Comparative analysis of anti-measles antibody detection of laboratory techniques:

Immunoserological assay development for the assessment of long-term measles/MMR vaccine efficacy with practical and theoretical benefit

Thesis (PhD)

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LIST OF ABBREVIATIONS

Abbreviation	Phrase
anti-CS	Anti-citrate synthase
anti-F4	Anti-dsDNA topoisomerase I fragment F4
AUROC	Area Under the Curve Receiver Operating Characteristics
BG	Background
HIT	Herd immunity threshold
ICPs	Immunological correlates of protection
IDSA	Infectious Diseases Society of America
IgM RB	IgM reducing buffer
IUPAC	International Union of Pure and Applied Chemistry
IVIg	Intravenous Immunoglobulin
LOD	Lower limit of detection
LOQ	And limit of quantification
MeV	Measles virus
mIU	Mili international unit
MMR	Measles, mumps, rubella
nAAb	Natural(auto)antibody
NIBSC	National Institute for Biological Standards and Control
NSB	Non-specific binding
OD	Optical density
OP	Operational protocol
PEG	Polyethylene glycol
PRNT	Plaque reduction neutralization test
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
R_0	Basic reproduction number
RA	Rheumatoid arthritis
SAIDs	Systemic autoimmune diseases
SD	Standard deviation
SLE	Systemic lupus erythematosus
SSc	Systemic sclerosis
VPD	Vaccine-preventable diseases
WB	Washing buffer
WHO	World Health Organization

1 SYNTHESIS

Infectious immunity has an outstanding importance today. Vaccination remains one of the safest and most effective interventions available in public health for the primary prevention of infectious diseases, resulting in both direct and indirect (herd immunity) immunity in individuals vaccinated (1–3). Even though in Europe a safe and effective two-dose measles/MMR vaccination schedule has been made available since the 1960s, the maintenance of high vaccine coverage is still difficult (4–7). Despite the fact that in Hungary the measles (nowadays MMR) vaccine is mandatory since 1969, and consequently the vaccine coverage is estimated at 99% (WHO), vaccination-group specific immunization gaps may exist (8–15). Suboptimal vaccine effectiveness in certain vaccination -or age- groups has a negative impact also on overall vaccination coverage. The small-scale 'measles outbreak of Makó and Szeged' (2017) confirmed that certain measles vaccines - applied during the early phases of the Hungarian vaccination history –, failed to elicit the desired immunological response. The resulting immunization gap(s) raise the concern of potential further outbreaks (10,15). Screening for immunity and effectiveness of vaccination against infectious diseases has increasing importance in the design of preventive public health strategies, especially today, when prompt testing is also emphasized by the ongoing COVID-19 pandemic. Measles has been already an issue worldwide, yet being aggravated by disrupted immunization protocols due to the COVID-19 burden (4–6,16). Immunity gaps arising from suspended immunization activities are an ominous precursor to a measles resurgence (4). Measles is extremely communicable with the basic reproduction number (R_0) estimated at 12-18 (compared to 2.9-3.4 of COVID-19, for example). Accordingly, we have developed a robust, time-saving, cost-effective and standardized 'triple' immunoserological assay for simultaneous detection of anti-measles, -mumps, and -rubella IgG antibodies in human sera. Since our test has been optimized for the screening of suboptimal antibody titers, it is able to operate reliably in the low measurement range, therefore can be readily used to delineate susceptible individuals and gaps of immunological protection.

In addition to the practical benefit, the present study also has a theoretical importance; comparison of adaptive antibody levels with natural (auto)antibody (nAAb) levels that for a long time have been thought to lack the capacity of dynamic adaptation. We intended to find quantitative data for a new approach in vaccination; natural autoantibodies may play a role in efficient vaccination (95), and the unforeseen benefit of immunization may lie in the potential enhancement of natural antibody pool (17). For this reason, we compared vaccine- (or pathogen-) induced antibody levels (elicited by the historical measles/MMR vaccine, with >99% of current vaccination coverage, strengthened by epidemics in the early decades of vaccination) with nAAbs (anti-citrate synthase [anti-CS]), anti-DNA topoisomerase I F4 fragment [anti-F4]) and double stranded DNA [anti-dsDNA], of patients with systemic autoimmune diseases (SAIDs) as SLE, RA and SSc. Simultaneously, we also investigated the potentially impaired memory B cell functions in autoimmune diseases by comparing the population-level anti-measles IgG antibody titers to that of patients with SAIDs.

2 BACKGROUND

2.1 Resurgence of measles cases

Despite the existence of an effective measles vaccine, resurgence in measles cases in the United States and across Europe has occurred, including in individuals vaccinated with two doses of the vaccine (18). Regarding EU/EEA countries, ECDC states that a large pool of measles susceptible individuals may be present, owing to low historical and current vaccination coverage. Among this group, there are unvaccinated children and teenagers born in the EU/EEA after 1999. A high burden of measles is seen among infants and adults. Almost half (45%) of all measles deaths were reported in infants. The continued potential of importations, which can worsen existing outbreaks or start new ones in communities where immunity gaps persist (19). ECDC emphasizes that the most important intervention is to ensure a high quality routine immunization program, reaching 95% vaccination coverage at subnational level. It is also important to increase the opportunities for checking vaccination status and offer vaccination as appropriate, and to offer supplementary immunization activities to close immunity gaps in older populations (20).

Despite the fact that in Hungary the MMR vaccine is mandatory and consequently the vaccine coverage is estimated to be at 99%, from literature data and from historical epidemiological reports it presumable that vaccination –group specific immunization gaps may exist in the Hungarian population (8–13). Moreover, suboptimal protection levels are supposed to be prevalent in vaccination-groups that represent a significant portion of active manpower of the country; e.g. health care workers (7). Questionable vaccine effectiveness in certain clusters of the population has a negative impact on overall vaccination coverage. Small-scale outbreaks confirm that certain measles vaccines - applied during the early phases of the Hungarian vaccination history – failed to elicit the desired immunological response. The resulting immunization gap(s) raise the concern of potential further outbreaks (10,15).

Between January 2017 and May 2019 there were 76 reported measles cases in Hungary (21), of which 54 cases were reported between 21 February and 22 March 2017 (22). Because of recent outbreaks worldwide, not only of measles, but also mumps and rubella (MMR)

infections, and because of waning of immunity over time after vaccination (23–26), the importance of continuous MMR sero-epidemiological screening is evident.

2.2 Waning immunity

Vaccine-preventable diseases (VPD) including measles and mumps have been re-emerging in countries with sustained high vaccine coverage. For mumps, waning immunity has been recognized as a major contributor to recent outbreaks (27). Measles outbreaks in countries with high vaccine coverage are mainly due to failure to vaccinate and importation; however, cases in immunized individuals exist and raise questions about suboptimal measles vaccine-induced humoral immunity and/or waning immunity (28). Cumulated serological and epidemiological evidence suggests that natural immunity induced by infection may be more durable compared to vaccine-induced immunity. As the proportion of population immunity via vaccination gradually increases and boosting through natural exposures becomes rare, risk of outbreaks may increase (27–29).

2.3 The MMR (measles, mumps, rubella) vaccine

Currently in Hungary the PRIORIX (GSK), PRIORIX-TETRA (GSK), ProQuad (MERCK) and the M-M-RVAXPRO (MSD Pharma) vaccines are used for vaccination of children (at age 15 months and 11 years) and adults (30). The vaccines contain live attenuated viruses.

Regarding immunocompromised individuals and children with contraindications, in Hungary also immunocompromised persons complete the recommended immunization series against vaccine preventable diseases (VPD), whenever possible. The vaccination practice follows international guidelines (2013 IDSA), and contemporaneously an individualized patient approach is also applied. This implies the involvement of a vaccination expert who performs case-to-case risk evaluation. As a general rule, live viral vaccines (e.g. polio, MMR, varicella) that may induce severe systemic reactions in immunocompromised individuals should not be administered to patients with severe immunosuppression and/or immune deficiency/autoimmunity. Nevertheless, important exceptions exist: certain live vaccines can be administered in some immune system disorders or when the benefit of the vaccine outweighs

the side effects, or major risk arising from the epidemiological environment (personal information from Dr. Andrea Kulcsár, Hungary, Vaccination Counseling expertise).

2.4 ELISA assays – Correlates or surrogates of protection?

According to the WHO's 'Guidelines on clinical evaluation of vaccines: regulatory expectations' the immunological correlates of protection (ICPs) are based on humoral immune response parameters that measure functional or total IgG antibody (31). According to Stanley A. Plotkin, regarding the four live vaccines commonly given in infancy (measles, mumps, rubella, and varicella) antibodies are certainly relevant to protection, especially in case of measles, where the role of antibodies in protection against is indisputable (however, there may be several other factors to take into account). However, if we talk about correlates of protection, probably plaque reduction neutralization test (PRNT) shows the best a correlation (32–34). As far as Plotkin's definitions are considered normative, indirect ELISA assays for anti-measles, -mumps and –rubella IgG titer detection are to be considered rather a good surrogate; a comparable, objective, well- quantified and standardized (based on international WHO standards) measure of immune response that substitutes for the true immunologic 'direct correlate' of protection (see Table1).

Table 1. Definitions of terms used by S.A. Plotkin as 'correlates of protection induced by vaccination' (35)

TERM	DEFINITION
Correlate	An immune response that is responsible for and statistically interrelated with protection
Absolute correlate	A specific level of response highly correlated with protection; a threshold
Relative correlate	A level of response variably correlated with protection
Co-correlate	One of two or more factors that correlate with protection in alternative, additive, or synergistic ways
Surrogate	An immune response that substitutes for the true immunologic correlate of protection, which may be unknown or not easily measurable

Although virus-neutralizing antibody remains the primary protection correlate (35,36) (and also the measurement of cell-mediated immune responses after vaccination may provide additional useful data (37)), in scientific literature it is commonly accepted to publish sero-epidemiological

results and sero-prevalence data using ELISA/EIA assays (38)(38–43). ELISA /EIA assays have the indisputable advantage of being easily available and easy-to-perform, do not imply the handling of living virus cultures, lack the inherent subjectivity of human-eye based result evaluation, and give quantified (unit or ratio expressed) data about the total humoral antigen load formed against a pathogen of interest. Despite all the analytical advantages, it must be mentioned that although a great effort has been devoted to achieve harmonization and comparability of ELISA/EIA tests, large differences in unitage between test kits may exist, despite standardization against an international or local standards (38)(39).

2.5 ELISA assays – standardization

Since different laboratories have their own favored assay platforms/SOPs and new assays are continually being developed, the widespread adoption of a standardized assay(s) is unlikely. Nevertheless, in order for serological test results to be compared among laboratories reference standards, especially reference antisera, are needed. To assist in this endeavor, the World Health Organization (WHO) maintains an Expert Committee on Biological Standardization as the scientific body responsible for establishing WHO reference standards. The WHO International Standard (IS) is recognized as the highest reference standard in which antibodies are assigned potencies in International Units (IU). The IS allows for comparison of assays from different laboratories, thereby helping to establish protective antibody levels after vaccination (13,36,37).

2.6 Assay development and optimization

When we would like to screen for insufficient antibody levels in order to point out individuals that might require revaccination, the elimination of false positivity from the assay is crucial. Inherent errors of the ELISA technique may interfere with seronegativity screening.

An ELISA has two major components. 1) Antigen –antibody reaction. This reaction is crucial and must be optimized in order for the assay to be reliable. 2) The surface to which antigens and antibodies are immobilized. The surface is an integral component of any assay due to its effect on biomolecules as they attach or do not attach to the matrix. Biomolecules attach to surfaces via a variety of mechanisms. This attachment phenomenon is controlled by the chemical properties of the surface, but can be influenced by (i) the physical properties of the containment vessel (96-well microplate) and (ii) other factors such as pH and temperature (38).

2.7 Hinges

Ideally, the capture, the detection reagents and the analyte of interest bind and only bind to each other without cross-reacting with any additional compound(s). In reality, endogenous matrix interferences can specifically or nonspecifically bind to capture/detection reagents or the analyte of interest and lead to an increase or decrease of the signal generated (39–42). For biomarker assays, specific matrix effects can be caused by endogenous molecules with similar structure to the target analyte or their natural ligands and ligand analogs (43). Typically, the potential interference compounds are not available in well-characterized forms and often the nature of the interference is not known, which makes it impossible to test of all of the interferences (42). To assay serum antibodies by indirect ELISA, it is critical to eliminate a variety of false positive and negative reactions attributed to the principle. These include 1) the background (BG) noise reaction caused by hydrophobic binding of immunoglobulin components in sample specimens to solid surfaces, 2) false positive reaction caused by non-specific binding of immunoglobulins to target-antigens by protein-protein interactions, and 3) other false positive and negative reactions caused by buffer components (38–42).

2.8 The role of immunization in the development of natural autoantibodies

It is proved (by animal studies) that immunization enhances the natural autoantibody (nAAb) repertoire (17,44–46). While the role of immunization in the production of antibodies directed against immunogens is widely studied, the role of immunization in the development of natural (auto)antibodies (in humans) has not been so deeply investigated (17,47). Potential associations among levels of vaccine (or wild-type infection) induced antibodies and natural (auto)antibodies - especially of IgG isotype-, are not yet in focus of research works of this field, therefore we were interested if we can find such relationships between the abovementioned antibodies.

We performed studies in serum sample groups of systemic autoimmune patients (SLE, SSc and RA), focusing on associations among levels of vaccine (or infection-induced) antibodies and naturally occurring autoantibodies. Serum levels of anti-measles IgG antibodies were compared with SLE-associated autoantibodies (natural and pathological anti-dsDNA IgG and IgM), and nAAbs (anti-citrate synthase [anti-CS]), anti-dsDNA topoisomerase I F4 fragment [anti-F4]), aiming to find an immuno-serological proof for the co-existence of IgG isotype pathogen or

disease -related antibodies and nAAbs. Secondly, we wanted to evidence the simultaneous presence of the known protective anti-dsDNA IgM autoantibodies and IgM isotype of anti-CS and anti- F4 in SLE patients; confirming their potential regulatory and beneficial role (47).

3 AIMS

I. Assay development: optimization of an efficient tool for the screening of suboptimal measles/MMR humoral antibody levels

II. Sero-epidemiology: large-scale screening of MMR antibodies at population level

III. Comparative study for the assessment of potentially impaired immune-regulatory functions in systemic autoimmune diseases: analysis of the potential link between naturally occurring (auto)antibodies, and vaccine –or infection– induced antibodies

4 MATERIALS AND METHODS

4.1 Assay development

4.1.1 Establishment of anti-measles, -mumps and -rubella IgG Indirect ELISA operational protocol (OP)

Development of the 'combined' or 'three-in-one' ELISA assay protocol was based on the former 'only' anti-measles IgG Indirect ELISA operational protocol, detailed thoroughly in our previous paper (15). For simplicity reasons, herein we detail only the improved operational protocol (OP), used for the latter MMR ELISA.

Table 2. Summary of major steps of the MMR indirect ELISA protocol

COATING ANTIGEN	**Bio-Rad PIP013 Measles virus, Edmonston strain**	**Bio-Rad PIP014 Mumps virus, Enders strain**	**Bio-Rad PIP044 Rubella virus, HPV-77 strain**
CONCENTRATION OF THE COATING ANTIGEN USED ON MICROPLATES	2.8 µg/mL	3 µg/mL	0.4 µg/mL
Antigens are dissolved in ELISA Coating Buffer (Bio-Rad BUF030), overnight at 4-6°C. Blocking ≥ 2 hours, RT with our in-house purely synthetic blocking buffer.			
STANDARD /QUALITY CONTROL REAGENT (S1-S5)	3rd WHO International Standard for Anti-Measles (NIBSC code: 97/648)	Anti-Mumps Quality Control Reagent Sample 1 (NIBSC code: 15/B664)	Anti-Rubella Immunoglobulin 1st WHO International Standard Human (NIBSC code: RUBI-1-94)
STARTING CONCENTRATION OF THE STANDARD /QUALITY CONTROL REAGENT	~ 5000 mIU/mL	~ 1000 'Mumps Assay Unit'/mL, arbitrarily assigned	1600 International Units per ampoule
NEGATIVE CONTROL (NC)	A sample found to be negative in a previous run		
POSITIVE CONTROL (PC)	A sample found to be positive in a previous run		
INCUBATION	3 x 15 minutes, 37°C		
COLOR DETECTION	Polyclonal anti-human IgG HRP-conjugated (Dako polyclonal rabbit anti-human IgG or equivalent) + TMB		
ADDITIONAL REAGENTS	Washing Buffer (WB), used also for sample dilution in combination with the IgM Reducing Assay Diluent (Bio-Rad BUF038), as previously described (Böröcz et al. 2019)		
AUTOMATION AND READING	Siemens BEP 2000 Advance System, λ = 450/620 nm		

4.1.2 Plate and coating buffer selection

In order to choose the optimal surface, we tested the following plates (using standards and serum samples as analytes): Nunc MaxisorpTM ELISA 96-well high-binding plates (442404 Sigma-Aldrich/Merck), 3D NHS and 3D Epoxy with covalent binding capacity, 705070 and 762070 with medium binding capacity, 705071 and 762071 with high binding capacity (Greiner Bio-One). Using these plates, we also tested different types of coating buffers: ELISA Coating Buffer (BUF030 Bio-Rad), PBS (pH 8.5), and for the Greiner Bio-One covalent binding plates; N-morpholino ethane-sulfonic acid (MES) buffer (25 mM, pH 6.0). After coating of plates with antigen, the results for different plates were compared to each other, as well as to Siemens Enzygnost kit (Siemens/Dade Behring, Marburg, Germany), known as the gold standard for measles ELISA assays.

4.1.3 Testing for potential coating-related interferences

For coating purity testing of the 'only' anti-measles IgG ELISA we used a recombinant nucleocapsid monoclonal antibody based sandwich ELISA, as a control system detailed in our previous paper (15).

For coating purity testing of the improved, combined 'three-in-one' MMR ELISA, we compared the 'conventional' assay system (of cell-culture derived, total virus antigen repertoire containing antigen coatings), to recombinant antigen based control system(s) (15). Accordingly, for the 'target' or 'conventional' assay we used purified, inactivated native virus preparations, derived from disrupted cells; measles Edmonston strain cultured in Vero cells (PIP013 Bio-Rad), mumps Enders strain cultured in BSC-1 cells (PIP014 Bio-Rad), rubella HPV-77 strain cultured in Vero cells (PIP044 Bio-Rad). Antigen preparations were sonicated before use, as per manufacturer's instruction. ELISA 96-well Maxisorp plates (Nunc) were divided vertically into three equal parts and each third was incubated overnight at 4-6 °C with measles, mumps and rubella antigens (100 µL/well), respectively (Figure 1, Table 2).

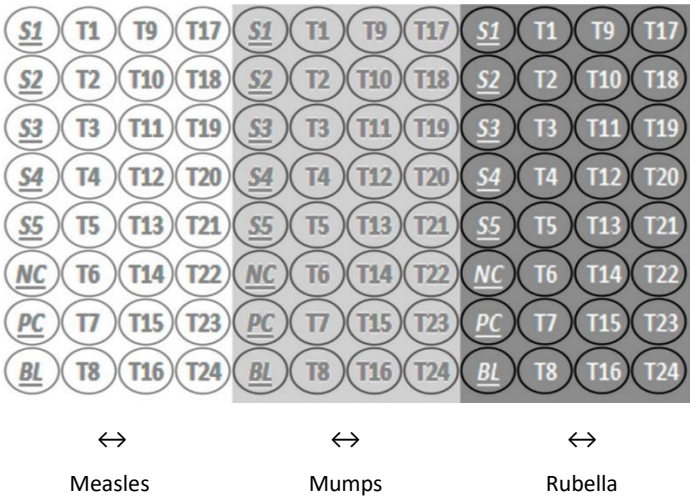


Figure 1. Abbreviations: S1—S5: Standards, PC: Positive control, NC: Negative control, BL:Blank (sample diluent). Color coding of antigen coatings: white: Measles virus, Edmonston strain, grey: Mumps virus, Enders strain, anthracit: (Rubella virus, HPV-77 strain)

Results obtained using the above represented setting were compared to purified recombinant viral capsid protein antigen based assays. To demonstrate the lack of interference when using the total antigen repertoire based coatings, plate-to-plate comparisons to purified, recombinant antigen based coatings were run. 'Control' microplates were coated with series of doubling, 4-point dilutions of recombinant antigens; measles virus Priorix, Schwarz strain nucleocapsid protein (Abcam ab74559, source: Saccharomyces cerevisiae) 1.66 - 0.207 µg/mL, mumps virus wild-type, Gloucester strain, nucleocapsid protein (Abcam ab74560, source: Saccharomyces cerevisiae) 0.832 - 0.104 µg/mL, recombinant rubella virus capsid protein (Abcam ab43034, source: E. coli) 2 - 0.25 µg/mL. Results of negative and low positive sample pools, international measles and rubella standards (3rd WHO International Standard for Anti-Measles, NIBSC code 97/648, Anti Rubella Immunoglobulin 1st WHO International Standard Human, NIBSC code RUBI-1-94), and the mumps quality control reagent (Anti-Mumps Quality Control Reagent Sample1) were applied in both 'target' and 'control' assay systems. Parallelism was tested to ascertain that the binding characteristic of the analyte (high and low antigen-titred sample pools) was the same, independent of the type of coating. For graphical representation, optical density (OD) values were linearized; dilution series of analytes were depicted as a function of common logarithm of both relative dilutions and OD values. Coating combinations with

sufficiently high R2 values of the linear fittings (with the same slope) were selected for further analysis of correlation between 'target' and 'control' assays, using Bland-Altman plots.

4.1.4 Blocking and diluent optimization

The basics of blocking and diluent use were set directly for the earlier 'only' anti-measles IgG ELISA, and were kept in the same way throughout the large-scale measurements, also when applying the latter MMR test.

The following assay diluents were tested: Hispec Assay Diluent (BUF049 Bio-Rad), ELISA Neptune Assay Diluent (BUF039 Bio-Rad), Block ACE (BUF029 Bio-Rad), ELISA General Assay Diluent (BUF037 Bio-Rad), ELISA IgM Reducing Assay Diluent applied without dilution, 2-fold, 4-fold, 8-fold dilutions (BUF038 Bio-Rad), and our own washing buffer (NaH2PO4 ×H2O 0.345 g + Na2HPO4×12 H2O 2.68 g + NaCl 28.675 g + Tween-20 1 mL for 1L, made in distilled water). Various blockers were also tested: Block ACE (BUF029 Bio-Rad), gelatin blocker (made from bovine skin), ELISA SynBlock (BUF034 Bio-Rad) and our purely synthetic PVA solution. Results were analyzed to obtain an optimal signal-to-noise ratio.

4.1.5 Calibration curve and serum antibody quantification

Milli-International Unit (mIU) content of samples was calculated based on absorbance measurements at 450 nm (620 nm reference) using a calibration curves prepared using the following reagents (Table 3). Extinction values (OD) were converted to quantified results (mIU) using 4-parameter logistic fitting to the generated sigmoid calibration curves (14,15).

Table 3. WHO standards and quality control reagent (for mumps). *Stocks of the 66/202 standard were exhausted and collaborative study was run in 2005/06 to establish a replacement. The 3rd International Standard was established by ECBS in 2006 and is available from NIBSC.

VIRUS	WHO NAME	NIBSC CODE
MEASLES	Anti-Measles Serum, Human and Anti-Poliovirus serum Types 1,2 and 3	66/202
*MEASLES**	*3rd International Standard for Anti-Measles**	*97/648**
MUMPS	QCRMUMPSQC1 - Anti-Mumps Quality Control Reagent Sample 1	15/B664
RUBELLA	Anti-Rubella Immunoglobulin, Human	RUBI-1-94

4.1.6 Optimal dilution of samples

High- and low-titer groups of samples were established based on preliminary measurements using well-established commercial kits. Low-titer sera were diluted 25-fold, while high-titer sera were diluted 50-fold in order to ensure that the OD values of these stock solutions fell within optimal range. These stock solutions were subsequently diluted in two-fold steps (9 times) until the absorbance values became indistinguishable from the background. The main criterion for selecting the dilution level of the sample was the ability to tell the difference between positive and negative samples, while staying in the optimal absorbance range (with acceptable signal-to-noise ratio) and using the lowest amount of standard stock solution for cost effectiveness. Using the same experimental setting, linearity and parallelism of dilution were also investigated (14,15).

4.1.7 Determination of cut-off values

For the earlier established high throughput 'only measles ELISA', the cut-off was determined as follows (based on arbitrary statistical method): intersection defined by the constant line (calculated by adding 3 SD to the mean OD values of negative samples), and the 4-parameter logistic curve (fitted to the dilution points of IS 66/202) was projected onto the X axis, denoting the concentration (14,15).

For the extended 'three-in-one' MMR ELISA, the determination of cut-off values was based on (a) Cohen's kappa statistics (κ), as an index of agreement between our assay and commercially available kits, (b) Area Under the Curve Receiver Operating Characteristics (AUROC) analysis (combined with Youden's J equation) - which in this case was used for comparing the performance of diagnostic tests -, and (c) the 'empirical approach' (already detailed above) (48).

In equivocal cases (and also to periodically check the assay performance), borderline and negative samples were measured using indirect immunofluorescence assays, using measles, mumps and rubella virus infected cells, IIF (IgG) (Euroimmun). In case of commercial assays, calculation of qualitative results was performed according to default thresholds specified by the manufacturers. AUROC results were analysed using Youden's formula (J = sensitivity + specificity -1), and the highest OD values were selected and transformed into units based on the standards (3rd WHO International Standard for Anti-Measles, Anti-Mumps Quality Control Reagent Sample

1, 1st WHO International Standard Human). For these transformations, sigmoid dose-response curves were fitted onto the dilution points of the standards (15).

4.1.8 Instrumentation platform

Measurements were performed on automated Siemens BEP 2000 Advance® platform (Siemens AG, Germany), using our self-developed ELISA assays validated by well-established commercial kits, as previously described. (14,15).Indirect immunofluorescent microscopy was used a reference (Euroimmun, Germany). Human sera were stored in the accredited laboratory of the Department of Immunology and Biotechnology (University of Pécs, Medical School, Pécs, Hungary) according to quality assurance criteria (ISO 17025) (7).

