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Measles and Rubella IgG-Seroprevalence in Luxembourg and Alternative Laboratory Approaches

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Abbreviations

A

ADP (Adenosine Diphosphate)

AU (Antibody Units)

AI (Antibody Index)

B

BCG (Bacille Calmette Guerin)

BSA (Bovine Serum Albumine)

BSL (Biosafety level)

C

CDC (Centers for Disease Control and Prevention)

CHO (Chinese Hamster Ovary)

CNER (Comité National d'Etique de Recherche)

CNPD (Commission Nationale pour la Protection des Données)

CPE (Cytopathic Effect)

CDw150 (Synonyme for hSLAM)

D

DMEM (Dulbecco's Modified Eagles Medium)

DMSO (Dimethylsulfoxid)

DNA (Deoxyribonucleic Acid)

DO (Densité optique – optical density)

DPBS (Dulbecco's Phospate Buffered Saline)

DTP (Diptheria, Tetanus Toxoids and Pertussis)

E

ECDC (European Centre for Disease Prevention and Control)

EEA (European Economic Area)

EPI (Expanded Program of Immunization)

EU (European Union)

F

FcR (Fc-Receptor)

FACS (Fluorescence-Activated Cell Sorting)

FBS (Fetal Bovine Serum)

FVO (Fractional Volume Occupancy)

G

GMRLN (Global Measles and Rubella Laboratory Network)

H

HBSS (Hank's Balanced Salt Solution)

HI (Heat-Inactivated)

I

ICA (Immunocolorimetric Assay)

IFN (Interferon)

IgG (Immunoglobulin G)

ITS (Insulin-Transferrin-Selenium)

IU (International Unit)

L

LNS (Laboratoire National de Santé)

LIH (Luxembourg Institute of Health)

M

MCV (Measles Containing Vaccine)

MeaNS (Measles Nucleotide Surveillance)

MMR (Measles Mumps Rubella)

MMRV (Measles Mumps Rubella Varicella)

MoH (Ministry of Health)

MV (Measles Virus)

N

NCP (National Cytometry Platform)

n.d. (no date)

NIBSC (National Institute for Biological Standards and Control)

O

OPV (Oral Polio Vaccine)

P

PCR (Polymerase-Chain-Reaction)

PBS (Phosphate Buffered Saline)

PBS-EDTA (Phosphate Buffered Saline – Ethylendiamintetraacetat)

PE (Phycoerithrin)

PFU (Plaque Forming Units)

Pen/Strep (Penicillin/Streptomycin)

PVP (Polyvinylpyrrolidone)

R

RCV (Rubella Containing Vaccine)

RNA (Ribonucleic Acid)

RPMI (Roswell Park Memorial Institute)

RV (Rubella Virus)

S

SLAM (Signaling Lymphocytic Activation Molecule)

SOP (Standard Operating Procedure)

SP-ORF (Structural Protein Open Reading Frame)

T

T-25, T-75 (Tissue Culture Flask, $25cm^2$, $75cm^2$)

$TCID_{50}$ (50% Tissue Culture Infective Dose)

TMB (3,3,5,5-Tetramethylbenzidine)

tRNA (transfer RNA)

U

UG (Ultraglutamin)

V

Vero/hSLAM (Vero Cells transfected with a plasmid encoding the human Signaling Lymphocytic Activation Molecule)

W

WHO (World Health Organization)

A Abstract (German and English)

Zusammenfassung

Noch immer werden Masern und Röteln häufig als harmlose Kinderkrankheiten verkannt. Mit Blick auf die Zahl der Todesfälle und die in einigen Fällen langfristigen Beeinträchtigungen nach einer durchgemachten Infektion, ist diese Sichtweise jedoch fraglich. Die Weltgesundheitsorganisation hat einen Plan erstellt, der die zukünftige Eliminierung von Masern und Röteln vorsieht. Dabei soll die Eliminierung auf nationaler Ebene schließlich in eine globale Eliminierung und somit Ausrottung dieser Viruserkrankungen münden. Um dieses Ziel zu erreichen, ist jedes Land dazu aufgerufen, seine Bevölkerung über die Impfung mit der Masern-Mumps-Röteln-Kombinationsimpfung zu informieren und diese zur Verfügung zu stellen. Des Weiteren sollte die Immunisierung der Bevölkerung regelmäßig kontrolliert und überwacht werden, um für eine ausreichende Durchimpfungsrate zu sorgen. Im epidemiologischen Teil der vorliegenden Arbeit wurde zum ersten Mal in Luxemburg ein retrospektives Format auf Basis bereits erhobener Daten verwendet, um die Anti-Masern/Anti-Röteln IgG-Seroprävalenz der Bevölkerung zu untersuchen. Ein Screening, das bis 2015 verpflichtend bei allen heiratswilligen Frauen in Luxemburg durchgeführt wurde, führte insbesondere in dieser Gruppe zu einem großen Datensatz und somit zu hoher Repräsentativität. Um weiterhin den Status Luxemburgs als eines der 33 Länder der Europäischen WHO Mitgliedsstaaten zu gewährleisten, die sowohl Masern als auch Röteln eliminiert haben, ist zu überlegen, die Impfanstrengungen zu intensivieren, um dabei insbesondere Personen zu erreichen, die mit höherer Wahrscheinlichkeit keine Immunität gegenüber Masern und Röteln aufweisen.

Das Luxembourg Institute of Health ist als WHO Regional Reference Laboratory for Measles and Rubella damit betraut, für WHO National Reference Laboratories in 22 Ländern Fälle und Ausbrüche zu bestätigen sowie Erregerstämme zu charakterisieren und molekularepidemiologisch zu untersuchen. Für diese und für andere wissenschaftliche Zwecke werden Virus-Varianten benötigt, die mittels Zellkultur isoliert werden können. Im Falle des Masern-Virus scheint die Isolation weniger häufig erfolgreich zu sein als im Falle des Röteln-Virus. Der experimentelle Teil der vorliegenden Arbeit beschäftigt sich mit Methoden, die zum einen Infektionsraten, zum anderen aber auch die Vermehrung des Masern-Virus in Vero/hSLAM Zellen steigern könnten. Mithilfe dieser Methoden soll erreicht werden, dass Masern-Virus Isolationen in Zellkultur insgesamt erfolgreicher werden.

Abstract

Measles and rubella are oftentimes underestimated as being insignificant childhood diseases. However, death rates and serious long-term complications in affected people of all ages paint a different picture. The World Health Organization has adopted goals for the elimination of measles and rubella. Systematic elimination on the national level will eventually result in global elimination. To get there, every single country needs to make efforts in promoting and administering measles-mumps-rubella-containing vaccine but also in surveilling immunization rates on a regular basis. The epidemiological part of the underlying study has, for the first time in Luxembourg, applied a retrospective format based on already existing data to assess the population´s anti-measles/anti-rubella IgG seroprevalence. A pre-marital screening, mandatory in Luxembourg until 2015 for all women planning to get married, has contributed to the huge dataset, particularly in the group of women at childbearing age. To further ensure Luxembourg´s status as one of 33 European WHO member states currently classified as having eliminated both measles and rubella, an increase in vaccination efforts might be considered, particularly in certain groups at risk of being IgG negative for measles and rubella.

The Luxembourg Institute of Health as WHO European Regional Reference Laboratory for Measles and Rubella is entrusted with case and outbreak confirmation, virus characterization and molecular epidemiology for WHO National Reference Laboratories in 22 countries. For these and other scientific purposes virus strains need to be isolated. However, oftentimes Measles Virus isolation seems to be less successful compared to Rubella Virus isolation. The experimental part of this study has therefore investigated methods to enhance viral infection and propagation in Vero/hSLAM cells.

B Introduction

1.1 Measles

A measles-like syndrome was first scientifically described by Abu Becr, known as Rhazes, in the 9th century. An increasing number of descriptions of measles is found in the 11th and 12th century (Furuse et al., 2010). Measles Virus was first isolated in 1954 by John F. Enders and Thomas C. Peebles. They infected cell cultures with viral material from a 12-year old, named David Edmonston. Continuous growth and attenuation of this initial virus strain has led to licensure of the first and successful live attenuated Measles Virus vaccine strain in 1963, subsequently called Edmonston vaccine strain. In 1974, the vaccine became part of the World Health Organization´s (WHO) Expanded Program of Immunization (EPI) along with BCG (Tuberculosis), OPV (Polio) and DTP (Tetanus, Diphtheria, Pertussis) (Katz, 2009). Before measles vaccine introduction, worldwide around 30 million cases of measles with more than 2 million deaths had occurred each year (Rota et al., 2016). Nowadays, still more than 250 000 measles cases and around 150 000 deaths (mostly children in developing countries) are reported each year, although a considerable decline (75%) of measles deaths was observed between 2000 and 2013 (Statistics Portal Grand Duchy of Luxembourg, n.d.).

1.1.1 Classification and structure

Within the family of Paramyxoviridae, Measles Virus belongs to the genus Morbillivirus, which includes Dolphin and Porpoise Morbillivirus, Phocid Distemper Virus, Canine Distemper Virus, Peste des Petits Ruminants Virus and Rinderpest Virus (RPV). Measles Virus is most closely related to the latter one, this being a pathogen of cattle. Calculations assume that Measles Virus has emerged from RPV in an environment of close proximity between human and cattle, probably around the 11th and 12th centuries (Furuse et al., 2010). Measles Virus is enveloped and has a non-segmented, negative-sense, single-stranded RNA genome consisting of approximately 16 000 nucleotides. Its genome is made of six genes, of which each one encodes a single structural protein (Furuse et al., 2010). Hemagglutinin (H) protein and Fusion (F) protein are envelope glycoproteins. H-protein mediates receptor binding, whilst F-protein is necessary for membrane fusion. The Phosphoprotein (P) gene encodes for Phosphoprotein (P) as well as for V and C proteins. The latter ones are non-structural proteins and responsible for antagonizing host interferon (IFN) response. Phosphoprotein (P) and Large protein (L) build the viral RNA-dependent RNA polymerase. Nucleocapsid (N) protein forms a helical capsid around the Measles Virus genome and associates with the RNA-dependent RNA polymerase to form the ribonucleoprotein complex. Matrix (M) protein links this complex with envelope glycoproteins during Measles Virus assembly (Iwasaki et al., 2009).

1.1.2 Clinical diagnosis and complications

After an incubation period of around 7-14 days, prodromal signs of Measles Virus infection can be observed. These may include fever, cough, coryza, conjunctivitis and malaise (Rota et al., 2016). Few days later, Koplik spots (clustered white lesions) can appear on the buccal mucosa but cannot be considered pathognomonic as they also come along with other viral infections (Kimura et al., 2019). The generalized maculopapular skin rash usually occurs around 4 days after onset of the prodromal phase.

Depending on age and nutritional status, the infection can lead to more or less severe complications, including encephalitis (1:1000) and pneumonia (1:20) with the latter one being the most common cause of death from measles in small children. Approximately one out of 1000 pediatric measles infections result in death, mostly due to secondary infections. Other complications include blindness, deafness (due to severe ear infections), as well as diarrhea and related dehydration (World Health Organization Regional Office for Europe: Measles in the WHO European Region, 2016). Around 134 000 measles-related deaths have been reported globally in 2015 (World Health Organization: Measles, 2017), mostly in children aged younger than five. Measles therefore remains a major cause of death in young children, mainly (> 95%) in countries with low per capita income and weak health infrastructures (World Health Organization Regional Office for Europe: Measles in the WHO European Region, 2016).

1.1.3 Laboratory diagnostic

1.1.3.1 Serology

Anti-measles IgM should be detectable around three days after onset of rash and persists for at least four weeks after rash (World Health Organization: Manual for the laboratory diagnosis of measles and rubella virus infection, Second edition, 2007), which is why IgM detection by ELISA is the most routinely used method for measles diagnostic in many laboratories. Serum samples as well as dried blood spots (DBS) and oral fluid (OF) serve as specimens to detect IgM. Two types of ELISA for measles serology can be distinguished: A capture assay with anti-human IgM in the solid phase binds IgM antibodies in the patient specimen. Subsequent addition of measles antigen and labelled antigen-specific antibodies will sort out only measles-specific IgM. The indirect assay, on the other hand, is based on immobilized antigen in the solid phase, binding specific IgM (Ratnam et al., 2000).

Due to nonspecific reaction, interference of rheumatoid factor, or other underlying conditions such as human parvovirus, rubella, or human herpesvirus 6 infections, IgM detection bears a risk of false-positivity (Hubschen et al., 2017). In some cases, IgM may only be detectable for some few days after rash onset, thereby potentially leading to false-negative IgM results if the sample was taken too early. In case of doubtful results, measles-

specific IgG antibodies may also be searched for. IgG avidity testing is further used to distinguish recent and earlier contact to the virus (Hubschen et al., 2017).

1.1.3.2 Viral RNA detection

Reverse transcription-PCR (RT-PCR) is an important tool for laboratory diagnosis of acute Measles Virus infection. A variety of clinical specimens, such as throat/nose swabs, nasopharyngeal aspirates, oral fluid, urine and peripheral blood mononuclear cells (PBMC) are used to detect viral RNA. In absence of cold chain maintenance and to facilitate transportation, specimens may be spotted on FTA® Elute Micro Cards.
Measles Virus load peaks about 4-5 days after onset of fever (Akiyoshi et al., 2010) and viral RNA in serum may be detected for up to 7 days after onset of rash and in most other specimens for up to two weeks or even longer (Hubschen et al., 2017). For sufficient amounts of RNA, samples should be collected timely within the first week after rash onset (Centers for Disease Control and Prevention: Epidemiology and prevention of vaccine-preventable diseases, 2019).

1.1.3.3 Virus isolation

Measles Virus isolation is less sensitive compared to detection of RNA and antibodies. However, isolation provides unlimited material for genome sequencing and is therefore highly valuable for genome characterization.
Laboratories taking part in the World Health Organization´s Measles and Rubella Laboratory Network (WHO LabNet) are recommended to use the Vero/hSLAM cell line for isolation purposes. This cell line has significantly improved Measles Virus isolation (Ono et al., 2001). Compared to B95a cells (persistently infected with Epstein Barr Virus) Vero/hSLAM cells are non-infectious and easy to handle. Working with Vero/hSLAM cells requires permission of Dr. Yusuke Yanagi, Kyushu University, Kukuyoka, Japan. Any use of this cell line must further quote the original publication (Ono et al., 2001).
More detailed information concerning virus isolation can be found in the following review:

Hübschen JM, Bork SM, Brown KE, Mankertz A, Santibanez S, Ben Mamou M, Mulders MN, Muller CP (2017) Challenges of measles and rubella laboratory diagnostic in the era of elimination. Clinical Microbiology and Infection. 23:511-515

1.1.4 Epidemiology

Before the vaccine became available in 1963, there was a chance of about 90% for children aged younger than 15 years to go through measles infection (World Health Organization: Global measles and rubella strategic

plan: 2012-2020, 2012). The success of measles vaccination is demonstrated by an estimated 17 million deaths prevented between 2000 and 2015, thereby reflecting the increase of global coverage with the first dose of measles-containing vaccine (World Health Organization Regional Office for Europe: Measles in the WHO European Region, 2016).

The application of the first dose of measles vaccine is usually recommended at the age of 12-15 months. The second vaccine dose should be given four weeks after the first one at the earliest. Measles vaccines contain live-attenuated strains of Measles Virus and are often combined with other live-attenuated vaccines, e.g. MMR vaccine. Adverse reactions to the vaccine were investigated intensively. In approximately 5-15% of vaccinated persons they appear in mild fever and rash around 1-2 weeks after vaccination (Rota et al., 2016). Serious allergic reactions are very rare (1:1 000 000). A link between the MMR vaccine and autism development in children has been disproved (Taylor et al., 2014).

1.2 Rubella

The first documented disease description originates from two German physicians, de Bergan in 1752 and Orlow in 1758 (Wesselhoeft, 1947). Considering rubella as a derivative of measles they have contributed to the term “German Measles”. When another German physician, George de Maton, claimed the illness to be a distinct entity in 1814, the term “Rötheln” became more popular (Wesselhoeft, 1947). In 1866, Henry Veale, a British Army surgeon, suggested to use the latin word “rubella” (Forbes, 1969).

Despite being confirmed as a viral disease in 1938 by Hiro and Tasaka (Forbes, 1969), rubella was rather neglected further on. It was not until 1941 that the general notion of its harmlessness was challenged. The Australian ophthalmic surgeon, Norman Gregg, had observed 78 mothers with children suffering from congenital cataracts. 68 of them had a history of rubella infection early in pregnancy (Gregg, 1991). Strikingly, the high incidence of congenital cataract seemed to coincide with a rubella epidemic in Australia in 1940 (Centers for Disease Control and Prevention: Epidemiology and prevention of vaccine-preventable diseases, 2019). Gregg made the link between maternal rubella infection and congenital cataract, giving rise to the hypothesis that viruses might be teratogenic agents. Other congenital defects caused by rubella infection during pregnancy were identified over the following years. In 1962, two groups (Parkman et al. and Weller and Neva) isolated Rubella Virus, hence building the fundament for vaccine development (Lee and Bowden, 2000). The first vaccine was licensed in 1969 (Centers for Disease Control and Prevention: Epidemiology and prevention of vaccine-preventable diseases, 2019).

1.2.1 Classification and structure

Rubella Virus belongs to the family of Togaviridae and is the only member of the genus Rubivirus. Enveloped by a host-derived lipid bilayer, the rubella virion contains an icosahedral nucleocapsid that surrounds the

single-stranded positive-sense RNA genome. The nucleocapsid is mainly composed of capsid protein-containing clusters of proline and arginine residues binding on the Rubella Virus genomic RNA (Lee and Bowden, 2000). The RNA genome encodes for only five proteins: 3 virion proteins (Capsid protein, CP, and two envelope glycoproteins, E1 and E2) and two non-structural replicase proteins (P90 and P150) (Abernathy et al., 2011). Glycoproteins E1 and E2 seem to contain epitopes of which some are associated with hemagglutination and neutralization. E1 builds the main protein on the viral surface and is involved in attachment and fusion with the host cell membrane (Lee and Bowden, 2000). The two non-structural replicase proteins, P90 and P150, are derived from the large replicate precursor P200. P150 mainly provides enzymatic functions for tRNA splicing (ADP-ribose phosphatase) and 5'-capping of mRNA (methylguanyl-transferase), whilst P90 is responsible for helicase function and contains the RNA-dependent RNA polymerase (Matthews et al., 2009).

1.2.2 Clinical diagnosis and complications

After an incubation period of around 14 days, 25-50% of infected individuals will show mild symptoms such as low grade fever, swollen and tender glands and maculopapular rash (Garcia, 2010). Often the infection appears mild or asymptomatic in children, whilst adults tend to develop fever and malaise. Women in particular frequently suffer from arthralgia/arthritis (White et al., 2012). Due to these rather unspecific symptoms, distinguishing rubella from other viral agents can be challenging, particularly from Parvovirus B19. Fever, rash and joint symptoms are common in both infections (Banatvala and Brown, 2004).
Whilst postnatally acquired rubella mostly results in few complications, an infection during the first trimester of pregnancy might seriously harm the fetus. Besides higher risk for miscarriage and stillbirth, a range of clinical abnormalities can be observed in the infant, also known as congenital rubella syndrome (CRS). They are classified as transient and permanent and include defects of the central nervous system as well as ocular, auditory and cardiovascular defects (Banatvala and Brown, 2004). Infants usually present with a combination of several features of CRS, but might as well only suffer from a single defect, with deafness being the most common single impairment (Lanzieri T et al., 2018).

1.2.3 Laboratory diagnosis

1.2.3.1 Serology

Routine laboratory confirmation of suspected rubella cases is mainly based on detection of virus-specific IgM in serum, using commercial enzyme immunoassay (EIA) kits. However, IgM testing might not be sufficient, especially in elimination settings, where the relative likelihood of having false-positive results is increased in comparison to countries where rubella is still endemic. Further, IgM antibody responses to viral infections

vary between individuals. Some patients fail to produce detectable amounts of IgM antibodies but respond with only IgG antibodies (Inouye et al., 1984). Therefore, in case of doubtful IgM test results it might be necessary to carry out supplemental tests, such as detection of specific IgG rise by collecting two serum specimens (acute and convalescent sera) and testing them in parallel, applying the same method (World Health Organization: Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome, 2018).

If only one serum sample is available, it may be tested for both IgM and IgG. Depending on the IgG avidity test kit, IgM antibodies in combination with low avidity IgG can be indicative for primary rubella infection, whilst IgM reactivity with high avidity IgG suggests primary infection at least 13 weeks prior (Bottiger and Jensen, 1997).

Non-reducing IgG immunoblot assays are another possibility to distinguish recent from past rubella infection. These assays detect rubella-specific IgG bound to the recombinant Rubella Virus glycoproteins CP, E1, E2 and E1E2 (Chen and Icenogle, 2007). Anti-E1 IgG usually appears within 4-6 days following contact, whereas E2-specific antibodies are not detectable until 3-4 months post-infection (Pustowoit and Liebert, 1998). The detection of E2-specific IgG therefore rules out primary infection and is indicative for reinfection or antibody persistence.

1.2.3.2 Viral RNA detection

Reverse-transcription (RT-) PCR is usually both utilized for laboratory confirmation of rubella infection and molecular characterization, with the real-time RT-PCR being particularly feasible for the detection of very small amounts of rubella RNA in clinical specimens. The low-priced endpoint assays, on the other hand, may be used for amplification of fragments for sequencing (Centers for Disease Control and Prevention: Rubella (German Measles, Three-Day Measles), 2017). A nested PCR including two primer sets can potentially increase the overall sensitivity. It requires two manipulations though, thereby increasing the risk of contamination. Hybridization probes (such as TaqMan®) allow for the exclusion of the nested step as their design as fluorescent DNA oligonucleotides ensures their binding downstream of the primer, whilst intercalating dyes (such as SYBR® Green) bind any and thus also unspecific double stranded DNA. In addition, due to the fluorescence signal of hybridization probes given throughout the reaction, subsequent agarose gel electrophoresis becomes obsolete, hence excluding one more source of contamination. However, the choice of PCR technique also depends on costs, laboratory equipment and training of staff (Hubschen et al., 2008).

Rubella Virus infection can most successfully be confirmed by combined RT-PCR and serology testing (Abernathy et al., 2009). To do so, a robust quality control program is required, for example by applying synthetic control RNAs resulting in amplimers of slightly different molecular weight as compared to the standard PCR product (Rota et al., 2011). Furthermore, to ensure high quality diagnostic, samples need to be

chosen and collected appropriately. Throat and nasopharyngeal swabs are excellent specimens. They allow for both detection of RNA for RT-PCR and virus isolation. A combined PCR for the simultaneous detection of measles and rubella (Hubschen et al., 2008) also takes into account the importance of distinguishing rubella from measles (Vaidya et al., 2016; Yasui et al., 2014).

