothelial brain cells, followed by the brain cells
Lethality	10-20%	100%	100%

Two different pathways are discussed for the MeV entry into the host. Although MeV is transmitted over the respiratory tract, the entry does not happen through the respiratory epithelium, which is explained by missing $CD150^+$ (CDw150 receptor) (20,21) and the lack of nectin-4 expression (22,23) on the apical surface of epithelial cells. After having contact with a contagious person, the primary infection takes place in the lower respiratory tract (20) through binding to $CD150^+$ alveolar macrophages and DC-$SIGN^+$ (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) dendritic cells (20,21,24). A second, but less important way for MeV entry is the infection of myeloid or lymphoid cells of the lamina propria in the conjunctiva (25,26).

After the primary infection, cells migrate to tertiary lymphoid tissues like the bronchus-associated lymphoid tissue (BALT) or draining lymph nodes (20,21,24).

Once MeV infected cells reach the lymphatic system, CD150$^+$ lymphocytes become infected with the virus and spread to all lymphoid tissues (24,27). As a consequence, up to 10% of T-lymphocytes and 30% of B-lymphocytes can be affected (24,27). During this viremia, MeV infected lymphocytes spread throughout the body and proliferate in various organs, including the lungs and skin, causing generalized lymph node swelling (7,20,24,27). The lung damage leads to cough and, due to the immunosuppression accompanying the infection, to a predisposition for superinfection with other pathogens (7,8,24). Similarly, the infection of CD150$^+$ alveolar macrophages and DC-SIGN$^+$ dendritic cells in gut-associated lymphoid tissue (GALT) leads to a predisposition for superinfections in the intestinal system (24). Nectin-4$^+$ dermal endothelial cells (28) and keratinocytes (24,29) get infected and the following virus-specific T-cell infiltration for clearance of the infected cells leads to the characteristic maculopapular rash (24,30). Thus, the rash marks an immune response to the MeV infection that has occurred and, if absent, is an indicator for a deficient immune system (13,16).

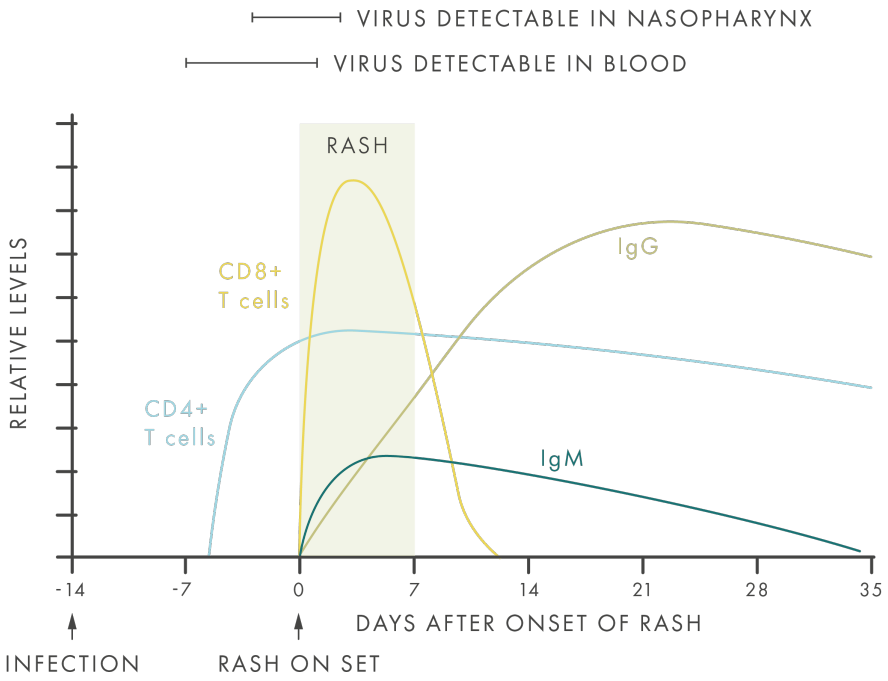

Figure 1 Clinical measles appearance and induced immune response. *The clinical appearance of measles as described in 1.1.1 with the characteristic exanthema and the time-related immune reaction can be seen here. A detailed description of the immune response and thus the function of CD4+/CD8+ T cells, IgM and IgG is further described in 1.3. A direct pathogen detection in case of disease by polymerase chain reaction (PCR, described in 2.2.5.5) is limited in time and ends around the onset of the rash. The figure is modified after (31–34).*

Furthermore, a transient immune suppression is described after MeV infections (6,9). Besides the predisposition for opportunistic infections in guts and airways (7,8,24), an immune deficiency was observed until weeks up to three years after the infection (7,9). Through various approaches, such as B- and T-cell depletion (24), measles disease leads to a kind of immune amnesia (7–9,24) of the previously established protection against pathogens after the acute symptoms have subsided. This can result in severe courses of infections with pathogens (7–9).

1.1.2 Therapy and prevention

Currently, there is no effective approved or off-label treatment for measles disease available (15). Therefore, the clinical management of MeV infections is symptomatic treatment, in addition to important interventions such as isolation and monitoring of the patient (17). For children under the age of five, an application of two doses vitamin A is recommended to prevent ophthalmic complications (17). If patients are not vaccinated, measles is one of the rare diseases for which an effective post-exposure prophylaxis (PEP) by passive immunization has been established (17,35,36).

The guidelines for Germany given from the Standing Committee on Vaccination (‘Ständige Impfkommission’, STIKO) and the Robert Koch-Institute (RKI) for PEP recommend the following (35,36): For the passive immunization, immunoglobulins obtained from human donors are applied intravenously with 1x 400mg/kg body weight dose. The application should follow as soon as possible, preferably within six days post-exposure. PEP is given as an off-label use and is developed for patients with contraindications for the vaccination. Concerned patient groups are pregnant women, patients with immunodeficiency, or infants under six months of age.

Infants between six and eight months of age can be actively vaccinated in off-label use. A vaccination should not be given for up to eight months after PEP (35,36).

The WHO recommends vaccination with two doses of the combined measles, mumps, rubella and varicella virus (MMR/V) vaccine to eradicate measles and to prevent severe courses (3,5,10,12,37,38). Several MeV vaccines are in use, which are all based on Edmonston wild-type MeV and contain attenuated live MeV strains (5,10,39). Attenuation was achieved by passaging in different cells (5,39). Edmonston wild-type MeV was first cultivated in 1954 and named after the blood donator and patient David

Edmonston (40,41). Following vaccine strains, including the common strain Edmonston-Schwarz (EdmS), were all based on Edmonston MeV strains, as described in 1994 (5,39). For complete active immunization, two doses of measles containing vaccine (MCV) are given, either subcutaneously or intramuscularly (10,36). The second dose (MCV2) is for patients with failed first dose (MCV1) vaccination (5). For sufficient immune protection of a population, called herd immunity, a vaccination rate with MCV2 of >95% is necessary (5,10).

In Germany, two vaccination doses are recommended by the STIKO and the RKI (36): The first dose is given at eleven months of age and the second dose at 15 months of age. The minimum interval between the two doses is four weeks. Vaccination for infants with nine months of age is recommended if the infant is either staying in a community facility or has had contact to a measles patient. Carefully decided, infants at six to eight months of age can be vaccinated with the first dose in off-label use (36).

1.1.3 Epidemiology

Between 2010 to 2012, the WHO aimed to not only reduce measles cases, but to eliminate measles by 2020 in five out of six WHO regions (3,11,37,42). By 2019, the WHO reported significant success, with declining measles incidence and deaths, while coverage with both MCV1 and MCV2 increased globally (2,43). The Global vaccine action plan (3) led to a 62% decrease of annual measles deaths in 2019 (43), while 85-86% (1,2,43) of children worldwide received MCV1 and 71% MCV2 (1,2,43). However, a significant turnaround was seen between 2016 and 2019, with 567% increase in measles incidence and nearly 50% in the mortality (43). Moreover, no WHO region worldwide has been able to achieve and maintain measles elimination in the long term (1,12). In addition, the COVID-19 pandemic starting in 2019 disrupted vaccination activities in many countries and measles surveillance became a lower priority (1,44), resulting in the risk of missing vaccination for over 178 million people (12). Subsequently, the elimination goal was repeatedly renewed (12,38,45,46) and despite the turnaround, seen over the last 21 years, measles vaccination prevented an estimated 56 million deaths (1).

In Germany, the Ministry of Health elaborated additional goals for elimination of measles in 2015:

- Increasing the acceptance of vaccination in the public and the general population
- Achieving 95% MCV1 coverage among infants at the age of 15 months
- Achieving 95% MCV2 coverage among children before school entrance
- Achievement and maintenance of measles elimination status through interruption of transmission chains
- Increasing of reported laboratory-confirmed measles and rubella cases up to 80% and improving communication between the local level and national surveillance (47).

Since March 2020, the measles protection act makes measles vaccination mandatory for most of the population groups in Germany: Children attending community facilities and adults born after 1970, who work or live in medical or community facilities, are supposed to be either proven immune or proven vaccinated (19).

Evaluating these numbers and efforts, MeV vaccination is an effective and economical method (2,3), preventing millions of deaths worldwide (1) and the co-related increase of other infectious diseases (7–9). The national and international efforts emphasize the importance of measles elimination to improve and maintain the global, especially children´s, health situation.

1.1.4 Genotyping and molecular epidemiology

To track the elimination process, the surveillance of circulating MeV variants is essential (48). Therefore, MeV genome sequence data are collected in the Measles Nucleotide Surveillance (MeaNS) database of the Global WHO Measles/Rubella Laboratory Network (GMRLN) (48–50). A standardized Nomenclature was developed by the WHO in 1998 and is updated regularly (49). As there is only one MeV serotype, the viruses are distinguished using a highly variable part of their genome (14,48,49). Based on 450 nucleotide sequence encoding the C-terminal part of the viral N protein, MeV are divided into eight clades (A, B, C, D, E, F, G, H) and 24 subclades (A, B1, B2, B3, C1, C2, D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, E, F, G1, G2, G3, H1, H2, and one provisional genotype D11) (14,48,49). MeV sequence subvariants of a genotype are

further stratified using a numerical code assigned to the respective N450 sequence stored in the MeaNS database (or in the case of globally dominant variants, using attributed naming of the first isolate) (48,49). The detection of genotype A indicates a measles vaccine virus, as wild-type viruses of this genotype are no longer circulating worldwide (48,49). Moreover, six genotypes (B1, C1, D1, E, F, and G1) have not been detected this century and are described as 'inactive' (49). Another five genotypes (D2, D3, D10, G2, and H2) have not been detected since 2006 and are described as presumably inactive (49). The genotype diversity has been further reduced to two types (B3 and D8) remaining in 2021 (1). A lesson learned from years of surveillance is, that shortly after large outbreaks, related genotypes are reported nearly worldwide (48).

1.2 Measles virus

Literature assumes that through the domestication of cattle, leading to the close coexistence of humans and cattle, the human-pathogenic MeV arose by splitting off from the most closely related and cattle-infested rinderpest virus in the 6th century BCE (51–53). The Persian physician Rahzes then delivered the first accurate clinical description of the measles disease in the 10th century CE (54).

1.2.1 Taxonomy and morphology

MeV is assigned to the subfamily Morbilliviruses (14,55). Morbilliviruses are part of the genus Orthoparamyxovirinae in the virus family of Paramyxoviridae (14,55). The family of Paramyxoviridae is characterized by 150-250 nm sized helices, 16,000-20,000 nucleotides (MeV has 15,892 nucleotides), and negative-sensed, single-stranded (ss) RNA (14). Viruses containing negative-sensed RNA cannot be directly translated into proteins (14). They require a RNA-dependent RNA-polymerase to first transcribe the RNA into complementary RNA molecules, which are translated into proteins or viral structures (14).

1.2.2 Proteins

The proteins of MeV are distinguished according to their function into two major functional groups.

The first group contains the nucleocapsid proteins, the L ('large'), the P ('phosphoprotein') and the N ('nucleocapsid') protein (14,18). The L and P proteins form the RNA-dependent RNA-polymerase and are, in combination with the N protein, necessary for the transcription of viral RNA (14,18). The N protein communicates with the M ('matrix') protein and protects the RNA from nucleases (14).

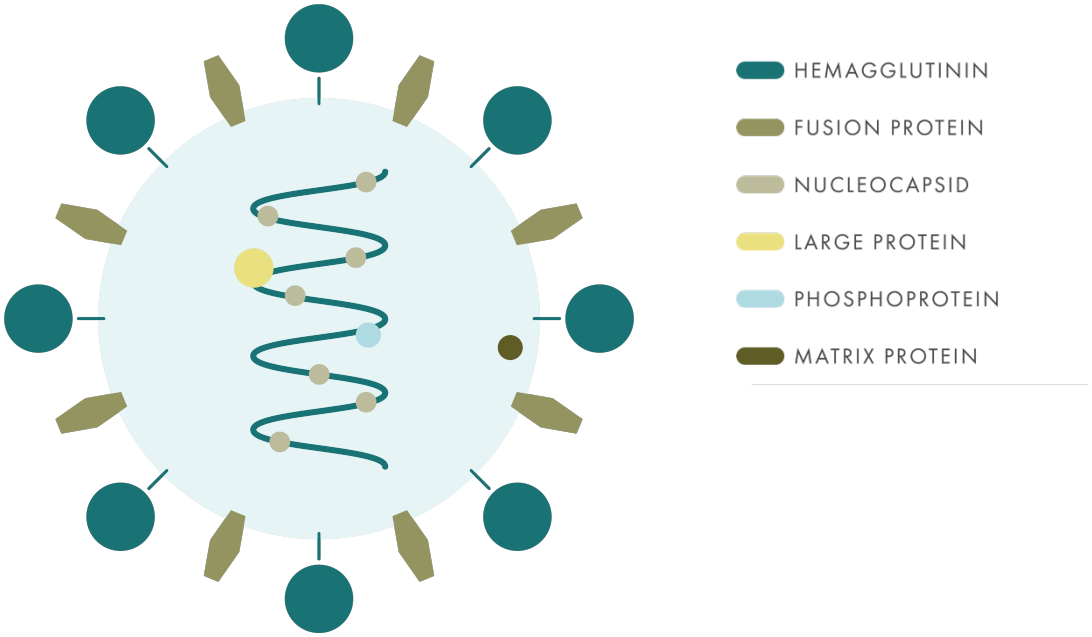

Figure 2 Measles virus morphology. *Schematic structure of measles virus (MeV) morphology and proteins. The spherical structure with associated H and F proteins on the outside of the surface, anchored in the lipid membrane, and M protein, coating lipid membrane on the inside, can be seen. Also shown is the viral ss-RNA, which is protected by the envelope (lipid bilayer) and associates with the P, L and N proteins. Scheme is modified after (5,14,30).*

The second group are the surface proteins F ('fusion'), H ('haemagglutinin'), and M (14,18). The F and H proteins are part of the envelope, being anchored in the lipid bilayer membrane (14,18). In comparison, the M protein covers the inside of the envelope and interacts with the N protein as part of the nucleoprotein complex (14,18,56). The F protein mediates the membrane fusion activity of the MeV and host cell membranes and consists of the two subunits F1 and F2 (14,18). The H protein binds to the receptor on the surface of the host cell and represents the major target for MeV-neutralizing antibodies (5,14,57).

1.2.3 Genome organization

The genome of all paramyxoviruses is, with small variants, organized as N-P-M-F-H/HN-L, seen in 3' to 5' direction (14). The MeV genome contains leading- and trailer-sequences, the leader sequence at the 3' end and the trailer sequence at the beginning, which are responsible for the initiation of transcription and replication (14). Between the genes, there are conserved sequences for regulation of correct transcription at the start and end sites (S and E sequences) of each gene, next to non-transcribed intergenic nucleotide sequences (Figure 3, (14)).

The genome replication of paramyxoviruses takes place in the cytoplasm of infected cells (14). N, P and L proteins bind to the genome and form a nucleocapsid with helical composition (14). For replication, genomic RNA is transcribed into mRNA initiated by the RNA-dependent RNA-polymerase activity of the L protein, passing over the intergenic regions (14). The synthesis of new RNA genomes for the replication is based on antigenomes, transcribed if enough N proteins are translated in the new cell (14).

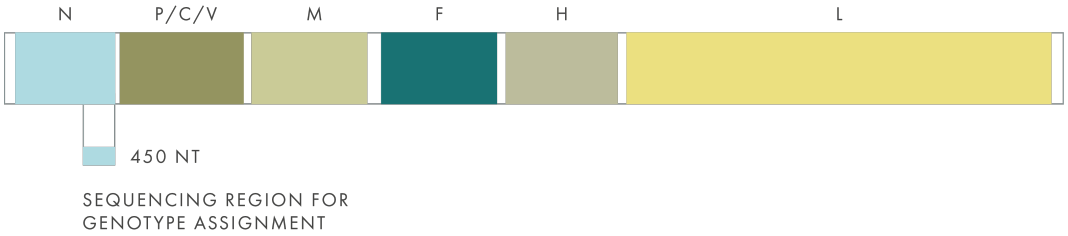

Figure 3 Measles virus genome. *Schematic arrangement of genomic and intergenomic sequences of a MeV genome. Scheme is modified after (14,30).*

1.2.4 Cellular receptors

Nectin-4 and hSLAM (called CDw150) are the identified cellular receptors used by MeV and therefore responsible for the infection of host cells and the pathogenesis (1.1.1, (20,24,58,59)). The related morbilliviruses rinderpest virus and canine distemper virus infect similarly using animal SLAM family receptors (14). While nectin-4 is expressed from the epithelia of the respiratory tract (59), hSLAM is found on the surfaces of B- or T- lymphocytes (24) and macrophages and dendritic cells (20). In contrast to the wild-type viruses, attenuated MeV strains, including vaccine viruses, bind to CD46 (membrane cofactor protein) as a receptor using the H protein, which leads to direct lysis by the complement system and stops the infection early (14,60,61). The ability of currently

circulating MeV to bind to CD46 receptors decreased since the amino acid 481 in the H protein changed from tyrosine to asparagine (61). Therefore, currently circulating MeV use the above described nectin-4 and hSLAM receptors for the host cell entry (1.1.1).

1.3 Immune response

Humans are protected against infectious pathogens through their individual immune system. The immune system of humans in general is based on two different immune sub-systems, the innate and the adaptive immune system (62).

1.3.1 Innate immune response

If a patient is infected with a new pathogen, the innate immune system forms the first response in three parts, the physical and chemical barriers, the cellular mechanisms, and the humoral mechanisms (62).

The epithelia and mucous membranes represent as physical and chemical barriers against the pathogens entry as first part of the innate immune response (62).

The cellular mechanisms, activated through human leucocyte antigen (HLA) receptors, form the second part of the innate immune response (62). If a host cell is infected with an intracellular pathogen (such as viruses), the major histocompatibility-complex-I (MHC-I), an HLA receptor, presents fragments of (e.g. viral) proteins for the cytotoxic T-cell reaction (62). Missing expression of MHC-I leads to the activation of natural killer cells (62). Both reactions are supposed to eliminate the pathogen directly (62). The MHC-II as a second HLA is found on antigen-presenting cells (APCs) like macrophages, dendritic cells, or B-lymphocytes (62). The MHC-II presents parts of phagocytosed pathogens for $CD4^+$ T-lymphocytes, which are part of the adaptive immune system (62).

Humoral mechanisms form the third part of the innate immune system and are based on three different kinds of plasma proteins: Firstly, the acute-phase response, activated by interleukins and in clinical practice often depicted as inflammatory reaction, characterized through leukocytosis, increased C-reactive protein (CRP) and increased blood sedimentation (62). Secondly, the proinflammatory cytokines like for example

interferons (IFN), tumor necrosis factor (TNF-α), interleukins or arachidonic acid derivatives (e.g., prostaglandins) (62). Thirdly, the complement system, as part of both the innate and adaptive immune system, supporting the immune system to destroy pathogens directly or to make them vulnerable to attack (62). Acute-phase-reaction and pro-inflammatory cytokines increase the inflammation and are therefore important markers to detect and monitor infections in clinical practice (62).

Summarized, the basic principle of the innate immune response is the identification of infections through the recognition of surface structures on either foreign particles or endogenous cells, followed by an activation of the first defense mechanisms (62).

1.3.2 Adaptive immune response

Despite the defense mechanisms of the innate immune system are sophisticated, pathogens may eventually overcome those obstacles. Therefore, humans have developed an additional slower, but more specific and effective adaptive immune system, capable of immunological memorizing and thus providing long-term immunity.

The adaptive immune response is based on cellular mechanisms, mainly activated via antigen-presentation of the innate immune system, with three major ways of cell-activation: First, the activation of cytotoxic ($CD8^+$) T-cells through antigen-presentation on MHC-I receptors, which initiates direct lysis or apoptosis of the respective cell (62). Second, macrophages stimulate T_H1 cells as an immune reaction to intracellular pathogens like viruses (62). The T_H1 cells consequently produce cytokines, stimulating macrophages and cytotoxic T-cells (62).

Third, as a reaction to extracellular pathogens like bacteria or parasites, B-cells phagocyte the pathogens through surface located immunoglobulins and present antigens on MHC-II receptors (62). T_H2 cells then interact with the described B-cells and activates them (62). The B-cells, now called plasma cells, subsequently start producing antibodies against the antigen (62).

1.3.3 Antibodies and adaptive immune response

Antibodies form a crucial part of the adaptive immune system. They are produced by B-cells for the recognition of foreign pathogens and therefore constitute an essential

part of the immunological memory (62). They can either be presented on B-cell surfaces, appearing as antigen receptors, or be secreted by activated B-cells, binding and neutralizing their targeted antigens (62,63).