4.1.9 Software, statistical data evaluation

Microsoft Excel, XLSTAT, MedCalc (MedCalc Software BVBA), Origin Pro (OriginLab), and SPSS were used for data evaluation. AUROC analysis, Youden's *J* equation, confidence interval comparison at 95% confidence level (prop test), and Bland-Altman plot were used as statistical methods.

4.1.10 Assay cost, and execution time

We compared our manufacturing costs to commercially available 'off the shelf' kit prices, in order to examine the cost-effectiveness of our system. Our self-developed ELSIAs are targeted for even larger dimension measurements (e.g. anti-measles IgG antibody screening of healthcare workers), therefore we had to assess if it is worth to transform these fine-tuned operational protocols to products. For the same reason we also compared the assay execution times (15).

4.2 Sero-epidemiology

4.2.1 Samples

A serum bank consisting of anonymous patient sera was established from routine laboratory samples at the University of Pécs, Clinical Centre (Ethical License number 2015/5726). We would like to note that the serum bank establishment and the assay development were synchronous tasks, therefore samples used for the earlier detailed trials of the new assays were selected from the serum samples detailed in this paragraph. For the high-throughput 'only measles ELISA' we used N= 1985 sera (14). Later on, since the trend of the results did not change with the addition of new measurements of new samples, these results were fused with subsequent measurements, and thus cumulated data were reported. Accordingly, the following sample numbers; N total measles = 3523 measles, N mumps = 1736 mumps, and N rubella = 1736 were used for result analysis in the subsequent report, describing the extended 'three-in-one' MMR ELISA assay (15). Our most recent sero-epidemiological study (7) was based on the following sample numbers: N total measles = 3919 measles, N mumps = 2132 mumps, and N rubella = 2132. The samples were considered representative, as clinical residual samples were randomly selected (with the exclusion of seriously immunocompromised patients) from the Department of Laboratory Medicine, University of Pécs, Medical School, which serves three counties (Baranya, Somogy, and Tolna, with a population of ~887 000), and receives laboratory examination requests from all over Hungary (7,14,15).

4.2.2 Categorization

Serum samples were from all age-groups (beginning from the era before the implementation of measles vaccine, through several different vaccine types, manufacturers and vaccination schedules, up to present), and were categorized based on past changes introduced in measles and MMR immunization schedules (Table 4). The vaccination- (or age-) group based division of serum samples was based on the landmarks of the Hungarian history of measles/MMR vaccination. Given the anonymous nature of samples, the only known data was the date of birth of the patients. Considering that we were interested in the differences between the various vaccination periods, dates of vaccination (instead of dates of birth) were chosen to define cluster boundaries. By knowing the dates of birth and the important milestones of the

Hungarian vaccination history (e.g. the first measles vaccine was introduced in Hungary in 1969, in 1990 the measles-rubella bivalent vaccines were introduced, and in 1991 the measles-mumps-rubella trivalent vaccine was introduced; for further details, (Table 4)), establishment of the vaccination based age-group matrix became feasible. Neonates and children under the age of vaccination were excluded from our study. As mentioned above, seriously immunocompromised patients were also excluded, however, patients with mild immunocompromised conditions may have been included (14,15).
(see Table 4.)

4.2.3

Population level antibody-titer assessment was performed in relation to the concept of herd immunity threshold (HIT) values (HIT Measles = 92–95%, HIT Mumps = 85–90%, HIT Rubella = 83–86). Considering that our sero-epidemiological study rely on entire virus antigen repertoire-based indirect ELISA method, it must be considered rather a good surrogate than an absolute correlate marker for immunity (35,49,50).

Table 4. Age-group categorization based on the Hungarian history of measles/MMR vaccination

Age-groups	Explanation, rationale
	Vaccination groups were defined by adding the number of months indicated for the first childhood vaccine (e.g. 15 months of age) to the dates of birth. For example, a person born in February 1990 was assigned to age-group *"Patients vaccinated between 1991- 1995"*, since this individual received the first measles (MMR) vaccine in May 1991.
Patients born before 1969	Unvaccinated patients, wild-type infections. 1969: introduction of measles vaccine in Hungary (live, attenuated Leningrad-16 strain produced in the Soviet Union).
Patients vaccinated between 1969 - 1977	From 1969 to 1974, a single dose of measles vaccine was administered in mass campaigns to persons 9-27 months of age. The recommended age for vaccination was 10 months until 1978, when it was changed to 14 months. After the 1980-81 epidemics, persons born between 1973 and 1977, who would have received vaccine when the recommended age was 10 months, were revaccinated. After 1989, children were re-vaccinated at the age of 11 years with monovalent measles vaccine in a scheduled manner. Consequently, the first individuals who received a reminder vaccine at the age of 11 were born in 1978. Thus, the cluster of 1969-77 was the last that did not receive a reminder vaccine at the age of 11 as a part of the official vaccine schedule.
Patients vaccinated between 1978 - 1987	These are the first individuals who benefited from the reminder monovalent measles vaccine at the age of 11. In 1999 the administration of trivalent vaccine was started in Hungary, consequently who received the first trivalent vaccine in 1999 were born in 1988.
Patients vaccinated between 1988 - 1990	In 1989 the rubella vaccine was introduced, and the monovalent measles reminder vaccine at age 11 was started. 1990: Introduction of measles-rubella bivalent vaccines.
Patients vaccinated between 1991 - 1995	The administration of the first vaccine at age 14 months lasted from 1978 to 1991. 1991: Measles-mumps-rubella trivalent vaccine 1992: MMR vaccine at age 15 months 1996: Introduction of MERCK MMR II - Enders' Edmonston strain (live, attenuated)
Patients born between 1996 – 1998	1996: Introduction of MERCK MMR II - Enders' Edmonston strain (live, attenuated) 1999: Measles-mumps-rubella re-vaccination (reminder shot) instead of monovalent measles vaccine 1999: Introduction of GSK PLUSERIX - Measles Schwarz Strain
Patients vaccinated between 1999 - 2002	1999: Introduction of GSK PLUSERIX - Measles Schwarz Strain 2003: Introduction of the GSK PRIORIX vaccine
Patients vaccinated in 2003	2003: Introduction of the GSK PRIORIX vaccine - attenuated Schwarz Measles
Patients vaccinated in 2004 – 2005	2004-2005: Administration of the MERCK MMR II
Patients vaccinated between 2006 - 2010	2006-2010 (5-year tender): GSK PRIORIX - attenuated Schwarz Measles
Patients vaccinated after 2011	Beginning from 2011 we use a Sanofi-MSD product; MMRvaxPro (Measles virus Enders' Edmonston strain, live, attenuated) for vaccination and re-vaccination of children; GSK PRIORIX is still on the market, commonly used for vaccination in adulthood.
Epidemics: **1973-74: large epidemics, affecting primarily unvaccinated 6-9-years-old children**(11) **1980-81: another significant epidemic, affecting primarily 7-10-years old children** (11) **1988-89: epidemic with high age-specific attack rates of 17-21 years old individuals , who had been vaccinated during the first years of the vaccination program in Hungary** (11) **2017-18: Smaller epidemics with few connected and sporadic cases, derived mainly from virus importation** (11)	

4.3 A potential link between natural (auto)antibodies and vaccine –or infection– induced antibodies

4.3.1 Samples

Serum samples of patients suffering from different systemic autoimmune diseases (SAIDs) were obtained from the serum bank of the University of Pécs, Clinical Center, Department of Rheumatology and Immunology. The samples were stored and analyzed anonymously in the laboratories of the Department of Immunology and Biotechnology according to quality assurance criteria (ISO 17025) (Ethical License: 2015/5726 by the Regional Research Ethics Committee, at the University of Pécs, Hungary). The number of sera derived from different systemic autoimmune patients was the following: systemic sclerosis (SSc) n=157, systemic lupus erythematosus (SLE) n =92, rheumatoid arthritis (RA) n=73, other=52 (total n=374). Mean age (rounded values; years) within sample groups was the following: SSc: 56, SLE: 44, RA: 59, 53(overall: 52) (47).

4.3.2 Comparative ELISA measurements

In order to investigate potential associations; vaccine-(or infection-) induced antibodies (anti-measles IgG), systemic lupus erythematosus (SLE)-related autoantibodies (anti-dsDNA IgG/M) and natural (auto)antibodies (anti-DNA topoisomerase-I [or anti-Scl-70] fragment F4 [anti-F4] IgG/M, anti-citrate synthase [anti-CS] IgG/M), were measured. For this purpose, we used the earlier published (51–53) self-developed anti-CS and anti-F4 indirect ELISA tests, with slight modifications in order to harmonize with the automated platform (47), and commercially available anti-dsDNA ELISA kits (ORG604G and ORG604M by Orgentec Diagnostika GmbH). ELISAs were executed on automated platform (Siemens BEP 2000 Advance® System, Siemens AG, Germany.

4.3.3 Statistical analysis

Statistical analysis was carried out in the undivided serum bank of SAD, and also in the individual SAD subgroups that had sufficient sample numbers to yield representative data (SSc, SLE, and RA). Statistical evaluation was performed using SPSS version 25.0 statistics package (IBM, Armonk, NY, USA). Spearman's correlation analysis, Mann–Whitney U and Kruskal-Wallis-tests were used as appropriate, p values < 0.05 were considered significant.

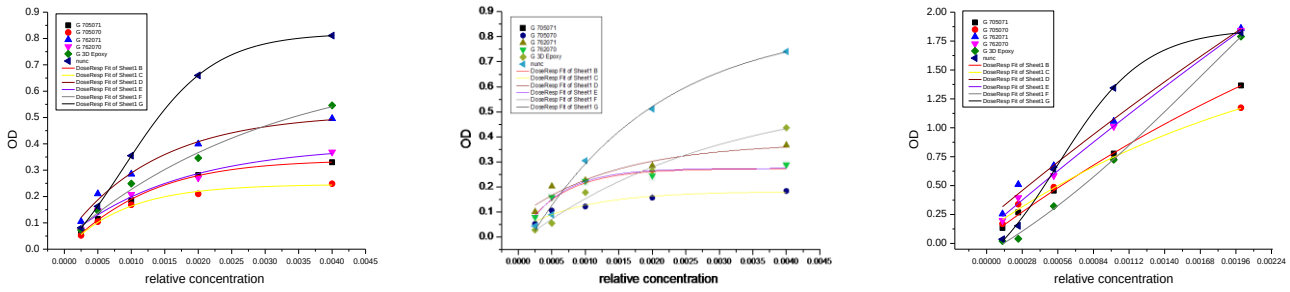
5 RESULTS I – ASSAY DEVELOPMENT

5.1 ELISA plate surface optimization

The ability of the surface to interact with proteins and other biomolecules is essential, however, non-specific binding (NSB) of other proteins or biomolecules to unoccupied spaces on the surface during subsequent steps of the assay can be detrimental to the specificity and sensitivity of the assay results. Therefore, the here presented results are to be interpreted in the context blocking ad diluent optimization trials. Of the below listed solid surfaces, Nunc Maxisorp high-binding plate was selected, because of negligible backgrounds, well-fitting (R^2) and adequately high standard curves (Figure 2), as well as successful parallelism test (represented as a supplementary figure of our paper (15)).

Standard curves

(a) measles (b) mumps (c) rubella



	Fitting (Adjusted R^2) (Sigmoidal dose response fit)		
Nunc Maxisorp	0.99	1	0.99
Greiner 3D Epoxy	0.96	0.98	1
Greiner 762071	0.96	0.89	0.99
Greiner 762070	0.93	0.9	0.99
Greiner 705070	0.98	1	0.97
Greiner 705071	1	0.82	0.99

Figure 2. Plates used for the comparison: Nunc MaxiSorp™ high-binding, Greiner Bio-One 762071 high -binding, Greiner Bio-One 762070 medium-binding, Greiner 96 well 1 x 8 strip plates 705071 high-binding, Greiner 96 well 1 x 8 strip plates 705071 medium-binding, Greiner Epoxy 3D - covalent binding. Analytes used for the comparison: 3rd International Standard for Anti-Measles (97/648), QCRMUMPSQC1 Anti-Mumps Quality Control Reagent Sample 1 (15/B664), anti-rubella Immunoglobulin (RUBI-1-94)

5.2 Antigen coating purity testing

Considering the 'three-in-one' MMR ELISA; total virus antigen repertoire based anti-measles, -mumps and –rubella IgG indirect ELISAs were considered as 'target', while recombinant, nucleocapsid based ELISAs (of the same viruses) were considered as 'control' tests. Assays were contrasted to check whether the cell culture derived, entire virus based coatings contain off-

target molecules that may lead to unwanted interference and consequent false-positive results. Based on the parallelism tests described in our paper (15), the following recombinant viral nucleocapsid antigen coatings were selected for further analysis: measles 0.83 µg/mL, mumps 0.416 µg/mL and rubella 1.0 µg/mL (R^2 standards ≥ 0.97, R2 samples ≥ 0.93). Bland-Altman plots were then generated; ratios of the results from the two techniques ('target' versus 'control' assay) were plotted against the averages. As shown in Figure 3, we obtained data points that fell within the range ± 1.96 SD (confidence interval 95%), with no observable trends, suggesting that the two methods are in agreement, thus demonstrating the adequate purity of the entire virus based coating system used in the 'target' assay (15).

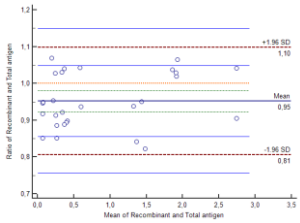
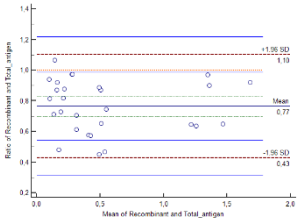
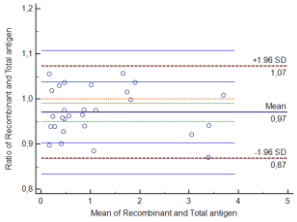
	(A) Measles	(B) Mumps	(C) Rubella
			
RECOMBINANT ANTIGEN BASED COATING	Measles virus Priorix, Schwarz strain nucleocapsid protein	Mumps virus wild-type, Gloucester strain nucleocapsid protein	Recombinant Rubella virus nucleoapsid protein
SELECTED RECOMBINANT ANTIGEN CONCENTRATION	0.83 µg/mL	0.416 µg/mL	1.0 µg/mL
INACTIVATED PATHOGEN-BASED COATING CONCENTRATION	2.8 µg/mL	3.0 µg/mL	0.4 µg/mL
SAMPLE NUMBER (N)	N=28 (Duplicates of the dilution series of positive and negative sample pools and quadruplicates of the dilution series of standards)		

Figure 3. Comparison of cell-culture grown, entire virus antigen repertoire based coatings and recombinant, antigen-specific ELISA coatings. Bland-Altman graphs display scatter diagrams of the ratios plotted against the averages of the two types of measurements. Sample number = 28 (duplicates of the dilution series of positive and negative sample pools and quadruplicates of the dilution series of standards). Limits of agreement (LoA) are defined as the mean difference ± 1.96 SD (95% confidence interval). Since data points do not exceed the maximum allowed difference between methods (dotted brown lines), and no pronounced trend is observable, the two methods (target: total antigen repertoire based coating versus control: recombinant antigen based coating) are in agreement and can be used interchangeably.

5.3 Blocking and diluent optimization

As described in our article (14), blocking solutions (protein-containing and protein-free) were tested on plates that had not been coated with antigen; only blocking solutions only were applied to 'coat' plates (overnight at 4-6 °C), than analytes were tested as per OP. Figure 4 shows the results when the IS 66/202 anti-measles serum was used at five different dilutions (range 10 mIUnit/mL - 2.5 mIUnit/mL). Results demonstrated that using Block ACE and bovine skin gelatin, the absorbance values reflect the increasing concentration of the standard, which suggests non-specific interactions. Such non-specific reactions were not observed in the case of SynBlock and our polyvinyl alcohol (PVA) based synthetic blocking solution, used as a cost-effective alternative of SynBlock. Therefore, for our subsequent experiments we used the PVA based synthetic blocking solution (14).

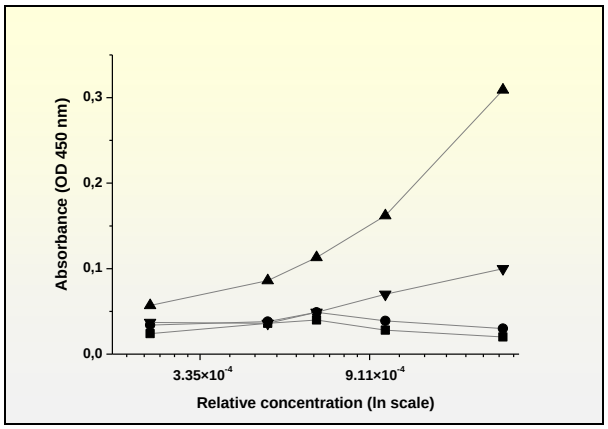


Figure 4. Effect of protein-free blocking

5.4 Background reduction

In a serological assay that is targeted for the detection of low, or suboptimal results, it is crucial to get rid of as many interference (and subsequent false-positivity) inducing factors, as possible. In the light of this principle we used the so called IgM reducing buffer (IgM RB) that resulted efficient not only in the elimination of IgM, but also in the reduction of unwanted uncategorized bindings. We observed a high background when using undiluted IgM reducing buffer (IgM RB) for diluting the serum samples without centrifugation. Using a two-step dilution process as described in the Materials and methods, a 2-fold and 4-fold final dilution of IgM RB the treatment was effective. Control experiments using 4-fold diluted IgM RB alone (without sera)

showed low levels of background (N=16 wells on 3 separate plates; OD mean +/-SD = 0.0384 +/- 0.0088). IgM RB treatments resulted in little or no change in absorbance of standards, applied at concentrations used for calibration curves. Using 2-fold diluted IgM RB, the absorbance values of patient sera decreased to 30% (70% decrease from the original value), while at 4-fold dilution of IgM RB the absorbance values to 40% of the original value (60% decrease) (Figure 5). The differences between the means of absorbance of the 2-fold and the 4-fold dilutions of IgM RB were statistically significant (P = 0.012, Student's t-test). Standard deviations were equal (P = 0.305, Levine's test/ F-test) suggesting that the less concentrated IgM RB was also effective at removing non-specific reactions. We also verified the effectiveness of IgM RB treatment on 10 randomly selected samples (of varying antibody titers) by adding polyclonal rabbit anti-human HRP-conjugated IgG and IgM (on two separate plates with the same layout) to the samples. The plate with IgM secondary antibody resulted in close to zero absorbance values. However, when using secondary anti-human IgG, the signal decreased, but it fell well within detectable range (Figure 5) (14).

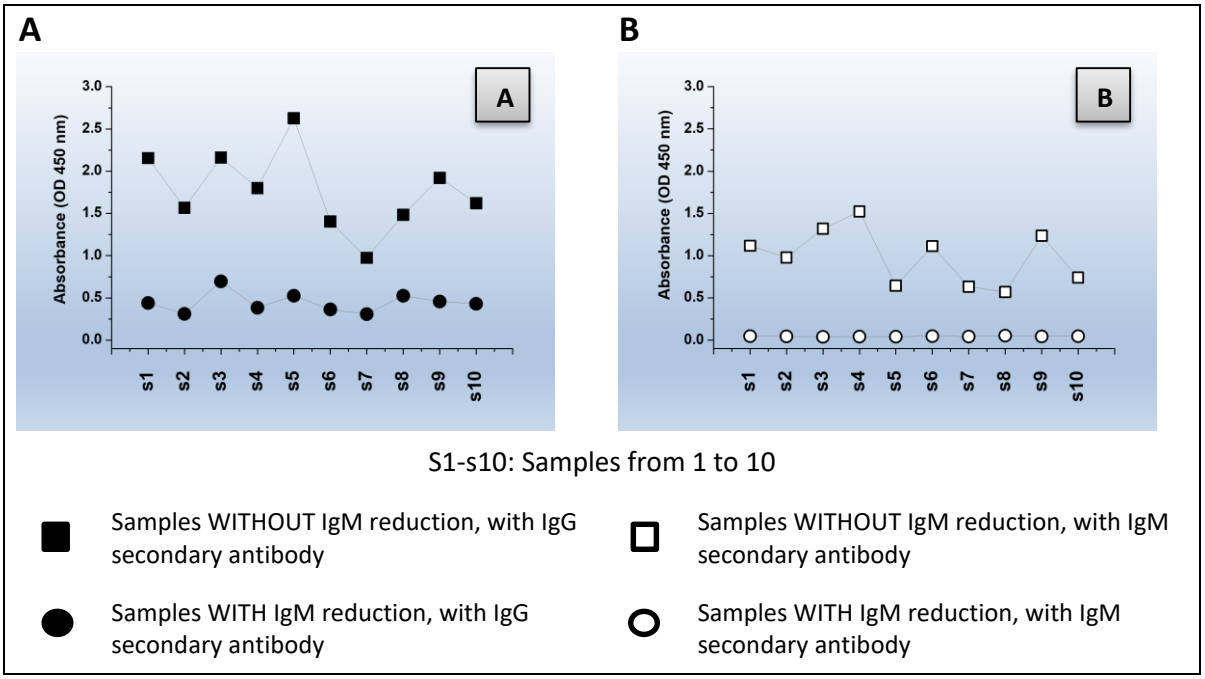
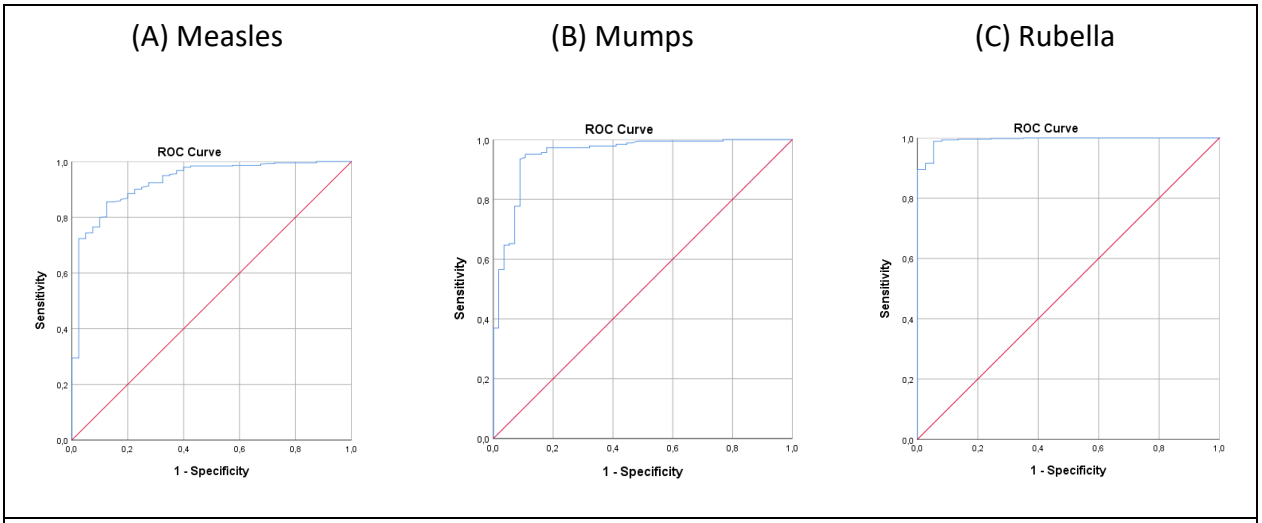


Figure 5. Effect of IgM reduction on absorbance values of serum samples used for anti-measles IgG detection. (A) Effect of IgM reduction with anti-IgG secondary antibody. (B) Effect of IgM reduction with anti-IgM secondary antibody.

5.5 Cut-off determination and assay precision

Although for the 'only' anti-measles IgG ELSIA the cut-off the value has been already set (14), the combined 'three-in-one' assay required correction, because three different antigens (measles, mumps, rubella) were bound on the same surface, while assay conditions (buffers, incubation times and temperatures) had to be uniform in order to enable a user-friendly, plain automated test execution. The combined setting inevitably implied slight modifications in the already detailed anti-measles ELISA OP, therefore all three (measles, mumps and rubella) cut-off values had to be (re)calculated (15).

Cohen's Kappa 'plate-to-plate κ statistics' gave 'substantial' to 'near perfect' agreement; $0.64 \leq \kappa \leq 0.92$. AUROC Areas were ≥ 0.92, for all three antigens (Figure 6). Based on the AUROC analysis, with the help of Youden's equation, the following sensitivity-specificity pairs were selected 0.985 – 0.975, 0.935 – 0.911, 0.989 – 0.946 for measles, mumps and rubella, respectively (15).

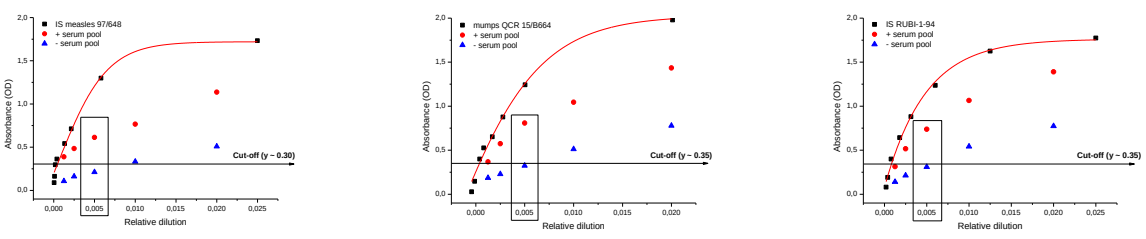


AREA	0.92	0.95	0.99
CUT-OFF (OD)	0.28 (~0.3)	0.36 (~0.35)	0.33 (~0.35)

(N = 474 samples from unselected diverse age-groups)

Figure 6. Determination of cut-off values using ROC analysis. The data obtained by our 'three-in-one' ELISA showed high agreement with the averaged qualitative results of commercially-available kits, used as reference (or the base of the binary classifier system) for AUROC calculations.