1.2.3.3 Virus isolation

Rubella Virus isolation is recommended by the WHO Measles and Rubella Labnet for the establishment of viral genetic baselines and thus the understanding of transmission patterns (Zhu et al., 2007). Rubella Virus grows in RK13, SIRC, baby hamster kidney cells, Vero and Vero/hSLAM cells, but only produces indistinct cytopathic effect in all cell lines (Figueiredo et al., 2000). Even with lab-adapted strains, it usually takes an incubation of about 5 days at 35°C to obtain visible plaques by conventional plaque assay. Using immuno-assays, a cell culture´s infection with Rubella Virus can be confirmed more rapidly, mostly after an incubation of 3-4 days. Immunocolorimetric assay (ICA) and immunofluorescence assay (IFA) are based on the same principle, but use different substrates (Vaidya et al., 2014). Stained foci visible for the naked eye are indicative for viral infection.
More detailed information can be found in the following review:

Hübschen JM, Bork SM, Brown KE, Mankertz A, Santibanez S, Ben Mamou M, Mulders MN, Muller CP (2017) Challenges of measles and rubella laboratory diagnostic in the era of elimination. Clinical Microbiology and Infection. 23:511-515

1.2.4 Epidemiology

In unvaccinated populations where rubella is endemic, epidemics occur every 6 to 9 years (Lanzieri T et al., 2018). Outbreaks are usually triggered by the accumulation of susceptible individuals, which is why in pre-vaccine settings mainly children are affected, leading to naturally acquired immunity in the majority of adults. With the introduction of the vaccine, a coverage of more than 80% of the population has to be reached to prevent an increase in the average age of infection (Lessler and Metcalf, 2013).
With seroconversion in at least 95% of vaccinated individuals after a single dose and probably lifelong immunity, rubella-containing vaccine (RCV) is highly effective. It is delivered subcutaneously or intramuscularly, with the first dose usually given at the age of 12-15 months. The time point for the second dose varies from country to country. In Luxembourg it is recommended at the age of 15 to 23 months (European Centre for Disease Prevention and Control: Vaccine Scheduler, 2019).
In 2014, rubella-containing vaccine had been introduced in 140 (72%) of the 194 WHO member states and the proportion of infants vaccinated against rubella was 46% (Grant et al., 2015). Although nowadays the vaccine

is widely available, rubella remains endemic in many countries and is responsible for an estimated 100 000 CRS cases per year (World Health Organization: Rubella, 2019).

1.3 Aim of the work

This work aims to contribute to:

i) The improvement of Measles Virus isolation in Vero/hSLAM cell culture.

ii) The assessment of anti-measles/-rubella IgG seroprevalence in the Luxembourg population.

Although no longer routinely used for case confirmation, virus isolation remains an important laboratory tool, particularly for Next Generation Sequencing (NGS) for genome characterization and the establishment of reference strains for research purposes. Simple methods for enhancement of initial viral infection and propagation might increase the chances for successful Measles Virus isolation.

The WHO European Region plans to eliminate both measles and rubella (World Health Organization Regional Office for Europe: Eliminating measles and rubella, 2014). Annual status reports of the National Verification Committees for Measles and Rubella elimination are used to identify a country as having achieved disease elimination or not. Luxembourg is one of 33 WHO European Region countries currently classified as having eliminated both measles and rubella (World Health Organization Regional Office for Europe: Seventh meeting of the European regional verification commission for measles and rubella elimination (RVC), 2019). However, a survey conducted in 2012 by the Ministry of Health indicates that 99% of 2 year-old children in Luxembourg have received the first dose of measles- and rubella-containing vaccine, but only less than 86% have also received the second dose (Krippler, 2012). Assuming this trend to continue for a few years, the 95% immunization coverage recommended for achieving and maintaining measles elimination might no longer be met (Funk, 2017). An increasing part of the population not being protected against measles and rubella implies a higher chance for disease outbreaks and even re-establishment of endemic transmission. This is even more the case in view of many European countries still reporting measles and rubella cases, including states bordering Luxembourg. The last study on measles and rubella IgG antibody prevalence in Luxembourg was conducted in 2000 and 2001 (Mossong et al., 2004), suggesting that more recent data are required to evaluate the current level of protection within the Luxembourg population.

The study on "anti-measles/-rubella IgG seroprevalence in the Luxembourg population" aims to identify measles and rubella immunization gaps in the Luxembourg population to allow for the implementation of targeted intervention strategies, such as supplementary immunization activities, vaccination promotion campaigns, etc.

I Experimental study "Enhancement of Measles virus infection in Vero/hSLAM cell culture"

2 Materials and Methods

2.1 Materials and methods for cell cultures

2.1.1 Cell culture materials

2.1.1.1 Equipment

The following equipment and disposables were used:

Devices: Airstream E-Series Class II Biological Safety Cabinet (ESCO), CelCulture® CO_2 Incubator (ESCO), Thermo Scientific SL 40R, Cat.-No.: 75004527 (ThermoFisher), Digital color camera (c-mount) for all applications, Leica DFC480 (Leica Microsystems), Julabo SW20 Water Bath (Julabo), Vacuum Pump SUE 30 (Heto)

Pipet-X Pipet Controller PX-100 (Mettler-Toledo), Mammalian Cell Counter, NucleoCounter® NC-100™ (ChemoMetec), Mr. Frosty™ Freezing Container (ThermoFisher), BD LSRFortessa™ (BD Biosciences), Nitrogen Tank

Disposable material: Greiner CELLSTAR® serological pipette, volume 10 ml, 25 ml (greiner bio-one), Greiner CELLSTAR® serological pipette, 2 ml aspiration (greiner bio-one), Greiner CELLSTAR® Filter Cap Cell Culture Flasks, 25 cm^2 , 75 cm^2 , 175 cm^2 (greiner bio-one), Greiner CELLSTAR® Cell Culture Multiwell Plates, 12 well format (greiner bio-one), Greiner Cell Scrapers, 40 cm length (greiner bio-one), Tips LTS 10 µL, 200 µL, 1000 µL Filter 960/10 RT-L200F (Mettler-Toledo), Greiner Centrifuge Tubes, 15 ml, 50 mL (greiner bio-one), 5 ml tubes (BD Biosciences)

2.1.1.2 Reagents and media

The following reagents (suppliers) were used to prepare the different media used:

DMEM (Lonza), FBS (Life Technologies), Pen/Strep (Lonza), Ultraglutamine (Lonza), Trypsin-EDTA (Lonza), DPBS (10x) without Ca^{2+}, Mg^{2+} (Lonza), EDTA (Sigma-Aldrich), Double distilled water (in-house), G418 (Geneticin) (Life Technologies), CD150 Monoclonal Antibody (A12 (7D4)), PE (eBioscience), OneCompensation beads (eBioscience), Zombie NIR™ Fixable Viability Kit for Live Cell/Dead Cell Discrimination (APC-Cy7A-stained) (Biolegend®), FACS-buffer (PBS + 2% FBS) (in-house)

The following cell culture media and other reagents were used:

Vero/hSLAM standard growth medium

500 ml DMEM, 35 ml FBS (HI), 5 ml Pen/Strep, 5 ml Ultraglutamine

ITS-DMEM/F-12-2%FBS

480 ml DMEM/F-12, 10 ml FBS (HI), 5 ml ITS (Insulin-Transferrin-Selenium), 5 ml P/S

CHO/hSLAM growth medium

460 ml RPMI with Glutamin, 5 ml P/S, 35 ml FBS (HI)

Vero/hSLAM growth medium with PVP40

Three different concentrations of PVP40 (Polyvinylpyrrolidone40) in standard Vero/hSLAM growth medium were prepared (9% v/v [10,75 mg/ml]; 0,9% v/v [1,075 mg/ml]; 0,09% v/v [0,1075 mg/ml]).

Calculation of the amount of PVP40 (kindly provided by Dr. Rafi Rashid from the National University of Singapore, NUS Graduate School for Integrative Sciences and Engineering, Faculty of Science, Centre for Bioimaging Sciences (CBIS), Department of Biomedical Engineering, Faculty of Engineering):

1. *Calculation of the volume of a single PVP40 molecule:* Hydrodynamic radius (R_H) of PVP40 = 5.1 nm; $V = 4/3\pi(R_H)^3 = 4/3\pi(5.1 \text{ x } 10^{-9})^3 = 5.56 \text{ x } 10^{-25} \text{ m}^3$

2. *Calculation of the number of PVP40 molecules needed in 1 ml:* [1 ml = 1 x 10^{-6} m^3]*;* 9% FVO = 9 x 10^{-8} m^3; Number of PVP40 molecules needed for 9% FVO, N = Occupied volume ÷ volume of 1 PVP40 molecule = 9 x 10^{-8} m^3 ÷ 5.56 x 10^{-25} m^3 = 1.62 x 10^{17}

3. *Calculation of the mass (m) of PVP40 that needs to be added to 1 ml:* [MW of PVP40 = 40,000 Da; 1 Da = 1 g/mol]*;* $m \times \frac{6.023 \times 10^{23}}{40000} = 1.62 \times 10^{17}; m = 10.75$ mg

4. *Addition of 10.75 mg of PVP40 to 1 ml of solution (e.g. PBS or water) to achieve a final concentration of 10.75 mg/ml.*

Vero/hSLAM freezing medium

Vero/hSLAM continuous culture medium, 30% FBS, 15% DMSO, 5 ml Ultraglutamine, 4 ml Geneticin

PBS 1x

50 ml DPBS without Ca^{2+} and Mg^{2+}, 450 ml autoclaved double distilled water

PBS-EDTA (10x)

1 g EDTA is dissolved in 500 ml PBS (10x) w/o Ca^{2+}, Mg^{2+} and aliquoted in 50 ml portions (stored at -20°C). Before use, one aliquot is diluted 10 times in 450 ml autoclaved double distilled water.

2.1.1.3 Cell lines

Vero/hSLAM cells

The Vero/hSLAM cell line was generated through transfection of African green monkey kidney cells with an expression plasmid encoding human CDw150 (Signaling Lymphocytic Activation Molecule – SLAM) measles virus receptor (Ono et al., 2001). In contrast to Epstein-Barr virus infected B95a cells, which as well can be used for measles virus isolation, Vero/hSLAM cells are non-infectious. Having a similar sensitivity to Rubella Virus infection as B95a cells they are recommended by WHO for use within the Global Measles Rubella Laboratory Network (GMRLN). The LIH keeps a stock of Vero/hSLAM cells in liquid nitrogen. They express both human SLAM and a neomycin resistance gene that makes them resistant against geneticin treatment. After 15 passages, one passage should contain geneticin to eliminate those cells not stably expressing SLAM and the neomycin resistance gene. Prior to freezing, multiple passaging with geneticin is recommended.

Vero cells

Vero cells are a mammalian continuous cell line derived in the 1960s from African green monkey kidney epithelial cells. This cell line is widely used in research fields such as virology, bacteriology and parasitology. Vero cells can be kept long-term either in liquid nitrogen or at -80°C. After starting a culture, it usually takes them 2-3 passages to reach their regular growth rate (Ammerman et al., 2008).

CHO/hSLAM cells

The CHO (Chinese Hamster Ovary) cell line has been derived in the 1950s from the ovaries of Chinese hamsters and is intensively used for research purposes in molecular biology as well as for the production of therapeutic proteins. The cells can be grown both as adherent cells or in suspension. Their deficiency in producing proline and expressing the epidermal growth factor receptor (EGFR) should be considered when choosing a cell culture medium.

CHO/hSLAM cells are CHO cells transfected with an expression plasmid encoding human CDw150 (SLAM), hence being applicable for measles virus growth and isolation (Tatsuo et al., 2000).

2.1.2 General conditions for cell culture

Vero/hSLAM cells were kept in a cell culture incubator at 37°C and 5% CO_2 located in a biosafety lab 1 (BSL1). A relative humidity of around 95% was maintained by evaporation from the water tank filled with distilled water and antimicrobial agent (Aqua Clean WAK-Chemie Medical GmbH) to prevent growth of germs and fungi. For manipulation, cell cultures were transferred into a biological containment cabinet (hood) providing strict aseptic conditions. All manipulations were done using disposable plastic pipettes. Cell culture media was kept at 4°C until date of expiry.

2.1.3 Seeding Vero/hSLAM cells

Vero/hSLAM cells are generally first seeded into T-25 flasks to ensure a proper ratio of cells to growth medium. T-25 flasks were prepared with 5 ml Vero/hSLAM growth medium before seeding the cells.
A vial of Vero/hSLAM cells was taken from the liquid nitrogen tank and directly transferred to a 37°C water bath. When 75% of liquid was melted, the vial was removed from the water bath and cleaned with 70% isopropanol. Afterwards, the entire content was transferred to the previously prepared flask and mixed gently. The flask was stored at 37°C and 5% CO_2 and checked for confluency the day after. If the cells were confluent, the culture was split as described below. If not, the medium was replaced by fresh pre-heated medium and checked for confluency the day after.

2.1.3.1 Removal of cryoprotectant from freshly-thawed cell vials

The freezing medium contains cryoprotectant DMSO (Dimethylsulfoxid), which may be harmful to cells at higher temperatures. To remove DMSO prior to further cell propagation, the cell suspension was transferred into a 15 ml conical tube containing 10 ml conventional growth medium and then centrifuged at 1200 rpm for 5 minutes at room temperature. Supernatant was removed and the pellet was re-suspended in 5-10 ml conventional growth medium. The cell suspension was transferred into a T-25 tissue culture flask and placed in the incubator for further propagation (Ammerman et al., 2008).

2.1.4 Splitting Vero/hSLAM cell cultures

For maintenance of Vero/hSLAM cells, cultures need to be split routinely, normally when reaching a confluency level of 75-80%. To rule out large temperature differences, the following reagents were preheated in a 37°C water bath: PBS 1x, Trypsin-EDTA, Vero/hSLAM growth medium.
After aspiration of culture fluid, the flasks were rinsed with 2-7 ml PBS 1x and afterwards incubated with 2-7 ml PBS-EDTA at 37°C and 5% CO_2 for 10 minutes. To completely detach the cells from the surface, it was sometimes necessary to softly percuss the flask. For neutralization of PBS-EDTA, culture medium was added in the same amount as PBS-EDTA and the liquid was transferred into a 50 ml tube for centrifugation at 1200 rpm, 21°C for 5 minutes. After aspiration of supernatant, the cell pellet was re-suspended in culture medium, depending on the size of the pellet. 1 ml of resuspension was brought into a new flask filled with either 9 ml Vero/hSLAM growth medium for T-25 flasks, 19 ml for T-75 flasks or 30 ml for T-175 flasks.

2.1.5 Freezing Vero/hSLAM cells

Two passages prior to freezing, geneticin should be added to the cell culture medium. The present experiments, however, were done using a stock of cells that were passaged only two times after seeding (Passage 11 + 2). As the expression of SLAM was assumed to be high enough in these cells, geneticin treatment was spared

prior to freezing. The cells were kept in T-75 flasks for two passages. According to the SOP "Cryopreservation of cell lines" (LIH) a freezing medium was prepared using Vero/hSLAM growth medium, 30% FBS and 15% DMSO.
At an estimated 90% confluency, the cell-monolayer was first rinsed with 5 ml PBS per T-75 flask. Afterwards the cells were incubated for 10 minutes at 37°C and 5% CO_2 with 5 ml PBS-EDTA per T-75 flask in order to detach them from the surface. After the incubation period, PBS-EDTA was neutralized using 5 ml Vero/hSLAM growth medium and the liquid was transferred into a 50 ml tube and centrifuged at 1200 rpm, 21°C for 5 min. The supernatant was trashed and the cell pellet was re-suspended in 5 ml Vero/hSLAM growth medium. Both 50 µl and 150 µl of resuspension were separately transferred into screw-cap vials and used to conduct cell counting applying a nucleocounter (Mammalian Cell Counter, NucleoCounter® NC-100™). After one more centrifugation at 1200 rpm, 21°C for 5 min, the cell pellet was re-suspended at a concentration of $5.0 \times 10^5 – 5.0 \times 10^6$ cells/ml in freezing medium. Aliquots of 1 ml were filled into cryo vials and directly stored in a freezing container (Mr. Frosty™ Freezing Container) at -80°C to ensure a slow freezing process. Within one week the vials were brought to the liquid nitrogen tank for long-term storage.

2.1.6 Flow cytometry

Flow cytometry was applied to determine the expression level of CDw150 on the surface of Vero/hSLAM cells and was performed at the National Cytometry Platform (NCP), Department of Infection and Immunity, LIH. The NCP provides two cell sorters, three analyzers, one imaging flow cytometer, one mass cytometer and one microscope. The following analysis was done using the BD LSR Fortessa Analyser (Lasers: 355nm, 405 nm, 488 nm, 561 nm, 640 nm, 18 colours and 2 scatters).
According to the antibody manufacturer's instructions, one test should be carried out with around 10^5 to 10^8 cells. For the present experiment 2.5×10^5 cells per 100 µl (including dead and live cells) were taken for each test throughout the experiments (centrifuge for 5 minutes at 500 g).

Protocol for establishment of CDw150 antibody working-concentration:
A working-concentration for the CDw150 antibody was established prior to analysis of Vero/hSLAM cells. The CDw150-manufacturer only gives a general advice of using 5 µl for each test. However, depending on the extent of receptor surface expression, the antibody concentration needs to be adjusted in order to avoid background problems. CHO/hSLAM-cells were used for titration of different antibody concentrations:

Antibody volume	**Number of cells**	**Concentration adapted to cells per test**
5 µl	10^6	1.25 µl / 2.5×10^5
4 µl	10^6	1 µl / 2.5×10^5
3 µl	10^6	0.75 µl / 2.5×10^5
2 µl	10^6	0.5 µl / 2.5×10^5

1 µl	10^6	0.25 µl / 2.5 x 10^5

Table 1. Panel of different antibody volumes used to establish the working-concentration for flow cytometry.

In addition, one negative control containing only Vero cells (to build the baseline) and one vial not containing antibody but instead only *Zombie Dye* (Zombie NIR™) were prepared.
The preparation of tubes was as follows: Seven vials were filled with each 100 µl of cell suspension. 0.25 µl *Zombie Dye* (1 µl per 10^6 cells – 0.25 µl per 2.5x10^5 cells) were then added to each vial, except for the unstained negative control. In a third step, antibody (in 5 different concentrations) and 5 µl FcR Blocking Reagent (Miltenyl Biotec) (to block unspecific antibody reactions) were given to five vials, followed by a 5-minutes incubation. All seven vials then received one drop of *OneCompensation bead*. After an incubation period at 4°C for 30 minutes the content of each vial was transferred into 5 ml tubes (BD Biosciences), followed by addition of 500 µl *FACS-buffer* per tube. The tubes were centrifuged at 500 g for 5 minutes to remove supernatant, followed by addition of each 200 µl *FACS-buffer* and subsequent analysis using the Fortessa Machine.

OneCompensation beads consist of bimodal spherical particles. One portion reacts with antibody of mouse, rat and hamster origin whilst the other portion reacts with none of them, thereby building positive and negative populations. These populations are used to set a range from 100% (positive population) to 0% (negative population) antibody capturing (Thermo Fisher Scientific: OneComp eBeads™ Compensation Beads, n.d.). The percentage of human SLAM expression by Vero/hSLAM cells will be somewhere in this range. *FACS-buffer* serves to minimize unspecific binding and to protect the cells from damage. *Zombie Dye* is used to enable live cell/dead cell discrimination. Zombie dyes are fixable dyes reacting with primary amine groups on proteins. Live cells cannot be entered by those dyes, so that only cell surface proteins will be labeled. Dead cells, on the other hand, are more permeable, hence making the dyes enter the cytoplasm, thereby increasing the amount of total protein labeling. As a result, dead cells will be significantly brighter for Zombie fluorescence than live cells (BioLegend: Live Cell/Dead Cell Discrimination, n.d.).

The antibody was successful in detecting CDw150 on the surface of CHO/hSLAM cells. An antibody concentration of 0.5 µl per 2.5x10^5 cells was found to be appropriate and hence chosen for a second analysis with Vero/hSLAM cells.

Protocol for testing of Vero-hSLAM cells:
Vero/hSLAM cells at different passages were tested for their CDw150-receptor surface expression: Vero/hSLAM SB (Passage 13+15), Vero/hSLAM SB (Passage 11+2), Vero/hSLAM EC (Passage 9+15), Vero/hSLAM EC (Passage 9+2).

CHO/hSLAM cells served as positive control and Vero cells (not transfected with an expression plasmid encoding human CDw150 (SLAM)) as negative control.

Twelve vials with each 100 µl cell suspension were prepared for FACS-screening, including negative controls. Except for the unstained negative controls, the cell suspensions were mixed with 0.25 µl Zombie Dye (1 µl per 10^6 cells – 0.25 µl per $2.5x10^5$ cells) per vial. Six vials (Vero/hSLAM SB (Passage 13+15), Vero/hSLAM SB (Passage 11+2), Vero/hSLAM EC (Passage 9+15), Vero/hSLAM EC (Passage 9+2), CHO/hSLAM, Vero) received CDw150 antibody. One drop compensation beads was given to the vial containing CHO/hSLAM cells. At 4°C all vials were incubated for 30 minutes and then transferred into 5 ml tubes (BD Biosciences). After addition of each 500 µl FACS-buffer, the tubes were centrifuged at 500 g for 5 minutes to remove supernatant, followed by the addition of each 200 µl FACS-buffer and subsequent analysis using the Fortessa Machine. FlowJo® was used for data analysis (leading analysis platform for single-cell flow cytometry analysis), provided by the National Cytometry Platform, LIH.

2.2 Materials and methods for virus cultures

2.2.1 Viral culture materials

2.2.1.1 Technical Equipment

The following equipment and disposables were used:

Devices: Orbital Shaker SSM1 (Stuart), Incubator, Megafuge 1.0 R (Heraeus), Pico 17 Centrifuge (Heraeus), Vortex Genie 2 (Scientific Industries), Microscope , Pipet-X Pipet Controller PX-100 (Mettler-Toledo), Pipetus (Hirschmann Laborgeräte)

Disposable material: Greiner CELLSTAR® serological pipette, volume 10 ml, 25 ml (greiner bio-one), Greiner CELLSTAR® serological pipette, 2 ml aspiration (greiner bio-one), Greiner CELLSTAR® Filter Cap Cell Culture Flasks, 25 cm^2, 75 cm^2, 175 cm^2 (greiner bio-one), Greiner CELLSTAR® Cell Culture Multiwell Plates, 12 well format (greiner bio-one), Greiner Cell Scrapers, 40 cm length (greiner bio-one), Tips LTS 10 µl, 200 µl, 1000 µl Filter 960/10 RT-L200F (Mettler-Toledo), Greiner Centrifuge Tubes, 15 ml, 50 ml (greiner bio-one)

2.2.1.2 Reagents and media

The following reagents (suppliers) were used to prepare the different media used:

DMEM (Lonza), FBS (HI) (Lonza), Pen/Strep (Lonza), DPBS (10x) (Sigma-Aldrich), Ultraglutamine (Lonza), HBSS (Lonza), BSA (Sigma-Aldrich), Sodium carboxymethyl cellulose (Sigma-Aldrich), WHO Reference Reagent for Measles Vaccine (Live), NIBSC code: 92/648 (NIBSC), Human Measles Mvi/Moscow Rus/1988 Genotype A Virus for Nucleic Acid Amplification Techniques (NIBSC), Human Measles MV 029 (Nigeria, B3) (in-house), Human Measles MV 030 (Kongo, B2) (in-house), Human Measles MV 501, Genotype A, Schwartz Vaccine Strain (in-house), Ms X Measles Nucleoprotein primary antibody (Merck Millipore), Anti-Mouse IgG (Fab specific)-Peroxidase antibody produced in goat (Sigma-Aldrich), 1-Step TMB Blotting (Life Technologies), Tween 20 P1379 (Sigma-Aldrich), BH195 Measles Hemagglutinin primary antibody (in-house), BNP146 Measles Nucleoprotein primary antibody (in-house), Acetone AR (Biosolve), Methanol (Biosolve), Ethanol for molecular biology (Merck), H_2O_2 (Merck), O-Dianisidine (Sigma-Aldrich), Milk powder (Nestle), MycoAlert™ Mycoplasma Detection Kit (Lonza), QIAamp Viral RNA Mini Kit (Qiagen), 2X Buffer (Invitrogen), $MgSO^4$ (Invitrogen), MVN1139-F (Forward-primer) (Hummel et al., 2006) (Eurogentec), MVN1213-R (Reverse-primer) (Hummel et al., 2006) (Eurogentec), MVNP1163-P (Probe) (Hummel et al., 2006) (Eurogentec), SuperScript™ III One-Step Quantitative Reverse Transcriptase / Platinum® Taq High Fidelity (Invitrogen), RNase OUT (Invitrogen)

The following cell culture media and other reagents were used:

Virus growth medium (VGM)

500 ml DMEM, 10 ml FBS (HI), 5 ml Pen/Strep, 5 ml Ultraglutamine

Virus transport medium (VTM)

500 ml Hank's Balanced Salt Solution (HBSS), 1 g BSA, 5 ml Pen/Strep

PBS (1x)

450 ml sterile water, 50 ml DPBS (10x)

Blotto (Blocking solution) for ICA

200 ml sterile PBS, 2 ml 10% Tween 20, 10 g milk powder

Staining solution

Acetone + Methanol (1:1)

Blocking solution for immunostain

PBS, 2% BSA

2.2.2 General conditions for viral culture

Measles Virus infected Vero/hSLAM cells were kept in a biosafety lab II (BSLII) in a cell culture incubator at 37°C and 5% CO2. A relative humidity of around 95% was maintained by evaporation from the water tank, which was filled with distilled water and antimicrobial agent (Aqua Clean WAK-Chemie Medical GmbH) to prevent growth of germs and fungi. For manipulation, the plates or flasks were transferred into a biological

containment cabinet (hood) maintaining strict aseptic conditions. All manipulations were done using disposable plastic pipettes. The virus room was only to be entered when wearing lab coat, hair coverage, goggles, 3M Aura disposable respirators, protection sleeves, overshoes and two pairs of gloves (one nitrile, the other latex). Virkon® disinfectant cleaner was used to wipe all surfaces potentially being in contact with viral material. No material was brought outside from the virus room unless it was properly cleaned and estimated harmless.