Antibodies are Y-shaped glycoproteins (Figure 4), which are composed of four major parts. The Fc region is formed by heavy chains and determines the respective isotype (e.g., IgM, IgG) (62). The chains are constant and communicate with immunological cells and the complement system (62). The Fab regions of an antibody, which are connected to the Fc region via disulfide bridges of the hinge region, consist of one heavy and one light chain, which again are connected to each other via disulfide bridges (62). Both chains of the Fab region consist of one constant and one variable domain (62). The variable regions of the Fab region are epitopes that bind antigens with high specificity (62). After B-cell maturation, the antibodies produced and secreted by a plasma cell bind only to one particular antigen (62).

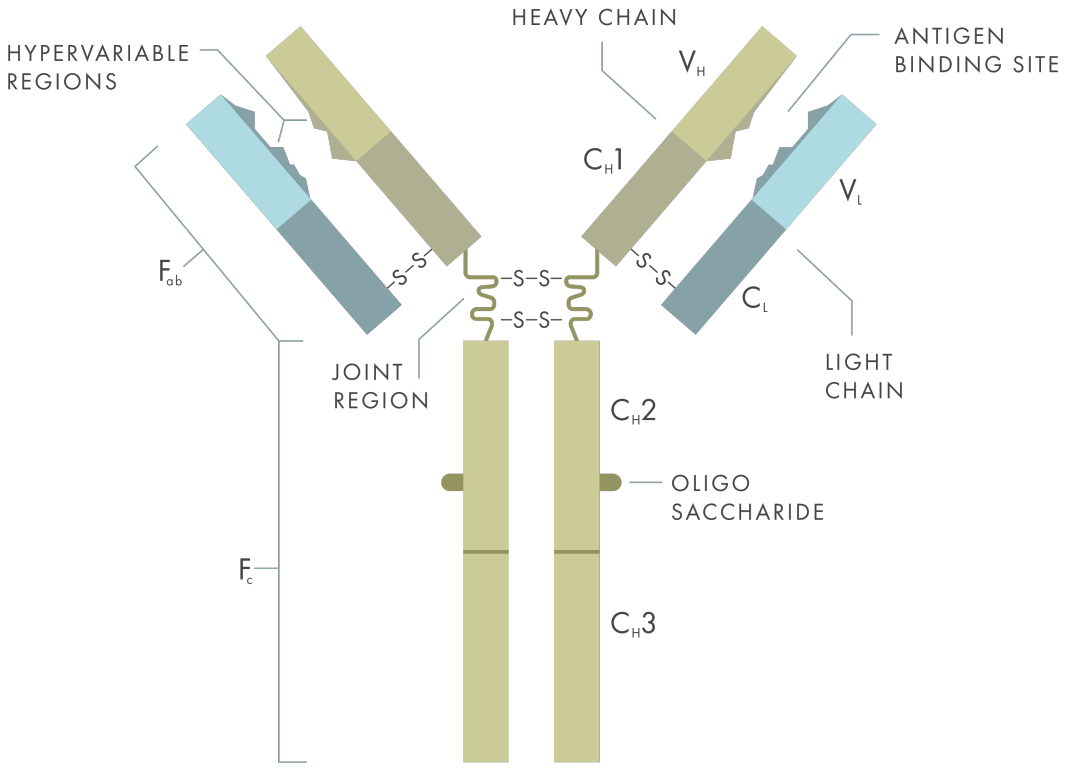

Figure 4 Antibody structure. *Schematic antibody structure as described in 1.3.3. Scheme is modified after Murphy et al., 2012 (62).*

IgM, IgG, IgA, IgE and IgD represent the five different isotypes of produced antibodies (Ig classes) of the humoral immune response (62). Class IgM antibodies are found in pentamers or monomers and eliminate new pathogens (62,64). IgA antibodies are secreted in mucosal surfaces and exist in mono- or dimers (62). IgD are exposed at B-cells, with their function not fully explored yet (62). Class IgE antibodies are monomers and essential for parasite defense (62). Moreover, they are part of allergic reactions through the induction of histamine secretion (62).

At the beginning of an infection with an unknown pathogen, plasma cells predominantly produce IgM antibodies, followed by increasing numbers of IgA, IgE and IgG antibodies as the infection progresses (62). The affinity of IgGs, defined as the strength of a single antibody-antigen binding, increases over time through somatic hypermutations of lymphocyte genes (62). This increases the avidity, defined as the summary of all bindings and therefore the binding strength of pathogen-immune system interactions, and makes the bonds more specific (62). The specific antibody response after MeV infections in humans is depicted in Figure 1.

1.3.4 Neutralizing antibodies, T cell reaction and long-life protection against MeV infection

As described in 1.1.1, the host cell receptors used by wildtype MeV for infection are hSLAM of lymphocytes and Nectin-4 of epithelial cells. For host cell infection, the surface H protein of MeV binds to hSLAM- or nectin-4- receptor and initiates, by interaction with the F protein, the fusion of the cell membranes and the subsequent virus entry into the host cell (14,65,66). To protect the organism against re-infection, class IgG antibodies targeting viral H and F proteins are produced (although antibodies against H protein are dominating here), and block the binding of the MeV H protein to the host cell, as well as the interaction between H and F proteins (57,67). During the next three to six months after the initial infection (68), the IgG avidity (1.3.3) matures (68,69) until the IgG are able to neutralize the MeV infection (67,69). Besides this neutralizing antibodies, preventing the initial cell-virus fusion and therefore the secondary infection, T-cell immunity plays a notable role in the protection of re-infection with MeV: $CD8^+$ and $CD4^+$ T-cells were detected over 34 years after the primary infection (70), at various time points during and after the infection (31), and are part of the adaptive immune system as they are communicating with MHC-I^+ and MHC-II^+ cells (71). It was

described, that CD8+ cells are found in higher titers than CD4+ cells in the long term (71). T-cells are not capable to protect against MeV infection, as neutralizing antibodies are. However, they eliminate MeV RNA and therefore play an essential role in fast RNA clearance after re-infection (31,72). For a schematic T-cell response after MeV infection in humans see Figure 1.

1.4 Detection of neutralizing antibodies

In the medical practice for patient care as well as in the scientific context, these neutralizing antibodies are measured to assess a person's immunity to MeV infection (67,73,74).

1.4.1 Foci Reduction Neutralization Assay

To measure the titer of neutralizing antibodies, the Foci Reduction Neutralization Assay (FRNT, (75)) or the Plaque Reduction Neutralization Assay (PRNT, (73)) are used in specialized laboratories, e.g. WHO global and regional reference laboratories (5,76). For the FRNT, sera are serially diluted and incubated with MeV (e.g., MeV Edmonston wild-type) (73,75). After an incubation period, the samples are placed on cell culture plates and incubated again for several days (73,75). The cells can be infected by the added virus, which amount is used constantly for all FRNTs (75). Depending on how many neutralizing antibodies are present in the serum of an individuum or patient, MeV is prevented from entering the cells, i.e., neutralized by antibodies (67,75). For the test end, an immuno-colorimetric assay is used in FRNTs to titrate the amount of neutralizing antibodies initially obtained in the patients serum (75,76). All infected cells expressing MeV specific antigens on their surface are coupled with an antigen-specific monoclonal antibody, bound by a second antibody, which converts the substrate added and triggers a color reaction (75,76). The foci of infection marked in this way are counted using a magnifying glass or the naked eye and a titer of neutralizing antibodies is calculated (75,76). If a standard, for FRNT the WHO 3rd International Standard (WHO IS 3rd, (77)), is used and measured parallel to the serum, the calculated titers of neutralizing antibodies are converted to International Units (IU) to unify the results (73,78).

The FRNT or PRNT is the gold standard for the measurement of neutralizing antibodies and therefore widely used and studied (5,73,76). However, there are numerous challenges to standardize the assay and transfer between laboratories (79). Since the endpoint determination with an immuno-colorimetric assay is obtained by counting the stained foci of infection using a magnifying glass or the naked eye (75,76), it is subjective as well. FRNT also is a laborious and time-consuming assay (79,80).

1.4.2 Neutralization assay with endpoint determination using RT-qPCR

To replace endpoint determination using immuno-colorimetric assay (75), the neutralization assay used in this work, called NT-qPCR, is performed using RT-qPCR (80). Instead of staining and counting infected foci of infection by hand, the wells of the cell culture plate are harvested. The MeV RNA contained in the harvested wells can be measured, as it is present in varying quantities depending on the titer of neutralizing antibodies contained in the used serum and subsequently on the level of infection (80).

Using RT-qPCR makes the endpoint determination objective and transferrable (80,81). The incubation period for viral infection can be reduced, since a smaller number of infected cells are sufficient for measurement with RT-qPCR. This enables high-throughput measurement with large numbers of sera (80).

1.4.3 Relative quantification in RT-qPCR

With measurement using conventional RT-qPCR, a statement can be made whether viral RNA was present in the sample or not (82,83). Thus, a rough estimation of the quantity of RNA in the measured sample is possible (83). However, to calculate a titer that can be compared with different instruments and measurement series, a more exact measurement of the RNA quantity is necessary (83). With this work, two standard curves were established: first, a MeV RNA standard, extracted from cells, as described previously (80). In comparison, a DNA standard was developed. For this purpose, a DNA fragment (2.1.12) was ordered that is compatible with the primers used in qPCR and is thus amplified in qPCR in the same way as the cDNA described above.

1.4.4 Patient sera, International Standard, and commercial Immunoglobulin

Several patient blood sera with positive and negative FRNT titers, as well as positive and negative ELISA IgG-Titers, were used for NT-qPCR testing. In addition, commercial immunoglobulin (50g/L, KEDRION) was tested with NT-qPCR, which had shown neutralizing capacity in FRNT before (84). WHO 3rd International Standard (77) was produced to standardize FRNT by converting measured neutralizing capacity into International Units (IU) as described in 1.4.1 and 2.2.4.4., and also performed in NT-qPCR.

1.5 Research aims and objectives

Although there is an efficient vaccine for measles available for 60 years (10), there are still groups at high risk of severe complications followed by the infection, especially amongst unvaccinated patients (12,36,37). In some cases, for example during immunodeficiency (10,85) or pregnancy (10,36,84), a vaccination with the attenuated measles virus (MeV), being a live vaccine, must be carefully considered. Since the immune status is not always known after a vaccine book check, antibodies as important part and correlate of immunity are measured (67,73,74). Therefore, an ELISA for detection and quantification of MeV-binding IgG antibodies can be performed with the patients serum (73,86). However, even if the measured value for IgG antibodies is categorized as negative or borderline, a patient can have immunity against MeV infection, since protective neutralizing antibodies might exist in sufficient quantity (67,69,73). The titer of neutralizing antibodies are determined with foci reduction neutralization assays (FRNT, (73,80)). Even if the ELISA value for IgG was categorized as negative, a seroprotective neutralizing antibody level correlating with life-long protection may be present, making a revaccination with live vaccine or passive immunization obsolete (5,73,74). It is also shown, that neutralizing antibody titer can be detected reliable and efficiently with plaque reduction or foci reduction neutralization assays (73,80,86). Established neutralization assays, however, require long incubation times and are technically difficult (79,80). In addition, the assays must be evaluated with a magnifying glass or the naked eye (75,76,80) and are difficult to standardize (79).

The neutralization assay presents an important tool for complementary diagnostic in clinical routine and to investigate research questions such as the correlation between immunoassays (ELISA) and neutralization assays (79,87). It might be used even more frequently, if throughput possibilities are increased and the assay time is shortened (80).

The aim of this work is to develop and establish a neutralization assay, based on the FRNT, which will be improved and shortened by endpoint determination using RT-qPCR for virus quantification.

2 Materials and Methods

2.1 Materials

2.1.1 Devices

Name:	Distributor:
Centrifuges:	
5415 D	Eppendorf AG, DE
Biofuge Fresco, Biofuge Stratos	Heraeus Holding, DE
Heraeus™ Fresco 17	Thermo Fisher Scientific Inc., US
PerfectSpin P	Peqlab Biotechnologie GmbH, DE
Mega Star 1.6R	VWR International, LLC.
mySPIN™	Thermo Fisher Scientific Inc., US
CO_2 Incubator	ThermoScientific HERAcell Vios160i/HERAcell 150
EUROIMMUN Analyzer I-4P	EUROIMMUN Medizinische Labordiagnostika AG, DE
Freezer	
Freezer -20°C	Liebherr, DE
Freezer -80°C	Thermo Fisher Scientific Inc., US
Fridge 4°C	Robert Bosch GmbH, DE
LightCycler® 480 II	Roche Diagnostics GmbH, DE
LUNA™ automated cell counter	Logos Biosystems, Inc., US
Microscopes:	
Nikon eclipse TS 100	VWR Mikroskop Technik Rathenow GmbH Nikon Instruments Inc., US
Pipettes:	Eppendorf AG, DE; Gilson, Inc., USA; Thermo Fisher Scientific Inc., US
Safety cabinets	Thermo Fisher Scientific Inc., US
ThermoMixer® comfort	Eppendorf AG, DE
VacusafeTM Comfort Aspiration system	Integra Bioscience AG, CH

Vortex shaker:	
IKA® MS 2 minishaker	IKA-Werke GmbH & Co. KG, D
IKA® MS 3 basic	IKA-Werke GmbH & Co. KG, D
VF2 (IKA®-Labortechnik)	Janke & Kunkel GmbH & Co. KG, D
Pipetboy pipette controller	Integra Bioscience AG, CH
Water bath	Haake Technik GmbH, DE; JULABO GmbH, DE

2.1.2 Chemicals and reagents

Name:	Distributor:
Bovine Serum Albumin (BSA), 30 % solution, > 99 %	PAN-Biotech GmbH, DE
Carboxymethylcellulose (CMC) sodium salt, ultra high viscosity, highly purified	Sigma-Aldrich Chemie GmbH, DE; nr. 21904
Dimethyl sulfoxide (DMSO)	VWR International GmbH, DE
Ethanol, 99.5%, extra pure	Carl Roth GmbH & Co. KG, DE
iScript RT-qPCR Sample Preparation Reagent	Bio-Rad Laboratories GmbH, DE; Nr. 170-8899
Magnesium sulfate ($MgSO_4$)	Thermo Fisher Scientific Inc., US
Methanol 99.5%	Electron Microscopy Sciences, US
Minimum Essential Medium Eagle, 10x (MEM)	Sigma-Aldrich Chemie GmbH, DE
Paraformaldehyde, 16 % aqueous solution (PFA)	Electron Microscopy Sciences, US
Sodium hydrogen carbonate ($NaHCO_3$)	Nährmedienzentrale RKI, DE
3,3',5,5'-Tetramethylbenzidine (TMB)	Mikrogen GmbH, DE
Trypan Blue Stain, 0.4%	Logos Biosystems, Inc., US
Tween® 20	Carl Roth GmbH & Co. KG, DE
Water	Sigma-Aldrich Chemie GmbH, DE

2.1.3 Buffers and solutions

Name:	Composition, Distributor:
λ-DNA	Thermo Fisher Scientific Inc., US Productnr. SD0011 500 µg, c = 0.3 µg/µL
λ-DNA-TE-buffer	c = 1ng/µL: 30µL λ-DNA 8,97 mL TE-buffer
Blocking buffer	PBS with: 2% BSA 1% FBS 0.1% Tween® 20
CMC 1%	1% CMC sodium salt in ultrapure water
CMC overlay	50% CMC 1% 6% 10xMEM 3% FBS 2.2% Sodium hydrogen carbonate (7.5%) 1% Penicillin/Streptomycin 37.8% D-MEM
Cell culture growth medium	D-MEM with: 10% FBS 1% Penicillin/Streptomycin 1% Glutamine 0.5% Geneticine ~ 0.6% Sodium hydrogen carbonate
Cell culture preservation medium	D-MEM with: 2% FBS 1% Penicillin/Streptomycin 1% Glutamine
DNA standard ‘gBlocks’	c = 10 ng/µL:

	1 tube (á 250ng) 25 µL pure water
FRNT Growth medium	D-MEM with 10% FBS 1% Penicillin/Streptomycin 1% Glutamine
FRNT preservation medium	D-MEM with 2% FBS 1% Penicillin/Streptomycin
FRNT medium	D-MEM pure
$NaHCO_3$ solution 7.5%	7.5% $NaHCO_3$ in ultrapure water
PFA 2%	12.5% PFA (16% ampoule) 87.5 % PBS
Primer-Probe MVNP1163-P-mix	In test concentration 0.25 µM, diluted in water
2xRXN (reaction mix)	Thermo Fisher Scientific Inc.; Kit: Super-script III One-Step qRT-PCR
Tris-EDTA/RNase free buffer (10mM Tris, 1mM EDTA, pH 8)	Thermo Fisher Scientific Inc., US; BP2473-100

2.1.4 Cell culture reagents

Name:	Distributor:
Dulbecco's Modified Eagle Medium (DMEM), Ph 7.4	Nährmedienzentrale RKI, DE
Fetal bovine serum (FBS)	PAN-Biotech GmbH, DE
Gibco™ Geneticin	Thermo Fisher Scientific Inc., US
Gibco™ L-Glutamine, 200mM	Thermo Fisher Scientific Inc., US
Gibco™ Penicillin/Streptomycin, 10,000 U/mL	Thermo Fisher Scientific Inc., US
Minimum Essential Medium Eagle, 10x (MEM)	Sigma-Aldrich Chemie GmbH, DE

Phosphate-Buffered Saline (PBS), excluded Ca++ and Mg++	Nährmedienzentrale RKI, DE
$NaHCO_3$ solution, 7.5%	Nährmedienzentrale RKI, DE
Trypsin-EDTA, 0.05%	Nährmedienzentrale RKI, DE

2.1.5 Eukaryotic cells

Name:	Organism:	Tissue:	Distributor:
Vero/hSLAM	Monkey African Green	Kidney/Fibroblast	Prof. Dr. Yusuke Yanagi; Department of Virology, Faculty of Medicine, Kyushu University, Kukuyoka, Japan

2.1.6 Measles virus strains

Name:	Distributor:
Edmonston wild-type Genotype A	Dr. Judy Beeler, Division of Viral Products, U.S. Food and Drug Administration (FDA), Bethesda, Maryland, U.S.A.
MVi/Offenburg.DEU/10.19 Genotype D8	Robert Koch-Institute, NRC MMR, FG12, MeV isolated from throat swab of measles patient

2.1.7 Enzymes and inhibitors

Name:	Distributor:
RNasin® Ribonuclease inhibitor	Promega GmbH, DE; N2518/N2511
Taq-Polymerase (= SuperScript® III RT/Platinum® Taq Mix)	Thermo Fisher Scientific Inc., US
Trypsin	Nährmedienzentrale RKI, DE
Trypsin-EDTA, 0.05%	Nährmedienzentrale RKI, DE

2.1.8 Antibodies

Name:	Distributor:
Goat anti-mouse IgG antibody, HRP conjugated, 1mg/mL	Thermo Fisher Scientific Inc., US Catalognumber # G-21040
Immunoglobulin 50 g/L Infusionslösung	KEDRION S.p.A., ITA
Specific monoclonal antibodies: Measles virus anti-nucleoprotein antibody, 'NP cl. 120'	ECACC Collection: Hybridoma cell line; antibody-collection RKI in-house from supernatant (ECACC 95040312)
WHO International Standard/3rd International Standard for Anti-Measles	NIBSC-code: 97/648; Version 2.0; dated 26/02/2008

2.1.9 Primers

Name:	Sequence (5' to 3'):	Concentration (μM)	Distributor:
3424 (MeVN1139-F), forward	5'- TGG CAT CTG AAC TCG GTA TCA C -3'	0.3	Metabion
3425 (MeVN1213-R), reverse	5'- TGT CCT CAG TAG TAT GCA TTG CAA -3	0.3	Metabion
3426 (MeVN1163-P), Probe	5'- FAM-CCG AGG ATG CAA GGC TTG TTT CAG A-BHQ1 -3'	0.25	Metabion

2.1.10 Consumables

Name:	Distributor:
Cell culture plates: 96-well	SARSTEDT AG & Co. KG, DE; nr. 83.3924

Cell culture flasks Nunc™ EasYFlask™: 25 cm^2, 75 cm^2, 175 cm^2	Thermo Fisher Scientific Inc., US
CELLSTAR® Cell culture plates: 48-well	Greiner Bio-One GmbH, AT; kat.nr. 677 180
Centrifuge tubes: 15 mL, 50 mL	SARSTEDT AG & Co. KG, DE
Combitips advanced®	Eppendorf AG, DE
Cover slips (Ø 12 mm)	Carl Roth GmbH & Co. KG, DE
Nunc™ Cryogenic tubes	Thermo Fisher Scientific Inc., US
Glasware	SCHOTT AG, DE
LightCycler® 480 Multiwell Plate 96, white	Roche Diagnostics GmbH, DE
LUNA™ Cell Counting Slides	Logos Biosystems, Inc., US
Microscope slides	Carl Roth GmbH & Co. KG, DE
Pipetboy pipette controller: consumables	Integra Bioscience AG, CH
Pipette tips	Thermo Fisher Scientific, Inc., US; Eppendorf AG, DE; Gilson, Inc., USA
Reaction tubes	Applied Biosystems, U.S.A.; Eppendorf, D; Greiner, D; Nunc, DK; TPP, CH
Serological pipette	Brand GmbH & Co. KG, DE; Hirschmann Laborgeräte GmbH & Co. KG, DE; SARSTEDT AG & Co. KG, DE
S-Monovette® Serum-Gel	SARSTEDT AG & Co. KG, DE

2.1.11 Kits

Name:	Distributor:
Anti-Measles Virus NP ELISA (IgM) + Anti-Measles Virus ELISA (IgG)	EUROIMMUN Medizinische Labordiagnostika AG, DE
QIAmp Viral RNA Mini Kit	QIAGEN GmbH, DE; Cat.No./ID: 52904 or 52906

QIAamp Viral RNA Mini Accessory Set	QIAGEN GmbH, DE; Cat.No./ID: 1048147
QIAshredder	QIAGEN GmbH, DE; Cat.No./ID: 79656
RNeasy® Mini Kit	QIAGEN GmbH, DE; Cat No./ID: 74104
Superscript® III One-Step qRT-PCR	Thermo Fisher Scientific Inc., US; nr. 12574018 (Invitrogen)

2.1.12 DNA standard

Name:	Distributor:	Sequence (5' to 3'):
gBlocks™ Gene Fragments in tubes, 186bp	Integrated DNA technologies, Inc., BEL	5' - TGA TCC AGC ATA TTT TAG ATT AGG GCA AGA GAT GGT AAG GAG GTC AGC TGG AAA GGT CAG TTC CAC ATT GGC ATC TGA ACT CGG TAT CAC TGC CGA GGA TGC AAG GCT TGT TTC AGA GAT TGC AAT GCA TAC TAC TGA GGA CAA GAT CAG TAG AGC GGT TGG ACC CAG ACA AGC CCA AGT ATC ATT - 3'

2.1.13 Software

Name:	Distributor:
MS Office 2003-2019	Microsoft, U.S.A.
GraphPad Prism 8/9	Graphad Software, US
LightCycler® 480 II Software	Roche Diagnostics GmbH, DE
EUROIMMUN Analyzer I	EUROIMMUN Medizinische Labordiagnostika AG, DE

2.2 Methods

2.2.1 Human blood sera, WHO 3rd International Standard, commercial Immunoglobulin

De-identified human blood sera from the Robert Koch-Institute (RKI) blood bank were used for this work, collected from volunteers after giving informed consent. The sera used were obtained by centrifugation, pipetting, and inactivation once at 56°C for 30 minutes before use. A commercial immunoglobulin (50 g/L, KEDRION) was also tested, which had shown neutralizing capacity in the foci reduction neutralization assay (FRNT) before (84). The World Health Organization (WHO) 3rd International Standard (IS 3rd, (77)) was produced to standardize the FRNT by converting the measured neutralizing capacity into International Units (IU) as described in 1.4.1 and 2.2.4.4., and also tested in the neutralization assays here.