According to the 'empirical approach' -already detailed in our earlier paper (14)-, cut-off values were set for all antigen types (measles, mumps, rubella) based on mean observed OD values belonging to diagnostically seronegative samples (3x15 samples, OD negative sample ≤ 0.28, 0.37, 0.34 for measles, mumps, and rubella, respectively; data not shown). Cut-off values calculated based on empirical results were concordant with the statistically computed values. Typical dose-response curves obtained for measles, mumps and rubella standards are shown in Figure 7. Analytical values, such as lower limit of detection (LOD) and limit of quantification (LOQ) are also represented in Figure 7 (14,15).



MODEL	Dose-response curve		
EQUATION	y = A1 + (A2-A1)/(1 + 10^((LOGx0-x)*p))		
ADJUSTED R^2	0.97	0.97	0.99
MEASUREMENT RANGE	0.025–12.5 mIU/mL	0.02–10.0 arbitrary U/mL	2.0 – 265 mU/mL
CUT-OFF	0.15 mIU/mL	0.15 arbitrary U/mL	9.5 mIU/mL
LOD (MEAN + 10SD)	0.08	0.10	0.08
LOQ (MEAN +10 SD)	0.20	0.23	0.20

Figure 7. Typical standard curves of MMR assay. Sigmoid dose-response curves of the dilution series of the standards were generated with optimal data fitting (R2 ≥ 0.97). Absorbance values are plotted in function of relative dilution (1/ dilution). These curves serve as the base for the conversion of OD values to units/mL. Rectangles show the optimal serum dilutions (200-fold) used in the final assay format.

5.6 Optimal dilution of samples

The optimal dilution of samples was found to be 200-fold (0.005 relative concentration), performed in two steps, combined with IgM reducing assay diluent (14). This method yielded an

acceptable signal and reproducible difference between positive and negative samples, with minimal use of stock solutions (14,15).

5.7 Parallelism testing

The measured absorbance (OD) of samples plotted versus relative concentration resulted in saturation curves similar to the calibration curve, thus 4-parameter logistic curves were fitted. Dilution curves of two, typical low-titer samples and two high-titer samples were linearized by taking the common logarithm of the dilution and the calculated concentration, as shown in Figure 8. Linear fit was performed with slope -1 (determined from previous assays) for each data set. R-square values were close to 1 (0.91-0.99), which suggested that the binding characteristic of the analyte (serum antibodies) to the antigen were co-measurable to the standard. A better linear fit was observed for higher titer samples, because of the better signal-to-noise ratio of the spectrophotometric method in the measured OD range (14).

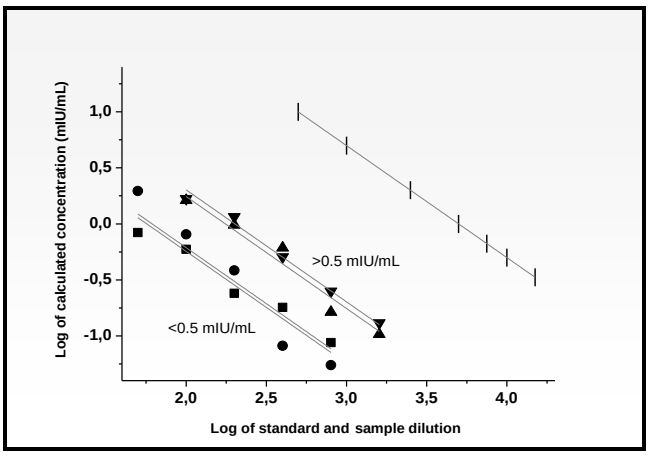


Figure 8. Linearity and parallelism testing for the high-throughput 'only measles' ELISA. Linearized dilutions of high and low titre samples with slope -1. Symbols ■, ●, ▲, ▼: samples, I: IS 66/202 (standard).

Similar analysis was performed also for the extended 'three-in-one' MMR assay. In this setting, dilution series of samples were also analyzed between entire virus antigen repertoire based coatings and purified recombinant viral capsid protein based coatings. This way, the parallelism testing could be also used to assess comparability of different coatings, and therefore to examine coating purity of the entire virus antigen repertoire based coatings. For linearization of logistic curves, the already described method was used; natural logarithm of the measured extinction results was plotted against the natural logarithm of the relative concentrations.

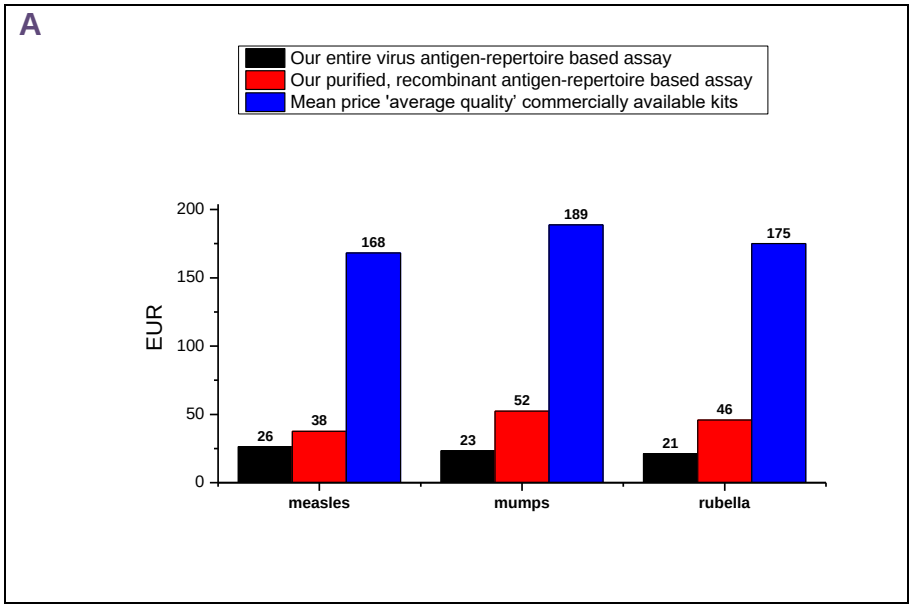
Linear fitting with the same slope (per viral antigen) was applied. This comparison of immune-reactivity between calibrators (WHO standards of known concentrations) to sample pools (of estimated concentrations) was successful; antibody-binding characteristics between standards and samples proved to be similar enough to allow the determination of analyte levels in the diluted samples (15).

5.8 Agreement between tests

In addition to the already detailed comparisons (also used for coating-purity testing) between the cell-culture derived, entire virus antigen repertoire based anti-measles IgG indirect ELISA and recombinant technology based control ELISAs (monoclonal nucleocapsid antibody based sandwich ELISA and nucleocapsid antigen based indirect ELISA)(14,15), comparability to commercially available kits was also investigated. Plate-to-plate Cohen's Kappa 'κ' statistics gave 'substantial' to 'near perfect' agreement; $0.64 \leq \kappa \leq 0.92$ (15).

5.9 Assay characteristics: cost, ease, and time

An important feature of our 'three-in-one' MMR ELISA assay is affordability; it costs only a fraction of the price of commercially available assays (Figure 9 A, B) (15).



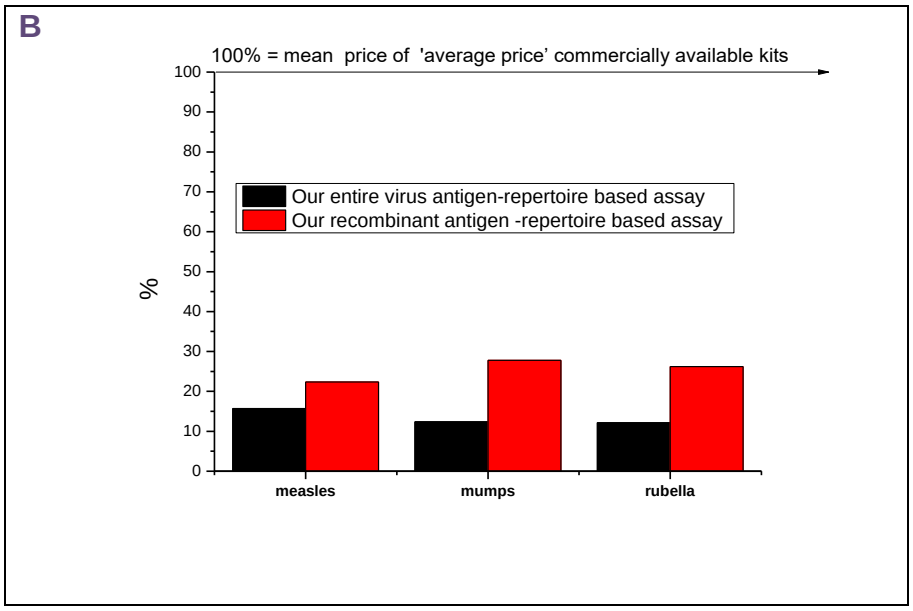


Figure 9. (A) Comparison of assay prices (commercial kits) and costs (our test) expressed in Euros, (B) Ratios of assay prices: "average price" commercial kits versus our test expressed in percentages. The average price of commercial kits was calculated based on the Hungarian distributor prices (VAT included), and included only those assays that we applied during the optimization and the test-to-test comparisons (Materials and methods). Siemens Enzygnost assays – belonging to a higher price-range – were excluded from the calculation.

Another important feature is the reduced assay duration time; compared to the ~ 1.5 / 2.5 hours of timeframe of commercially available tests (used for parallel and justificatory measurements) our test can be performed within 1 hour (Figure 10).

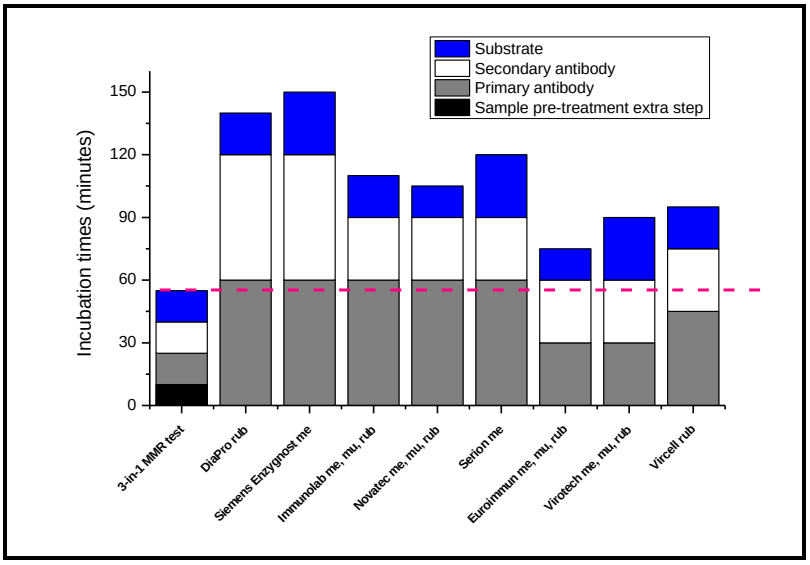


Figure 10. Comparison of incubation times of our test (3-in-1 MMR) to different commercial kits. (me = measles, mu = mumps, rub = rubella)

6 RESULTS II – SERO-EPIDEMIOLOGY

6.1 Vaccination history timeline

Changes and historical data regarding epidemics in the Hungarian measles/MMR vaccination schedule (8,54,55) were plotted on a timeline, in order to evaluate sero-epidemiological data accordingly. Figure 11 shows changes in measles and MMR vaccination schedules in Hungary, since the introduction of the vaccine (1969). High age specific attack rates characterizing major epidemics (1980-81 and 1988-89) along with 93% - 99% of vaccine coverage evidence insufficiencies of the early vaccination program (8)(11).

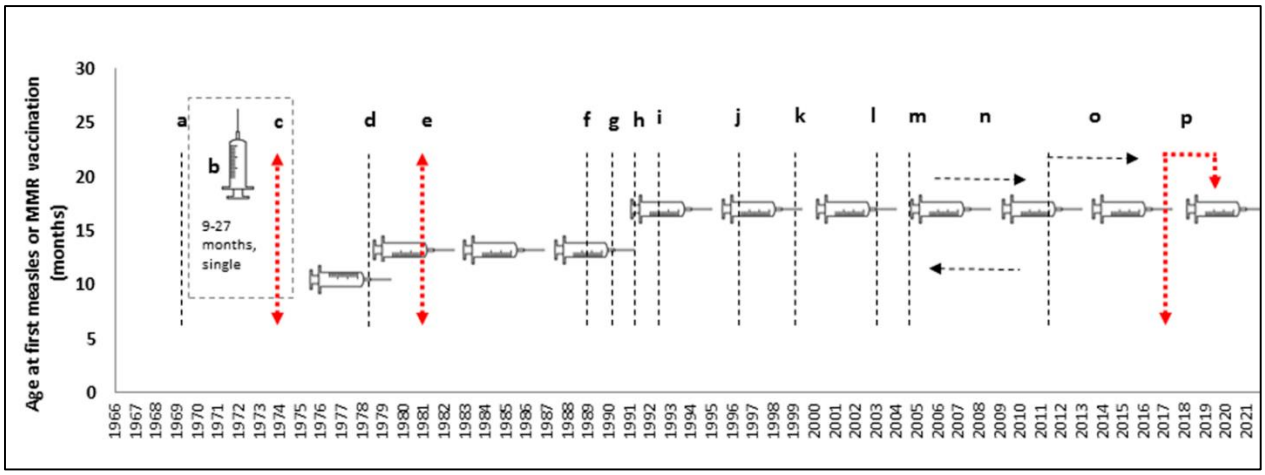


Figure 11. Measles and MMR vaccination schedules in Hungary (7)

(a) Vaccination against measles was introduced in Hungary in 1969. (b) From 1969 to 1974, a single dose of measles vaccine was administered in mass campaigns to persons 9-27 months of age. (c) After vaccination was implemented, the incidence rate decreased until 1973-74, when large epidemics occurred primarily in unvaccinated 6-9-year-olds. (d) The recommended age for vaccination was 10 months until 1978, when it was changed to 14 months. (e) After the 1980-81 epidemic, persons born between 1973 and 1977, who would have received vaccine when the recommended age was 10 months, were revaccinated. (f) The 1988-89 epidemic mainly affected persons 17-21 years of age, who had been targeted to receive vaccine during mass campaigns in the first years of the vaccination program in Hungary. After 1989, children were re-vaccinated at the age of 11 years with monovalent measles vaccine in a scheduled manner. Also in 1989 the rubella vaccine was introduced. (g) In 1990 measles-rubella bivalent vaccines were introduced. (h) The administration of the first vaccine at age 14 months lasted from 1978 to 1991. Also in 1991 the measles-mumps-rubella trivalent vaccine was introduced. (i) In 1992 the administration of the first MMR vaccine was shifted to 15 months of age. (j) In 1996 the MERCK MMR II vaccine (Enders' Edmonston strain, live attenuated) was introduced. (k) In 1999 measles-mumps-rubella re-vaccination replaced the monovalent measles vaccine. Also in 1999 the GSK PLUSERIX vaccine (Measles Schwarz Strain) was introduced. (l) In 2003 the GSK PRIORIX vaccine was introduced. (m) Between 2004 and 2005 the MERCK MMR II vaccine was used. (n) Between 2006 and 2010 the GSK PRIORIX vaccine in use. (o) Beginning from 2011 we use a Sanofi-MSD product; MMRvaxPro (Measles virus Enders' Edmonston strain, live, attenuated) for vaccination and re-vaccination of children. GSK PRIORIX is still on the market, commonly used for vaccination in adulthood. (p) Between January 2017 and December 2019 there were 76 reported measles cases in Hungary (according to ECDC Surveillance reports).

(Source of information: MMWR Weekly October 06, 1989 / 38(39); 665-668, International Notes Measles – Hungary, http://www.vacsatc.hu, https://www.ecdc.europa.eu)

6.2 Determination of age-groups with highest frequencies of seronegativity

Considering the relative herd immunity threshold (HIT) values (HIT Measles = 92–95%, HIT Mumps = 75–86%, HIT Rubella = 83–86), anti- measles IgG seropositvity ratios proved to be inadequate in certain clusters of the population (Figure 12,13). The lowest seropositvity ratios (N total measles = 3523 serum samples) were detected in clusters 'Vaccinated between 1978 and 1987' (~80% of seropositvity) and 'Vaccinated between 1969-77' (~90% of seropositvity). Analyzing the vaccination period-dependent confidence intervals of seronegativity; the group 'Vaccinated between 1978 and 1987' showed significant differences from the flanking age-groups; 'Vaccinated between 1969-1977 and 1988-1990' ($p = 0.00004$ and $p = 0.0015$, respectively). In the case of rubella (N = 1736 serum samples), the least protected groups were vaccinated during 1969-1977 (~85% of seropositvity) and 1988-1990 (~85% of seropositvity). Significant differences were observed between the group born before 1969 (not vaccinated) and vaccinated during 1969-1977 ($p = 0.00008$), and between groups 1988-1990 and 1991-1995 ($p = 0.009$). (Figure 12).

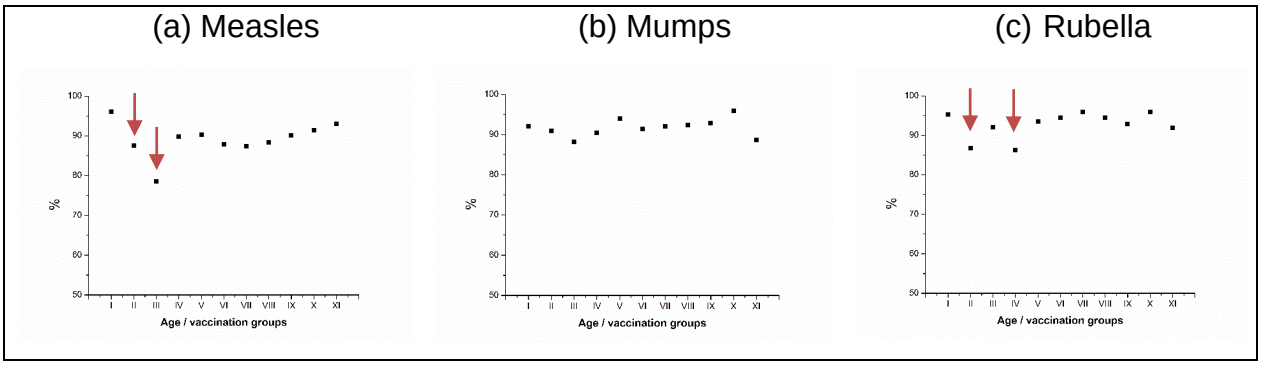
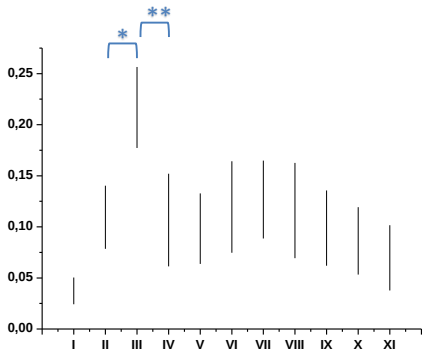


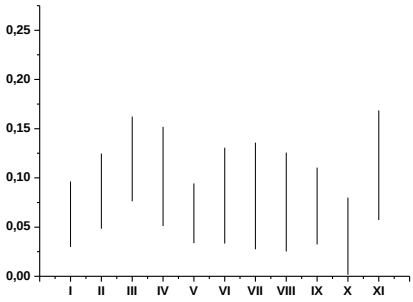
Figure 12. Measles, mumps and rubella seropositivity ratios according to vaccination groups. N measles = 3523, N mumps, rubella = 1736 serum samples. Age / vaccination groups: (I) Individuals born before 1969. (II) Individuals vaccinated between 1969 – 1977. (III) Individuals vaccinated between 1978 – 1987. (IV) Individuals vaccinated between 1988 – 1990. (V) Individuals vaccinated between 1991 – 1995. (VI) Individuals vaccinated between 1996 – 1998. (VII) Individuals vaccinated between 1999 – 2002. (VIII) Individuals vaccinated in 2003. (IX) Individuals vaccinated in 2004 – 2005. (X) Individuals vaccinated between 2006 – 2010 (XI) Individuals vaccinated after 2011. The lowest seropositivity ratio (78.48%) was observed in the anti-measles antibody titers (IgG) in group 'Vaccinated between 1978 – 1987' (7)

(A) Measles

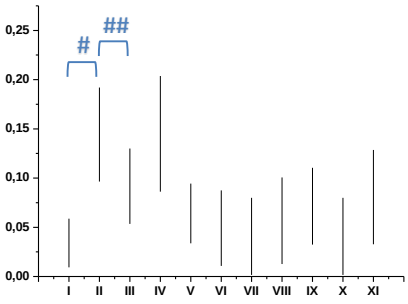


I	Born before 1969 (no vaccine)
II	Vaccinated between 1969-77
III	Vaccinated between 1978-87
IV	Vaccinated between 1988-90
V	Vaccinated between 1991-95
VI	Vaccinated between 1996-98
VII	Vaccinated between 1999-2002
VIII	Vaccinated in 2003
IX	Vaccinated between 2004-2005
X	Vaccinated between 2006-2010
XI	Vaccinated between 2011-2015

(B) Mumps



(C) Rubella



Statistically significant differences between adjacent age- (or vaccination-) groups

	Age-groups	p-value
*	Measles; Vaccinated between 1969-77 and 1978-87	0.00003841
* *	Measles; Vaccinated between 1978-1987 and 1988-90	0.0015
#	Rubella; born before 1969 (not vaccinated) and vaccinated during 1969-1977	0.00008437
# #	Rubella; Vaccinated between 1988-90 and 1991-95	0.008532

Figure 13. Relative frequencies of measles-, mumps- and rubella-specific seronegativity dependent on the period of vaccination. N measles = 3523, N mumps, rubella = 1736 serum samples.Vertical lines indicate 95% confidence intervals. Significant differences between the antibody levels of the critical age-groups and their flanking age-groups are marked with * and #.

In case of measles, mumps and rubella cumulative results, the seropositvity ratios were 89.97%, 91.60% and 92.58%, respectively, as shown in Figure 14. In practical terms, it means that in case of measles, due to cluster-specific inadequacy of IgG levels, also the overall seropositivity ratio (measles = 89.97%) failed to reach the herd immunity threshold (HIT Measles = 92–95%). Considering mumps and rubella, herd immunity – in terms of humoral antibodies –was reached. (Figure 14) (7).

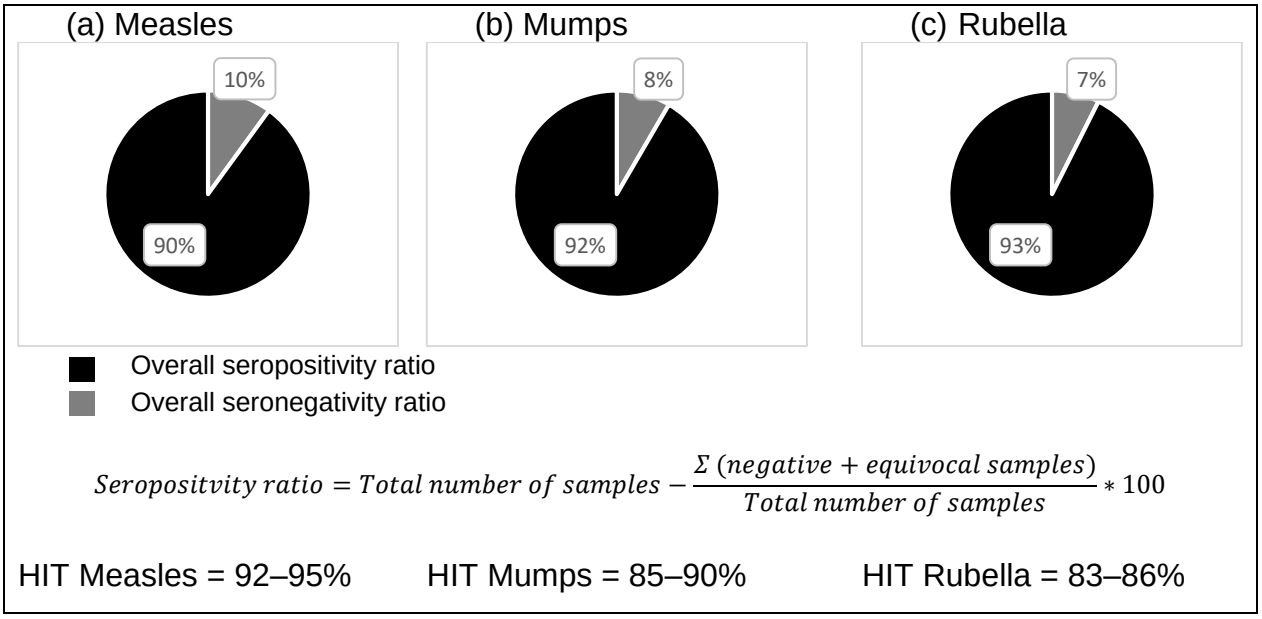


Figure 14. Overall seropositivity and seronegativity ratios. N measles = 3919; N mumps, rubella = 2132. In case of measles, mumps and rubella cumulative results the seropositvity ratios were 89.97%, 91.60% and 92.58%, respectively. The overall ratio of seropositive samples was lowest in the 'measles' group, where it remained under the threshold value (15).

7 RESULTS III - A POTENTIAL LINK BETWEEN NATURAL (AUTO)ANTIBODIES AND VACCINE –OR INFECTION– INDUCED ANTIBODIES

7.1 Vaccine– (or infection–) induced anti-measles IgG seropositvity ratios in systemic autoimmune diseases

Anti-measles IgG levels in samples obtained from all SAD patients (total n=374) were measured, then seronegativity ratios were also evaluated. As it is already described in epidemiological literature, and in accordance with our previously published findings (8,11,14,15,54); measles seronegativity ratios showed significant correlation with age ($p<0.001$ correlation coefficient; 0.323), which is a general phenomenon, not linked to autoimmunity. Considering the seronegativity ratios; between the earlier detailed population-level 'overall' anti-measles (IgG) result (10.03% of seronegativity) and the current autoimmune disease focused sample multitude (8.82% of seronegativity) we found no remarkable difference (Figure 16).

