UNIVERSITY OF TURKU

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Development of serological dual-mode multiplex microarray immunoassay and analysis of influenza and respiratory syncytial virus disease burden in early childhood

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To my family ♥

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ABSTRACT

Respiratory syncytial virus (RSV) and influenza A (IAV) and B (IBV) viruses infect humans throughout their life with exceptionally high rates of re-infection. Serological assays are commonly used to diagnose and characterize host immune responses against microbial pathogens acquired by natural infection or vaccination. The current methods are mostly based on enzyme immunoassay (EIA) or other conventional methods in a single analyte format. Presently, there is a need to shift from single analytical methods to microarray format which enables the detection of antibodies against multiple targets in a rapid and cost-effective manner.

In this study, a highly sensitive single analyte EIA method was used for serological analyses in children's sera. In addition, multiplex microarray immunoassay (MAIA) methods were developed for rapid and simultaneous detection of IgG antibodies against seven viral antigens (H1N1pdm09 vaccine ag, IAV H1N1, IAV H3N2, IBV Victoria, IBV Yamagata, RSV and adenovirus hexon protein). We found out that MAIA is well suitable for large-scale serosurveillance and vaccine immunity studies. We followed-up virus-specific immunity by MAIA in response to natural infection and vaccination in a large cohort of 0-2 year old children. Our serological findings showed a high rate of respiratory virus infections and reinfections in young children.

The applicability of MAIA in vaccine immunity studies was also analysed. We developed a specific, sensitive, sample and antigen saving assay for simultaneous detection of IgM and IgG antibodies against H1N1pdm09 vaccine ag and IBV Yamagata. MAIA showed excellent correlation with EIA and a good correlation with hemagglutination inhibition assay in measurement of vaccine-induced antibodies in sera of Pandemrix-vaccinated adults.

KEYWORDS: multiplexing, microarray, immunoassay, serology, RSV, influenza, pandemic, vaccine, antibodies, epidemiology

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TIIVISTELMÄ

Respiratory syncytial virus (RSV) ja influenssa A- (IAV) ja B(IBV)-virukset ovat merkittäviä ylähengitystieinfektioiden aiheuttajia koko ihmisen elinkaaren ajan. RSV:n ja influenssan aiheuttamat infektiot voivat joskus olla vakavia tai jopa henkeä uhkaavia erityisesti pienillä lapsilla ja ikäihmisillä. Nykyiset serodiagnostiset menetelmät perustuvat entsyymi-immunologisiin (EIA) ja muihin määritysmenetelmiin, joissa immuunivastetta voidaan tutkia kerrallaan vain yhtä taudinaiheuttajaa kohtaa. Tällä hetkellä on seerumin vasta-ainetutkimusten osalta suuri tarve siirtyä monianalyyttisiin menetelmiin, jotka olisivat nopeampia ja kustannustehokkaampia kuin nykyiset tutkimusmenetelmät.

Tässä tutkimuksessa on käytetty herkkiä EIA-menetelmiä pienten lasten ja influenssarokotettujen henkilöiden seerumien virusspesifisen immuunivasteen tutkimiseen. Työssä on myös kehitetty monianalyyttinen mikrosiruperusteinen immunomääritysmenetelmä (MAIA) samanaikaiseen seerumin IgG-luokan vasta-aineiden mittaamiseen eri influenssavirusantigeeneja, RSV:tä ja adenoviruksen heksoniproteiinia kohtaan. Tutkimuksemme osoittivat, että hengitysteiden virusinfektiot ja yllämainittujen virusten aiheuttamat uusintainfektiot ovat erittäin tavallisia pienillä lapsilla. MAIA-menetelmää sovellettiin myös aikuisten influenssarokotevasteiden analyysiin. Menetelmää kehitettiin edelleen siten, että näytteestä voitiin samalla kertaa mitata seerumin IgG- ja IgM-luokan vasta-aineita eri IAV- ja IBV-virusantigeeneja kohtaan. Menetelmä osoittautui erittäin spesifiseksi, herkäksi ja näytettä ja antigeeneja säästäväksi ja tulokset olivat hyvin yhtenevät muiden perinteisten vasta-ainetutkimusmenetelmien kanssa. Kehittämämme MAIA-menetelmä toimi erittäin hyvin ja sen voitiin osoittaa sopivan erinomaisesti laajojen väestön seerumiaineistojen ja virusrokotevasteiden analyysiin.

HAKUSANAT: monianalytiikka, mikrosiru, immunomääritysmenetelmät, serologia, RSV, influenssa, pandemia, virusrokotteet, vasta-aineet, epidemiologia

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Abbreviations

Ab	antibody
Ag	antigen
Anti-hIgG	anti-human immunoglobulin G
Anti-hIgM	anti-human immunoglobulin M
ARI	acute respiratory infection
BSA	bovine serum albumin
CCD	charge-coupled device
CF	complement fixation
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
hAdV	human adenovirus
hIgG	human immunoglobulin G
hIgM	human immunoglobulin M
HA	hemagglutinin
HI	hemagglutination inhibition
HSA	human serum albumin
IAV	influenza A virus
IBV	influenza B virus
IFA	immunofluorescence assay
Ig	immunoglobulin
MAIA	microarray immunoassay
NA	neuraminidase
PCR	polymerase chain reaction
RADT	rapid antigen detection tests
RBC	red blood cell
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SRH	single radial haemolysis
TMB	3,3’,5,5’-Tetramethylbenzidine

UCNP	upconverting nanoparticle
UCP	upconverting phosphor
UV	ultraviolet
VN	virus neutralization

List of original publications

The thesis is based on the following original publications, referred to in the text by Roman numerals (**I-III**), and on some unpublished data presented.

I Kutsaya, A.*, Teros-Jaakkola, T., Kakkola, L., Toivonen, L., Peltola, V., Waris, M., & Julkunen, I. (2016). Prospective clinical and serological follow-up in early childhood reveals a high rate of subclinical RSV infection and a relatively high reinfection rate within the first 3 years of life. Epid. Infect. 144(8):1622-33. doi: 10.1017/S0950268815003143. *Anna Kazakova (née Kutsaya).

II Kazakova, A., Kakkola, L., Päkkilä, H., Teros-Jaakkola, T., Soukka, T., Peltola, V., Waris**,** M., & Julkunen**,** I. (2019). Serological array-in-well multiplex assay reveals a high rate of respiratory virus infections and reinfections in young children, *mSphere* 2019, 4(5). doi: 10.1128/mSphere.00447-19.

III Kazakova, A., Kakkola, L., Ziegler, T., Syrjänen, R., Päkkilä, H., Waris, M., Soukka, T. & Julkunen, I. Pandemic influenza A(H1N1pdm09) vaccine induced high levels of influenza-specific IgG and IgM antibodies as analyzed by enzyme immunoassay and dual-mode multiplex microarray immunoassay methods, 2019 Sep. *Manuscript, submitted*

1 Introduction

Large-scale screening and estimation of immunity and exposure to viral pathogens are essential for understanding the risk factors of infection and population-scale patterns of transmission (Arnold *et al.*, 2018). Additionally, the impact of prior immunity in a population is important for estimation of the need for subsequent vaccinations. Improved evaluation of vaccines has impact on public health. The determination of antibody concentration in sera is considered the gold standard method to estimate humoral immunity to natural infection or vaccination (Plotkin, 2010). Enzyme immunoassays (EIA) are broadly used to measure antibody responses against different viruses. Nowadays, in most clinical laboratories, multiple single analyte EIAs are needed to determine antibody responses to different viruses.

Influenza A viruses are highly variable and virus mutations pose a risk to overcome species barriers causing human infections with animal influenza (Cauldwell *et al.*, 2014). Such infections with antigenically novel viruses have led to worldwide pandemics. It is very difficult to predict the time of future pandemics. The ongoing influenza epidemics highlight the need for constant monitoring of circulating virus strains and virus-host interactions. Two of the most common methods for measuring serum antibody to influenza and evaluation the effects of influenza vaccines are the hemagglutination inhibition (HI) and virus neutralization (VN) assays (Truelove *et al.*, 2016). With these methods large-scale serosurveillance or vaccine studies for influenza immunity are very laborious and time consuming. In order to facilitate the analysis of population immunity and the rapid assessment and comparison of vaccination efficacy, new multiplex high-throughput assays are highly desirable to supplement standard methods. New diagnostic and analytical methods will improve infectious disease surveillance and lead to a better understanding of vaccine and natural infection induced immunity. In the last decade, various microarray assays for infectious disease research have been developed and they show great potential to achieve these goals (Yuk *et al.*, 2004; Tang *et al.*, 2005; Negm *et al.*, 2015; Schepp *et al.*, 2019).

Multiplexing technology emerged about 20 years ago and has developed extensively since then. Currently, multiplex assays are widely used in clinical

diagnostics (Navidad *et al.*, 2013; Hanson and Couturier, 2016; Kim *et al.*, 2016), biomedicine research (Schaffer *et al.*, 2015), food safety assessment (Yoon and Kim, 2012), and environmental monitoring (Yu *et al.*, 2011; Hanson and Couturier, 2016). Multiplex assays have been actively developed also for applications in the fields of immunology, microbiology, and virology (Cannon *et al.*, 2010; Zhang *et al.*, 2013; Villar-Vázquez *et al.*, 2016). Multiplex protein-based immunoassays are used to analyse antibody responses to infectious diseases (Talha *et al.*, 2016) and nucleic acid-based multiplex assays are used for virus detection and genotyping (Martínez *et al.*, 2015; Quiñones *et al.*, 2017). Multiplex assays substantially improve the detection and control of viral respiratory and other types of infections and also facilitate the rapid characterization of new viruses.

In contrast to EIAs, a considerable amount of information can be generated from a single sample aliquot in a single analysis by multiplex immunoassay. Multiplex immunoassays can simultaneously detect different antibody types against multiple analytes in a single sample (Gomez *et al.*, 2010; Liu *et al.*, 2013). They facilitate high sample throughput, reduce labour and require small sample volumes and antigen amounts. Rapid and reliable screening of a large panel of respiratory viruses is of great importance for monitoring epidemic waves and the immune status in the population. Simultaneous detection of several different virus-specific antibodies on one platform should result in faster data accumulation. The method has important application in vaccine efficacy studies and studies of infectious diseases in young children. Efficient large-scale screening of respiratory virus antibodies in young children is important for estimating the need for vaccination and the potential use of antiviral drugs. In vaccine development, serological surveys facilitate adequate decisions on immunization schedules and targeted age groups.

2 Review of the literature

2.1 Traditional serodiagnostic methods

Serology enables the assessment of immunological status by measuring antibody levels against microbial pathogens in serum samples. Serological assays are essential for vaccine evaluation and the diagnosis of an infection. Serological surveys are also frequently used to determine the incidence or prevalence of an infectious disease in a human population or in animals. Such epidemiological data indicates the susceptibility and immunity level in the target population. The presence of antibodies in a serum sample can indicate past exposure to a virus or vaccination as well as cross-reaction to related viruses. The measurement of antigen-specific antibodies is the most widely used parameter to evaluate the immunogenicity of vaccines. Antibodies play an essential role in the protection of a host from infectious diseases. Neutralizing antibodies block the infection and they thus correlate with protection. For most vaccines induction of vaccine antigen-specific antibodies is commonly considered as a good parameter correlating with host protection and vaccine efficacy (Plotkin, 2010). The determination of antibody isotype allows distinguishing recent exposure to the pathogen from past immunity. IgG isotype antibodies correlate with immunological memory and they are the most commonly examined immune markers in vaccine research. IgA and IgM isotypes, however, also play an important role in immunity induced by vaccines. The presence of antivirus IgM antibodies indicates current or recent infection as they are produced early after infection or vaccination (James, 1990).

Serological methods are based on the analysis of a specific interaction between antigen and antibody. There is a wide range of serological techniques used in the diagnostics of infectious diseases: enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition (HI) test, single radial haemolysis (SRH) assay, immunofluorescence assay (IFA), multiplex immunoassays, complement fixation (CF) test, immunoblotting, virus neutralization (VN) assay, radioimmunoassays and latex agglutination.

Immunostaining is one of the techniques where enzyme-antibody conjugates are used to stain specific molecules on cells to visualise them in a tissue or in cells

to examine intracellular structures (Maity, Sheff and Fisher, 2013). In immunoblotting or immunoblot assays, enzyme-antibody conjugates are used to identify specific antigens, e.g. viral proteins that have been transferred to an absorbent membrane or to detect antibodies from patient samples specific to the immobilised antigens on the membrane (Mahmood and Yang, 2012). A patient serum sample is incubated with the membrane, followed by incubation with anti-human antibody conjugated with an enzyme. Antibody-antigen binding is visualized by the addition of a substrate and a colour reaction. The assay is commonly used as a confirmatory assay, especially for diagnosing HIV infection (Wald and Ashley-Morrow, 2002). Immunofluorescence assay (IFA) is used for the detection of IgM and IgG antibodies against virus-infected cells on glass microscope slides. Bound antibodies from patient samples are detected by fluorescein-conjugated anti-human antibodies using a fluorescence microscope (Malan *et al.*, 2003).

The complement fixation test (CF) is applicable to a large number of viral infections (Rice, 1961; Swack, Gahan and Hausler, 1992). In the CF test, virus antigen, sheep red blood cells, anti-sheep red blood cells antibodies and fixed amount of complement are added to a diluted serum sample. If patient does not have antibodies against antigen of interest, the complement will cause haemolysis of sheep red blood cells and the solution turns pink colour. However, the CF test is time consuming, labour intensive and not sensitive (Bannai *et al.*, 2013; Shibata *et al.*, 2013). Comparison of CF test and hemagglutination inhibition assay showed that the test lack strain specificity for influenza antibody responses and is not sensitive enough to assess the antibody responses to influenza vaccination (Prince and Leber, 2003). Nowadays, the method is not very commonly used in clinical diagnostics due to its complexity compare to other serological methods and has been replaced by newer and more sensitive techniques. Serological methods frequently used in research to determine the immunological response to influenza vaccine and/or natural infection include haemagglutination inhibition (HI) test, virus neutralization (VN) assay, single radial haemolysis (SRH) assays and enzyme immunoassay-based testing (Landolt, Townsend and Lunn, 2014; Medicines Agency, 2016; Trombetta *et al.*, 2018).

2.1.1 Hemagglutination inhibition assay

Humoral immune responses to influenza vaccination are primarily analysed by the hemagglutination inhibition (HI) assay (Trombetta, Perini, Mather, Temperton, Montomoli, *et al.*, 2014). HI test is frequently used in diagnostic laboratories and essentially performed according to a WHO standard protocol (Hirst, 1942; World Health Organization. and WHO Global Influenza Surveillance Network., 2011). HI

assay is primarily used for influenza viruses but also the assay is an important tool to study other groups of viruses − for example, paramyxoviruses, togaviruses, flaviviruses, and bunyaviruses (L'vov *et al.*, no date; Rösler *et al.*, 2013). The principle of the hemagglutionation inhibition assay is shown in **Figure 1**. Virus surface proteins (e.g. hemagglutinin on the surface of influenza virus) have the ability to bind to sialic acid-containing molecules (receptors) on the surface of red blood cells (RBCs). Haemagglutination is a process known as a formation of lattice-like structure in a suspension in the presence of virus that can agglutinate RBCs (Pedersen, 2014). Antibodies specific to the virus surface proteins are able to inhibit the hemagglutination process. Following influenza vaccination, antibodies specific to hemagglutinin are produced. Haemagglutinin specific antibodies bind to the influenza virus and prevent the attachment of the virus to the RBCs. The process is called hemagglutination inhibition. Haemagglutination will not be observed if antibodies to the virus are present until the antibodies are fully diluted. The main benefit of the assay is its relative simplicity and low cost. The assay does not require special equipment as the results are visually read. Hemagglutionation inhibition is observed when RBCs sediment to the bottom of the well and form a small pellet. A fixed amount of influenza virus is added to the wells, usually 4 hemagglutinin (HA) units/25 µL or 8 HA units/50 µL. Serum samples are prepared in two-fold serial dilutions and added along a row of wells. The highest antibody dilution which is able to clearly inhibit hemagglutionation is considered as the HI antibody endpoint titre.

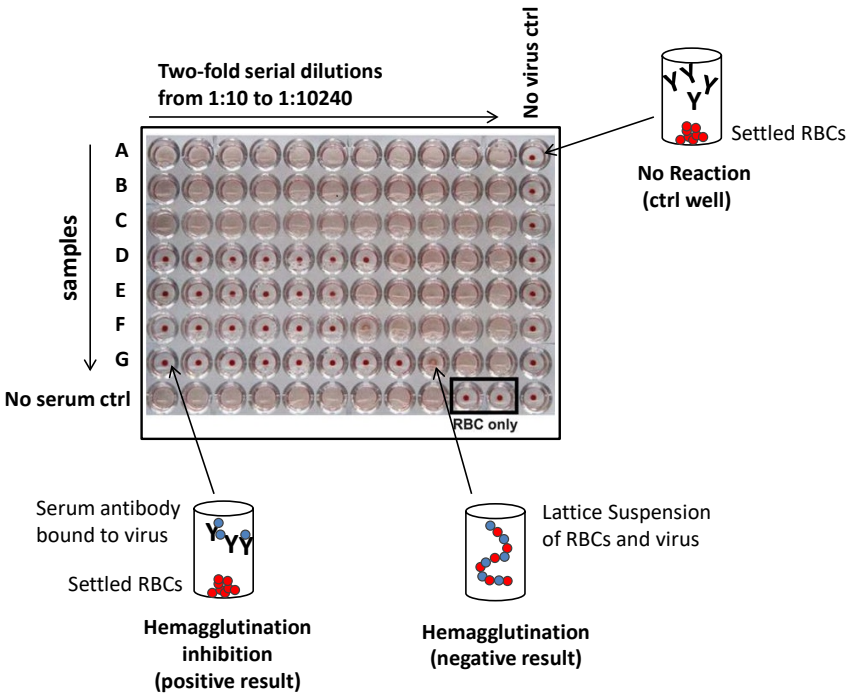

Figure 1. Schematic principle of a hemagglutionation inhibition assay. Serial dilutions of serum samples are added into wells in a 96-well plate. Anti-influenza antibodies present in serum samples prevent virus binding to RBCs causing haemagglutination inhibition. The result is read visually by eye observing a small pellet of sedimenting RBCs at the bottom of the well.

Despite its wide use and benefits, the HI test has many limitations. First of all the interpretation of the HI test can be quite subjective and there is significant variation from one laboratory to another (Wagner *et al.*, 2012; Wood *et al.*, 2012). Researchers assess the outcome of the test visually and it is sometimes hard to accurately determine the final antibody dilution that inhibits hemagglutionation as the results may be unclear due to partial inhibition of hemagglutination. Species selection and freshness of RBCs is critical for reliable results (Wibawa *et al.*, 2012; Ovsyannikova *et al.*, 2014). Since samples can only be tested in serial dilutions, a high degree of specificity cannot be achieved for the determination of antibody concentrations. Multiple replicates are needed to improve the accuracy of the results, which makes the assay quite labour intensive. The assay is not very suitable for large-scale studies as serial dilutions of only 7 serum samples can be tested in one 96-well microtiter plate. The failure of the assay to differentiate between

immunoglobulin isotypes is an additional disadvantage. Poor hemagglutination of recent and contemporary avian influenza viruses and low sensitivity for influenza B viruses are further limitations of the HI assay (Rowe *et al.*, 1999; de Jong *et al.*, 2003; Stephenson *et al.*, 2009; Trombetta *et al.*, 2018).

Antibody endpoint titers determined by the HI test are known to correlate well with protective immunity and allow the estimation of a risk for influenza infection and thus the efficacy of the vaccine (Noah *et al.*, 2009). An HI titre of 1:40 or higher is generally accepted as a protective level and it corresponds to a 50% reduction in the risk of contracting influenza infection in a susceptible population (FOX *et al.*, 1982; Ng *et al.*, 2013). High HI antibody titres are strongly associated with the protection against clinical influenza infection (Hobson *et al.*, 1972; Plotkin, 2001; de Jong *et al.*, 2003). Coudeville *et al.*, 2010 have revealed a significant and positive relationship between HI titre and clinical protection against influenza using a meta-analytical approach where HI data from multiple studies were analysed (Coudeville *et al.*, 2010). The results indicate that the immunogenicity data allows the prediction of the efficacy of inactivated influenza vaccines. However, HI antibody titre of 1:40 remains a theoretical immunological correlate of protection. It is well known that, influenza infection can sometimes be acquired even by a vaccinated individual with a theoretically protective antibody level (Plotkin, 2008). Nevertheless, according to the guidelines for pandemic influenza vaccines, HI titre of 1:40 or higher is currently the best available parameter to estimate vaccine induced protective immunity (Noah *et al.*, 2009).

2.1.2 Virus neutralization (VN)

Virus neutralization (VN) test is a reliable method to detect antiviral immunity as it only detects antibodies that can neutralise the virus and block virus replication (Payne, 2017). The method is not used for routine diagnosis but has been applied to determine neutralizing serum antibody titers to some viral vaccines e.g. measles (Kontio *et al.*, 2016) and poliovirus types 1, 2 and 3 (Resik *et al.*, 2017), to quantify serum antibodies to influenza A and B and parainfluenza 1 and 2 viruses (Frank *et al.*, 1980), to differentiate among highly related arboviruses (Kuno, 2003) and enteroviruses serotyping (Zhu *et al.*, 2018). VN assay is often used for measuring anti-influenza antibodies as an alternative method to HI assay (Truelove *et al.*, 2016). VN assay is also called a microneutralization assay when performed in 96-well microtiter plate (Okuno *et al.*, 1990). VN assay is more resource intensive, time consuming and laborious to perform compared to the HI assay. While HI assay measures the ability of antibodies to bind to the virus and inhibit

RBCs agglutination, VN assay measures antibodies that can bind to the virus and block its ability to infect target cells.

VN is performed in a similar manner to HI, i.e. incubating serial dilutions of serum samples with fixed amount of virus to determine which serum dilution will inhibit virus replication. In VN assay a live virus is used and the killing of the cells by the virus is determined as a plaque formation. Antibodies that block virus infectivity and as a result prevent the killing of the cells, are called neutralizing antibodies. Neutralizing antibodies are able to inhibit viral attachment, entry, and the release of progeny virions (Trombetta, Perini, Mather, Temperton, Montomoli, *et al.*, 2014). VN titre is the highest serum dilution that induces a 50% inhibition of virus growth.