2.2.2 Cell biological methods

2.2.2.1 Cultivation of Vero/hSLAM cells

Vero/hSLAM cells (88) were cultivated at 5% CO_2 and 37°C. Therefore, the growth medium was produced from D-MEM including 10% FBS, 1% Penicillin/Streptomycin, 1% Glutamine, 0.5% Geneticin and 0.6% Sodium hydrogen carbonate. The T-25 cell culture flasks with confluent adherent cells were first poured off and washed with 5 mL Trypsin-EDTA. For detachment, the cells were incubated with 5 mL Trypsin-EDTA for two minutes at room temperature, 4.5 mL of incubated Trypsin-EDTA was pipetted off and the cells were incubated again for six minutes at 5% CO_2 and 37°C. The cells were resuspended in 5 mL medium. 2.5 mL of the cell suspension were diluted in 45 mL medium. The volumes used from the received suspension for further cell cultivation are shown in Table 2.

Table 2. *Volumes for cell cultivation*

Cell culture vessel	Volume cultivation (mL)
25 cm^2 flask	10 mL
75 cm^2 flask	30 mL
48-well plate	0.5 per well
96-well plate	0.2 per well

2.2.2.2 Thawing of Vero/hSLAM cells

The cells are stored at -150°C in CoolCell® boxes. After the cryo tubes were put in a 37°C water bath for thawing, 1 mL cell suspension was diluted in 20 mL D-MEM in 25 cm^2 flasks. The suspension was transferred into two 25 cm^2 flasks á 10 mL. After 24 h, the medium was changed to wash DMSO off. After three days, if the cells were confluent, they were passed into new flasks.

2.2.2.3 Freezing of Vero/hSLAM cells

Three days after, the cells were passed 1:10. Therefore, the cells from one 25 cm^2 flask were collected (2.2.2.1) and diluted in 100 mL D-MEM. The cells were then pipetted into three 75 cm^2 flasks á 30 mL and passed as previously described in 2.2.2.1. After three days incubated at 5% CO_2 and 37°C, the cells were collected (2.2.2.1) and diluted in 900 mL D-MEM. The cells were passed in 175 cm^2 flasks á 70 mL for another three days. Afterwards, the cells were collected (2.2.2.1), pipetted in 1.8 mL aliquots in cryo tubes, put into CoolCell® box, frozen for 4h at the -80°C freezer, and stored in a -150°C freezer for long-term storage.

2.2.2.4 Cell counting

The cells were counted using LUNA™ automated cell counter. Therefore, 20 µl of the reagent of interest were diluted with 20 µl of trypan blue staining solution in a reaction tube. Afterwards, 10 µl of the received reagent were transferred to LUNA™ cell counting slide and put into LUNA™ automated cell counter. Numbers are given as cell count per mL, the dead and alive cells could be distinguished by the device through the trypan blue staining.

2.2.3 Enzyme Linked Immunosorbent Assay: Detection of MeV-specific antibodies

Quantitative measurement of IgG or IgM antibodies binding specifically to MeV antigen was performed using an Enzyme Linked Immunosorbent Assay (ELISA, (86)). The EUROIMMUN Anti-Measles Virus ELISA (Euroimmun Medizinische Labordiagnostika

AG) automat was therefore used: Immunoglobulin titers were analyzed using fully automated (79) 'EUROIMMUN Analyzer I-4P'. With the associated reagents and program 'EUROIMMUN Analyzer I', IgM or IgG titers could be quantitatively determined following the user instructions.

2.2.4 Virological methods

2.2.4.1 Growing measles virus

First, Vero/hSLAM cells were passed into 75 cm^2 flasks á 30 mL. Three incubation days at 5% CO_2 and 37°C later, when the cells are confluent, they were collected (2.2.2.1) and re-suspended in 300 mL medium. 175 cm^2 flasks, each with 70 mL of the suspension, were prepared and incubated for one day at 5% CO_2 and 37°C. The cells were infected with 0.5 mL virus material (from previous virus cultivation) and 2 mL preservation medium (2.1.3), incubated for 30 minutes at 5% CO_2 and 36°C. 40 mL medium was added and flasks were incubated at 5% CO_2 and 36°C. Measles virus (MeV) of two different strains was grown, one with strain Edmonston wild-type (genotype A) and one with strain MVi/Offenburg.DEU/10.19 (genotype D8). Three days post infection (100% CPE), the flasks infected with MVi/Offenburg.DEU/10.19 were harvested and five days post infection (100% CPE), the flasks with Edmonston wild-type. Therefore, the cells were scraped off the bottoms, transferred to centrifugation tubes and centrifuged at 3000 U and 4°C for ten minutes. The received supernatant was then collected, mixed carefully, and pipetted in cryo-tubes. Virus aliquots were stored either in -20°C freezers or -80°C freezers for long-term storage.

2.2.4.2 Determination of virus concentrations (virus titer)

For virus titration, Vero/hSLAM cells were passed 1:10 and seeded in 48-well cell culture plates (2.2.2.1). One day later, if the cells were confluent, they were infected with MeV of either strain Edmonston wild-type or strain MVi/Offenburg.DEU/10.19. Different virus concentrations obtained by 10-fold serial dilution of the virus stock suspension were used to infect the cells. Starting with a dilution of 1E-1, 450 µL D-MEM medium and 50 µl virus stock suspension was mixed in reaction tubes. Five more tubes were prepared with 450 µL D-MEM medium each. Then, a 10-fold dilution series was made transferring 50 µL from concentration 1E-1 up to 1E-6 by pipetting and discarding 50

µL from the last dilution step. As positive control, a standard with known titer was used, as negative control preservation medium. For infection of the cell culture plates, the medium from the wells was removed and 100 µL of diluted virus suspensions or controls per well pipetted. The plate was incubated for 1h at 5% CO_2 and 36°C, afterwards 0.5 mL CMC overlay (2.1.3) per well was added and the incubation continued for another five days.

For evaluation and visualization of the MeV-infected cells, the protocol for the FRNT was followed as previously described from *Finsterbusch* et al. (75): Briefly, the plate was washed, and the cells were fixed and stained using an immuno-colorimetric assay (73,75,76).

The cells were washed with 0.5 mL/well PBS. 200 µL/well cold PFA were added, and plates were incubated for 30 minutes in the fridge. The cells were washed again with 0.5 mL/well PBS and 200 µL/well cold Methanol, which was stored in a -20°C freezer before, was added and the plate incubated for 10 minutes at -20°C. After the cells were fixed, they were washed once with 0.5 mL/well PBS and incubated with 200 µL/well blocking buffer (2.1.3) at room temperature for 30 minutes. The blocking buffer contains proteins that can bind to unspecific binding sites. The plate could be stored at this stage for four to eight weeks. For staining of MeV-antigen, the MeV-specific monoclonal antibody ‘Ab NP cl. 120’, a HRP-conjugated goat anti-mouse IgG (secondary) antibody and the HRP-substrate TMB were used (2.1.8). ‘Ab NP cl. 120’ binds to viral nucleoproteins expressed in MeV-infected cells. For a 1:50 dilution, 100 µL of ‘Ab NP cl. 120’ was diluted with 5 mL blocking buffer. The blocking buffer was removed from the plate, 100 µL/well of diluted ‘Ab NP cl. 120’ was added, and the plate was incubated for 30 minutes at room temperature. Afterwards, the plates were washed twice with 0.5 mL/well blocking buffer. For a 1:1000 dilution, 5 µL of HRP conjugate was diluted with 5 mL blocking buffer. Then 100 µL/well HRP-conjugated secondary antibody mix was added, and the plate was incubated for 30 minutes at room temperature. The HRP conjugated secondary antibody binds to the mouse IgG ‘Ab NP cl. 120’. The plates were washed again twice with 0.5 mL/well blocking buffer and 100 µL/well substrate was pipetted for staining the foci of MeV-infection through bonded monoclonal and HRP antibodies and enzymatic reaction of HRP. After 5 minutes incubation at room temperature, the enzymatic reaction was stopped with isotonic water and therefore the foci of MeV-infection became visible as blue spots.

For the calculation of virus concentrations, referred to as virus titer and given in PFU/mL, foci per well corresponding to 100 µL of diluted virus suspension were counted. Provided, the negative control showed no infection and the positive control showed infection. The wells with numbers of foci of infection ranging from 20 to 40 were considered for calculating the virus concentration as follows:

$$c\ in\ PFU/mL = (number\ of\ foci\ counted \times dilution\ factor \times 10) : 1mL$$

2.2.4.3 Pre-test for Foci reduction neutralization assay

To determine the volume of virus for use in the FRNT with a new virus stock, several pre-tests were done. Similarly, as for virus titration (2.2.4.2), Vero/hSLAM cells were seeded in 48-well plates and incubated for one day. Depending on the virus concentration determined previously, several dilutions were made from the virus stock suspensions. For each dilution, three replicates were made and for each replicate (of each dilution), four wells were pipetted. As a negative control, 300 µL medium and as a positive control, 300 µL medium containing virus suspension, diluted 1:1, was used. Virus dilutions and controls were incubated at 37°C in a water bath for 1 h. Samples were pipetted in the cell culture plate with 100 µL volume/well, incubated at 36°C and 5% CO_2, 0.5 mL/well CMC overlay was added, and the plates again incubated at 36°C and 5% CO_2. After four days of incubation, the reaction was stopped, the cells were fixed and stained as done for the virus titration (2.2.4.2, (75)). Mean number of foci per dilution were counted and further pre-testing with varied dilutions was done with the same protocol, until a concentration for the virus control was chosen.

2.2.4.4 Foci reduction neutralization assay

The foci reduction neutralization assay (FRNT) was performed as described from *Finsterbusch* et al. (75). Therefore, an immuno-colorimetric assay (75,76) is used to measure the inhibition of viral replication and therefore MeV infection in a cell culture plate by human serum antibodies. Vero/hSLAM cells (88) were seeded with a volume of 0.5 mL/well in a 48-well cell culture plate (2.2.2.1) and incubated at 37°C and 5% CO_2 for 24 h. The collected sera (2.2.3) were thawed and inactivated for 30 minutes at 56°C once before use in FRNTs.

The following four steps were necessary in preparation of the cell culture plate infection:

First, a virus aliquot with MeV Edmonston wild-type was thawed, and a dilution was made. Pre-tests depicted the concentration c = 1E-3 as reasonable for the virus control. Therefore, first the concentration c = 1E-2 was prepared with 0.1 mL virus suspension and 9.9 mL D-MEM medium. After mixing gently, c = 1E-3 was pipetted with 1 mL from the obtained virus mix and another 9 mL of medium.

Second, the controls were prepared. For the cell control (CC, negative control), D-MEM medium was used. Since the serum samples will be mixed 1:1 with virus material, the virus control (VC) is prepared by 1:1 dilution of virus suspension (of the previously adjusted concentration c = 1E-3) with D-MEM medium. To calculate neutralizing antibody titer values in IU, the WHO IS 3rd (78) was used in the FRNT. Therefore, a 2-fold serial dilution of the WHO IS 3rd was prepared. One ampoule of WHO IS 3rd contains 3 IU of anti-measles activity (78), which is reconstituted in 1 mL of distilled water, resulting in 3 IU/mL (3000 mIU/mL). The WHO IS 3rd was then diluted 1:50 and 150 µL of diluted standard serum were mixed 1:1 with 150 µL D-MEM medium resulting in a dilution of 1:100. Dilution series were made preparing first tubes with each 150 µL medium. Then 150 µL were transferred and mixed starting with the received 1:100 dilution up to 1:3200 and discarding 150 µL from the last dilution.

Third, dilution series of the patient sera were made. Eight tubes, each one containing 150 µL medium D-MEM, were pipetted. Only for the first dilution 1:4, another 75 µL medium were added, which makes 225 µL. Afterwards, 75 µL serum was mixed gently for the first 1:4 dilution. A 2-fold dilution series was made up to 1:512, transferring each time 150 µL to the next dilution and discarding 150 µL from the last.

Fourth, the neutralization of MeV, which means binding of serum antibodies to the virus, was aimed. Therefore, both dilution series, with serum and with WHO IS 3rd, were diluted 1:1 by pipetting 150 µL virus mix (c = 1E-3) and mixed gently. All prepared samples and the controls were incubated in the water bath at 37°C for 1 h.

After those four steps, the prepared samples were pipetted to the cell culture plate for susceptible MeV infection (Table 3). The medium from the cell culture plate was absorbed and 100 µL from each prepared sample including the controls were transferred

into a well of the cell culture plate. The plate was incubated for 1 h at 36°C and 5% CO_2, then CMC overlay was added with 0.5 mL/well and the incubation continued.

Table 3. *Pattern for infection on 48-well plates for a foci reduction neutralization assay (FRNT).*

Each sample consists of equal volumes of diluted serum and virus suspension (1:1).

	1	2	3	4	5	6	7	8
Serum	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Serum	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
WHO IS 3rd	1:100	1:200	1:400	1:800	1:1600	1:3200		
WHO IS 3rd	1:100	1:200	1:400	1:800	1:1600	1:3200		
	VC 1	VC 1	VC 2	VC 2	CC	CC		
	VC 1	VC 1	VC 2	VC 2	CC	CC		

For visualization of the MeV-infected foci, the virus replication was stopped after five days of incubation, the cells were fixed and stained using an immuno-colorimetric assay afterwards, followed by the calculation of neutralization dose (ND) titer (73,75,89).

For calculation of the MeV-neutralizing antibody titer, the foci were counted for each well. The interim 50% foci reduction titer (ND50) is calculated by the following formula (89):

$$Interim\ ND50\%\ titer = (0{,}5 \times (a \times b \div c \pm d \times b \div e))$$

$a = reciprocal\ dilution\ of\ serum\ with\ 50\%\ foci\ reduction$

$d = reciprocal\ dilution\ of\ serum\ with\ 50\%\ foci\ reduction$

$b = 0{,}5 \times number\ of\ foci\ in\ mean\ virus\ control$

$c = number\ of\ foci\ from\ a$

$e = number\ of\ foci\ from\ d$

Since all samples were diluted 1:1 with virus suspension, the final titer is calculated as follows:

$$ND50\%\ titer = 2 \times Interim\ ND50\%\ titer$$

Both steps are important and therefore 90% foci reduction titer is calculated as follows:

$$Interim\ ND90\%\ titer = (0{,}9 \times (a \times b \div c \pm d \times b \div e))$$

$$ND90\%\ titer = 2 \times Interim\ ND90\%\ titer$$

A ND titer of ≥ 8 is interpreted as positive, which means that MeV-neutralizing antibodies were detected by FRNT (73).

The ND50 and ND90 titers were transformed using the WHO 3rd IS into international units (IU/mL) as follows (73,78,89):

$$ND\ in\ IU/mL = (ND\ titer\ sample \times 3\ IU/mL) \div ND\ titer\ WHO\ IS\ 3rd$$

2.2.4.5 Foci reduction neutralization assay with endpoint determination using RT-qPCR

The NT-qPCR established by this work is based on endpoint determination of FRNT and thus the measurement of neutralizing antibodies by Real time quantitative reverse transcription polymerase chain reaction (RT-qPCR). The basic design of the assay is similar to the FRNT (75), except that the immuno-colorimetric assay detection of MeV infection is replaced by MeV-specific RT-qPCR for the test evaluation (80).

For NT-qPCR, Vero/hSLAM cells were seeded in 96-well cell culture plates (2.2.2.1). First, human sera were inactivated at 56°C for 30 minutes (2.2.1). Then dilution series of sera and WHO IS 3rd, also cell and virus controls were prepared (2.2.4.4). All serum samples were then diluted 1:1 with the prepared virus mix and incubated in water bath at 37°C for 1 h (2.2.4.4). The medium was aspirated from the cell culture plate and 40 µL/well pipetted of each reagent. Plates were incubated at 36°C and 5% CO_2 for 24 h. In comparison, for FRNT, 48-well plates were used and the incubation period was five days.

After 24 h incubation of the cell culture plate, cells were harvested and therefore viral RNA collected with one of two given different variants, either one-step lysis using iScript SPR lysate (Bio-Rad) or extraction of viral RNA by hand using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Both methods are described in 2.2.5.2.

Once the samples were harvested using one of the respective methods, they could either be stored frozen until further use (2.2.5.2) or analyzed directly by RT-qPCR (2.2.5.5). Through RT-qPCR with relative quantification using a DNA standard, the amount of RNA per sample is given in copy numbers as described in 2.2.5.3.

ND titers are now calculated either through simple linear regression, or through non-linear regression:

For calculation using a simple linear regression model, the titers are calculated as described before (2.2.4.4) for FRNT. Besides, instead of foci numbers, copy numbers are used as values. The results are given in copy numbers, since the relative quantification is performed at every RT-qPCR run using a DNA standard (1.4.3). Copy numbers itself are then calculated using the LightCycler480 program. For calculation using a non-linear regression model, the values in copy numbers were used for a non-linear regression model in GraphPad Prism, where a 'dose-response-inhibition curve' was selected. ND titer were then interpolated using the non-linear regression model at 50% or 90% of the mean virus control.

The experimental results must meet the following requirements:

1. Is there any contamination in the cell culture? Is the medium clear? Otherwise, the whole experiment should be repeated.
2. Microscope: The cells should be confluent besides single plaques, where the cells got infected, and the cell control wells show an intact monolayer. Before the infection of cells all wells should show an intact monolayer. No infection in the cell control should be seen at any time point.
3. Positive control for RT-qPCR is positive (Ct > 35). Otherwise, the RT-qPCR run is not considered valid.

4. Negative control (H_2O) for RT-qPCR is negative in RT-qPCR measurement. Otherwise, the well (or more wells) could be contaminated during pipetting the RT-qPCR-plate, thus the RT-qPCR run is not considered valid.
5. The DNA standard curve resulted in $R^2 \geq 0{,}99$. Otherwise, the calculated amount of copy numbers is not considered valid, and the run should be repeated with a new DNA standard curve.
6. Cell control in RT-qPCR is negative. Otherwise, a contamination caused either by pipetting the qPCR-plate or pipetting the cell culture-plate is assumed.
7. Virus control (VC) in RT-qPCR is positive (Ct = 27 +/-3). Otherwise, either the RT-qPCR run or the whole experiment should be repeated.
8. RT-qPCR-efficiency should be > 90%.

2.2.5 Molecular biological methods

2.2.5.1 Isolation of viral RNA from liquids

Isolation of MeV RNA from liquids for use in amplification procedures was conducted using the QIAcube with the QIAmp Viral RNA Mini Kit (Qiagen) according to the manufacturer´s instructions: Viral RNA can be obtained from a wide variety of liquid samples, such as throat and nasal swabs, urine, plasma, or serum. For this purpose, the sample is lysed, which enables the binding of the viral RNA to the silica-based membrane of the spin columns. The obtained RNA is dissolved in RNAse-free buffer and can then be further used for amplification or stored frozen.

After loading the designated vacancies with AVL, AW1, AW2, AVE, and AVE + cRNA (carrier RNA) buffers (Table 4) along with 100% ethanol, and filling tip racks and 1.5 mL Eppendorf tubes in the automat, 140 µL of each patient sample and control were placed in 2 mL micro-screw tubes in the QIAcube. Afterwards, QIAmp viral RNA Mini Kit program was performed with the QIAcube. As negative control, 140 µL D-MEM and as positive control, 140 µL of measles-/mumps-/rubella-vaccine-virus-mix with known PFU titers (25 PFU/mL for MeV) was used.

Table 4. *Used AVE buffer and AVE + cRNA dilutions for RNA extraction with QIAcube.*

Number of samples	AVE buffer [in µL]	cRNA + AVE buffer [in µL]
2	256	28 + 97
3	364	33.6 + 116.4
4	472	39.2 + 135.8
5	580	44.8 + 155.2
6	688	50.4 + 174.6
7	796	56 + 194
8	904	61.6 + 213.4
9	1012	67.2 + 232.8
10	1120	72.8 + 252.2
12	1336	84 + 291

2.2.5.2 Isolation of viral RNA from cells

For the extraction of MeV RNA from collected cells, two different variants were used as described before (2.2.4.5):

For one-step lysis with iScript SPR lysate (Bio-Rad), after thawing the lysate, medium was aspirated by pipetting and the cells were washed with 200 µL/well PBS carefully. Then, 100 µL/well of iScript one-step sample preparation reagent (iScript SPR lysate, Bio-Rad) was added, and the plate incubated for one minute. The samples were collected and can be stored at -20°C (2.2.4.5).