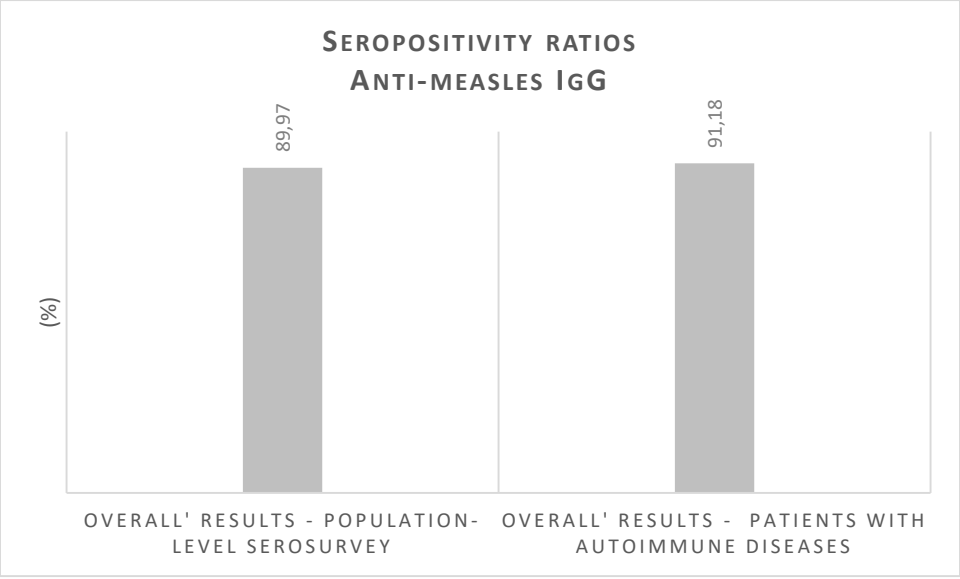


Figure 16. Comparison of cumulative anti-measles (IgG) seropositivity ratios of the population-representative sero-epidemiological survey (N = 3919) and the autoimmune disease focused study (N = 374)

7.2 Comparison of natural autoantibody and vaccine– or infection– induced antibody levels

Based on previous findings nAAbs (IgG/M) against the mitochondrial inner membrane enzyme citrate synthase and topo I F4 could be detected in sera of healthy individuals and patients with SSc, SLE and in other autoimmune rheumatic diseases (51). When analyzing the undivided totatlity of SAD samples, the same trend was observed as in case of the three accentuated

disease groups (SSc, SLE, RA). Considering all SAD samples together (total n=374); significantly higher anti-CS IgG titers were detected in the anti-measles IgG seropositive patient group (p=0.011) compared to the anti-measles IgG seronegative individuals (Figure 17) (47).

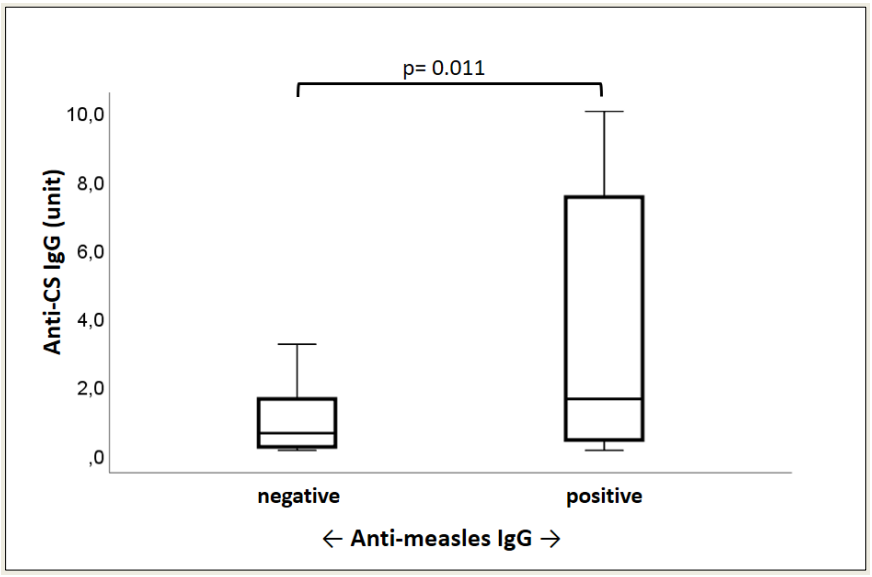


Figure 17. Relationship between anti-measles IgG and anti-CS IgG antibody titers in the undivided serum bank of systemic autoimmune diseases (SAD) serum samples. (n SAD=374= n_{RA} 73 + n_{SLE} 92 + n_{SSc} 157 + n_{Other} 52] Qualitative (positive, negative) anti-measles IgG results were compared to quantitative anti-citrate synthase IgG results (expressed in arbitrary units, based on our in-house standard), using Mann–Whitney U analysis. Significantly higher anti-CS IgG titers were detected in the anti-measles IgG seropositive compared to seronegative SAD patients. Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians.

Analyzing the association between vaccine or virus- -induced (anti-measles IgG) and natural (anti-CS IgG) antibody titers in the individual autoimmune diseases (RA, SLE and SSc), the same trend described above was observable; in all three groups the anti-measles IgG seropositive samples showed significantly higher anti-CS IgG titers (Figure 18). Similar, but statistically not significant trend was observed in case of anti-F4 IgG nAAbs (figure not shown) (47).

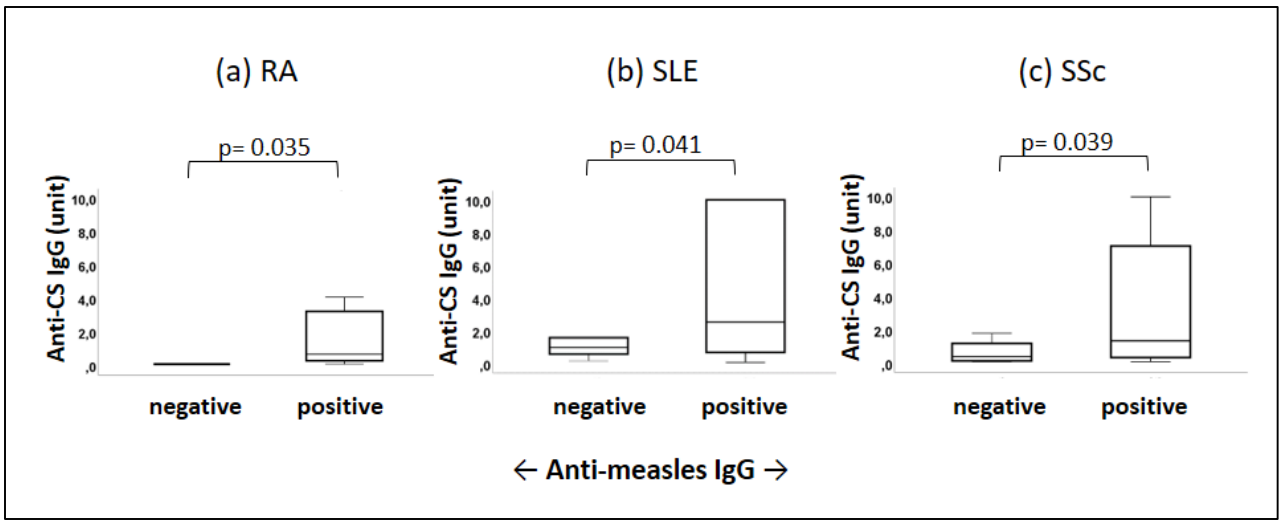


Figure 18. Relationship between anti-measles IgG and anti-CS IgG natural autoantibody titers in SSc, SLE and RA disease groups. (nRA=73; nSLE=92; nSSc=157) Qualitative (positive, negative) anti-measles IgG results were compared to quantitative anti-citrate synthase (anti-CS) IgG results (expressed in arbitrary units, based on our in-house standard), using Mann–Whitney U analysis. Significantly higher levels of natural anti-CS IgG were detected in anti-measles IgG seropositive compared to seronegative samples in rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc). Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians.

7.3 Relationship between IgG natural autoantibody levels and anti-dsDNA IgG in SLE patients

In order to investigate the association between autoimmune disease-specific pathological autoantibodies and IgG isotype nAAbs, anti-dsDNA IgG measurement was chosen, as anti-dsDNA IgG is a highly specific disease marker in SLE. In those SLE patient samples that proved to be positive for the disease specific marker; anti-dsDNA IgG, significantly higher levels of anti-F4 IgG ($p=0.001$) and anti-CS IgG ($p<0.001$) nAAbs were measured, as shown in Figure 19. Anti-dsDNA IgG and nAAbs levels also showed significant correlation (p / correlation coefficient: 0.006 / 0.321 and 0.000 / 0.510 for anti-F4 IgG and anti-CS IgG, respectively) (47).

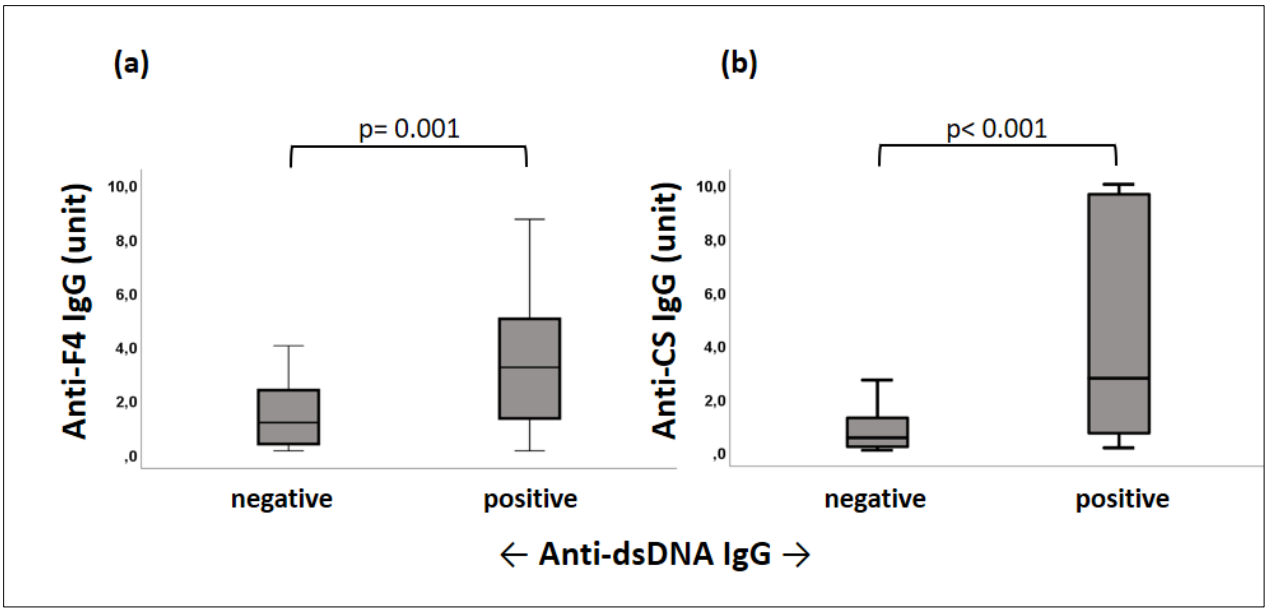


Figure 19. Relationship between disease-specific anti-dsDNA IgG autoantibody levels and natural anti-F4 and anti-CS IgG natural autoantibody titers in SLE patients. (n SLE=92) Qualitative (positive, negative) anti-dsDNA IgG results were compared to quantitative anti-DNA topoisomerase I F4 fragment (anti-F4) IgG and anti-citrate synthase (anti-CS) IgG results (expressed in arbitrary units, based on our in-house standard), using Mann–Whitney U analysis. The level of anti-F4 and anti-CS IgG antibodies were significantly increased in anti-dsDNA IgG positive compared to anti-dsDNA IgG negative SLE patients. Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians.

7.4 Anti-dsDNA IgM and natural IgM autoantibody levels showed association in SLE

Previous reports proposed that anti-dsDNA IgM antibodies may play a protective role in lupus nephritis (56–58). Herein we compared anti-dsDNA IgM levels with nAAb titers in SLE patients. Figure 20 shows that in anti-dsDNA IgM positive SLE patient samples significantly higher levels of anti-F4 IgM and anti-CS IgM nAAbs were detectable (p=0.002 and 0.016, respectively) as shown in Figure 4. Anti-dsDNA IgM titers and nAAb levels also showed significant correlation (p/ correlation coefficient: 0.002 / 0.344 and 0.018 / 0.252 for anti-F4 IgM and anti-CS IgM, respectively) (47).

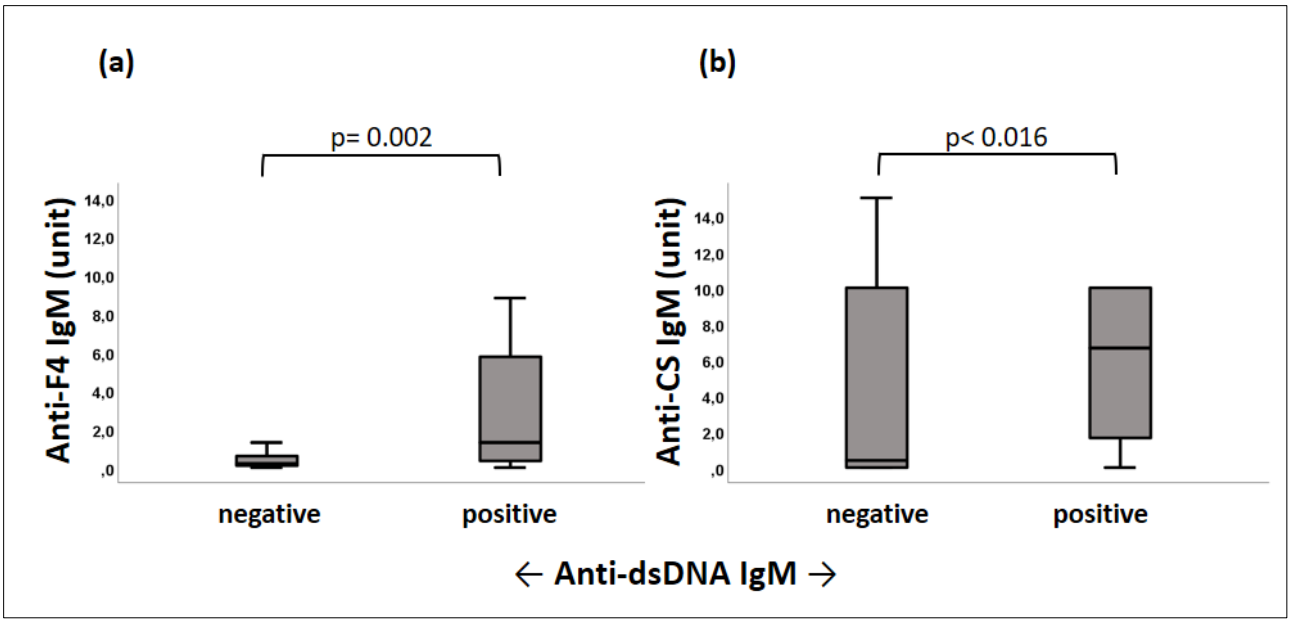


Figure 20. Relationship between anti-dsDNA IgM autoantibody levels and anti-F4, anti-CS IgM natural autoantibody titers in SLE patients. (n SLE =92) Qualitative (positive, negative) anti-dsDNA IgM results were compared to quantitative anti-DNA topoisomerase I F4 fragment (anti-F4) IgM and anti-citrate synthase (anti-CS) IgM results (expressed in arbitrary units, based on our in-house standard), using Mann–Whitney U analysis. The level of anti-F4 and anti-CS IgM natural antibodies were significantly elevated in anti-dsDNA IgM positive compared to anti-dsDNA IgM negative SLE patients. Boxes show interquartile ranges (IQR), whiskers indicate lowest and highest values, horizontal lines represent medians.

8 DISCUSSION

8.1 Assay development and optimization

8.1.1 Background reduction and blocking

As it was mentioned in the introduction, the elimination of false positivity inducing reactions – that might be considered as 'innate errors' of the ELISA method –is a key question, especially when the aim is the detection of negative or suboptimal antibody levels.

Substances that alter the measurable concentration of the analyte or alter antibody binding can potentially result in immunoassay interference. Interfering, endogenous substances that are natural, polyreactive antibodies or autoantibodies (heterophiles), or human anti-animal antibodies together with other unsuspected binding proteins that are unique to the individual, can interfere with the reaction between analyte and reagent antibodies in immunoassay. Lipemia, cross-reactivity, and exogenous interferences due to pre-analytical variation, matrix and equipment reaction also affect immunoassay. Interfering substances may lead to falsely elevated or falsely low analyte concentration. The prevalence of interference is generally low in assays containing blocking agents that neutralize or inhibit the interference but is often higher in new, untested immunoassays (38,40,43,59).Interference may be grouped as: a) background noise, due to hydrophobic binding of immunoglobulin components in sample specimens to solid surfaces, b) the false positive reaction caused by non-specific binding of immunoglobulins to target-antigens by protein-protein interactions, c) non-specific binding of secondary antibody to off-target molecules, and d) uncategorized positive and negative reactions caused by buffer components.(40,43).

No current blocking agents can perfectly eliminate these reactions, therefore antibody assay results vary significantly depending on the buffer system used (60). To address these fundamental problems, we used a combination of washing, blocking and sample pre-treatment methods and buffers, in order to minimize unspecific biomolecule binding, and optimize the signal-to-noise ratio. In case of our ELISA - that was targeted for the detection of suboptimal or 'under the threshold' antibody levels - blocking was a key factor to prevent non-specific binding antibodies. On high binding polystyrene surfaces using a simple non-ionic detergent, such as

Tween-20 or Triton-X, is not enough and a protein blocking step is required. Usually a diluted solution of bovine serum albumin (BSA) or skimmed milk can be applied, but especially in case of human sera these may give a substantial inhomogeneous background due to cross-reactions with patient sera and/or with the secondary antibody. In assays where the antibodies of interest are likely to interact with these agents, use of a synthetic blocker is necessary (38). BSA cannot be an efficient blocking agent for samples of human sera due to the high background caused by the immunoglobulins present in BSA (60). Polyvinylpyrrolidone (Povidone, PVP), polyethylene glycol (PEG) or polyvinyl alcohol (PVA) are typically recommended synthetic blockers, although it was proven that the latter is preferable. We developed our PVA-containing synthetic blocker (Figure 4) based on ELISA tests using SynBlock buffer (Bio-Rad) in preliminary experiments. Thompson et al. found that wells blocked with PVA gave comparable results to a commercially available premium synthetic blocker in ELLA (Enzyme-Linked Lectin Assay) experiments (61). Our PVA based blocker contained 5 g/L PVA dissolved in PBS buffer. PVA of MW 124-186 kDa has been recommended (62), although some investigators used PVA-50 (MW 50 kDa) or PEG-360 (MW 360 kDa) (63). Our synthetic blocker contained PVA of different molecular weight (MW ~72 000 kDa). Conditions of preparation, such as temperature and stirring, may also affect the effectiveness of the blocker as PVA shown poor solubility in water. To prevent degradation of the blocker a preservative was added (14).

8.1.2 Elimination of interfering antibodies

With regard of background reduction, another important step in the elimination of nonspecific binding of proteins was the introduction of sample pre-treatment with IgM Reducing Assay Diluent (BioRad). It is well known that serum IgM antibodies can decrease specificity because both pathogen- specific and natural (low affinity, multi-specific) IgM antibodies are present in human serum. Autoantibodies present in patients with autoimmune disease can potentially interfere with the results. Cross reaction caused by rheumatoid factor and heterophilic antibodies can also occur (64–66). To solve these problems, interference reducing methods have been suggested in the literature, such as pre-incubation of sera with animal serum or immunoglobulin (66,67). We used IgM Reducing Assay Diluent, a buffer enriched by mammalian

proteins and recommended for matrix equalization to eliminate “sticky” or non-specific IgM from assays (68) (Figure 5).

8.1.3 Parallelism

Parallelism experiments help to understand assay relative accuracy. Testing of dilution effects on the quantitation of endogenous analyte(s) in matrix, selectivity, matrix effects, minimum required dilution are crucial to establish a precise test (42). Ligand-binding assays (LBA) quantitate macromolecules by comparing immune-reactivity of calibrators of known concentrations to the samples of unknown concentration. For a well-developed LBA with an appropriate logistic regression approach, the calibration curve should be parallel to support the assumption that the antibody-binding characteristics are similar enough to allow the determination of analyte levels in the diluted samples (42). As suggested by the literature (42)(69–71), a ‘relative quantitative approach’ was used for the development of the current assay (anti-measles indirect ELISA); WHO standard(s) were available and were adequately characterized (specially the 66/202 anti-measles serum), and blank matrices free of analyte were substituted with low (or close to zero) titer samples, obtained from children (of ~0.5-1 year of age) that fell in the gap ‘already without maternal antibodies, but not yet immunized’. The experiment was based on the principle that if standard curves and samples are prepared under the same circumstances, matrix effects are normally similar between standards and samples. In bioanalysis it is known and accepted that in case of LBAs, the correlation between signal and concentration/dilution normally is not linear. Hence, linear plotting cannot accurately reflect the parallelism among samples. Four or five parameter logistic (4PL or 5PL) regression with or without weighting is suggested to achieve the best curve fit with the least variance. Log/log plot provides good linearity for the low to medium range of the concentrations (The higher end of the range tends to lose linearity) (70,72). Examples for LBA parallelism assessment are provided in our articles discussing the ‘only measles’ and the upgraded, ‘three-in-one’ MMR ELISA assays (14,15). We plotted the natural logarithm of the measured extinction results against the natural logarithm of the relative concentrations. Linear fitting with the same slope (per viral antigen) were applied (Figure 8).

8.2 Sero-epidemiology

8.2.1 Vaccination-group specific immunity gaps

In 1969 the measles vaccine was introduced in Hungary in the form of live, attenuated Leningrad-16 strain vaccine, produced in the former Soviet Union. Between 1969 and 1974, a single dose of vaccine was administered in campaigns to individuals of 9-27 months (11). After vaccination had been implemented, the incidence rate decreased until 1973-1974, when large epidemics occurred primarily in unvaccinated 6-9-year-olds (11), questioning the effectiveness of the early vaccination programme. Regarding post-vaccination humoral immune response, heterogeneous data are available in the literature. It is generally accepted that the success of vaccination in children is dependent on the presence (or absence) of inhibitory maternal antibodies and the immunologic maturity of the recipient, as well as on the dose and vaccine strain. It is also recognized that the age of ≥12 months is a milestone in the development of an efficient immune response (15).

According to our sero-epidemiological data (7,14,15,47), the most susceptible clusters are those of individuals vaccinated between '1978-1987' (>20% of seronegativity) and '1969-1977' (>10% of seronegativity). These finding are in agreement with a similar report from our colleagues; analyzing the results of screening more than 2000 healthcare workers (10).The gap detected in herd immunity is also supported by the already known insufficiencies during the initial vaccination era (8,11). These individuals were vaccinated during the early 1970s, when the optimal age of vaccination was not well defined, and the thermolability of the reconstituted vaccine was not fully characterized (11). These relatively high measles seronegativity ratios may have been a consequence of vaccine inefficiency, which seems to be supported by historical data: after the starting of the immunization campaign in 1969, a decade later, in 1978 the recommended age for vaccination was changed from 10 to 15 months. The 1988-1989 epidemics affected individuals (16-22 years of age), who were vaccinated in the early era with a singular measles vaccine. Following the 1988-89 epidemics, persons born between 1973 and 1977 were revaccinated (8,11). Even though a significant portion of individuals belonging the 'problematic' group (vaccinated between 1978-87) have been re-vaccinated and/or have contracted the wild-type measles infection, in this cluster we found the highest ratio of

seronegativity. This finding supports the ineffectiveness of the early vaccination system. Additional verification for this hypothesis is the high age-specific attack rate during the 1988-1989 outbreak that affected the population with ≥ 93% vaccine coverage. After the introduction of the trivalent MMR vaccine (1991), we detected a statistically significant improvement in the anti-measles seropositvity ratios (Figure 13). The group “Vaccinated between 1988-1990” has significantly better humoral response compared to the group “Vaccinated between 1978-1987”, reflecting the effectiveness of the trivalent reminder vaccine at age 11. Nowadays, in Hungary the MMR vaccination coverage is ideal, due to the mandatory administration of safe and modern trivalent vaccines. Nevertheless, dubious immunization practices in some of our neighboring countries, aggravated by the detrimental effect of the COVID-19 pandemic and subsequent suspension of measles vaccination campaigns, may facilitate the occurrence of smaller importation-related MeV outbreaks in susceptible cohorts. A smaller measles outbreak during 2017 (‘outbreak of Makó and Szeged)’ (73,74), which started from imported cases, has made it obvious that despite of a good vaccination coverage, latent immunization gaps are in fact, present in the Hungarian population (7).

8.3 A potential link between natural (auto)antibodies and vaccine –or infection– induced; pathological antibodies

The presence of specific natural antibodies both in healthy individuals and in patients with SAIDs has been widely described (52,53,75–77). The majority of these antibodies was thought to be of IgM isotype, but nowadays it is known that also IgG and IgA are abundant, and play an important role (78). Today it is well-known that natural IgM autoantibodies convey protection from pathologic autoimmune reactions (56,58,79–84), and their impaired anti-apoptotic function of may lead to insufficient elimination of dying cells and the consequent maintenance of autoinflammation (79,85,86). Since its discovery, the role of natural IgG, which pre-exists in neonates and uninfected individuals, has remained unclear due to the common view that natural antibodies lack affinity for pathogens (87) . Because of their weak interactions with self-antigens, IgG type NAbs have often been neglected as the so-called background serum antibodies without significant relevance (88,89).The potential role of natural IgGs in controlling inflammation has been demonstrated (90,91). Many autoimmune diseases are initiated by the appearance of IgG autoantibodies to specific cellular and tissue components (92–94), for instance, by the presence of pathological anti-dsDNA IgG antibodies in SLE . Moreover, these were found to play a role in transplantation related complications, such as graft-injury (95) which further confirms their adaptive immune system–derived nature and role in pathological conditions (47).