Although the VN assay provides a better correlation to protective immunity, the assay is more time-consuming and expensive and it is considered to be difficult to standardize between the laboratories (Stephenson *et al.*, 2009; Wood *et al.*, 2012). There are many studies comparing the performance of HI and VN methods for measuring anti-influenza antibodies. In general, HI and VN assay comparisons have shown a good correlation between the two methods especially for seasonal influenza A virus antibodies (Truelove *et al.*, 2016; Haveri *et al.*, 2019). However, a study on the reproducibility tests for H1N1 pandemic influenza A virus antibodies revealed up to 6 or 7-fold inter-laboratory variation in HI and VN test results, respectively (Wagner *et al.*, 2012). Poor reproducibility of the VN assay and the lack of standardization poses a problem on the reliability of the method for the determination of influenza vaccine efficacy (Laurie *et al.*, 2015). The investigation of VN assay inter-laboratory variation for detection of anti-influenza antibody was performed in 11 laboratories from eight countries (Stephenson *et al.*, 2007). As there is no established common protocol for VN assays, the laboratories used own established in-house protocols. VN assays displayed significant inter-laboratory variability. The between laboratory geometric coefficients of variation were 256 –359 % depending on the virus strain.

Despite its sensitivity and specificity, the VN assay is unsuitable for large-scale studies. The need to handle wild-type virus and cells, poor reproducibility and the time required for the assay performance are the main limitations of the assay. Presently, VN titre value which correlates with protection is undefined. VN titre equal to a HI titre of 1:40 varies between laboratories and cannot be easily standardised (Stephenson *et al.*, 2007). VN is also unable to discriminate different immunoglobulin classes in antibody responses. VN is useful in analysing humoral immune responses to avian strains of influenza, especially when HI test fails to detect antibodies against these viruses (Rowe *et al.*, 1999; Ansaldi *et al.*, 2004). However, studies of highly pathogenic avian strains such as H5 and H7, require work in biosafety level 3 (BSL-3) laboratory, additional safety precautions and

extensive training for laboratory personnel which are associated with high running costs.

2.1.3 Single radial haemolysis assay

Single radial haemolysis (SRH) was developed in 1975, combining the benefits of two different methods. The accuracy of SRH is combined with the sensitivity of the HI test (Russell, McCahon and Beare, 1975; Schild, Pereira and Chakraverty, 1975). SRH assay was developed for virus infections such as rubella, mumps, influenza, adenovirus, dengue and Japanese encephalitis virus (Russell *et al.*, 1978; Fulton *et al.*, 1984; Chan *et al.*, 1985; Trombetta *et al.*, 2015). However, the main use of this technique remains in the screening for influenza antibodies (Trombetta, Perini, Mather, Temperton, Montomoli, *et al.*, 2014). The principle of the SRH assay is shown in **Figure 2**. The technique relies on the passive haemolysis of virus-treated red blood cells by virus-specific antibody and complement.

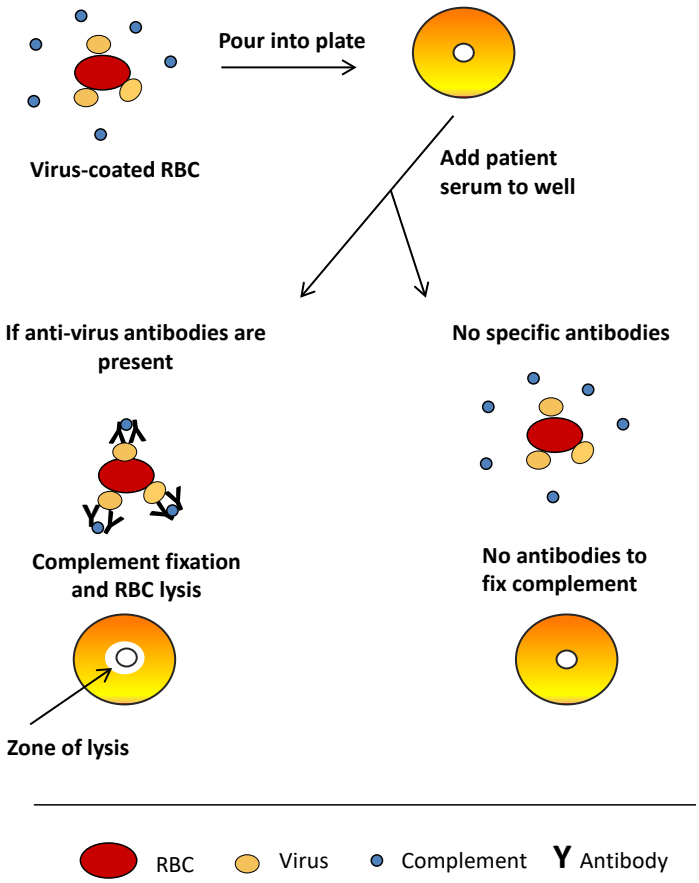

Figure 2. Schematic principle of single radial haemolysis assay. Virus-coated sheep RBCs and guinea pig complement are mixed with agar and poured in a plate. A heat-inactivated serum sample is added to the well punched in the agarose gel. If there are virus-specific antibodies in the serum, there will be antigen-antibody complexes following complement fixation. Fixed complement causes RBC haemolysis. The result is read visually by eye observing a zone of lysis around the well. Virus-specific antibody amount present in the test serum corresponds to the measured zone of lysis (in mm^2).

SRH can be used for the antigenic characterization of influenza viruses as it detects strain-specific antihaemagglutinin antibody (Lu *et al.*, 2014). Although the assay is more laborious than HI assay, the SRH assay is more reproducible than HI assays and more sensitive than CF test (Katz, Hancock and Xu, 2011). The greatest advantage of the SRH method is its safety as the assay does not require purified or concentrated virus. It is performed with inactivated virus and it can be safely conducted under BSL-2 conditions (Wood *et al.*, 2001). The HI test, VN and SRH have shown a good agreement with each other for analysing anti-influenza virus antibodies (Morley *et al.*, 1995; Trombetta *et al.*, 2018). However, for influenza B viruses, the sensitivity of SRH was higher than that of the HI test in detecting

antibodies. The great advantage of SRH is its ability to detect antibodies against avian influenza viruses in contrast to the HI test. A previous study showed that SRH and VN tests provide sensitive determination of vaccine-induced antibodies against avian influenza viruses (Stephenson *et al.*, 2003). Meanwhile, the HI test was shown to underestimate antibody responses against avian influenza viruses.

2.1.4 Enzyme immunoassays

For many virus infections, enzyme immunoassay (EIA) is the most commonly used serological assay. EIA enables accurate and sensitive detection of the antigen or antibody and it is widely used in clinical laboratories and biomedical research. A wide variety of EIA kits for different applications are commercially available. There are many different variations of EIAs, but all of them use the enzyme-conjugated antibody as a detection molecule (John R. Cowther, 2009). The addition of a substrate for the enzyme allows visualisation and quantification of the bound antibody to the target molecules. The substrate may be either a chromogen, a colourless molecule that is converted into a coloured end product or a fluorogen, a nonfluorescent molecule that is transformed into a fluorescent form by the enzyme. The assay is typically performed on a 96-well plate format and optical density values are read by a plate reader.

The enzyme-linked immunosorbent assays (ELISAs) are the most widely used EIAs. ELISAs were first introduced in the 1971 by Engvall and Perlmann (Engvall and Perlmann, 1971) and Van Weemen and Schuurs (Van Weemen and Schuurs, 1971). ELISA can be performed to evaluate the presence of and quantify specific analytes (e.g. antigens, antibodies, proteins, hormones, peptides, etc.) in biological samples such as serum, plasma, urea, stool and cell culture supernatants (Demerdash *et al.*, 2011; Song *et al.*, 2016; Tsai *et al.*, 2018). Currently, direct, indirect, sandwich and competition variations of ELISA exist (John R. Cowther, 2009). The principles of the ELISA systems are illustrated in **Figure 3**.

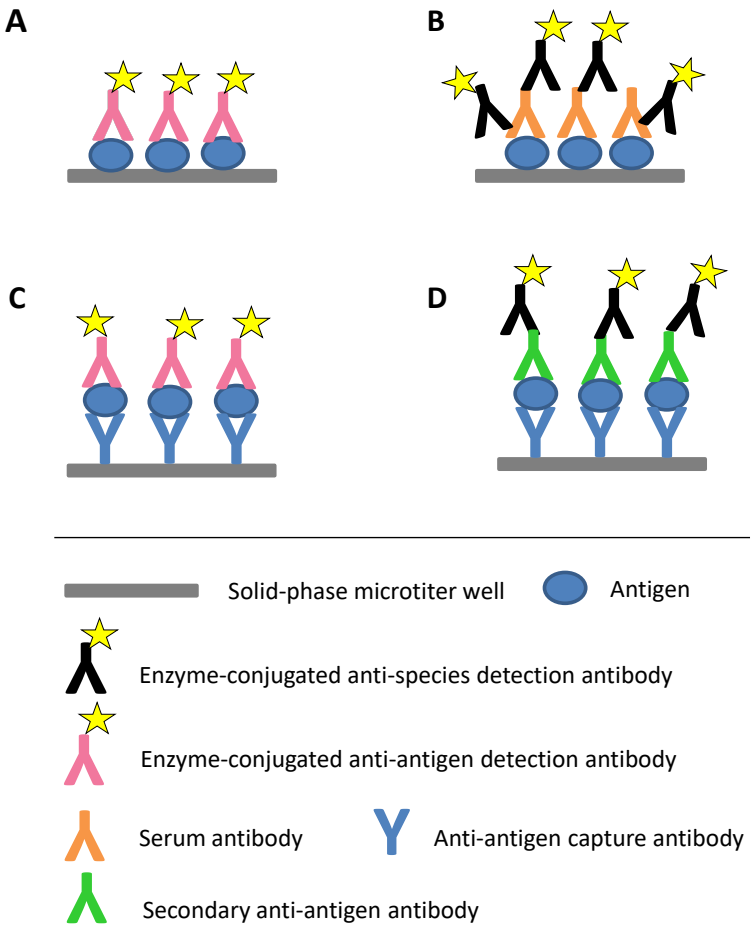

Figure 3. Schematic overview of ELISA techniques. (A) Direct ELISA; (B) Indirect ELISA; (C) direct sandwich ELISA; (D) indirect sandwich ELISA.

Direct ELISA **(A)** is the simplest form and the base style for other types of ELISA. Antigens from the patient sample are attached to a surface of a microtiter plate by passive adsorption. After the antigen is immobilised, the plate is washed and the surface is blocked with blocking buffer containing detergents and other proteins (e.g., albumin, gelatine, casein, and skimmed-milk). The specific to that particular antigen enzyme-conjugated antibody will bind followed by colour development with addition of appropriate substrate. Alternatively, an antibody from a sample immobilized on the surface of microtiter plate can be detected with corresponding enzyme-conjugated antigen. Direct ELISA is suitable only for the qualitative analysis since the method of antigen or antibody immobilization is not specific (Sakamoto *et al.*, 2018). A major disadvantage of the direct ELISA is in immobilisation of all proteins from a patient sample, not only analyte of interest.

Thus, small concentrations of target antigen or antibody in the sample must compete with other proteins when binding to the well surface. An indirect and sandwich ELISA systems solve this problem by using capture antigen or antibody specific for the target analyte in patient sample.

In an indirect ELISA **(B)**, serum antibodies from a patient sample against an antigen are measured. Fixed amount of the antigen is immobilized in the well of a 96-well microtiter plate. Antibodies specific to the antigen are bound from the serum sample. Secondary enzyme-conjugated antibodies are added to detect bound antibodies. The colour end product is produced after incubation with the substrate. Produced colour intensity is proportional to the quantity of antigen-specific antibodies present in serum sample of a patient.

Sandwich ELISA systems are used to detect sample antigen and can be divided into a direct sandwich ELISA and an indirect sandwich ELISA. In the direct sandwich ELISA **(C)**, an antigen from a patient sample binds to immobilised capture antibodies specific for the test antigen. The antigen is then detected using enzyme-labelled antibodies specific to the antigen. In the indirect sandwich ELISA **(D)**, an antigen from a sample is also captured by immobilized antibodies but the detection is different. Second antibody from a different species to solid phase antibody but specific for the antigen is added and form "antibody-antigen-antibody sandwich". Detection is performed by applying enzyme-conjugated antibodies specific for species from which the second antibody was prepared. The direct sandwich ELISA is technically simpler than indirect method, but the last has certain advantages. Production of enzyme-conjugated antibodies against every antigen of interest is expensive process. It is avoided in indirect sandwich ELISA by using the same enzyme-conjugated anti-species antibodies in a variety of antigen detection tests.

The indirect ELISA is the most commonly used method to detect antigen-specific antibodies to assess responses to an infection or vaccination. A wide range of ELISAs have been developed and they are commercially available for measuring antibodies against different viruses. ELISA methods are versatile, simple to perform, sensitive and quantifiable. The assay can be performed in many laboratories as it requires only equipment with optical scanner to read the results. It is possible to measure any antibody isotype (e.g. IgG, IgM, IgA) by ELISA. As serum titration is not performed, the quantity of antibodies in a sample can be measured more precisely than e.g. in the HI test. High assay reproducibility is an additional advantage of the ELISA assays. However, there are certain limitations of the assay. Serum samples are required to be properly diluted in order to fall within a relatively small dynamic range of the absorbance and antibody quantity (S. X. Leng *et al.*, 2008). The major weakness of this method is that only one antibody type and antibodies against only one antigen can be measured at the same time,

which makes the assay relatively labour-intensive. To facilitate screening of a large number of samples, multiplex immunoassays have been actively developed during the last decade.

2.1.5 Serology and influenza vaccines

In the Unites States, vaccines licensure involves approval from the Food and Drug Administration (FDA). After the FDA approves the vaccine, the Advisory Committee on Immunization Practices (ACIP) within the Centres for Disease Control and Prevention (CDC) considers the risks and benefits of the vaccine and develop official federal guidelines for the use of vaccines in the Unites States (Grohskopf *et al.*, 2019). In European Union, vaccines are monitored by the Committee for Proprietary Medicinal Products (CPMP) under the European Medicines Agency (EMA) (Medicines Agency, 2012). In addition to national surveillance organizations, international vaccines monitoring is established by the World Health Organization (Trombetta, Gianchecchi and Montomoli, 2018).

Influenza vaccines are needed to fulfil the criteria of immunogenicity based on the HI and SRH assays according to the international licensing criteria of the EMA and FDA (Trombetta, Perini, Mather, Temperton and Montomoli, 2014). Specifically, the vaccines have to clearly increase the geometric mean antibody titers and induce seroconversion and establish antibody titers to levels that correlate with seroprotection. According to the international licensing criteria of EMA, in the age group of 18-60 year-olds a vaccine can be licensed when it induces an antibody titre mean fold rise >2.5, a seroconversion rate in ≥40% or seroprotection rate in ≥70% of the vaccines (European Committee for Proprietary Medical Products, 1997). In adults over 60 years of age, an antibody titre mean fold rise >2.0, a seroconversion rate ≥30% and seroprotection rate ≥60% are required. FDA established criteria for efficient vaccine-induced responses as a seroconversion rate in ≥40% (lower limit of 95% CI) or seroprotection rate in ≥70% (lower limit of 95% CI) in the vaccinated adults < 65 years of age and in the paediatric population (Food and Drug Administration, 2007). For adults ≥ 65 years of age, a seroconversion rate in ≥30% (lower limit of 95% CI) or seroprotection rate in ≥60% (lower limit of 95% CI) are required. If pre-vaccination sample is negative, seroconversion means an increase in the HA antibody titers ≥40 in the HI test or that the serum haemolysis area is greater than ≥25mm^2 in the SRH assay. In case of a positive pre-vaccination serum sample, at least a fourfold increase in the HI titre or higher than 50% increase in haemolysis area is required. The seroprotection rate is counted as a proportion of individuals with HI titre ≥40 or an SRH titre ≥25mm^2 that is considered to correlate with protection.

The serological criteria described above for the influenza vaccine immunogenicity assessment have been the cornerstone for many years (Wijnans and Voordouw, 2016). There has been enhanced awareness that it is not the most suitable strategy to rely on a single serological cut-off (HI titre ≥40 or an SRH titre ≥25mm^2) for all age groups and different influenza vaccine types (Treanor and Wright, 2003; Ohmit *et al.*, 2011; Pfleiderer *et al.*, 2014). This cut-off is well defined in healthy adults but not in children. A few studies questioned whether the HI assay is the best option to assess the influenza vaccination efficacy in children. As young children have little to no previous exposure to influenza vaccination or natural influenza infection, higher HI titre levels are required to be set as a protective level. Black *et al.,* 2011 showed that HI titre of 1:110 was associated with the conventional 50% clinical protection rate against influenza infection in children, while the conventional HI titre value of 1:40 was associated with only 22% protection (Black *et al.*, 2011). In addition, one study in the elderly suggested that cell-mediated immunity rather than humoral immunity correlates better with the protection against influenza in this age group (McElhaney *et al.*, 2006).

In 2014, the European Committee for Medicinal Products for Human Use (CHMP) incorporated changes to guidelines for the influenza vaccine licensing in Europe (Wijnans and Voordouw, 2016). CHMP works under the European Medicines Agency (EMA) which is an agency of the European Union. In addition to standard HI antibody titre determinations, CHMP requires that neutralizing antibodies are quantitated by VN assay and cell-mediated immunity is evaluated in a subset of elderly individuals. Moreover, it is required that clinical studies in children aged 6–36 months must be conducted for all new influenza vaccines. Enhanced monitoring of vaccine safety and efficacy is needed. It is an open question whether neuraminidase-specific antibody responses need to be analysed. While anti-HA antibodies are known to inhibit viral infection and replication, neuraminidase antibodies (anti-NA) were discovered to play a role in the prevention of a clinical disease (Murphy, Kasel and Chanock, 1972). Identifying the role of anti-NA response to vaccination is also important, even though the quantity of NA protein in current influenza vaccines has not been standardized. It has been reported that in the absence of well-matching anti-HA antibodies, NA-specific antibodies can still provide protection against clinical influenza illness (Marcelin, Sandbulte and Webby, 2012; Jagadesh *et al.*, 2016). Different techniques may be applied to quantitate NA. Accelerated Viral Inhibition with NA (AVINA) assay was shown to be advantageous to predict vaccine efficacy and the assay is relatively simple to perform (Hassantoufighi *et al.*, 2010). A great advantage of the assay is its potential to simultaneously quantify hemagglutinin and neuraminidase-inhibiting antibody responses. However, the most used

technique to measure NA-specific antibodies is an Enzyme-Linked lectin Assay (ELLA) (Lambré *et al.*, 1990).

2.2 Multiplex technologies

The onset of multiplex era opens up new research possibilities. To date, numerous multiplex assays used in diagnostics, cohort screening and research setups have been described (Engin, 2019). Multiplex assays overcome the limitations of conventional serological assays enabling simultaneous detection of multiple analytes in a single sample. High sample throughput, simultaneous detection of several analytes, low cost and the requirement of small sample volumes make multiplex immunoassays well suitable for testing large numbers of samples from clinical vaccine trials and serosurveillance studies. Multiplex technologies can be divided into two main categories of bead-based and microarray assays, described in the following sections.

2.2.1 Bead-based multiplex assays

Bead-based multiplex assays utilise polystyrene (Moss *et al.*, 2004), glass (Ostendorff *et al.*, 2013) or magnetic sets of beads with different fluorescent codes (Zhu, Duan and Publicover, 2010). The beads are coated with either specific antibodies or antigens. A single sample can be analysed against multiple analytes with multiple sets of color-coded beads. There are two major types of bead-based assays: Luminex and cytometric bead assays (CBA) (Ayling, Vedhara and Fairclough, 2018). The CBA system from BD Biosciences (www.bdbiosciences.com) does not require expensive equipment. A standard clinical flow cytometric device can be used to read the results. However, CBA uses one fluorescent colour per bead set that limits the number of measured analytes. Luminex multi-analyte profiling (xMAP) is a customizable commercial technology from Luminex which has a broader multiplex capacity (www.luminexcorp.com; later referred as Luminex; based on original publications of (Dunbar and Li, 2010; Angeloni *et al.*, 2014). Up to 100 analytes per well in a 96-well microtiter plate can be analysed simultaneously by Luminex. The procedures of all bead-based multiplex assays are similar and rely on bead sets coded with fluorescent dyes and flow cytometry.

The principle of the Luminex multiplex assay is shown in **Figure 4**. The specific antigen of interest is conjugated onto the surface of polystyrene beads. Each well in 96-well microtiter plate contains multiple bead sets with different fluorescent signatures and antigens. A serum sample is added to a mixture of bead sets and antibodies bind to their specific antigens. The detection is based on

biotinylated anti-species antibodies and phycoerythrin (PE)-conjugated streptavidin which binds to biotin. Dual-laser flow-based detection instrument, such as the Luminex® 100™ analyser, is used to read signals for each bead set. One laser classifies the beads on the basis of their fluorescent signatures and determines to which antigen antibodies are bound. PE-derived signal is determined by a second laser. The signal intensity is proportional to the amount of bound antibodies.