For hand extraction using the RNeasy Mini Kit (Qiagen), first the medium was aspirated by pipetting and the cells were washed with 200 µL/well PBS. The samples were proceeded following the QIAshredder (Qiagen) and the RNeasy Mini Kit protocols: The cells were collected by adding 350 µL/well buffer RLT and pipetting the lysate. Samples were then homogenized using QIAshredder. Therefore, the received lysate (up to 700 µL) was pipetted onto 2 mL collection tubes of the QIAshredder kit and centrifuged for two minutes at maximum in a microcentrifuge. After the homogenization, 350 µL of 70% Ethanol per sample was added and up to 700 µL were loaded in 2 mL collection tubes of the RNeasy Mini Kit. The samples were centrifuged at ≥ 8000 x g for 15 seconds and the flow-through was discarded. 700 µL buffer RW1 was pipetted on the

columns, the samples were again centrifuged at ≥ 8000 x g for 15 seconds and the flow-through was discarded. Then, 500 µL buffer RPE was pipetted on the columns, the samples were centrifuged at ≥ 8000 x g for 15 seconds and the flow-through was discarded. For the last washing step, 500 µL buffer RPE was pipetted on the columns, the samples were centrifuged at ≥ 8000 x g for two minutes and the flow-through was discarded. The samples were then centrifuged on new 2 mL collection tubes at full speed for one minute to dry the membrane. For elution of the received RNA from the membrane, spin columns were put in new 2 mL collection tubes, 50 µL RNase-free water was added directly to the spin column membrane and the samples were centrifuged at ≥ 8000 x g for one minute. After extraction of the viral RNA from collected cells, the RNA samples can be stored at -20°C or used for further testing (2.2.4.5).

2.2.5.3 Relative quantification of MeV RNA in RT-qPCR using RNA standard

Here, MeV is grown on cells, the cells and the supernatant are harvested, and then the MeV RNA is extracted (2.2.5.1, 2.2.5.2). Subsequently, the extracted RNA can be used as RNA standard in RT-qPCR (90). The standard curve with predefined concentrations was generated running with each measurement. Quantitative concentrations of viral RNA can now be calculated from the LightCycler480 program by counter-calculation against the standard curve (80,90). For this purpose, it is not the concentration of each step, but the ratio between the dilution levels that is necessary. However, the standard is used in a 10-fold dilution series and concentrations are determined manually in the LightCycler480 program. After measuring samples and the standard curve in one RT-qPCR run, quantitative concentrations of viral RNA in each sample are counter-calculated (90) with the LightCycler480 program and given in copy numbers per sample. Copy numbers here describe the number of MeV RNA fragments contained in the measured sample and are further used for calculation of neutralization titers as described in 2.2.4.5.

For the preparation of a MeV RNA standard, Vero/hSLAM cells were seeded in 25 cm^2 flasks (2.2.2.1). With ≥ 80% confluence, the medium was discarded, and the cells were infected with a reference MeV. The inoculum was prepared by adding 100 µL suspension containing MeV Edmonston wildtype (c = 1E6 PFU/mL) to 900 µL D-MEM and

pipetted on the cells. Flasks were incubated at 36°C and 5% CO_2 for 30 minutes, then 10 mL preservation medium was added.

After 24. 48 or 72 h incubation at 36°C and 5% CO_2, cells and supernatant were collected mechanical and centrifuged at 3000 U and 4°C for 10 minutes. The supernatant was removed and extracted using a QIAcube automat (Qiagen). The cells were extracted using the RNeasy Mini Kit (Qiagen) with 600 µL of each buffer RLT and 70% Ethanol for further use in NT-qPCR.

2.2.5.4 Relative quantification of MeV RNA in RT-qPCR using DNA standard

A DNA standard can also be used in RT-qPCR for relative quantification. The DNA fragment used is complementary to the used primers in RT-qPCR (2.2.5.5), diluted with λ-DNA-TE-buffer for stabilization, and used as standard curve (2.2.5.3).

For λ-DNA-TE-buffer with c = 1 ng/µL, 30 µL λ-DNA (0.3 µg/µL) were diluted with 8.97 mL Tris-EDTA-buffer and mixed well. The λ-DNA-TE-buffer can be stored at -20°C in the freezer.

Table 5. *'gBlocks' DNA dilution steps.*

Dilution step	Source of fragment DNA	Volume of fragment DNA [in µL]	Volume of diluent [in µL]	Concentration [in copy numbers]
1	reconstituted gBlocks DNA stock with c = 10 ng/µL	19.06	80.94	1E10/µL
2	dilution 1	10.00	990.00	1E8/µL
3	dilution 2	10.00	990.00	1E6/µL
4	dilution 3	50	50	1E6/2 µL

Then, the lyophilized DNA standard was reconstituted in RNase-free water. Therefore, 25 µL water was added to one tube gBlocks DNA with 250 ng for c = 10 ng/µL and the reagent was incubated at 50°C in the heater for 20 minutes. The DNA was then diluted

in DNase and RNase free water up to c = 1E6 copy numbers/2 µL in four steps (Table 5). The DNA was stored at -20°C in the freezer.

In order to set up a standard curve for relative quantification in RT-qPCR, a 10-fold serial dilution in λ-DNA-TE-buffer was prepared starting with 1E6 gBlock DNA copies/2 µL. Six dilution steps from 1E6/2 µL to $1x10^1$/2 µL, all in λ-DNA-TE-buffer, were pipetted and performed (double determined) as standard curve in each RT-qPCR run (2.2.5.5). The prepared dilution series was stored at -20°C in the freezer and re-used several times (3.2.2).

2.2.5.5 Real time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

For the real time quantitative polymerase chain reaction (RT-qPCR), the MeV RNA was first transcribed into cDNA (complementary DNA) with a RNA-dependent DNA polymerase, a so-called reverse transcriptase (82,83). This RT-qPCR is used to amplify DNA fragments until they are present in an amount that can be measured by fluorescence as described below. The cDNA template is amplified using added desoxyribonucleosid-triphosphates (dNTPs), primers, and a temperature-stable DNA polymerase (here Taq DNA polymerase, (82,83)). Two different primers, a Forward and a Reverse primer (2.1.9), both specifically generated for the MeV cDNA sequence, ensure that the DNA target sequence can be read and synthesized in both directions (82,83).

TaqMan probes are used for the fluorescence measurement. A probe consists of a specific complementary DNA-polymerase, which has a reporter and a quencher at each end (82,83). After attachment to the DNA, the reporter fluoresces, but the signal is suppressed by the quencher (82,83). By degradation of the 5'-end (through 5'-3'-Exonuclease-activity of Taq DNA-polymerase) during synthesis, the fluorescence signal of the reporter can be measured (82,83). With increasing fluorescence, proportional to the increasing amount of DNA amplicons, the measured fluorescence signal exceeds a so-called Cycle threshold (Ct, (83)). The Ct value indicates the number of cycles to amplify DNA that are required until the fluorescence exceeds the threshold at which there is sufficient fluorescent light for the automat to measure a signal and for the sample to be designated as positive by the program (83). Since optimal reaction conditions dominate during the exponential phase of the amplification, where the

amount of the DNA product and therefore the signal ideally doubles after each cycle, the measurement takes place here (82,83). Compared to conventional PCR, the fluorescence signal in quantitative real-time PCR (qPCR) is measured at the end of each amplification cycle (82,83). In addition, since a one-step RT-qPCR was performed for this study in the LightCycler480 II, the reverse transcription and the qPCR measurement were performed without an intermediate step in one RT-qPCR run.

Performing RT-qPCR, first a master mix was prepared by mixing the reagents (Table 6). The RNasin® Ribonuclease inhibitor and the Taq-polymerase (= SuperScript® III RT/Platinum® Taq Mix) were pipetted on ice and not thawed, the primer/probe mix FAM was thawed for maximal three times.

Table 6. *Pipetting scheme for master mix for real time quantitative polymerase chain reaction (RT-qPCR).*

Component	Volume per reaction [in µL]	Volume for one fully loaded 96-well plate [in µL]
RNase-free water	2.145	205.92
2xRXN (= 2x reaction mix)	6	576
$MgSO_4$	0.48	46.08
primer/probe mix FAM	1	96
RNasin® Ribonuclease inhibitor	0.125	12
Taq-Polymerase (= SuperScript® III RT/Platinum® Taq Mix)	0.25	24
= volume mastermix	10	960
+ added sample	2	192
= volume all	12	1152

Afterwards, 10 µL per well of the master mix was pipetted into the LightCycler480 Multiwell plates 96, followed by 2 µL per well of each sample. A preparation of viral RNA of the measles vaccine virus Leningrad 16 (L 16), adjusted by dilution to a Ct value of 27 +/- 3, served as positive control and RNase-free/DNase-free water was used as negative control. Positive and negative controls were included in each RT-qPCR run.

The controls and the RNA or DNA standard were pipetted with 2 µL per well into the plate, which was afterwards sealed with the belonging plastic foil. The LightCycler480 machine was then started, the prepared plate centrifuged at 1000 rpm for two minutes and put into the machine. A RT-qPCR run was performed with the LightCycler480 automat and the selected programs were FAM = 450-520 nm, Cy5 = 650-680 nm and 12 µL reaction volume. For the temperature profile see Table 7. After the RT-qPCR run, the RNA or DNA standard was set as standard and concentrations 1E6 to 1E1 set by hand for each DNA dilution step into the program. Values in copy numbers are then calculated automatically (2.2.5.4).

Table 7. *LightCycler480 program.*

Cycler program	Temperature	Time	Additional steps
Program 1	48 °C	30 minutes	
Program 2	95 °C	5 minutes	
Program 3	95°C 60°C	15 seconds 30 seconds	45 cycles (Analysis mode: Quantification Acquisition mode: 1. None, 2. Single)

2.2.5.6 Foci reduction neutralization assay with endpoint determination using RT-qPCR

NT-qPCR was performed as described before (2.2.4.5).

3 Results

Standardized measles virus (MeV) neutralization assays are usually performed as foci reduction neutralization assays (FRNT). For the FRNT performed in this study, MeV is incubated with serially diluted patient serum samples and cell layers are inoculated with the virus-antibody-mixture and incubated (75). The foci of infection on the cell layer are then stained using an immuno-colorimetric assay and counted, to calculate the 50% or 90% MeV neutralization titer (73,75,89). In principle, it is possible to use Real time quantitative reverse transcription polymerase chain reaction (RT-qPCR) for endpoint determination (80). This thesis aims to establish the use of RT-qPCR for endpoint determination of MeV FRNTs, as well as the use of Vero/hSLAM (human signaling lymphocytic activation molecule, (88)) cells for cell culture methodology. This newly developed neutralization assay allows a high-throughput and rapid determination of MeV-neutralizing antibodies in human sera and could therefore be applied in the NRC MMR (National reference center for measles, mumps, and rubella) at the RKI (Robert Koch-Institute).

If not stated otherwise, for all experiments performed with RT-qPCR, the positive controls ranged between Ct = 27 +/-3, while the negative control and cell controls showed no signals.

3.1 Validation of virus controls

Preceding all experiments, MeV Edmonston low passage wild-type (genotype A, isolated in 1954) and a current MeV of genotype D8 isolated from an acute case in 2019 (WHO MeV strain name: MVi/Offenburg.DEU/10.19), were cultivated on cells and harvested. After the titration of the virus stocks thus obtained, several preliminary tests were performed to determine the optimal quantity of MeV to adjust the virus control (VC). This virus control was adopted for all subsequent FRNTs, as well as NT-qPCRs.

The number of foci of MeV infection in the wells of the cell plate is used in FRNT for the virus control and a measure for plaque forming units (PFU). These foci indicate MeV-infected cells displaying MeV-specific viral antigens which are visualized by an immuno-colorimetric assay (75,76). The number of stained foci can then be counted with a magnifying glass or the naked eye (75,76). If insufficient cells are infected and subsequently the number of foci in the virus control is too low, the assay cannot be

validated. However, if too many cells are infected, the foci cannot be distinguished from each other, and the cell lawn can detach from the cell culture.

Therefore, a permitted range of foci in the virus control wells should be defined, determined through a specific range of PFU used in the virus control wells. In addition, the calculation using linear regression used for neutralization titers in FRNT is limited, which, in combination with the described counting limitations, leads to the permitted VC = 14.28-36.8 foci per well (mean number of foci obtained from several wells) for this work. The degree of infection and thus the number of visible foci per virus control well correlates with the concentration of MeV (PFU) used and with the chosen incubation time. Both variables were tested until the optimal virus concentration and incubation time for the obtained virus stocks were found. As has been set for the laboratory of the NRC MMR at the RKI inhouse, the mean foci number of virus control wells is accepted at VC = 24-30, resulting in a mean half VC = 12-15.

Performing MeV titration as described in 2.2.4.2, a concentration of c = 1E6.7 PFU/mL for the wild-type MeV Edmonston (genotype A) and c = 1E6 PFU/mL for the MVi/Offenburg.DEU/10.19 (genotype D8) virus stock was determined. Since the tests were running primarily with Edmonston wild-type, the respective virus stock was tested and an incubation time of four days resulted in an intact cell lawn and foci numbers of 24-30. The concentration used to infect the cell layer at each well was set at c = 1E-3 PFU/mL, since this quantity had resulted in a mean VC = 27. For the genotype D8 MeV, a three-day incubation time and concentrations ranging from c = 7.5E-4 to 5E-4 PFU/mL were chosen for further experiments.

Once the virus control has been established successfully, patient serum samples were measured in FRNT. For a FRNT, patient sera are serially diluted and incubated with the Edmonston wild-type concentration used for the virus control. The virus-antibody-mix, as well as cell controls and virus controls, are incubated in cell culture plates. Endpoint determination is performed using an immuno-colorimetric assay (76) and the foci of infection are counted for the controls and for the serum dilution series. Subsequently, the number of foci for serum dilutions is related to the virus control. A neutralization titer of 50% is interpolated and displays a dilution factor (73,75,89).

With this work, however, the neutralization assay was evaluated by RT-qPCR. Here, all cell culture wells were harvested and measured individually using RT-qPCR.

The titers were counter-calculated using standard curves for relative quantification and, as for the FRNT, calculated in relation to the virus control.

3.2 Performance of RT-qPCR and relative quantification using standard curves

For the endpoint determination of neutralization assays with RT-qPCR, relative quantification of the RT-qPCR Ct results needed to be established. This work used standard curves for counter-calculation. Following, two different materials were analyzed to generate the standard curve: 1) MeV Edmonston wild-type, called RNA standard, as also described from *Alvarado-Facundo* et al. (80) and 2) DNA, called DNA standard, of the target sequence, which was ordered compatible to the primer set. After establishing the standards, the standard curve, either RNA or DNA standard, is measured parallel to the NT-qPCR endpoint determination with RT-qPCR. The results for NT-qPCR are calculated in copy numbers of MeV RNA per well of the cell culture plate using the LightCycler480 program.

3.2.1 RNA standard for relative quantification and MeV replication kinetics

For the establishment of a RNA standard, the replication kinetics of MeV after infection of a Vero/hSLAM cell lawn was analyzed at different time points. In addition, the concentration of MeV in the supernatant as well as in the cell pellet containing the sedimented cells was compared. Using MeV from the supernatant is preferred here, since there are fewer procedure steps needed to collect the RNA.

As seen in Figure 5, MeV could be cultivated on Vero/hSLAM cells and successfully collected either from the supernatant or the sedimented cell pellet. Extracted MeV from the cell pellet showed, at 24, 48, and 72 h post-infection, Ct values ranging from 10 to 16. Extracted MeV from the supernatant at 24, 48 and 72 h post-infection showed Ct values ranging from 15 to 18.

Since MeV RNA could be cultivated, collected, extracted, and measured with RT-qPCR at all incubation times, either from the cell pellet or the supernatant, MeV RNA extracted from the supernatant with an incubation time of 24h was chosen to produce all future RNA standards.

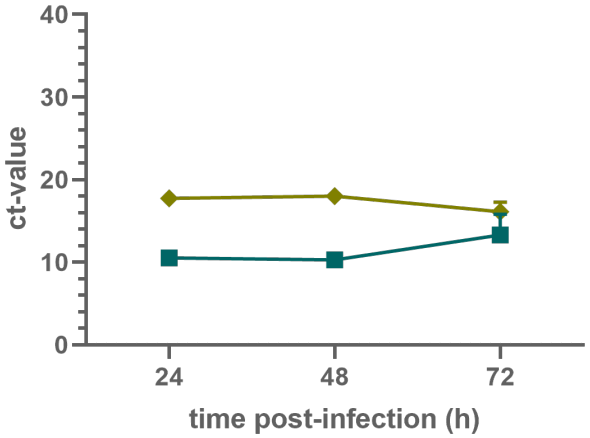

Figure 5 Replication kinetics of MeV. *Vero/hSLAM cells were seeded in 25cm² flasks. At 80-100% confluence, cells were infected with Edmonston wildtype measles virus (MeV; 100µL with concentration 10E6,7 PFU/mL). 24 h, 48 h and 72 h post-infection flasks were harvested and centrifuged at 3000 U for 10 min at 4°C. The cell pellet was extracted using RNeasy Mini Kit, the supernatant using RNeasy Mini Kit according to the QIAGEN protocol. MeV RNA was measured using real time quantitative reverse transcription polymerase chain reaction (RT-qPCR). Each point represents the mean (n = 2) with corresponding range (± SD) of MeV-infected Vero/hSLAM cells then extracted from the cell pellet (□) and from the supernatant (◇). For all graphs, lines represent linear regression curves and curves represent nonlinear regression curves; R² relates to each regression model.*

3.2.2 DNA standard for relative quantification

As an alternative to the RNA standard, a DNA standard was designed. When using the DNA standard, the quantity of nucleic acid expressed as DNA copy numbers at each dilution step of the standard curve is known, since the quantity of DNA inserted in the qPCR can be calculated using the producer's protocol. The through calculation estimated concentrations of the used standard curve can be entered in the LightCycler480 program. In addition, the DNA standard is freely available and therefore more standardized and the results comparable between laboratories. For the DNA standard, a DNA fragment (2.1.12) was ordered complementary to the primers used in the RT-qPCR and is thus amplified in the same way as cDNA fragments obtained by reverse transcriptase (2.2.5.4, 2.2.5.5).

The DNA standard could be successfully established for the use in RT-qPCR (Figure 6). To shorten working hours and investigate the requirements for storage and stability of the DNA standard, varying conditions regarding storage and reuse of pre-prepared DNA standard were tested. As seen in Figure 6, each standard curve had a very low variance from the regression line model ($R^2 > 0.99$) and was stable to varying conditions such as different diluents like water or stabilizer, freeze/thaw cycles or storage.

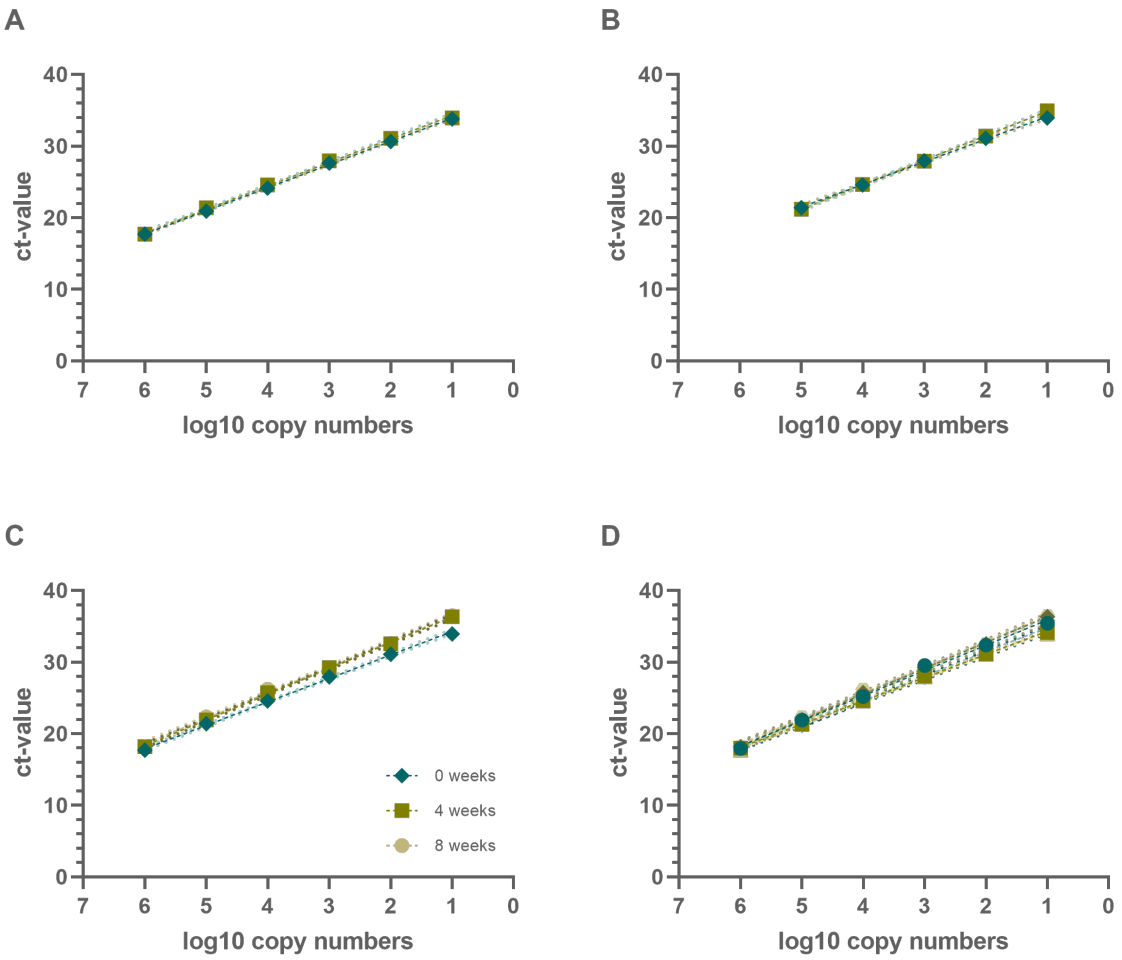

Figure 6 Reproducibility, storage, and freeze-thaw cycles with DNA standard. *After a DNA standard was established successfully, several basic conditions were tested. Therefore, DNA standard 10-fold dilution series were made as previously described. First (A) RNAse-free water (◇) and Lambda-DNA (□) were compared as diluents for the DNA standard. DNA standard with Lambda-DNA as a diluent was then tested (B) by freezing and thawing 15 times (□) compared to direct measurement (◇). For (C), storing at -20°C for 4 (□) and 8 (○) weeks was compared to direct measurement (◇). Reproducibility was tested by preparing DNA standard with Lambda-DNA as a diluent six times (D). Each point represents the mean (n = 2) with corresponding range (± SD), except for (B) with mean of n = 4 ± SD.*

3.2.3 Standards for relative quantification in comparison

After establishing the RNA and DNA standard, both standards were compared against each other to choose one for use in the NT-qPCRs. Since the dilution steps are logarithmic (10-fold dilution series) and the amount of cDNA per sample doubles with each qPCR cycle, a difference of around Ct = 3.3 is expected between the successive dilution steps. Accordingly, the slopes should be comparable. As a condition for using the standard for relative quantification, the variance was defined to be $R^2 > 0.99$ after creating a linear regression model for each standard series.