We found significant associations between infection- or vaccine-induced anti-measles IgG and anti-CS IgG natural autoantibodies in the undivided sample multitude of SAID samples, and also in the individual autoimmune disease groups (SLE, SSc, RA). The concomitance of pathogen- or vaccine-associated and natural antibodies is also verified by literature; enhancement of natural antibody repertoire by immunization has been already described in animal models (17,96), moreover, a significant correlation between levels of naturally occurring autoantibodies and response to vaccination in elderly, physically active individuals has been also reported (97).

Based on our immunoserological findings (supported by cumulative scientific data), it can be hypothesized that natural autoantibodies play a role in efficient vaccination and in the

subsequent formation of long lasting immunological memory (89), and vice versa; vaccination may also broaden the natural antibody repertoire (17,44–46).

The connection between disease-specific anti-dsDNA IgG and the Ig isotypes of antibodies of natural origin in SLE samples may support the notion regarding the adaptive nature of the IgG isotype nAAbs. According to previous findings, titers of anti-CS antibodies with IgG isotype are fluctuating over time, consequently the presence of these antibodies can be a result of an adaptive-like immune response (52,77). Recent publications suggest that IgM autoantibodies may play a protective role in SLE (56–58,83,98,99). As we expected, IgM isotypes of natural anti-F4 and anti-CS autoantibody levels showed correlation with anti-dsDNA IgM titers, supporting the hypothesis that these IgM autoantibodies are part of the natural immune repertoire in SLE.

The herein presented serological investigations harmonize with literature data (100,101)(51,52,77)(51,52,77), regarding the mechanisms that underlie the secretion of Nabs. Accordingly, NAb B-cell is pre-existing and secretes IgM NAb in steady state conditions, but it is also able to differentiate into IgG (or IgA) secreting plasma cells after repeated antigenic stimulation (77). This view is supported by the finding that IgG NAb against citrate synthase in the pericardial fluid (PF) correlated with antibody titers against pathogens associated with cardiovascular diseases, whereas anti-CS IgM NAb were not (77). This also implies that only IgM antibodies could be defined as 'conventional, protective' NAbs according to the classical definition. The current immunoserological data shows associations between IgG isotype nAbs and antigen specific (infectious) humoral antibodies. This finding may corroborate the notion that natural antibodies do not only play an important role in the first-line of defense of the immune system, but also contribute to an effective adaptive response (100,102,103) against pathogens (or vaccines). Consequently, nAbs can in fact, act as a mediator between the innate-like and the adaptive arms of the natural immune system and they are able to modify effectiveness of vaccination (52,90,100).

9 CONCLUSION

We have developed a high throughput, time-saving, cost-effective immunoserological assay that relies on international standards, for simultaneous detection of anti-measles, -mumps, and -rubella IgG antibodies in human sera. This test – to the difference of many commercially available immunoserological kits – has been optimized with the pronounced purpose of maximal background and interference removal, in order to enable reliable detection of suboptimal antibody titers. Our 'triple' or 'three-in-one' assay uses the same reagent load with uniform, short incubation times and equally pre-treated samples, enabling the three-parametric screening of 24 samples per plate within one hour, manually, or in an automated platform. In high throughput automated settings, separate testing of the three antigen types is also feasible, thus allowing the measurement of 80 samples per run.

We conclude that the importance of sero-epidemiological surveys is confirmed by recent outbreaks of measles, mumps, and rubella infections in several countries (23,25,26,104–109). Vaccine effectiveness monitoring is especially important nowadays, when the already dubious immunization practices in some of our neighboring countries aggravated by the detrimental effect of the COVID-19 pandemic, and the subsequent suspension of measles vaccination campaigns may facilitate the occurrence of smaller importation-related MeV outbreaks in susceptible cohorts (4–6,16). We analyzed serum samples (N total measles = 3919 measles, N mumps = 2132 mumps, and N rubella = 2132). Considering the HIT values, suboptimal anti-measles seropositivity ratios were detected in certain clusters of the early vaccination era (≈80 % of sufficient anti-measles IgG antibody titers among individuals vaccinated between '1978 and 1987'). This finding – in accordance with recent publication (10) and previous literature data (11) - suggests the existence of age-specific immunization gaps in the Hungarian population. For mumps and rubella, our data shows satisfactory immunity levels. We would like to emphasize that today in our country, the MMR vaccination coverage is ideal, due to the mandatory administration of safe and modern trivalent vaccines. The revealed gaps at population-level humoral immunity (IgG) are attributable to early vaccination periods, and are not a general phenomenon relative to current immunization practices (7,14,15).

Regarding nAAbs, our results – supported by literature data (59,90,60,98) -, suggest that the natural antibody pool, which was thought to be constant over time, is in fact capable of a certain degree of dynamic adaptation, which also implies the recognition of evolutionarily fixed epitopes not only of self, but also of foreign antigens (87,100). Today it is supposed that natural antibody repertoire is inherently linked to the host biome (17,110). It may explain, how vaccination - one of the main pillars of modern medicine-, induces not only the formation of memory B-cells and antibodies that confer immunity to disease causing pathogens, but also has an unintended impact on the natural antibody repertoire (17,97). The unforeseen benefit of vaccination; the enhancement of natural antibody networks, has been also reported (17). Our measurements demonstrate that there is a significant positive connection between IgG isotypes of vaccine- (or pathogen-) induced antibody levels and natural antibody levels, hence may serve as a confirmation of this theorem.

Considering nAAbs, still many paradox findings are described by literature. These may be, at least, partly explained by the many diverse approaches, each focusing on different research targets. The long-term vaccine effectiveness monitoring - using historical, well-established vaccines, as objects - may serve as a tool to resolve these contradictions. By pragmatic means; using immunoserology -based comparisons of vaccine- (or pathogen-) induced 'adaptive' IgG levels to nAAb titers, we can answer yet debated questions regarding the natural human IgG antibody repertoire (e.g. life-long stability of reactivity towards self-antigens in contrast with age-dependent diversification of reactivity against foreign antigens (111)).

The dogma that high-affinity IgG response is the major goal of immunization and low-affinity Abs should be avoided, has positively contributed to the lack of information regarding the role of nAbs in vaccination (100). However, it has been recently proposed that nAbs may serve as potential screening targets to predict the strength of antigen-induced immune response (89).

10 SUMMARY OF NOVEL RESULTS

1. We have developed a high throughput, time-saving, cost-effective and standardized 'triple' immunoserological assay for simultaneous detection of anti-measles, -mumps, and -rubella IgG antibodies in human sera. Since our test has been optimized for the screening of suboptimal antibody titers, it can be readily used to delineate susceptible individuals and potential gaps of immunological protection.
2. The methodology we have developed has also gained a secondary benefit in the meantime; it can be used as a base for a similar assay development in the respect of COVID-19 vaccine assessment, and in the screening for suboptimal antibody titers and non-responder vaccinees.
3. Our 'triple' or 'three-in-one' assay uses the same reagent load with uniform, short incubation times and equally pre-treated samples, enabling the three-parametric screening of 24 samples per plate within one hour, manually, or in an automated platform.
4. Using our self-developed assay(s) (verified by well-established commercially available kits), we have specified the potentially susceptible cohorts (who have received the measles vaccine during the early times of vaccination; between 1969-77 and between 1979-87) in the Hungarian population.
5. We have demonstrated - using the historical, widely used measles vaccine –induced immunological memory as a 'tool'- that there is a significant positive connection between IgG isotypes of vaccine- (or pathogen-) induced antibody levels and natural antibody levels, giving a functional evidence to the theory regarding the adaptive nature of IgG isotype natural antibodies.
6. We found a quantifiable, numerical (measurable antibody levels) proof demonstrating that the natural antibody pool, which was thought to be constant over time, is in fact capable of a certain degree of dynamic adaptation
7. Our assay – applied for post-vaccination follow-up studies - can be used as a functional test of potentially impaired memory B cell functions in patients with autoimmune diseases

8. We found an easily feasible, cheap serological method to answer yet debated questions regarding the natural human IgG antibody repertoire (e.g. life-long stability of reactivity towards self-antigens in contrast) with age-dependent diversification of reactivity against foreign antigens

11 REFERENCES

1. Torre G La, Saulle R, Unim B, Meggiolaro A, Barbato A, Mannocci A, et al. The effectiveness of measles-mumps-rubella (MMR) vaccination in the prevention of pediatric hospitalizations for targeted and untargeted infections: A retrospective cohort study. Hum Vaccin Immunother [Internet]. 2017 [cited 2019 Aug 1];13(8):1879. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5557224/

2. Rosian-Schikuta I, Fröschl B, Habl C, Stürzlinger H. The Measels-Mumps-Rubella Vaccination from a health political and economical point of view. GMS Health Technol Assess [Internet]. 2007 Nov 28 [cited 2019 Aug 1];3:Doc12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21289946

3. Plans-Rubió P. Evaluation of the establishment of herd immunity in the population by means of serological surveys and vaccination coverage. Hum Vaccin Immunother [Internet]. 2012 Feb 27 [cited 2019 Aug 1];8(2):184–8. Available from: http://www.tandfonline.com/doi/abs/10.4161/hv.18444

4. COVID-19's Impact on Measles Vaccination Coverage [Internet]. [cited 2021 Mar 8]. Available from: https://www.cdc.gov/globalhealth/measles/news/covid-impact-on-measles-vaccination.html

5. Durrheim DN, Andrus JK, Tabassum S, Bashour H, Githanga D, Pfaff G. A dangerous measles future looms beyond the COVID-19 pandemic. Nat Med [Internet]. 2021 Feb 15 [cited 2021 Mar 8]; Available from: http://www.nature.com/articles/s41591-021-01237-5

6. Suspension of vaccination due to COVID-19 increases the risk of infectious diseases outbreaks - UNICEF and WHO [Internet]. [cited 2021 Mar 8]. Available from: https://www.unicef.org/ukraine/en/press-releases/suspension-vaccination-due-covid-19-increases-risk-infectious-diseases-outbreaks

7. Böröcz K, Markovics Á, Zsuzsanna C, Joseph N, Timea B, Németh P. Imported infections versus herd immunity gaps ; a didactic demonstration of compartment models through the example of a minor measles outbreak in. Southeast Eur Med J [Internet]. 2021;5(1):1–16. Available from: http://seemedj.mefos.unios.hr/index.php/seemedj/article/view/199/94

8. Agócs MM, Markowitz LE, Straub I, Dömök I. The 1988-1989 measles epidemic in Hungary: assessment of vaccine failure. Int J Epidemiol [Internet]. 1992 Oct [cited 2019 May 5];21(5):1007–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1468837

9. Lengyel G, Ánosi N, Marossy A, Mátyus M, Bosnyákovits T, Orosz L. Az európai és a magyarországi kanyaróhelyzet összefoglalása és tanulságai. Orv Hetil [Internet]. 2019 May 12 [cited 2019 Jul 31];160(20):767–73. Available from: https://www.akademiai.com/doi/10.1556/650.2019.31397

10. Lengyel G, Marossy A, Ánosi N, Farkas SL, Kele B, Nemes-Nikodém É, et al. Screening of more than 2000 Hungarian healthcare workers' anti-measles antibody level: results and possible population-level consequences. Epidemiol Infect [Internet]. 2019 Sep 11 [cited 2019 May 15];147:e7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30201057

11. International Notes Measles -- Hungary [Internet]. [cited 2018 May 3]. Available from: https://www.cdc.gov/mmwr/preview/mmwrhtml/00001472.htm

12. Molnár Z, Szomor K, Huszti G, Ozsvárné Csepregi É. Local mumps outbreak in Hungary, 2007. Wkly releases [Internet]. 2007 Mar 29 [cited 2019 Aug 20];12(13):3167. Available from: http://www.eurosurveillance.org/content/10.2807/esw.12.13.03167-en

13. Is AT, He C, Dr NAN, Ws E, Kafatos G, Nardone A, et al. Standardization of measles, mumps and rubella assays to enable comparisons of seroprevalence data across 21 European countries and Australia. Epidemiol Infect. 2007;135:787–97.

14. Böröcz K, Csizmadia Z, Markovics Á, Mészáros V, Farkas K, Telek V, et al. Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human sera. J Immunol Methods. 2019 Jan 1;464:1–8.

15. Böröcz K, Csizmadia Z, Markovics, Farkas N, Najbauer J, Berki T, et al. Application of a fast and cost-effective “three-in-one” MMR ELISA as a tool for surveying anti-MMR humoral immunity: The Hungarian experience. Epidemiol Infect. 2020;148.

16. WHO Western Pacific Region. WHO Western Pacific Region: Countries and areas. Measles-Rubella Bull [Internet]. 2021;15(1):1–10. Available from: http://www.wpro.who.int/countries/en/

17. Beinart D, Ren D, Pi C, Poulton S, Holzknecht ZE, Swanson C, et al. Immunization enhances the natural antibody repertoire. EXCLI J. 2017 Jul 10;16:1018–30.

18. Haralambieva IH, Kennedy RB, Ovsyannikova IG, Whitaker JA, Poland GA. Variability in Humoral Immunity to Measles Vaccine: New Developments. Trends Mol Med [Internet]. 2015 Dec [cited 2019 Aug 2];21(12):789–801. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26602762

19. ECDC: Insufficient vaccination coverage in EU/EEA fuels continued measles circulation [Internet]. [cited 2019 Jul 24]. Available from: https://ecdc.europa.eu/en/news-events/ecdc-insufficient-vaccination-coverage-eueea-fuels-continued-measles-circulation

20. ECDC. Review of outbreaks and barriers to MMR vaccination coverage among hard-to-reach populations in Europe [Internet]. [cited 2021 Mar 21]. Available from: www.ecdc.europa.eu

21. ANTSZ - Tájékoztatás a kanyarós megbetegedésekről [Internet]. [cited 2021 Mar 4]. Available from: https://www.antsz.hu/felso_menu/temaink/jarvany/jarvany_archivum/kanyaro/tajekoztataskanyarosmegbetegedesekrol.html

22. Epidemiological update: Measles - monitoring European outbreaks, 7 July 2017 [Internet]. [cited 2021 Mar 4]. Available from: https://www.ecdc.europa.eu/en/news-events/epidemiological-update-measles-monitoring-european-outbreaks-7-july-2017

23. Lambert N, Strebel P, Orenstein W, Icenogle J, Poland GA. Rubella. Lancet (London, England) [Internet]. 2015 Jun 6 [cited 2019 Aug 1];385(9984):2297–307. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25576992

24. Moss WJ. Measles. Lancet [Internet]. 2017 Dec 2 [cited 2018 Apr 13];390(10111):2490–502. Available from:

https://www.sciencedirect.com/science/article/pii/S0140673617314630?via%3Dihub

25. Hviid A, Rubin S, Mühlemann K. Mumps. Lancet [Internet]. 2008 Mar 15 [cited 2019 Feb 21];371(9616):932–44. Available from: https://www.sciencedirect.com/science/article/pii/S0140673608604195?via%3Dihub

26. Lewnard JA, Grad YH. Vaccine waning and mumps re-emergence in the United States. Sci Transl Med [Internet]. 2018 Mar 21 [cited 2019 Feb 18];10(433):eaao5945. Available from: http://stm.sciencemag.org/content/10/433/eaao5945

27. Yang L, Grenfell BT, Mina MJ. Waning immunity and re-emergence of measles and mumps in the vaccine era. Vol. 40, Current Opinion in Virology. Elsevier B.V.; 2020. p. 48–54.

28. Haralambieva IH, Kennedy RB, Ovsyannikova IG, Schaid DJ, Poland GA. Current perspectives in assessing humoral immunity after measles vaccination [Internet]. Vol. 18, Expert Review of Vaccines. Taylor and Francis Ltd; 2019 [cited 2021 Mar 19]. p. 75–87. Available from: /pmc/articles/PMC6413513/

29. Bitzegeio J, Majowicz S, Matysiak-Klose D, Sagebiel D, Werber D, Bitzegeio juliabitzegeio J. Estimating age-specific vaccine effectiveness using data from a large measles outbreak in Berlin, Germany, 2014/15: evidence for waning immunity. [cited 2021 Mar 19];1. Available from: www.eurosurveillance.org

30. Vacsatc - Oltásbiztonság | Szakmai oldalak | Oltóanyagok csoportosítása betegségek szerint [Internet]. [cited 2020 Dec 10]. Available from: http://www.oltasbiztonsag.hu/?Olt%F3anyagok-csoportos%EDt%E1sa-betegs%E9gek-szerint&pid=73

31. WHO. Guidelines on clinical evaluation of vaccines: regulatory expectations POST ECBS version Page 2. 2016;WHO TRS 92(October):17–21. Available from: https://www.who.int/biologicals/expert_committee/Clinical_changes_IK_final.pdf%0Ahttp://www.who.int/biologicals/expert_committee/Clinical_changes_IK_final.pdf

32. Orenstein WA, Albrecht P, Herrmann KL, Bernier R, Bart KJ, Rovira EZ. The Plaque-Neutralization Test as a Measure of Prior Exposure to Measles Virus. J Infect Dis [Internet]. 1987 Jan 1 [cited 2021 Jan 6];155(1):146–9. Available from: https://academic.oup.com/jid/article-lookup/doi/10.1093/infdis/155.1.146

33. Chen RT, Markowitz LE, Albrecht P, Stewart JA, Mofenson LM, Preblud SR, et al. Measles Antibody: Reevaluation of Protective Titers. J Infect Dis [Internet]. 1990 Nov 1 [cited 2021 Jan 6];162(5):1036–42. Available from: https://academic.oup.com/jid/article-lookup/doi/10.1093/infdis/162.5.1036

34. Albrecht P, Herrmann K, Burns GR. Role of virus strain dm conventional and enhanced measles plaque neutralization test. J Virol Methods. 1981 Dec 1;3(5):251–60.

35. Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol [Internet]. 2010 Jul [cited 2019 Aug 30];17(7):1055–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20463105

36. Andrews N, Pebody RG, Berbers# G, Blondeau$ C, Crovari% P, Davidkin& I, et al. The European Sero-Epidemiology Network : standardizing the enzyme immunoassay results for measles, mumps

and rubella. Epidemiol Infect [Internet]. 2021;125:127–43. Available from: https://doi.org/10.1017/S0950268899004173

37. Bentley M, Christian P, Heath A. EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION Geneva - 8 to 12 October 2007 Report on a Collaborative Study to Investigate the Relationship between the 1 st IRP and the 2 nd and 3 rd International Standards for Anti-Measles Serum / Plasma , in both ELISA. 2007;(October).

38. Gibbs J. Immobilization Principles – Selecting the Surface. Corning Inc Life Sci. 2001;(1):1–8.

39. Gorovits B, McNally J, Fiorotti C, Leung S. Protein-based matrix interferences in ligand-binding assays [Internet]. Vol. 6, Bioanalysis. Future Science Ltd; 2014 [cited 2021 Mar 28]. p. 1131–40. Available from: https://pubmed.ncbi.nlm.nih.gov/24830897/

40. Tate J, Ward G. 5-3 Interferences in immunoassay - Google Scholar. Clin Biochem Rev [Internet]. 2004 [cited 2021 Mar 28];25(May):105–20. Available from: https://scholar.google.com/scholar_lookup?author=JTate&author=GWard&title=Interferences+in+immunoassay&pages=105-120&volume=25&publication_year=2004

41. Ward G, Simpson A, Boscato L, Hickman PE. The investigation of interferences in immunoassay. Vol. 50, Clinical Biochemistry. Elsevier Inc.; 2017. p. 1306–11.

42. Tu J. Parallelism experiments to evaluate matrix effects, selectivity and sensitivity in ligand-binding assay method development: pros and cons.

43. Schwickart M, Vainshtein I, Lee R, Schneider A, Liang M. Interference in immunoassays to support therapeutic antibody development in preclinical and clinical studies [Internet]. Vol. 6, Bioanalysis. Future Science Ltd; 2014 [cited 2021 Mar 28]. p. 1939–51. Available from: https://pubmed.ncbi.nlm.nih.gov/24806787/

44. Devalapalli AP, Lesher A, Shieh K, Solow JS, Everett ML, Edala AS, et al. Increased levels of IgE and autoreactive, polyreactive IgG in wild rodents: Implications for the hygiene hypothesis. Scand J Immunol [Internet]. 2006 Aug [cited 2021 Mar 26];64(2):125–36. Available from: https://pubmed.ncbi.nlm.nih.gov/16867157/

45. Pi C, Allott EH, Ren D, Poulton S, Ryan Lee SY, Perkins S, et al. Increased biodiversity in the environment improves the humoral response of rats. PLoS One [Internet]. 2015 Apr 8 [cited 2021 Mar 26];10(4). Available from: https://pubmed.ncbi.nlm.nih.gov/25853852/

46. Kearney JF, Patel P, Stefanov EK, King RG. Natural Antibody Repertoires: Development and Functional Role in Inhibiting Allergic Airway Disease. Annu Rev Immunol [Internet]. 2015 Mar 21 [cited 2021 Mar 25];33(1):475–504. Available from: http://www.annualreviews.org/doi/10.1146/annurev-immunol-032713-120140

47. Böröcz K, Simon D, Erdő-Bonyár S, Kovács KT, Tuba É, Czirják L, et al. Relationship between natural and infection-induced antibodies in systemic autoimmune diseases (SAD): SLE, SSc and RA. Clin Exp Immunol. 2020;

48. Griner PF, Mayewski RJ, Mushlin AI GP. Selection and interpretation of diagnostic tests and procedures. Principles and applications. - PubMed - NCBI. Ann Intern Med [Internet]. 1981 [cited 2019 Oct 22];94(4):557–92. Available from:

https://www.ncbi.nlm.nih.gov/pubmed/?term=selection+and+interpretation+griner

49. Plotkin SA. Complex correlates of protection after vaccination. Clin Infect Dis. 2013;56(10):1458–65.

50. Plotkin SA, Gilbert PB. Nomenclature for immune correlates of protection after vaccination. Clin Infect Dis [Internet]. 2012 Jun [cited 2019 Aug 30];54(11):1615–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22437237

51. Simon D, Czömpöly T, Berki T, Minier T, Peti A, Tóth E, et al. Naturally occurring and disease-associated auto-antibodies against topoisomerase I: A fine epitope mapping study in systemic sclerosis and systemic lupus erythematosus. Int Immunol. 2009;21(4):415–22.

52. Czömpöly T, Olasz K, Simon D, Nyárády Z, Pálinkás L, Czirják L, et al. A possible new bridge between innate and adaptive immunity: Are the anti-mitochondrial citrate synthase autoantibodies components of the natural antibody network? Mol Immunol [Internet]. 2006 Apr 1 [cited 2019 Dec 5];43(11):1761–8. Available from: https://www.sciencedirect.com/science/article/pii/S0161589005003937

53. Simon D, Gilicze O, Farkas N, Najbauer J. Correlation of natural autoantibodies and cardiovascular disease-related anti-bacterial antibodies in pericardial fluid of cardiac surgery patients. 2018;55–63.

54. MMWR Weekly. MMWR Publications | MMWR [Internet]. [cited 2019 May 5]. Available from: https://www.cdc.gov/mmwr/publications/index.html

55. Hungary V. Vacsatc - Oltásbiztonság | Nyitólap [Internet]. [cited 2020 Mar 31]. Available from: http://www.oltasbiztonsag.hu/

56. Jiang C, Zhao ML, Scearce RM, Diaz M. Activation-induced deaminase-deficient mrl/lpr mice secrete high levels of protective antibodies against lupus nephritis. Arthritis Rheum. 2011 Apr;63(4):1086–96.

57. Ehrenstein MR, Notley CA. The importance of natural IgM: Scavenger, protector and regulator. Vol. 10, Nature Reviews Immunology. Nature Publishing Group; 2010. p. 778–86.

58. Witte T. IgM antibodies against dsDNA in SLE. Clin Rev Allergy Immunol. 2008 Jun 21;34(3):345–7.

59. Every S. Corning ® ELISA Microplates for Biochemical and Cell-based Applications.

60. Waritani T, Chang J, McKinney B, Terato K. An ELISA protocol to improve the accuracy and reliability of serological antibody assays. MethodsX [Internet]. 2017 [cited 2018 Jan 2];4:153–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28417081

61. Thompson R, Creavin A, O'Connell M, O'Connor B, Clarke P. Optimization of the enzyme-linked lectin assay for enhanced glycoprotein and glycoconjugate analysis. Anal Biochem [Internet]. 2011;413(2):114–22. Available from: http://dx.doi.org/10.1016/j.ab.2011.02.013

62. Rodda DJ, Yamazaki H. Poly(Vinyl Alcohol) as a Blocking Agent in Enzyme Immunoassays. Immunol Invest [Internet]. 1994 Jan 7 [cited 2018 Feb 5];23(6–7):421–8. Available from: http://www.tandfonline.com/doi/full/10.3109/08820139409066836

63. Studentsov YY, Schiffman M, Strickler HD, Ho GYF, Pang YS, Schiller J, et al. Enhanced enzyme-linked immunosorbent assay for detection of antibodies to virus-like particles of human papillomavirus. J Clin Microbiol [Internet]. 2002;40(5):1755–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11980956%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC130949

64. Bolstad N, Warren DJ, Nustad K. Heterophilic antibody interference in immunometric assays. Best Pract Res Clin Endocrinol Metab [Internet]. 2013 Oct 1 [cited 2018 Mar 5];27(5):647–61. Available from: https://www.sciencedirect.com/science/article/pii/S1521690X13000778

65. Hennig C, Rink L, Fagin U, Jabs WJ, Kirchner H. The influence of naturally occurring heterophilic anti-immunoglobulin antibodies on direct measurement of serum proteins using sandwich ELISAs. J Immunol Methods [Internet]. 2000 Feb 21 [cited 2018 Mar 7];235(1–2):71–80. Available from: https://www.sciencedirect.com/science/article/pii/S0022175999002069

66. Kragstrup TW, Vorup-Jensen T, Deleuran B, Hvid M. A simple set of validation steps identifies and removes false results in a sandwich enzyme-linked immunosorbent assay caused by anti-animal IgG antibodies in plasma from arthritis patients. Springerplus [Internet]. 2013 Dec [cited 2018 Mar 4];2(1):263. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23875127

67. Sturgeon CM, Viljoen A. Analytical error and interference in immunoassay: minimizing risk. Ann Clin Biochem [Internet]. 2011 Sep 1 [cited 2018 Mar 5];48(5):418–32. Available from: http://acb.sagepub.com/lookup/doi/10.1258/acb.2011.011073

68. Datasheet: BUF038A Description. ELISA IgM REDUCING ASSAY DILUENT Datasheet BUF038A Description. Vol. 50. 2018. p. 2–3.