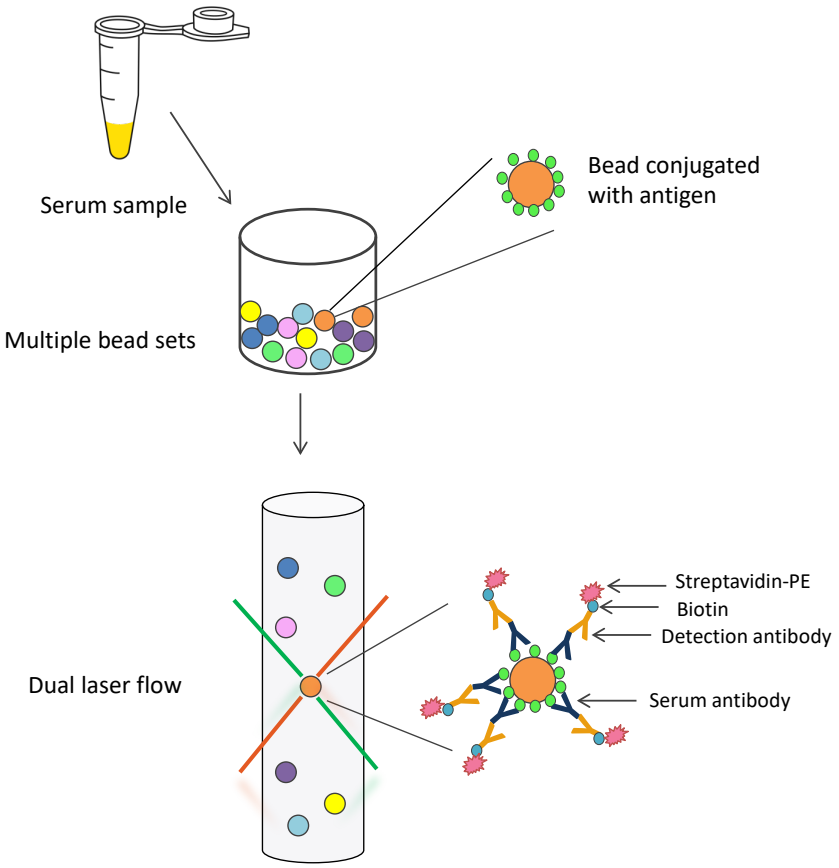

Figure 4. The principle of Luminex polystyrene bead-based multiplex assay.

Similar to all multiplex assays, the main advantage of bead-based assays is the ability to measure simultaneously multiple analytes and antibody isotypes. The assays require smaller sample volumes and they are highly efficient in terms of time and costs. The assays are widely used in different fields of biosciences, such as studies on inflammation, diabetes, immunology and virology (S. X. Leng *et al.*, 2008; Martins, Litwin and Hill, 2008; Purohit, Sharma and She, 2015). The bead-based multiplex assay detection systems are based on fluorescent signals and have

a large dynamic range compared to ELISAs. Currently, magnetic bead-based immunoassays displace polystyrene bead-based assays. Magnetic bead-based assays enable separation analytes during washing steps which improves the ability for automatization (Houser, 2012).

Good correlations between ELISA and bead-based multiplex assays have been reported (Khan *et al.*, 2004; Elshal and Mccoy, 2006). Multiplex fluorescent microsphere immunoassay for the determination of antibodies e.g. against three Epstein-Barr virus (EBV) antigens showed a good correlation with ELISA (83.8%-92.8%%) and even better correlation with IFA (92.8%-98.1%) (Martins, Litwin and Hill, 2008). The degree of correlation between bead-based multiplex assays and ELISA depends on how the comparisons are made and whether similar reporter antibodies and diluents are used (Elshal and Mccoy, 2006). Bead-based multiplex assays results have been reported to be much higher than ELISA values (Ray *et al.*, 2005). This can be explained by the fact that in bead-based multiplex assays all reactions take place in solution, while in traditional ELISA antigens/antibodies are bound to solid-phase plastic. However, cross-reactions are possible and potential biomolecule interactions in the same assay solution must be considered. There are, however, some technical problems with multiplex assays and caution with the interpretation of the results is needed when compared with ELISA data.

The total costs of multiplex assays are lower if compared to the costs of separate ELISA assays used to obtain the same amount of data. Even though the multiplex technology is more cost effective, it requires an initial investment in expensive equipment and software. "For example, Luminex technology-based systems require a dedicated analyser with a price tag of ca. $60,000 and the price of multiplexed ELISA systems (Randox Laboratories and MesoScale Discovery) range from $90,000 to $140,000."(Sean X Leng *et al.*, 2008). From the other side, despite the fast growing field of multiplex assays, single analyte detection tests could stay in use for decades. Current multiplex assays cannot be tailored to the individual patient. Clinicians or patients might desire to be screened only for one or a few targets, but with the multiplex systems they are forced to be screened against all analytes included in the multiplex assays.

2.2.2 Microarray-based multiplex assays

The multiplex chip or microarray-based assays have been widely used in proteomics to study protein functions and interactions, and in genetic research to examine the expression of multiple genes (Schena *et al.*, 1995). Microarrays consist of biomolecules immobilized to a solid phase, usually plastic, glass, silicone or nylon (Pastinen, 2000; Sachse *et al.*, 2005; Cretich *et al.*, 2006).

Immobilized biomolecules can be oligonucleotides, PCR products, proteins, peptides, carbohydrates, or other small molecules. Many studies have demonstrated microarray assays to be a useful tool in biochemical research. Most microarrays are based on nucleic acids or proteins. In infectious disease studies, nucleic acid-based microarrays are used to identify microbial pathogens whereas protein microarrays are used to identify specific antibodies against different pathogens. Protein microarrays may have either antibodies or antigens immobilized in an array format. Protein microarray assays have shown a broad application range: testing of antibody specificity, studies of protein function, identification of the protein targets of small molecules, screening for protein-protein interactions, identification of antigenic proteins and immune responses to them (Michaud *et al.*, 2003; Qiu *et al.*, 2005; Selvarajah *et al.*, 2014; Negm *et al.*, 2015).

The first microarrays detecting viral genetic material were described in the early 2000s (Chizhikov *et al.*, 2002; Wilson *et al.*, 2002; Sengupta *et al.*, 2003). Nucleic acid microarray methods are based on oligonucleotides which capture PCR products and use different visualization approaches (Wang *et al.*, 2002; Lovmar *et al.*, 2003; Boriskin *et al.*, 2004; Albrecht *et al.*, 2006). Multiple viral antigens can be detected simultaneously from a single sample by microarray assays. This makes the microarray technology very well suitable tool for large-scale screening purposes. Presently, microarray assays are widely used in virology to detect and genotype influenza-, RSV-, adeno-, corona-, flavi-, zoonotic, rota-, noro-, astro-, entero-, papilloma- , hepatitis and other viruses (Gemignani *et al.*, 2004; Coiras *et al.*, 2005; Nordström *et al.*, 2005; Chou *et al.*, 2006; Gauthier *et al.*, 2010; Díaz-Badillo *et al.*, 2014; Wang *et al.*, 2017; Erickson *et al.*, 2018; Kim *et al.*, 2018; Xiao *et al.*, 2019; Nybond *et al.*, 2019).

Currently, many nucleic acid-based microarray technologies are being developed. The development of new primers facilitates the simultaneous detection of a wide range of multiple pathogens and subtypes. For instance, Takizawa *et al.,* (2013) developed a simultaneous pathogen detection system for viruses, such as human hepatitis C virus (HCV), human hepatitis B virus (HBV), human parvovirus B19 (PVB19), and West Nile virus (WNV), and human immunodeficiency virus (HIV) (Takizawa *et al.*, 2013). Chemiluminescence (CL) detection oligonucleotide microarray was developed for the detection and genotyping of different strains of influenza A and B viruses, including avian influenza A H5N1 and H7N9 subtypes (Zhang *et al.*, 2016). The authors reported 91.1 % sensitivity of the microarray as compared to real-time PCR.

Several commercial microarray technologies for the detection of virus infections are available. Clart PneumoVir and Clart FluAVir kits (Genomica, Madrid, Spain) are commercial technologies allowing simultaneous detection and identification of 21 different types and subtypes of human respiratory viruses

(influenza A/H1N1 and A/H3N2 viruses, influenza B and C viruses, RSV A and B, PIV-1, PIV-2, PIV-3, PIV-4, PIV-4 A and B, coronavirus E-229, HRVs, HMPV A and B, HBoV, adenoviruses, and enterovirus species B). Renois *et al.,* (2010) described a study where they compared generic real-time RT-PCR assay for the detection of influenza A viruses with commercially available microarray assays (Renois *et al.*, 2010). The study confirmed that microarray technology allows a rapid and accurate detection of viral nucleic acids and it has a great potential for routine virological diagnosis.

DNA microarrays are capable of simultaneously detect hundreds of viruses. The first viral microarray was described in 1999 for human cytomegalovirus (Chambers *et al.*, 1999). In 2002, Wang *et al.* described a microarray assay for the detection of 140 viruses (Wang *et al.*, 2002). Such high throughput multiplex technologies are important to provide not only epidemiological and virological information but also the opportunity to understand the emergence of new virus strains. Furthermore, Wang et al. (2003) designed a DNA microarray which is able to identify and characterise existing and novel viruses. They combined array hybridization with direct sequencing of viral genomes. With the established methodology a previously uncharacterized coronavirus was discovered (Wang *et al.*, 2003). Chou *et al.,* (2006) designed a microarray for identification of emerging viruses at genus level using the principle of conserved probe design. The detection is based on the finding that highly similar sequences can hybridize with all viruses of a particular genus but not with other genera viruses (Chou *et al.*, 2006).

2.2.3 Serological multiplex microarray assays

Recently, multiplex protein microarrays have become widely used in serology to detect antibodies against multiple microbial antigens. This type of microarray is called antigen microarrays. They are widely used in research to measure antibody responses against different microbes or their structural components and to profile antibody responses to different proteins in order to evaluate promising candidates for future subunit vaccines and/or diagnostic antigens. The generalized principle of antigen microarrays is shown in **Figure 5**.

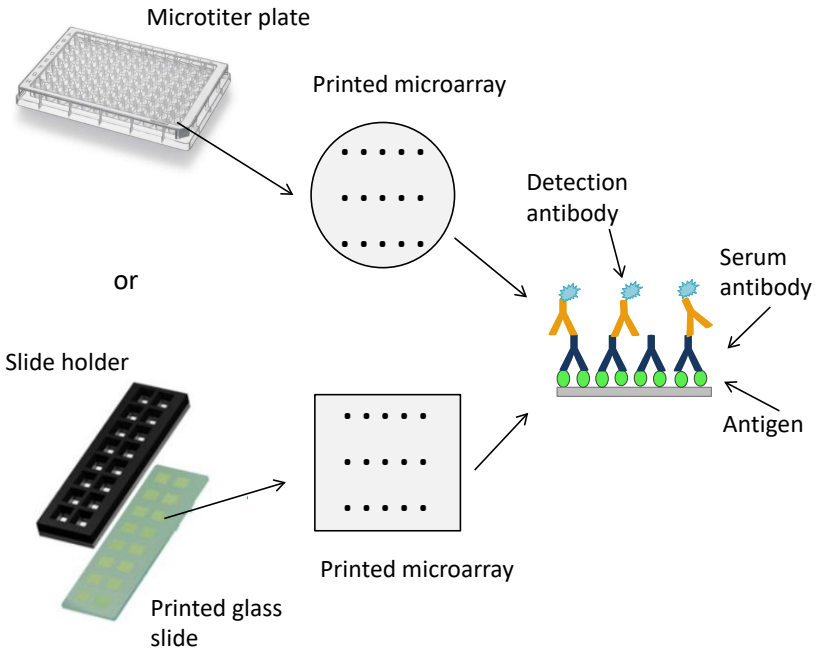

Figure 5. Schematic principle of multiplex antigen microarray immunoassays.

Tiny spots of antigens of interest and protein controls are printed onto a surface of a functionalised glass slide (Selvarajah *et al.*, 2014) or plastic wells of a microtiter plate (Gehring *et al.*, 2008). The spots are printed by a robotic array printer creating spots <200 µm in diameter. Then blocking solution is added to prevent further nonspecific binding. Antibodies from diluted serum samples bind to specific antigen spots and positive control spots. After washing, labelled anti-human antibodies are added. Typically, the detection system is based on fluorescence (Andrew D. Livingston *et al.*, 2005). Detection antibodies are labelled with a small molecule biotin that can be recognized by the protein streptavidin (Ayling, Vedhara and Fairclough, 2018). Then a fluorescent dye-conjugated streptavidin is added and it binds with high specificity to the biotinylated antibody. Upon excitation with a laser, fluorescence is generated. The slides or plates are read by a laser scanner. Antibody responses are determined based on antigen spots position. Microarray data are pre-processed with specially designed software that finds spots and counts average signal intensity in a pre-defined spot area. As a rule, all data are filtered with pre-defined criteria and aberrant spots are removed. Obtained fluorescence intensity values are proportional to the amount of detected antibodies. The antibody concentration is quantified based on a standard curve.

Microarray assays are frequently called ”miniaturised ELISAs”. Compared with traditional ELISAs, multiplex microarray immunoassays have a number of

advantages including: 1) simultaneous detection of multiple analytes; 2) simultaneous detection of different antibody isotypes; 3) requirement of small sample volumes; 4) time and cost efficiency; 5) accurate detection of different antibodies with a wide dynamic range. The last advantage is especially important in vaccine studies where high levels of antigen-specific antibodies are found in post-vaccination samples. ELISA has a quite narrow dynamic range and multiple sample dilutions may be need to be analysed. A limiting factor of the microarray assays is the requirement of expensive machinery. In addition, aberrant spots are known to occur during array printing stage. These spots are typically of inadequate morphology including size and shape. In addition, the purity of the protein, its native conformation after immobilization, and storage conditions may lead to technical variation in the microarray assay. Thus, the assay requires multiple internal controls and good expertise of the staff running the assays.

A wide range of microarray immunoassays for detection of antibodies against different viruses, such as herpesviruses, measles, rubella, mumps, HIV, Epstein-Barr virus, cytomegalovirus, coronavirus, vaccinia virus and for five hepatitis viruses (HAV, HBV, HCV, HDV and HEV) in human sera have been already developed (Mezzasoma *et al.*, 2002; Davies *et al.*, 2005; Qiu *et al.*, 2005; Xu *et al.*, 2007; Kwon *et al.*, 2008; Jääskeläinen *et al.*, 2009; Feron *et al.*, 2013; Sivakumar *et al.*, 2013). Microarrays can be set up with virus recombinant proteins or whole viruses as antigens. Some microarray assays have even been reported to be more specific than ELISAs. For instance, a sol-gel-based protein microarray developed for the detection of hepatitis C virus antibodies showed 98.78% specificity whereas ELISA showed 81.71% (Kwon *et al.*, 2008). Several influenza virus antibody-specific multiplex microarray assays have been designed and they have shown a great potential in studies for profiling of humoral immune responses to influenza viruses and characterization of influenza vaccine induced humoral responses (Gall *et al.*, 2009; Koopmans *et al.*, 2012; Desbien *et al.*, 2013; Price *et al.*, 2013; Freidl *et al.*, 2014). Influenza virus protein microarrays for measurement of antibody responses to influenza A in animals and poultry have been started to be developed (Freidl *et al.*, 2014; Meade *et al.*, 2017). A multiplex microarray based on five RSV proteins for the determination of antibody levels against RSV was designed and it showed a good correlation with microneutralization test (Schepp *et al.*, 2019). Multiplex microarray assays for simultaneous detection of different antibody classes have also been developed. Liu *et al*., 2013 developed a recombinant antigen-based microarray assay for simultaneous detection of IgM and IgG antibodies against herpes simplex virus type 1 and 2, cytomegalovirus and rhinovirus in a single sample (Liu *et al.*, 2013). For vaccine studies multiplex assay have recently been started to be developed. Multiplex poliovirus assay has been successfully used in large-scale vaccine studies and the assay showed a great

potential as a safe assay without the need to use live polioviruses (Schepp *et al.*, 2017). A multiplex assay for simultaneous detection of antibodies against hepatitis B, *Haemophilus influenzae* B, diphtheria, tetanus, pertussis rubella, and RSV demonstrated its applicability in studying immune responses against common childhood vaccines (Itell *et al.*, 2018).

Antibodies bound to different antigens on the microarray can be detected by different methods. In general, multiplex microarray assays are based on either chemiluminescent or fluorescent detection systems. Chemiluminescence technology has been considered to be more sensitive than chromogenic detection in traditional ELISA assays. Multiplex immunoassays employing electrochemiluminescence technology are also commercially available, e.g. kits from Meso Scale Discovery (www.mesoscale.com). The electric wired microplate is coated with specific antibodies. A tag in detection antibodies is excited by an electric field. Time-dependent signal decay-related problems are avoided when electrochemiluminescence-based assays are used (Sean X Leng *et al.*, 2008). In fluorescence based detection systems a cyanine-labelled secondary antibody are commonly used (Lu *et al.*, 2005).

There has also been a successful development of label-free serological microarrays. For instance, a label-free immunoassay for flavivirus detection was developed based on a principle of Reflective Phantom Interface (RPI) technology (Tagliabue et al., 2017). Arrayed Imaging Reflectometry (AIR) is commonly used in such microarray sensors. The detection is based on perturbation of an antireflective coating on the surface of a silicon chip when antibody binds to an immobilized target antigen. When serum antibody is captured, a film thickening gives rise to a signal generation in the form of reflected light. Multiple probe/target interactions may be simultaneously monitored without any requirement for secondary antibodies or labelling. The amount of antibodies present in a sample is quantified proportionally to the reflectance changes. Label-free biosensor technique is able to provide quantitative information up to 100 of analytes simultaneously.

Such a label-free optical biosensor based on the AIR detection system was developed for analysing immune responses against influenza viruses using a panel of recombinant hemagglutinin proteins (Mace *et al.*, 2011). The assay can be performed in the field conditions and it demonstrated the capability to dramatically simplify influenza surveillance. Another multiplex hemagglutinin microarray based on AIR technology also proved to be rapid and sensitive for the detection of influenza-specific antibodies in both human and avian serum samples (Bucukovski *et al.*, 2015). Serological assessment of influenza exposure in the field is especially important when anti-HA antibody titers in avian serum are analysed. Avian species

are the primary reservoir of type A influenza viruses and a rapid detection of the exposure to avian influenza is needed (Webster, 2002).

In recent years, microarray technology has been actively developed and currently microarray assays are based not only on immobilised nucleic acids and proteins but also cells, glycans, and carbohydrates (Andrew D Livingston *et al.*, 2005). For instance, Rider and co-workers engineered a light-emitting B cell-based system which is able to detect pathogens within 3 minutes (Rider *et al.*, 2003).

2.2.4 Commercially available multiplex immunoassays

Several planar multiplex microarray assays and bead-based suspension assays are commercially available. Most of these assays have not been validated for *in vitro* diagnostics and they are currently available for research use only. The largest number of commercially available multiplex assays are designed for studies on autoimmunity, allergy and cytokine and chemokine profiling (Tighe *et al.*, 2015). However, a substantial proportion of commercial multiplex assays are also available for cardiovascular, angiogenesis and infectious disease studies. **Table 1** shows the currently available commercial multiplex assays for serum antibody detection in patients with infectious diseases.

Table 1. Commercially available multiplex immunoassays for antibody detection suitable for infectious disease research and diagnostics

Company	Product	Platform/format
Zeus Scientific	AtheNA Multi–Lyte® Test System	Luminex
Bio–Rad Laboratories	Bio–Plex® 2200 Autoimmune and Infectious Disease Panels	Luminex
Biomérieux	VIDAS® range	VIDAS
Arrayit Corporation	Pathogen Antigen Microarrays	Planar array
DiaMex	Optiplex Borrelia	Luminex
Focus Diagnostics	Plexus™ HerpeSelect®	Luminex

Bead-based suspension assays are currently the dominant technology among FDA-approved multiplex protein assays in clinical settings (Ellington *et al.*, 2010). Bead-based multiplex assays are based on flow cytometry and easily adaptable for automation and for large-scale screening. However, planar microarray immunoassays are more affordable as they are relatively inexpensive and simple to perform. In addition, planar microarray-based immunoassays are less susceptible to cross-reactivity as capture proteins are immobilised on a solid phase (Tighe *et al.*, 2015). It has been reported that cross-reactivity increases considerably with the number of targets in suspension multiplex assays (Pla-Roca *et al.*, 2012). In addition, such systems are highly sensitive to changes and inclusion of new analytes to the panel requires optimisation of the whole assay.

Despite the relatively high expenses of currently available commercial systems, serological microarrays have the potential to replace some of the presently used assays and be used as a fast screening tool in the future. The multiplex techniques are likely to be further adapted to fulfil the routine diagnosis criteria of viral infections and commercial applicable assays will be available in the nearest future.

2.3 Selected viruses and antigens for multiplex immunoassay

2.3.1 Respiratory syncytial virus

The respiratory syncytial virus (RSV) was discovered in 1956 when the virus was isolated from chimpanzees (Blount, Morris and Savage, 1956). RSV is a member of the *Orthopneumovirus* genus, of the family *Pneumoviridae* (Rima *et al.*, 2017). The virus is an enveloped, non-segmented virus with a single negative-sense RNA genome. The viral genome encodes 11 proteins including three envelope proteins, the fusion (F) and the attachment (G) glycoproteins, and a small hydrophobic (SH) protein (Borchers *et al.*, 2013). The G protein is responsible for the virus attachment on the host cell surface and the F protein participates in cell penetration and facilitates transmission between cells by syncytia formation. Antibodies targeted against either G or F protein play a major role in protective immunity against human RSV (Ascough, Paterson and Chiu, 2018). These antibodies have the ability to neutralize virus infectivity. The F protein induces widely cross-reactive antibody response against different RSV strains, whereas the G protein induces neutralizing antibodies that only function against the viruses of the same antigenic group. RSV has two major antigenic subgroups A and B. The classification is based on antigenic differences detected with monoclonal antibodies specific for the G protein. Only 53% of the G protein sequence is conserved between RSV A and B groups. Both subgroups co-circulate in the

community during the epidemics and either one of subgroup viruses may be predominant (Gilca *et al.*, 2006). The evolutionary rate of RSV is relatively slow (Tan *et al.*, 2012). Studies on RSV protein sequence variability reported the G glycoprotein to be the most variable protein. The attachment protein G has 2 to 12% sequence variability within the subgroup, whereas other RSV proteins have 0 to 5% variability between the consensus sequences of type A and type B RSV strains (Tan *et al.*, 2013). The mean substitution rates of the RSV A and B subgroups are similar, with 7.76 ($\times 10^{-4}$ substitutions/site/year) for RSV-B and 6.47 ($\times 10^{-4}$ substitutions/site/year) for RSV-A (Tan *et al.*, 2013)

RSV causes substantial epidemics with 1-2 epidemic periods each year all over the world (Haynes *et al.*, 2013). When suspected, RSV infection may be confirmed using a number of diagnostic methods such as RT-PCR, rapid antigen detection tests (RADT), ELISA, IFA and viral culture. Several RADTs for RSV are available in the form of immunochromatographic tests, enzyme immunoassays and optical immunoassays with varying (70% to 90%) sensitivity (Prendergast and Papenburg, 2013). RSV infection poses a high risk for severe disease in infants during the first year of life (Weisman L., 2003). It has been proved to be the most important cause of pneumonia and bronchiolitis in infants and young children (Piedimonte and Perez, 2014). Approximately, 1~3% of children under 1 year of age are hospitalized due to RSV infection (Leader and Kohlhase, 2002). The peak incidence is in children under 1 year of age, but RSV infection also causes a substantial disease burden in children one to three years of age (Hall *et al.*, 2009). RSV is associated with higher rates of outpatient care visits and hospitalizations in young children compared to that caused by influenza infection (Bourgeois *et al.*, 2009). Generally, all children older than 3 years of age have experienced one or more RSV infections (Ogra, 2004). The study by Glezen *et al*., (1986) showed that two-thirds of infants are infected during their first year of life, practically all children are infected by the age of 2 years and 76% of children are reinfected during the second year of life (W P Glezen *et al.*, 1986). Primary RSV infection causes cold-like symptoms including fever, runny nose, cough, wheezing and tachypnea (Eiland, 2009). Acute otitis media (AOM) is a common complication of RSV respiratory infection. RSV is the most common virus associated with AOM as it complicates about 50% of the cases (Patel *et al.*, 2007). The symptoms can progress to bronchiolitis or pneumonia in 25-40% of cases and even cause deaths (Shay *et al.*, 2001; Thompson, 2003; Zhou *et al.*, 2012). RSV coinfection with other respiratory viruses, such as rhinovirus and metapneumovirus is known to occur frequently and the coinfection increases the risk of complications (Calvo *et al.*, 2015). Very severe RSV infection can sometimes lead to long-term complications including the development of wheezing and asthma later in life (Welliver, 1998; Carroll *et al.*, 2009). However, in the majority of cases the

recovery from RSV infection is complete. In older children and adults the infection symptoms are relatively mild.