2.2.3 Virus inoculation

Inoculation of Measles Virus was conducted in a biosafety cabinet in the biosafety lab II (BSLII). The cell culture plates or flasks were transferred from biosafety lab I (BSLI) into biosafety lab II (BSLII) as well as the virus growth medium for dilution of Measles Virus. Vials containing virus were taken from the -80°C freezer and kept at room temperature until thawed. An appropriate amount of virus was mixed with viral growth medium to obtain the desired dilution. Afterwards the dilution was vortexed thoroughly.
Following the removal of cell growth medium from the plates or flasks, an appropriate amount of virus dilution was added and the cells were kept in the incubator for 1 hour to enable proper virus attachment. Finally, virus growth medium was added and the cells were stored in the incubator at 37°C and 5% CO_2.

2.2.4 Measurement of virus infection in culture

Before starting to compare different methods used to enhance the success of Measles Virus isolation, a sensitive method for measuring the extent of viral infection in cell culture was needed.
A first step was to sort out a virus concentration at which virus is only just growing sufficiently to make the infection detectable. This virus concentration was determined applying the $TCID_{50}$ method.
As a second step the extent of virus infection in cell culture was measured applying an immunostaining assay. PCRs were done to support the results of the immunostaining.

2.2.4.1 Cytopathic effect (CPE)

Vero/hSLAM cells are permissive cells since they support viral replication. In doing so, the host cell is killed, which is why the infection by Measles Virus is called cytolytic. Viruses being cytolytic usually drive characteristic changes in cell morphology (Albrecht et al., 1996), called cytopathic effect (CPE). Measles Virus is known to induce giant cell growth followed by the formation of multinucleated syncytia (Enders and Peebles, 1954; Heneen et al., 1967). Depending on virus concentration, distinct CPE can be seen 5-6 days after virus inoculation (Vaidya et al., 2014), sometimes even earlier.

CPE was observed through a microscope in BSL2. The extent of CPE was expressed in percent of cell monolayer exhibiting characteristic changes in cell morphology.

2.2.4.2 50% Tissue culture infective dose ($TCID_{50}$)

$TCID_{50}$ is based on quantification of plaques resulting from cytopathic effects.
Several viral dilutions were mixed with Vero/hSLAM cells and incubated in 96-well plates. The dilution at which 50% of infected wells show cytopathic effects after 5 days of incubation, contains the 50% tissue culture infective dose ($TCID_{50}$) of virus.
The assay was done in BSL2 according to the SOP "Titration of Measles virus by $TCID_{50}$" established at the LIH (former CRP Santé and LNS) in 2011.
Two T-75 flasks with Vero/hSLAM cells were grown in BSL1 until a confluency of about 90% was reached. Afterwards, the cell layers were detached, centrifuged and re-suspended, according to the SOP "Vero and Vero/hSLAM culture". Both 50 µl and 150 µl of re-suspension were transferred separately into screw-cap vials and used to determine the amount of live cells applying a nucleocounter (Mammalian Cell Counter, NucleoCounter® NC-100™). The volume of re-suspension was adjusted until reaching a concentration of 7.5 x 10^4 live cells/ml. Re-suspension and an adequate aliquot of Vero/hSLAM growth medium were transferred to BSL2. The vial(s) of Measles Virus chosen to be tested, were taken from either the -80°C freezer or the nitrogen tank. After thawing, the virus was pre-diluted 10 times in Vero/hSLAM medium (dilution ratio 1:10). Two 96 well plates (for *one* $TCID_{50}$ assay of *one* virus strain) were prepared for inoculation by filling them with 100 µl Vero/hSLAM medium per well. Afterwards, 100 µl of virus dilution were put into each of the 8 wells of plate 1 column 1 (dilution ratio 1:20). With an 8-channel pipette the volume of column 1 was mixed up by pipetting up and down 5 times. 100 µl from each well of column 1 was transferred into column 2. After change of tips, the volume was mixed up by pipetting up and down before transfer to the third column. Here again, tips were changed prior to mixing. When all wells of plate 1 were filled, 100 µl virus dilution were transferred from plate 1, column 12, to plate 2, column 1. The procedure was repeated with plate 2 until reaching column 11. 100 µl taken from each well of column 11 were discarded due to column 12, plate 2, serving as a negative control with non-infected cells. Afterwards, all wells were filled with each 100 µl Vero/hSLAM cell resuspension. This step was done using only one set of tips, but starting from the lowest dilution in order to prevent contamination of low virus concentration wells with traces of higher virus concentration wells.
After incubation, results of $TCID_{50}$ assay were analyzed applying a $TCID_{50}$ calculator (© Marco Binder, Dept. Infectious Diseases, Molecular Virology, Heidelberg University). This calculator uses the Spearman & Kärber algorithm to generate $TCID_{50}$.

2.2.4.3 Polymerase chain reaction (PCR)

PCRs were performed to quantify the amount of virus in the six well plates (i) after two days and (ii) after five days of incubation period. A reference reagent with known concentration of Measles Virus was purchased from NIBSC and used as reference point for every PCR run.
RNA-extraction was performed with the QIAamp Viral RNA Mini kit protocol according to the manufacturer's protocol.
The threshold cycle (Ct) is a relative measure of DNA and RNA templates in a sample. It depicts the cycle number at which the amount of templates is high enough to switch from the linear phase to the exponential phase of amplification. The earlier this happens the more template was available at the starting point of amplification. Hence, the smaller the Ct-value, the higher the initial amount of template in the sample.
For more detailed information, see 2.4.4.

2.2.4.4 Immunocolorimetric assay (ICA)

The protocol as well as aliquots of the secondary antibody and the TMB blotting solution for this indirect immunoassay were kindly provided by Dr. Min-hsin Chen from the Centers for Disease Control and Prevention (CDC) in Atlanta, US (Chen et al., 2007).
Vero/hSLAM cells were grown in 12-well, 48-well and 96-well plates until reaching a confluency of approximately 90%. The cells were then inoculated with an appropriate amount of virus dilution and incubated for 2 days in an incubator at 37°C and 5% CO_2.
As a first step methanol was pre-chilled in the -20°C freezer and the NeA-Blue TMB precipitating substrate was pre-warmed in the incubator.
After removal of virus growth medium, the cells were washed with PBS one time. Cold methanol was added and incubated for 30 minutes to fix the cells. After removal of methanol, the cells were again washed with PBS one time and afterwards overlaid with blotto for 30 minutes to block nonspecific antibody reactions. Meanwhile, the primary antibody was diluted in blotto (different dilutions were tested, ranging from 1:200 to 1:1000) and added to the cells after removal of blotto and two washing steps with PBS. 30 minutes later and following two more washing steps with PBS, the diluted secondary antibody (1:200-1:1000) was added and incubated for 30 minutes. After two more washing steps, the pre-warmed NeA-Blue TMB precipitating substrate was given onto the cells. The substrate was removed when the colour appeared after 2-5 minutes. To stop the reaction, the wells were rinsed with distilled water. Foci of infection should appear as blue stained dots, visible to the naked eye.

2.2.4.5 Immunostaining

Both immunostaining and ICA are based on the indirect detection of specific antigens of the Measles Virus, hence the same antibodies were used for both methods. The protocol was taken from the SOP "Culture and Titration of MVA (Modified Vaccinia Ankara) expressing Measles Virus (MV) proteins", 2012 (Axel Dubois, Laboratoire National de Santé, Institute of Immunology).

Special attention is required when preparing the dianisidine-solution. O-dianisidine is potentially hazardous and must be handled under a safety hood.

Vero/hSLAM cells were grown in 6 well plates until reaching a confluency of 90%. The cells were inoculated with 1 ml virus dilution per well and incubated for 1-2 days in an incubator at 37°C and 5% CO_2. After the incubation period, the medium was removed from the infected wells and the cells were fixed for 2 minutes with 1 ml fixing solution at room temperature and air-dried afterwards at open lid. The following incubation steps were carried out at room temperature on an orbital shaker at 60 rpm:

1 ml primary antibody (1:500) was incubated for 60 minutes on the wells and after removal, the plates were washed three times with 1 ml PBS 1x (5 minutes each time). The same amount of secondary antibody (1:500) was given to the wells and being incubated for 45-60 minutes, followed by 3 washing steps with PBS 1x. During the incubation period, the o-dianisidine solution was prepared. The following volumes are sufficient for 12 wells (using 6 well-plates): O-dianisidine was mixed with 500 µl ethanol. After 2 minutes vortexing, incubation for 5 minutes at 37°C and centrifugation at maximum speed for 30 seconds, the solution was checked for saturation. Saturation was assumed in case of a visible dianisidine-pellet on the ground of the tube. 240 µl of the saturated dianisidine solution were mixed with 12ml PBS and this mixture was supplemented with 12 µl H_2O_2 (tube wrapped in aluminium foil to avoid reactions with light). The substrate solution was then immediately brought onto the cells followed by an incubation period of 15-30 minutes (wrapped in aluminium foil). After the staining was fully developed, the dishes were washed with water and overlaid with 1 ml of water. Foci of infection appeared as brown-yellow dots visible to the naked eye.

2.2.5 Freezing viruses

Viruses can be stored long-term in liquid nitrogen. For the experiments, aliquoted vials of virus were stored in a -80°C freezer in the virus room.

It was further useful for the experiments to produce a stock of viruses with similar concentrations. For this purpose, Vero/hSLAM cell cultures in T-75 flasks were infected by Measles Virus. Five days after virus inoculation and after the occurrence of significant cytopathic effects, cells and viruses were removed from the flask by scraping the cells into the viral growth medium. The medium was then transferred into a sterile 50 ml tube and stored at -80°C overnight to destroy the cell membrane and release virus particles from the cells.

For preparation of aliquots, the harvested material was defrosted the following day and centrifuged for 15 minutes at 3000 rpm at 4°C. Supernatant was used to make aliquots of different volumes.

2.3 Pretests

2.3.1 Reagents

To search for toxic effects of Polyvinylpyrrolidone (PVP), ITS-DMEM/F-12-2%FBS and Virus-production serum-free medium (VP-SFM) on Vero/hSLAM cells, these reagents were either added to the standard cell growth medium (like PVP) or used as a substitute of it (like ITS-DMEM/F-12-2%FBS and Virus-production serum-free medium). Five T-75 flasks were each filled with 1 ml cell suspension and 19 ml growth medium. A variety of five growth media was tested:

1. Standard growth medium (500 ml DMEM, 35 ml FBS, 5 ml Pen/Strep, 5 ml Ultraglutamine)
2. Growth medium with reduced FBS (480 ml DMEM/F-12, 5 ml Pen/Strep, 5 ml ITS, 10 ml FBS)
3. Virus production serum-free medium (490 ml VP-SFM, 5 ml Pen/Strep, 5 ml Ultraglutamine)
4. Growth medium with 100% PVP 40 (2,28 g PVP 40, filled up with standard growth medium to 19 ml)
5. Growth medium with 9% PVP 40 (204,25 mg PVP 40, filled up with standard growth medium to 19 ml)

After two days of incubation at 37°C and 5% CO_2, the flasks were checked for cell growth. Both the flask with standard growth medium (flask 1) and the one with FBS-depleted medium (flask 2) were found to show good cell growth with 80 % confluency and cells being homogenously distributed. Cells grown in the Virus-production serum free medium (flask 3) appeared to have a similar confluency, however, their bodies were stretched out leaving large free spaces between each other. The flask with 204 mg PVP 40 (flask 5) in 19 ml standard growth medium showed 75% confluency with small and round cell bodies. Only 60% confluency was observed for the medium containing 2,28 g PVP 40 (flask 4). Here, the cells were obviously not able to stretch sufficiently and initiate cell division of neighbouring cells, probably due to lack of nutrients (as nutrients could be superseded by the relatively high amount of PVP 40).
Two days later, flasks 1, 2 and 5 had grown satisfactorily, whereas flasks 3 and 4 were still found containing underdeveloped and unhealthy cells, indicating that the cells were neither able to adapt to virus-production serum free medium nor to huge amounts of PVP 40. These precursory findings led to the exclusion of VP-SFM and large concentrations of PVP 40 from the following experiments.

2.3.2 Mycoplasma

Mycoplasma are very small-sized prokaryotes lacking a cell wall, hence not being vulnerable to treatment with antibiotics that interfere with cell wall synthesis, such as penicillin (Jean et al., 2017). Due to their size and plasticity they pass through 0,2 µm filters and are not visible by microscopy, even at high concentrations ($>10^7$

cfu/ml) (Lonza: Mycoplasma, n.d.). Since they compete with cells for the nutrients in culture media, one of the first signs of mycoplasma infection might be a slowdown in cell proliferation. Subtle effects not being directly visible, include DNA fragmentation due to mycoplasma nuclease, changes in cell membrane antigenicity, disruption of nucleic acid synthesis and chromosomal aberration. These effects on cell cultures make them a serious threat for culture systems.

Infection with mycoplasma is mostly due to cross-contamination derived from other infected cultures but also from the researcher himself as a possible carrier.

To avoid interferences with cell culture, standard testing for mycoplasma is an important quality control. All virus strains and cultures of Vero/hSLAM cells used during the experiments, were tested for mycoplasma, applying the MycoAlert™ Mycoplasma Detection Kit (Lonza).

The MycoAlert™ assay measures the activity of mycoplasmal enzymes prior to and after the addition of a certain substrate. Enzymes reacting with the substrate causes ADP to convert into ATP. A luminescent signal serves as an indirect measurement of ATP increasing in the sample.

The Vero/hSLAM cells and the virus strains chosen for the definite experiments, were Mycoplasma-negative. Only passages x+2, x+3 and x+4 of *Human Measles Mvi/Moscow Rus/1988 Genotype A Virus for Nucleic Acid Amplification Techniques* were colonized by Mycoplasma and hence only used for pretest purposes.

2.4 Enhancement of virus infection in culture

2.4.1 Plate preparation

6-well plates were prepared with Vero/hSLAM cells (Passage 13+2) one day prior to virus inoculation according to the following experimental setup:

	Immunostaining	**PCR 1**	**PCR 2**	**Total**
Centrifugation				
(2000 rpm, 1 hour)	(1 plate)	(1 plate)	(1 plate)	**6 plates**
1500 rpm, 30 minutes	1 plate	1 plate	1 plate	
Rolling				
60 rpm, 5 days	1 plate	1 plate	1 plate	**6 plates**
60 rpm, 1 hour	1 plate	1 plate	1 plate	
Temperature				
33°C 1 hour, 35°C 5 days	1 plate	1 plate	1 plate	**6 plates**
33°C 1 hour, 37°C 5 days	1 plate	1 plate	1 plate	
Conventional procedure				
37°C 5 days	1 plate	1 plate	1 plate	**3 plates**

Table 2. Experimental setup with number of 6-well plates by method.

For reasons of time and logistics, the effect of macromolecular crowding and serum-free medium was not investigated in the course of these experiments, although included in the pretests.
In total, 21 plates were prepared and each well was filled with $3x10^5$ live Vero/hSLAM cells to ensure 80% confluency by the next day.

2.4.2 Inoculation

Virus strain *MV 029 (B3) Nigeria* was used for inoculation. Previous testing of several dilutions (1:1000, 1:2000, 1:5000, 1:10 000, 1:20 000, 1:40 000, 1:80 000) and measurement of infection using immunostaining had identified dilutions 1:2000 and 1:10 000 as suitable for the experiments. Pairing each dilution on the plates allows for replicate testing (Figure 1).

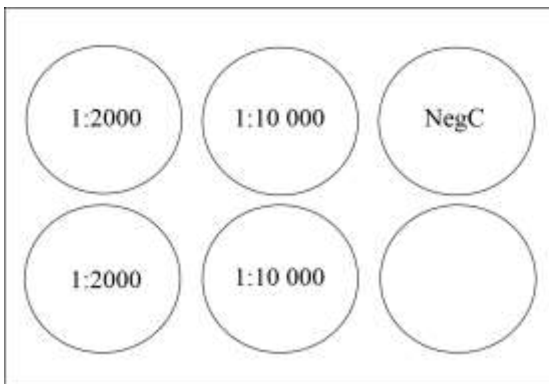


Figure 1. 6 well plate seeded with Vero/hSLAM cells and incubated with couplets of two different dilutions (1:2000 and 1:10 000). Negative control (NegC) in the fifth well.

The plates were inoculated one after another, starting with the six plates for i) centrifugation. First, the Vero/hSLAM growth medium was removed and 1 ml virus dilution was brought onto each cell layer. The outer surfaces were then disinfected and the plates put into plastic zip lock bags to avoid spillage during centrifugation. As the plastic zip lock bags were slightly damaged after centrifugation, speed was reduced from 2000 rpm to 1500 rpm. For reasons of safety, only those three plates planned for centrifugation at 1500 rpm for 30 minutes were further used for the experiments, the other ones were trashed. Following centrifugation, the plates were incubated at 37°C for another 30.
Another three plates serving as ii) negative control were inoculated and incubated at 37°C for one hour.
Six plates were used to examine the effects of iii) rolling on viral infection. After inoculation, all six plates were placed on the orbital shaker at 60 rpm. For the experiments the orbital shaker was installed in the 37°C incubator, so the plates would spend the one-hour incubation period at 37°C.
For assessment of the effect of iv) temperature variation on viral infection, six plates were kept at 33°C for one hour.

The exact time point of virus inoculation was noted for every single method. According to these time points, 2 ml virus growth medium were added exactly one hour later to each well. Afterwards, i) centrifugation plates were stored at 37°C, as well as ii) negative control plates, iii) rolling plates and three iv) temperature plates. The other three iv) temperature plates kept on staying at 35°C. Only three iii) rolling plates were placed on the orbital shaker at 60 rpm for the following incubation period, the other ones were kept under stationary conditions.

2.4.3 Immunostaining

Immunostaining was performed 48 hours after virus inoculation. One plate for each method was used to stain foci of infection, hence six plates in total.
After fixation with acetone and methanol (1:1) and addition of 1 ml primary antibody solution per well, the plates were carefully cleaned and then transferred to the orbital shaker in BSL2. All incubation steps on this orbital shaker were carried out at 60 rpm. The primary antibody BH195 used for the immunostaining was available in-house. It detects C-terminal peptides of the hemagglutinating and neutralizing epitope (HNE) of Measles Virus (Ziegler et al., 1996) and was used at a dilution of 1:500 for the experiments. The secondary anti-mouse IgG (Fab specific)-peroxidase antibody produced in goats is commercially available and was also used at a dilution of 1:500. Dots were manually counted when foci of infection became visible for the naked eye.

2.4.4 RNA extraction and Polymerase chain reaction (PCR)

The viral cultures of 14 six-well plates were being investigated by PCR in order to support the immunostaining results. By the time the immunostaining was done, seven of 14 plates were brought to the -80°C freezer. The remaining 7 plates were kept for 3 more days at incubation conditions and then also transferred to the freezer. Freezing destroys cell membranes and releases virus particles from the cells. 13 days later (10 days later respectively), RNA extraction and PCR were performed both on the same day.

RNA extraction:
The QIAamp Viral RNA Mini kit spin protocol for purification of viral RNA (Quiagen: QIAamp Viral RNA Mini Handbook, 2018) was used for isolation of Measles Virus RNA. The extraction was performed in the virus room of biosafety lab 2 (BSL2). Four runs were conducted in total and on the same day.
Prior to extraction, samples were taken from the -80°C freezer and equilibrated to room temperature. The lids of the six well plates were replaced by new ones to remove condensation water. The volume of each well was transferred into 15 ml falcon tubes and centrifuged for 15 minutes at 3000 rpm and 4°C, according to the SOP "Measles virus culture from clinical specimen using Vero/hSLAM cells" (LIH) from 2007. 140 µl of

supernatant were then transferred into 1.5 ml microcentrifuge tubes and subsequently mixed with 560 µl buffer AVL (viral lysis buffer). After pulse-vortexing for 15 seconds, the mixture was incubated at room temperature for 10 minutes, followed by the addition of each 560 µl ethanol (96-100%). After pulse-vortexing and short-spin centrifugation to remove drops from the lids of the microcentrifuge tubes, each 630 µl of the solutions were applied to the QIAamp Mini columns hold in 2 ml collection tubes. The columns were then centrifuged at 8000 rpm for 1 minute and placed in fresh 2 ml collection tubes. After addition of each 500 µl buffer AW1 (wash buffer 1), the columns were again centrifuged at 8000 rpm for 1 minute and placed in fresh 2 ml collection tubes, followed by addition of each 500 µl buffer AW2. The columns were now centrifuged at full speed (14 000 rpm) for 3 minutes and then again placed in fresh collection tubes. After another centrifugation at full speed for 1 minute, collection tubes were trashed and columns were placed in previously labelled clean 1.5 ml microcentrifuge tubes. 60 µl of buffer AVE (elution buffer) were added to each column and incubated at room temperature for 1 minute, followed by centrifugation at 8000 rpm for 1 minute. Afterwards, the column was trashed. 2.5 µl of each sample were kept for PCR. The remaining RNA was frozen at -80°C.

Polymerase chain reaction:

The SuperScript™ III One-Step Quantitative RT-PCR System with Platinum® Taq High Fidelity for rapid detection and quantification of Measles Virus was used for the experiments. The combination of SuperScript™ III Reverse Transcriptase and Platinum® Taq DNA Polymerase High Fidelity allows for a one-step formulation with both cDNA synthesis and PCR amplification in a single tube. SuperScript™ III Reverse Transcriptase can synthesize cDNA at a temperature range of 45°C to 60°C and is inactivated by the following denaturation step at 95°C. The Platinum® Taq antibodies blocking the Taq DNA polymerase at ambient temperatures, are denatured as well, thereby allowing for activation of the polymerase function. The inclusion of a TaqMan™ probe makes the amount of synthesized DNA directly visible.