69. Cummings J, Raynaud F, Jones L, Sugar R, Dive C. Fit-for-purpose biomarker method validation for application in clinical trials of anticancer drugs [Internet]. Vol. 103, British Journal of Cancer. Nature Publishing Group; 2010 [cited 2021 Mar 28]. p. 1313–7. Available from: /pmc/articles/PMC2990602/

70. Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, et al. Fit-for-purpose method development and validation for successful biomarker measurement. In: Pharmaceutical Research [Internet]. Pharm Res; 2006 [cited 2021 Mar 28]. p. 312–28. Available from: https://pubmed.ncbi.nlm.nih.gov/16397743/

71. Chau CH, Rixe O, McLeod H, Figg WD. Validation of analytic methods for biomarkers used in drug development [Internet]. Vol. 14, Clinical Cancer Research. NIH Public Access; 2008 [cited 2021 Mar 28]. p. 5967–76. Available from: /pmc/articles/PMC2744124/

72. Desilva B, Smith W, Weiner R, Kelley M, Smolec J, Lee B, et al. Recommendations for the Bioanalytical Method Validation of Ligand-binding Assays to Support Pharmacokinetic Assessments of Macromolecules. 2003.

73. ECDC. Epidemiological update: Measles - monitoring European outbreaks, 7 July 2017 [Internet]. [cited 2020 Mar 31]. Available from: https://www.ecdc.europa.eu/en/news-events/epidemiological-update-measles-monitoring-european-outbreaks-7-july-2017

74. ANTSZ. ANTSZ - Public information on measles epidemics [Internet]. [cited 2020 Mar 31]. Available from:

https://www.antsz.hu/felso_menu/temaink/jarvany/jarvany_archivum/kanyaro/tajekoztataskanyarosmegbetegedesekrol.html

75. Zhang LY, Lu YP, Yang L, Luo GH, Song J, Li YP. Detection of Citrate Synthase Autoantibodies in Rats with Chronic Allograft Nephropathy. Transplant Proc. 2009 Dec;41(10):4366–8.

76. Petrohai Á, Nagy G, Bősze S, Hudecz F, Zsiros E, Paragh G, et al. Detection of citrate synthase-reacting autoantibodies after heart transplantation: an epitope mapping study. Transpl Int. 2005 Feb 15;

77. Czömpöly T, Olasz K, Nyárády Z, Simon D, Bovári J, Németh P. Detailed analyses of antibodies recognizing mitochondrial antigens suggest similar or identical mechanism for production of natural antibodies and natural autoantibodies. Autoimmun Rev. 2008;7(6):463–7.

78. Saalmueller A, Guzman E, Binder CJ, IJsbrand Reyneveld G, J Savelkoul HF, Parmentier HK. Current Understanding of Natural Antibodies and Exploring the Possibilities of Modulation Using Veterinary Models. A Review. Front Immunol | www.frontiersin.org [Internet]. 2020;1:2139. Available from: www.frontiersin.org

79. Mũoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. Vol. 6, Nature Reviews Rheumatology. Nature Publishing Group; 2010. p. 280–9.

80. McMahon M, Skaggs B. Autoimmunity: Do IgM antibodies protect against atherosclerosis in SLE? Vol. 12, Nature Reviews Rheumatology. Nature Publishing Group; 2016. p. 442–4.

81. Grönwall C, Silverman GJ. Natural IgM: Beneficial autoantibodies for the control of inflammatory and autoimmune disease. J Clin Immunol. 2014 Apr 2;34(SUPPL. 1):12–21.

82. Grönwall C, Akhter E, Oh C, Burlingame RW, Petri M, Silverman GJ. IgM autoantibodies to distinct apoptosis-associated antigens correlate with protection from cardiovascular events and renal disease in patients with SLE. Clin Immunol. 2012 Mar 1;142(3):390–8.

83. Grönwall C, Vas J, Silverman GJ. Protective roles of natural IgM antibodies. Vol. 3, Frontiers in Immunology. Frontiers; 2012. p. 66.

84. Grönwall C, Hardt U, Gustafsson JT, Elvin K, Jensen-Urstad K, Kvarnström M, et al. Depressed serum IgM levels in SLE are restricted to defined subgroups. Clin Immunol. 2017 Oct 1;183:304–15.

85. Tarlinton DM, McLean M, Nossal GJV. B1 and B2 cells differ in their potential to switch immunoglobulin isotype. Eur J Immunol [Internet]. 1995 [cited 2020 Jul 22];25(12):3388–93. Available from: https://pubmed.ncbi.nlm.nih.gov/8566028/

86. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG. Natural IgG Autoantibodies Are Abundant and Ubiquitous in Human Sera, and Their Number Is Influenced By Age, Gender, and Disease. PLoS One. 2013 Apr 2;8(4).

87. Panda S, Zhang J, Tan NS, Ho B, Ding JL. Natural IgG antibodies provide innate protection against ficolin-opsonized bacteria. EMBO J. 2013 Nov 13;32(22):2905–19.

88. Kazatchkine MD, Kaveri S V. Immunomodulation of Autoimmune and Inflammatory Diseases with

Intravenous Immune Globulin. Mackay IR, Rosen FS, editors. N Engl J Med [Internet]. 2001 Sep 6 [cited 2021 Apr 19];345(10):747–55. Available from: http://www.nejm.org/doi/10.1056/NEJMra993360

89. Kohler H, Bayry J, Nicoletti A, Kaveri S V. Natural autoantibodies as tools to predict the outcome of immune response? Vol. 58, Scandinavian Journal of Immunology. 2003. p. 285–9.

90. Panda S, Ding JL. Natural Antibodies Bridge Innate and Adaptive Immunity. J Immunol. 2015 Jan 1;194(1):13–20.

91. Palma J, Tokarz-Deptuła B, Deptuła J, Deptuła W. Natural antibodies – Facts known and unknown. Cent Eur J Immunol. 2018;43(4):466–75.

92. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLoS One. 2012 May 25;7(5).

93. Pisetsky DS. Antinuclear Antibodies in Rheumatic Disease: A Proposal for a Function-Based Classification. Scand J Immunol [Internet]. 2012 Sep 1 [cited 2020 May 1];76(3):223–8. Available from: http://doi.wiley.com/10.1111/j.1365-3083.2012.02728.x

94. Bournia VK, Vlachoyiannopoulos PG. Subgroups of Sjögren syndrome patients according to serological profiles. J Autoimmun. 2012 Aug 1;39(1–2):15–26.

95. Zorn E, See SB. Polyreactive natural antibodies in transplantation. Vol. 22, Current Opinion in Organ Transplantation. Lippincott Williams and Wilkins; 2017. p. 8–13.

96. Gudmundsdó S, Gudmundsdó BK. Specific and natural antibody response of cod juveniles vaccinated against Vibrio anguillarum. 2008;

97. Bachi ALL, Suguri VM, Ramos LR, Mariano M, Vaisberg M, Lopes JD. Increased production of autoantibodies and specific antibodies in response to influenza virus vaccination in physically active older individuals. Results Immunol. 2013 Jan 1;3:10–6.

98. Shoenfeld Y, Toubi E. Protective autoantibodies: Role in homeostasis, clinical importance, and therapeutic potential. Arthritis Rheum. 2005;52(9):2599–606.

99. Hahn BH. Antibodies to DNA. Vol. 338, New England Journal of Medicine. 1998. p. 1359–68.

100. Maddur MS, Lacroix-Desmazes S, Dimitrov JD, Kazatchkine MD, Bayry J, Kaveri S V. Natural Antibodies: from First-Line Defense Against Pathogens to Perpetual Immune Homeostasis. 2016 [cited 2020 Jul 13]; Available from: https://doi.org/10.1007/s12016-019-08746-9

101. Reyneveld GIj, Savelkoul HFJ, Parmentier HK. Current Understanding of Natural Antibodies and Exploring the Possibilities of Modulation Using Veterinary Models. A Review [Internet]. Vol. 11, Frontiers in Immunology. Frontiers Media S.A.; 2020 [cited 2021 Apr 20]. p. 2139. Available from: /pmc/articles/PMC7511776/

102. Notley CA, Brown MA, Wright GP, Ehrenstein MR. Natural IgM Is Required for Suppression of Inflammatory Arthritis by Apoptotic Cells. J Immunol [Internet]. 2011 Apr 15 [cited 2020 Jul 13];186(8):4967–72. Available from: https://pubmed.ncbi.nlm.nih.gov/21383247/

103. Maddur MS, Kaveri S V., Bayry J. Circulating Normal IgG as Stimulator of Regulatory T Cells: Lessons from Intravenous Immunoglobulin [Internet]. Vol. 38, Trends in Immunology. Elsevier Ltd; 2017 [cited 2020 Jul 13]. p. 789–92. Available from: https://pubmed.ncbi.nlm.nih.gov/28916232/

104. Ujiie M. Rubella Resurgence in Japan 2018–2019. J Travel Med [Internet]. 2019 Sep 2 [cited 2021 Feb 1];26(6). Available from: https://academic.oup.com/jtm/article/doi/10.1093/jtm/taz047/5523888

105. WHO | New measles surveillance data from WHO. WHO [Internet]. 2019 [cited 2019 Aug 22]; Available from: https://www.who.int/immunization/newsroom/new-measles-data-august-2019/en/

106. Wadman M. Measles epidemic in Ukraine drove troubling European year. Science (80-) [Internet]. 2019 Feb 15 [cited 2019 May 16];363(6428):677–8. Available from: http://www.sciencemag.org/lookup/doi/10.1126/science.363.6428.677

107. The Lancet. Measles, war, and health-care reforms in Ukraine. Lancet [Internet]. 2018 Sep 1 [cited 2019 May 16];392(10149):711. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30191812

108. Ukraine: Measles Outbreak - Jan 2019 | ReliefWeb [Internet]. [cited 2019 Aug 22]. Available from: https://reliefweb.int/disaster/ep-2019-000017-ukr

109. Balbi AM, Van Sant AA, Bean EW, Jacoby JL. Mumps: Resurgence of a once-dormant disease. J Am Acad Physician Assist [Internet]. 2018 May 1 [cited 2021 Feb 1];31(5):19–22. Available from: https://pubmed.ncbi.nlm.nih.gov/29642091/

110. Haghighi HR, Gong J, Gyles CL, Hayes MA, Zhou H, Sanei B, et al. Probiotics stimulate production of natural antibodies in chickens. Clin Vaccine Immunol [Internet]. 2006 Sep 1 [cited 2021 Apr 19];13(9):975–80. Available from: http://cvi.asm.org/

111. Lacroix-Desmazes S, Mouthon L, Coutinho A, Kazatchkine MD. Analysis of the natural human IgG antibody repertoire: life-long stability of reactivities towards self antigens contrasts with age-dependent diversification of reactivities against bacterial antigens. Eur J Immunol [Internet]. 1995 Sep 1 [cited 2021 Apr 19];25(9):2598–604. Available from: http://doi.wiley.com/10.1002/eji.1830250929

1. Torre G La, Saulle R, Unim B, Meggiolaro A, Barbato A, Mannocci A, et al. The effectiveness of measles-mumps-rubella (MMR) vaccination in the prevention of pediatric hospitalizations for targeted and untargeted infections: A retrospective cohort study. Hum Vaccin Immunother [Internet]. 2017 [cited 2019 Aug 1];13(8):1879. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5557224/

2. Rosian-Schikuta I, Fröschl B, Habl C, Stürzlinger H. The Measels-Mumps-Rubella Vaccination from a health political and economical point of view. GMS Health Technol Assess [Internet]. 2007 Nov 28 [cited 2019 Aug 1];3:Doc12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21289946

3. Plans-Rubió P. Evaluation of the establishment of herd immunity in the population by means of serological surveys and vaccination coverage. Hum Vaccin Immunother [Internet]. 2012 Feb 27 [cited 2019 Aug 1];8(2):184–8. Available from: http://www.tandfonline.com/doi/abs/10.4161/hv.18444

4. COVID-19's Impact on Measles Vaccination Coverage [Internet]. [cited 2021 Mar 8]. Available from: https://www.cdc.gov/globalhealth/measles/news/covid-impact-on-measles-vaccination.html

5. Durrheim DN, Andrus JK, Tabassum S, Bashour H, Githanga D, Pfaff G. A dangerous measles future looms beyond the COVID-19 pandemic. Nat Med [Internet]. 2021 Feb 15 [cited 2021 Mar 8]; Available from: http://www.nature.com/articles/s41591-021-01237-5

6. Suspension of vaccination due to COVID-19 increases the risk of infectious diseases outbreaks - UNICEF and WHO [Internet]. [cited 2021 Mar 8]. Available from: https://www.unicef.org/ukraine/en/press-releases/suspension-vaccination-due-covid-19-increases-risk-infectious-diseases-outbreaks

7. Böröcz K, Markovics Á, Zsuzsanna C, Joseph N, Timea B, Németh P. Imported infections versus herd immunity gaps ; a didactic demonstration of compartment models through the example of a minor measles outbreak in. Southeast Eur Med J [Internet]. 2021;5(1):1–16. Available from: http://seemedj.mefos.unios.hr/index.php/seemedj/article/view/199/94

8. Agócs MM, Markowitz LE, Straub I, Dömök I. The 1988-1989 measles epidemic in Hungary: assessment of vaccine failure. Int J Epidemiol [Internet]. 1992 Oct [cited 2019 May 5];21(5):1007–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1468837

9. Lengyel G, Ánosi N, Marossy A, Mátyus M, Bosnyákovits T, Orosz L. Az európai és a magyarországi kanyaróhelyzet összefoglalása és tanulságai. Orv Hetil [Internet]. 2019 May 12 [cited 2019 Jul 31];160(20):767–73. Available from: https://www.akademiai.com/doi/10.1556/650.2019.31397

10. Lengyel G, Marossy A, Ánosi N, Farkas SL, Kele B, Nemes-Nikodém É, et al. Screening of more than 2000 Hungarian healthcare workers' anti-measles antibody level: results and possible population-level consequences. Epidemiol Infect [Internet]. 2019 Sep 11 [cited 2019 May 15];147:e7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30201057

11. International Notes Measles -- Hungary [Internet]. [cited 2018 May 3]. Available from: https://www.cdc.gov/mmwr/preview/mmwrhtml/00001472.htm

12. Molnár Z, Szomor K, Huszti G, Ozsvárné Csepregi É. Local mumps outbreak in Hungary, 2007. Wkly releases [Internet]. 2007 Mar 29 [cited 2019 Aug 20];12(13):3167. Available from: http://www.eurosurveillance.org/content/10.2807/esw.12.13.03167-en

13. Is AT, He C, Dr NAN, Ws E, Kafatos G, Nardone A, et al. Standardization of measles, mumps and rubella assays to enable comparisons of seroprevalence data across 21 European countries and Australia. Epidemiol Infect. 2007;135:787–97.

14. Böröcz K, Csizmadia Z, Markovics Á, Mészáros V, Farkas K, Telek V, et al. Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human sera. J Immunol Methods. 2019 Jan 1;464:1–8.

15. Böröcz K, Csizmadia Z, Markovics, Farkas N, Najbauer J, Berki T, et al. Application of a fast and cost-effective "three-in-one" MMR ELISA as a tool for surveying anti-MMR humoral immunity: The Hungarian experience. Epidemiol Infect. 2020;148.

16. WHO Western Pacific Region. WHO Western Pacific Region: Countries and areas. Measles-Rubella

Bull [Internet]. 2021;15(1):1–10. Available from: http://www.wpro.who.int/countries/en/

17. Beinart D, Ren D, Pi C, Poulton S, Holzknecht ZE, Swanson C, et al. Immunization enhances the natural antibody repertoire. EXCLI J. 2017 Jul 10;16:1018–30.

18. Haralambieva IH, Kennedy RB, Ovsyannikova IG, Whitaker JA, Poland GA. Variability in Humoral Immunity to Measles Vaccine: New Developments. Trends Mol Med [Internet]. 2015 Dec [cited 2019 Aug 2];21(12):789–801. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26602762

19. ECDC: Insufficient vaccination coverage in EU/EEA fuels continued measles circulation [Internet]. [cited 2019 Jul 24]. Available from: https://ecdc.europa.eu/en/news-events/ecdc-insufficient-vaccination-coverage-eueea-fuels-continued-measles-circulation

20. ECDC. Review of outbreaks and barriers to MMR vaccination coverage among hard-to-reach populations in Europe [Internet]. [cited 2021 Mar 21]. Available from: www.ecdc.europa.eu

21. ANTSZ - Tájékoztatás a kanyarós megbetegedésekről [Internet]. [cited 2021 Mar 4]. Available from: https://www.antsz.hu/felso_menu/temaink/jarvany/jarvany_archivum/kanyaro/tajekoztataskanyarosmegbetegedesekrol.html

22. Epidemiological update: Measles - monitoring European outbreaks, 7 July 2017 [Internet]. [cited 2021 Mar 4]. Available from: https://www.ecdc.europa.eu/en/news-events/epidemiological-update-measles-monitoring-european-outbreaks-7-july-2017

23. Lambert N, Strebel P, Orenstein W, Icenogle J, Poland GA. Rubella. Lancet (London, England) [Internet]. 2015 Jun 6 [cited 2019 Aug 1];385(9984):2297–307. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25576992

24. Moss WJ. Measles. Lancet [Internet]. 2017 Dec 2 [cited 2018 Apr 13];390(10111):2490–502. Available from: https://www.sciencedirect.com/science/article/pii/S0140673617314630?via%3Dihub

25. Hviid A, Rubin S, Mühlemann K. Mumps. Lancet [Internet]. 2008 Mar 15 [cited 2019 Feb 21];371(9616):932–44. Available from: https://www.sciencedirect.com/science/article/pii/S0140673608604195?via%3Dihub

26. Lewnard JA, Grad YH. Vaccine waning and mumps re-emergence in the United States. Sci Transl Med [Internet]. 2018 Mar 21 [cited 2019 Feb 18];10(433):eaao5945. Available from: http://stm.sciencemag.org/content/10/433/eaao5945

27. Yang L, Grenfell BT, Mina MJ. Waning immunity and re-emergence of measles and mumps in the vaccine era. Vol. 40, Current Opinion in Virology. Elsevier B.V.; 2020. p. 48–54.

28. Haralambieva IH, Kennedy RB, Ovsyannikova IG, Schaid DJ, Poland GA. Current perspectives in assessing humoral immunity after measles vaccination [Internet]. Vol. 18, Expert Review of Vaccines. Taylor and Francis Ltd; 2019 [cited 2021 Mar 19]. p. 75–87. Available from: /pmc/articles/PMC6413513/

29. Bitzegeio J, Majowicz S, Matysiak-Klose D, Sagebiel D, Werber D, Bitzegeio juliabitzegeio J. Estimating age-specific vaccine effectiveness using data from a large measles outbreak in Berlin,

Germany, 2014/15: evidence for waning immunity. [cited 2021 Mar 19];1. Available from: www.eurosurveillance.org

30. Vacsatc - Oltásbiztonság | Szakmai oldalak | Oltóanyagok csoportosítása betegségek szerint [Internet]. [cited 2020 Dec 10]. Available from: http://www.oltasbiztonsag.hu/?Olt%F3anyagok-csoportos%EDt%E1sa-betegs%E9gek-szerint&pid=73

31. WHO. Guidelines on clinical evaluation of vaccines: regulatory expectations POST ECBS version Page 2. 2016;WHO TRS 92(October):17–21. Available from: https://www.who.int/biologicals/expert_committee/Clinical_changes_IK_final.pdf%0Ahttp://www.who.int/biologicals/expert_committee/Clinical_changes_IK_final.pdf

32. Orenstein WA, Albrecht P, Herrmann KL, Bernier R, Bart KJ, Rovira EZ. The Plaque-Neutralization Test as a Measure of Prior Exposure to Measles Virus. J Infect Dis [Internet]. 1987 Jan 1 [cited 2021 Jan 6];155(1):146–9. Available from: https://academic.oup.com/jid/article-lookup/doi/10.1093/infdis/155.1.146

33. Chen RT, Markowitz LE, Albrecht P, Stewart JA, Mofenson LM, Preblud SR, et al. Measles Antibody: Reevaluation of Protective Titers. J Infect Dis [Internet]. 1990 Nov 1 [cited 2021 Jan 6];162(5):1036–42. Available from: https://academic.oup.com/jid/article-lookup/doi/10.1093/infdis/162.5.1036

34. Albrecht P, Herrmann K, Burns GR. Role of virus strain dm conventional and enhanced measles plaque neutralization test. J Virol Methods. 1981 Dec 1;3(5):251–60.

35. Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol [Internet]. 2010 Jul [cited 2019 Aug 30];17(7):1055–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20463105

36. Andrews N, Pebody RG, Berbers# G, Blondeau$ C, Crovari% P, Davidkin& I, et al. The European Sero-Epidemiology Network : standardizing the enzyme immunoassay results for measles, mumps and rubella. Epidemiol Infect [Internet]. 2021;125:127–43. Available from: https://doi.org/10.1017/S0950268899004173

37. Bentley M, Christian P, Heath A. EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION Geneva - 8 to 12 October 2007 Report on a Collaborative Study to Investigate the Relationship between the 1 st IRP and the 2 nd and 3 rd International Standards for Anti-Measles Serum / Plasma , in both ELISA. 2007;(October).

38. Gibbs J. Immobilization Principles – Selecting the Surface. Corning Inc Life Sci. 2001;(1):1–8.

39. Gorovits B, McNally J, Fiorotti C, Leung S. Protein-based matrix interferences in ligand-binding assays [Internet]. Vol. 6, Bioanalysis. Future Science Ltd; 2014 [cited 2021 Mar 28]. p. 1131–40. Available from: https://pubmed.ncbi.nlm.nih.gov/24830897/

40. Tate J, Ward G. 5-3 Interferences in immunoassay - Google Scholar. Clin Biochem Rev [Internet]. 2004 [cited 2021 Mar 28];25(May):105–20. Available from: https://scholar.google.com/scholar_lookup?author=JTate&author=GWard&title=Interferences+in+immunoassay&pages=105-120&volume=25&publication_year=2004

41. Ward G, Simpson A, Boscato L, Hickman PE. The investigation of interferences in immunoassay.

Vol. 50, Clinical Biochemistry. Elsevier Inc.; 2017. p. 1306–11.

42. Tu J. Parallelism experiments to evaluate matrix effects, selectivity and sensitivity in ligand-binding assay method development: pros and cons.

43. Schwickart M, Vainshtein I, Lee R, Schneider A, Liang M. Interference in immunoassays to support therapeutic antibody development in preclinical and clinical studies [Internet]. Vol. 6, Bioanalysis. Future Science Ltd; 2014 [cited 2021 Mar 28]. p. 1939–51. Available from: https://pubmed.ncbi.nlm.nih.gov/24806787/

44. Devalapalli AP, Lesher A, Shieh K, Solow JS, Everett ML, Edala AS, et al. Increased levels of IgE and autoreactive, polyreactive IgG in wild rodents: Implications for the hygiene hypothesis. Scand J Immunol [Internet]. 2006 Aug [cited 2021 Mar 26];64(2):125–36. Available from: https://pubmed.ncbi.nlm.nih.gov/16867157/

45. Pi C, Allott EH, Ren D, Poulton S, Ryan Lee SY, Perkins S, et al. Increased biodiversity in the environment improves the humoral response of rats. PLoS One [Internet]. 2015 Apr 8 [cited 2021 Mar 26];10(4). Available from: https://pubmed.ncbi.nlm.nih.gov/25853852/

46. Kearney JF, Patel P, Stefanov EK, King RG. Natural Antibody Repertoires: Development and Functional Role in Inhibiting Allergic Airway Disease. Annu Rev Immunol [Internet]. 2015 Mar 21 [cited 2021 Mar 25];33(1):475–504. Available from: http://www.annualreviews.org/doi/10.1146/annurev-immunol-032713-120140

47. Böröcz K, Simon D, Erdő-Bonyár S, Kovács KT, Tuba É, Czirják L, et al. Relationship between natural and infection-induced antibodies in systemic autoimmune diseases (SAD): SLE, SSc and RA. Clin Exp Immunol. 2020;

48. Griner PF, Mayewski RJ, Mushlin AI GP. Selection and interpretation of diagnostic tests and procedures. Principles and applications. - PubMed - NCBI. Ann Intern Med [Internet]. 1981 [cited 2019 Oct 22];94(4):557–92. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=selection+and+interpretation+griner

49. Plotkin SA. Complex correlates of protection after vaccination. Clin Infect Dis. 2013;56(10):1458–65.

50. Plotkin SA, Gilbert PB. Nomenclature for immune correlates of protection after vaccination. Clin Infect Dis [Internet]. 2012 Jun [cited 2019 Aug 30];54(11):1615–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22437237

51. Simon D, Czömpöly T, Berki T, Minier T, Peti A, Tóth E, et al. Naturally occurring and disease-associated auto-antibodies against topoisomerase I: A fine epitope mapping study in systemic sclerosis and systemic lupus erythematosus. Int Immunol. 2009;21(4):415–22.