RSV is a pathogen with an exceptionally high rate of reinfections and those may occur throughout the life and the same RSV serotype can re-infect both children and adults. Reinfection is suspected to occur because infection does not induce complete long-lasting protective immunity. A study on the correlation between the age and susceptibility to RSV infections demonstrated a lower risk of severe disease with increasing age but no protection from reinfections (Ohuma *et al.*, 2012). RSV infection normally induces host immune responses including the activation of both humoral and cell-mediated immunity. Anti-RSV IgG levels play an important role in the protection against subsequent infections (Roca *et al.*, 2002). Generally, immune response to a primary infection is relatively weak but in reinfection(s) a significant booster effect is observed in serum. A study by Welliver *et. al.* published in 1980 on the kinetics of class-specific antibody responses to primary and secondary RSV infection showed that anti-RSV antibodies wane relatively rapidly after a primary infection. IgG antibodies were at a low level or absent one year after the primary infection (Welliver *et al.*, 1980). Another study also reported that a rapid antibody decline after a primary RSV infection seems to facilitate repeated infections (C J Sande *et al.*, 2013). This study showed that in young children neutralizing antibodies against RSV declined to pre-infection levels within 3 months after the infection. Second RSV infection induces boosted immune response with higher antibody levels in all three immunoglobulin classes and the antibodies persist for longer periods after reinfection. RSV studies suggest that a basic level of antibodies is needed for the protection against lower respiratory tract infection. The effect of aging was demonstrated to reduce antibody production in response to RSV infection. A study comparing neutralizing antibodies to RSV in healthy young individuals (20–60 years age) and in elderly (>80 years of age) showed that only 36.21% of aged individuals had RSV neutralizing antibodies compared with 92.5% in the youngest group (Terrosi *et al.*, 2009). In general, adaptive immune response to respiratory virus infections are known to decrease with age and elderly people are more susceptible to RSV infection and disease complications (Fulton and Varga, 2009). Also very young children and immunosuppressed individuals, show reduced ability to mount a protective immunity to RSV. A study in cotton rats showed that old cotton rats are cured from RSV infection slower than the young ones (Guichelaar *et al.*, 2014). Reduced virus clearance at the older age was found in the rat lungs and in the upper airways. However, the relationship between antibody levels induced by RSV in humans and age is still a subject of speculation.

Presently, there is no good specific antiviral treatment for the RSV infection apart from antibodies. The treatment for severe RSV infection is usually limited to

supportive care. At hospital, severe RSV disease is managed by oxygen supplementation, continuous positive airway pressure and mechanical ventilation. Ribavirin is the only approved drug for the treatment of severe RSV (Krilov, 2011). However, due to conflicting data on efficacy and side effects of the drug, the use of ribavirin is very limited (Mazur *et al.*, 2015; Drysdale, Green and Sande, 2016). Humanized monoclonal antibody product Synagis® (palivizumab) is available for prophylaxis against severe disease caused by RSV infection (Welliver, 1998). However, in view of high costs it is warranted only in selected high risk and prematurely born infants. RSV vaccines would be highly desirable but currently they are still in the developmental phase. A formalin inactivated crude, whole virus vaccine was tried in 1960, but this vaccine failed to produce protective immunity and vaccination led to a more severe disease during subsequent natural infection. Despite intensive efforts to design an effective RSV vaccine, a licensed vaccine is still not available (Piedra, 2003). Annual vaccination against RSV would likely be needed despite the genetically stable virus because serum antibodies wane rapidly.

2.3.2 Influenza viruses

Influenza viruses are classified into four types, A, B, C and D viruses (Long *et al.*, 2019). Influenza B and C are human viruses and influenza D infects only some animals. A wide variety of host species are infected by influenza A viruses. The dominant influenza A virus hosts are humans, birds, and pigs (Mostafa *et al.*, 2018). Influenza A (IAV) is the most important member of the influenza virus group and IAV causes worldwide yearly epidemics and occasional pandemics. Influenza A virus has a remarkable capacity to change its antigenic structures (Das *et al.*, 2013). Animal influenza A viruses, especially avian influenza may overcome species barriers and initiate new pandemic in humans. Due to susceptibility of influenza A viruses to mutations, animal influenza may establish occasional interactions with humans at any time. If a novel animal virus adapts to a human host, it may become transmissible between the humans which may result in the emergence of a new pandemic virus. Avian influenza is usually asymptomatic in birds but it may be dangerous and highly pathogenic when transmitted to another species. There has been documented cases of human infections caused by avian influenza viruses of H5, H6, H7, H9 and H10 subtypes (Long *et al.*, 2019).

Influenza viruses are divided into subtypes according to antigenic differences of the viral envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (Sautto, Kirchenbaum and Ross, 2018). These antigenic differences take place either by point mutations and short deletions or insertions leading to changes in the amino acid composition of the antigenic determinants of HA or NA proteins. This

phenomenon is called the antigenic drift. However, influenza A viruses evolve also by a genetic reassortment called antigenic shift, i.e. where a whole segment of the genome is transferred from one virus subtype to another. The antigenic shift has not been observed in influenza B and C viruses because they infect only humans (Jernigan and Cox, 2013) and these viruses tend to show slower antigenic variation compared to influenza A viruses. Influenza A virus is an enveloped virus with a diameter of 100-150 nm (Bouvier and Palese, 2008). The envelope is composed of HA and NA molecules embedded in a lipid bilayer derived from the plasma membrane of the host cell. Underneath the membrane there is a protein layer that consists of matrix protein (M). Nucleoprotein (NP) is the major nucleocapsid protein that covers the segmented RNA genome (8 segments) of the virus. Viral polymerase proteins, PB2, PB1 and PA are also components of the viral nucleocapsid structures. Amino acid changes in the major antigenic regions may alter the antigenic structure of the hemagglutinin giving rise to a new virus variant (antigenic drift) (Das *et al.*, 2013).

There is 25-40% conservation in the amino acid sequences between influenza A and B virus HAs. Influenza virus HA binds to sialic acid receptors onto host cell surface (Hensley *et al.*, 2009). Species-specific susceptibility to influenza virus infection is determined by the structure of these receptors and the ability of virus HA to attach to them. The receptor binding specificity is a key determinant of the virus transmissibility (Tumpey *et al.*, 2005). Antibodies against HA can prevent the attachment of the virus onto cellular receptors or they can prevent the fusion of viral and cellular membranes. The extent of HA glycosylation and its changes are known to be important in the ability IAV to cross-infect different species (Herfst *et al.*, 2012). In NA the antigenic drift and antigenic shift also occur and considerable variation between the strains is observed. NA catalyses the cleavage of terminal sialic acids of the sugar residues of cellular glycoproteins enabling the release of progeny virions (Matrosovich *et al.*, 2004). Antibodies against NA are known to give some protection, but they cannot fully neutralise the virus.

Currently, several different approaches are available for diagnosis of influenza infections, including viral culture, immunofluorescence assays, serological assays, nucleic acid amplification tests and immunochromatography-based rapid diagnostic tests (Vemula *et al.*, 2016). However, Centres for Disease Control and Prevention (CDC) recommend diagnosing influenza infections only by nucleic acid amplification tests (e.g. reverse transcription-polymerase chain reaction (RT-PCR) test) and antigen detection tests (including rapid influenza diagnostic tests (IRDT) and immunofluorescence assays) in clinical settings (Centres for Disease Control and Prevention (CDC) Seasonal Influenza (Flu), 2016). Influenza vaccine responses are usually analysed by HI, VN, and SRH tests. High mutation rates allow influenza viruses to escape from host immune responses. Therefore, the

effectiveness of influenza vaccines are relatively low compared to that of other viral vaccines. Influenza vaccines are annually reformulated in order to cope with the changes observed in seasonal circulating strains. To date, whole inactivated virus vaccines, split virus and subunit vaccines, live-attenuated and recombinant HA-based influenza virus vaccines have been used (Krammer, 2019). All of these vaccines are required to be reformulated each year to match well with the antigenicity of circulating viruses. Serum antibody response to a natural influenza infection is usually strong, the response is HA and NA-specific and very long-lived or even lifelong (Krammer, 2019). Split virus or subunit and recombinant HA-based vaccines induce strong serum antibody response but weak or undetectable mucosal antibody response and the persistence of vaccine-induced antibodies is often of short duration (Wong and Webby, 2013). In addition, the strength of individual responses to influenza vaccine antigens varies tremendously. Despite major efforts to control influenza infections, the efficacy and effectiveness of seasonal vaccines in children have varied a lot (Osterholm *et al.*, 2012; Jefferson *et al.*, 2018). There are great efforts to design a broadly protective influenza vaccine, so called universal influenza vaccine, to avoid the need of annual influenza vaccinations (Yamayoshi and Kawaoka, 2019). Currently, universal influenza virus vaccines based on original antigenic sin phenomenon are in clinical trials (Krammer, 2019).

2.3.3 Influenza 2009 pandemic vaccine antigen

During the last one hundred years influenza A viruses have caused five pandemics. The swine-origin influenza A 2009 virus likely originated from Mexico and it spread all over the world causing the most recent pandemic. Influenza A H1N1 subtype virus have caused three pandemics: in 1918-1920, 1977-1979 and in 2009-2010 (Allwinn *et al.*, 2010). H2N2 and H3N2 subtypes caused the 1957 and 1968 pandemics, respectively (Kilbourne, 2006). The latest pandemic was caused by an H1N1 reassortant virus that emerged from swine and it was relatively mild with an estimated mortality of ca. 0.02% (Van Kerkhove *et al.*, 2013). However, due to ongoing virus evolution there is a threat of a more severe pandemic in the future as it was with “spanish flu” in 1918-1920. A new variant of emerged pandemic influenza A virus in 2009 had substantial antigenic differences in relation to the seasonally circulating H1N1 viruses (Zhang, Song and Wang, 2012). Although it was shown that elderly people had some cross-reacting antibodies, most of the population had no herd immunity against the new emerged influenza (Hancock *et al.*, 2009; Ledford, 2009). In Finland, influenza A (H1N1)pdm09 viruses predominated during the influenza seasons 2009-2010, 2012-2013, 2013-2014 and 2015-2016 (Lyytikäinen *et al.*, 2011). Influenza A (H1N1)pdm09 virus displaced

the previous seasonal H1N1 virus and currently H1N1 and H3N2 viruses co-circulate together with two lineages of influenza B viruses and cause seasonal epidemics.

In 2009, seasonal H1N1 viruses and pandemic H1N1 virus had high antigenic mismatch (Saxena *et al.*, 2009), which allowed the virus to spread rapidly in the whole population. When the pandemic was declared by WHO, the production of seasonal vaccines was replaced by the production of the pandemic vaccine (Johansen, Pfeifer and Salisbury, 2018). To prevent the spread of the pandemic, Pandemrix™ vaccine was used in Europe in 2009-2011 (European centre for disease prevention and control, 2013). Pandemrix is an AS03-adjuvanted influenza A (H1N1)pdm09 vaccine produced by GlaxoSmithKlein, Rixenart, Belgium (Tsai, 2011). In 20 countries more than 30.5 million people were vaccinated. A similar AS03-adjuvanted influenza A (H1N1)pdm09 vaccine (Arepanrix) against 2009 pandemic influenza was produced in Canada where it was used locally (Ahmed and Steinman, 2016). There were also other nonadjuvanted vaccines developed and produced to prevent the pandemic. Pandemrix vaccine was highly immunogenic and effective against infections caused by this virus (Syrjänen *et al.*, 2014). Several studies reported that the vaccine was most effective in children and adults aged 18–64 years (Lansbury *et al.*, 2017). Overall, the vaccination has successfully prevented influenza-related hospitalization. However, there were increased cases of narcolepsy in children reported and associated with the pandemic influenza vaccination in some European countries (Jefferson *et al.*, 2018). The possible biological mechanism in Pandemrix-associated narcolepsy has remained unresolved.

2.3.4 Human adenoviruses

Human adenoviruses (hAdVs) belong to the genus *Mastadenovirus* in the *Adenoviridae* family (Davison, Benko and Harrach, 2003). HAdVs are divided into seven sub-groups (HAdV-A to G) based on phylogenetic analysis, genomic organization, growth characteristics and oncogenicity (Robinson *et al.*, 2013). Currently, HAdVs account for over 70 different genotypes (Radke and Cook, 2018). However, most human infections are associated with only one third of these viruses. HAdVs are icosahedral, non-enveloped, linear double-stranded DNA viruses. Hexon (n=240) and penton (n=12) proteins constitute the two major outer virion proteins. The penton and hexon proteins define the hAdV serotype. Most detection methods and the typing of the viruses are based on conserved (C1 to C4) and variable (V1 to V3) sequences of the hexon gene (Biere and Schweiger, 2010).

Adenoviruses periodically cause global infection outbreaks. Clinical manifestations of the infection depend on the HAdVs subgroup. HAdVs can cause

conjunctival, upper and lower respiratory tract and gastrointestinal tract infections (Radke and Cook, 2018). The virus is known to cause persistent infections in humans that can last for months or years and severe disease forms are seen in immunocompromised patients (Lion, 2014). Occasionally, the viruses can cause disease in the liver, urinary tracts, pancreas or in the central nervous system in immunocompromised individuals (Niemann *et al.*, 1993). The most common HAdV infections are keratoconjunctivitis and upper or lower respiratory tract infections. HAdV can cause large and prolonged outbreaks as the virus is extremely resistant to different physical factors and chemical agents and the virus may remain viable for many days (Radke and Cook, 2018). Respiratory tract infections caused by HAdV are usually mild and self-limiting in healthy individuals. However, newly emerged hHAdV with viral mutations are known to cause severe respiratory infections leading to pneumonia and rarely to acute respiratory distress syndrome (ARDS) (Cook and Radke, 2017). Respiratory tract infections are usually caused by adenovirus species C and B1 in infants and young children and by species E in military recruits.

The transmission of adenovirus diseases varies from sporadic to epidemic. A large number of completely asymptomatic adenovirus infections have been documented (Ketler A, Hall CE, Fox JP, Elveback L, 1969). Most infections in young children have been reported to be transmitted through fecal-oral route. Adenoviruses account for about 5% to 10% of the respiratory infections in young children. The usual symptoms include nasal congestion, fever, chills, headache, coryza and cough. According to the study of Cooper *et al.,* (2000), children of less than 5 years of age was the largest group suffering from adenovirus infections. Among adenovirus infected individuals 61% were under 5 years of age, 6% were 5 to 15 year-olds and 24% were adults (Cooper *et al.*, 2000). In about 10% of infected children adenovirus infection may lead to pneumonia.

The molecular basis for adenovirus pathogenesis and differences in disease severity among adenovirus subtypes are still unresolved. Serological surveys are crucial for studies on adenovirus epidemiology. Serological studies estimate the adenovirus seroprevalence and determine the most prevalent serotypes (Ludwig *et al.*, 1998; Mast *et al.*, 2010). Predominant subtypes differ between countries but the most commonly circulated adenovirus subtypes are HAdV-C1, -C2, -C5, -B3, -B7, -B21, -E4, and -F413 (Lion, 2014). In children, antibodies against HAdV-C1, -C2, -C5 subtypes are the most common ones and antibody prevalence varies from 40% to 60% in children (Brandt *et al.*, 1969). Respiratory infections caused by adenovirus are common among military personnel. These infections can effectively be controlled by vaccination with oral enteric-coated and live vaccines against Ad4 and Ad7 (Top *et al.*, 1971). Although the adenovirus vaccines are effectively used in the military, they are not licensed for administration for the general population

and therefore the vaccines cannot be used for susceptible children. In the mid-1990s, the vaccine production was stopped which lead to increased hAdV outbreaks in the military. In 2011, when vaccination in military was restarted, adenovirus disease rates dramatically decreased again (Hoke and Snyder, 2013).

Many adenovirus infections are indistinguishable from other respiratory virus infections or even from some bacterial infections. To identify hAdV infections, RT-PCR, viral isolation, detection of adenovirus antigens or nucleic acid from clinical samples is carried out. ELISA is the most commonly used method to detect antibody responses to adenovirus. For instance, anti-hAdV antibodies are analysed by ELISA based on a panel of bacterially expressed adenovirus proteins (Bauer *et al.*, 2005). There is need for new adenovirus tests for better surveillance of the infections and genotyping of the viruses identified in different disease spectra. Additionally, there is an increasing interest on adenoviruses used as vectors for the delivery of therapeutic proteins to humans e.g. in the treatment of cancer (Wold and Toth, 2014).

3 Aims of the Study

The overall aim of the study was to develop a novel multiplex microarray immunoassay (MAIA) for simultaneous detection of IgM and IgG antibodies against multiple viral antigens for large-scale serological and vaccine studies.

We intended to develop an assay that requires less serum and is less time and labour consuming as compared to traditional enzyme immunoassay, for determination of serum antibodies against RSV, adenovirus hexon protein, IAV H1N1, IAV H3N2, IAV H1N1 pdm09 vaccine ag, IBV Victoria and IBV Yamagata. The technology has a strong potential to improve the survey of infection diseases and vaccine immunity studies. To demonstrate the functionality of MAIA we carried out a large-scale serological study and estimated respiratory infection disease burden in early childhood with the established method. We also studied influenza vaccine-induced immune responses in a cohort of adults by three different methods (HI, EIA, and MAIA) in order to demonstrate the feasibility of MAIA in vaccine immunity studies.

The specific research objectives were:

1. To develop a multiplex antigen-based microarray assay for rapid and simultaneous detection of antibodies against multiple viral antigens
2. To follow-up RSV, adenovirus and influenza specific immunity in response to natural infection and vaccination with the newly established microarray method
3. To identify RSV, adenovirus and influenza infection, re-infection rates and the duration of protective immunity in early childhood
4. To develop a multiplex microarray immunoassay for the simultaneous detection of IgM and IgG antibodies against influenza A virus antigens
5. To estimate influenza A vaccine induced immunity with the established method and test the suitability of the novel method for diagnostic and research purposes

4 Materials and methods

4.1 Reagents and samples

4.1.1 Clinical specimens (I-III)

In Study **I-II** child serum specimens were used for serological follow-up of respiratory infections by in-house EIA and MAIA. The samples were collected from children participating in a birth-cohort study, called the Steps to the Healthy Development and Well-being of Children (i.e. the STEPS study) (Lagstrom *et al.*, 2013). Children (n=1827) born in the Hospital District of Southwest Finland between 2008 to 2010 were recruited. The samples were collected and tested with the informed consent of the parents. Children were followed-up from birth until 3 years of age. Annually serum samples were collected from each individual at ages 13, 24 and 36 months. The sera were stored frozen at −80°C until tested.

A subgroup of children was followed more intensively until the age of 2 years. Within this cohort, 4728 nasal swabs from 876 children were collected during acute symptoms of respiratory infection episodes and when the infection was considered significant and required the attention of a study nurse or a doctor. Nasal swabs from the children were collected either by trained parents at home or during the study clinic visit. Also, 2270 nasal swab samples were taken at scheduled study clinic visits at 2, 13 and 24 months of age regardless of respiratory illness symptoms. The samples were stored at − 80°C. Respiratory pathogens were detected by PCR.

In Study **I,** we have analysed a series of three serum specimens, collected at 13, 24 and 36 months of age from a total of 291 children. The samples were analysed for anti-RSV and anti-IAV H1N1 vaccine antigen IgG by in-house EIA. From the studied group, 186 children had symptoms of acute respiratory infection and their nasal swab samples were analysed by PCR.

In Study **II** we tested a larger amount of child serum samples against seven virus antigens (RSV, IAV H1N1 vaccine antigen, IAV H1N1, IAV H3N2, IBV Victoria, IBV Yamagata, and AdV-C5 hexon protein) simultaneously by MAIA. Serum samples collected from 13 months of age (n=768) and 24 months of age (n=714) children were analysed. The subgroup that included 13- and 24- months

samples from 291 children analysed in Study **I** by EIA was also tested by MAIA to validate the method.

In Study **III** an adult cohort of individuals vaccinated with pandemic influenza A H1N1 virus vaccine was analysed. A cohort of healthy volunteers was vaccinated between January and August 2010 in Tampere, Finland by intramuscular injection of Pandemrix™ (GlaxoSmithKlein, Rixenart, Belgium) vaccine. We collected serum samples at (day 0), four weeks (day 28) and 6 months (day 180) after the vaccination and determined anti-IAV H1N1 vaccine antigen antibody endpoint titers by three methods: HI, EIA, and MAIA. Virus-specific IgM and IgG antibodies were detected by EIA and MAIA in full series of three serum samples from 60 individuals.