This protocol has been established for the detection of Measles Virus by Aurélie Sausy and Elsa Tisserand in 2017 based on a protocol from the Centers for Disease Control and Prevention, Atlanta (CDC). The system is sensitive enough to detect RNA targets of 100-200 base pairs (bp). It takes two hours to get results which makes the protocol a rapid tool for diagnostic detection of Measles Virus RNA.

The reaction mix was prepared for an amount of 100 wells in PCR hood 1 in the mastermix room, using the following components:

Component	Volume per well	Volume per 100 wells
H2O	7 µl	700 µl
2X buffer	12.5 µl	1250 µl
MgSO4 (5 mM)	0.5 µl	50 µl

MVN1139-F (25 µM) (forward primer)	0.75 µl	75 µl
MVN1213-R (25 µM) (reverse primer)	0.75 µl	75 µl
MVNP1163-P (25 µM) (TaqMan™ probe)	0.25 µl	25 µl
SuperScript™ III qRT/Platinum® Taq Mix	0.5 µl	50 µl
RNase OUT	0.25 µl	25 µl

Table 3. Composition of reaction mix for PCR.

94 wells were each filled with 22.5 µl of reaction mixture. In a second PCR hood, 2.5 µl RNA template were added to each well. Instead of template, water was added to the negative control well (Negative Control (NC) PCR). MVi/Moscow.RUS/1988 was used as a positive control for the PCR (Positive Control (PC) PCR). The RNA template of NIBSC standard (reference reagent with known concentration of Measles Virus) was diluted six times up to 10^{-7} to create a standard curve. These seven dilutions of NIBSC standard RNA were also brought onto the well plate. After vortexing and short spin to remove drops from the lid, the well plate underwent polymerase chain reaction in a real-time cycler.

3 Results

3.1 Pre-arrangements

3.1.1 Viral titer determination by TCID50

Three $TCID_{50}$ assays were conducted in total.

The first two ones were conducted to determine the titer of *Human Measles Mvi/Moscow Rus/1988 Genotype A Virus for Nucleic Acid Amplification Techniques.* This virus was used for all pretests that included the use of viral material and was passaged three times in order to prepare a sufficient amount of virus for pretest purposes. The passages were named x+2, x+3 and x+4. $TCID_{50}$ was applied on passages x+2 and x+3. All wells of the 96-well plates showing CPE after the 5-days incubation period were counted positive (+) for MV infection, while the others were counted negative (-).

The third assay determined the titer of *MV029 (B3, Nigeria).*

A free of charge online $TCID_{50}$ calculator was used for calculation (© Marco Binder, Dept. Infectious Diseases, Molecular Virology, Heidelberg University), based on the Spearman & Kärber algorithm as described in Hierholzer & Killington (1996), Virology Methods Manual, p. 374. The $TCID_{50}$/ml for *MV029 (B3, Nigeria)* was calculated as 2,79 x 10^4. 1 ml of virus dilution 1:27900 hence causes infection in 50% of inoculated cell cultures.

The 50% tissue culture infective dose was used to find a viral concentration at which virus is just sufficiently growing to make the infection detectable. A much higher concentration would probably result in overall infection and numerous foci in the immunostaining. A much lower concentration, on the other hand, might not lead to any foci of infection at all. Considering that adequate amounts of foci facilitate the interpretation of immunostaining, the virus dilution should be somewhat lower than 1:27900. For the experiments *MV029 (B3, Nigeria)* was hence diluted up to 1:2000 and 1:10 000, respectively.

3.1.2 Immunocolorimetric assay to measure virus infection in culture

Due to background problems, immunocolorimetric assay was not found to be suitable for the experiments.

3.1.3 Immunostaining to measure virus infection in culture

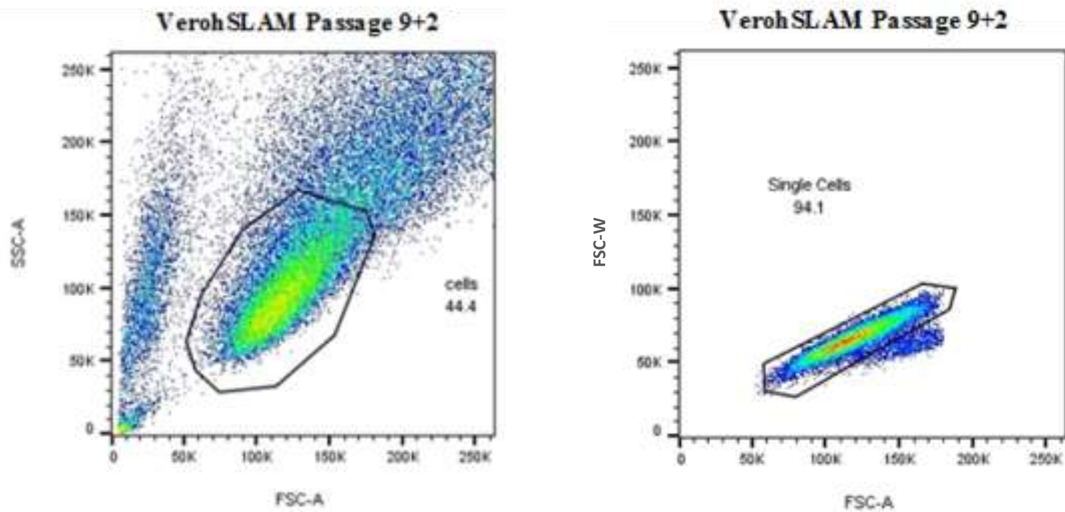
The immunostaining protocol proved to be fast and reliable. Only six well plates were used for this assay and the yellow-brown foci clearly stood out against a white background. The concepts of immunocolorimetric assay and immunostaining are similar. Both assays use the same antibodies. However, other than the

immunocolorimetric assay, the results of the immunostaining were clearly detectable as the readout was not complicated by background problems.
Immunostaining was therefore the method of choice to make viral infection visible throughout the experiments.

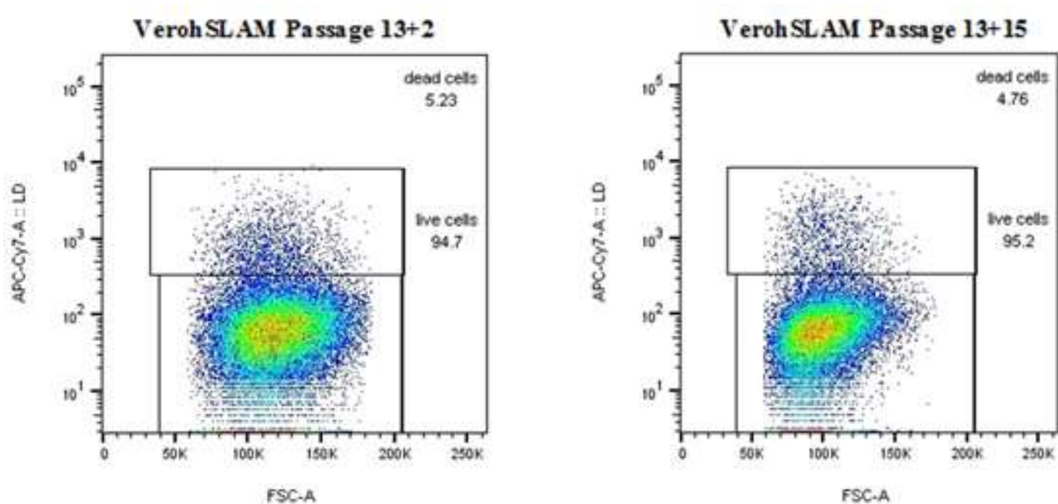
3.1.4 FACS-analysis of SLAM-expression

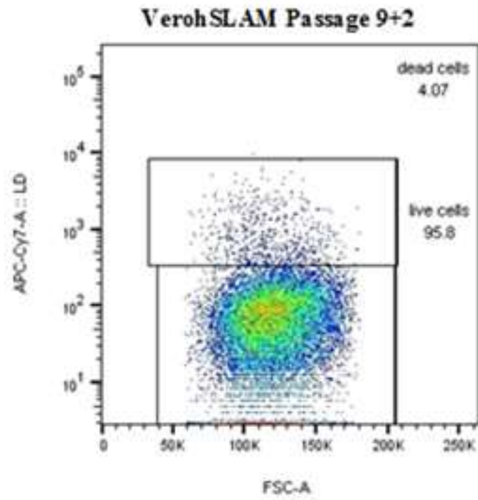
Vero/hSLAM cells can be passaged up to 15 times without addition of geneticin. After 15 passages geneticin should be added to selectively eliminate those cells not stably expressing CDw150 and the neomycin resistance gene. In the scope of this study FACS-analysis was done to search for possible decline in CDw150 expression after multiple passages without geneticin and to investigate the effect of geneticin-treatment prior to freezing.

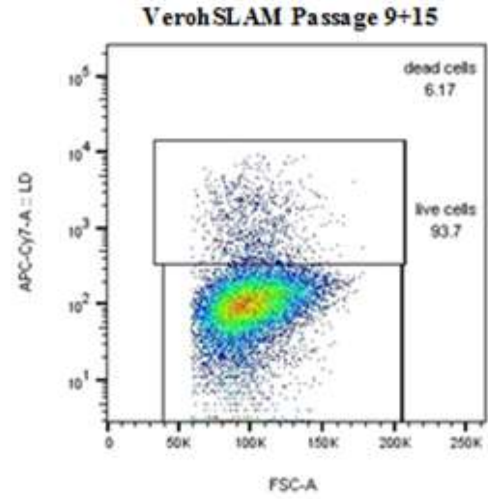
A)



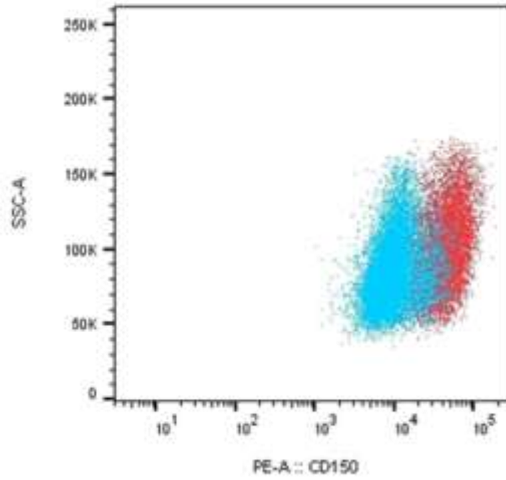
B)





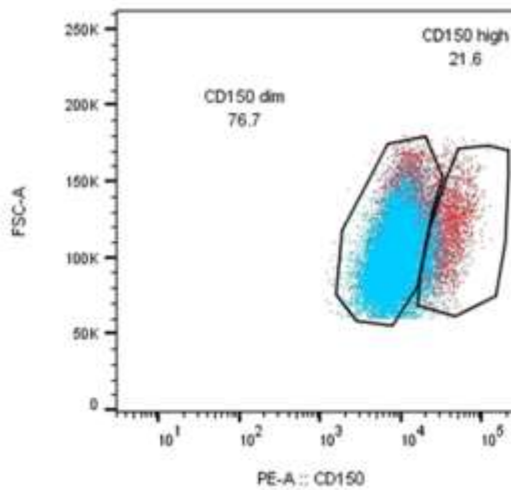


C) Vero/hSLAM Passage 13+15 (blue) compared to 13+2 (red)



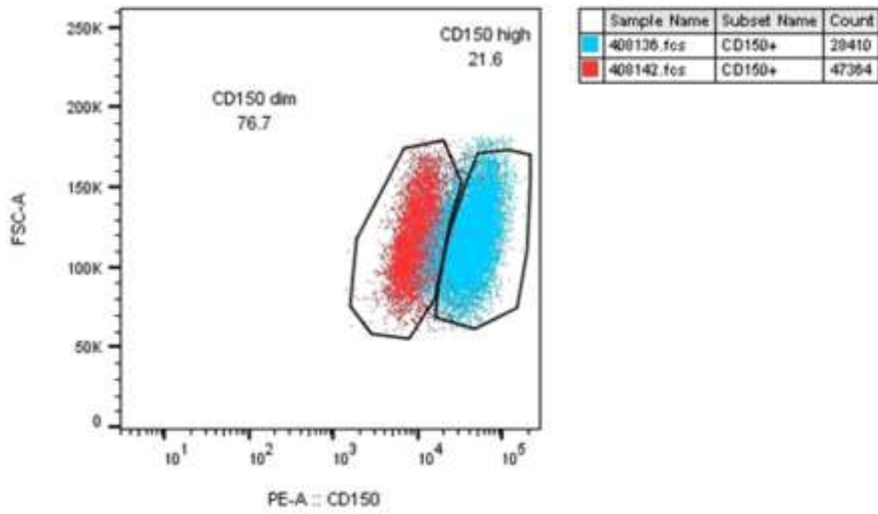
	Sample Name	Subset Name	Count
blue	408144.fcs	CD150+	23101
red	408136.fcs	CD150+	28410

VerohSLAM Passage 9+2 (red) compared to 9+15 (blue)

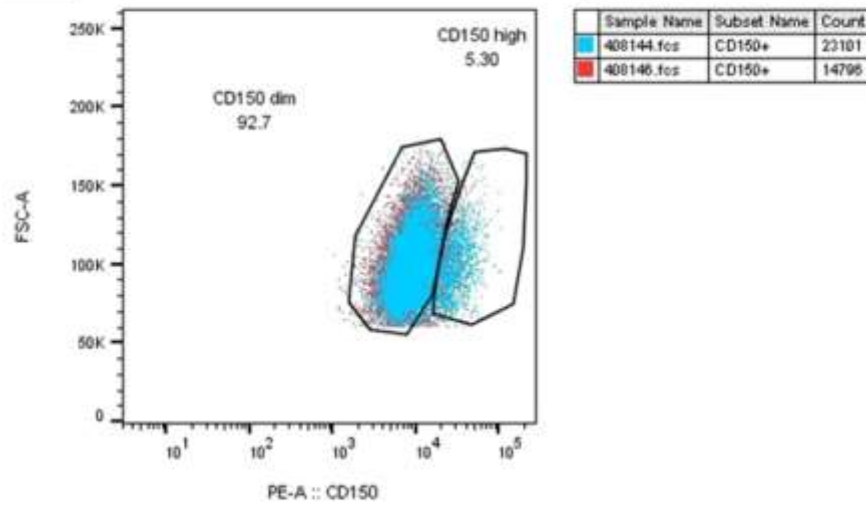


	Sample Name	Subset Name	Count
blue	408146.fcs	CD150+	14796
red	408142.fcs	CD150+	47364

VerohSLAM Passage 9+2 (red) compared to 13+2 (blue)



VerohSLAM Passage 9+15 (red) compared to 13+15 (blue)



Vero cells Passage x+7 CDw150 negative control

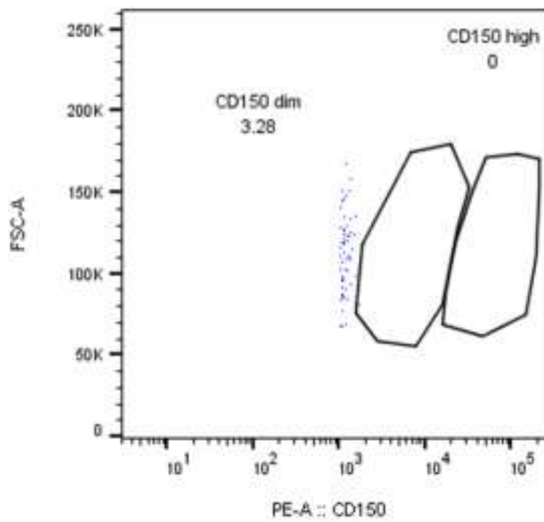


Figure 2. FACS-analysis of SLAM-expression. A) Forward/sideward scatter analysis (left) to distinguish live cells from debris and doublets (cells bound together and counted as one event) and forward/forward scatter (right) to distinguish singles from doublets only by cell size. B) Live and dead cell discrimination using APC-Cy7-A to stain amines relatively higher expressed on dead cells than on live or dying cells. C) Staining with PE-labeled CD150 Monoclonal Antibody (A12 (7D4)) and comparison of two cell passages within one graph. Vero cells were used as negative control (no expression of CDw150). Starting point for the detection of CDw150 is where depiction of Vero cells stops (roughly 10^3). The black gate was used in every graph except for the first one to outline two populations of CDw150-expression (dim and high expression). The expression of SLAM on Vero/hSLAM cells is hence depicted only in relative measures.

Passages 9+2 and 13+2 are taken from two separate Vero/hSLAM cell stocks. One week prior to FACS a culture was started and passaged two times (starting passage 9+0 and 13+0). There was no significant difference in the amount of dead cells with regard to live/dead cell discrimination (Figure 2). The same situation was found for multiple passages (9+15 and 13+15) and when comparing those passaged two times with those passaged multiple times (9+2 and 9+15, 13+2 and 13+15). A huge proportion of dead cells would have been indicative for increasing cell death due to natural senescence (mainly affecting multiple passages, e.g. 9+15 and 13+15) or other factors, such as remaining cryoprotectant (DMSO) after transferring the cells from frozen into live conditions (concerning new passages in particular, e.g. 9+2 and 13+2).

The comparison of two and multiple passages (13+2 and 13+15, 9+2 and 9+15) showed a clear decrease in the expression of CDw150 with increasing passage number. Comparing only the cells gone through two passages (13+2 and 9+2), the passage frozen without prior geneticin-treatment (13+2) turned out having a higher expression level of CDw150 than the geneticin-treated passage (9+2). 13 passages later, however, the expression level of both passages (13+15 and 9+15) had adjusted.

3.1.5 Removal of cryoprotectant from freshly-thawed cell vials

Freezing medium for cryopreservation of cell lines contains 15% of cryoprotectant DMSO (Dimethylsulfoxid) (SOP "Cryopreservation of cell lines"). Low hydrophilicity and molecular weight allows for free permeation of cell membranes. DMSO is assumed to form hydrogen bonds with water in the cell, hence inhibiting the formation of ice crystals throughout both cooling and warming processes (Chen et al., 2016). With toxicity of DMSO increasing at higher temperatures (Best, 2015), it becomes necessary to dilute and remove DMSO prior to further propagation in plates or tissue culture flasks (Ammerman et al., 2008). According to the SOP "Vero and Vero/hSLAM culture" (LIH) from 2007, cells are directly transferred from cryovial into flask. However, during the experiments an initial centrifugation at 200 x g for 5 minutes was found beneficial for further cell growth, which is probably due to DMSO accumulating in the supernatant that is later discarded. Without initial centrifugation, it took around four passages for Vero/hSLAM cells to reach proper cell growth, whereas cells grown after initial centrifugation adapted faster to live conditions, showing proper cell growth already after around 2 passages.

3.2 Methods for enhancement of virus infection in culture

3.2.1 Immunostaining

Plates not used for RNA extraction underwent immunostaining. The wells were photographed (Figure 3) and the brown-yellow stained foci (CPE) were manually counted.

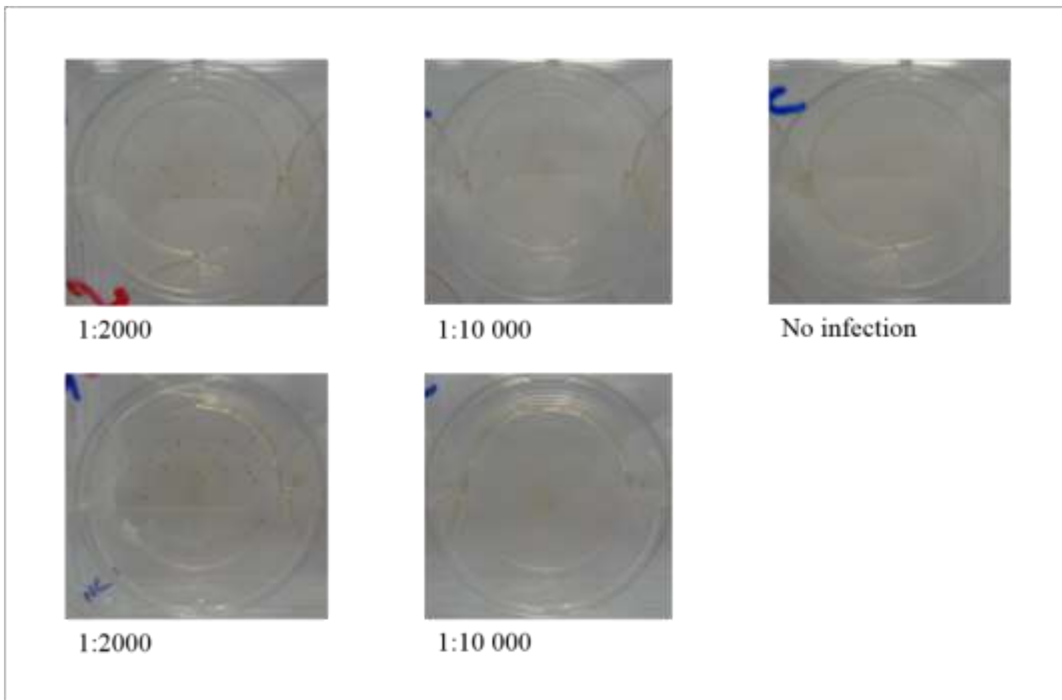


Figure 3A. Immunostaining conventional procedure plate.

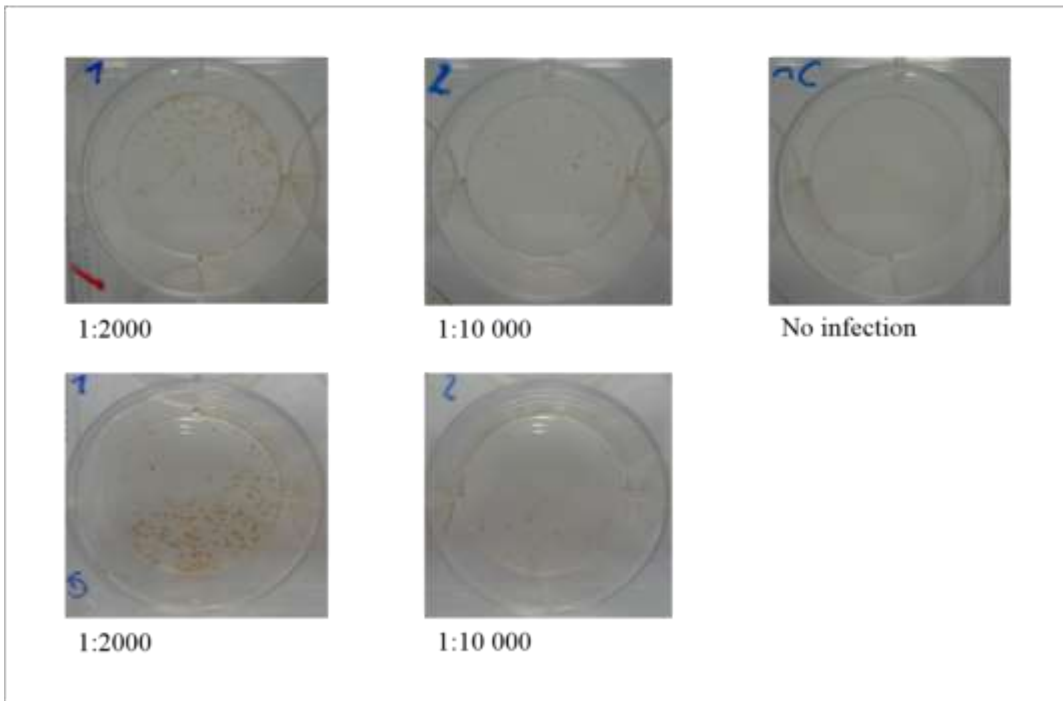


Figure 3B. Immunostaining centrifugation plate.