Unlike for the DNA standard, both RNA standards showed variation at high dilution levels (1E-7 to 1E-10) and thus undercut the specified maximum variation of $R^2 > 0.99$ (Figure 7). In addition, growing MeV in cell flasks and extracting the RNA by hand is time consuming and expensive as well as less reproducible. The DNA standard was therefore used for further NT-qPCRs.

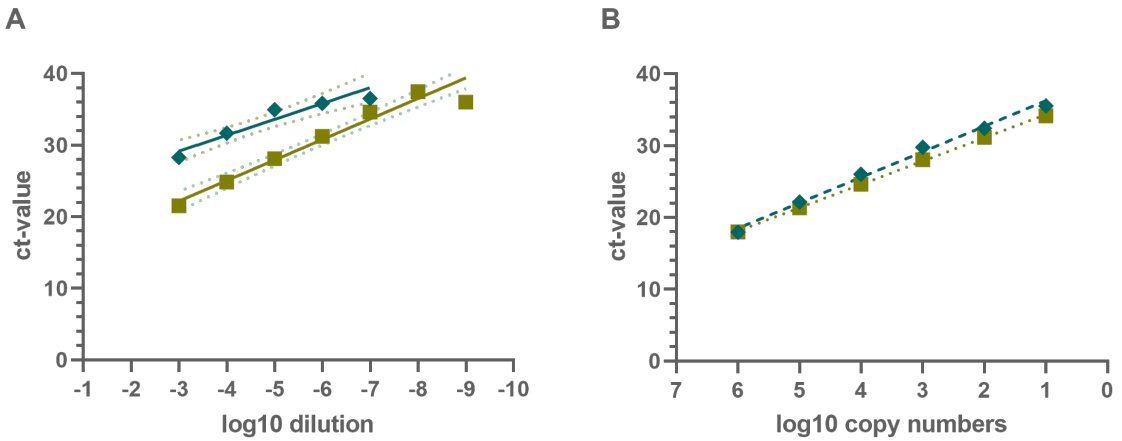

Figure 7 Regression analysis for RNA and DNA standard. *First the MeV RNA standard (Figure 5) was measured, intended to use it for relative quantification in RT-qPCR. Two standards were made with RNA, harvested, and extracted after 24 h (Figure 5), one from the cell pellet and one from the supernatant material. Therefore a 10-fold dilution series was made from 1E-3 to 1E-10. Second a Standard curve was made with DNA standard. Therefore a 10-fold dilution series was made from 1E6 to 1E1 copy numbers per 2 µL. The figure shows (A) RNA standard in 10-fold dilution series, with the MeV RNA extracted either from the cell pellet (□) or the supernatant (◇). And (B) DNA standard in 10-fold dilution series with two repetitions. Each Graph shows linear regression curves for the standards; (A) also with the 95% CI for each linear regression curve. Each point represents the mean (n = 2) with corresponding range (± SD).*

3.3 Isolation of MeV RNA from cells for endpoint determination by RT-qPCR

After establishing the replication of MeV in cell culture plates on Vero/hSLAM cells and RT-qPCR measurement with relative quantification for endpoint determination of the NT-qPCR, two variants for harvesting the cells from the cell culture plates and extracting the MeV RNA from the cells prior to measurement and quantification with RT-qPCR were compared: A one-step lysis of the MeV-infected cells using SPR one-step lysate, which should allow direct use of the lysate in RT-qPCR without RNA extraction. And a stepwise extraction process with isolation of MeV RNA by hand using RNeasy Mini Kit.

Performing NT-qPCRs with one-step lysis allows high-throughput measurement of patient blood sera, since the one-step lysis of harvested cells needs less than five minutes until the lysate can directly be measured in RT-qPCR for endpoint determination.

In comparison, the isolation of MeV RNA from the harvested cells through stepwise extraction is expensive and time consuming, with several protocol steps and hours needed per cell culture plate. However, harvesting the MeV-infected cells with one-step lysate, many components of the sample like the lysed cells and the lysis buffer are added to the RT-qPCR, which may influence the reverse transcription, the amplification, and the fluorescence measurement of the RT-qPCR. In comparison, the stepwise extraction of MeV RNA from cells clears the cell components and isolates the viral RNA, being diluted in RNase and DNase free water.

3.3.1 Performance of RT-qPCR using one-step lysis

To investigate the influence of one-step lysis on the RT-qPCR results, lysed Vero/hSLAM cells (mock-infected cells, without infection by MeV RNA), were measured in the RT-qPCR. Therefore, the DNA standard was diluted in a suspension containing lysed cells and one-step lysis in a 10-fold dilution series was performed. Additionally, it was analyzed whether storage or multiple use of those dilution series influences the RT-qPCR measurement.

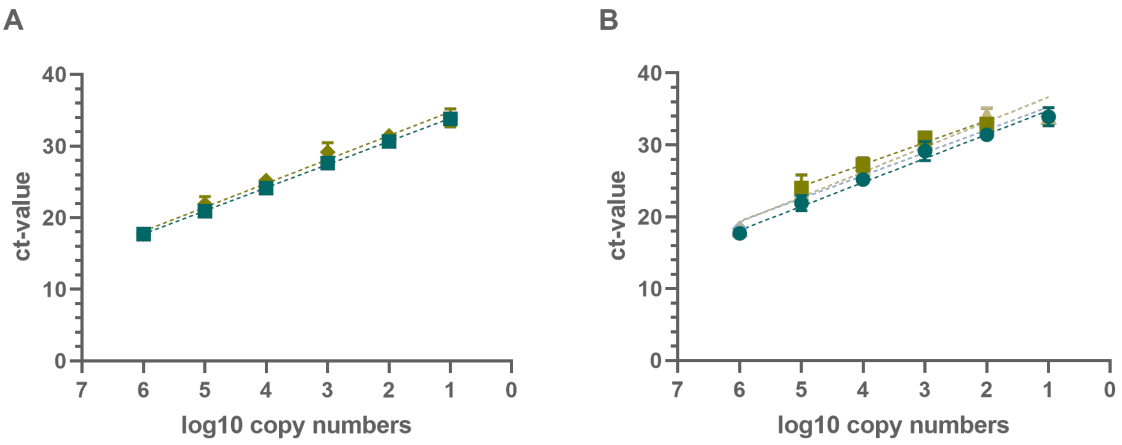

Figure 8 RT-qPCR measurement and regression analysis for one-step lysis. *Before performing a foci reduction neutralization assay (FRNT) using endpoint determination by RT-qPCR, it must be chosen how to harvest the MeV RNA from the cell culture plate. Therefore, the performance of one-step lysis with SPR iScript lysate was tested: (A) by RT-qPCR using DNA standard diluted in mock cells (◇) or water (□). For (B) with the same framework but stored at -20°C for 0 weeks (○), 4 weeks (△), 8 weeks (◇) and frozen/thaw 15 times (□). Data are presented as mean (n = 2, dilution 1E6 for (A) with N = 5) ± SD of each value.*

As seen in Figure 8A, the measurement of the DNA standard diluted with water shows, compared to the measurement results for DNA dilution series (3.2.2), equally low variance when generating a regression curve (R^2 = 0.99) and low standard deviation of the mean values of dilution steps (SD = 0.03-0.2). In comparison, for the DNA standard measured with the mock cells lysed with the SPR one-step lysate, deviations (R^2 = 0.98, SD = 0.3-1.3) in the accuracy of the measurement were observed.

According to the results shown in Figure 8B, storing DNA dilution series with lysed mock cells affects the accuracy of the RT-qPCR results, since the results differ for the measurement immediately after sample preparation (R^2 = 0.98, SD = 0.3-1.3), storage at -20°C of the samples for four weeks (R^2 = 0.95, SD = 0.1-1.3), storage for eight weeks (R^2 = 0.97, SD = 0.2-0.9), or thaw/freeze cycles without storage (R^2 = 0.92, SD = 0.1-1.8).

The variance between DNA dilution series with water or mock cells as diluent differs with R^2 = 0.99 (water) and R^2 = 0.98 (mock cells). The use of the lysed cells directly for RT-qPCR influences the measurement in a range, that is accepted for NT-qPCRs in this work. If the lysed cells are thaw/frozen or stored at -20°C for weeks, the variance contains wide ranges from R^2 = 0.92 to 0.98. Since the immediate measurement after lysis is possible for the NT-qPCR protocol, the one-step lysis is used for NT-qPCRs. Thaw/freeze cycles or storing are not part of the NT-qPCR procedures.

3.3.2 Performance of RT-qPCR using extraction or lysis

After testing one-step lysis on non-infected cells and the effect on RT-qPCR measurement, Vero/hSLAM cells were infected with MeV in 10-fold dilution series (1E-1 to 1E-8). To further compare the performance of RT-qPCR using both harvesting ways with MeV infected cells, the MeV RNA was isolated either by one-step lysis and immediate use in RT-qPCR or by stepwise extraction of the MeV RNA, followed by measurement with RT-qPCR.

As shown in Figure 9, the amount of amplified DNA obtained by RT-qPCR from the MeV RNA dilution series for the series harvested with one-step lysis is lower (mean Ct = 16-30) than the results of the extraction process (mean Ct = 12-25), comparing dilution steps 1E-1 to 1E-5.

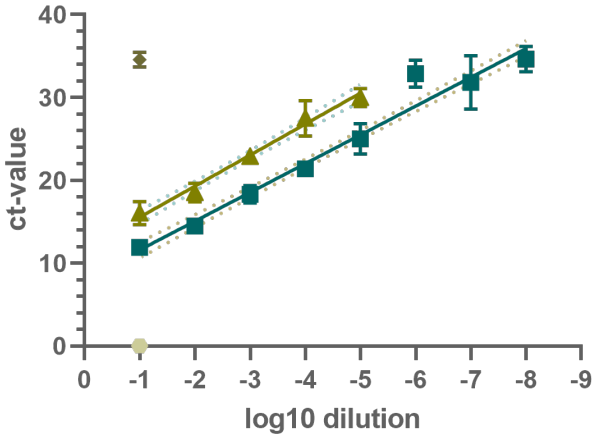

Figure 9 RT-qPCR measurement with one-step lysis vs. extraction by hand. *The performance of two variants for isolation of MeV RNA for NT-qPCR endpoint determination with RT-qPCR was tested: MeV was seeded in 10-fold dilution series (1E-1 to 1E-8) and harvested either by RNeasy Mini Kit (□ ;n = 4) or by SPR iScript one-step lysate (△ ;n = 8) with cell control for extraction (◇) and one-step lysis (○). For all graphs, the lines represent the linear regression curve, and a 95% CI is shown. Data are presented as mean ± SD of each value.*

Furthermore, when harvested with the one-step lysis, many samples had negative Ct values (52 %), which was not the case for the same dilutions after extraction (2 %). High dilution steps 1E-6 to 1E-8 were negative for lysed dilution series (Figure 9).

According to the higher Ct values obtained from RT-qPCR (mean Ct = 16-30), the higher proportion of wells with a negative result of RT-qPCR (52 %), especially for dilution steps 1E-6 to 1E-8, the output for wells with one-step lysis is lower than for wells with extraction. Although the measurement using one-step lysis has a lower output, the shortened working hours for harvesting and the less expensive lysis kit allow high-throughput and serial multiple measurements of sera and were therefore used for the following NT-qPCR.

After the cell culture, the viral input for infection, the harvesting of MeV RNA from wells with infected cell culture, and the RT-qPCR were set, human blood sera were measured with NT-qPCR. The NT-qPCRs were performed with both harvesting ways to further investigate the effect of one-step lysis or stepwise extraction on the measurement with RT-qPCR, especially if human sera are used.

3.4 NT-qPCR with human blood sera

In this experiment, NT-qPCRs with extraction and with one-step lysis, as described in 2.2.4.5, were performed for two human blood sera. The experiment for NT-qPCR with

stepwise extraction additionally compares the copy number of viral RNA extracted from MeV-infected cells cultured in two different types of cell culture plates, 48- and 96-well plates.

The 48-well plate is established for in-house FRNTs. Since the RT-qPCR is used to determine the endpoint titer of MeV neutralization for NT-qPCR, there are no foci of MeV infection to be counted and there is no need to use plates with wells of a higher diameter. The same NT-qPCRs were therefore performed using 96-well plates, which would allow a higher throughput and is more resource-efficient.

For the set-up of the NT-qPCRs, two sera from a 24-year-old female patient from the collection of the NRC MMR with different characteristics were used: Sample 20-00762 was submitted with two MMR vaccinations in childhood, while sample 20-00848 was taken 13 days after application of the 3rd MMR vaccination to the same 24-year-old patient. The patient was given the 3rd MMR vaccination after the IgG-Titers were classified as negative in ELISA for the first sample 20-00762 (IgG = 132 IU/L; Table 8). Although the ELISA IgG-Titer was negative for serum 20-00762, the serum contained MeV-neutralizing antibodies (ND50 = 55), detected with a FRNT. For this work, sera with negative, borderline, or positive ELISA IgG-Titer and FRNT ND50-Titer were chosen to test the ability for the NT-qPCR to measure neutralizing antibodies in a broad linear range. The NT-qPCR for this experiment was performed for sera with FRNT positive, while ELISA negative or positive, titer. Serum 20-00848 is expected to have a higher neutralization titer in NT-qPCR than serum 20-00762, since serum 20-00848 showed more neutralizing capacity in the FRNT (ND50 = 115; Table 8) than serum 20-00762 (ND50 = 55) and the initial ELISA IgG-Titer for serum 20-00762 before the 3rd MMR vaccination was negative (IgG = 132 IU/mL) and positive for serum 20-00848 (IgG = 330 IU/mL) after vaccination.

As seen in Figure 10 and Figure 11, the copy numbers per well measured with RT-qPCR in correlation to the dilution steps of the virus-antibody-mix follow a non-linear regression model with sigmoidal form. Therefore, dose-response-inhibition curves following a non-linear regression model were chosen as an approximation for the calculation of regression models and neutralization titers. Neutralization (antibody) titers itself are calculated in two ways: using either simple linear regression (SLR) or non-linear regression (NLR), each followed by interpolation of ND50 (2.2.4.5). If the NT-

qPCR results from different experiments are shown in a graph with copy numbers instead of Ct values, those are approximations showing relative proportions and not exact results.

Table 8. *Antibody titers of used human blood sera.*

Serum	IgG-Titer in ELISA [in IU/L]	Neutralization titer in FRNT [in ND50]
20-00762	132 (negative)	55 (positive)
20-00848	330 (positive)	115 (positive)

The virus control indicates the mean value of all wells with virus controls (N = 4-8; calculated from at least 4 wells in parallel). For negative controls, mock-cell control (cell control) and water as RT-qPCR negative control were used.

3.4.1 NT-qPCR using extraction

As described in 3.4, for this experiment first NT-qPCRs for human blood serum were performed. For serum 20-00762 and serum 20-00848, the FRNT was positive (ND50 = 55 for 20-00762 and ND50 = 115 for 20-00848), where the ELISA IgG-Titer was negative for serum 20-00762 (IgG = 132 IU/L) and positive for serum 20-00848 (IgG = 330 IU/L).

Using the assumed dose-response-inhibition curve with sigmoidal form (3.4), a neutralization titer was calculated for both serum 20-00762 and 20-00848 (Table 9, Table 10, Table 11). The copy numbers of viral RNA per well (log10) in correlation to the dilution steps of virus-serum-mix (log2) fill in the assumed non-linear regression model as described in 3.4 (Figure 10A-C) and display the kinetics of MeV growing in each cell culture well of the dilution series, depending on the amount of neutralizing antibodies in each dilution step. If the amount of MeV RNA in a dilution step reaches the mean amount of MeV RNA, that was collected and measured in the virus control wells, for example for the dilution steps 1E-5 to 1E-9 for serum 20-00762 grown in 48-well plates (Figure 10A), the inoculated MeV infected the cell layer without being neutralized by antibodies.

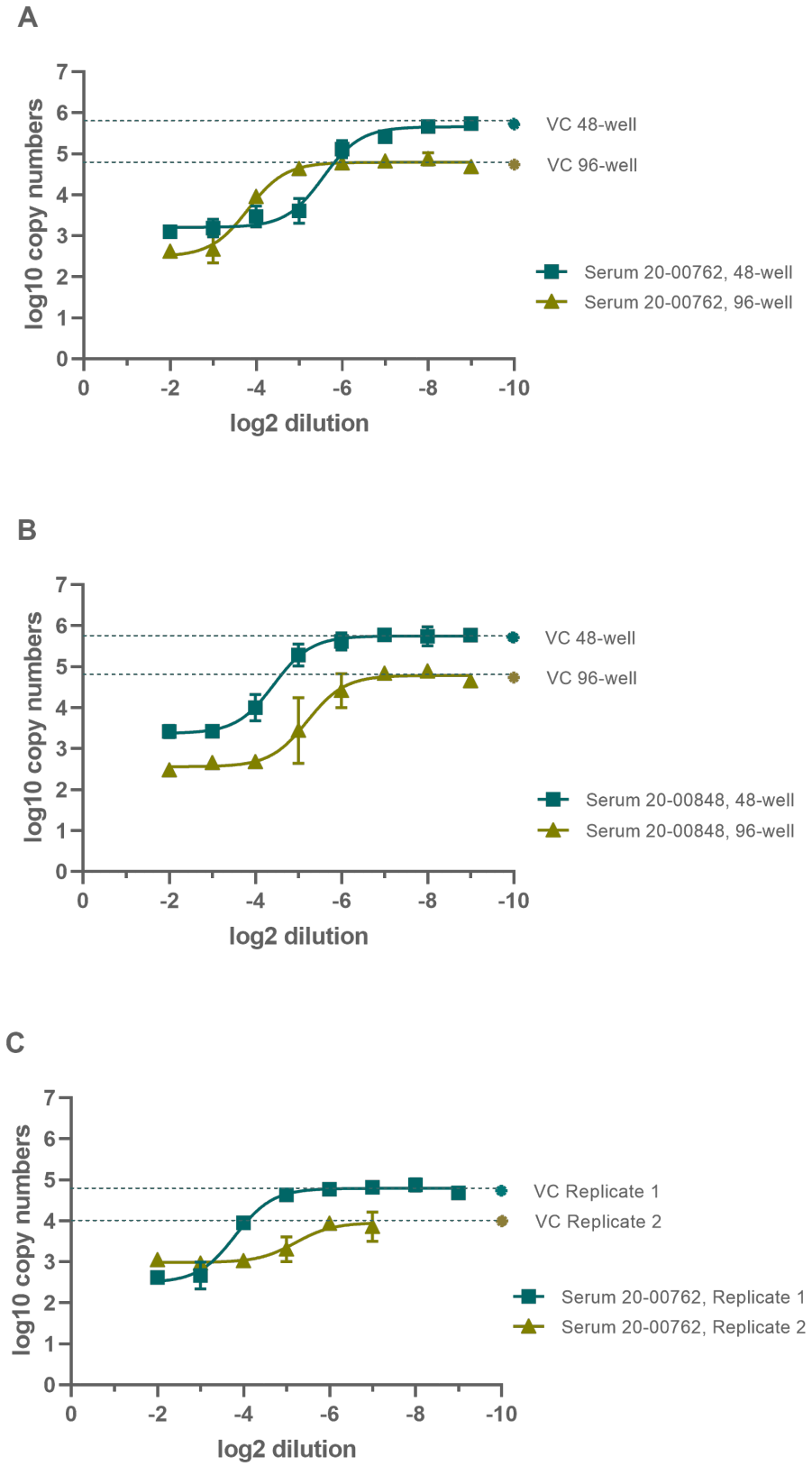

Figure 10 NT-qPCR for serum 20-00762 and 20-00848 with non-linear regression analysis. *For NT-qPCR, Vero/hSLAM cells were seeded in different plates and incubated for 24h at 36°C. The blood sera were prepared in 2-fold dilution series (starting with 1:4) using DMEM as diluent. MeV (Edmonston wild-type) was prepared (e.g., with dilution 2E-3) and mixed with the sera dilution series 1:1. After incubated at water and 37°C for 1h, the cells were infected, and the plates were incubated for 24h at 36°C. At 24h p.i. the cells were harvested using RNeasy*

Mini Kit and measured using relative quantification by RT-qPCR. For figure A-C, NT-qPCRs were performed with extraction, each point represents a mean value ± SD (n = 2) and analyzed using a non-linear regression model. Figure 10 shows NT-qPCRs with two different sera neutralizing MeV Edmonston wild-type; (A) shows a RT-qPCR with serum 20-00762 neutralizing MeV in 48-well and in 96-well cell culture plates, (B) RT-qPCR for serum 20-00848 neutralizing MeV in 48-well and 96-well cell culture plates, (C) RT-qPCR for serum 20-00762 neutralizing MeV, repeated in 96-well cell culture plates (replicate 2) compared to the RT-qPCR results from the first NT-qPCR in 96-well plates, seen also in Figure 10A (replicate 1).