4.1.2 RSV and influenza viruses (I-III)

RSV subgroup A (Randall strain) whole virus was used as an antigen in EIA and MAIA in Studies (**I-II**). The prototype of RSV was initially obtained from the Diagnostic Service Unit of the Department of Virology, University of Turku. RSV was propagated in Vero cells with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 10mM HEPES and 10 μg/ml gentamicin. The virus was grown for four days until an extensive cytopathic effect was observed. RSV antigen collection and purification were performed as described previously (Meurman, Waris and Hedman, 1992).

In Studies (**II-III**) several influenza A and B viruses were used as antigens in MAIA. Influenza virus strains that circulated in Finland during the epidemic seasons in 2009-2012 were grown for antigen production. A/Turku/10/2009 (H1N1), B/Finland/51/2011 Victoria-like lineage and B/Finland/58/2011 Yamagata-like lineage were propagated in MDCK cells, and A/Finland/208/2012 (H3N2) was propagated in MDCK-Siat cells with DMEM supplemented with 0,2% BSA, 5% TPB, TPCK-Trypsin, HEPES, fungizone and antibiotics. The viruses were partially purified through a sucrose cushion pelleting (30% w/w) and ultracentrifugation at 25600 rpm for 90 minutes at +4°C (Beckman, rotor SW41).

All virus antigen pellets were resuspended in a small volume of phosphate-buffered saline, pH 7.3 (PBS), aliquoted and stored at -60℃. Produced and partially purified RSV and influenza viruses antigens were further verified by Western blotting with Coomassie blue staining. The antigen concentrations were measured with Pierce™ BCA protein assay kit (Thermo Scientific™).

In Study **III,** H1N1pdm09 (A/California/07/2009 strain; provided by the National Institute for Health and Welfare, THL, Finland) vaccine virus was propagated in chicken eggs. The virus was utilized as an antigen in the HI test to estimate influenza vaccine-induced immunity in the cohort of vaccinated adults.

4.1.3 Influenza A H1N1pdm09 vaccine (II-III)

In Studies (II-III) we used pandemic influenza A H1N1pdm09 vaccine antigen (Pandemrix, GlaxoSmithKline Biologicals S.A., Germany). The vaccine is a monovalent AS03 adjuvanted vaccine and is composed of split influenza virus, inactivated and equivalent to A/California/7/2009 (H1N1)v-like strain (X-179A).

In Study III the cohort of adult volunteers was vaccinated with one dose of H1N1pdm09 Pandemrix vaccine. Also in Studies (II-III) H1N1pdm09 vaccine was used as a source of capture antigen in EIA and MAIA. Initially, the vaccine was concentrated with Amicon 10K filter centrifugal concentrator tubes (Merck Millipore) using PBS and according to the manufacturer guidelines.

4.1.4 HAdV type C05 hexon protein (II)

In Study II, hAdV type C05 hexon protein was used as a capture antigen in MAIA. The hAdV type C05 hexon protein was obtained from a previous research project at the Department of Virology, University of Turku. Originally, the hAdV C05 prototype was kindly provided by the Centres for Disease Control and Prevention (Atlanta, GA, USA). The virus was propagated in Hela cell culture and hexon protein was purified by anion-exchange chromatography as previously described (Waris and Halonen, 1987).

4.1.5 Controls and detection antibodies (I-III)

In Studies (II-III) purified human IgG (hIgG) (Sigma-Aldrich, St.Louis, MO) and rabbit anti-hIgG (Thermo Scientific, Rockford, IL, USA) were used as positive controls in MAIA. Human serum albumin (HSA), (Sigma-Aldrich, St.Louis, MO) was used as a negative control in MAIA. In Study III, additionally purified human IgM (hIgM) (Jackson ImmunoResearch Laboratories Inc.) was used as a positive control.

Polyclonal rabbit anti-human IgG/HRP (Dako, Denmark; Studies I-III) and polyclonal rabbit anti-human IgM/HRP (Dako, Denmark; Study III) were used for the determination of human IgG and IgM, respectively in EIAs.

The anti-hIgG coated green-emitting UCNPs (upconverting nanophosphors; Er-UCNP-anti-hIgG; Studies II-III) and the anti-hIgM coated blue-emitting UCNPs (Tm-UCNP-anti-hIgM; Study III) was used for the detection of human IgG and IgM, respectively in MAIAs. The green-emitting NaYF4:Yb^{3+},Er^{3+} (Er-doped UCNP; 30–40 nm) and blue-emitting NaYF4:Yb^{3+},Tm^{3+} (Tm-doped UCNP; 35–40 nm) UCNPs were synthesized as described earlier (Kale *et al.*, 2013, 2016; Ylihärsilä *et al.*, 2013).

Polyclonal rabbit anti-human IgG (Dako, Denmark) and polyclonal rabbit anti-human IgM (Dako, Denmark) were conjugated onto the surface of Er-UCNPs and Tm-UCNPs, respectively as has been described before (Kale *et al.*, 2016).

4.2 Assay methods

4.2.1 Enzyme immunoassay (I-III)

In-house enzyme immunoassay was used for the detection of anti-RSV IgG antibodies (in Studies **I-II**) and anti-H1N1pdm09 vaccine antigen IgM and IgG antibodies (in Studies **II-III**). The enzyme immunoassay principle is shown in **Figure 6**.

In Study **I**, anti-RSV IgG antibodies were analysed in 867 child serum samples diluted 1/300 (samples collected at 13, 24 and 36 months from 289 individuals) by EIA. In Study **II**, anti-H1N1pdm09 vaccine antigen IgG antibodies were analysed in 576 child serum samples diluted 1/300 (samples collected at 13 and 24 months from 288 individuals) by EIA. In Study **III**, anti-H1N1pdm09 vaccine antigen IgM and IgG antibodies were analysed in 180 Pandemrix-vaccinated adult serum samples (samples collected at day 0 and 28 and 180 days after vaccination from 60 individuals) by EIA. The samples were tested at 1/100, 1/300, 1/1000, 1/3000 and 1/10000 dilutions for IgM detection, and at 1/1000, 1/3000, 1/10000, 1/30000 and 1/100000 dilutions for IgG detection.

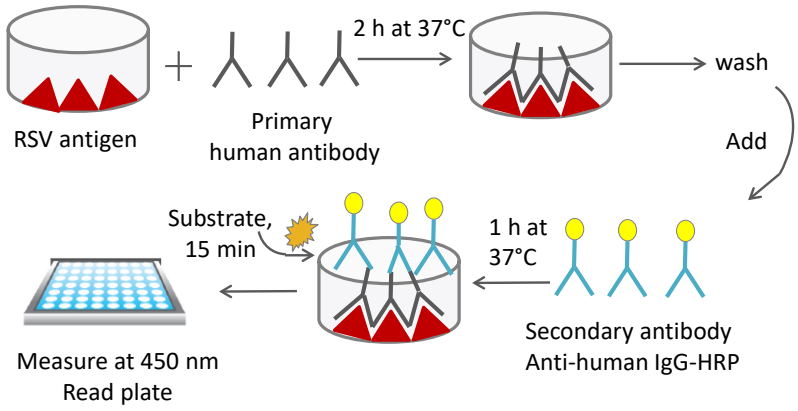

Figure 6. RSV-specific antibodies in the specimen react with solid-phase bound RSV antigen. IgG antibodies bound to the antigen are detected with anti-human IgG labelled with peroxidase catalysing colour formation in a substrate reaction. The intensity of the colour is measured spectrophotometrically at 450 nm.

The virus antigen was dissolved in phosphate-buffered saline (PBS), pH 7.2 (2,5 µg/ml for RSV or 1,25 µg/ml for H1N1pdm09 vaccine antigen, 100 µl/well) and adsorbed onto the wells of polystyrene microtiter plates (Combiplate, 96-well format, Thermo Scientific, USA) at room temperature for 24 h and then stored at +4°C before use. Assay optimisation and stability tests showed that the antigen coated plates (the antigen is under PBS and plates are sealed) can be stored up to 6 months at +4°C without the loss of assay sensitivity and assay quality. Before using the plates were washed once with 400 ul/ well of washing buffer (PBS containing 0.5% Tween 20). Briefly, 100 µl of the 1/300 diluted serum sample in assay buffer containing 5% normal swine serum and 0.5% tween 20 in PBS was added in duplicate to the plate well and incubated for 2 h at 37°C. After the incubation, the plate was emptied and washed row by row for three times. 100 µl/well of either polyclonal rabbit anti-human IgG/HRP (diluted 1/2000 in assay buffer) or polyclonal rabbit anti-human IgM/HRP (diluted 1/1000 in assay buffer) were added to the plate wells depending on the detection of bound serum IgG or IgM antibodies. The plate was incubated for 1 h at 37°C. After the incubation, unbound antibodies were washed out by three times with 400 µl/well of washing buffer. After washing, 100 µl/well of fresh substrate solution (0.3% 1,2-phenylenediamine and 0.02% hydrogen peroxide in citrate-phosphate buffer, pH 5.5) was added and plates were incubated in dark at room temperature during 15 min. The reaction was terminated by adding 100 µl/well of 1 M hydrochloric acid. The absorbance was measured by a multilabel plate reader (VICTOR 3 V – 1420 Multilabel Counter, Perkin Elmer) at 490 nm.

Since there is some inter-assay variation in original absorbance values obtained in individual antibody determinations we standardised the assay by converting the raw absorbance data to EIA units. Each EIA plate included control sera which were prepared as a pool of earlier tested patient sera. Negative control serum is a pool of sera with a result of <1 EIA units. Positive control serum is a pool of high positive serum specimens (100 EIA units). Accuracy control serum is a patient serum with intermediate EIU value (60-70 EIA units). All specimens were tested in duplicates. The raw absorbance values (A_{450}) of samples were converted to EIA units using the Negative Control (denoted as 0 EIA units) and Positive Control (denoted as 100 EIA units) as standards to make the calibration linear plot and to calculate the unit values of individual samples. The positivity cut-off level was calculated as the mean of Negative Control + 3 standard deviations (SD).

4.2.2 RSV detection and typing by PCR (I-II)

Nasal swabs collected from the children with respiratory infection symptoms or during scheduled control clinic visits were suspended in phosphate buffered saline and subjected to automated virus nucleic acid extraction by utilising NucliSense easyMag (BioMerieux, Boxtel, Netherlands) or MagnaPure 96 (Roche, Penzberg, Germany) instrument. Virus RNA extraction was followed by reverse transcription and cDNA amplification using real-time qPCR assay designed for RSV, rhinoviruses, and enteroviruses as previously described (Toivonen *et al.*, 2015; Kutsaya *et al.*, 2016).

In Study **I**, those samples that were RSV positive as detected by initial PCR test were subjected to repeated RNA extraction and RSV-N A/B group typing PCR. Random hexamer primers from the QuantiTect Reverse Transcription kit (Qiagen, The Netherlands) and 5 µl of extracted RNA were used to perform reverse-transcription reactions. The amplification protocol, primers, and probes were described in detail earlier (Hu *et al.*, 2003; Kutsaya *et al.*, 2016).

4.2.3 Multiplex microarray immunoassay (II-III)

Multiplex microarray immunoassay principle and array layout (Study **II**) are shown in **Figure 7**.

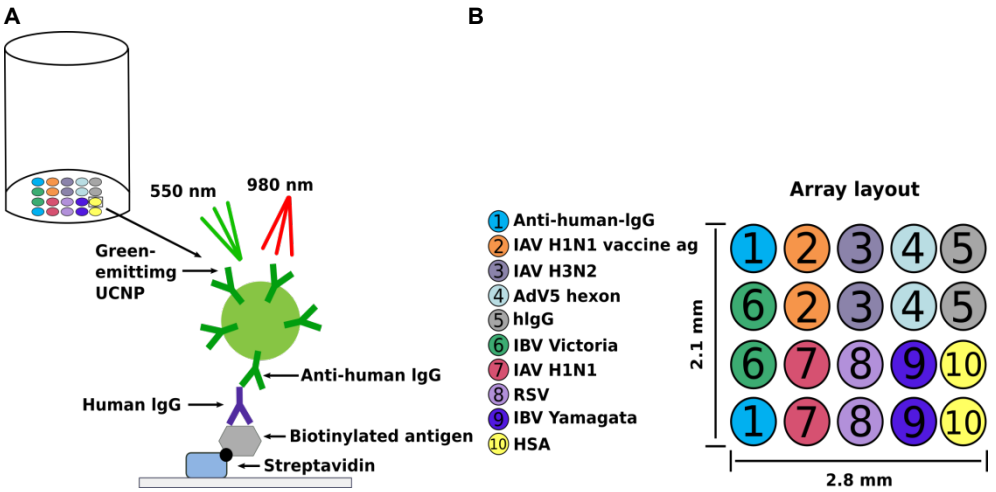

Figure 7. (A) Multiplex microarray immunoassay principle (II). Biotinylated virus antigens and control proteins are printed in array-in-well 4x5 format on streptavidin-coated plates. Human serum IgG antibodies binding to the corresponding antigens and positive controls are detected by secondary anti-hIgG coated UCNPs. Upon 980 excitation by laser diode, green upconversion luminescence is emitted and spots signal intensities are measured by photoluminescence imager. Antibody responses to different antigens are differentiated based on the spot position in the array. (B) Microarray layout with printed in duplicates antigens (H1N1pdm09 vaccine ag, IAV H1N1, IAV H3N2, IBV Victoria, IBV Yamagata, RSV, hAdV type C05 hexon protein), positive controls (hIgG, anti-hIgG) and negative control (HSA) spots.

In both Studies **II** and **III**, virus antigens, as well as control proteins, were biotinylated with biotin isothiocyanate (BITC) as described in detail in Study **II**. The biotinylated virus antigens and control proteins were printed on the bottom of the 96-well streptavidin-coated KaiSA microplates (Kaivogen Co., Turku, Finland) using a non-contact Nano-Plotter 2.1 array printing instrument (Gesim, Germany).

In Study **II**, a multiplex microarray immunoassay for the detection of IgG antibodies against seven virus antigens (H1N1pdm09 vaccine ag, IAV H1N1, IAV H3N2, IBV Victoria, IBV Yamagata, RSV, hAdV type C05 hexon protein) was developed. The biotinylated virus antigens (n=7) and controls (hIgG, anti-hIgG, HSA, n=3) were printed in duplicates forming 4x5 array-in-well consisting of 20 spots. With the MAIA, 1482 child serum samples were screened. MAIA procedure was described in detail previously (Kazakova *et al.*, 2019). Briefly, 50 µl of the 1/100 diluted serum sample in assay buffer was added in duplicate to the pre-washed microarray-containing well and incubated for 2 h at room temperature. After the incubation, the wells were washed three times. A 50 µl/well of anti-hIgG coated UCNPs (Er-UCNP-anti-hIgG) were added to the wells and incubated for 40 min at room temperature with shaking. The wells were washed four times and dried for 2 h before the wells were imaged. For multianalyte detection, an anti-Stokes photoluminescence imager was constructed (**Figure 9**). For the descriptions of the instrument used see below. To evaluate the inter-assay variation and determination of antibody concentrations in MAIA units, negative and positive control samples were included in each plate. The MAIA results were compared to the reference RSV IgG and H1N1pdm09 vaccine ag IgG EIAs for 576 serum samples.

In Study **III**, a multiplex microarray immunoassay was developed for the simultaneous detection of IgM and IgG antibodies against two virus antigens (H1N1pdm09 vaccine ag and IBV Yamagata). The assay principle and array layout are shown in **Figure 8**. Biotinylated virus antigens (n=2) and controls (hIgM, hIgG, anti-hIgG, HSA, n=4) were printed in duplicates forming a 3x4 array-in-well consisting of 12 spots. With the MAIA, 180 Pandemrix-vaccinated adult serum samples were screened. Five serum dilutions (1/100, 1/300, 1/1000, 1/3000 and 1/10000) were added in duplicates in each assay run with the purpose to determine the antibody end-point titre to H1N1pdm09 vaccine ag. The assay was performed as described earlier for MAIA in Study II with the exception that IgM antibodies were detected in addition to IgG antibodies. A 50 µl/well of each type of detection antibodies (Er-UCNP-anti-hIgG and Tm-UCNP-anti-hIgM) were added simultaneously. The plates were incubated for 40 min at room temperature with shaking. After washing and drying two images of each well were produced with anti-Stokes photoluminescence imager at the green 550 nm and blue 470 nm channels. Negative and positive control calibrator sera were included in each plate and used to convert microarray specific signal counts into MAIA IgM and IgG

units. The MAIA results were compared to the reference H1N1pdm09 vaccine ag IgM and H1N1pdm09 vaccine ag IgG EIAs run for the same samples. The cut-off levels and the determination of antibody endpoint titers were described in detail in Study **III**.

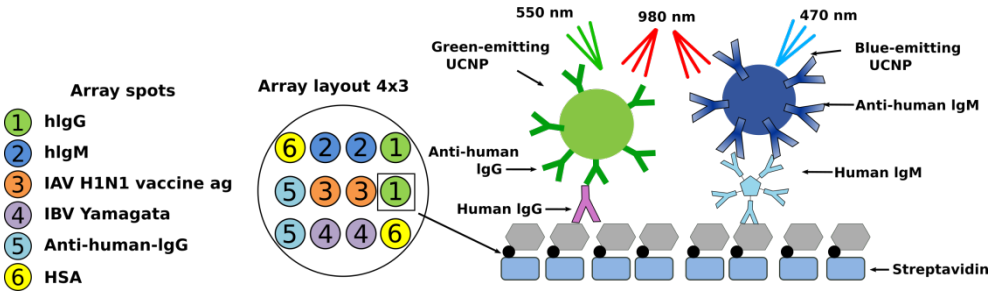

Figure 8. Dual mode (IgG and IgM) multiplex microarray immunoassay principle and array layout. The microarray consists of 12 spots including antigens (H1N1pdm09 vaccine ag and IBV Yamagata), positive controls (hIgM, hIgG, anti-hIgG) and negative control (HSA). Human serum IgM and IgG antibodies binding to the corresponding antigens and positive controls are detected simultaneously by secondary anti-hIgM and anti-hIgG coated UCNPs in the same well. Upon 980 excitation by laser diode, blue and green upconversion luminescence is emitted and spot signal intensities are measured by photoluminescence imager. Antibody responses to different antigens are differentiated spatially based on the spot position. Antibody classes are differentiated spectrally based on blue or green colour of the signal.

The microarray immunoassays (**II-III**) were measured with the microtiter plate reader (Hidex Oy, Turku, Finland) modified for the anti-Stokes photoluminescence imaging (**Figure 9**). The detection is based on the ability of UCNPs to convert low-energy infrared radiation to high-energy visible light. The plate wells were excited with an infrared laser at the wavelength of 976 ± 2 nm with 7 W optical power. The laser beam passes through a long-pass filter and is reflected to the well from a hot mirror. UCNPs emit anti-Stokes photoluminescence which is collected with 50 mm camera lens. Two short-pass filters block scatter laser radiation. The upconversion photoluminescence is imaged using short-pass filters (Chroma Technology, Rockingham, VT, USA). The green-emitting Er-UCNP-anti-hIgG and blue-emitting Tm-UCNP-anti-hIgM are imaged through a 550 nm and 470 nm channels, respectively. The images are produced with a cooled CCD camera with a 50 mm objective lens using the following parameters: 2x binning, 2.2 s exposure time per well. The instrument is described in more detail in a previous publication (Ylihärsilä *et al.*, 2011).

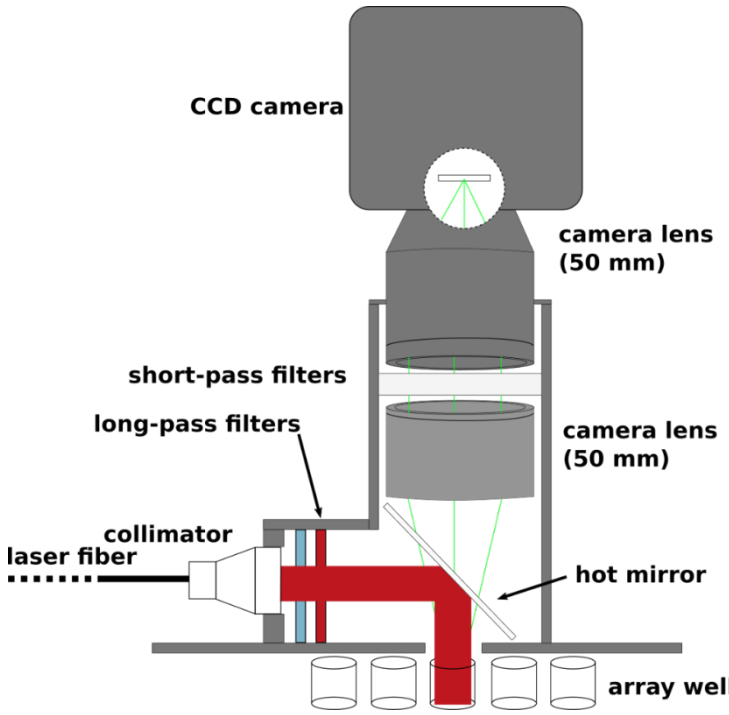

Figure 9. Anti-Stokes photoluminescence imager construction for multianalyte detection in MAIA (II-III) (Modified from Ylihärsilä et. al., 2011).