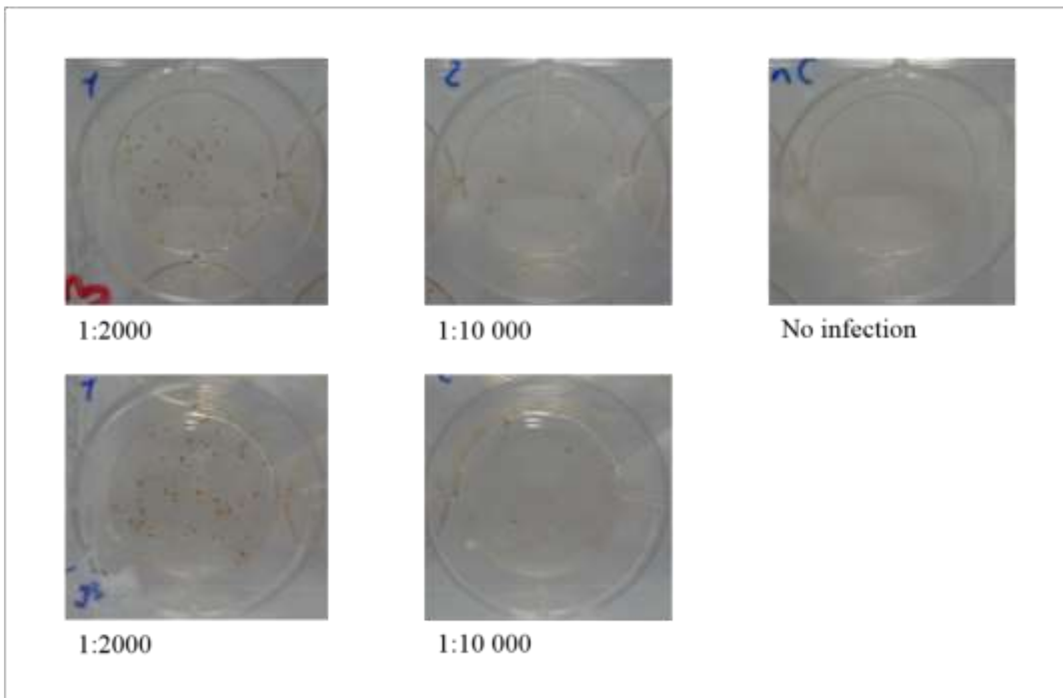


Figure 3C. Immunostaining rolling plate.

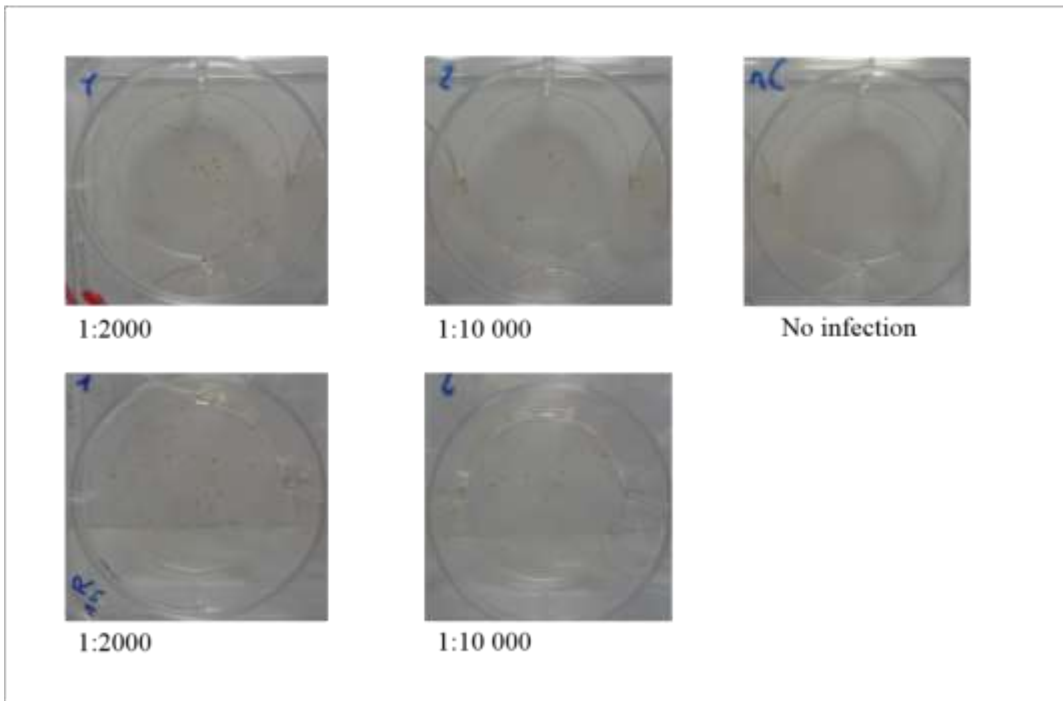


Figure 3D. Immunostaining rolling plate.

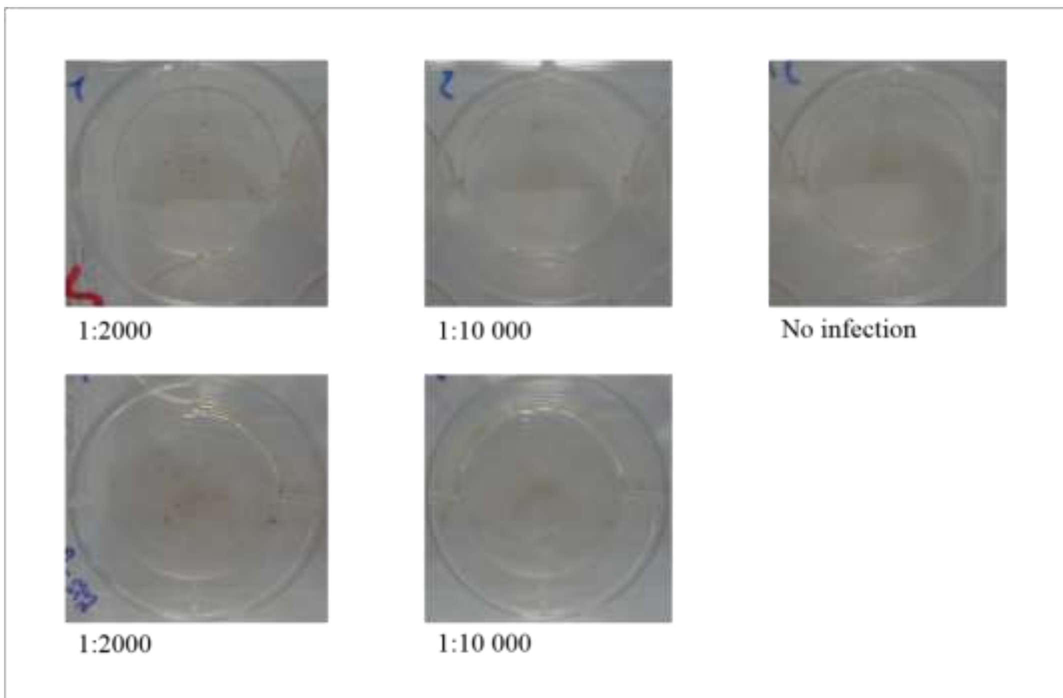


Figure 3E. Immunostaining temperature plate.

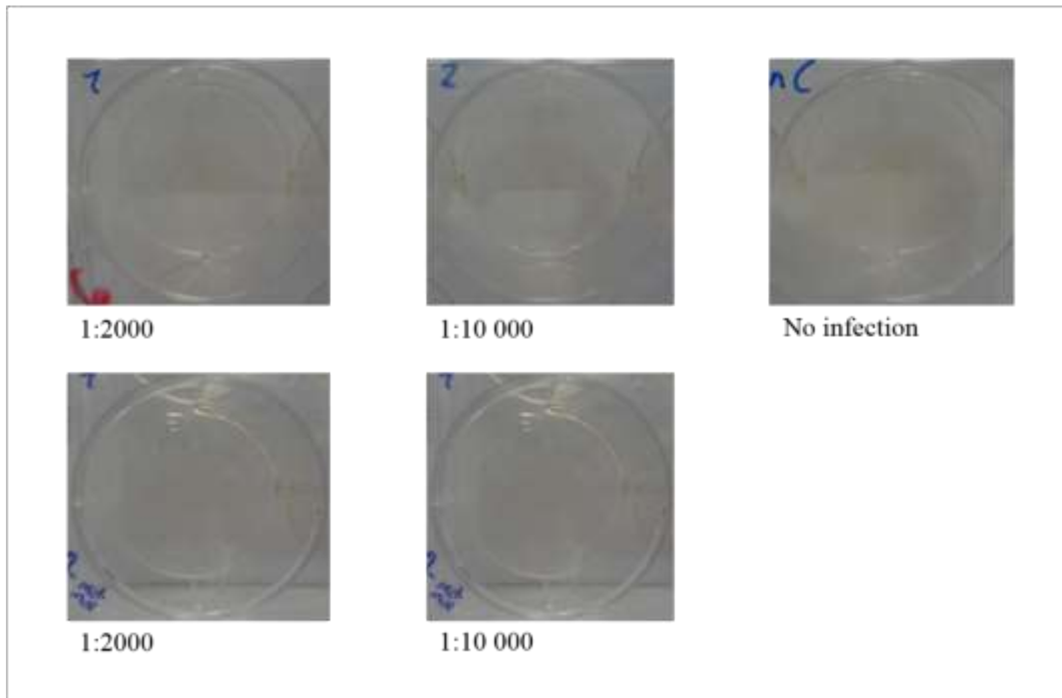


Figure 3F. Immunostaining temperature plate.

Figure 3. Immunostaining of plate treated by (A) Conventional procedure, (B) Centrifugation, (C, D) Rolling, (E, F) Temperature variation. After the one-hour incubation period at 37°C, each well of the conventional procedure plate (A) was overlaid with 2 ml virus growth medium and then incubated for 48 hours at 37°C. The centrifugation plate (B) was centrifuged at 1500 rpm for 30 minutes after virus inoculation and afterwards stored for 30 more minutes at 37°C. After addition of 2 ml virus growth medium the plate was kept for 48 hours at 37°C under stationary conditions. The rolling plates (C, D) were rolled at 60 rpm on an orbital shaker in the 37°C incubator for one hour after virus inoculation. After addition of 2 ml virus growth medium per well one plate (C) was kept for 48 hours on the orbital shaker at 60 rpm (37°C). The other plate (D) was kept for 48 hours at stationary conditions (37°C). The temperature plates (E, F) were first incubated at 33°C for one hour after virus inoculation. Following addition of 2 ml virus growth medium per well, one plate (E) was kept at 35°C for 48 hours under stationary conditions. Another plate (F) was kept at 37°C for 48 hours under stationary conditions.

3.2.2 CPE and PCR results

	CPE (Number of stained foci 48 h after virus inoculation)					Ct-values PCR 1 (48 h after virus inoculation)					Ct-values PCR 2 (5 days after virus inoculation)				
	1:2000		1:10 000			1:2000		1:10 000			1:2000		1:10 000		
	Duplicate well a	Duplicate well b	Duplicate well a	Duplicate well b	Negative Control	Duplicate well a	Duplicate well b	Duplicate well a	Duplicate well b	Negative Control	Duplicate well a	Duplicate well b	Duplicate well a	Duplicate well b	Negative Control
Conventional Procedure: 37°C under stationary conditions	46	50	13	8	blank	25.02	23.52	23.98	28.35	negative	16.98	18.09	17.29	15.91	38.52
Centrifugation: 1500 rpm, 30 min	212	149	39	34	blank	18.81	18.90	22.12	22.46	negative	17.10	17.84	16.79	17.23	36.98
Rolling: Entire incubation period	157	116	11	37	blank	20.31	20.63	23.14	23.27	negative	19.97	19.49	17.35	17.21	negative
Rolling: 1 hour directly after inoculation	85	94	22	25	blank	23.36	24.44	25.59	25.23	negative	17.66	18.01	16.24	15.78	39.46
Temperature: 33°C followed by 35°C	50	60	11	37	blank	21.29	21.28	24.08	24.17	negative	18.14	17.99	19.43	21.39	negative
Temperature: 33°C followed by 37°C	46	22	4	2	blank	25.25	25.61	27.09	29.40	negative	19.34	21.02	18.39	20.78	negative

Table 4. CPE (number of stained foci after a 48 h incubation period) and Ct-values of PCR 1 (conducted on cells incubated for 48 h following virus inoculation) and PCR 2 (conducted on cells incubated for 5 days following virus inoculation).

	CPE (Number of stained foci 48 h after virus inoculation)	
	1:2000	1:10 000
Centrifugation: 1500 rpm, 30 min	180.5	36.5
Rolling: Entire incubation period	136.5	24
Rolling: 1 hour directly after inoculation	89.5	23.5
Temperature: 33°C followed by 35°C	55	24
Conventional Procedure: 37°C under stationary conditions	48	10.5
Temperature: 33°C followed by 37°C	34	3

Table 5. Different methods for Measles Virus isolation and corresponding CPE gained 48 h after inoculation through immunostaining. Average number of stained foci from well a and well b. Arranged from highest to lowest.

	Ct-values PCR 1 (following 48 h incubation period)	
	1:2000	1:10 000
Centrifugation: 1500 rpm, 30 min	18.855	22.29
Rolling: Entire incubation period	20.47	23.205
Temperature: 33°C followed by 35°C	21.285	24.125
Rolling: 1 hour directly after inoculation	23.9	25.41
Conventional Procedure: 37°C under stationary conditions	24.27	26.165
Temperature: 33°C followed by 37°C	25.43	28.245

Table 6. Different methods for Measles Virus isolation and corresponding Ct-values gained 48 h after inoculation through PCR 1. Average Ct-value of well a and well b. Arranged according to Ct-value. Lowest Ct-value on top.

	Ct-values PCR 2 (following 5-days incubation period)	
	1:2000	1:10 000
Rolling: 1 hour directly after inoculation	17.835	16.01

Conventional Procedure: 37°C under stationary conditions	17.535	16.6
Centrifugation: 1500 rpm, 30 min	17.47	17.01
Rolling: Entire incubation period	19.73	17.28
Temperature: 33°C followed by 35°C	18.065	20.41
Temperature: 33°C followed by 37°C	20.18	19.585

Table 7. Different methods for Measles Virus isolation and corresponding Ct-values gained 5 days after inoculation through PCR 2. Average Ct-value of well a and well b. Arranged according to Ct-value. Lowest Ct-value on top.

Cytopathic effect

Numbers of stained foci on plates treated with different temperatures at dilution 1:2000 (55 foci, 34 foci) and dilution 1:10 000 (24 foci, 3 foci) do not differ significantly from the ones found on the conventionally treated plate (48 foci at dilution 1:2000 and 10.5 foci at dilution 1:10 000) (Table 5). Centrifugation and rolling plates, on the other hand, exhibit higher numbers of stained foci compared to the conventionally treated plate. Rolling for one hour after inoculation results in about two times higher numbers (89.5 foci at dilution 1:2000 and 23.5 foci at dilution 1:10 000 compared to 48 foci at dilution 1:2000 and 10.5 foci at dilution 1:10 000), rolling for the entire incubation period in about three times higher numbers (136.5 foci at dilution 1:2000 and 24 foci at dilution 1:10 000 compared to 48 foci at dilution 1:2000 and 10.5 foci at dilution 1:10 000) and centrifugation in three to four times higher numbers (180.5 foci at dilution 1:2000 and 36.5 foci at dilution 1:10 000 compared to 48 foci at dilution 1:2000 and 10.5 foci at dilution 1:10 000) (Table 5).

Ct-values

Whilst two days after virus inoculation the difference between lowest and highest Ct-value equals 6.575 at dilution 1:2000 and 5.955 at dilution 1:10 000, the difference is only 2.645 at dilution 1:2000 and 3.575 at dilution 1:10 000 five days after inoculation (Tables 6 and 7). After the five-days incubation period the Ct-values seem to have assimilated.

Conventional procedure: 37°C under stationary conditions

The low viral yield (Ct: 24.27 and 26.165) (Table 6) found after the two-days incubation period turns to a relatively higher yield after the five-days incubation period, reflected by the low Ct-values found by PCR 2 (Ct: 17.535 and 16.6) (Table 7).

Centrifugation: 1500 rpm, 30 min

Initially (PCR 1, following 48 h of incubation period) the highest viral yield was found in the centrifugation culture, reflected by the lowest Ct-values both in dilution 1:2000 (Ct: 18.855) and dilution 1:10 000 (Ct: 22.29) (Table 6). However, after the five-days incubation period the Ct-values of both dilutions (Ct: 17.47 and 17.01) nearly equate the ones found in the conventionally treated culture (Ct: 17.535 and 16.6), hence being relatively lower than expected (Table 7). The presumably larger number of virus particles (as compared to the other procedures) having entered the cells initially, has apparently not led to an overall higher yield in the end.

Rolling: Entire incubation period and Rolling: 1 hour directly after inoculation

After the two-days incubation period the Ct-values for rolling plates were found to be higher (Ct: 20.47 and 23.205, 23.9 and 25.41) than for the centrifugation plate (Ct: 18.855 and 22.29), hence indicating a lower viral yield in these plates (Table 6). When comparing both rolling methods, mechanical agitation for the entire

incubation period initially results in higher viral yields (Ct: 20.47 and 23.205 compared to 23.9 and 25.41). However, after five days this is reversed, as now the plate agitated for only one hour shows the lowest Ct-value (Ct: 16.01) compared to all other methods (Table 7).

Temperature: 33°C followed by 35°C and Temperature: 33°C followed by 37°C

Incubation at 33°C for one hour after virus inoculation and subsequent storage at 37°C has led to the highest Ct-values both after 2 (Ct: 25.43 and 28.245) and after 5 days (Ct: 20.18 and 19.585), hence indicating low viral yields achieved through this method (Tables 6 and 7). Storage at 35°C (PCR 1) was found to have higher yields (Ct: 21.285 and 24.125) as compared to storage at 37°C. However, the Ct-values have nearly equated after the five-days incubation period (Table 7).

Negative Controls

PCR after an incubation period of 5 days has revealed traces of Measles Virus RNA in the negative control wells of three plates: Conventional procedure plate, centrifugation plate, rolling plate (for one hour after inoculation) (Table 4). In all three cases, the Ct-values found in these negative control wells was close to 40, suggesting very low amounts of viral RNA. Contamination throughout virus inoculation is likely to be responsible for virus propagation in the above mentioned negative control wells.

Summary of main results

The study on improvement of Measles Virus isolation compares the effect of several physical methods on the isolation success of Measles Virus using Vero/hSLAM cells. Centrifugation, orbital shaking and temperature variation were applied in several formats after virus inoculation. The effect on viral infection was measured using an immunoassay as well as RNA-detection by PCR at two different time points.

After two days of incubation period, more virus was detected particularly in centrifuged and rolling cultures as compared to the conventionally treated culture. After five days of incubation period, however, this difference had disappeared. Only the plate that was rolled for one hour after virus inoculation still had a slightly higher viral yield compared to the conventionally treated culture, especially in dilution 1:10 000.

Rolling and centrifugation are most effective in increasing the viral yield. Rolling for one hour after virus inoculation might even increase the overall viral yield (measured five days of incubation period) as compared to the conventional method, but the difference was not significant.

From the different methods tested, centrifugation and rolling appear to be the most promising ones to increase viral infection rates and propagation in Vero/hSLAM cells.

II “Seroprevalence of Measles and Rubella IgG antibodies in the Luxembourg population”, an Epidemiological Study

In the 12-months period from July 2018 to July 2019, 483 cases of rubella were reported by 11 EU/EEA member states (European Centre for Disease Prevention and Control: Monthly measles and rubella monitoring report, 2019). From January 2018 to December of the same year, 82 596 people in 47 of 53 European countries were affected by measles, which is the highest number of Measles Virus infections in the European Region of this decade. This is despite the historically highest coverage of 90% for the second dose of measles-containing vaccine (typically MMR vaccine) in 2017 (World Health Organization Regional Office for Europe: Measles in Europe: record number of both sick and immunized, 2019). Seen from the global perspective, the ambitious goal set by WHO to eliminate measles and rubella in at least 5 WHO regions by 2020, will most likely not be met, as also other WHO Regions are still frequently reporting cases and outbreaks (Orenstein et al., 2018). Nevertheless, remarkable efforts in implementation and propagation of measles- and rubella-containing vaccine have been made globally in the past few years. Between 2012 and 2015 global coverage with the second dose of Measles-containing vaccine has increased from 48% to 61%, whilst global coverage with Rubella-containing vaccine has increased from 39% to 46% in the same time (Orenstein et al., 2018). These numbers result from achievements on the national and regional level, being finally summed up on the global level. However, sometimes the large-scale perspective does not sufficiently depict the situation on the subnational level. High rates of immunization coverage on the regional and national level and at the same time high numbers of people affected by the disease, hint towards immunization gaps on the local level (World Health Organization Regional Office for Europe: Measles in Europe: record number of both sick and immunized, 2019). These gaps are shelters for Measles and Rubella Virus. Working towards a world without these infectious diseases, immunization gaps need to be identified and closed.

From the 53 WHO European Region countries, 33 are currently classified as having eliminated both measles and rubella on the national level, with Luxembourg being one of them (World Health Organization Regional Office for Europe: Eighth meeting of the European regional verification commission for measles and rubella elimination (RVC), 2019). These countries have reported at least 36 months without endemic transmission of measles and rubella (World Health Organization Regional Office for Europe: Eighth meeting of the European regional verification commission for measles and rubella elimination (RVC), 2019), thereby not including sporadic cases, which might still occur. From this perspective it is crucial to maintain elimination and prevent sporadic cases from emerging into local outbreaks.

Due to its central location in Europe, its function as one of the three institutional seats of the European Union and its huge demand of employees in the tertiary and quaternary sector, about 170 000 cross-border commuters are heading to Luxembourg every day, most of them coming from the neighboring countries (Wijsbek, 2018). With measles and rubella being currently endemic in France and Germany (World Health Organization

Regional Office for Europe: Eighth meeting of the European regional verification commission for measles and rubella elimination (RVC), 2019), commuting poses a significant risk of disease introduction to Luxembourg. Furthermore, being an immigration country, Luxembourg frequently registers refugees or asylum seekers who may not be sufficiently vaccinated, thereby adding to the risk of disease introduction but also to a higher number of permanent residents being susceptible to vaccine-preventable diseases. A study from 2012 could show that 58% of newcomers arriving between May and September 2012 did not have antibodies against at least one of the measles/mumps/rubella vaccine components (Hübschen et al., 2018), suggesting that the number of susceptible individuals might increase if not specifically targeted and vaccinated. Another threat to the vaccination coverage of Luxembourg inhabitants are anti-vaccination movements that grew stronger in the past few years, especially through social media. To further maintain elimination of measles and rubella in Luxembourg, it will be necessary to control the population´s immunity status on a regular basis. At least for measles, there have been estimations that transmission stops when a herd-immunity threshold of 93% to 95% is met, which is why a target of 95% vaccination coverage is often referred to when talking about achieving and maintaining measles elimination (Funk, 2017). The last study that determined the antibody status in the Luxembourg population used anti-measles/-rubella IgG-test results from samples taken between 2000 and 2001 (Mossong et al., 2004). It could be demonstrated that the 95%-immunization target was met for both measles and rubella. In 2012, however, a survey conducted by the MoH had revealed that less than 86% of the 2-year-old children in Luxembourg had received two doses of measles- and rubella-containing vaccine, suggesting a risk factor for immunization gaps (Krippler, 2012). After the first vaccination dose between 12 and 15 months of age, the second dose is recommended in Luxembourg at the age of 15 to 23 months (European Centre for Disease Prevention and Control: Vaccine Scheduler, 2019).