This experiment was also performed to investigate the replication kinetics of MeV RNA with serum 20-00762 and 20-00848 in NT-qPCR in 48- or 96-well plates. The 48-well plates have higher diameter for each well and therefore more cells in each well grown, which are infected with a higher amount of MeV, since the same concentration with more volume was used than for 96-well plates.

Table 9. *Neutralization titers for serum 20-00762 from NT-qPCR (Figure 10A).*

NT-qPCRs for serum 20-00762 with cell culture plate:	Neutralization titer			
	NLR		SLR	
	ND50	ND90	ND50	ND90
NT-qPCR 48-well	172	108	238	78
NT-qPCR 96-well	47	30	44	22
ND differ with a ratio of	3.9	3.6	5.4	3.6

As seen in Figure 10A and Figure 10B, for 48-well plates the copy numbers of MeV RNA per well at each dilution step and the copy numbers per well for the mean virus control was higher than in 96-well plates. The amount of viral RNA in 96-well plates was even though measurable for RT-qPCR and the calculation of neutralization titers was successful. Since the neutralization titer displays a dilution factor, that is interpolated from the NLR curve and the titer is calculated in relation to the value of the mean virus control, the neutralization titer values should not be affected by this difference.

Differing ND50 and ND90 titer values were obtained for serum 20-00762 in 48- and 96-well plates (Table 9): for example for serum 20-00762 with ND50 = 182 (SLR: ND50 = 238) in a 48-well plate and ND50 = 47 (SLR: ND50 = 44) in a 96-well plate. The ND50 calculated with NLR differ with a ratio of 3.9, where the ND50 calculated with SLR differ with a ratio of 5.4. For serum 20-00848 the ND50 titer differed with ND50 = 74 (SLR: ND50 = 71) for 48-well and ND50 = 122 (SLR: ND50 = 109) for 96-well and

a ratio of 1.7 for NLR and 1.5 for SLR. Since the ND50 for serum 20-00762 differed with a ratio of 3.9 between 48-well and 96-well plates and serum 20-00848 differed with a smaller ratio of 1.7, the NT-qPCR for serum 20-00762 was repeated to confirm the ND50 titer in 96-well plates (Figure 10C).

Table 10. *Neutralization titers for serum 20-00848 from NT-qPCR (Figure 10B).*

NT-qPCRs for serum 20-00848 with cell culture plate:	Neutralization titer			
	NLR		SLR	
	ND50	ND90	ND50	ND90
NT-qPCR 48-well	74	46	71	36
NT-qPCR 96-well	122	77	109	64
ND differ with a ratio of	1.7	1.7	1.5	1.8

The repeated NT-qPCR confirmed a ND50 = 99 (SLR: ND50 = 80) for serum 20-00762 (replicate 2), differing with a ratio of 2.1 from the first NT-qPCR (replicate 1) with the ND50 = 47 (SLR: ND50 = 44). As described in 3.4 and seen here, the ND50 and ND90 are calculated in two ways: Either using simple linear regression (SLR), as it is standardized for FRNTs, or with non-linear regression (NLR), using a statistic program. As seen in this experiment, the neutralization titers differ for both calculation variants. The neutralization titers are interpolated and display a dilution step with 50% or 90% reduced amount of viral RNA compared to the virus control. The calculation is compared for all NT-qPCRs in this work.

Table 11. *Neutralization titers for serum 20-00762 from NT-qPCR (Figure 10C).*

Repeated testing with NT-qPCR for serum 20-00762:	Neutralization titer			
	NLR		SLR	
	ND50	ND90	ND50	ND90
Replicate 1	47	30	44	22
Replicate 2	99	13	80	20
ND differ with a ratio of	2.1	2.3	1.8	1.1

In summary, serum 20-00762 and serum 20-00848 were successfully measured with NT-qPCR. Either with SLR or with NLR and dose-response-inhibition-curves used for interpolation, neutralization titers were calculated for NT-qPCRs in 48-well and 96-well plates. A repeated measurement for serum 20-00762 with NT-qPCR differs with a ratio of 2.1 (for SLR: 1.8), as seen in Table 11. Variations of ND50 titer up to a ratio of 3 can be tolerated between repeated runs of a PRNT (plaque reduction neutralization test) (73). This value can be applied to the FRNT that only differs from PRNT by the method used for detection of foci of MeV infection in the cell monolayer and may be applied for NT-qPCRs as well.

3.4.2 NT-qPCR using one-step lysis

After the neutralization titers were measured for serum 20-00762 and serum 20-00848 with NT-qPCR and stepwise extraction, a NT-qPCR with one-step lysis was performed. The influence of extraction or one-step lysis as ways of harvesting the cells and isolating MeV RNA for the measurement with RT-qPCR were discussed and tested without human blood sera (3.3). To compare the performance of RT-qPCR measurement and the neutralization titers calculated from both variants of harvesting MeV RNA for the RT-qPCR, the same sera used for NT-qPCR with extraction were tested in NT-qPCR with one-step lysis.

For the NT-qPCRs, neutralization titers were calculated for serum 20-00762 and 20-00848 using NLR and SLR. For serum 20-00762, the ND50 tested with NT-qPCR using one-step lysis was ND50 = 51 (SLR: 40) and a repeated NT-qPCR with one-step lysis showed a ND50 = 38 (SLR: 28), differing with a ratio of 1.3 (1.4 for SLR) from the first NT-qPCR (Table 12). For serum 20-00848, the ND50 was 106 (SLR: 89) and the ND50 from the repeated NT-qPCR was 132 (SLR: 94), differing with a ratio of 1.3 (SLR: 1.1) from the first NT-qPCR with one-step lysis (Table 13).

The ND50 titer calculated either with NLR or SLR and the ratios between the ND50 from NT-qPCR repetitions differ, as seen here and for the calculation with NLR and SLR in 3.4.1.

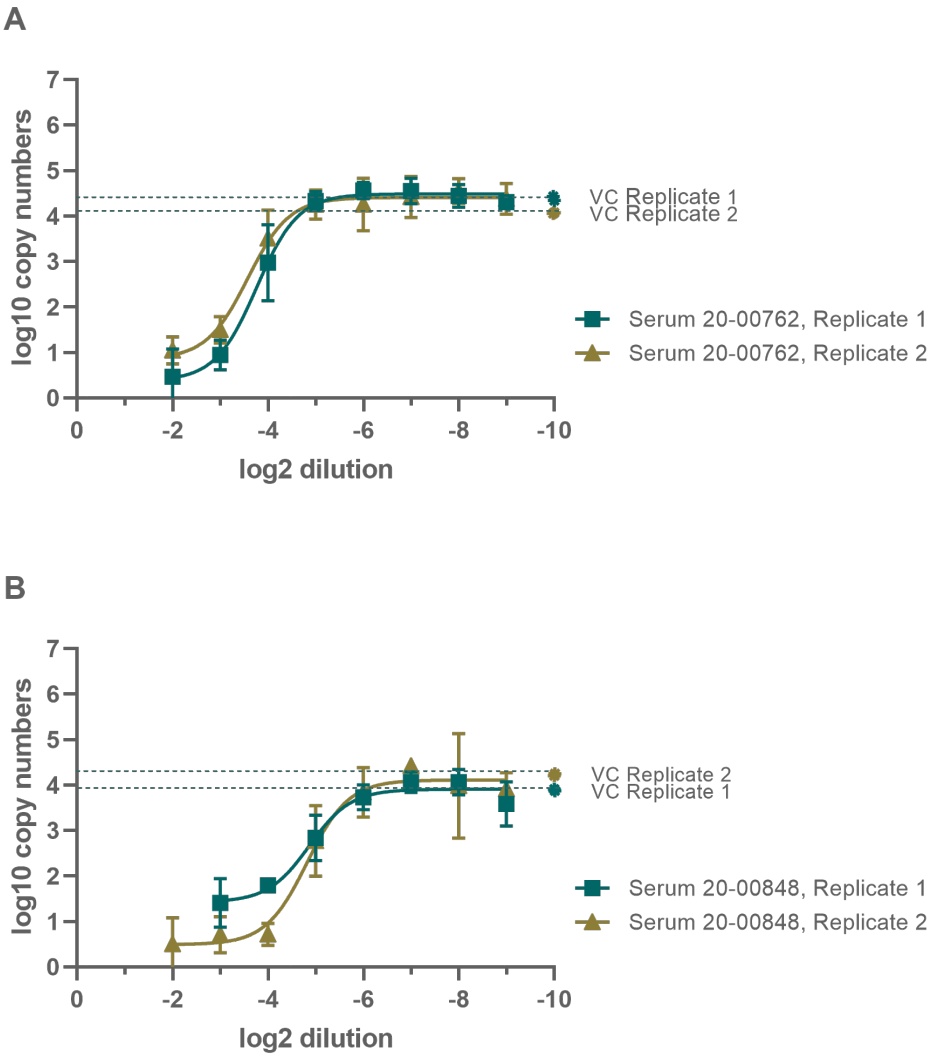

Figure 11 NT-qPCR for serum 20-00762 and 20-00848 with non-linear regression analysis. *After a NT-qPCR was performed using stepwise extraction by hand as a way of harvesting cells from the cell culture plate and isolating MeV RNA for RT-qPCR measurement, sera 20-00762 and 20-00848 were tested in NT-qPCR using one-step lysis instead of extraction by hand. NT-qPCR for each serum was performed twice (Replicate 1, 2). Figure (A) shows RT-qPCR results for serum 20-00762 neutralizing MeV, (B) shows RT-qPCR results for serum 20-00848 neutralizing MeV. Data are presented as mean ± SD (n = 4) and analyzed using a non-linear regression model.*

The amounts of MeV RNA detected with RT-qPCR after lysis of the cells in each well are overall lower than with using stepwise extraction, as seen in Figure 10 and Figure 11. This was also seen in the direct comparison of stepwise extraction and one-step lysis as in Figure 9 and 3.3.2.

The experiments described under 3.3.2 and 3.4.1 demonstrated that RT-qPCR involving extraction by hand was not only feasible but gave satisfying results. It must however be taken into account that it is a cost-expensive and laborious method.

Table 12. *Neutralization titers for serum 20-00762 from NT-qPCR (Figure 11A).*

Repeated NT-qPCR for serum 20-00762:	Neutralization titer			
	NLR		SLR	
	ND50	ND90	ND50	ND90
Replicate 1	51	36	40	28
Replicate 2	38	28	28	17
ND differ with a ratio of	1.3	1.3	1.4	1.7

To save these resources, for this experiment NT-qPCRs were done using SPR iScript one-step lysis, which allows to lyse samples directly in the cell culture plate well and perform RT-qPCR without further steps with the lysate. ND titer obtained for serum 20-00848 is higher (ND50 = 106) than for serum 20-00762 (ND50 = 51).

Table 13. *Neutralization titers for serum 20-00848 from NT-qPCR (Figure 11B).*

Repeated NT-qPCR for serum 20-00848:	Neutralization titer			
	NLR		SLR	
	ND50	ND90	ND50	ND90
Replicate 1	106	66	89	46
Replicate 2	132	79	94	65
ND differ with a ratio of	1.3	1.2	1.1	1.4

This agreed with further expectations described in 3.4, since the neutralization titer measured with FRNT was also higher for serum 20-00848 (ND50 = 115) than for serum 20-00762 (ND50 = 55).

3.5 Varied NT-qPCR procedures

NT-qPCRs were successfully performed testing human blood sera, with stepwise extraction and with one-step lysis to harvest MeV RNA from the cell culture plates before measuring the viral RNA in RT-qPCR. Although the results of the NT-qPCR with one-step lysis demonstrated the usefulness of this approach, the measurement with RT-qPCR showed, that the amount of viral RNA obtained with one-step lysis from each

well was less than with stepwise extraction (3.4.2). Moreover, more wells of the cell culture plate showed negative results in RT-qPCR measurement after harvesting MeV with one-step lysis than with extraction (3.3.2). To raise the amount of MeV RNA obtained with one-step lysis and investigate the influence of other factors, the procedure steps for NT-qPCR and the inserted concentration of MeV for NT-qPCR were varied in the following experiments.

3.5.1 NT-qPCR using different one-step lysis procedures

To vary the NT-qPCR procedure, the formerly used serum 20-00762 was tested and the RT-qPCR measurement results and ND50 titers were compared after different procedures for handling lysed cells. The lysed dilution series harvested from the cell culture plate were either centrifuged (Figure 12A), centrifuged and vortexed (Figure 12B), or pooled (Figure 12C) prior to the RT-qPCR measurement, each experiment in parallel to a dilution series immediately tested in RT-qPCR after lysis. Serum 20-00762 was tested in these experiments to allow comparison of the experiment to former NT-qPCRs (3.4.1, 3.4.2).

As seen in Table 14 and Figure 12A, serum 20-00762 was measured in NT-qPCR and the harvested cells from the cell culture plate were either measured immediately in RT-qPCR or centrifuged and afterwards measured in RT-qPCR. For the NT-qPCR with serum 20-00762 and immediate measurement, which is the procedure that was used for the NT-qPCR with serum 20-00762 in 3.4.2, neutralization titers were calculated with NLR (ND50 = 24) and SLR (ND50 = 21). The neutralization titers differ from the formerly calculated (3.4.2) GMT (ND50) = 44 (SLR: GMT (ND50) = 34) for serum 20-00762 with a ratio of 1.8 (SLR: 1.6). For serum 20-00762 tested in NT-qPCR, the harvested lysed cells were centrifuged and the contained MeV RNA per well was measured in RT-qPCR and the neutralization titers could not be calculated (Table 14). Although a ND90 = 893 was calculated with SLR, it differs with a ratio of 53 from the ND90 = 17, calculated for the NT-qPCR with serum 20-00762 with immediate measurement in RT-qPCR without centrifugation of the lysed cells, and it also differs with a ratio of 41 to the GMT (ND90) = 22 from former NT-qPCRs for serum 20-00762 with one-step lysis described in 3.4.2.

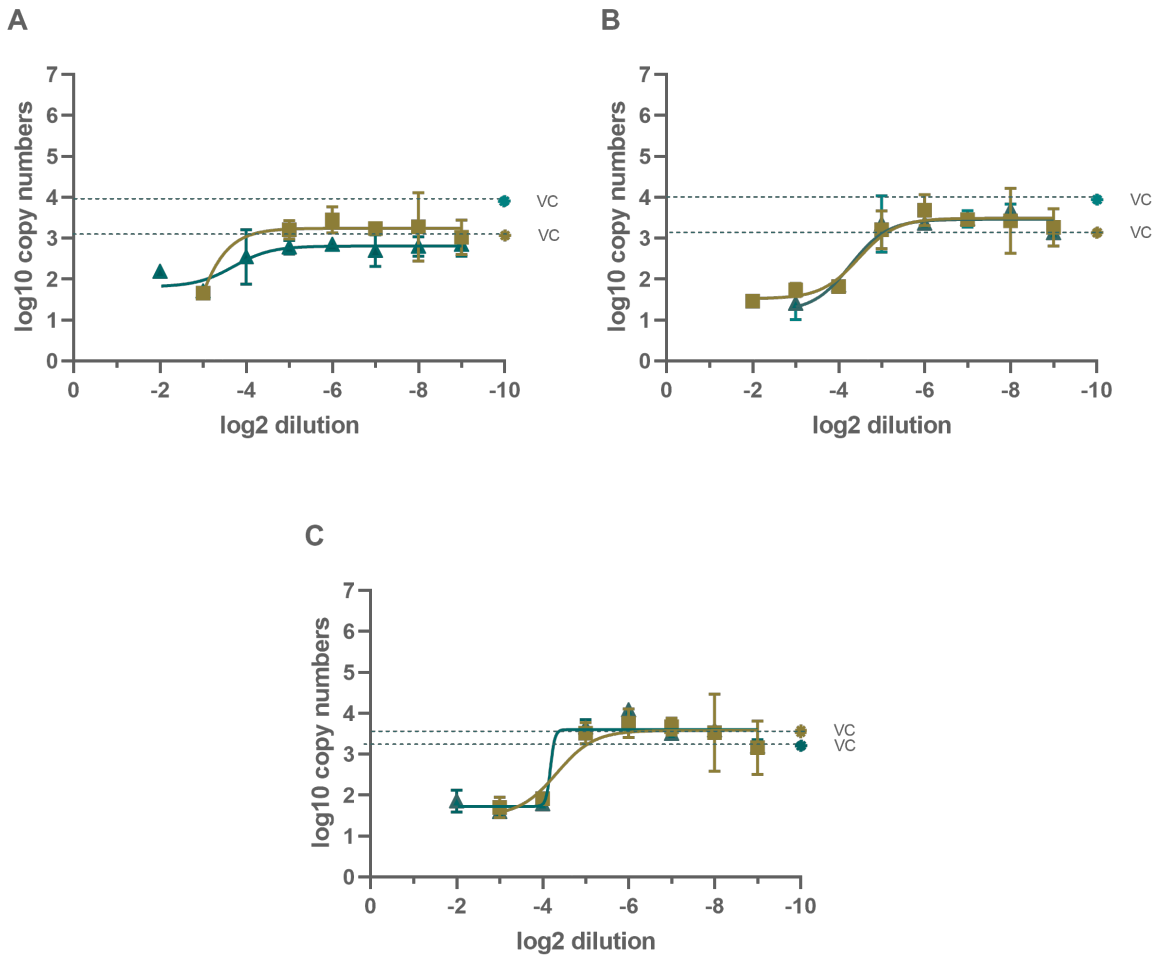

Figure 12 NT-qPCR for serum 20-00762 with non-linear regression analysis. *The NT-qPCR protocol with one-step lysis was varied: After harvesting the wells of the cell plate with iScript one-step lysis, two dilution series of the lysed samples were either (A) centrifuged (△), (B) centrifuged and vortexed (△), or (C) pooled (△) prior to the RT-qPCR measurement of obtained MeV RNA per sample. The RT-qPCR measurement is then compared with two dilution series, tested with immediate measurement in RT-qPCR (□; A-C) as previously described for NT-qPCRs (3.4.2), to each protocol variant. The data are presented as mean ± SD copy number per well (n = 2) and analyzed using a non-linear regression model.*

Since the ND50 titer for serum 20-00762 tested in NT-qPCR with centrifugation of the harvested cells prior to RT-qPCR measurement could not be calculated and the ND90 interpolated with SLR differed with a wide ratio of 39 to 53 from other NT-qPCRs with one-step lysis for serum 20-00762, this procedure variant for NT-qPCRs was no longer performed.

As a second variant of the NT-qPCR procedure, the cells harvested with one-step lysis from the cell culture plate were centrifuged and vortexed, and the MeV RNA was measured with RT-qPCR.

Table 14. *Neutralization titers for serum 20-00762 from NT-qPCR (Figure 12A).*

NT-qPCR for serum 20-00762 with procedure variants:	Neutralization titer			
	NLR		SLR	
	ND50	ND90	ND50	ND90
Immediate measurement of the lysed cells in RT-qPCR	24	18	21	17
Lysed cells are centrifuged prior to RT-qPCR measurement	-	-	-	893
ND differ with a ratio of	-	-	-	53

A NT-qPCR was performed with this harvesting variant for serum 20-00762 and a NT-qPCR for serum 20-00762 without procedure variations, where the lysed cells were immediately used for RT-qPCR measurement of the MeV RNA, to compare both variants. For both NT-qPCR variants, neutralization titers were calculated with NLR and SLR. For the NT-qPCR with centrifugation and vortexing of the lysed cells, the ND50 could not be calculated with NLR. However, a ND50 = 60 was calculated with SLR, differing with a ratio of 1.6 from the ND50 = 37 for the NT-qPCR with immediate measurement in RT-qPCR after one-step lysis, and differing with a ratio of 1.4 from the GMT (ND50) = 44 (SLR: GMT (ND50) = 34, ratio = 1.8) of former NT-qPCRs for serum 20-00762 (3.4.2). Although the neutralization titers were successfully calculated for serum 20-00762 with both NT-qPCR variations, the variant with centrifugation and vortexing of the lysed cells did not reach a higher amount of copy numbers per well in the RT-qPCR measurement (Figure 12B) than the NT-qPCR without variations. Since the NT-qPCR variation with centrifugation and vortexing did not improve the RT-qPCR measurement through higher copy numbers per well, this variant was not used for further NT-qPCR testing.

As a third variation of the NT-qPCR procedure to improve RT-qPCR measurement with one-step lysis, the lysed cells from the cell culture plate of the NT-qPCR were pooled from two biological replicates and measured with RT-qPCR. The neutralization titer and the RT-qPCR measurement were compared with a NT-qPCR for serum 20-00762 without variations and direct measurement of the lysed cell samples in RT-qPCR.

Table 15. *Neutralization titers for serum 20-00762 from NT-qPCR (Figure 12B).*

NT-qPCR for serum 20-00762 with procedure variants:	Neutralization titer			
	NLR		SLR	
	ND50	ND90	ND50	ND90
Immediate measurement of the lysed cells in RT-qPCR	54	35	37	33
Lysed cells are centrifuged and vortexed prior to RT-qPCR measurement	-	56	60	35
ND differ with a ratio of	-	1.6	1.6	1.1

As shown in Table 16, the ND50 were calculated for the pooled NT-qPCR for serum 20-00762 with ND50 = 50 (SLR: 35), differing only with a ratio of 1.3 (ND50 with SLR: 1.2) from the ND50 = 65 (SLR: 41) for serum 20-00762, tested with a NT-qPCR with immediate RT-qPCR measurement. The ND50 for the pooled variation also differs with a ratio of 1.1 (for ND50 with SLR: 1.03) from former NT-qPCR results for serum 20-00762 (3.4.2). The ND50 titers for serum 20-00762 with both procedure variations of NT-qPCR differed with small ratios of 1.03 to 1.3. However, the RT-qPCR did not measure higher amounts of copy numbers per well for the pooled NT-qPCR, so the NT-qPCRs were performed without pooling of the lysed cells.