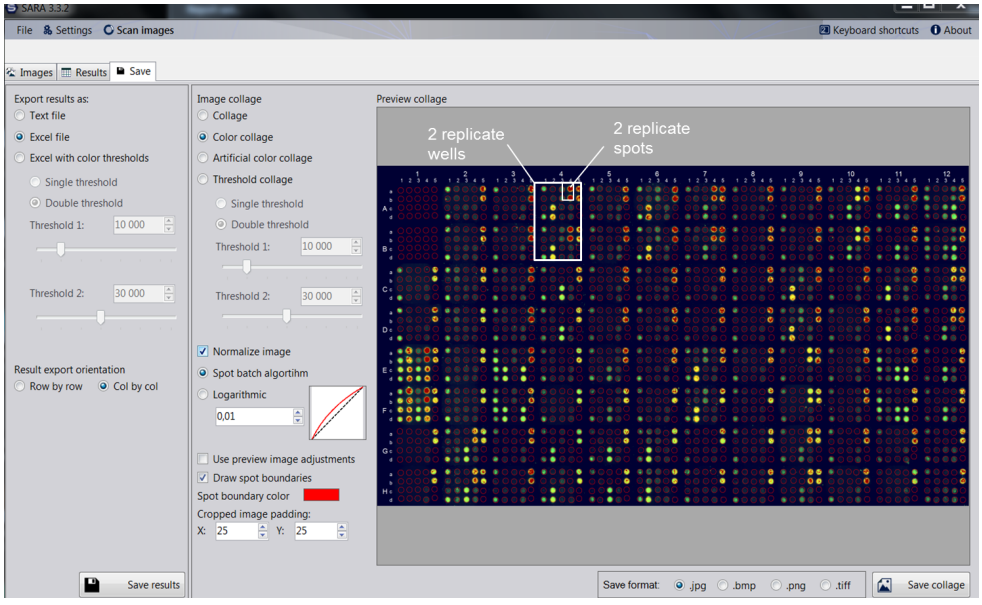

Figure 10. Fluorescent colour collection of MAIA 96-well plate.

UCNPs signal counts were obtained from the microarray well image and spot detection by calculating the mean intensity of the pixels within the spot using ImageJ software version 1.43n (**Figure 10**). The images were saved in 16-bit sif-file format. Since each analyte spot was printed in duplicates and each serum sample was tested in two replicate wells, average signal counts for each analyte were calculated as a mean signal from 2 replicate spots in 2 replicate wells. The specific signal of each analyte spot was calculated in Excel by subtracting HSA spot signal (assay background) from the average signal of the analyte spot.

4.2.4 Hemagglutination inhibition assay (III)

In Study III, serum samples obtained from Pandemrix-vaccinated adults were analysed by HI to determine antibody endpoint titers and vaccination efficacy. The HI test essentially was performed according to WHO guidelines (World Health Organization. and WHO Global Influenza Surveillance Network., 2011). H1N1pdm09 (A/California/07/2009 strain) vaccine virus was used in the HI test. The test was conducted on U-based 96-well microtiter plates using 0.5%/vol turkey erythrocytes. The serum samples were serially diluted from 1/10 to 1/1280. The serum samples with HI titers <10 were assigned a titer value of 5.

5 Results and discussion

5.1 Serological follow-up of respiratory infections in early childhood

In publications **I** and **II**, serological studies were conducted on respiratory virus infections and reinfections in young children. **Figure 11** provides a general description of the number of samples, assay methods and study design. In publication **I**, RSV disease burden in early childhood was analysed. The analysis was based on identifying viral pathogens by RT-PCR from nasal swabs taken during acute infection episodes and detecting anti-RSV IgG antibodies by EIA in child serum specimens taken annually at scheduled visits at the study clinics. We estimated primary RSV infection and reinfection rates in children 0-3 years old. RSV-positive acute respiratory infections (ARIs) were analysed to compare proportions of primary infections and reinfections with RSV group A and B strains. In publication **II**, we described the development of the multiplex microarray immunoassay for the simultaneous detection of IgG antibodies against RSV, adenovirus and influenza viruses. With the established assay we conducted a large serological study in the young children and compared the microarray assay results to reference EIA methods.

5.1.1 RSV enzyme immunoassay optimisation (I-II)

Anti-RSV IgG antibodies were analysed by an in-house EIA in serum samples collected at 1 year, 2 years and 3 years from 291 children. Anti-RSV IgG antibody levels were determined from altogether 873 serum specimens and the seropositivity status of serum samples was determined to estimate RSV disease burden in early childhood. To obtain low background levels of nonspecific binding (OD < 0.200) for seronegative samples and buffer controls, and high binding of RSV-specific IgG antibodies, and to prevent nonspecific adsorption of the anti-IgG conjugate, optimal EIA parameters were invesigated. During the development of the assay following parameters were evaluated: i) antigen concentrations, ii) different serum dilutions, iii) conjugate concentrations.

The plates were coated and tested with the following RSV antigen concentrations: 5µg/ml, 2,5µg/ml, 1,25µg/ml and 0,625µg/ml. The optimal RSV

concentration of 2,5µg/ml (0,25 ug/well in 100 ul) was chosen for the following analysis. Anti-human IgG-peroxidase conjugate has been tested at 1:1000, 1:2000 and 1:3500 dilutions. The conjugate dilution of 1:2000 was chosen.

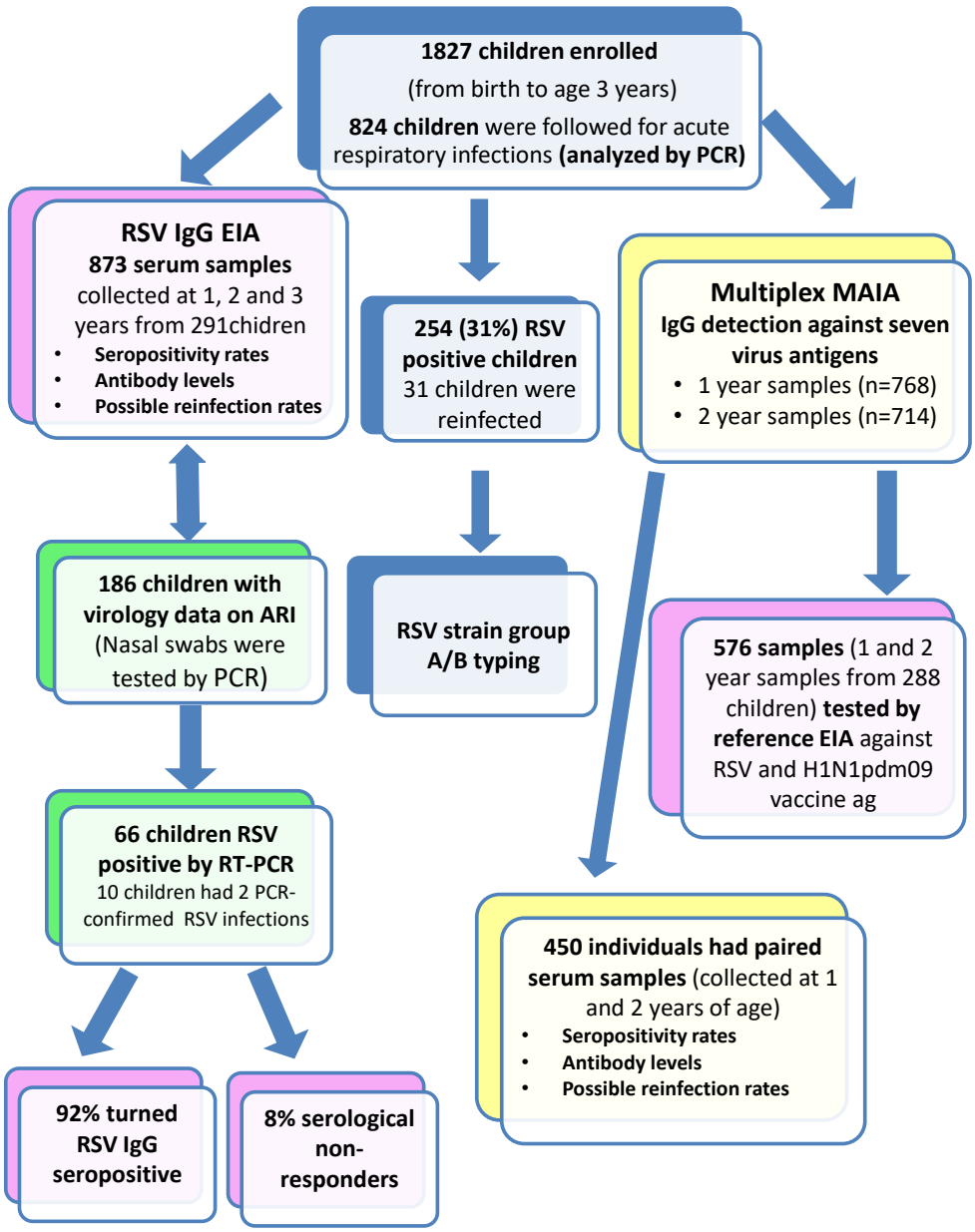

Figure 11. The workflow of respiratory infections follow-up in the birth-cohort of children 0-3 years of age (Studies **I-II**).

Serum specimens of young children contain much lower levels of immunoglobulins than adults and the IgG concentrations gradually increase with increasing age reaching adult values at 5-10 years of age (Johansson SG and Berg, 1967). Since serum IgG levels were considerably lower in the studied very young children as compared to those in adults, we modified the original assay and analysed child sera in lower serum dilutions of 1/300 (**I-II**) in contrast to 1/1000 dilution designed for adults (**III**). In this manner, the absorbance values in the analysis were at a higher level and the dynamic range of the RSV IgG EIA assay became wider and more suitable for analyzing anti-RSV IgG levels in young children (**Figure 12**).

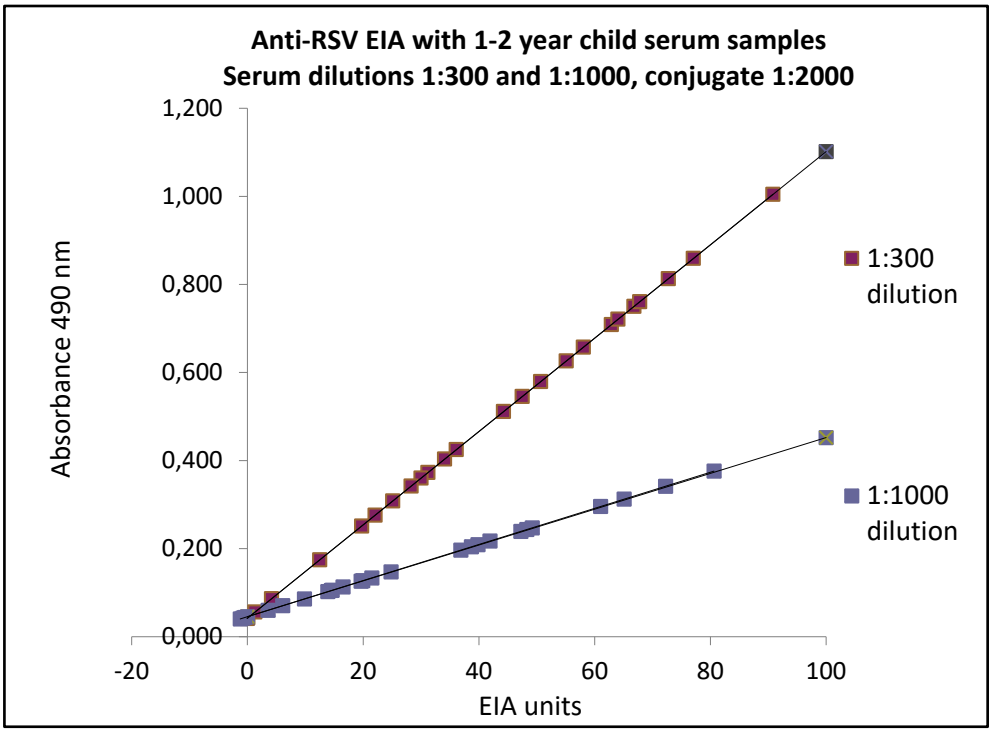

Figure 12. RSV IgG antibody analysis of random STEPS study samples in different serum dilutions by EIA. Serum dilutions of 1:300 and 1:1000 have been used. Antigen coating concentration 2,5 ug/ml (0,25 ug/well in 100 ul) and anti-human IgG-peroxidase conjugate dilution of 1:2000 has been used.

Table 2 describes the interassay variation in 25 individual anti-RSV IgG EIA determinations and **Figure 13** describes the variation of an intermediate (60-70 EIA units) control sample between the assays. The percentage of the coefficient of variation (CV%) of absorbance values was 30% for high positive control specimens and 16% for negative control specimen. The mean EIA IgG unit values

for the intermediate positive sample were 64, SD was 9 and CV% 13. Thus the conversion of original absorbance values to EIA IgG unit values reduces the variation significantly and makes the comparison of the data from one assay to another more reliable.

Table 2. Inter-assay variation characteristics of anti-RSV IgG determinations.

Plate no.	Pos ctrl Absorbance	Neg ctrl Absorbance	Cut-off Absorbance	Intermediate ctrl IgG units
1	0.748	0.048	0.099	55
2	0.765	0.063	0.133	75
3	0.822	0.065	0.121	71
4	0.673	0.085	0.149	65
5	0.445	0.072	0.118	50
6	0.833	0.065	0.136	64
7	0.538	0.074	0.125	75
8	0.544	0.084	0.133	77
9	0.550	0.099	0.146	74
10	0.818	0.074	0.111	72
11	0.915	0.084	0.144	52
12	0.748	0.075	0.129	62
13	0.789	0.075	0.113	70
14	0.837	0.075	0.141	57
15	1.052	0.077	0.137	76
16	1.216	0.073	0.129	74
17	1.168	0.068	0.111	66
18	0.901	0.074	0.130	72
19	1.019	0.085	0.140	46
20	1.328	0.094	0.155	78
21	1.444	0.105	0.168	67
22	0.975	0.069	0.174	66
23	1.037	0.077	0.134	69
24	1.189	0.079	0.138	68
25	1.312	0.094	0.170	63
Mean	0.882	0.075	0.131	64
SD	0.261	0.012	0.018	9
CV %	30	16	14	13

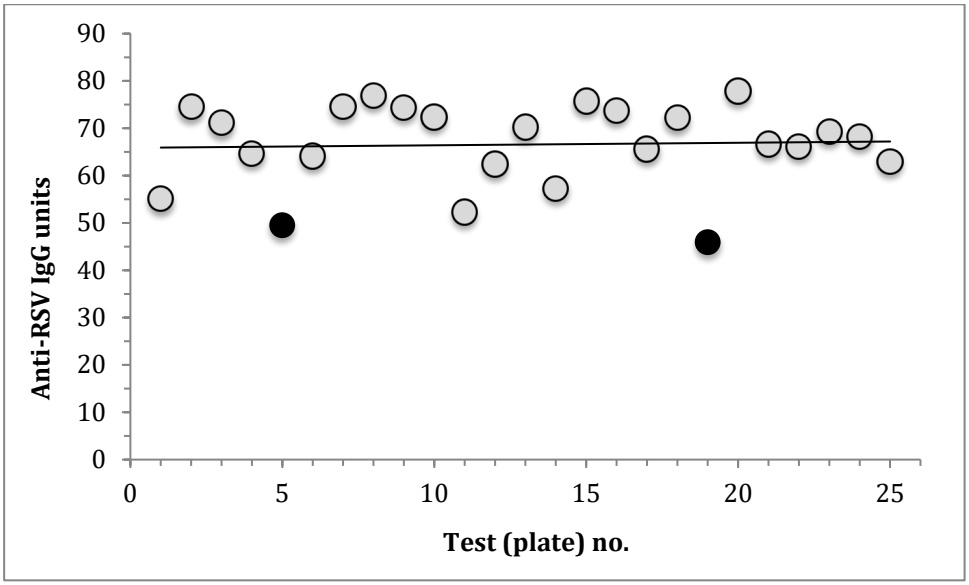

Figure 13. Interassay variation of anti-RSV IgG determination. Intermediate control of pooled serum samples included in each test. The mean ± SD was 64 ± 9 with a CV of 13%. Black dots indicate values outside the acceptance criteria of expected value ± 20%.

Since respiratory infections in children are common and many infection episodes remain subclinical or mild, clinically recognized infections do not allow a reliable estimation of the disease burden of any respiratory infection including RSV infection. Thus analysing the anti-RSV-IgG serostatus of young children provides a better estimate of RSV disease burden than the identification of clinical infections with PCR detection of RSV nucleic acids. However, since in young children the immune system is not fully developed, some children may show weak or undetectable induction of anti-RSV antibodies during RSV infection. The individual variation in the humoral immune response is generally known to be wide. In our study material it was possible to compare the RSV PCR-positivity and serum anti-RSV IgG levels (at 13 and 24 months) in 186 children of whom nasal swab specimens had been collected during the first 2 years of life. Children with a previous positive RSV PCR test obtained at different times (at ages 1 to 12 months of age) before the collection of the 13 months serum specimens showed clearly higher EIA IgG unit values as compared to those collected from RSV PCR negative children (**Figure 14**). However, many PCR negative children were RSV seropositive. All individual test series included the seronegative control sample (0 unit value) and three seronegative child sera. The cut-off for RSV seropositivity was determined as the mean absorbance value of negative control sera and 3 seronegative child sera + 3 SD units. In most assays the sample was considered

seropositive if the RSV IgG EIA unit values were higher than 5. In different assays the seropositivity cut-off value corresponded to EIA unit values between 4 and 7, depending on negative controls values and SD.

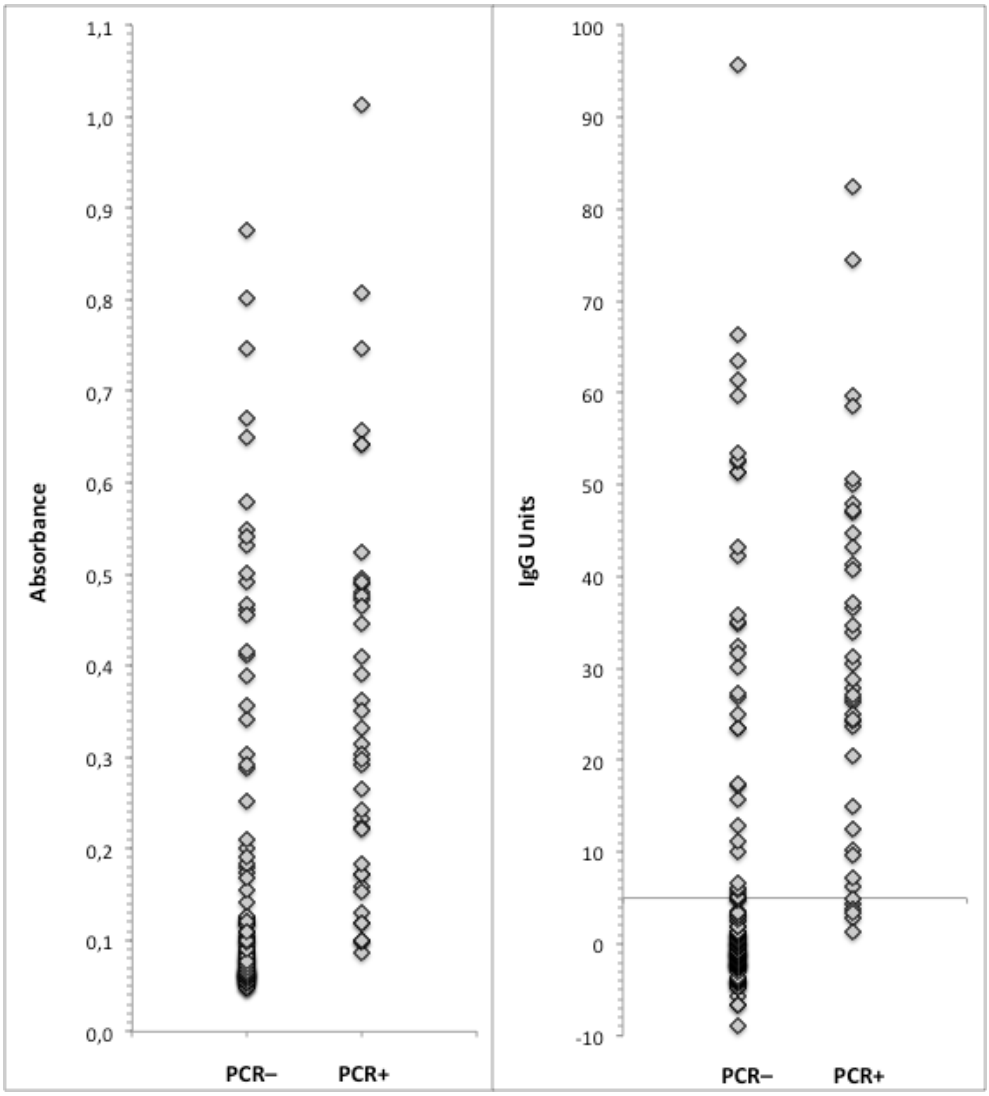

Figure 14. Distribution of serum anti-RSV IgG EIA values in 13 months old children with or without a history of PCR confirmed RSV infection (N=186). PCR analyses were done at different times (1-12 months) before the collection of serum samples. For IgG units, the X-axis is set at an arbitrary cut-off level of 5 units.

There were very few samples from PCR positive children that showed values lower than 5 EIA IgG units and they were very young children of less than 9 months of

age. The overall analysis of serum specimens indicated that serum specimens collected from those children whose nasal swab samples had been RSV nucleic acid positive at some point before sample collection showed EIA unit values higher than 5. Further statistical analyses strongly support the concept that a previous RSV infection detected by RT-PCR strongly correlates with seropositivity (**Figure 15**).

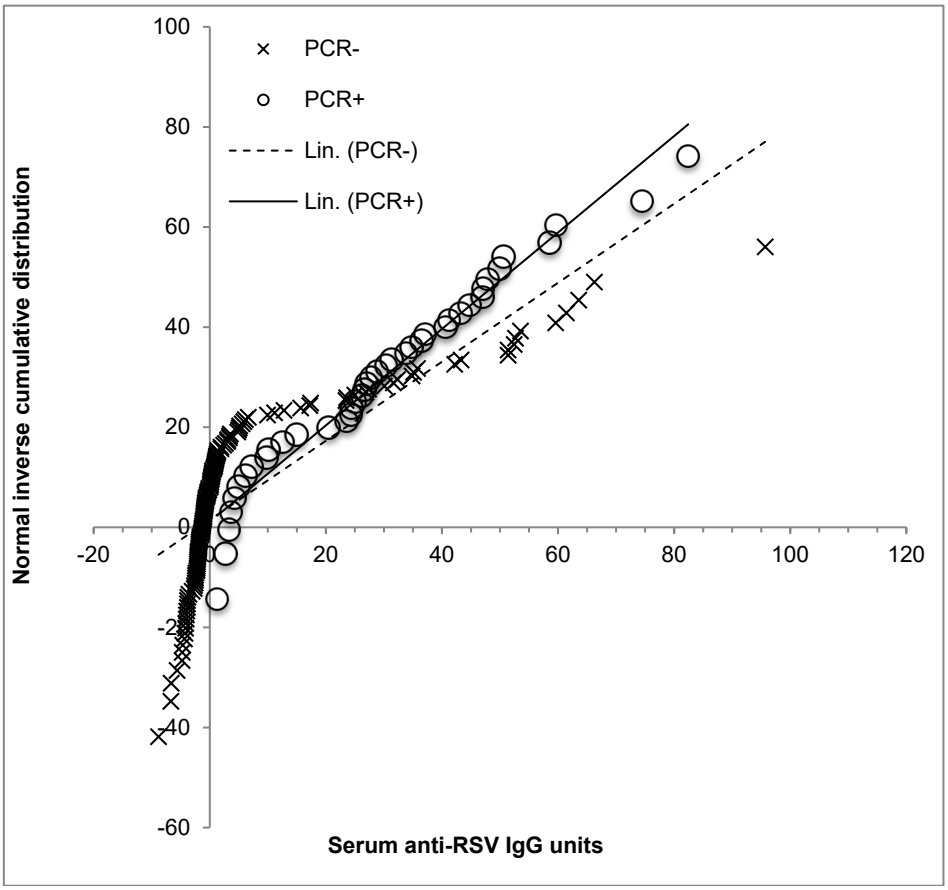

Figure 15. Normal distribution analysis of serum anti-RSV IgG EIA values in 13 months old children with or without a history of PCR confirmed RSV infection (N=186).