The underlying study was initiated to assess the current immunization coverage in Luxembourg and to identify possible immunization gaps in certain age groups to be able to design targeted vaccination strategies. Applying a retrospective design, the study is based on already existing anti-measles/anti-rubella IgG test results, which are commonly used as an estimate of protection against infectious diseases as they confer long-lasting humoral immunity.

4 Materials and Methods

4.1 Ethical approval

The Comité National d'Ethique de Recherche Luxembourg (CNER), a member of the European Network of Research Ethics Committees (EUREC), is watching human and personal rights in research projects. A new study project must be evaluated by CNER and only its ethical approval gives the permission to start the project. For the CNER meeting in January 2017, several documents were submitted in advance, including the project description, the CNER application form as well as a certificate giving proof of the Commission Nationale pour la Protection des Données Luxembourg (CNPD) being informed about the project. Ethical approval to conduct the study was given early in 2017.

4.2 Data assessment

Data were obtained by inviting laboratories in Luxembourg to participate in the study and share their anti-measles/-rubella IgG test results from a time range between 2006 to 2015. They were further asked to provide information about age, sex, place of residence of the people tested, date of testing and about the applied test system for all anti-measles/-rubella IgG test results from between 2006 and 2015. Peoples´ identities were pseudonymized using IDs given by the laboratories. The datasets were compiled in Excel format and, depending on the laboratory, contain test results from timespans between 2006 and 2015.
The following six laboratories had kindly agreed to participate:

Laboratoires d'analyses médicales Ketterthill, Belvaux, Luxembourg
Département de Microbiologie, Laboratoire National de Santé, Dudelange, Luxembourg
Laboratoires Réunis, Junglinster, Luxembourg
Laboratoire de bactériologie/microbiologie, Centre Hospitalier de Luxembourg, Luxembourg
Laboratoires d'analyses médicales, Hôpitaux Robert Schuman, Luxembourg, Luxembourg
Laboratoire Centre Hospitalier du Nord, Ettelbruck, Luxembourg

For reasons of anonymity they are arbitrarily named LAB 1 to 6 in the following.

4.3 Data processing

When a particular ID was found several times within one year or within several years, the corresponding test results were manually checked for consistency in age, year of birth and year of measurement, in order to ensure they would originate from the same person. Apparently, these persons were tested multiple times by the same

laboratory and are hence referred to as “multiple test results”. Sometimes not only ID, age, sex and place of residence were identical, but also the IgG test result and the date of testing. These cases are referred to as “multiple entries” in the following.

Multiple entries were excluded except for one of each person concerned. Also multiple test results were excluded except for the oldest entry of each specific person concerned. If persons were tested in different laboratories between 2006 and 2015, it was not possible to identify them as multiple test results due to different ID systems applied by the laboratories.

From the initial 94 081 anti-measles and anti-rubella IgG test results, 2908 multiple entries and test results without age/gender/place of residence were excluded. 2797 of these were anti-rubella IgG test results and 111 anti-measles IgG test results. From the remaining 91 173 test results, 19 248 anti-rubella IgG and 118 anti-measles IgG results were excluded because they were multiple test results. The final dataset consists of 69 046 anti-rubella IgG and 2761 anti-measles IgG test results, summed up to a total dataset of 71 807 test results, all representing individuals due to prior exclusion of multiple test results and multiple entries (Table 8).

The year of birth of every single individual was calculated from the age and date of testing. If not already done by the laboratories, their IgG titers were translated into qualitative test results (positive/negative/equivocal), applying the cut-off concentrations of the test systems used (Table 9).

The area of the Grand Duchy of Luxembourg can be divided into northern, southern, eastern and central region (Figure 4). This division is identical to the division in constituency regions. Depending on the place of residence, all data were assigned to these four regions, using a sorting function of the statistics program R. For this purpose, a list of all Luxembourgish communities was prepared. Due to several spelling varieties in Luxembourg, all places of residence were listed in German, Luxembourgish and French. All locations that could not be allocated to the regions of Luxembourg by R were checked one by one and either assigned to one of the Luxembourg regions, one of the neighboring countries (Belgium, France, Germany) or registered as “others” (other countries than Luxembourg and the neighboring ones).

Finally, all IgG test results (qualitative and quantitative), all information about age, year of birth, sex, place and region of residence of the person tested, date of testing and the applied test system from all participating laboratories were compiled in one Excel file for further analysis. However, separate files for each laboratory were kept in order to also analyze them one by one.

Laboratory	Data time range	Number of test results in the initial dataset	Number of test results after exclusion of multiple entries, multiple test results and test results without age/gender/place of residence
LAB 1	Measles (12/2012 – 2015)	718	707
	Rubella (2007 – 2015)	40 576	27 708
LAB 2	Measles (2010 – 2015)	1 361	1227
	Rubella (2010 – 2015)	14 671	11 496
LAB 3	Rubella (2006 – 2015)	10 590	9049
LAB 4	Measles (2008 – 2015)	911	827
	Rubella (2008 – 2015)	13 081	12545
LAB 5	Rubella (2006 – 2015)	5200	2561
LAB 6	Rubella (2007 – 2015)	6 973	5687
In total	Rubella	91 091	69 046
	Measles	2990	2761
	Measles and Rubella	94 081	71 807

Table 8. Number of test results in the initial dataset and number of test results after exclusion of multiple entries, multiple test results and test results without age/gender/place of residence.

Measles

Laboratory	Test System and Cut-off Concentration	
LAB 1	Platelia® Measles IgG BIO-RAD (2012 – 06/2014) Positive > 1.2 AI Equivocal > 0.8 and ≤ 1.2 AI Negative ≤ 0.8 AI	Automate DIASORIN - Liaison XL (from 06/2014 onwards) Positive ≥ 16.5 AU/ml Equivocal ≥ 13.5 et < 16.5 AU/ml Negative < 13.5 AU/ml
LAB 2	Analyzer (Euroimmun) (03/2010 – 12/2015) Negative < 0.8 Equivocal ≥ 0.8 and < 1.1 Positive ≥ 1.1	
LAB 4	Rougeole IgG (Dade-Behring) (08/2008 – 01/2009) Measurement of extinction in the photometer: Negative < 100 Equivocal 100 – 200 Positive > 200	Rougeole IgG (EIA-Virtech) (01/2009 – 12/2015) Negative DO < 9 Equivocal DO 9 – 11 Positive DO > 11

Rubella

Laboratory	Test System and Cut-off Concentration		
LAB 1	Abbott AXSYM (2007 – 09/2008) Positive > 10 IU/ml Equivocal 5 – 10 IU/ml Negative < 5 IU/ml	Abbott ARCHITECT-C (from 09/2008 onwards) Positive > 10 IU/ml Equivocal 5 – 10 IU/ml Negative < 5 IU/ml	
LAB 2	Architect (03/2010 – 05/2014) Negative < 5 IU/ml Equivocal 5 – 10 IU/ml Positive > 10 IU/ml	Modular E (Roche) (05/2014 – 07/2015) Negative/Equivocal ≤ 10 IU/ml Positive > 10 IU/ml	Cobas 8000 (Roche) (04/2015 – 12/2015) Negative/Equivocal ≤ 10 IU/ml Positive > 10 IU/ml
LAB 3	Rubéole IgG (ARCHITECT)		

	Negative 0.0 – 4.9 IU/ml Equivocal 5.0 – 9.9 IU/ml Positive ≥ 10 IU/ml	
LAB 4	Rubéole IgG (ARCHITECT) Negative 0.0 - 4.9 IU/ml Equivocal 5.0 - 9.9 IU/ml Positive ≥ 10 IU/ml	
LAB 5	Vidas NCII (2006 – 2013) Negative <10 IU/ml Equivocal 10 – 14 IU/ml Positive ≥ 15 IU/ml	Liaison XL (2013 – 2015) Negative 0.00 - 4.99 IU/ml Equivocal 5.00 - 9.99 IU/ml Positive ≥ 10.00 IU/ml
LAB 6	VIDAS (Bio Mérieux) (2007 – 02/2009) Positive > 10 IU/ml Equivocal 5 – 10 IU/ml Negative < 5 IU/ml	Rubéole IgG, CLIA, Liaison (DiaSorin) (02/2009 – 2015) Positive > 9 IU/ml Equivocal 5 – 9 IU/ml Negative < 5 IU/ml

Table 9. Test systems applied by participating laboratories and corresponding cut-off concentrations for anti-measles/anti-rubella IgG negative/equivocal/positive. Abbreviations: IU = International Unit, DO = Densité Optique (optical density), AU = Antibody Units, AI = Antibody Index

Figure 4. Grand Duchy of Luxembourg. Division into northern (green), central (beige), eastern (orange) and southern (blue) region. Map from *d-maps.com*.

4.4 Data analysis

1. Basic analysis was done using Excel 2013-functions.
2. The analysis focused on differences and discrepancies in the qualitative IgG test results (positive/equivocal/negative) according to age, year of birth, sex and region of residence of the people tested. This was done to identify possible immunization gaps with regard to certain regions, age groups or gender.
3. Anti-measles/-rubella IgG equivocal test results are interpreted as IgG negatives, in order to avoid an overestimation of IgG positives (if IgG equivocals were counted as IgG positives).
4. Information about the Luxembourg population (size of birth cohorts between 2006 and 2015) were obtained from the statistics portal of the Grand Duchy of Luxembourg run by the National Institute of Statistics and Economic Studies of the Grand Duchy of Luxembourg (STATEC). It provides a publicly accessible statistical information service (Statistics Portal Grand Duchy of Luxembourg, n.d.).

5 Results

5.1 Data set

After cleaning, a total of 71 807 IgG test results were used for analysis. For years 2006 to 2009, less than 8000 datasets by year were available, whilst there were more for years 2010 to 2015 (Figure 5).

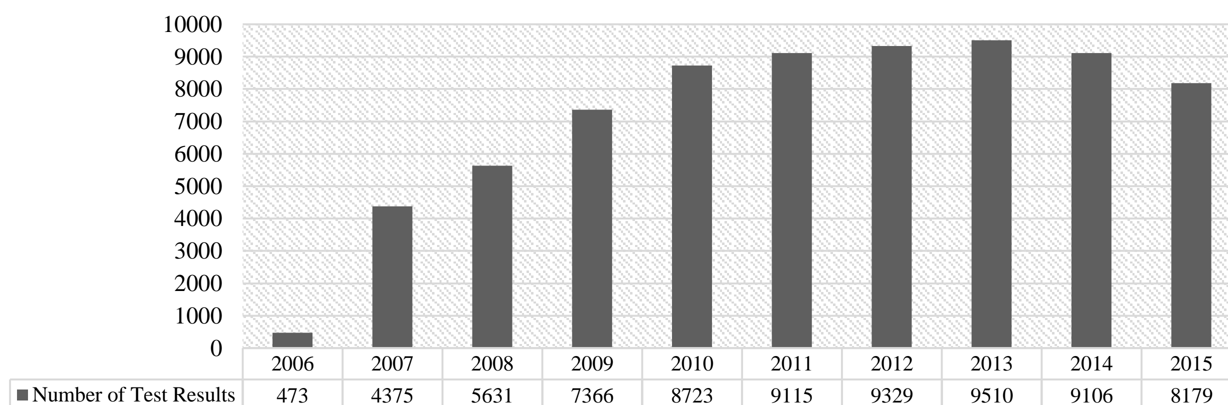


Figure 5. Dataset after cleaning. Number of test results by year.

The majority of datasets (n=67 386; 93.84%) were from women, most of them at childbearing age (n=64 322; 95.45%) at the time of testing. The median age for women in the dataset is 31 years with a range from 0 to 111. The median age for men is 35 years with a range from 0 to 90.

89.77% (n=64 458) of datasets were from Luxembourg residents, 9.93% (n=7128) from people living in neighboring countries (Germany, Belgium, France) and 0.3% (n=221) from persons with other places of residence. Most datasets from Luxembourg residents were linked to the Center (n=23 006; 35.69%) and the South (n=22 817; 35.4%), followed by the North (n=10 996; 17.06%) and the East (n=7639; 11.85%).

69 046 persons were tested for anti-rubella IgG and 2761 for anti-measles IgG. Differences can be observed in the percentage of IgG positives/equivocals/negatives when determined separately for each laboratory. The lowest rate (89.43%) of anti-rubella IgG positives differs by 3.99% from the highest rate (93.42%). 5.38% is the difference between the highest (7.43%) and lowest rate (2.05%) of anti-rubella IgG equivocals. The highest rate (7.57%) of anti-rubella IgG negatives is 4.44% higher than the lowest rate (3.13%) (Table 10). With 7.71% between the highest (83.67%) and lowest (75.96%) anti-measles IgG positive rate, the discrepancies are higher for measles than for rubella. Anti-measles IgG equivocal rates (highest 7.99%; lowest 2.11%) differ in 5.88%, whilst the IgG negative rates (highest 16.05%; lowest 11.49%) differ in 4,56% (Table 11).

Rubella

Laboratory	IgG Positives	IgG Equivocals	IgG Negatives	Total

LAB 1	24 949 (90.04%)	1368 (4.94%)	1391 (5.02%)	27 708
LAB 2	10 591 (92.13%)	336 (2.92%)	569 (4.95%)	11 496
LAB 3	8287 (91.58%)	186 (2.05%)	576 (6.37%)	9049
LAB 4	11 720 (93.42%)	329 (2.62%)	496 (3.95%)	12 545
LAB 5	2293 (89.50%)	75 (2.93%)	194 (7.57%)	2562
LAB 6	5086 (89.43%)	423 (7.43%)	178 (3.13%)	5687

Table 10. Number and percentage of anti-rubella IgG negative/equivocal/positive tested persons by laboratory.

Measles

Laboratory	IgG Positives	IgG Equivocals	IgG Negatives	Total
LAB 1	582 (81.86%)	15 (2.11%)	110 (15.47%)	707
LAB 2	932 (75.96%)	98 (7.99%)	197 (16.05%)	1227
LAB 4	692 (83.67%)	40 (4.84%)	95 (11.49%)	827

Table 11. Number and percentage of anti-measles IgG negative/equivocal/positive tested persons by laboratory.

5.2 Rubella seroprevalence

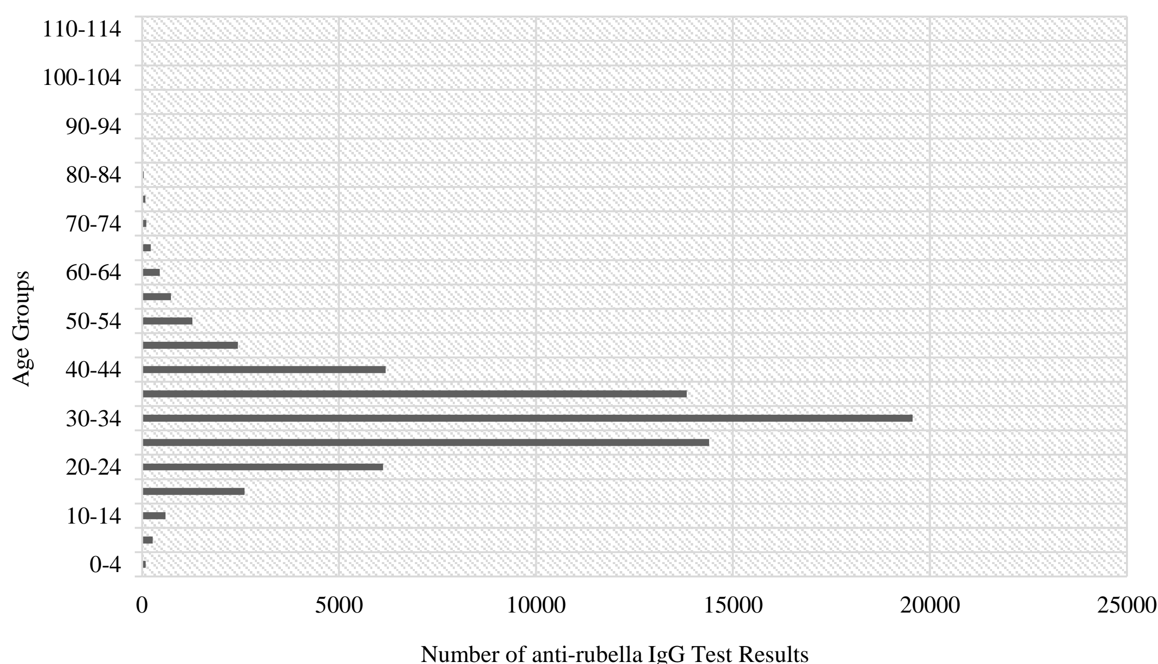


Figure 6. Number of anti-rubella IgG test results by age group.

From the 69 046 persons tested, 65 575 (94.97%) were women and 3471 (5.03%) were men. The median age of men and women in the dataset was 31.5 years. Most persons (94.32%; n=65123) were tested between 15

and 49 years of age, few persons were younger than 15 (1.39%; n=961) or older than 49 (4.29%; n=2962) (Figure 6). Female individuals aged between 15 and 49 were considered being at childbearing age at the time point of testing. Making up for 81.5% (n=56239) of rubella test results, they are largely represented in the dataset.

Similar to the entire dataset with measles and rubella test results, 89.73% (n=61952) of individuals tested for anti-rubella IgG were from the Grand Duchy of Luxembourg, 9.98% (n=6888) from neighboring countries (Germany, Belgium, France) and 0.29% (n=206) from other countries. With 32.05% (n=22129) and 31.85% (n=21990) central and southern region are largely represented, whilst northern (15.33%, n=10583) and eastern (10.5%, n=7250) regions are less represented in the dataset. The majority of test results from the four Luxembourg regions, France, Germany, Belgium and other countries originates from women. In relative numbers, slightly less women were tested in the South of Luxembourg (92.39%; n=20 317) than in the North (96.43%; n=10 205), East (96.54%; n=6999) and Center (96.34%; 21 319). Women from France, Germany, Belgium and other countries represent 96.60% (n=2953), 95.91% (n=1455), 92.49% (n=2140) and 90.77% (n=187) of the total (men and women).

A total of 62 974 (91.02%) of the 69 046 anti-rubella IgG tested individuals were positive for anti-Rubella Virus antibodies, 2729 (3.86%) were equivocal and 3343 (5.12%) were negative. Individuals born after 1982 present with higher percentages of anti-rubella IgG equivocal test results than older age groups (Figure 7). Datasets of age groups born between 1960 and 1982 do not exceed a proportion of 3.07% equivocals, whilst the rates of equivocals in age groups born between 1983 and 2000 range between 4.12% and 9.72%, with the highest proportions found in the cohort born between 1986 (7.24%) and 1994 (9.57%). After 1994, the rates of IgG equivocal test results decline from 7.63% to 5.56% and 2% in age groups born in 1996, 2000 and 2004 (Figure 7). The proportions of anti-rubella IgG positive test results stably exceed the 90% mark in age groups born between 1946 and 1983, whilst in age groups born after 1983 the proportions constantly decline to 83.86% in 1994, until they increase again rapidly in 1995 to 88.82% (Figure 7). Subsequent age groups, similar to age groups born before 1946, present with highly oscillating proportions of IgG positives.

When combining anti-rubella IgG equivocal and negative test results, the percentage of negatives and equivocals together was lower in older age groups, namely <9.85% for people born before 1983, whilst the percentages for birth cohorts born after 1983 were higher than 10% (Figure 7). With less than 500 test results by age group born prior to 1964 and after 1994, the percentages of anti-rubella IgG negative and equivocal test results in these age groups vary widely (Figure 7).

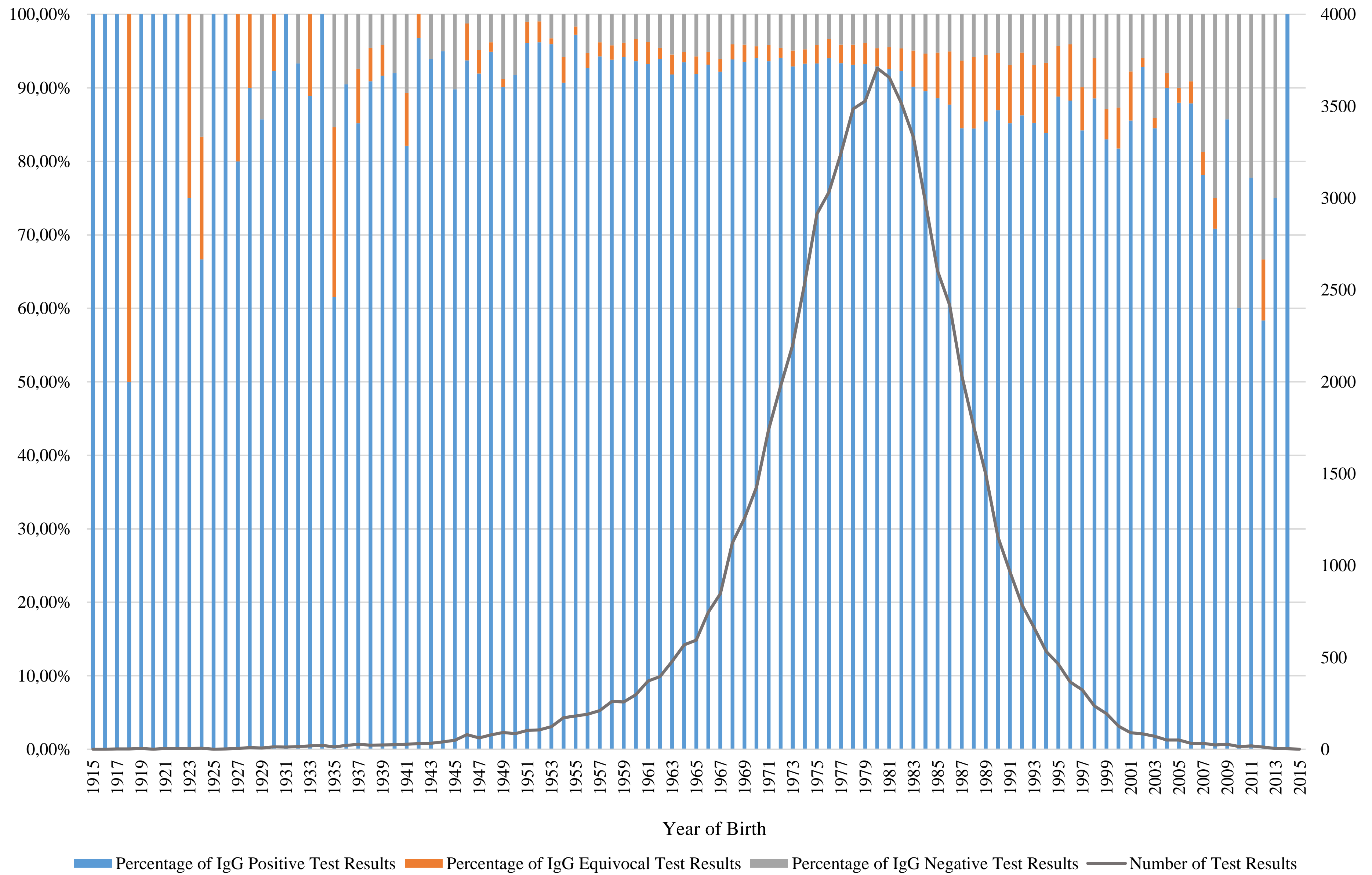


Figure 7. Seroprevalence of anti-rubella IgG by birth year (1915 – 2015) and numbers of test results.