Table 16. *Neutralization titers for serum 20-00762 from NT-qPCR (Figure 12C).*

NT-qPCR for serum 20-00762 with procedure variants:	Neutralization titer			
	NLR		SLR	
	ND50	ND90	ND50	ND90
Immediate measurement of the lysed cells in RT-qPCR	65	40	41	33
Lysed cells are pooled prior to RT-qPCR measurement	50	32	35	32
ND differ with a ratio of	1.3	1.3	1.2	1.03

Although two out of three handling variants for the NT-qPCRs with serum 20-00762 were feasible and the neutralization titers showed similar results as for direct measurement of the lysed cells, the procedure variants for NT-qPCR did not show an increase in the amount of copy numbers of MeV RNA per well measured with RT-qPCR. The following NT-qPCRs were performed without any handling variations and the lysed cells from the cell culture plates were directly measured in RT-qPCR.

3.5.2 NT-qPCR with varied virus concentrations

Three procedure variations for the lysed cells harvested from the cell culture plates with one-step lysis for NT-qPCR were tested with the objective to increase the amount of MeV RNA per well so that higher amounts of copy numbers of DNA per well can be obtained with RT-qPCR measurement described in 3.5.1 and not added to the NT-qPCR procedure. For this experiment, a NT-qPCR for serum 20-00762 was performed with increased viral input for the infection of the cell culture plates to reach higher MeV RNA concentrations in the cell culture wells. If MeV is used in a higher concentration, the dilution series of the blood serum as well as the virus control contains higher concentrations of MeV when the samples are inserted in the cell culture plate and the cells incubated for infection. After harvesting the cells from the cell culture plate, the amount of MeV RNA obtained in each well after 24 h incubation time is expected to be higher and therefore the copy numbers of DNA per well measured with RT-qPCR should increase. For previous NT-qPCRs, a concentration of 2E-3 with MeV Edmonston wild-type (c = 1E6.7 PFU/mL) in DMEM was used. For the NT-qPCR in this experiment, the concentration was increased up to 7E-3. Since the bottom values represent dilution steps for serum 20-00762 and virus-mix with high concentrations of neutralizing antibodies, they range in low copy numbers of DNA per well measured with RT-qPCR. Those results are often found in regions with less sensitive and less precise RT-qPCR measurement.

As seen in Figure 13, the NT-qPCR with a higher concentration of MeV showed higher amounts of MeV RNA in each well in the RT-qPCR measurement compared to the NT-qPCR with virus concentration = 2E-3. This leads to the conclusion, that the used system for NT-qPCRs is stable for different virus concentrations and can be varied in future experiments.

For the NLR model used for NT-qPCR, the top line of the curve indicates high dilution steps, with a decreasing serum concentration per dilution step leading to a decreasing number of neutralizing antibodies. If the measured amount of viral RNA per well equals the amount of viral RNA in the virus control wells for this dilution steps, there is no MeV-neutralizing activity visible. As seen for the NT-qPCRs shown in Figure 13, the copy numbers of cDNA measured with RT-qPCR after reverse transcription and amplification of viral RNA are apparently due to an unspecific inhibition of viral replication: The amount of copy numbers per well for those high dilution steps do not equal the virus control signal, even though the copy numbers do not decrease with the following dilution steps and therefore indicate free replication without any neutralizing activity.

This effect is also seen for the NT-qPCR with a viral input (c = 2E-3) and serum 20-00762, which was tested in those settings before (3.4.2), where no inhibitory effect was seen. Since the inhibitory effect was first seen for the NLR model evaluation with GraphPad Prism for NT-qPCRs in this experiment, it was decided to investigate the effect with other blood sera.

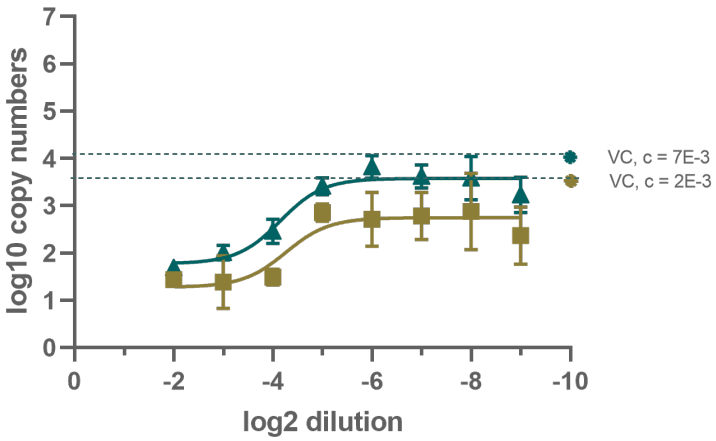

Figure 13 NT-qPCR for serum 20-00762 with non-linear regression analysis. *The NT-qPCR was performed with two different viral concentrations inserted for infection of the cell culture plates. MeV (Edmonston wild-type; c = 1E6.7 PFU/mL) was used diluted in D-MEM to two different concentration, c = 2E-3 or 7E-3. Shown are the RT-qPCR measurement results of serum 20-00762 neutralizing MeV either with concentration = 2E-3 (□) or c = 7E-3 (△). Data are presented as mean values ± SD (n = 4) and analyzed using a non-linear regression model.*

3.6 NT-qPCRs for serum 20-00762 and serum 20-00848 in comparison

Several NT-qPCRs were performed with serum 20-00762 and 20-00848 comparing two ways of harvesting cells and isolating MeV RNA from the harvested cells for RT-qPCR measurement, stepwise extraction of MeV RNA from the harvested cells or direct measurement of the cells lysed with one-step lysis. To compare both methods, lysis or extraction as ways of harvesting, the ND50 titers for serum 20-00762 and 20-00848 are shown in Figure 14.

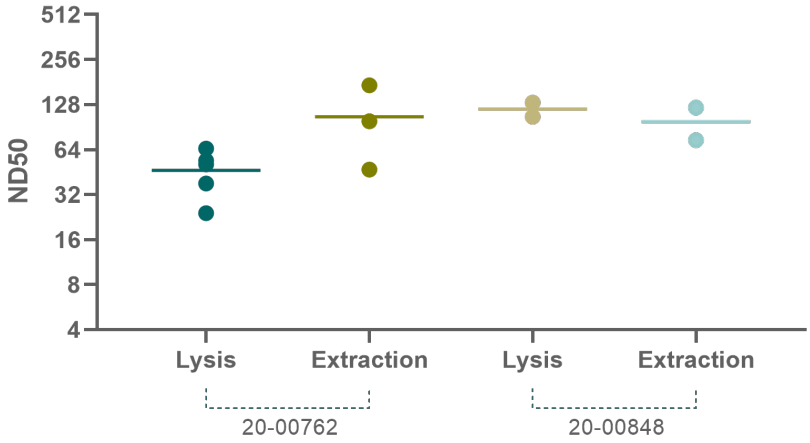

Figure 14 ND50 titer from NT-qPCR for human sera in comparison. *Several NT-qPCRs were performed with both serum 20-00762 and serum 20-00848. ND50 titers are shown in comparison for all performed NT-qPCRs depending on two different methods tested for NT-qPCR procedures: Two ways of harvesting cells from the cell culture plate prior to the RT-qPCR measurement are compared, stepwise extraction and isolation of MeV RNA from the harvested cells ("extraction") or direct use of the lysed cells for RT-qPCR measurement of MeV RNA ("lysis").*

Table 17 *Neutralization titers for serum 20-00762 and serum 20-00848 from NT-qPCR (Figure 14)*

NT-qPCR for serum:	Neutralization titer						ND differ with a ratio of
	Lysis			Extraction			
	ND50	SD	N	ND50	SD	N	
20-00762	44	16	5	93	63	3	2.3
20-00848	118	11	2	95	34	2	1.1

3.7 Human blood sera tested in NT-qPCR

Varied NT-qPCR procedures, including the use of 48- and 96-well cell culture plates, different ways of harvesting cells and isolating MeV RNA from the cells, as well as different approaches to increase the amount of copy numbers of RNA per well measured with RT-qPCR were tested with two human blood sera, serum 20-00762 and

serum 20-00848. After evaluating the results from all experiments, the NT-qPCR performed in 96-well plates, with one-step lysis as way of harvesting cells from the plate and the immediate measurement of MeV RNA per well in RT-qPCR without further procedure steps was found to be feasible, cost- and timesaving and demonstrated the usefulness of this approach.

Table 18. *Neutralization titers for human blood sera tested with NT-qPCR, FRNT and ELISA.*

NT-qPCR for human sera:	Neutralization titer				FRNT [in ND50]	ELISA [IgG in IU/L]
	NLR		SLR			
	ND50	ND90	ND50	ND90		
20-00056	>521	292	506	257	>256	539 (pos.)
20-00059	>521	223	562	261	>256	643 (pos.)
17-00858	248	94	150	42	128	203 (pos.)
11-00914	-	23	-	19	36	144 (neg.)
15-00805	-	36	151	30	52	201 (pos.)
16-00110	23	14	18	-	64	132 (neg.)
19-00043	127	66	77	56	58	767 (pos.)

Proceeding with the chosen NT-qPCR protocol, more human blood sera were tested in NT-qPCRs to test the NT-qPCR ability for the detection of MeV-neutralizing antibodies. For all used human sera, tested with ELISA for the IgG titer and with FRNT for the ND50, neutralizing antibodies in different concentrations were detected with FRNT, where the ELISA showed negative or positive IgG titer.

For six of the seven blood sera tested in NT-qPCR, neutralization titer could successfully be calculated (Table 18). As shown in Table 18, ND50 and ND90 (14%), both with NLR and SLR, could not be calculated. Compared to all NT-qPCRs performed with human blood sera, 9% of the ND50/ND90 titers (out of 88 total) could not be calculated either with NLR or SLR. For five out of seven NT-qPCRs for the blood sera a probably unspecific inhibition of viral replication, as described in 3.5.2, was seen for a NLR model evaluation with GraphPad Prism. The calculated neutralization titers were in close agreement with the neutralization titers determined by the use of a FRNTs, differing with ratios less than 3.0.

3.8 Immunoglobulin in NT-qPCR

NT-qPCRs were not only performed with human sera, but also with an immunoglobulin product for intravenous administration representing the cross section of sera obtained from a high number of plasma donors (1.4.4, 2.1.8). NT-qPCRs for a commercially available immunoglobulin product instead of blood sera were performed either using extraction or lysis as ways of harvesting the cells and isolating MeV RNA prior to RT-qPCR measurement.

Table 19. *Neutralization titers obtained for an immunoglobulin product by NT-qPCR.*

Harvesting variation for NT-qPCR:	Neutralization titer			
	NLR		SLR	
	ND50	ND90	ND50	ND90
Stepwise extraction	2604	1217	4137	749
One-step lysis	1144	864	1003	553
ND differ with a ratio of	2.3	1.4	4.1	1.4

For both NT-qPCRs, either with stepwise extraction or with one-step lysis, the neutralization titer was calculated and differed with a ratio of 2.3 (for SLR: 4.1) for the ND50 and 1.4 (for SLR: 1.4) for the calculated ND90. For this work, both ways of NT-qPCR procedures, either stepwise extraction or one-step lysis, were successfully performed and could be applied to determine the MeV-neutralizing capacity of immunoglobulin products.

3.9 3rd WHO International Standard in NT-qPCR

When a standardized conventional FRNT is used, neutralizing antibody titers are determined in comparison to the 3rd WHO International Standard for anti-measles serum/plasma (WHO IS 3rd) containing 3 IU/ml (78). The use of WHO IS 3rd is further described in 2.2.4.4 and 1.4.4. The international standard is important to establish comparability across laboratories and in surveillance programs, particularly with regard to vaccine studies within the WHO measles elimination program.

Table 20. *Neutralization titer for WHO IS 3rd in NT-qPCR.*

Harvesting variation for NT-qPCR:	Neutralization titer [in ND50, N = 2]	
	NLR	SLR
Stepwise extraction	3353	3822
One-step lysis	530	360
ND differ with a ratio of	6.3	10.6

The neutralization titer for WHO IS 3rd tested with NT-qPCR differ with a wide ratio of 6.3 (calculated with NLR) up to 10.6 (calculated with SLR) between NT-qPCRs with different ways of harvesting cells and isolating MeV RNA prior to RT-qPCR measurement. The results must be re-evaluated in further experiments.

4 Discussion

Although an effective, safe and inexpensive vaccine against measles virus (MeV) has been available for 60 years now (10), measles is still a widespread infectious disease with high prevalence worldwide, responsible for a high number of severe courses and deaths (1,2). Since the WHO adopted measles elimination and a 95% coverage with the first and second dose of measles containing vaccines (MCV) regionally and internationally (10,11), various strategies and resolutions have been developed (3,12,38). As a result, major achievements have been made, including a 83% reduction of estimated annual measles deaths from 2000 to 2021, decreasing from 761,000 to 128,000 annually, preventing an estimated 56 million deaths by vaccination (1). Nevertheless, since 2016, the measles incidence and thus the mortality have been rising significantly worldwide again for several years now (12,43). The COVID-19 pandemic was also a severe disruption to vaccination strategies worldwide (1,12,43). Regular laboratory-based global surveillance of transmission was interrupted, and approximately 178 million people could not be regularly vaccinated in 2020 (12). For this reason, measles elimination efforts, particularly laboratory surveillance of measles cases and population immunity questions, needs to be strengthened in the coming years.

Neutralizing antibodies are the best correlate for protection against measles and hence the neutralization assay is considered as the gold standard for the assessment of measles immunity (5,73,76). The ELISA measures antibody titers (IgG, IgM) reliably, quickly, and inexpensively (79,86,87). In contrast to the neutralization assay it does not distinguish between functional neutralizing antibodies and total antigen-binding antibody titers (74,79,86). The neutralization assays for MeV measure the titer of MeV neutralizing antibodies binding to the viral proteins H and F, mainly the H protein, of MeV particles (57,67) in patient serum samples and thus represent a correlate of the immunity status against re-infection (73,74,76). The procedure of a neutralization assay is however time-consuming and labor-intensive, and the test can only partially be standardized (79,80).

The aim of this study was to establish a neutralization assay for the National Reference Centre for Measles, Mumps and Rubella in Germany (NRC MMR) at the Robert Koch-Institute (RKI), based on the foci reduction neutralization assay (FRNT), but shortened and improved by endpoint determination using real time quantitative reverse

transcription polymerase chain reaction (RT-qPCR) and relative quantification instead of the regular immuno-colorimetric assay (75,76) commonly used in the FRNT. The study is based on a previous report (80), describing a microneutralization assay with RT-qPCR endpoint determination (MN-RT-qPCR).

Working time can be strongly shortened and in the long term a more economical and objective alternative to conventional neutralization assays can be established (80). Especially regarding future challenges in the measles elimination phase this assay can contribute to research, immunity surveillance, and clinical practice.

4.1 Foci reduction neutralization assay or endpoint determination using RT-qPCR

The MeV-neutralizing activity of a patient's blood serum is measured by the determination of reduced virus replication in cell cultures after incubating the virus suspension with the serially diluted blood serum (73,75).

The more neutralizing antibodies the serum contains, the more MeV can be neutralized by the antibodies. Hence, fewer cells of the cell culture plate are infected. As a result, there are fewer foci of infection seen after infection and incubation. These foci of infection appear as blue spots after the measles antigens of infected cells of the cell culture plate are stained with an immuno-colorimetric assay (73,76). A neutralization titer can be calculated by the number of foci in relation to the number of foci in a virus control with free virus replication. If, in addition to the controls, a reference serum as e.g. WHO 3rd International Standard for anti-measles serum (WHO IS 3rd, (77,78)) is used as a standard in the FRNT, the neutralization titer can be expressed in International Units (IU) and thus be compared with other results (78).

FRNTs offers many advantages, it has been established for many years and is standardized (73). Due to the standardization the results are comparable between laboratories worldwide and a statement on whether a determined neutralizing titer indicates seroprotection can be made: A titer of >120 mIU/mL may be correlated with protection against measles disease in vaccinated individuals (10,73). However, the FRNT is a very labor-intensive test: A run takes at least six days at a stretch with preparation of the cell culture plates and five days of incubation after infection (73). In addition, repetitions are needed if the results of an assay run do not meet the validation criteria and the standardization is difficult (73,79). Moreover, high-throughput is made difficult by

the labor-intensive assay process involved in fixing and staining cells on 48-well plates and long incubation periods (73,79,80). Moreover, the evaluation of a neutralization titer by counting the stained foci using a magnifying glass or the naked eye is subjective (75,76). Therefore, a rapid and profound answer to the clinical questions of immunity status is difficult to provide.

A neutralization assay with endpoint determination by RT-qPCR (NT-qPCR) was performed in this work. After 24 h incubation, the infection is stopped, and the amount of viral RNA is measured by RT-qPCR for each well of the cell culture plate. Thereupon, the titer of neutralizing antibodies is calculated with the amount of viral RNA per well measured by RT-qPCR and given in copy numbers by relative quantification, instead of the number of stained foci of infection counted with a magnifying glass or the naked eye.

To establish the NT-qPCR, this study investigated the influence of cell type, MeV genotype, cell culture plates, reagents, incubation, ways of harvesting viral RNA and RT-qPCR measurement with quantification, as well as differences in test evaluation and calculation of neutralization titers using different mathematical models.

4.2 Patient blood serum samples

For this work, sera with negative, borderline, or positive ELISA IgG-titer and FRNT ND50-titer were chosen to test the ability of the NT-qPCR to measure neutralizing antibodies in a wide range. The NT-qPCR was performed for sera with a positive titer in FRNT, while the ELISA IgG-titer was negative, equivocal, or positive. Sera 20-00762 and 20-00848 were selected to perform the initial experiments since a sufficient serum volume was available. Serum 20-00762 was tested negative with ELISA for IgG (IgG = 132 IU/L), although neutralizing antibodies were present (ND50 = 55). Serum 20-00848 is from the same 24-year-old patient. Since the IgG titer was negative in the first place after having received two doses of MCV, the patient was vaccinated with a third MCV and a positive IgG titer was measured (IgG = 330 IU/mL) afterwards, as well as a higher titer for neutralizing antibodies in FRNT (ND50 = 115). For ELISA EUROIMMUN interpretation of findings see Table 21.

Table 21. *Interpretation of ELISA IgG results for EUROIMMUN analyzer.*

IgG-titer [in IU/L]	Interpretation
< 200	Negative
≥ 200 until < 275	Equivocal
≥ 275	Positive

4.3 Cell culture methodology in NT-qPCR

As described (1.2.4), MeV use the cellular receptors CD46 (membrane cofactor protein, (60,61)) and hSLAM (human signaling lymphocytic activation molecule, (20,21,24)) as targets to infect humans. Vaccine viruses can infect both Vero cells (with the CD46 receptor) and Vero/hSLAM cells (expressing the receptor hSLAM in addition to CD46) (61,88). Vice versa, wild-type MeV, including currently circulating genotypes, use hSLAM as a receptor to infect the cells of the immune system, B- or T-lymphocytes (24), dendritic cells and macrophages (20), but not the CD46 receptor (88).

Alvarado-Facundo et al. (80) used Vero cells (ATCC CCL-81) lacking hSLAM receptor for their MN-RT-qPCR. This can be justified for work with MeV Edmonston using CD46 receptor for the infection (60,88). Since the hSLAM receptor is present on the immune cells and used for their infection by MeV, those antibodies that prevent binding of MeV the hSLAM receptor are an important part of immunity of patients against MeV infection. In neutralization assays with Vero cells lacking hSLAM receptor like the neutralization assay *Alvarado-Facundo* et al. (80) established, those neutralizing antibodies that are targeted specifically to the hSLAM binding domain of the MeV H protein are not measured, which is a disadvantage compared to this work.

To analyze the neutralizing capacity of vaccinated and infected individuals against MeV of currently circulating genotypes, cells expressing the hSLAM receptor should be used. Therefore, Vero/hSLAM cells are used in the laboratories of the WHO Global Measles/Rubella Laboratory Network (GMRLN) to isolate and passage MeV (88,91). A FRNT using Vero/hSLAM cells has been established in the NRC MMR at the RKI (75) and was used in this work for all NT-qPCRs and FRNTs.

A limitation for the NT-qPCR from this work, as well as for the microneutralization assay from *Alvarado-Facundo* et al. (80), is that the neutralization assays have only been performed with the MeV Edmonston wild-type belonging to the earlier circulating

genotype A. For this purpose, a MeV of genotype D8 (MVi/Offenburg.DEU/10.19) was successfully isolated and passaged in Vero/hSLAM cells and is used in the FRNT. The next step is to adapt the NT-qPCR to the currently circulating MeV genotypes D8 and also B3 (1) and investigate the ability of NT-qPCR to measure neutralizing capacity of human blood sera against different genotypes.

4.4 RT-qPCR methodology in NT-qPCR

RT-qPCR with relative quantification was successfully used for endpoint determination in NT-qPCR. The RT-qPCR has been validated by the NRC MMR according to the RKI internal quality management standards, testing for inter-assay and intra-assay precision, linearity, specificity, sensitivity, matrix effect and limit of detection, which was determined to be 50 copies per test.