ROC curve analysis and statistical compilation of the EIA unit values also show that with a seropositivity cut-off of 5 EIA units the sensitivity and specificity of the assay are excellent (**Figures 16 and 17**). The data strongly indicates that EIA values higher than 5 are seropositive with very high certainty, values from 1 to 5 are border line positive/negative and values less that1 unit are considered seronegative.

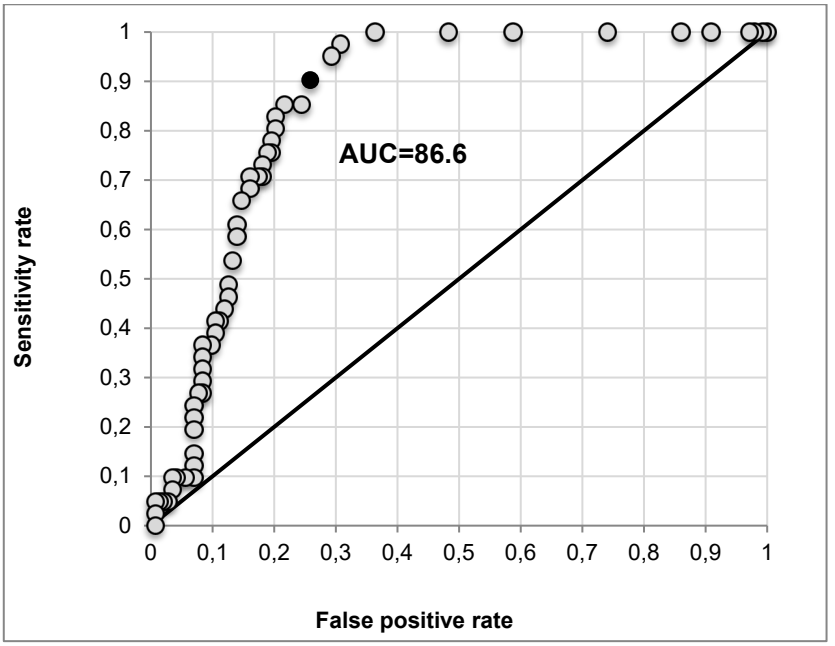

Figure 16. ROC curve of serum anti-RSV IgG EIA values in 13 months old children with or without a history of PCR confirmed RSV infection (N=186).

The sensitivity indicates the rate of anti-RSV IgG detection in a child with a history of PCR confirmed RSV infection. False positive indicates the detection of anti-RSV IgG in a child without a history of PCR confirmed RSV infection. Data points were calculated for all possible cut-off IgG values with 1 unit interval (descending from left to right). The black dot indicates the cut-off value of 5 units.

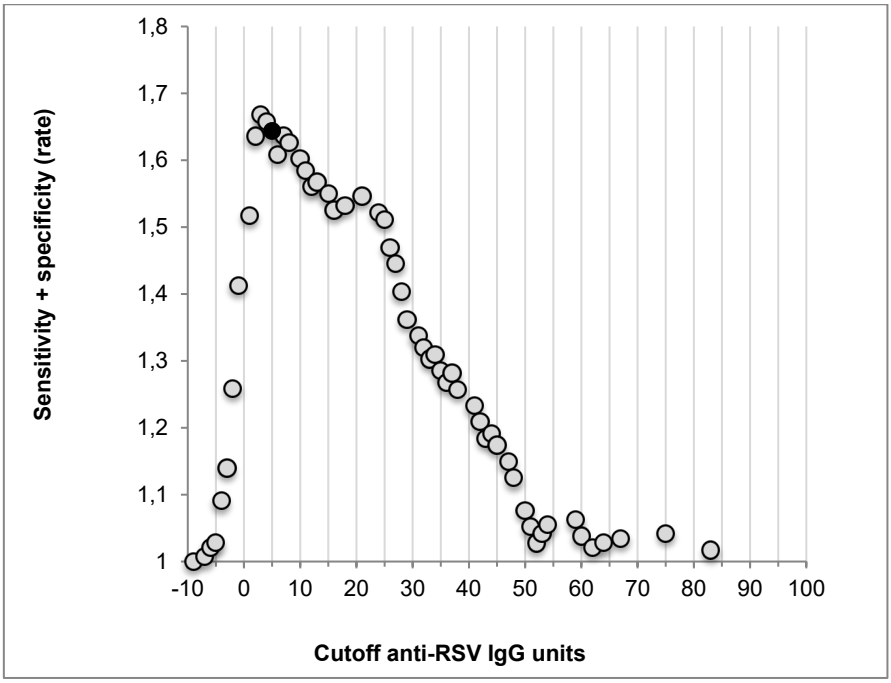

Figure 17. Effect of cut-off on the anti-RSV IgG assay performance in 13-month old children (n=186). Sensitivity and specificity were calculated against an earlier RSV-PCR result. The black dot indicates the cut-off value of 5 units.

5.1.2 RSV primary infection and reinfection rates (I-II)

Study I revealed that RSV infections are very common in early childhood and many children suffer from repeated RSV infections during their first years of life. We analysed anti-RSV IgG levels in the children at 1, 2 and 3 years of age and estimated seropositivity rates, reinfection rates, mean RSV IgG levels after primary and repeated infections and the rate of RSV IgG antibody decline.

Based on the positive anti-RSV IgG EIA result we determined RSV seroprevalence in the studied population. **Figure 18** shows anti-RSV IgG seropositivity and reinfection rates in serially collected serum samples from 291 children. We found that 37% of children (n=109) were RSV IgG seropositive by the age of 1 year. RSV seropositivity increased to 68% (n=198) at the age 2 years and to 86% (n=250) at the age 3 years. Out of 291 children, only 35 individuals (12%) remained RSV seronegative during the 3 years of follow-up. The discrepancy between the overall number of children and finally seropositive/seronegative children comprised 6 individuals who were initially seropositive but turned seronegative by 3 years of age.

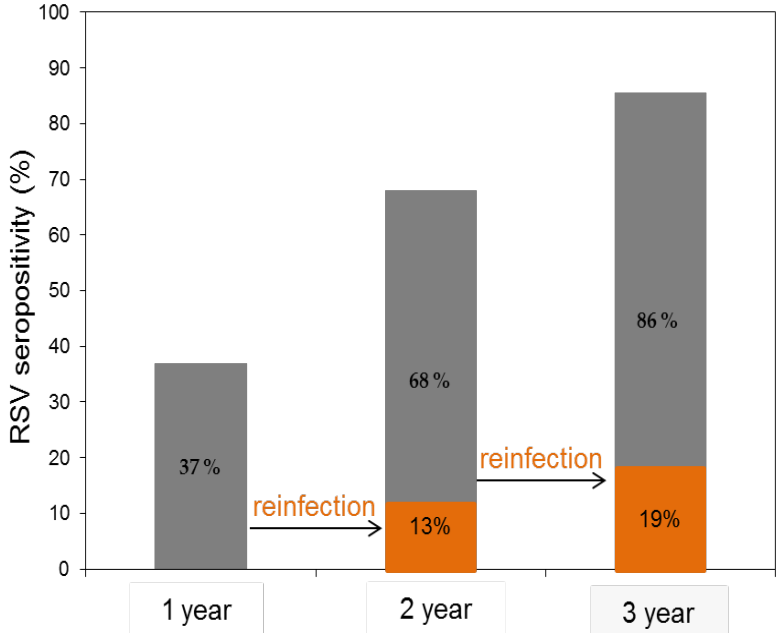

Figure 18. RSV IgG seropositivity and reinfection rates by the age of children (n=291).

Our findings indicate a relatively high RSV disease burden during the first year of life. Moreover, a high proportion of children were reinfected. A case of reinfection was defined when a rise in IgG levels of >25 EIA units was observed between the serial samples. Of children who were seropositive by the age of 1 year (n=109), 40 individuals were likely reinfected (13% out of 291). The estimated RSV reinfection rate in seropositive children between 0-2 years was 19% (out of 291) by the age of 3 years.

Out of 109 children seropositive at the age of 1 year, 65 children (60%) were finally reinfected by the age of 3 years. One individual among them acquired two RSV reinfections. Out of 95 children who acquired primary RSV infection between 1 and 2 years of age, 30 (32%) children became reinfected by the age of 3 years.

Our serological findings on the high RSV primary infection and reinfection rates are supported by a clinical study conducted in the same study group (Studies **I-II**) and are consistent with previous studies (W. Paul Glezen *et al.*, 1986; Hall *et al.*, 1991; Law, Carbonell-Estrany and Simoes, 2002).

Among this cohort (n=291), 186 children were monitored for acute respiratory infections (ARI) and provided a nasal swab sample during the infection episode. A symptomatic RSV infection was detected at least once in 66 (35%) of the 186 children during the 0-2 years follow-up. Of the 66 RSV RT-PCR positive individuals, 92% developed anti-RSV IgG antibodies. Only five (8%) very young

children did not demonstrate anti-RSV IgG response. An RSV-positive ARI was documented twice in 10 children and three times in one individual. A rise in RSV IgG antibodies of >10 EIA units was revealed in those individuals. Our serological findings indicate that there was a high rate of mild or asymptomatic RSV infections.

Out of 923 children followed more intensively for respiratory tract infection, data on acute respiratory tract infections were received from 824 children. Most of ARIs were caused by rhinovirus (63.5%). However, our study revealed RSV to be the most common viral cause of medically attended visits for ARIs in young children.

At least one symptomatic RSV infection was detected in 254 (31%) of 824 children at the age of 0-24 months (Toivonen *et al.*, 2019). Among 254 children, 223 had only one infection, 27 children had one reinfection and 4 children had two reinfections. Toivonen and colleagues reported an estimated rate of 37 RSV infections per 100 children per year. The reported RSV infection rate was higher than in other studies among children aged <3 years (Nokes *et al.*, 2008; Simpson *et al.*, 2016; Heikkinen *et al*., 2017). However, our serological findings support this data and show a high rate of subclinical RSV infection within the first 3 years of life.

The clinical study conducted by our colleagues shows that RSV infections are associated with substantial morbidity. Even though the RSV-associated hospitalization is very low (1-2%) as documented in many studies (Forster *et al.*, 2004; Iwane *et al.*, 2004; Simpson *et al.*, 2016; Scheltema *et al.*, 2017; Toivonen, 2019), the majority of RSV infections require treatment at home or at outpatient clinics. The study of our colleagues (Toivonen *et al.*, 2019) demonstrated that RSV infections in this child cohort were more severe than other ARIs, with an acute otitis media being diagnosed more often (44%) and a high rate of prescribed antibiotics. Of 824 children 188 (23%) had physician visits due to RSV infection and 7 (0.8%) very young children (<11 months of age) had RSV-associated hospitalizations. The frequency of RSV infections increased with age from 10-16% before 5 months of age, to 32% at the age of 6-11 months, and to 42% at the age of 12-24 months (Toivonen *et al.*, 2019).

RSV-positive ARI samples were identified as either group A or group B by RSV-N A/B group typing by PCR (Study **I** and unpublished data). We have typed samples from 248 RSV positive individuals (In 6 cases the determination failed due to a low amount of viral RNA in the samples). The results are presented in **Figure 19**.

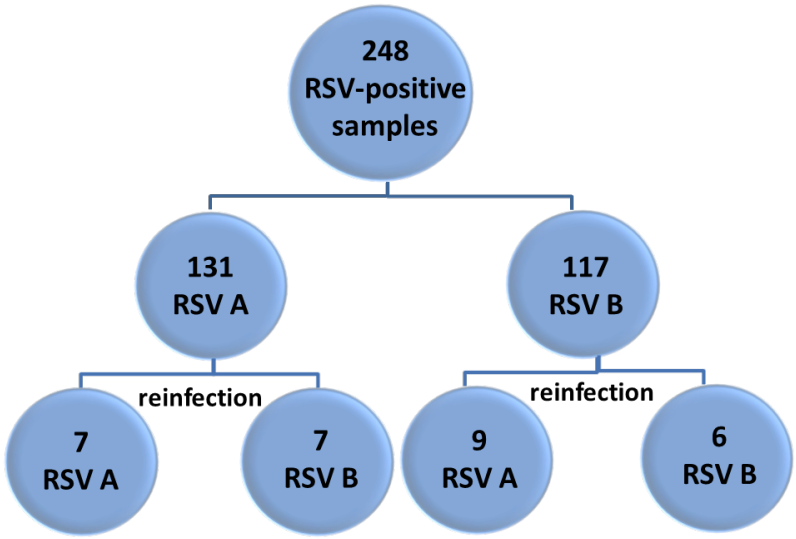

Figure 19. An RSV-positive ARI samples typing in 248 children.

Among 248 children with primary RSV infection, 131 and 117 children were infected with RSV group A and RSV group B, respectively at the age of 0-24 months. Out of 248 children, reinfection was documented in 29 children (12%). There were a few cases with three infections but some samples were missing or the sample quality was inadequate for virus RNA determination. Of 131 children with RSV group A infection being their primary infection, seven had a group B and seven had a group A infection during the reinfection episode. Of 117 children with a group B infection as their primary infection, six had a group B and nine had a group A reinfection. Our data suggest that both strains are equally pathogenic and reinfections occur with either homologous or heterologous strains. However, there have been suggestions that RSV A viruses are more prevalent and slightly more pathogenic compare to RSV B viruses (Melero and Moore, 2013). Moreover, RSV neutralizing antibodies were reported to be highly group-specific, so the incorporation of strains or antigens of both RSV groups (A and B) in future RSV vaccine may be optimal (Charles J. Sande *et al.*, 2013).

5.1.3 The development and validation of multiplex microarray immunoassay (II)

In study **II**, a multiplex antigen-based microarray-in-well immunoassay (MAIA) was developed for seven virus antigens. The assay was developed for simultaneous detection of IgG antibodies against partially purified IAV H1N1, IAV H3N2,

influenza B virus (IBV) Victoria, IBV Yamagata, and RSV whole viruses, purified AdV-C5 hexon protein, and IAV H1N1pdm09 vaccine antigen.

Initially, the produced viruses and available antigens were tested in EIA with no-sample, two negative, two low positive, two medium positive and two high positive child serum samples. Chess board titration method was applied to determine the optimal antigen, conjugate and serum sample dilutions. Then the antigens were biotinylated and tested in EIA on streptavidin-coated plates with a series of serum samples. Thereafter multiplex immunoassay was developed with the biotinylated antigens. First, biotinylated antigens were tested in a singleplex whole well assay format where one antigen was plated to cover the whole well surface. Then, the microarray 5x4 format was designed including 7 antigens and controls. The microarray immunoassay results were compared to the reference EIA assays at all stages.

During the multiplex microarray immunoassay development different manufacturer's streptavidin-coated 96-well plates were tested for their suitability in MAIA tests. White flat bottom 96-well streptavidin-coated KaiSA microplates (Kaivogen Co.,Turku, Finland) were used in further experiments for which the best signal to background ratio and better spot morphology were observed. To optimise the printing conditions different printing buffer compositions, concentrations of virus antigens and blocking buffers were used (data not shown). The visual examination of array spots were performed during microarray images analysis. The spot signal was excluded from the mean antigen signal in case of one inadequate spot out of four spot replicates (two duplicate antigen spots in two duplicate wells). If more than one antigen spot was printed with inadequate morphology a sample test was repeated.

In the preliminary experiments, weaker spot signals and suboptimal spot morphology were obtained with new partially purified whole virus antigens in contrast to previously used AdV-C5 hexon protein. The reason for that appeared to be excessive biotinylation and free unbound biotin in the antigen stock solution. Therefore, either the antigens with insufficient performance were further purified with Amicon Ultra 10K centrifugal filter device (Merck Millipore) or newly biotinylated antigens were produced. Also, different incubation times, at different temperatures (RT or at +37°C), with or without shaking were tested to achieve higher signal intensities in the assay. We extended the incubation time with serum samples at RT until 2 hours in contrast to the previous assay protocol.

Despite all modifications and improvements to obtain higher signal intensity, the bottleneck was in the high assay background. Since the multiplex immunoassay was developed to detect IgG antibodies in very young children <3 years of age, it required to be highly sensitive to detect low levels of immunoglobulins and discriminate negative samples from samples with low positivity. For high

specificity and for low background in MAIA test different concentrations and compositions of the assay buffer were evaluated. The addition of 1% FCS to the Kaivogen assay buffer gave the highest ratio between the IgG positive signal and IgG negative signal values. The hypothesis was that very young children have anti-bovine antibodies due to high milk consumption. Bovine serum albumin was used for plate blocking and apparently child serum IgG antibodies tend to bind to the plate and cause non-specific background.

The performance of the developed MAIA has been demonstrated in **Figure 20**. The array well without serum samples shows reagent background. Only assay buffer containing secondary antibodies was added to that well. Fluorescent signals come only from hIgG control spots which were positive control spots for binding of secondary antibodies labelled with UCNPs. With a negative serum sample signal from hIgG control spots obtained along with anti-hIgG control spots. When the positive control serum sample was included, all antigens and control spots except HSA emitted green light due to the detection of bound serum IgG antibodies. A test serum sample with anti-RSV and anti-hAdV5 hexon IgG antibodies gave a signal from the corresponding antigen spots. Fluorescent colours were related to signal intensity: green colour corresponded to 7000-35000 signal counts, yellow to 35000-45000 and red colour to 45000-65000 signal counts. The signal measured from the HSA spot indicates the assay background and it corresponded to approximately 5000 signal counts. Antigen specific signals were calculated by subtracting the HSA signal from the average signal of viral antigen spots. The specific signal was converted to MAIA units by linear correlation with negative and positive controls.

With the established method, we tested 768 serum samples from 1-year-olds and 714 serum samples from 2-year-olds (Study **II**). To validate the MAIA method we compared the results to reference EIA where IgG antibodies to RSV and H1N1pdm09 vaccine ag were determined in 576 samples from 1 and 2 years old children (n=288). We conducted a receiver operating characteristic (ROC) curve analysis to estimate MAIA performance and to determine the cut-off values for antibody positivity (**Figure 21**). The sensitivity and specificity of the assay were calculated against an earlier EIA result. The intersection of the sensitivity and specificity lines indicated the optimal ratio and cut-off value of 12 and 8 units for RSV and IAV H1N1 vaccine ag, respectively. The cut-offs for other antigens were calculated as the means of negative controls plus 6 SD.

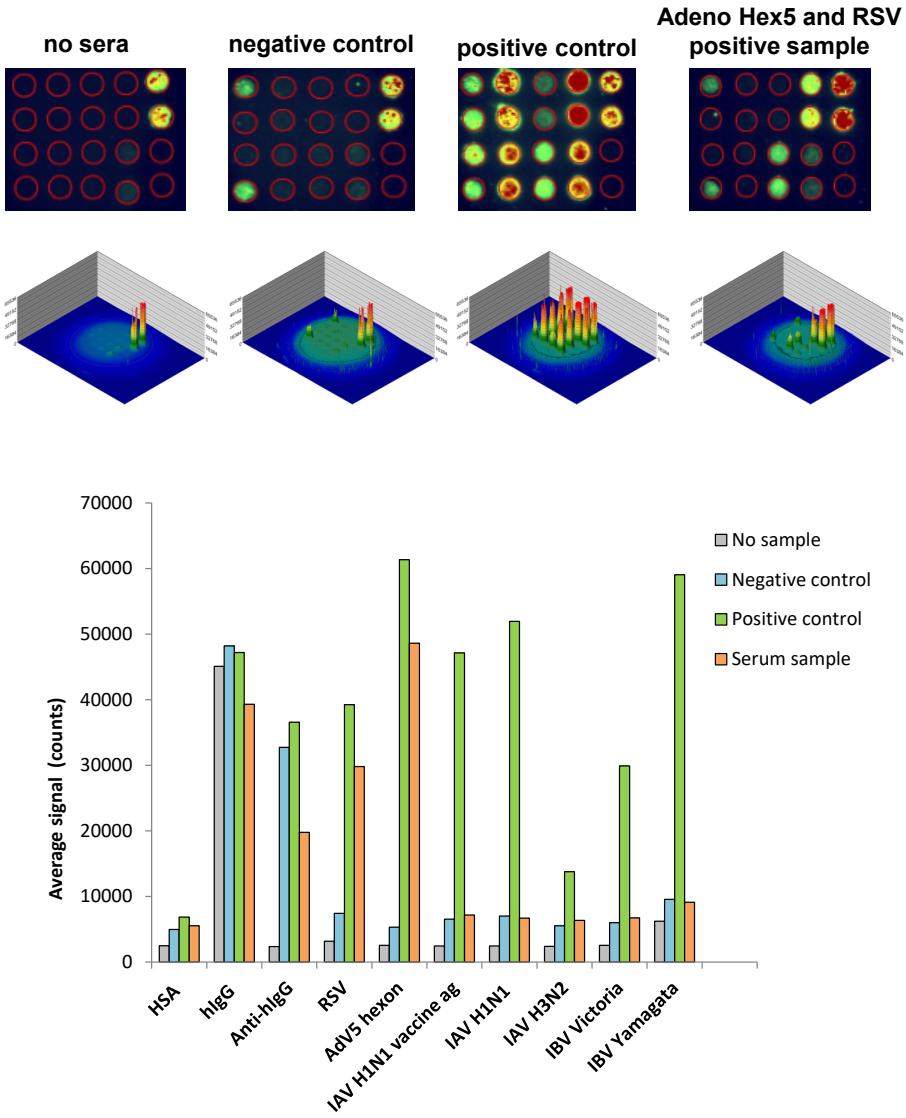

Figure 20. Fluorescent array-in-well images, their 3D models and signal intensity counts.