7.13% of negative and equivocal test results are made up of male test results, 92.87% of female test results. 8.6% of all female anti-rubella IgG test results and 12.47% of all male anti-rubella IgG test results were either IgG negative or equivocal.

The rates of male and female anti-rubella IgG negatives + equivocals vary between the Luxembourg regions and range from 7.96% (n=577) (East) and 8.40% (n=1859) (Center) to 8.89% (n=941) (North) and 9.50% (n=2089) (South). The percentages of male and female IgG negative + equivocal test results of residents from other than the neighboring countries (9.22%; n=19), residents from Belgium (9.12%; n=211) and French residents (8.77%; n=8.77%) are similar and slightly lower in German residents (7.12%; n=108). When comparing the proportion of male anti-rubella IgG negative + equivocal test results to the total dataset of anti-rubella IgG negative + equivocal (of both men and women) (Figure 8), male individuals make up 9.86% (n=206), 7.27% (n=42), 5.40% (n=101) and 4.35% (n=41) of all negatives + equivocals from the South of Luxembourg, the East, the Center and the North. 26.31% (n=5), 9.47% (n=20), 5.22% (n=14) of IgG negatives + equivocals from Belgium, France and Germany respectively and 3.70% (n=4) from other than the given neighboring countries, originate from male individuals.

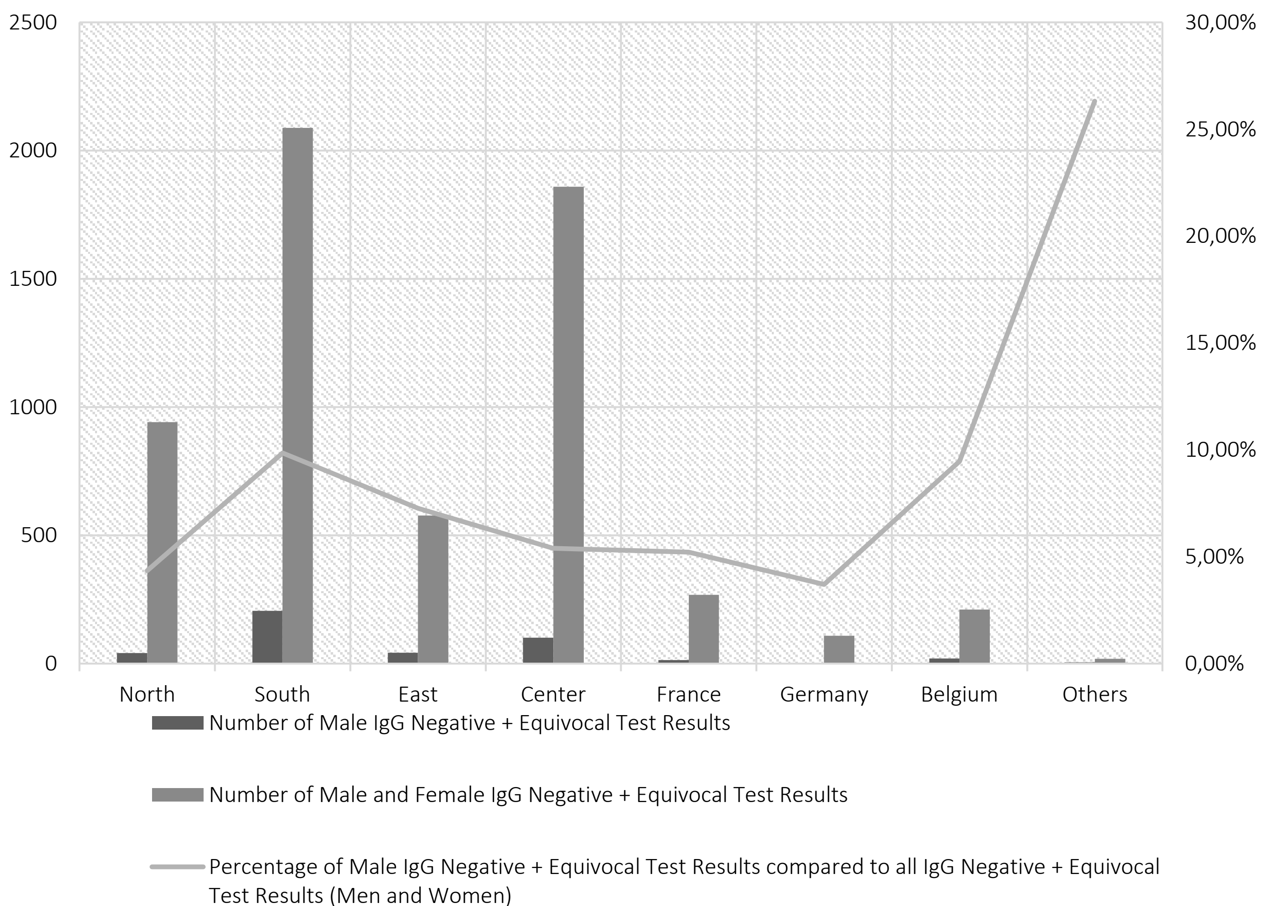


Figure 8. Proportion of male anti-rubella IgG negative + equivocal test results from all anti-rubella IgG negative + equivocal test results of both men and women.

Main results anti-rubella IgG dataset

From 69 046 individuals tested for anti-rubella IgG, 94.97% were women and 5.03% were men. The majority of test results (89.73%) originates from Luxembourg residents. 91.02% of test results were anti-rubella IgG positive, 3.86% IgG equivocal and 5.12% IgG negative. Individuals born after 1983 were more often tested IgG negative/equivocal compared to those born prior to 1983.

5.3 Measles seroprevalence

A total of 2761 datasets (3.85% of all test results left after cleaning) were linked to anti-measles IgG tests and 1811 (65.6%) of them were from women. The median age of men and women in the dataset is 30.5 years. Similar to the entire dataset containing both measles and rubella test results, 90.77% (n=2506) of individuals tested for anti-measles IgG were from the Grand Duchy of Luxembourg, 8.69% (n=240) were from neighboring countries (Germany, Belgium, France) and 0.54% (n=15) from other countries. With 31.76% (n=877) and 29.96% (n=827) central and southern region are largely represented, whilst northern (14.96%, n=413) and eastern (14.09%, n=389) region are less represented in the dataset. The majority of test results from the four Luxembourg regions, France, Germany, Belgium and other countries originates from women. Out of all test results from northern, central, southern and eastern regions, 66.42% (n=273), 65.91% (n=578), 65.54% (n=542), 64.43% (n=250) were from female individuals. The datasets from countries other than Luxembourg are made up of 72.58% (n=45), 67.5% (n=81), 63.79% (n=37), 26.67% (n=4) of women from France, Germany, Belgium and other countries respectively.

A total of 2206 (79.90%) of the 2761 anti-measles IgG tested individuals were positive for anti-Measles Virus antibodies, 153 (5.54%) were equivocal and 402 (14.56%) were negative. Whilst the majority of anti-measles IgG positivity rates from age groups born prior to 1969 stably exceeded 95%, they drop below the 95% mark in the 1969 birth cohort (81.25%) and from then on constantly decline (Figure 9). At the same time, the proportions of IgG equivocal test results increase from 3.45% (1970) over 8.11% (1985) to 13.85% (1990) and peak with 17.50% in the age group born in 1998 (Figure 9).

The highest number of test results (n=119) was linked to people born in 1983 (Figure 9). With each less than half of this number of test results (less than n=59), age groups born before 1973 and after 1992 are underrepresented (Figure 14).

The percentage of anti-measles IgG negative and equivocal test results is higher in younger age groups. In birth cohorts born after 1986 the rates are constantly higher than 15%. The percentages of birth cohorts born before 1986 are mainly lower than 15%, intermittently interrupted by peaks (Figure 9). Rates of anti-measles IgG negative and equivocal test results vary widely throughout the entire anti-measles IgG dataset (Figure 9), attributable to the relatively small number of test results obtained from each birth cohort.

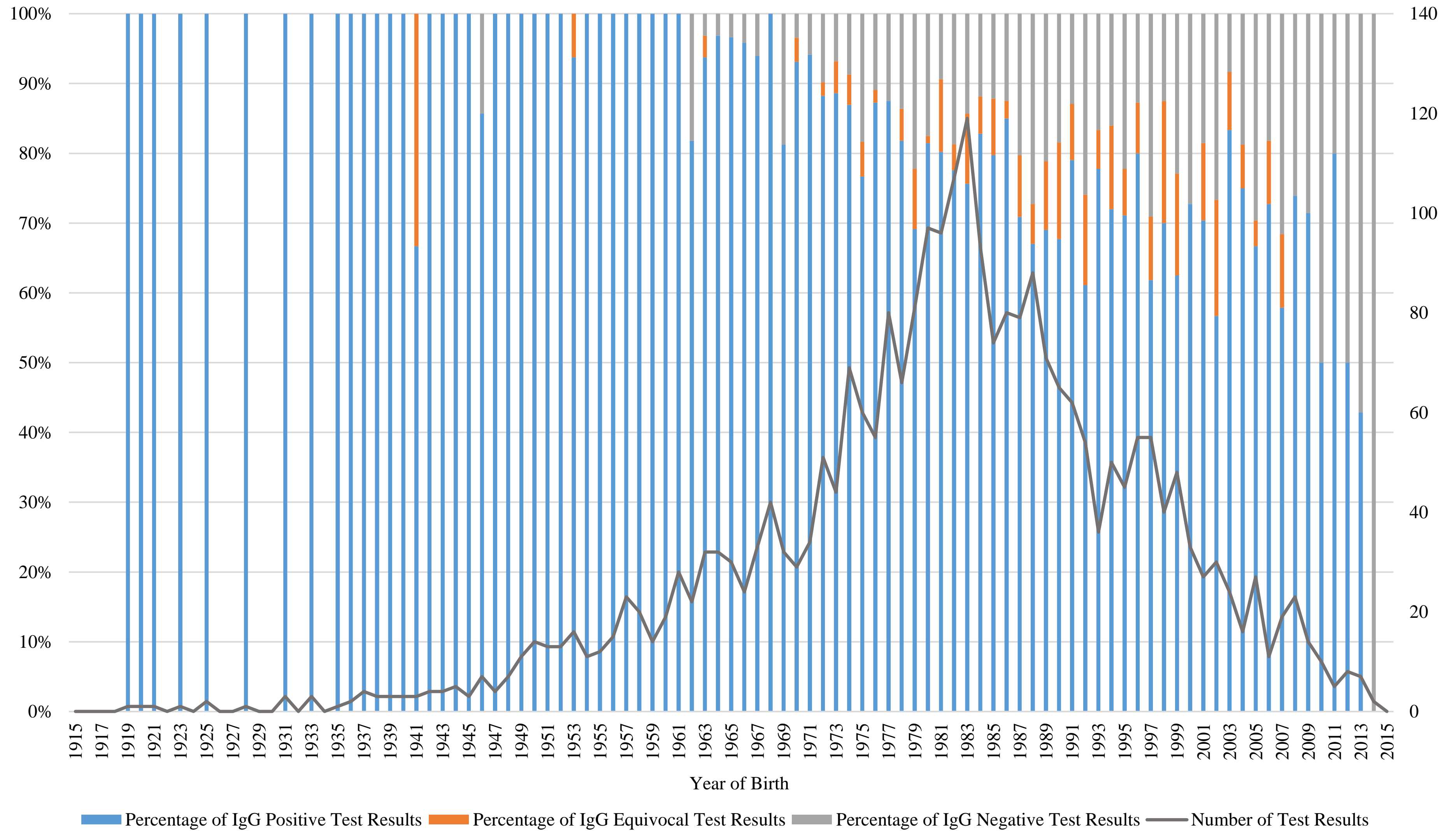


Figure 9. Seroprevalence of anti-measles IgG by birth year (1915 – 2015) and numbers of test results.

32.61% of negative and equivocal test results were made up of male test results, 67.39% by female test results. 20.65% of all female test results and 19.10% of all male test results were either IgG negative or equivocal. The rates for anti-measles IgG negatives + equivocals between the Luxembourg regions range from 19.23% (n=159) (South) and 19.61% (n=172) (Center) to 21.31% (n=88) (North) and 24.16% (n=94) (East). The percentage of IgG negative + equivocal test results from French residents (19.35%; n=12%) is within that range, whilst residents from Germany (16.67%; n=20), Belgium (15.52%; n=9) and other countries (6.67%; n=1) present lower rates. When comparing the proportion of male anti-measles IgG negative + equivocal test results to the total dataset of anti-measles IgG negatives + equivocals (of both men and women) (Figure 10), male individuals make up 37.50% (n=33), 36.17% (n=34), 31.98% (n=55) and 28.30% (n=45) of all negatives + equivocals from the North of Luxembourg, the East, the Center and the South. 100% (n=1) of IgG negatives + equivocals from other than the neighboring countries, 44.44% (n=4) from Belgium and 40.00% (n=8) from Germany respectively and 8.33% (n=1) from France, originate from male individuals.

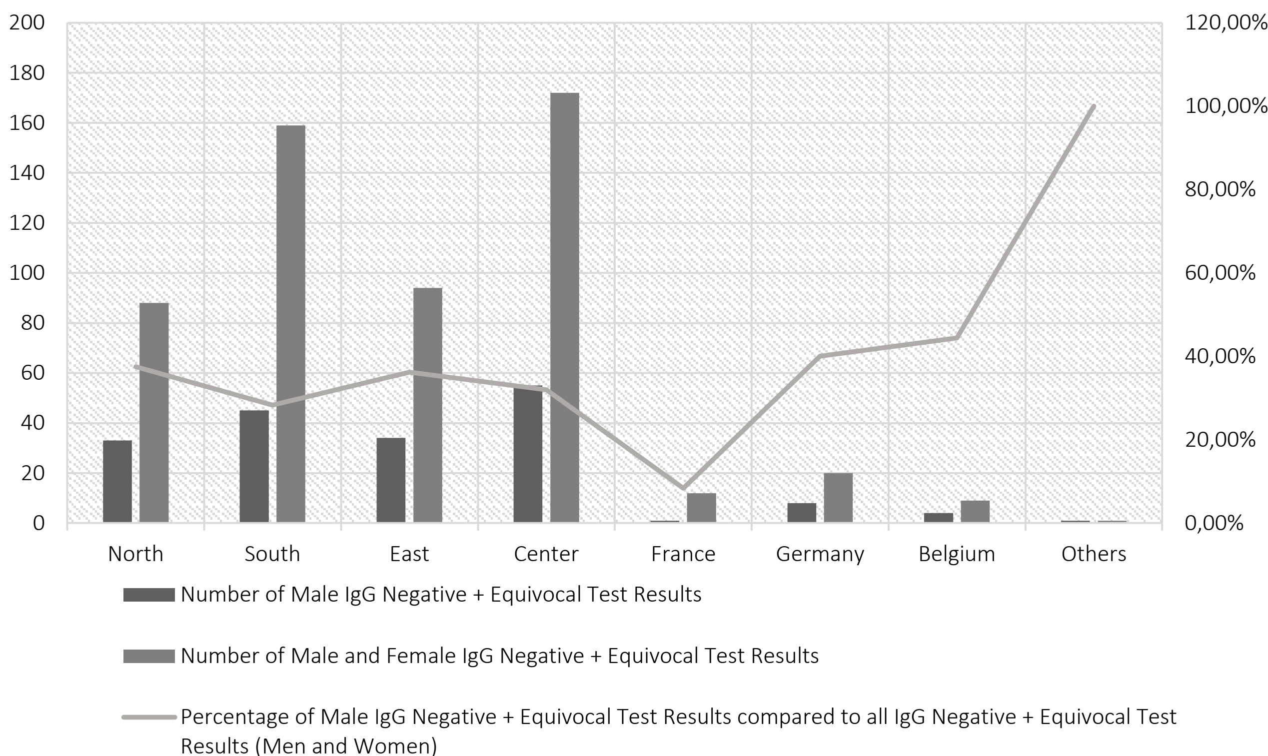


Figure 10. Proportion of male anti-measles IgG negative + equivocal test results from all anti-measles IgG negative + equivocal test results of both men and women.

Main results anti-measles IgG dataset

From 2761 individuals tested for anti-measles IgG, 65.6% were women and 34.4% were men. The majority of test results (90.77%) originates from Luxembourg residents. 79.90% of test results were anti-measles IgG positive, 5.54% IgG equivocal and 14.56% IgG negative. Individuals born after 1986 were more often tested IgG negative/equivocal compared to those born prior to 1986.

5.4 Measles and Rubella seroprevalence

2165 (78.41%) of 2761 individuals tested for anti-measles IgG antibodies were also tested for anti-rubella IgG antibodies. 2165 (3.13%) of 69 046 anti-rubella IgG tested individuals were also tested for anti-measles IgG antibodies. The majority of anti-rubella IgG tests was hence done without additional anti-measles IgG testing.

Individuals having received both tests are mostly (72.84%) anti-measles and anti-rubella IgG positive. 1.99% are double negative. Those being double positive were mainly (75.07%) born prior to MMR vaccine introduction in Luxembourg in 1986. 88.36% of those being double negative, however, were born after 1986 (Table 12). Only 4.2% are anti-measles IgG positive and anti-rubella IgG negative, whilst the other way around 12.15% are anti-rubella IgG positive but anti-measles IgG negative (Table 12). About two third (66.54%) of those being anti-rubella IgG positive and anti-measles IgG negative were born prior to 1986 and only about one third after 1986. Also for those tested negative for anti-rubella IgG and positive for anti-measles IgG, the majority (78.02%) was born prior to 1986. Two third (66.67%) of those tested double equivocal were born after MMR vaccine introduction (Table 12).

		Anti-rubella IgG positive	**Anti-rubella IgG equivocal**	**Anti-rubella IgG negative**
Anti-measles IgG positive		n=1577 72.84%	n=51 2.36%	n=91 4.2%
	Average birth year	1977	1983	1978
	Gender distribution	Female (F): 83.13% Male (M): 16.87%	F: 78.43% M: 21.57%	F: 76.92% M: 23.08%
	Born prior to 1986	n= 1184 75.07%	n= 24 47.06%	n= 71 78.02%
	Born in/after 1986	n= 393 24.93%	n= 27 52.94%	n= 20 21.98%
Anti-measles IgG equivocal		n=109 5.03%	n=3 0.14%	n=8 0.37%
	Average birth year	1982	1987	1980
	Gender distribution	F: 88.07% M: 11.93%	F: 66.67% M: 33.33%	F: 75.0% M: 25.0%
	Born prior to 1986	n= 60 55.05%	n= 1 33.33%	n= 5 62.5%
	Born in/after 1986	n= 49 44.95%	n= 2 66.67%	n= 3 37.5%

Anti-measles IgG negative		n=263 12.15%	n=20 0.92%	n=43 1.99%
	Average birth year	1981	1987	1996
	Gender distribution	F: 78.71% M: 21.29%	F: 85.0% M: 15.0%	F: 67.44% M: 32.56%
	Born prior to 1986	n= 175 66.54%	n= 7 35%	n= 5 11.64%
	Born in/after 1986	n= 88 33.46%	n= 13 65%	n= 38 88.36%

Table 12. Individuals tested for both anti-measles and anti-rubella IgG.

6 Discussion

6.1 Virus inoculations

Discussion of methods

Centrifugation, orbital shaking and temperature variation were conducted under exact equal conditions to ensure comparability. Methods were chosen depending on their applicability in laboratories with cell culture and virus culture facilities. For reasons of reproducibility they were meant to be financially and logistically feasible for any such laboratory.

Some methods applied in previous studies have focused on processing the original sample (e.g. throat swab, serum) in a way that would allow the recovery of more virus particles to be transfered into cell culture (Hall et al., 2014). However, previous observations made by LIH staff indicate that the amount of Measles Virus particles on clinical specimens is usually high enough (provided adequate sample collection and transportation) and even higher compared to rubella samples, suggesting that other reasons must be responsible for the occasional failure of Measles Virus isolation.

The focus of the present experiments was therefore on viral infection and virus propagation within the cells. An increased concentration of virus particles on cell surface as well as prevention of rapid virus degradation might lead to more viruses entering a single cell. This, in turn, might enhance virus propagation as more viral RNA should lead to faster production of viral proteins, hence enhancing viral assembly.

Centrifugation has been previously shown to enhance viral infection through centripetal force (Hughes, 1993; Sundin and Mecham, 1989; Weiss et al., 2012). Minnich et al. have studied the influence of centrifugation on Measles Virus infection in cultures of A549 cells (Minnich et al., 1991). However, so far there are no specific investigations on the effect of centrifugation on Measles Virus isolation using Vero/hSLAM cells.

Danger of spillage might explain why centrifugation is not yet broadly used for virus isolation purposes. The present experiments have tried to avoid spillage by placing viral culture plates in plastic zip lock bags during centrifugation. This, in turn, has led to speed limitations to prevent damage to the zip lock bags. Further investigations might benefit from safety centrifuge cups.

Continuous motion, such as rolling, can enhance virus-cell interaction and viral cell-to-cell spread (Hughes, 1993). Several studies have pointed out the benefit of rolling or continuous orbital motion for viral detection in cell culture (Mavromoustakis et al., 1988; Price et al., 1959). To apply this method, it was necessary to find an orbital shaker that would be applicable under warm and humid conditions as it needs to be placed inside the incubator. At 60 rpm the risk of spillage is low, hence allowing for the plates to be left without zip lock bags.

For temperature variation two incubators were required. One that would be run at 37°C and the other one for temperatures lower than 37°C. Measles Virus is highly temperature sensitive and rapidly degrades at 37°C (Black, 1959), hence suggesting that lower temperatures either only shortly after virus inoculation or throughout the entire incubation period might prevent virus particles from degrading, thereby increasing the chance for viruses to enter cells.

Besides the above mentioned physical methods, change in the composition of virus culture medium was part of the experiments, at least in the beginning. As a regular component of virus culture medium, fetal bovine serum (FBS) is associated with contaminants of animal origin. In terms of animal rights its production is often criticized (Sargent, 2012). Other drawbacks of FBS are growth inhibiting factors potentially interfering with purification and isolation of cell culture products (Arora, 2013). Whilst conventional virus growth medium for Measles virus culture only consists of 2% FBS, growth medium used for Vero/hSLAM cells contains 7.5%. As a provider of basic nutrients, several growth factors and hormones, several binding proteins (such as albumin and transferrin), protease inhibitors and minerals, it is essential. It increases the medium's viscosity, hence protecting the cells from damage caused by physical forces, and it acts as a buffer. The present experiments were planned to maintain the positive effects of FBS and reduce the negative ones, mainly by keeping FBS at a low concentration of 2% and compensatory adding of essential substitutes to the medium, such as insulin, transferrin and selenium (Sargent, 2012).