52. Czömpöly T, Olasz K, Simon D, Nyárády Z, Pálinkás L, Czirják L, et al. A possible new bridge between innate and adaptive immunity: Are the anti-mitochondrial citrate synthase autoantibodies components of the natural antibody network? Mol Immunol [Internet]. 2006 Apr 1 [cited 2019 Dec 5];43(11):1761–8. Available from: https://www.sciencedirect.com/science/article/pii/S0161589005003937

53. Simon D, Gilicze O, Farkas N, Najbauer J. Correlation of natural autoantibodies and cardiovascular

disease-related anti-bacterial antibodies in pericardial fluid of cardiac surgery patients. 2018;55–63.

54. MMWR Weekly. MMWR Publications | MMWR [Internet]. [cited 2019 May 5]. Available from: https://www.cdc.gov/mmwr/publications/index.html

55. Hungary V. Vacsatc - Oltásbiztonság | Nyitólap [Internet]. [cited 2020 Mar 31]. Available from: http://www.oltasbiztonsag.hu/

56. Jiang C, Zhao ML, Scearce RM, Diaz M. Activation-induced deaminase-deficient mrl/lpr mice secrete high levels of protective antibodies against lupus nephritis. Arthritis Rheum. 2011 Apr;63(4):1086–96.

57. Ehrenstein MR, Notley CA. The importance of natural IgM: Scavenger, protector and regulator. Vol. 10, Nature Reviews Immunology. Nature Publishing Group; 2010. p. 778–86.

58. Witte T. IgM antibodies against dsDNA in SLE. Clin Rev Allergy Immunol. 2008 Jun 21;34(3):345–7.

59. Every S. Corning ® ELISA Microplates for Biochemical and Cell-based Applications.

60. Waritani T, Chang J, McKinney B, Terato K. An ELISA protocol to improve the accuracy and reliability of serological antibody assays. MethodsX [Internet]. 2017 [cited 2018 Jan 2];4:153–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28417081

61. Thompson R, Creavin A, O'Connell M, O'Connor B, Clarke P. Optimization of the enzyme-linked lectin assay for enhanced glycoprotein and glycoconjugate analysis. Anal Biochem [Internet]. 2011;413(2):114–22. Available from: http://dx.doi.org/10.1016/j.ab.2011.02.013

62. Rodda DJ, Yamazaki H. Poly(Vinyl Alcohol) as a Blocking Agent in Enzyme Immunoassays. Immunol Invest [Internet]. 1994 Jan 7 [cited 2018 Feb 5];23(6–7):421–8. Available from: http://www.tandfonline.com/doi/full/10.3109/08820139409066836

63. Studentsov YY, Schiffman M, Strickler HD, Ho GYF, Pang YS, Schiller J, et al. Enhanced enzyme-linked immunosorbent assay for detection of antibodies to virus-like particles of human papillomavirus. J Clin Microbiol [Internet]. 2002;40(5):1755–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11980956%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC130949

64. Bolstad N, Warren DJ, Nustad K. Heterophilic antibody interference in immunometric assays. Best Pract Res Clin Endocrinol Metab [Internet]. 2013 Oct 1 [cited 2018 Mar 5];27(5):647–61. Available from: https://www.sciencedirect.com/science/article/pii/S1521690X13000778

65. Hennig C, Rink L, Fagin U, Jabs WJ, Kirchner H. The influence of naturally occurring heterophilic anti-immunoglobulin antibodies on direct measurement of serum proteins using sandwich ELISAs. J Immunol Methods [Internet]. 2000 Feb 21 [cited 2018 Mar 7];235(1–2):71–80. Available from: https://www.sciencedirect.com/science/article/pii/S0022175999002069

66. Kragstrup TW, Vorup-Jensen T, Deleuran B, Hvid M. A simple set of validation steps identifies and removes false results in a sandwich enzyme-linked immunosorbent assay caused by anti-animal IgG antibodies in plasma from arthritis patients. Springerplus [Internet]. 2013 Dec [cited 2018 Mar 4];2(1):263. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23875127

67. Sturgeon CM, Viljoen A. Analytical error and interference in immunoassay: minimizing risk. Ann Clin Biochem [Internet]. 2011 Sep 1 [cited 2018 Mar 5];48(5):418–32. Available from: http://acb.sagepub.com/lookup/doi/10.1258/acb.2011.011073

68. Datasheet: BUF038A Description. ELISA IgM REDUCING ASSAY DILUENT Datasheet BUF038A Description. Vol. 50. 2018. p. 2–3.

69. Cummings J, Raynaud F, Jones L, Sugar R, Dive C. Fit-for-purpose biomarker method validation for application in clinical trials of anticancer drugs [Internet]. Vol. 103, British Journal of Cancer. Nature Publishing Group; 2010 [cited 2021 Mar 28]. p. 1313–7. Available from: /pmc/articles/PMC2990602/

70. Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, et al. Fit-for-purpose method development and validation for successful biomarker measurement. In: Pharmaceutical Research [Internet]. Pharm Res; 2006 [cited 2021 Mar 28]. p. 312–28. Available from: https://pubmed.ncbi.nlm.nih.gov/16397743/

71. Chau CH, Rixe O, McLeod H, Figg WD. Validation of analytic methods for biomarkers used in drug development [Internet]. Vol. 14, Clinical Cancer Research. NIH Public Access; 2008 [cited 2021 Mar 28]. p. 5967–76. Available from: /pmc/articles/PMC2744124/

72. Desilva B, Smith W, Weiner R, Kelley M, Smolec J, Lee B, et al. Recommendations for the Bioanalytical Method Validation of Ligand-binding Assays to Support Pharmacokinetic Assessments of Macromolecules. 2003.

73. ECDC. Epidemiological update: Measles - monitoring European outbreaks, 7 July 2017 [Internet]. [cited 2020 Mar 31]. Available from: https://www.ecdc.europa.eu/en/news-events/epidemiological-update-measles-monitoring-european-outbreaks-7-july-2017

74. ANTSZ. ANTSZ - Public information on measles epidemics [Internet]. [cited 2020 Mar 31]. Available from: https://www.antsz.hu/felso_menu/temaink/jarvany/jarvany_archivum/kanyaro/tajekoztataskanyarosmegbetegedesekrol.html

75. Zhang LY, Lu YP, Yang L, Luo GH, Song J, Li YP. Detection of Citrate Synthase Autoantibodies in Rats with Chronic Allograft Nephropathy. Transplant Proc. 2009 Dec;41(10):4366–8.

76. Petrohai Á, Nagy G, Bősze S, Hudecz F, Zsiros E, Paragh G, et al. Detection of citrate synthase-reacting autoantibodies after heart transplantation: an epitope mapping study. Transpl Int. 2005 Feb 15;

77. Czömpöly T, Olasz K, Nyárády Z, Simon D, Bovári J, Németh P. Detailed analyses of antibodies recognizing mitochondrial antigens suggest similar or identical mechanism for production of natural antibodies and natural autoantibodies. Autoimmun Rev. 2008;7(6):463–7.

78. Saalmueller A, Guzman E, Binder CJ, IJsbrand Reyneveld G, J Savelkoul HF, Parmentier HK. Current Understanding of Natural Antibodies and Exploring the Possibilities of Modulation Using Veterinary Models. A Review. Front Immunol | www.frontiersin.org [Internet]. 2020;1:2139. Available from: www.frontiersin.org

79. Mũoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of

apoptotic cells in systemic autoimmunity. Vol. 6, Nature Reviews Rheumatology. Nature Publishing Group; 2010. p. 280–9.

80. McMahon M, Skaggs B. Autoimmunity: Do IgM antibodies protect against atherosclerosis in SLE? Vol. 12, Nature Reviews Rheumatology. Nature Publishing Group; 2016. p. 442–4.

81. Grönwall C, Silverman GJ. Natural IgM: Beneficial autoantibodies for the control of inflammatory and autoimmune disease. J Clin Immunol. 2014 Apr 2;34(SUPPL. 1):12–21.

82. Grönwall C, Akhter E, Oh C, Burlingame RW, Petri M, Silverman GJ. IgM autoantibodies to distinct apoptosis-associated antigens correlate with protection from cardiovascular events and renal disease in patients with SLE. Clin Immunol. 2012 Mar 1;142(3):390–8.

83. Grönwall C, Vas J, Silverman GJ. Protective roles of natural IgM antibodies. Vol. 3, Frontiers in Immunology. Frontiers; 2012. p. 66.

84. Grönwall C, Hardt U, Gustafsson JT, Elvin K, Jensen-Urstad K, Kvarnström M, et al. Depressed serum IgM levels in SLE are restricted to defined subgroups. Clin Immunol. 2017 Oct 1;183:304–15.

85. Tarlinton DM, McLean M, Nossal GJV. B1 and B2 cells differ in their potential to switch immunoglobulin isotype. Eur J Immunol [Internet]. 1995 [cited 2020 Jul 22];25(12):3388–93. Available from: https://pubmed.ncbi.nlm.nih.gov/8566028/

86. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG. Natural IgG Autoantibodies Are Abundant and Ubiquitous in Human Sera, and Their Number Is Influenced By Age, Gender, and Disease. PLoS One. 2013 Apr 2;8(4).

87. Panda S, Zhang J, Tan NS, Ho B, Ding JL. Natural IgG antibodies provide innate protection against ficolin-opsonized bacteria. EMBO J. 2013 Nov 13;32(22):2905–19.

88. Kazatchkine MD, Kaveri S V. Immunomodulation of Autoimmune and Inflammatory Diseases with Intravenous Immune Globulin. Mackay IR, Rosen FS, editors. N Engl J Med [Internet]. 2001 Sep 6 [cited 2021 Apr 19];345(10):747–55. Available from: http://www.nejm.org/doi/10.1056/NEJMra993360

89. Kohler H, Bayry J, Nicoletti A, Kaveri S V. Natural autoantibodies as tools to predict the outcome of immune response? Vol. 58, Scandinavian Journal of Immunology. 2003. p. 285–9.

90. Panda S, Ding JL. Natural Antibodies Bridge Innate and Adaptive Immunity. J Immunol. 2015 Jan 1;194(1):13–20.

91. Palma J, Tokarz-Deptuła B, Deptuła J, Deptuła W. Natural antibodies – Facts known and unknown. Cent Eur J Immunol. 2018;43(4):466–75.

92. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLoS One. 2012 May 25;7(5).

93. Pisetsky DS. Antinuclear Antibodies in Rheumatic Disease: A Proposal for a Function-Based Classification. Scand J Immunol [Internet]. 2012 Sep 1 [cited 2020 May 1];76(3):223–8. Available from: http://doi.wiley.com/10.1111/j.1365-3083.2012.02728.x

94. Bournia VK, Vlachoyiannopoulos PG. Subgroups of Sjögren syndrome patients according to serological profiles. J Autoimmun. 2012 Aug 1;39(1–2):15–26.

95. Zorn E, See SB. Polyreactive natural antibodies in transplantation. Vol. 22, Current Opinion in Organ Transplantation. Lippincott Williams and Wilkins; 2017. p. 8–13.

96. Gudmundsdó S, Gudmundsdó BK. Specific and natural antibody response of cod juveniles vaccinated against Vibrio anguillarum. 2008;

97. Bachi ALL, Suguri VM, Ramos LR, Mariano M, Vaisberg M, Lopes JD. Increased production of autoantibodies and specific antibodies in response to influenza virus vaccination in physically active older individuals. Results Immunol. 2013 Jan 1;3:10–6.

98. Shoenfeld Y, Toubi E. Protective autoantibodies: Role in homeostasis, clinical importance, and therapeutic potential. Arthritis Rheum. 2005;52(9):2599–606.

99. Hahn BH. Antibodies to DNA. Vol. 338, New England Journal of Medicine. 1998. p. 1359–68.

100. Maddur MS, Lacroix-Desmazes S, Dimitrov JD, Kazatchkine MD, Bayry J, Kaveri S V. Natural Antibodies: from First-Line Defense Against Pathogens to Perpetual Immune Homeostasis. 2016 [cited 2020 Jul 13]; Available from: https://doi.org/10.1007/s12016-019-08746-9

101. Reyneveld GIj, Savelkoul HFJ, Parmentier HK. Current Understanding of Natural Antibodies and Exploring the Possibilities of Modulation Using Veterinary Models. A Review [Internet]. Vol. 11, Frontiers in Immunology. Frontiers Media S.A.; 2020 [cited 2021 Apr 20]. p. 2139. Available from: /pmc/articles/PMC7511776/

102. Notley CA, Brown MA, Wright GP, Ehrenstein MR. Natural IgM Is Required for Suppression of Inflammatory Arthritis by Apoptotic Cells. J Immunol [Internet]. 2011 Apr 15 [cited 2020 Jul 13];186(8):4967–72. Available from: https://pubmed.ncbi.nlm.nih.gov/21383247/

103. Maddur MS, Kaveri S V., Bayry J. Circulating Normal IgG as Stimulator of Regulatory T Cells: Lessons from Intravenous Immunoglobulin [Internet]. Vol. 38, Trends in Immunology. Elsevier Ltd; 2017 [cited 2020 Jul 13]. p. 789–92. Available from: https://pubmed.ncbi.nlm.nih.gov/28916232/

104. Ujiie M. Rubella Resurgence in Japan 2018–2019. J Travel Med [Internet]. 2019 Sep 2 [cited 2021 Feb 1];26(6). Available from: https://academic.oup.com/jtm/article/doi/10.1093/jtm/taz047/5523888

105. WHO | New measles surveillance data from WHO. WHO [Internet]. 2019 [cited 2019 Aug 22]; Available from: https://www.who.int/immunization/newsroom/new-measles-data-august-2019/en/

106. Wadman M. Measles epidemic in Ukraine drove troubling European year. Science (80-) [Internet]. 2019 Feb 15 [cited 2019 May 16];363(6428):677–8. Available from: http://www.sciencemag.org/lookup/doi/10.1126/science.363.6428.677

107. The Lancet. Measles, war, and health-care reforms in Ukraine. Lancet [Internet]. 2018 Sep 1 [cited 2019 May 16];392(10149):711. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30191812

108. Ukraine: Measles Outbreak - Jan 2019 | ReliefWeb [Internet]. [cited 2019 Aug 22]. Available from: https://reliefweb.int/disaster/ep-2019-000017-ukr

109. Balbi AM, Van Sant AA, Bean EW, Jacoby JL. Mumps: Resurgence of a once-dormant disease. J Am Acad Physician Assist [Internet]. 2018 May 1 [cited 2021 Feb 1];31(5):19–22. Available from: https://pubmed.ncbi.nlm.nih.gov/29642091/

110. Haghighi HR, Gong J, Gyles CL, Hayes MA, Zhou H, Sanei B, et al. Probiotics stimulate production of natural antibodies in chickens. Clin Vaccine Immunol [Internet]. 2006 Sep 1 [cited 2021 Apr 19];13(9):975–80. Available from: http://cvi.asm.org/

111. Lacroix-Desmazes S, Mouthon L, Coutinho A, Kazatchkine MD. Analysis of the natural human IgG antibody repertoire: life-long stability of reactivities towards self antigens contrasts with age-dependent diversification of reactivities against bacterial antigens. Eur J Immunol [Internet]. 1995 Sep 1 [cited 2021 Apr 19];25(9):2598–604. Available from: http://doi.wiley.com/10.1002/eji.1830250929

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Böröcz K, Csizmadia Z, Markovics Á, Mészáros V, Farkas K, Telek V, Varga V, Maloba G O, Bodó K, Najbauer J, Berki T, Németh P. Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human sera. J Immunol Methods. 2019 Jan 1; 464:1–8.

Böröcz K, Csizmadia Z, Markovics, Farkas N, Najbauer J, Berki T, Németh P. Application of a fast and cost-effective “three-in-one” MMR ELISA as a tool for surveying anti-MMR humoral immunity: The Hungarian experience. Epidemiol Infect. 2020; 148: 1-12.

Böröcz K, Simon D, Erdő-Bonyár S, Kovács KT, Tuba É, Czirják L, Németh P., Berki T. Relationship between natural and infection-induced antibodies in systemic autoimmune diseases (SAD): SLE, SSc and RA. Clin Exp Immunol. 2020; 203/1.: 32-40.

Böröcz K, Markovics Á, Zsuzsanna C, Najbauer J, Berki T, Németh P. Imported infections versus herd immunity gaps; a didactic demonstration of compartment models through the example of a minor measles outbreak in. Southeast Eur Med J. 2021;5(1):1–16.

13 LIST OF OTHER PUBLICATIONS

Bodo, K; Boros, A ; Rumpler, E ; Molnar, L ; Borocz, K ; Nemeth, P ; Engelmann, P. Identification of novel lumbricin homologues in Eisenia andrei earthworms. DEVELOPMENTAL AND COMPARATIVE IMMUNOLOGY 90 pp. 41-46. , 6 p. (2019)

Hayden, Z ; Böröcz, K ; Csizmadia, Z ; Balogh, P ; Kellermayer, Z ; Bodó, K ; Najbauer, J ; Berki, T-. Single-center study of autoimmune encephalitis-related autoantibody testing in Hungary. BRAIN AND BEHAVIOR 9 : 12 Paper: e01454 , 7 p. (2019)

Hayden, Z; Böröcz, K ; Csizmadia, Z ; Kellermayer, Z ; Balogh, P ; Berki, T. A paraneoplaziás neurológiai szindrómák laboratóriumi diagnosztikája és immunológiai vonatkozásai. MAGYAR ONKOLÓGIA 63: 3 pp. 261-267. , 7 p. (2019)

Molnar, T; Borocz, K; Berki, T ; Szapary, L ; Szolics, A ; Janszky, J ; Illes, Z ; Csecsei, P. Subacute Elevation of Plasma Level of Caspase-Cleaved Cytokeratin-18 is Associated with Hemorrhagic Transformation and Functional Outcome in Ischemic Stroke. JOURNAL OF STROKE AND CEREBROVASCULAR DISEASES 28 : 3 pp. 719-727. , 9 p. (2019)

Berki, T; Hayden, Z ; Böröcz, K ; Csizmadia, Z ; Kellermayer, Z ; Balogh, P. Immunmediált kórképek diagnosztikája : laboratóriumi szakemberek és a klinikusok együttműködése. NEUROLÓGIAI PRAXIS 1 : 4 pp. 14-15. , 2 p. (2018)

Borocz, K; Hayden, Z; Meszaros, V ; Csizmadia, Z ; Farkas, K ; Kellermayer, Z ; Balogh, P ; Nagy, F ; Berki, T. Az autoimmun encephalitisek laboratóriumi vizsgálati lehetőségei. ORVOSI HETILAP 159 : 3 pp. 107-112. , 6 p. (2018)

14 SCIENTIFIC ACTIVITIES

Occasion	Date	Place	Poster/ presentation	Title
46th Membrane-Transport Conference	17-20 May, 2016	Sümeg, Hungary	Poster and presentation	**Diagnostic value of anti-receptor autoantibodies in immune-mediated neurological diseases** *Böröcz K, Mészáros V, Kellermayer Z, Berki T*
45th Annual Roving Conference of the Hungarian Immunological Society	19-21 October, 2016	Velence, Hungary	Poster	**Large-scale immunoserological efficacy study of measles vaccination** *Böröcz K, Mészáros V, Farkas K, Csizmadia Z, Katz Z, Németh P*
University of Pécs **Center for Immunological Excellence - Seminars**	11 May, 2017	University of Pécs, Pécs, Hungary	Presentation	**Obstacles and successes during the development of an automated ELISA test, used for the detection of anti-measles antibody detection** *Böröcz K – Németh P*
47th Membrane-Transport Conference	16-19 May, 2017	Sümeg, Hungary	Poster	**Modeling the conformation sensitivity of antigen-antibody binding on artificial surfaces** *Böröcz K, Csizmadia Z, Mészáros V, Berki T, Markovics Á, Németh P*
46th Annual Roving Conference of the Hungarian Immunological Society	18-20 October, 2017	Velence, Hungary	Presentation	**Measles vaccine efficacy study-** **identifying potentially susceptible cohorts in Hungary** *Böröcz K, Ouma M G, Csizmadia Z, Farkas K, Németh P*
5th Autoimmunity Seminar – Shedding a new light on autoimmunity	13-15 November, 2017	Barcelona, Spain	Presentation	**Comparison of autoimmune routine diagnostic laboratory elisas with their corresponding chemiluminescent assays** **- a study based on our endeavors towards assay modernization in the light of quality control tendencies** *Böröcz K, Csizmadia Z, Berki T*
National Community of Young Biotechnologists (FIBOK)	28-29.March, 2018	ELTE, Budapest, Hungary	Poster	**Opening a new door to cost-effective largescale vaccine effectiveness studies** *Böröcz K, Csizmadia Z, Mészáros V, Berki T, Markovics Á, Németh P*
University of Pécs **World Immunization Week, International Roundtable Conference**	28-30 April, 2018	University of Pécs, Hungary	Presentation	**New challenges: monitoring the long-term efficacy of vaccines** *Böröcz K, Németh P*
MIT-MLDT Congress, 2018 **University of Pécs**	30 August – 1 September, 2018	Pécs, PTE ÁOK	Poster	**Development of a combined high throughput and cost-effective indirect ELISA for MMR vaccine efficacy screening** *Böröcz K, Csizmadia Z, Varga V, Telek V, Berki T, Németh P*
MIT-MLDT Congress, 2018	30 August – 1	Pécs, PTE ÁOK	Poster	**Comparison of chemiluminescence and conventional ELISA techniques in autoantibody detection**

University of Pécs	September, 2018			*Csizmadia Z, Böröcz K, Telek V, Varga V, Berki T*
5th European Congress of Immunology - ECI Amsterdam	2-5 September, 2018	Amsterdam, Netherlands	Poster	**Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human serum** *Böröcz K, Csizmadia Z, Mészáros V, Markovics Á, Farkas K,Telek V, Varga V, Ouma M G, Bodó K, Najbauer J, Berki T B, Németh P*
MIT MLDT **Professional Development Course**	3 October, 2018	Budapest, Hungary	Presentation	**New possibilities of autoantibody detection** *Berki T, Böröcz K*
University of Pécs **World Immunization Week, International Roundtable Conference**	30 April, 2019	University of Pécs, Hungary	Presentation	**Monitoring of long term efficacy of the MMR vaccination in the Hungarian population** *Böröcz K, Németh P*
1st Pécs-Osijek PhD Symposim	10 May, 2019	University of Pécs, Hungary	Presentation	**Vaccination state of the Hungarian population** *Böröcz K, Németh P*
49th Membrane-Transport Conference	14-17 May, Sümeg	Sümeg, Hungary	Poster and presentation	**A quick trivalent ELISA assay for the detection of MMR vaccine effectiveness, and for seronegativity screening** *Böröcz K, Csizmadia Z,Markovics Á, Farkas K, Najbauer J , Berki T B, Németh P*
WHO Consensus Meeting	8 October, 2019	University of Pécs, Hungary	Presentation	**Conclusions of a measles/MMR serosurvey in the Hungarian population** *Böröcz K, Németh P*
5th International Consensus on ANA pattern (ICAP) Workshop **4th International Autoantibody Standardization (IAS) Workshop**	10-13 September, 2019	Dresden, Germany	Poster	**Comparison of commonly used dsDNA assay types in order to better support an accurate clinical diagnosis** *Böröcz K, Csizmadia Z, Varga V, Berki T* **Evaluation of three years of maternal infertility - related autoantibody test requests** *Csizmadia Z, Böröcz K, Varga V, Balázs N, Berki T*
EWRR	13 Ferbruary, 2020	Leuven, Belgium	Poster	**Infection (or vaccine)-induced antibody and natural autoantibody levels may show association in systemic lupus erythematosus (SLE) patients** *Böröcz K, Simon D, Erdő-Bonyár S,Csizmadia Z, Kovács A, Czirják L, Németh P, Berki T*
Health Innovation Cluster of Pécs (PEIK) Conference	27 October, 2020	Pécs, Hungary	Presentation	Vaccine Effectiveness Monitoring (early warning) *Böröcz K, Németh P*

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Relationship between natural and infection-induced antibodies in systemic autoimmune diseases (SAD): SLE, SSc and RA

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Summary

Infection or vaccine-induced T cell-dependent immune response and the subsequent high-affinity neutralizing antibody production have been extensively studied, while the connection between natural autoantibodies (nAAbs) and disease-specific antibodies has not been thoroughly investigated. Our goal was to find the relationship between immunoglobulin (Ig) M and IgG isotype nAAbs and infection or vaccine-induced and disease-related autoantibody levels in systemic autoimmune diseases (SAD). A previously described indirect enzyme-linked immunosorbent assay (ELISA) test was used for detection of IgM/IgG nAAbs against citrate synthase (anti-CS) and F4 fragment (anti-F4) of DNA topoisomerase I in 374 SAD samples, with a special focus on systemic lupus erythematosus (SLE) (n = 92), rheumatoid arthritis (n = 73) and systemic sclerosis (n = 157) disease groups. Anti-measles IgG and anti-dsDNA IgG/IgM autoantibodies were measured using commercial and in-house indirect ELISA tests. In all SAD groups the anti-measles IgG-seropositive cases showed significantly higher anti-CS IgG titers (P = 0·011). In anti-dsDNA IgG-positive SLE patients, we detected significantly higher levels of anti-CS and anti-F4 IgG nAAbs (P = 0·001 and < 0·001, respectively). Additionally, we found increased levels of IgM isotypes of anti-CS and anti-F4 nAAbs in anti-dsDNA IgM-positive SLE patients (P = 0·002 and 0·016, respectively). The association between IgG isotypes of pathogen- or autoimmune disease-related antibodies and the IgG nAAbs may underscore the immune response-inducible nature of the diseases investigated. The relationship between protective anti-dsDNA IgM and the IgM isotype of anti-F4 and anti-CS may provide immunoserological evidence for the beneficial roles of nAAbs in SLE patients.