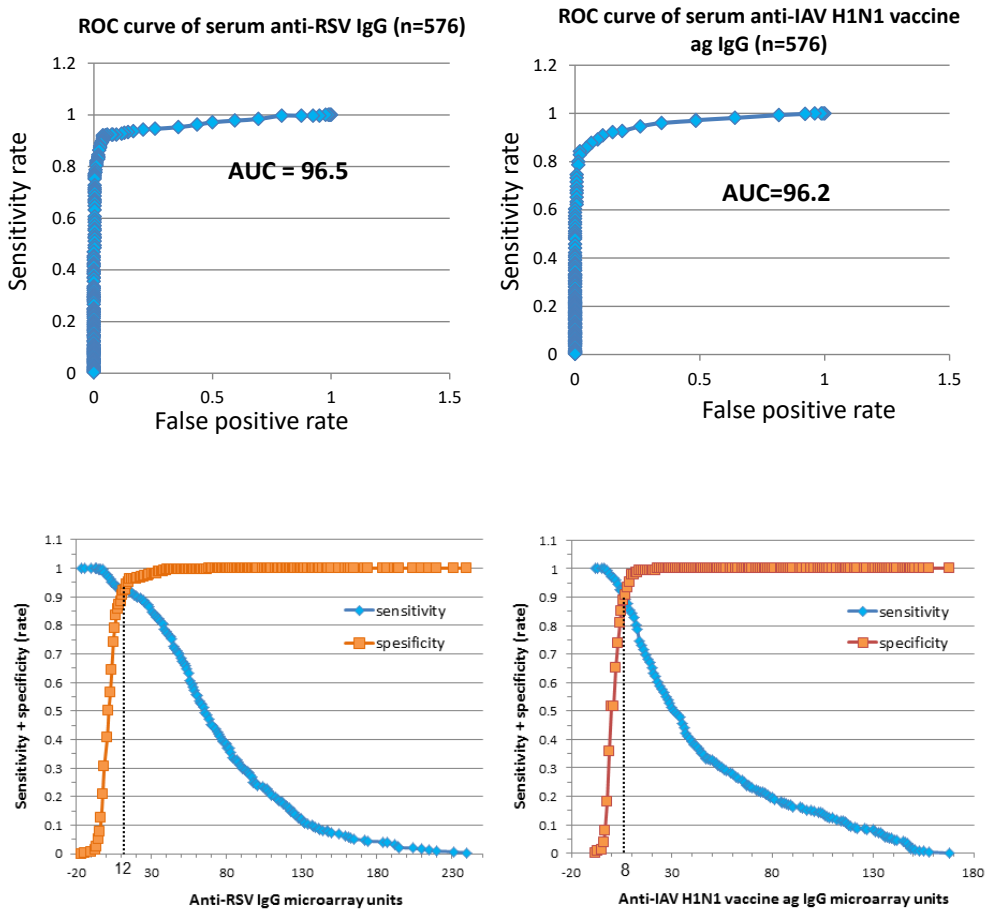

Figure 21. ROC curves and the effect of cut-off on anti-RSV IgG and anti-IAV H1N1 vaccine ag MAIA performance in 1-2 year old children (n=576).

At age 1 year, 37% (n=106) and 38% (n=109) children had anti-RSV IgG antibodies as detected by EIA and MAIA, respectively. By the age of 2 years, seropositivity rates increased to 68% (n=196) and 69% (n=199) as detected by EIA and MAIA, respectively. Anti-H1N1pdm09 vaccine ag IgG seropositivity rates detected by EIA and MAIA were also at similar levels: 58% (n=167) and 57% (n=164) in 1-year-olds, and 83% (n=240) and 72% (n=290) in 2-year-olds, respectively. A high degree of correlation was observed between EIA and MAIA results which confirm that MAIA is well suitability for serological studies. MAIA has advantages compared to EIA based on its ability to detect antibodies against several antigens simultaneously in one well. MAIA saves the antigens, serum sample volumes and the labour time. Gageldonk has estimated that the multiplex

immunoassay is cost-effective compared to enzyme immunoassay when more than three different antigens are measured in one assay (Gageldonk *et al.*, 2008).

5.1.4 Serological follow-up by multiplex microarray immunoassay in young children (II)

In publication **II**, we have estimated the seropositivity rates by MAIA against seven virus antigens in serum samples collected from 1 (n=768) and 2-year-old (n=714) children (**Figure 22**).

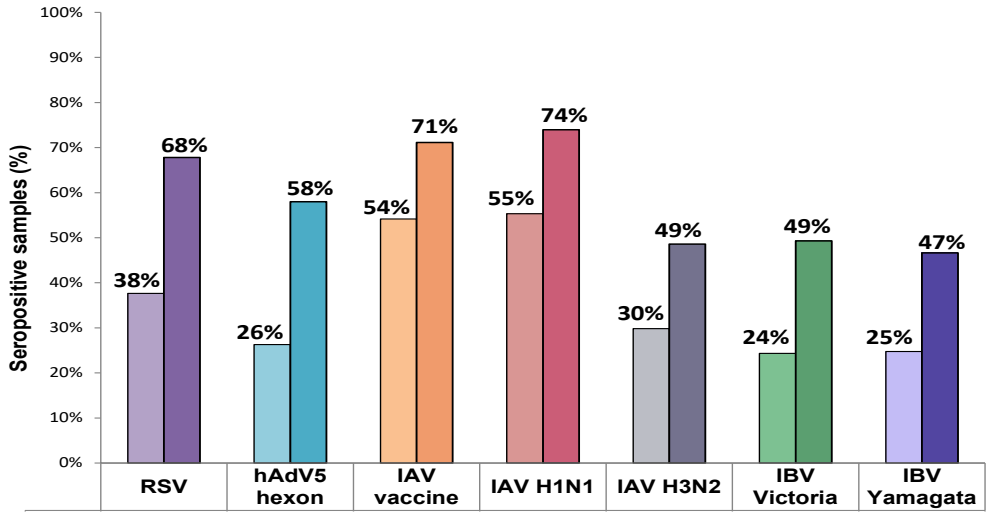

Figure 22. Seropositivity percentage for RSV, AdV-C5 hexon protein, IAV H1N1pdm09 vaccine antigen, IAV H1N1, IAV H3N2, IBV Victoria and IBV Yamagata in 1 year (lighter colour) (n=768) and 2 year (darker colour) (n=714) serum samples by MAIA.

Seropositivity rates against all antigens increased 1.3-2.07 fold by 2 years of age. Interestingly, very good agreement was found when comparing the results from RSV seropositivity rates detected by MAIA in a larger children group (>700 individuals) against the previously detected seropositivity rates by EIA in a cohort of 291 individuals (Study **I**). Based on the serology data, RSV infection was observed at least in 38% of children during their first year of life and in 68% by the age of 2 years. The findings validate that MAIA with multiple analytes detection agrees extremely well with a single analyte detecting EIA.

The seroprevalence against other respiratory virus antigens and IAV H1N1 vaccine ag were also relatively high during the first year of life and increased against all antigens by the age of 2 years. We thoroughly analysed antibody levels during 1 and 2 year against each antigen and estimated possible reinfection rates.

Mean antibody levels against IAV H1N1pdm09 vaccine ag, IAV H1N1, IAV H3N2, and IBV Yamagata stayed at a rather similar level between 1 and 2 year samples indicating that there were likely no reinfections. In addition, a low rate of influenza infections was detected by PCR, when our colleagues analysed nasal swabs taken at the onset of acute respiratory infections in this study group (Teros-Jaakkola *et al.*, 2017). Even though the study children were potentially exposed to influenza viruses during 3 epidemic seasons, the incidence of influenza infection was 2.7-5.1% and influenza cases were mostly non-severe in 0-2 years old children. Nevertheless, that can be explained by the high influenza vaccination coverage in the study population. In their study, Teros-Jaakkola and co-workers (2019) found that 22–47% of 6-23 month-old children were vaccinated with a seasonal influenza vaccine and 80% with IAV H1N1pdm09 vaccine (Pandemrix) in the epidemic season 2009–2010 (time line when 1 year samples were collected). High vaccination coverage was supported by the MAIA data since we observed 54% and 71% seropositivity rates against IAV H1N1pdm09 vaccine ag at the age of 1 and 2 years. The vaccine effectiveness against pandemic influenza was claimed to be 97% in this study group (Teros-Jaakkola *et al.*, 2019). The results from this serological study by MAIA (Study **II**) and the Teros-Jaakkola *et al.*, (2019) data support the conclusion that the vaccine against the pandemic influenza was highly effective in young children.

In contrast to influenza, RSV infections caused a high disease burden in the studied children. The incidence of medically attended RSV infection was consistently higher than that for influenza. The rate of RSV primary infections and reinfections remained high throughout the first 2 years of life and the infection frequently required healthcare visits. Among the MAIA tested samples, 450 paired 1 and 2 year samples from the same individuals were selected and analysed for the possible reinfection rates. Of the selected children, 38% (n=170) acquired the primary RSV infection during the first year of life and 40% were likely reinfected (n=68) by the age of 2 years. In the course of this work, we discovered that children who developed ~ 60% higher antibody level after primary infection were likely protected from reinfections. Very young children (< 1 year) developed lower antibody levels against respiratory viruses. We found that when reinfection occurs, children develop significantly higher antibody levels. High RSV disease burden in young children emphasizes the need for RSV vaccines and antiviral drugs.

5.2 Simultaneous measurement of influenza A virus-specific IgM and IgG antibodies by multiplex microarray immunoassay in Pandemrix vaccinated adults (III)

In Study **III**, we describe the development of a multiplex microarray immunoassay for the simultaneous detection of IgM and IgG antibodies in serum samples against two virus antigens (IAV H1N1pdm09 vaccine ag and IBV Yamagata). We investigated the feasibility of MAIA method in vaccine studies and analysed pandemic influenza A(H1N1pdm09) vaccine induced humoral immunity. Virus-specific antibodies are commonly regarded as markers of partial or complete immunoprotection however, most studies concentrate on IgG antibody class when the immunogenicity of a vaccine is investigated. We determined two classes of antibodies (IgM and IgG) in serum samples obtained from adult individuals before and after vaccination with a pandemic influenza A H1N1pmd09 vaccine. Healthy volunteers aged 18-65 years (n=60, median age 23 years) were investigated for antibodies against H1N1pdm09 vaccine antigen by HI, EIA, and MAIA. Altogether 180 serum samples from 60 individuals drawn before the vaccination (day -3 - 0) and on an average of four weeks (day 28) and 6 months (day 180) after the vaccination were analysed by each of three methods.

MAIA was developed to measure viral antigen-specific IgM and IgG antibodies against several analytes simultaneously in one assay well. The detection was based on secondary anti-human IgM and IgG antibodies coated on the surface of two types of UCNPs (Tm-UCNP-anti-hIgM and Er-UCNP-anti-hIgG). The ability of UCNPs to emit photoluminescence at different wavelengths allows the simultaneous detection of different serum immunoglobulin classes. In a previous study (Kale *et al.*, 2016), the spectral cross-talk and cross-reactivity between UCNPs were tested and they were found to very low of non-existing.

We have adapted the assay method for measuring antibodies against IAV H1N1pdm09 vaccine antigen and partially purified IBV Yamagata. As we analysed influenza A vaccine-induced antibody responses, the influenza B virus was included in the microarray as an additional control to confirm that the rise of antibodies is solely vaccine antigen-specific. The presence of one UCNP-conjugate type in a single well did not interfere with the measurement of another UCNP-conjugate type. **Figure 23** shows that there was no cross-reactivity between the secondary antibodies and both IgM and IgG antibody classes can be reliably detected simultaneously.

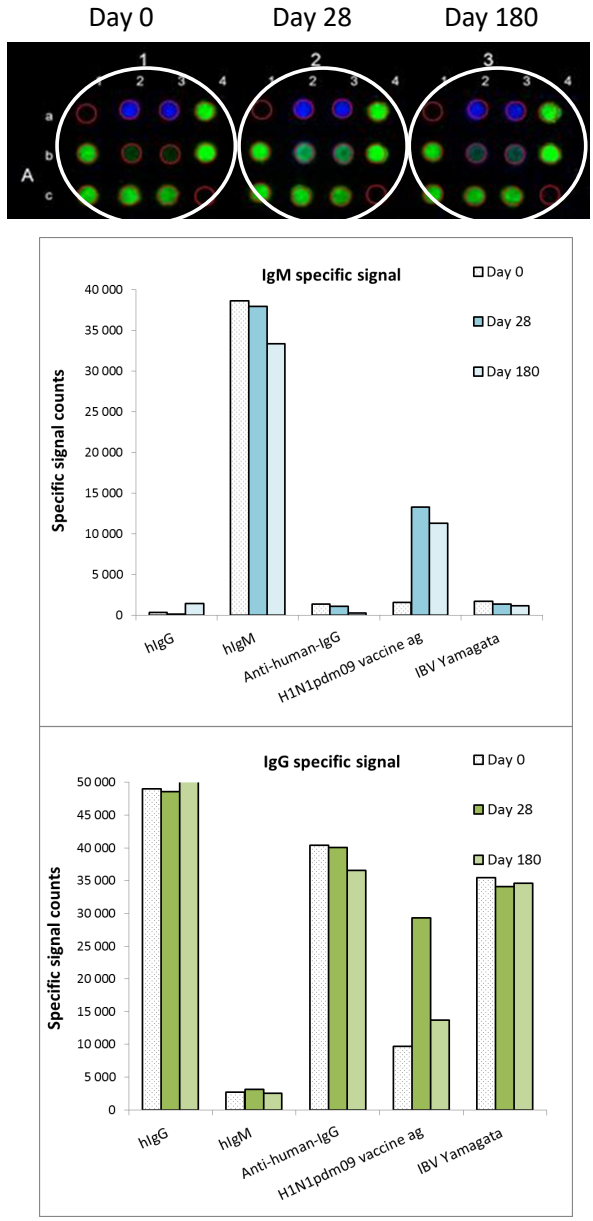

Figure 23. Fluorescent microarray-in-well images and IgM and IgG specific signal counts of samples at day 0, day 28 and day 180 before and after the vaccination from the same individual.

To validate the MAIA assay we performed EIA and determined anti-H1N1pdm09 vaccine antigen IgM and IgG antibody levels and endpoint titers in 60 individuals. Serum samples were tested at 5 dilutions and endpoint titers were calculated with 4

Parameter Logistic (4PL) curve. EIA absorbance was converted to EIA IgM and IgG units and MAIA specific signal counts to MAIA IgM and IgG units. The unit values were calculated from the linear plot using negative and positive control calibrator samples in each assay run. The detailed description of the assays and seropositivity cut-off values has been provided in publication **III**.

We observed practically no pre-existing IgM antibodies before the vaccination. There was a significant rise in IgM (8.9 to 12.2-fold) and IgG (2.9 to 4.2-fold) antibodies against H1N1pdm09 vaccine antigen in EIA and MAIA, respectively, in four weeks after the vaccination, confirming vaccine-induced immunity. A rise in the mean IgM and IgG antibodies against IBV Yamagata was not observed in post-vaccination samples. Anti-IBV Yamagata IgM antibodies were at a low level and pre-existing IgG antibodies stayed at an equally high level of 123 MAIA units in all three samples before and after the vaccination with influenza A vaccine. EIA and MAIA showed a strong agreement in both IgM and IgG unit values and endpoint titers (Figures are published in Study **III**).

In the HI assay, we tested 180 serum samples in serial dilutions and determined endpoint titers before and after vaccination. We found a statistically highly significant positive correlation between IgM endpoint titers determined by EIA or MAIA and HI titers ($p<0.001$). IgG endpoint titers determined by EIA or MAIA also correlated very well with the HI titers ($p<0.001$). Four weeks after the vaccination, a significant increase in geometric mean titers was observed in each of the three assays (**Table I**, Publication **III**). The study by Trombetta *et. al* (2018) has also shown a good positive correlation between EIA and "classical" assays for immunological response to influenza A and B strains (HI, SRH and VN) (Trombetta *et al.*, 2018).

The HI test is commonly used in influenza vaccine studies and it has proved to correlate well with clinical protection. It is generally considered, that HI titers of ≥40 provide protection against severe influenza illness. Based on this knowledge, we calculated the corresponding theoretical protection titers in the EIA and MAIA, and seroprotection rates by all three methods. In publication **III**, we compared endpoint titers and seroprotection rates before and after vaccination with IAV H1N1pdm09 vaccine as measured by the HI test, EIA and MAIA. Before the vaccination, 8 individuals (13%) showed an antibody titre of at least 1:40 in the HI test. Four individuals (7%) demonstrated theoretical "protective" IgM antibody titre by EIA and MAIA. Before the vaccination protective IgG antibody titers were documented in 11 (18%) and 12 (20%) of the individuals by EIA and MAIA, respectively. It has been shown in different age groups that some individuals, especially the elderly people had pre-existing humoral immunity to H1N1pdm09 influenza virus before the pandemic (Ikonen *et al.*, 2010). Since our study group was recruited between December 2009 and September 2010 when the pandemic

was ongoing, pre-existing immunity can indicate either a recent infection or cross-reacting antibodies induced by previous influenza A virus infections. At four weeks after vaccination, our theoretical seroprotection rates (**Table II**, Publication **III**) increased and they ranged from 90 to 98% as determined by MAIA, EIA and the HI test. Six months after the vaccination, in 80% individuals (by HI test) and in 73-83% (by EIA and MAIA) antibodies remained at theoretically seropotective level.

We showed that the H1N1pdm09 vaccine induced very high seroprotection rates against the H1N1pdm09 virus in adults. As some previous studies show, seasonal influenza vaccines can induce little or no cross-reactive antibody response to new emerged influenza viruses (Hancock *et al.*, 2009). When an antigenetically very different new influenza virus emerges (e.g. a pandemic of a novel seasonal influenza strain) it is important to estimate which fraction of the population has pre-existing immunity and evaluate the need and potential efficacy of seasonal vaccines. Since the immunoprotection against influenza virus is preferentially mediated by antibodies it is essential to follow-up influenza-specific antibody levels in the population. At present, the most common and widely used method for anti-influenza antibodies is the HI assay. The HI test is frequently used in diagnostic and research laboratories but it has certain limitations. HI assays are insensitive to agglutinate certain avian influenza A virus hemagglutinins and cannot detect human antibody responses to avian influenza viruses. Even high antibody titers after avian influenza infection or vaccination are failed to be detected by HI test (Rowe *et al.*, 1999; Nicholson *et al.*, 2001; Stephenson *et al.*, 2009). Beside this, inherently the reading of the HI test is subjective and the assay shows a large variation in HI endpoint titers between different laboratories (Hobson *et al.*, 1972; Wood *et al.*, 2012). In Study **III** we developed a multiplex immunoassay that has certain advantages compared to EIA and HI test in clinical vaccine trials and serosurveillance studies. The simultaneous detection of virus specific IgM and IgG in a single serum specimen tested at one dilution is a valuable tool in the diagnosis of virus infections and vaccine immune responses.

6 Conclusions

Serological studies are widely used to diagnose viral infections and to measure vaccine immune responses. Multiplexing technology is a globally emerging trend while enzyme immunoassay may stay as a conventional assay for certain more specific serodiagnostic applications. In the present study we developed a less time and labour consuming and cost effective multiplex immunoassay to carry out a large-scale serological follow-up of common respiratory infections in early childhood as well as simultaneous detection of anti-influenza IgG and IgM antibodies in an adult vaccination cohort. The study verifies the advantages and the potential of multiplex microarray immunoassay in large scale serosurveillance and vaccine immunity studies.

The main conclusions are as follows:

1. RSV accounts for a substantial proportion of respiratory infections in the child population. The burden of RSV infection is particularly great among young children aged <3 years. RSV reinfection rates are high and anti-RSV antibody levels decrease relatively rapidly. Young children 0-2 years are an important target group for the development of RSV vaccines and antiviral drugs.
2. The multiplex microarray immunoassay described in the present study is a specific, sensitive and rapid method for the simultaneous detection of multiple anti-viral antibodies in serum samples. We proved that the correlation of MAIA and EIA data is very high and therefore the MAIA is a promising alternative for the detection of antiviral antibodies in large-scale immunosurveillance studies.
3. A great advantage of the MAIA is the possibility to determine antibodies to several virus antigens in a single microtiter plate well with small sample volumes and use very small amounts of antigens as compared to EIA. The performance of the MAIA was shown to be more cost-effective than EIA.

4. We observed a high respiratory virus infection burden in very young children as evidenced by seropositivity in the MAIA assay. The rates of symptomatic infections, with the exception RSV, were relatively low based on virus-specific PCR assay. We can thus assume that a high proportion of infections are asymptomatic or relatively mild and the real rate of infection tends to be underestimated by PCR diagnostics. The lack of influenza A virus positive samples was partially explained by a high influenza vaccination coverage, which likely provided good protection against influenza A virus infection.
5. We developed an MAIA method for the simultaneous detection of IgM and IgG antibodies against several antigens in a single well. Our study demonstrated the ability of MAIA to effectively measure influenza A virus vaccine-induced IgM and IgG responses. MAIA data correlated very well with the ones of conventional EIA and HI assays and showed that MAIA is a good alternative to measure vaccine-induced immunity in large-scale vaccine studies.
6. The MAIA method has the potential to be used in large scale epidemiological and seroprevalence studies to simultaneously detect immune response against multiple viral antigens and to differentiate acute infection with IgM positivity from past infection with only IgG positivity. In addition, the method can likely be used for detecting vaccine-induced immune responses basically to any vaccine antigen. MAIA is a new highly useful tool for diagnostics, seroepidemiological and vaccine immunogenicity studies that provides a lot of information on humoral immunity in a cost-effective manner.

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