In the course of pretest experiments, Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A) was used as an additive to the Vero/hSLAM growth medium, together with DMEM/F-12. The latter was used instead of ordinary DMEM to make up for components that were probably lacking due to reduction of FBS. DMEM/F-12 is a 1:1 mixture of DMEM and Ham's F-12. As it has no proteins, lipids and growth factors, it cannot serve as a sole substitute but needs to be accompanied by FBS and ITS (Thermo Fisher Scientific: DMEM/F-12, n.d.).

Applying the above mentioned growth medium variations initially led to Vero/hSLAM cells growing as usual. However, after a few passages their growth appeared less efficient compared to cells grown in conventional medium. This might be due to adaptation processes to the new medium. If so, it was unclear how long the cells would take to fully adapt to the newly composited medium. For time reasons, further investigations on the effect of the new medium on virus entry and virus propagation were hence stopped in the scope of the present study.

A similar situation was found for commercially available serum-free medium. Although initially considered an alternative to FBS-containing medium, it was ruled out due to decreased growth rates of Vero/hSLAM cells right from the beginning.

Macromolecular crowding with Polyvinylpyrrolidone as another method pre-tested on Vero/hSLAM cells is meant to hinder diffusion, thereby increasing the effective solute concentration, in this case the concentration

of virus close to the cells. Crowding probably positively affects intracellular transport, receptor-ligand interactions and metabolism (Rashid et al., 2014). Macromolecular crowders might prevent extracellular virus particles from spreading far within the virus growth medium, thereby rather concentrating them on the surface of the cells and increasing the chance of infection. However, this method also hinders distribution of nutrients, which might explain the decreased growth rates of Vero/hSLAM cells observed during pretest experiments. At some point the cells might adapt to the new conditions but for time reasons macromolecular crowding was excluded from further experiments.

Discussion

Two days after virus inoculation, centrifugation and rolling had led to a higher viral yield as compared to the conventionally treated virus culture. This was particularly demonstrated by the approximate three- to four-fold increase in the amount of foci of infection on the centrifugation plates and a two- to three-fold increase on the rolling plates, as compared to the conventionally treated virus culture. These findings were further supported by the Ct-values of those plates incubated for two days after inoculation.

Centrifugation has effectively increased the initial viral yield, demonstrated both through immunostaining and PCR. A study from 1989 (Sundin and Mecham, 1989) has applied a similar duration and speed for centrifugation, but has used Bluetongue virus and baby hamster kidney cells (BHK-21). After an incubation period of 24 hours the authors report a 10- to 20-fold increase in the production of plaque forming units (PFU) as compared to non-centrifuged cultures. Although not directly comparable due to different cells and viruses, the experimental setup described in this study was helpful when choosing duration and speed for the present experiments. Further investigations using Vero/hSLAM cells might similarly benefit from plaque forming units as a measurement for viral infection, even more so as this method is less expensive and easier to conduct compared to immunostaining assays.

Based on studies of centrifugal enhancement of cytomegalovirus infectivity (Hudson, 1988; Hudson et al., 1976), Sundin and Mecham state that more virus might be attached and absorbed through centrifugation. With reference to Hudson et al., 1976, they further hypothesize that changes on the molecular level of viral interaction with the cellular membrane or cytoskeleton might be responsible for enhanced penetration and replication (Sundin and Mecham, 1989). The present study was not designed to support or weaken one of these hypotheses. Both concepts may be possible, perhaps also in combination. However, in terms of human SLAM receptor expression, the FACS-results show that the receptor itself is most likely not responsible for the occasional failure of Measles Virus isolation. After several passages a distinct decrease in the expression level of CDw150 is observed, but the receptor-expression of Vero/hSLAM cells is still log-fold higher as compared to the in-house stock of CHO/hSLAM cells, suggesting that a sufficient amount of receptor is available even after multiple passages.

The cells' vitality is another crucial factor possibly influencing viral infectivity. Geneticin (G418) treatment, for instance, turned out having adverse effects on growth rates and nutrient utilization of certain cell lines (Yallop et al., 2003). It is therefore reasonable to follow the suggestions given by WHO, about maintaining Vero/hSLAM cell cultures for approximately 15 passages without addition of geneticin and preferably infecting them with Measles Virus before reaching passage 15 (World Health Organization: Manual for the laboratory diagnosis of measles and rubella virus infection, Second edition, 2007). In doing so, the risk for over-treatment with Geneticin and associated adverse effects on the cells can be kept low.

Cultures that were mechanically agitated through rolling showed an approximate two- to three-fold increase in the amount of infectious foci as compared to the conventionally treated cultures. Same as centrifugation, rolling seems to effectively increase the initial viral yield.
Both in the case of temporary rolling (one hour after inoculation) and permanent rolling (entire incubation period), the orbital shaker was run at 60 rpm. Other than that, a study from 1988 (Mavromoustakis et al., 1988) has used 2 rpm, 96 rpm and 383 rpm when infecting Vero cells with Herpes Simplex virus. For 96 rpm they have registered the highest increase in the number of infectious foci, whilst the positive effect was diminished at 383 rpm. Follow-up experiments might further investigate the speed that leads to highest viral infection rates.

Contrary to centrifugation and rolling, temperature variation was not found to be particularly beneficial for Measles Virus infectivity using Vero/hSLAM cells. The present experiments, however, only investigate the effect of initial decrease and subsequent increase in temperature. Reversely, a United States patent from 2013 (U.S. Pat. No. 0286307) describes the positive effect of a two-temperature profile that increases and then decreases in temperature. The inventors argue that starting with higher temperatures might accelerate the formation of infectious virus particles. According to them, proceeding at decreased temperatures then allows for formation of stable antigen and maintenance of high viral titers (Reiter et al., 2013). Contrary to this patent, the idea of starting with lower than usual temperatures for the present experiments is based on studies suggesting that decreased temperatures might lead to alteration of temperature-dependent host cell proteases essential for processing virus polypeptides (Udem, 1984). Other findings report higher yields of MV Hallé and Edmonston strains when incubating cultures at 33°C instead of 37°C (Scott and Choppin, 1982). This effect might be due to increased viral entry but also due to improved growth conditions. Also the present study cannot differentiate between one cause or the other. Continuous viral RNA measurement throughout the process of incubation might help when further investigating the exact molecular processes. However, in the scope of the present experiments viral RNA assessment was meant to quantitatively support the rather subjective immunostaining results and for this purpose two PCR runs (day two and day five after virus inoculation) were regarded sufficient. Nevertheless, even this limited number of PCR runs could reveal that, after the five-days incubation period, the differences in viral yield between the methods are less pronounced. The Ct-values have

assimilated, suggesting a maximum production capacity of Vero/hSLAM cells that cannot be scaled up despite assumingly enhanced viral infection rates and propagation.
Yet, this does not explain why Measles Virus isolation is oftentimes less successful than Rubella Virus isolation. Lower viral yields in comparison to all other methods suggests that temperature variation creates the most challenging environment for virus to propagate. However, despite these challenging conditions virus isolation as such was successful. To really find out whether the application of centrifugation and rolling (the most promising methods in the present experiments) would ultimately decide whether isolation works or not, the viral concentration that builds a turning point between isolation success and isolation failure needs to be identified more accurately. This viral concentration would lead to isolation failure in conventionally treated cultures. In cultures undergoing centrifugation and rolling, however, isolation would be successful.

The present experiments can be seen as preliminary findings suggesting promising methods for initial enhancement of viral infection and propagation. It is not yet clear, however, whether the initial scale up in viral yield is due to more virus particles entering the cells or, on the other hand, improved conditions for virus propagation. Further experiments will need to differentiate between one cause or the other. As a second step, the most promising methods (rolling and centrifugation) should be tested on clinical samples. A systematic comparison between conventional treatment on one hand and rolling or centrifugation on the other hand will further clarify the effect of new methods on Measles Virus isolation success in cell culture.

6.2 Measles and Rubella seroprevalence

Dataset

The retrospective study design of collecting already existing data was responsible for the huge dataset, mainly consisting of test results from Luxembourg residents, and thereby providing robust information about the population-based anti-measles/anti-rubella IgG seroprevalence in Luxembourg.
The large dataset is also thanks to the pre-marital screening that was mandatory until 2015 for all couples in Luxembourg prior to marriage. Whilst men were only tested for syphilis and tuberculosis, women were additionally tested for toxoplasmosis and rubella (Mossong et al., 2006), thereby accounting for the large anti-rubella IgG dataset. Due to the dominance of female test results, the study gives reliable information about the IgG seroprevalence of women at childbearing age at the time point of testing, but is less informative concerning the IgG seroprevalence of the rest of the Luxembourg population. However, most findings apply for both men and women, based on the assumption that there is no difference between males and females in terms of vaccination uptake and the risk of natural infection. Both for measles and rubella the results show a higher percentage of IgG negative/equivocal test results after the introduction of the MMR vaccine in Luxembourg in 1986. This may seem surprising, but it hints at a phenomenon that Mossong has already described in a prospective study from 2001. After vaccine introduction, populations experience a shift from naturally

acquired immunity (by going through the infection) to vaccine-derived immunity (Mossong et al., 2004). Whilst vaccination can be refused or forgotten, nobody can fight off the actual infection when confronted with it. Older age groups were highly prone to get infected at some point in their lives (usually before reaching adulthood), hence more likely being IgG positive, which is also reflected in the results of the present study. Prior to vaccine introduction, immunity against measles and rubella used to be relatively stable in Luxembourg, reflected by high proportions of IgG positives. Due to infection with wild type virus in the pre-vaccine era, people were more likely to develop a strong immune response with consecutive higher anti-measles/-rubella IgG titers (Okada et al., 2001).

Together, the noticeable drop in IgG positives and the increasing number of IgG equivocals in age groups born after the introduction of the MMR vaccine in 1986, hint at antibody waning. This observation is consistent with previous studies demonstrating that immunity built as a response to the anti-measles/anti-rubella vaccine components is more likely prone to antibody waning (Kremer et al., 2006).

Interestingly, when aligning positives and equivocals in the rubella dataset and interpreting both as the percentage of vaccine-recipients (best case scenario), a vaccination coverage of 95% would be met in most age groups. Although not directly visible from the results, the same can be assumed for the measles dataset, due to the MMR vaccine containing both measles and rubella components. However, the proportions of true positives only range between 80% and 90% in the rubella dataset and go even lower in the measles dataset. Assuming an equal chance for individuals to develop IgG antibodies against both measles and rubella (due to the combined MMR vaccine), the impact of antibody waning is hence more drastic for the measles than for the rubella vaccine component, which was also shown in other studies (Kontio et al., 2012). This observation is further supported by those individuals in the dataset tested both for anti-measles and anti-rubella IgG. Although rare in the dataset, these double-tested individuals are of great interest in the course of this study. It turned out that more double-tested people were positive for rubella but negative for measles than the other way around. Most individuals being negative for one and positive for the other were born prior to MMR vaccine introduction in 1986. However, those born after 1986 might have received the vaccine without response to the measles component or alternatively with anti-measles IgG antibody waning. An interesting observation, visible in the rubella dataset, is that the proportions of IgG equivocals were particularly high in the period from 1986 to 1994 when only one dose of MMR vaccine was administered, whilst the proportion of true IgG positives increases directly after 1994 when a second dose was introduced at the age of 5 to 7 years (European Centre for Disease Prevention and Control: Vaccine Scheduler, 2019). This observation is consistent with previous studies demonstrating lower IgG titers and faster waning after one dose of MMR vaccine compared to the application of two doses (Christenson and Bottiger, 1994). In the measles dataset, however, the rates of IgG positives continue to decline even after introduction of the second dose. This further supports the observation that vaccine-induced immunity against measles is probably more intensely prone to antibody waning than vaccine-induced immunity against rubella (Kontio et al., 2012). Also, there are hints that at least after the first dose of MMR vaccine, the effectiveness of the rubella component might be higher

than for the measles component (Centers for Disease Control and Prevention: Measles, Mumps, and Rubella (MMR) Vaccination: What Everyone Should Know, 2019), probably due to lower immunogenicity of the measles vaccine component (Vynnycky et al., 2019).

Besides evidence for anti-measles/-rubella antibody concentration waning (Kremer et al., 2006), antibody avidity waning after two-dose vaccination seems to be another mechanism of decreasing vaccine-induced protection and was found in anti-measles IgG but not in anti-rubella IgG (Kontio et al., 2012). Measles and rubella infections attributable to concentration and avidity waning of vaccine-induced antibodies are referred to as secondary vaccine failure as the person concerned presents with non-protective immunization levels despite previous vaccination (Paunio et al., 2000).

With regard to the above mentioned differences in immunity against measles and rubella, conclusions from the anti-rubella IgG dataset about the anti-measles IgG dataset should be drawn carefully in the scope of this study. It should further be considered that the younger the age group, the fewer test results were provided by the laboratories, probably because these groups were less likely to be addressed by pre-marital screening. It is possible that age groups born after 1986 (hence children or young adults between 2006 and 2015) were preferably tested for anti-measles/-rubella IgG when they were presented with measles-/rubella-like symptoms and unclear or incomplete immunization status. This might be an important source of bias, which is particularly pronounced in the measles dataset as it is smaller than the rubella dataset.

Test systems

The participating laboratories used different test systems and also changed these systems internally between 2006 and 2015. Whether this led to significant discrepancies in the number of positive, negative and equivocal test results, which might affect the comparability of results, will be further analyzed in the following paper in preparation:

Sonja M. Bork, Pierre Weicherding, Patrick Hoffmann, Stéphane Gidenne, Marion Freichel, Bernard Weber, Chantal Tsobo Blistain, Annick Martin, Jaqueline Parmentier, Oliver E. Hunewald, Maude Pauly, Emilie Charpentier, Claude P. Muller, Judith M. Hübschen; Measles and Rubella IgG Antibody Prevalence in the Luxembourg Population – A Retrospective Analysis. In preparation.

The interpretation of IgG equivocal test results as negatives might underestimate the real IgG antibody prevalence in the Luxembourg population. By further investigating differences in the interpretation of test results obtained by different test systems, the paper in preparation will also examine what difference it makes whether IgG equivocal test results are considered as IgG positives or negatives.

Vaccination policy

In February 2009, the recommendation for administering the second dose of measles- and rubella-containing vaccine in Luxembourg was changed from the age of 5 to 7 years to the age of 15 to 23 months. Despite this new recommendation, a survey carried out by the Ministry of Health Luxembourg in 2012 showed that only

86% of 2-year-old children in Luxembourg had received both doses of measles- and rubella-containing vaccine at the time and that the others had missed out on the second one (Krippler, 2012). It is unclear why the uptake of the second dose is lower, but a conceivable explanation could be that it is oftentimes just simply forgotten. This would at least partially explain why IgG negativity rates remain relatively high even after the introduction of the second vaccine shot. To further investigate this case, it would be interesting and highly relevant for future studies to quantify the uptake of vaccination in the Luxembourg population. Differences in vaccination uptake could for example be related to ethnical background, place of residence, social status and income (Crocker-Buque et al., 2017).

Origin and place of residence

Differences, at least in the rates of IgG negatives + equivocals, can be seen between the four Luxembourg regions. For measles as well as for rubella, the northern region is among the places of residence (including neighboring and other countries) with the highest percentage of IgG negatives + equivocals, which is consistent with the findings of a prospective study from 2001 (Mossong et al., 2004). For rubella, only the South exhibits higher rates than the North, whilst for measles the southern region has the lowest rate of IgG negatives/equivocals amongst the four Luxembourg regions. Interestingly, the proportion of IgG negatives/equivocals in the South correlates with the proportion of test results from male residents. For rubella, the rate of male test results was observed to be higher in the southern region compared to the other Luxembourg regions. This coincides with the South having a noticeably higher rate of anti-rubella IgG negative + equivocal test results in total. For measles, however, the rate of male test results is smaller, which in turn might be responsible for the South exhibiting the smallest rate of anti-measles IgG negatives + equivocals as compared to the other Luxembourg regions. An influence of male test results on the overall rate of IgG negatives + equivocals is further supported by the fact that the measles dataset shows higher rates of IgG negatives + equivocals than the rubella dataset and is made up to approximately one third of male test results, whilst men are a minority in the rubella dataset. However, with regard to measles, men and women probably contribute similarly to the higher rate of IgG negatives/equivocals as about the same percentage of IgG negatives + equivocals is found in the female part as well as in the male part of the measles dataset. In the rubella dataset, by contrast, the percentage of IgG negatives + equivocals is noticeably higher in the male than in the female dataset.

For rubella, only a minority of test results was labelled originating from individuals with foreign address (other than French, German and Belgian residents) and only about 10% of them were male. These males, however, contribute to at least one fourth of the IgG negatives + equivocals in all anti-rubella IgG test results from foreign nationals (other than French, German and nationals from Belgium). Anti-rubella IgG negative + equivocal tested individuals from Belgium were also about 10% men, which is noticeably more than in the datasets from Germany and France. The only individual from a foreign country (other than France, Germany and Belgium) tested IgG negative for measles was male.

The above made observations point out that the focus of attention should not only be drawn to Luxembourg residents, but also to the influx of people coming to Luxembourg either temporarily or permanently. Also, despite the predominance of female test results in the dataset, the importance of watching the seroprevalence status of male individuals should not be forgotten. Even more so as men and women are equally coming to Luxembourg, be it as seasonal workers or commuters, for holidays or business trips. As foreign nationals they were not concerned by the premarital screening, hence leaving foreign men and women with equal chances to be tested for anti-measles/anti-rubella IgG. Although not representing the Luxembourg population as such, knowledge about their seroprevalence status is yet highly relevant, in particular in terms of long-term or permanent stays.

With 47.5% of Luxembourg residents in 2019 being of foreign origin, the Grand Duchy is a country of immigration. Since 2011 the influx of immigrants coming to Luxembourg has increased to about 10 000 arrivals per year (Statistics Portal Grand Duchy of Luxembourg, n.d.). Due to differences in immunization strategies and access to health care in the countries of origin, there is a potential risk of individuals entering the country without or with insufficient anti-measles/anti-rubella IgG-seroprevalence. Normally, this is targeted by medical examinations for immigrants: Upon their arrival, non-EU nationals are holding a temporary authorization to stay and may apply for a residence permit within three months. For their residence permits to be further processed, they need to undergo a medical examination (Just Arrived: Informations pour s´installer, vivre et travailler au Luxembourg, n.d.) that contains a vaccination check to make sure that vaccinations are up to date (Guichet Public: Health & Safety / Social Security, n.d.). Physicians are left free to decide the scope of the vaccination check and a blood test is not explicitly recommended in the online description (Guichet Public: Health & Safety / Social Security, n.d.).

It is also concerning that newcomers to Luxembourg coming from EU member states do not undergo an initial medical examination. For their residence permit they can simply request an "attestation of registration". Hence, in case of being IgG negative or having non-protective antibody levels, these individuals probably add to the emergence of hidden immunization gaps. The next chance for detection of natural or vaccine-induced immunity would then be a medical examination when starting a new job. As many commuters and newcomers to Luxembourg are looking for work and employment, many of them might undergo this medical examination, organized by the employer. However, according to the online descriptions (Guichet Public: Health & Safety / Social Security, n.d.), a blood test is not necessarily included and the new employee only has to present the vaccination record.

Prospect

With regard to the above mentioned points it is doubtful whether screening for anti-measles/anti-rubella IgG antibodies is a good means to identify and reach out to potentially unimmunized individuals. In their study on IgG antibody prevalence in newcomers to Luxembourg, Hübschen et al. argue that offering vaccination to people without reliable proof of previous immunization is cheaper and more efficient than conducting a blood

test first (Hübschen et al., 2018). Vaccination is not mandatory in Luxembourg and, in the event of a negative test result for anti-measles/anti-rubella IgG, it is not guaranteed that the person concerned would come back to receive vaccination. Further, an initial blood test followed by another appointment for vaccination is associated with more time investment for the physician.

In a comparison of cost effectiveness of MMR vaccination with and without prior serology in new health care workers in a hospital in Great Britain, Giri et al. have found vaccination without prior serology to be significantly cost-saving (Giri et al., 2013). They suggest offering vaccination to health care workers with no evidence of prior immunization. The setting is comparable to pre-employment examinations in Luxembourg, where presentation of the vaccination record but not necessarily serology is advised. In case of missing vaccination record or incomplete vaccination status, precautious vaccination could be offered to new employees, thereby saving costs for serology and time for an additional appointment. The good safety profile of the MMR vaccine, even in case of additional doses, supports this approach (Chen et al., 1991). This strategy could also apply to immigrants attending the medical check-up that is mandatory for residence permission.

With vaccination being optional in Luxembourg, it will most likely not become an obligation to vaccinate without prior serology in case of non-sufficient proof of immunization, neither for physicians nor for individuals concerned. However, communicating the benefits could at least raise awareness for this strategy.

From the psychological perspective, a less hesitant and instead more proactive attitude in dealing with the vaccine is more likely to comfort people when considering vaccination. Through propagation of a systematic and reasonable vaccination policy that emphasizes the importance of individual immunization for the sake of public safety, people are probably more strongly encouraged to contribute to herd-immunity. Their willingness to do so is not necessarily depending on preceding blood tests.

Summary of main findings and conclusions

1. In the best case scenario (counting equivocals as positives) and at least in terms of rubella, an immunization coverage of 93% to 95% is met in most age groups born before 2000. Age groups born after 2000 seem not to reach the 93% to 95% immunization coverage.
2. In the worst case scenario (counting equivocals as negatives), high vaccination rates in the Luxembourg population could mask a considerable proportion of IgG equivocal individuals potentially being susceptible to Measles and Rubella Virus despite previous vaccination (attributable to antibody waning).
3. Luxembourg faces enormous migration and commuting activities bearing the risk of reintroduction of measles and rubella, especially on the assumption of the worst case scenario (see 2.). Unimmunized individuals either migrating or traveling to Luxembourg on a regular basis (e.g. commuters) could further add to the emergence of immunization gaps in the Luxembourg population, thereby endangering herd-immunity.

4. Foreign individuals with unknown or incomplete vaccination status should be offered measles- and rubella-containing vaccine in the course of medical examinations for migrants and new employees. Skipping serology and directly offering vaccination is time- and cost-saving and could ultimately ensure sufficient immunization coverage in those coming to either work or stay in Luxembourg.
5. In the Luxembourg population the percentage of IgG negative/equivocal test results is higher in age groups born after the introduction of the MMR vaccine in 1986, especially in those born between 1986 and 1994, when only one dose of MMR vaccine was administered. Special attention should be paid to this cohort and in case of yet incomplete vaccination status, additional MMR vaccination should be offered.

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C Appendix

C.1 Acknowledgements

Writing about a topic in health sciences inevitably brings one to reflect about health itself. Besides genome characteristics, our behavior certainly plays a big role when it comes to maintenance but also recovery of health. I believe that health promoting behavior can be both apparent and invisible. For example, eating green vegetables is generally perceived as being healthy. This is something we take in from the outside and is apparent to others. Rather invisible, however, is what comes from inside ourselves. Health promoting feelings, memories, perceptions, beliefs, attitudes. Some might call it self-evident and I agree that this concept is not a recent discovery. Thousands of spiritual teachings and numerous scientific works over decades have pointed out its great importance. Yet, compared to all great achievements in the medical field, our immanent strategies for health sometimes fall behind. One of these invisible health promoting factors is surely gratitude – which I believe is salutary for both sides, sender and recipient. I wish I was at least sometimes able to express it when I was standing next to the people I was lucky enough to meet and spend time with in the past few years:
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« Il faut être toujours ivre, tout est là. C´est l´unique question. […] Mais de quoi ? De vin, de poesie, d´amour, ou de vertu, [ou des amis]. » *(Charles Baudelaire)* Merci pour ce temps partagé.

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