4.4.1 Primers, RT-qPCR machines, and RT-qPCR fluorescence measuring

4.4.1.1 RT-qPCR machine

For RT-qPCR measurement in their MN-RT-qPCR, *Alvarado-Facundo* et al. (80) decided to use a one-step RT-qPCR with the CFX-96 machine (Bio-Rad). Similar studies used either CFX-96 automates (81,92,93) or 7500 real-time PCR instruments (Applied Biosystems) with two-step RT-qPCR (94,95) for viral amplification and quantification in neutralization assays with RT-qPCR endpoint determination. Two-step RT-qPCRs offer advantages: Both reactions, the reverse transcription and the real-time PCR are optimized separately (83). The methodology is more sensitive since the reaction conditions and reagents do not have to be a compromise for both reactions and reaction temperatures (83). In comparison, the one-step RT-qPCR also offers certain advantages: It minimizes the contamination risk since the additional tubes and pipetting steps between the reverse transcription and the real-time PCR are omitted (90,96). Another disadvantage of the two-step RT-qPCR is, that through those additional pipetting steps, the methodology is time-consuming, next to the described increased contamination risk (83,96). Since the aim of this work is to perform high-throughput NT-qPCRs and to reduce working hours, a one-step RT-qPCR was performed for NT-qPCR using the LightCycler480 automated machine, since the LightCycler480 was already used at, and the methodology validated for the NRC MMR at the RKI.

4.4.1.2 RT-qPCR fluorescence measurement

Several studies for neutralization assays with RT-qPCR endpoint determination (80,81,92,93), including *Alvarado-Facundo* et al. (80), decided to use SYBR Green as detection method in qPCR. As a detection method, SYBR Green offers advantages, including a wide range of applications and high signal strength (83). The signal is measured after the SYBR Green I molecules bind to the resulting double-stranded DNA - the more binding molecules, the higher the intensity (82). However, this results in artificial fluorescence measurement, which considerably reduces the accuracy of the method, especially for the aimed relative quantification (82). In comparison, the fluorescence in TaqMan probes is enhanced by the binding of oligonucleotides to the DNA: As the fluorescence signal from the reporter is suppressed by the quencher when the reporter and quencher are attached at 5' and 3', the fluorescence increases as soon as the probe attaches along the DNA (83). Therefore, the fluorescence enhances parallel with the increasing amplified DNA, and small amounts of copy numbers can be measured (97). Choosing a TaqMan probe as the detection method in RT-qPCR for this work makes the method more expensive (90). Nevertheless, TaqMan RT-qPCR can increase the specificity of results, because the TaqMan probes do not bind non-specifically to double-stranded DNA like SYBR Green I, but rather bind directly to the selected sequences of single-stranded DNA (83) and quantify low amounts of cDNA more reproducible (97).

4.4.1.3 Primers for RT-qPCR

Alvarado-Facundo et al. (80) chose primers NBP1/NBP2 targeting the N gene of MeV (Table 22) used with the SYBR Green kit (Bio-Rad). After considering the amplification efficiency, the specific Forward and Reverse primers located in the MeV-N gene ((98); seen in Table 22) are used in the NRC MMR and were adopted for this work.

Table 22. *Primers for one-step RT-qPCR in comparison.*

Name:	Sequence (5' to 3'):
3424 (MeVN1139-F), Forward	TGG CAT CTG AAC TCG GTA TCA C
3425 (MeVN1213-R), Reverse	TGT CCT CAG TAG TAT GCA TTG CAA
3426 (MeVN1163-P), Probe	FAM-CCG AGG ATG CAA GGC TTG TTT CAG A-BHQ1
NPB1	GATCCGCAGGACAGTCGAAGGTC
NPB2	AGGGTAGGCGGATGTTGTTCT
from *Alvarado-Facundo* et al. (80), used with SYBR Green kit (Bio-Rad)	

4.4.2 Relative quantification

To measure the quantity of MeV RNA inserted in RT-qPCR for the NT-qPCR endpoint determination, relative quantification using counter-calculation against standard curves was successfully established. Two methods were tested, and both were established for the relative quantification of viral RNA, a RNA and a DNA standard.

As described in previous studies (80,81,93), RNA of the same virus and genotype as tested in the neutralization assay was often included as a standard in each RT-qPCR run. For this purpose, the virus was grown in cell culture, harvested, and pipetted into the qPCR plates in 10-fold dilution series (80). Estimated units of RNA were then entered manually after the qPCR run in the program and thus used as a calibration curve (80). The concentrations 1E6 to 1E1 copy numbers were chosen for the RNA standard with MeV Edmonston wild-type before (80). In this work, the relative quantification using MeV RNA was successfully established. For this purpose, RNA was extracted from the supernatant as well as from the cells and both variants were used in RT-qPCR.

However, the relative quantification was established using a DNA standard, which was ordered compatible to the primer set. It is reproducible since the DNA can be easily reordered and used in dilution series for other laboratories. In addition, the exact amount of DNA obtained is known before amplification in the RT-qPCR and was chosen 1E6 to 1E1 copy numbers in 10-fold dilution series. The addition of Lambda-DNA

with a protective effect to the DNA standard was used to keep the amount of DNA constant in dilution for storage and multiple thawing and freezing.

The DNA standard established in this work therefore offers consistent quality and concentrations and can be used for diagnostic routines.

4.4.3 Ways of harvesting MeV RNA

For the NT-qPCR, in this work two methodological variants of harvesting the cells and isolating the viral RNA for the RT-qPCR measurement, one-step lysis (Method 1) and extraction (Method 2), were performed: After the cultivation of the cells and infection of the cell culture plates with virus-antibody-mix, the infected cell layers in each well and the controls were harvested. Following the aspiration of medium and washing with PBS, for Method 1 the cells were incubated with SPR iScript lysate for one minute and the lysate containing MeV, but also the lysed cells and the SPR lysis buffer, directly used for RT-qPCR measurement. This approach was compared to Method 2, the extraction of the viral RNA per well from the cell culture plate by hand with RNeasy Kit and the isolation of the viral RNA.

A difference between one-step lysis and extraction of viral RNA is the cost factor. The lysis for one 96-well plate costs $186 (where four replicates of each sample were measured), the extraction for the NT-qPCR for one 96-well plate $576 (where two replicates of each sample were measured). The cost for the NT-qPCR with one-step lysis can further be reduced by the establishment of an in-house lysis buffer (99). The cost for an NT-qPCR with extraction can only be reduced using less serum dilution steps from the 2-fold dilution series for the endpoint measurement, for example four dilution steps instead of the eight currently used, resulting in a less precise calculation of neutralization titer.

Another aim of this work was to reduce working hours of the time-consuming FRNT, which takes over six days (73). Due to the reduced incubation time of one day, the NT-qPCR length is successfully reduced from six to seven to only two days. However, there is a difference in required working time between the two tested harvesting ways. The harvesting with one-step lysis takes one minute for the incubation and the samples are directly measured by RT-qPCR. In comparison, due to the numerous washing steps of the RNeasy Mini Kit manufacturer´s protocol, as well as the handling with

different tubes and buffers, the method of extracting viral RNA takes two to three hours and is therefore very time and material consuming. A solution for the extraction with the RNeasy Mini Kit is the possibility of automatizing the process by using the QIAcube or QIAcube connect automates, skipping the labor-intensive extraction by hand and reducing the risk of contamination (100).

4.4.3.1 Extraction of viral RNA with RNeasy Mini Kit

A major advantage of the RNA extraction is, that lysed cell residues, buffers and other materials are washed away and only the isolated pure viral MeV RNA remains on the membrane used in the kit (83). The MeV RNA is then eluted in RNase and DNase-free water and measured with RT-qPCR. Ideally, the sample with extracted RNA measured in the RT-qPCR does not contain any cell lysate, RNases, or buffer to either influence the amplification and the fluorescence measurement or to reduce the amount of viral RNA (83). As described in *Wilson* et al. (95), neutralization assays with RT-qPCR end-point determination for Zika virus and Dengue virus have been described using an extraction kit (QIAcubeHT, Qiagen). In this work, the NT-qPCR with MeV was successfully performed with RNA extraction using RNeasy Mini Kit for human blood sera, a commercial immunoglobulin product (KEDRION Biopharma GmbH) and the WHO IS 3rd.

4.4.3.2 One-step lysis with SPR lysate

A major advantage of one-step lysis is the incubation time of one minute, recommended by the manufacturer, leading to reduced working time of the RNA harvesting for the NT-qPCR. Although the harvesting with one-step lysis is time-effective and was used for neutralization assays in some works (80,81,92,93), there are notable disadvantages. After the cells infected with MeV RNA are lysed with SPR one-step lysate, the viral RNA in the sample is directly measured by RT-qPCR according to the manufacturer´s instructions. As described above, the sample measured with RT-qPCR not only contains MeV RNA, but also contaminations like the lysed cells and buffers, the reagents used during harvesting the RNA (90). This can interfere with the reverse transcription, the amplification, and the fluorescence measurement and lead, especially for the quantitative RT-qPCR measurement, to underestimation of the contained RNA

(90). The obtained samples were tested before and after storage at -20°C and the results suggest that storage as well as freezing and thawing have little effect on the results.

After NT-qPCRs with different human sera were performed using one-step lysis, an inhibitory effect was detected in samples with human blood serum compared to the respective virus controls (3.5.2, 3.7). Analysis with GraphPad Prism and a non-linear regression model gave a detailed view and underlined the effect. Similarly to our work, a nonspecific inhibitory effect was also described from *Varada* et al. (81) for antibody depleted sera, which is defined by a significantly reduced qPCR signal. A reduction of qPCR values up to 30% was described at high serum dilutions (81). *Alvarado-Facundo* et al. (80), who had also worked with Vero cells, Edmonston wild-type MeV, SPR lysate, and one-step RT-qPCR, did not describe anything similar. Experimental approaches with antibody depleted sera were mentioned from *Alvarado-Facundo* et al. (80), but no inhibitory effects were described. The work published by *Alvarado-Facundo* et al. (80) differs from our work in some aspects: cells, without hSLAM receptor were used, human sera from different patients were analyzed, the SYBR Green analysis instead of the use of TaqMan probes, and the use of a CFX-96 (Bio-Rad) automated machine instead of a LightCycler480 (Roche).

The quantitative RT-qPCR measurement of viral RNA might be influenced by the use of different cell lines, different procedures preparing the blood sera, the RT-qPCR measurement itself or the method used for the release of viral RNA (extraction and one-step lysis). The cells used in this work and those used by *Alvarado-Facundo* et al. (80) only differ in the presence of the hSLAM receptor on the cells surface, which should not influence the RT-qPCR measurement nor did it influence the measurement of viral controls, which are grown on the same cell line. The blood sera are an unlikely reason, they were prepared and proceeded in the same way for this work and by *Alvarado-Facundo* et al. (80). Since the inhibitory effect occurs at all serum dilutions, the concentration of the serum does not seem to influence the results. The most plausible cause might be the use of the one-step lysate and the remaining contamination in the measured samples. A counterargument to this would be that, in contrast to the dilution series with blood sera, no inhibitory effect was seen measuring the virus control containing the same lysis buffer and remaining contaminations. Furthermore, *Alvarado-Facundo* et al. (80) calculated the neutralization titer using simple linear regression.

Without the analysis using non-linear regression and a statistical program as done in this work, the neutralization titer is interpolated between two dilution steps without investigating a regression model and an inhibitory effect may not be seen.

Detailed testing with antibody depleted and MeV-naive sera with both harvesting variants, extraction and one-step lysis, in correlation to inhibition of the RT-qPCR, should be performed to investigate non-specific effects on quantitative measurement of viral RNA.

4.5 WHO 3rd International Standard and intravenous Immunoglobulin in NT-qPCR

4.5.1 Intravenous Immunoglobulin

The use of commercially available immunoglobulin products is recommended from the STIKO ('Ständige Impfkommission') as post-exposition prophylaxis (PEP) after having had contact with contagious measles patients and a contraindication for MCV (35,36). For this purpose, immunoglobulins for intravenous administration (IGIV) are obtained from plasma from human donors (84). Since the amount of preserved neutralizing antibodies in human plasma after measles infection is significantly higher than after vaccination (101) and the MCV coverage rate of the population has increased in the last decades with declining measles infections (1,84), the amount of preserved neutralizing antibodies in donor plasma and thus in IGIV may vary or even decrease (84,101). It is therefore necessary to monitor the MeV neutralizing capacity of commercially available IGIV products to adjust the recommended dosage accordingly (84). For this reason, IGIV have been analyzed several times in neutralization assays (84,101–103). Since this study aimed to investigate whether a switch from the use of FRNT to NT-qPCR can be recommended, an IGIV product available in Germany (Immunoglobulin 50 g/L, KEDRION) was included as previously described (84) for NT-qPCR.

Recently, a GMT (ND50) of 2588 for MeV Edmonston wild-type that corresponded to 21.8 IU/mL, respectively, were measured for the neutralizing capacity of this IGIV product (84). Obtained ND50 titers by the NT-qPCR in this work showed a GMT (ND50) = 1726 (SLR: GMT (ND50) = 2037) and varied from the previous GMT (ND50) = 2588 with a ratio of 1.5 (SLR: 1.3). Future responsibilities here would be to serially repeat NT-qPCRs with IGIV using both harvesting methods, extraction and one-step lysis.

4.5.2 WHO 3rd International Standard Anti-Measles serum 97/648

As described from *PF Wright* (104), international standards are crucial for the global evaluation of serological analysis between different laboratories, describe a minimum standard of diagnostic performance, and provide material for the calibration of test methods. The WHO IS 3rd has been used to convert neutralizing titers obtained through FRNTs to international units per mL (IU/mL, (77)). The results can ensure comparability between different approaches and laboratories worldwide (73,77,104).

For the standardization of the NT-qPCR, the WHO IS 3rd was tested in parallel to the blood sera. The values obtained for the NT-qPCR with extraction and RT-qPCR give a GMT (ND50) = 3353 (SLR: GMT (ND50) = 3822). Thus, an inhibitory effect can be assumed as described in 4.4.3.2. Similar effects are not described in *Alvarado-Facundo* et al. (80), who performed NT-qPCRs with SPR lysate for the WHO IS 3rd. For a standardization with WHO IS 3rd, further NT-qPCRs with varying reaction conditions need to be performed. Moreover, possible inhibitory effects when using SPR lysate for the NT-qPCR should be evaluated.

4.6 Calculation of MeV neutralization titers

For the FRNT, the calculation of neutralizing titers after obtaining the number of foci is standardized using simple linear regression, the corresponding formula is described in 2.2.4.4. The neutralizing capacity is calculated in this work by GraphPad Prism using copy numbers obtained by RT-qPCR measurement. RT-qPCR is a sensitive methodology (98), which makes an improved calculation methodology reasonable.

The calculation with simple linear regression (80,89), e.g. using MS Excel, offers the advantage of not requiring an expensive statistics program like GraphPad Prism. Nevertheless, factors, e.g. non-specific inhibitory effects, may remain undetected (4.4.3.2). A pure calculation with copy numbers for the simple linear regression calculation without a visual representation of the curve can lead to a false interpretation of the measured values. Furthermore, during linear regression a linear relationship is established between two values, which in case of doubt lie in an exponential slope and a straight line without a slope (105). Hence, the assumed behavior of the curve is straightened with the linear regression and thus an approximation (105).

Therefore, this work established a non-linear regression model using GraphPad Prism. For this purpose, a dose-response-inhibition model was chosen, which should correspond to the assumed curve. Various statistical tests were run in parallel to check if the data aligns with the associated regression model.

An ND50 or ND90 titer was interpolated after inserting the values into the model: For this purpose, the copy numbers for 50% or 90% of the virus control were calculated manually and entered to the model. The corresponding X-values were automatically interpolated and converted into titers.

4.7 Future challenges

There are still challenges to overcome to establish a functional neutralization assay with endpoint determination by RT-qPCR. This includes the testing of currently circulating MeV genotypes D8 and B3 (1). The method of cell harvesting (4.4.3) should be further improved and possible inhibitory effects as described in 4.4.3.2 should be explored. Measurement of WHO IS 3rd should be repeated in NT-qPCR with the objective to establish an acceptance range that can be used as a validation criterion in NT-qPCR assay runs. The WHO IS 3rd should be used in parallel to human sera or IGIV to convert the neutralization titer in International Units (IU) per mL. Furthermore, more human blood sera should be tested in NT-qPCR and acceptance criteria such as the maximum difference between calculated titers, inter- or intra-assay specificity, and the efficiency should be explored. Even though some open questions remain, this work is an important step towards the establishment of a new neutralization assay with endpoint determination using RT-qPCR.

4.7.1 Luminescence based pseudoviral assay

In recent years, various approaches have been developed to modify and improve testing for neutralizing antibodies, lately especially in the context of the SARS-CoV-2 pandemic with the SARS-CoV-2 virus (106–109). Herein, the use of pseudoviruses gained popularity since the pseudovirus based neutralization assays can be carried out in biosafety level 2 laboratories instead of the biosafety level 3 laboratories prescribed for the use of real SARS-CoV-2 (106,107). The enhanced throughput of pseudoviral neutralization assays enabled the testing of high numbers of patient samples in

seroepidemiological SARS-CoV-2 studies for neutralizing antibody titers (110–112). Therefore, what is mainly considered for this work on improvement of neutralization assays with MeV, a rather high throughput could be achieved by endpoint determination using luminescence measurement (113).

In those assays, a different virus, such as lentivirus (107–109) or vesicular stomatitis virus (108,109,114), is used as a non-replicable and modified backbone for the pseudovirus (107,109). Plasmids expressing the surface proteins of the targeted virus, e.g. spike for SARS-CoV-2 (114), are then transfected (107,109,115). In addition, the pseudovirus contains genes that express the luciferase enzyme (108,109,115). The level of luciferase expression from pseudovirus-infected cells can then be measured and is considered as a measure for the degree of infection (107,109,115), theoretically corresponding with the quantitative RT-qPCR measurement for endpoint determination (80) used in this work.

These pseudovirus-based neutralization assays with endpoint determination by luminescence measurement have been established not only for SARS-CoV-2 (106–109), but also for various other viruses (116–120). Regarding MeV, *Frecha* et al. (121) could produce pseudoviruses modified with the MeV surface proteins H and F and depicted to infect cells through the described MeV receptors CD46 and hSLAM, while *Dautzenberg* et al. (122) could proof their sufficient stability. Therefore, the pseudoviral neutralization assay or parts of those established systems could provide a well established alternative for the testing of MeV-neutralizing antibodies and endpoint determination by luminescence could represent another opportunity to improve MeV neutralization assays, including even shorter working times combined with a cost-saving method and increased throughput.

5 Appendix

5.1 List of Abbreviations

Ab	Antibody
ADEM	Acute para-/postinfectious or autoimmune encephalitis
AM	Alveolar macrophages
APC	Antigen presenting cells
b	Base
BALT	Bronchus-associated lymphoid tissue
bp	Base pairs
BSA	Bovine Serum Albumin
cc, CC	Cell control
CD	Cluster of differentiation
CD46	Membrane cofactor protein
CD150	Signalling lymphocyte-activation molecule
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CMC	Carboxymethylcellulose
CO_2	Carbon dioxide
CPE	Cytopathic effect, cytopathogenic effect
cRNA	Carrier RNA
Ct	Cycle threshold
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
D-MEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
Edm	Edmonston
Edm-S	Edmonston-Schwarz
Edm-Z	Edmonston-Zagreb

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
F	Fusion protein
FBS	Fetal bovine serum
FG 12	Fachgebiet 12
FRNT	Foci reduction neutralization test, foci reduction neutralization assay
g	Gramm
GALT	Gut-associated lymphoid tissue
h	Hours
H	Haemagglutinin protein
HLA	Human leukocyte antigen
HRP	Horseradish Peroxidase
hSLAM	Human signaling lymphocytic activation molecule
Ig	Immunoglobulin
IGIV	Intravenous immunoglobulin
IL	Interleukin
IU	International units
k	Kilo
L	Liter
L	Large protein
m	Meter
m	Milli
m	Monoclonal
M	Matrix protein
M	Molar
μM	Mikromolar
MCP	Membrane cofactor protein
MCV	Measles containing vaccines
MeV	Measles virus
MEM	Minimum Essential Medium Eagle
mg	Milligram

$MgSO_4$	Magnesium sulfate
MHC-I	Major histocompatibility complex 1
MHC-II	Major histocompatibility complex 2
MIBE	Acute progressive infectious encephalitis/ acute inclusion body encephalitis
min.	Minutes
mL	Milliliter
mM	Milli Molar
MMR	Measles, mumps, and rubella virus
MMR/V	Measles, mumps, rubella, and varicella virus
mRNA	Messenger RNA
MV	Measles virus
n	Nano
N	Nucleocapsid protein
nAB	Neutralizing antibody
$NaHCO_3$	Sodium hydrogen carbonate
nc, NC	Negative control
ND	Neutralizing dose
NLR	Non-linear regression
NP	Nucleoprotein
nr.	Number(s)
NRC	National reference centre
NRC MMR	National reference centre for measles, mumps and rubella
NRZ	Nationales Referenz Zentrum
NRZ MMR	Nationales Referenz Zentrum für Masern, Mumps, Röteln
NT	Neutralization test, neutralization assay
NT-qPCR	Neutralization test with endpoint determination using RT-qPCR, neutralization assay with endpoint determination using RT-qPCR

P	Phosphoprotein
PBNA	Pseudovirus-based neutralization assay
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PFU	Plaque forming units
p.i.	Post infection
pc, PC	Positive control
PV	Pseudovirus
PVF	Primary vaccine failure
P/S	Penicillin/Streptomycin
qPCR	Real time quantitative polymerase chain reaction
RKI	Robert Koch-Institute
RNA	Ribonucleic acid
RT	Reverse transcription
RT	Room temperature
RT-qPCR	Real time quantitative reverse transcription polymerase chain reaction
RXN	Reaction mix
SLAM	Signaling lymphocytic activation molecule
SRL	Simple linear regression
SSPE	Subacute sclerosing panencephalitis
STIKO	Ständige Impfkommission
SVF	Secundary vaccine failure
TE	Tris-EDTA-buffer
TMB	3,3',5,5'-Tetramethylbenzidine
vc, VC	Virus control
WHO	World Health Organization
µ	Micro

5.2 List of Figures

5.3 List of Tables

6 References

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