Keywords: autoimmunity, autoantibodies, antibodies, vaccination, systemic lupus erythematosus

Introduction

Since the discovery of natural autoantibodies, a great effort has been devoted to describing their generation, regulation and function [1]. It has been determined that natural immunoglobulin (Ig)M antibodies are present prior to antigen stimulation, and that their reactivity profiles are highly conserved between individuals [2]. The presence of natural antibodies recognizing citrate synthase (CS) both in healthy individuals and in patients with systemic autoimmune disease has already been demonstrated in our previous study. The majority of these antibodies proved to be of IgM isotype. Their presence in infants and their unaltered serum level during ≥ 5 years in adults indicates that these antibodies belong to the natural autoantibody (nAAb) repertoire established early in postnatal life [3]. Natural IgM autoantibodies have been also proposed to convey protection from pathological autoimmune reactions [4–8]. The impaired anti-apoptotic function of natural IgM

autoantibodies, may, leading to their pathological elimination of dying cells and subsequent maintenance of autoinflammation [5]. Natural IgM antibodies are produced by the relatively class-restricted B1 cells, while IgG antibodies are known to be produced via the T cell-dependent interactions of follicular B2 cells [2,9]. Although natural and pathological antibodies of IgG isotype may show mutual characteristics, it is important to differentiate between them. Since its discovery, the role of natural IgG, which pre-exists in neonates and uninfected individuals, has remained unclear due to the common view that natural antibodies lack affinity for pathogens [10]. Although it is already known that one of the most prevalent functions of nAAbs is the ability to provide protection against bacterial, viral and fungal infections, the connection between immunization and nAAbs has not yet been thoroughly investigated.

The potential role of natural IgGs in controlling inflammation has been also demonstrated [11,12]. Many autoimmune diseases are initiated by the appearance of IgG autoantibodies to specific cellular and tissue components [13–15]; for instance, by the presence of pathological anti-dsDNA IgG antibodies in systemic lupus erythematosus (SLE). Like those of pathological origin, levels of IgG isotype nAAbs have been proved to fluctuate over time [3,16], and are reported to be abundant and ubiquitous in human sera. Their levels are influenced by age, gender and disease [2,3,17]. Moreover, they were found to play a role in transplantation-related complications, such as graft injury [18], which further confirms their adaptive immune system-derived nature and role in pathological conditions.

Herein we describe our investigations in SLE, systemic sclerosis (SSc) and rheumatoid arthritis (RA) sample groups, focusing on associations among levels of vaccine- or infection-induced antibodies [pathological; anti-measles IgG, SLE-associated autoantibodies (naturally and pathological; anti-dsDNA IgG and IgM) and nAAbs (anti-CS), anti-dsDNA topoisomerase I F4 fragment (anti-F4)] aiming to find an immunoserological proof for the co-existence of IgG isotype pathogen- or disease-related antibodies and nAAbs. Secondly, we wanted to evidence the simultaneous presence of the known protective anti-dsDNA IgM autoantibodies and IgM isotype of anti-CS and anti-F4 in SLE patients, confirming their potential regulatory and beneficial role.

Materials And Methods

Samples

Serum samples of patients suffering from different systemic autoimmune diseases (SAD) were obtained from the serum bank of the Department of Rheumatology and Immunology, University of Pécs (Hungary). The samples were stored and analyzed anonymously in the laboratories of the Department of Immunology and Biotechnology (University of Pécs Medical School) according to quality assurance criteria (ISO 17025) (Ethical License: 2015/5726 by the Regional Research Ethics Committee at the University of Pécs). The number of sera derived from different systemic autoimmune patients comprised the following: SSc n = 157, SLE n = 92, RA n = 73, other = 52 (total n = 374). Mean age (rounded values; years) within sample groups was the following: SSc, 56; SLE, 44; RA, 59, 53 (overall, 52).

Methods

In order to investigate associations among vaccine-induced antibodies (anti-measles IgG), SLE-related autoantibodies (anti-dsDNA IgG/M) and natural (auto) antibodies [anti-DNA topoisomerase-I (or anti-Scl-70) fragment F4 (anti-F4) IgG/M, anti-CS IgG/M] indirect enzyme-linked immunosorbent assay (ELISA) tests were performed using manual sample dilution followed by programmed assay execution on automated Siemens BEP 2000 Advance® platform (Siemens AG, Frankfurt, Germany).

Anti-F4 IgG/M ELISA

We used the recombinant fragment-4 (F4) of topoisomerase I [amino acid (AA) 450–600] as antigen for detection of IgG and IgM nAAbs. Similarly, as described earlier [3,19], 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with recombinant topo I F4 fragment or with maltose-binding protein (MBP) on the other half of the plate at 2·5 μg/ml in ELISA coating buffer (Bio-Rad BUF030) (50 μl/well, 4–6°C, overnight; Biorad, Hercules, CA, USA). Plates were washed with washing buffer (WB) [100 mM phosphate-buffered saline (PBS), pH 7.4 + 1 ml/l Tween 20, (350 μl/well] and blocked with 0·5 m/m% polyvinyl alcohol (PVA) (~72 000 Mw, 300 μl/well, room temperature, ≥ 2 h). Serum samples were incubated in 100-fold dilution in WB (IgG: 50 μl/well, 37°C, 35 min/IgM: 50 μl/well, 37°C, 35 min). Standards, blanks (wells containing only WB), high and low controls (positive and negative samples identified in a previous run) were processed as patient sera, and were also automatically assigned to both plate halves. Subsequently, plates were incubated with horseradish peroxidase (HRP)-conjugated anti-human-IgG or anti-human IgM secondary antibody (Dako, Glostrup, Denmark) for 30 min at 37°C. Color reaction was developed using 3,3′,5,5′-tetramethylbenzidine (TMB) (Bio-Rad BUF056A); finally, 0·18 M H_2SO_4 stop solution was applied (50 μl/well). Reading was performed at λ = 450/620 nm.

To achieve a better comparability between vast numbers of results, the formerly used operational protocol was optimized to the automated setting. The former extinction [optical density (OD)]-based result evaluation was replaced by the conversion of optical densities into quantitative results, using an in-house anti-F4 standard made of pooled known positive sera. Doubling, five-point dilution series of standard points were applied in triplicate (starting dilution point: 50-fold). For the calculation of the standard curve, a four-point sigmoid fit was applied.

As described by Simon *et al.* [19], the F4 fragment of DNA topoisomerase I (topo I) was expressed as recombinant maltose-binding protein (MBP) fusion protein. Consequently, results obtained for the MBP antigen coating were used to measure the potential background. The final result calculation in terms of absorbance (OD) was performed as follows:

OD sample (anti-topo I F4 fragment) = OD sample F4 well–OD sample MBP well.

Anti-CS IgG/M ELISA

Anti-CS (IgG/M) indirect ELISA measurements were also performed as described earlier [3,16,17,19] with the already detailed slight modifications, due to high sample numbers and the need of signal-to-noise ratio optimization required by the automated setting. Nunc MaxiSorp™ ELISA plates were coated with CS from porcine heart (Sigma-Merck C3260) at a concentration of 2·25 μg/ml in coating buffer (Bio-Rad BUF030) (50 μl/well, 4–6°C, overnight). Plates were washed and blocked as described above. Serum samples were incubated in 100-fold dilution in WB for 35 min at room temperature (RT). Standards, blanks, high and low controls were processed as patient sera. After three washing steps, anti-human IgM or IgG secondary antibody (Dako) was incubated for 30 min followed by TMB substrate for 15 min and H_2SO_4 stop solution (50 μl/well). Automation and reading was performed as described earlier. A five-point dilution series of our in-house anti-CS standard was used for result quantitation, with subsequent four-point sigmoid curve fitting.

Anti-measles IgG ELISA

Anti-measles antibody (IgG)-level data were considered an adequate model for pathogen-derived (or infection-induced) antibodies that are present in the great majority of the Hungarian population. Based on our previous findings [20,21] and in accordance with data of other Hungarian colleagues [22,23], the overall ratio of the Hungarian population in possession of sufficient anti-measles IgG antibody levels is ≈90%, with a well-characterized, more susceptible cluster being responsible for the lagging ≈10%. Age group categorization based on historical changes in the measles, mumps and rubella (MMR) immunization schedule, recurring epidemics, subsequent revaccination protocols and shifts between vaccine valency (mono-, bi- or trivalent) and manufacturers have been thoroughly detailed previously [20,21,24]. Considering that we already had a population-level result [more-fold verified by multiple measurement techniques (ELISA, IIF)] and independent research groups] [20,21,23,25] focusing on anti-measles IgG antibody titers, it was obvious to use anti-measles data as one of the means of our comparisons. Anti-measles antibody (IgG) measurements were performed using our self-developed ELISA assays validated by well-established commercial kits (Novalisa, Immunolab, Euroimmun, Sekisui-Virotech, Serion, Siemens Enzygnost), as previously reported [20,21]. For the anti-measles (IgG) in-house ELISA, Nunc MaxiSorp™ plates were coated with measles virus Edmonston strain (Bio-Rad PIP013) as antigen at a concentration of 2·8 μg/ml in coating buffer (Bio-Rad BUF030) at 4–6°C overnight. After three washing steps, plates were blocked with PVA at RT overnight. After pretreatment of sera with IgM reducing assay diluent (Bio-Rad BUF038) and subsequent centrifugation, 25 μl of supernatant was transferred to a microplate prefilled with 75 μl of WB, resulting in a further fourfold dilution. The Third WHO International Standard for Anti-Measles (NIBSC code: 97/648) was used in five-point doubling dilutions (starting concentration: ~5000 mIU/ml). Standards, blanks, high and low controls were processed as patient sera, and were automatically assigned to plates. Primary and secondary antibodies (analytes and the Dako, rabbit anti-human HRP-conjugated polyclonal IgG) were incubated for 20–20 min (100 μl/well, 37°C). Color reaction development and stopping were carried out as detailed above. Automation and reading were performed using the Siemens BEP 2000 Advance System, λ = 450/620 nm. For the qualitative evaluation, cut-off values were based on area under the receiver operating characteristic (AUROC) analysis, as described earlier [21].

Anti-dsDNA IgG/M ELISA

Anti-dsDNA IgG/M measurements were performed using commercial anti-dsDNA ELISA kits (ORG604 and ORG604M; Orgentec Diagnostika GmbH, Mainz, Germany). In the test, human recombinant double-stranded DNA (dsDNA) is bound to microwells. Quantitative anti-dsDNA antibody titer evaluation was performed as per the manufacturer's instructions; optical density values were read at 450 nm (reference 600 690 nm). Qualitative (positive/negative) evaluation of anti-dsDNA IgM results was carried out as per the kit manual. For the anti-dsDNA IgG qualitative result evaluation a corrected cut-off was used (30 IU/ml) that had been optimized based on the past 5 years' clinical–practical feedbacks.

Statistical analysis

Statistical analysis was carried out in the undivided serum bank of SAD, and also in the individual SAD subgroups who had sufficient sample numbers to yield representative data (SSc, SLE and RA). Statistical evaluation was performed using SPSS version 25.0 statistics package (IBM, Armonk, NY, USA). Spearman's correlation analysis, Mann–Whitney *U* and Kruskal–Wallis tests were used as appropriate; *P*-values < 0·05 were considered significant.

The data that support the findings of this study are available from the corresponding author (S.D.) upon reasonable request

Results

Infection- or vaccine-induced anti-measles antibody levels in systemic autoimmune diseases

In our previous studies [20,21] we investigated the immunological protection against measles infection at population-level in healthy individuals categorized into age or vaccination groups, based on changes introduced in measles immunization schedules and in vaccine components since the introduction of the first measles vaccine in Hungary. In the current study, in order to explore relationships between infection- or vaccine-induced antibodies and natural antibodies of IgG isotype, anti-measles IgG levels in samples obtained from SAD patients (total n = 374) were measured. Anti-measles IgG seronegativity ratios in different SAD groups are listed in Table 1. As already described in the epidemiological literature, and in accordance with our previously published findings [20–22,24,26], measles seronegativity ratios showed significant correlation with age (P < 0·001 correlation coefficient; 0·323). Accordingly, enhanced seronegativity ratios were detected in patient groups with mean ages connected to the early vaccination era, which was characterized by an incomplete measles/MMR immunization routine and vaccine inefficiency. The highest seronegativity ratio was found in SLE patients, with a mean age of 44 years.

Comparison of natural autoantibody and infection-induced antibody levels

Based on previous findings nAAbs (IgG/M) against the mitochondrial inner membrane, enzyme CS and topo I F4 could be detected in sera of healthy individuals and patients with SSc, SLE and in other autoimmune rheumatic diseases [19]. Natural autoantibodies form a network in healthy individuals, but at the same time can also recognize other antigens (nucleosome) in systemic autoimmune patients [3]. When analyzing the undivided totality of SAD samples, the same trend was observed as in the case of the three accentuated disease groups (SSc, SLE, RA). Considering all SAD samples together (total n = 374), significantly higher anti-CS IgG titers were detected in the anti-measles IgG-seropositive patient group (P = 0·011) compared to the anti-measles IgG seronegative individuals (Fig. 1). Analyzing the association between virus- or vaccine-induced (anti-measles IgG) and natural (anti-CS IgG) antibody titers in the singularly investigated autoimmune diseases (RA, SLE and SSc), the same trend described above was observable; in all three groups the anti-measles IgG-seropositive samples showed significantly higher anti-CS IgG titers (Fig. 2). A similar, but statistically non-significant, trend was observed in the case of anti-F4 IgG nAAbs. No association was found between infection- or vaccine-induced IgG and IgM isotype nAAbs.

Relationship between IgG natural autoantibody levels and anti-dsDNA IgG in SLE patients

In order to investigate the association between autoimmune disease-specific pathological autoantibodies and IgG isotype nAAbs, anti-dsDNA IgG measurement was chosen, as anti-dsDNA IgG is a highly specific disease marker in SLE. In those SLE patient samples that proved to be positive for the disease-specific marker anti-dsDNA IgG, significantly higher levels of anti-F4 IgG (P = 0·001) and anti-CS IgG (P < 0·001) nAAbs were measured, as shown in Fig. 3. Anti-dsDNA IgG and nAAbs levels also showed significant correlation (*P*/correlation coefficient: 0·006/0·321 and

Table 1. Age division and ratio of anti-measles (IgG)-seronegative samples in systemic autoimmune disease groups

SAD	No. of sera	Mean age (rounded years)	No. of anti-measles IgG seronegative sera	Ratio (%)
SSc	157	56	13	8·28
SLE	92	44	13	14·13
RA	73	59	3	4·10
Other*	52	53	4	7·69
Total	374	52	33	8·82

*Other = myositis, non-differentiated collagen disease, arthritis psoriatica, mixed connective tissue disease, morphea, primary Raynaud syndrome, secondry Raynaud syndrome, Sjögren's syndrome.

Ig = immunoglobulin; SAD = systemic autoimmune diseases; SLE = systemic lupus erythematosus; SSc = systemic sclerosis; RA = rheumatoid arthritis.

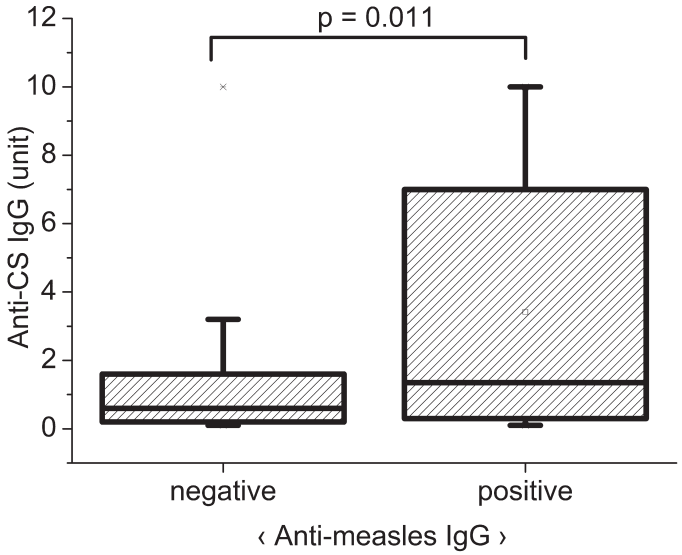

Fig. 1. Relationship between anti-measles immunoglobulin (Ig)G and anti-citrate synthase (CS) IgG antibody titers in the undivided serum bank of systemic autoimmune diseases (SAD) serum samples (n_{SAD} = 374 = n_{RA} 73 + n_{SLE} 92 + n_{SSc} 157 + n_{other} 52). Qualitative (positive, negative) anti-measles IgG results were compared to quantitative anti-CS IgG results (expressed in arbitrary units, based on our in-house standard), using Mann–Whitney *U* analysis. Significantly higher anti-CS IgG titers were detected in the anti-measles IgG-seropositive compared to -seronegative SAD patients. Boxes show interquartile ranges (IQR); whiskers indicate lowest and highest values; horizontal lines represent medians.

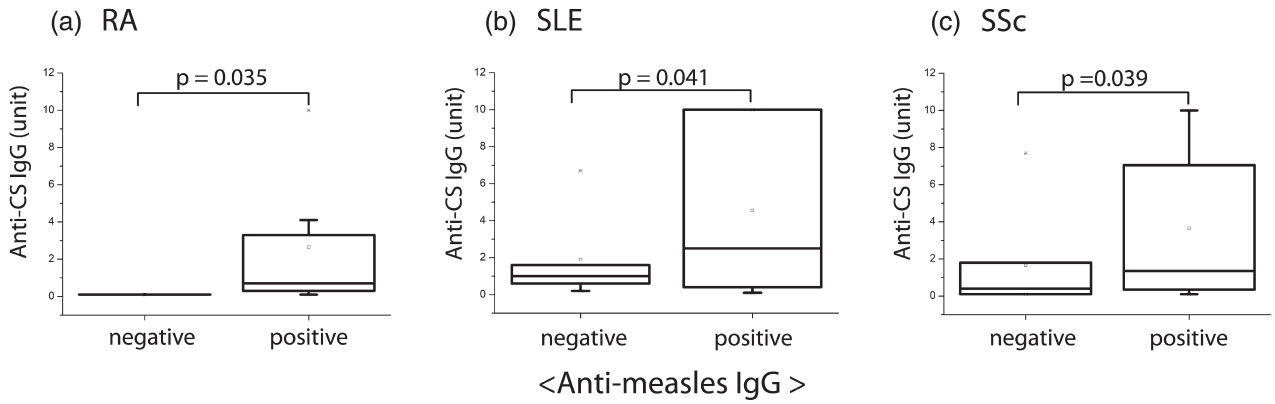

Fig. 2. Relationship between anti-measles immunoglobulin (Ig)G and anti-citrate synthase (CS) IgG natural autoantibody titers [in systemic sclerosis (SSc), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) disease groups (n_{RA} = 73; n_{SLE} = 92; n_{SSc} = 157]. Qualitative (positive, negative) anti-measles IgG results were compared to quantitative anti-CS IgG results (expressed in arbitrary units, based on our in-house standard), using Mann–Whitney *U* analysis. Significantly higher levels of natural anti-CS IgG were detected in anti-measles IgG-seropositive compared to seronegative samples in RA, SLE and SSc. Boxes show interquartile ranges (IQR); whiskers indicate lowest and highest values; horizontal lines represent medians.

0·000/0·510 for anti-F4 IgG and anti-CS IgG, respectively). No correlation was found between IgM nAAbs and disease-specific anti-dsDNA IgG autoantibody levels.

Anti-dsDNA IgM and natural IgM autoantibody levels showed association in SLE

Previous reports have proposed that anti-dsDNA IgM antibodies may play a protective role in lupus nephritis [7,8,27]. Herein we compared anti-dsDNA IgM levels with nAAb titers in SLE patients. Significantly higher levels of anti-F4 IgM and anti-CS IgM nAAbs were detectable (P = 0·002 and 0·016, respectively) in anti-dsDNA IgM-positive SLE patient samples, as shown in Fig. 4. Anti-dsDNA IgM titers and nAAb levels also showed significant correlation (*P*/correlation coefficient: 0·002/0·344 and 0·018/0·252 for anti-F4 IgM and anti-CS IgM, respectively).

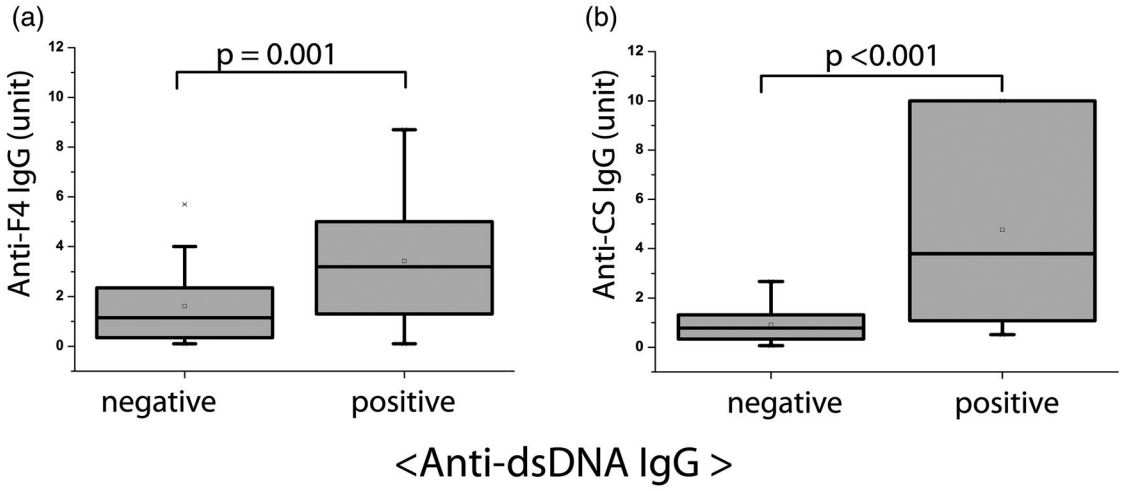

Fig. 3. Relationship between disease-specific anti-dsDNA immunoglobulin (Ig)G autoantibody levels and natural anti-F4 and anti-citrate synthase (CS) IgG natural autoantibody titers in systemic lupus erythematosus (SLE) patients (n_{SLE} = 92). Qualitative (positive, negative) anti-dsDNA IgG results were compared to quantitative anti-DNA topoisomerase I F4 fragment (anti-F4) IgG and anti-CS IgG results (expressed in arbitrary units, based on our in-house standard), using Mann–Whitney *U* analysis. The levels of anti-F4 and anti-CS IgG antibodies were significantly increased in anti-dsDNA IgG-positive compared to anti-dsDNA IgG-negative SLE patients. Boxes show interquartile ranges (IQR); whiskers indicate lowest and highest values; horizontal lines represent medians.

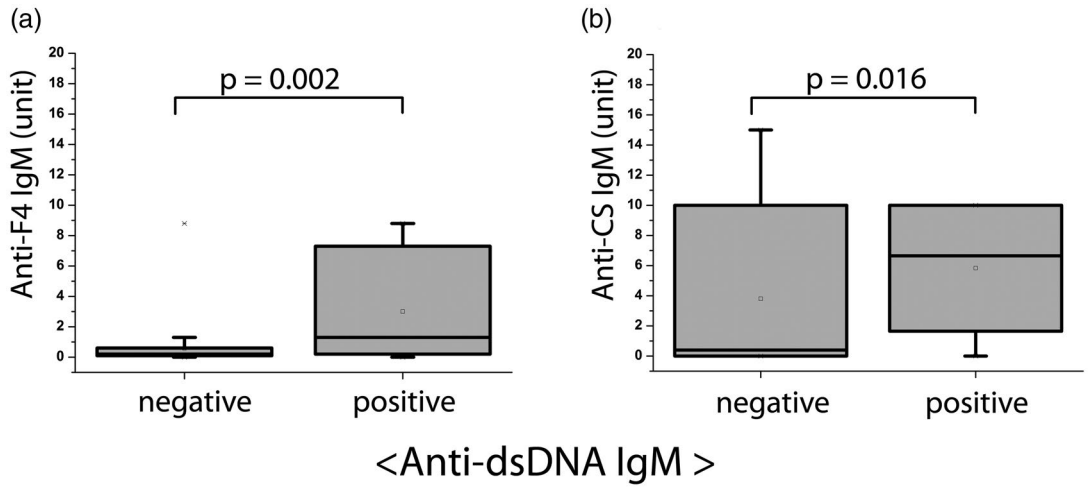

Fig. 4. Relationship between anti-dsDNA immunoglobulin (Ig)M autoantibody levels and anti-F4, anti-citrate synthase (CS) IgM natural autoantibody titers in systemic lupus erythematosus (SLE) patients (n_{SLE} = 92). Qualitative (positive, negative) anti-dsDNA IgM results were compared to quantitative anti-DNA topoisomerase I F4 fragment (anti-F4) IgM and anti-CS IgM results (expressed in arbitrary units, based on our in-house standard), using Mann–Whitney *U* analysis. The levels of anti-F4 and anti-CS IgM natural antibodies were significantly elevated in anti-dsDNA IgM-positive compared to anti-dsDNA IgM-negative SLE patients. Boxes show interquartile ranges (IQR); whiskers indicate lowest and highest values; horizontal lines represent medians.

Discussion

Regarding the evaluation of anti-measles antibody titers, we would like to note that all examined samples belonged to individuals who were aged a minimum of 15 years [the age range of the undivided serum bank (n = 374) was from 15 to 81 years]. Since 1992, the MMR trivalent vaccine has been administered in Hungary at age 15 months, and the reminder vaccine is given at age 11 years. Consequently, the problem of questionable sero-epidemiological data due to inadequate seroconversion as a consequence of an immature immune response or a scarce time interval between the measles (or MMR) vaccination and the measurement of the antibody titers can be excluded.

According to known epidemiological data, the early measles/MMR vaccination era was characterized by poorly defined age at vaccination. Moreover, disregarded thermolability of the inoculum and the lack of suitable adjuvants may have also contributed to vaccine inefficiency [22,24]. Based on our previous findings [21], and in accordance with recent publication data [25], immunization gaps have been formed in the age group of individuals vaccinated between 1978 and 1987 (≈20% of seronegativity) [21]. To this potentially susceptible cluster belong today's 35–42-year-old adults, explaining the high ratio of samples with low anti-measles antibody (IgG) titers. SLE typically affects young or middle-aged individuals; the mean age of SLE patients in our serum bank was 44 years, justifying the higher anti-measles seronegativity ratio in this patient group. We found a significant association between infection- or vaccine-induced anti-measles IgG and anti-CS IgG natural autoantibodies in the undivided sample multitude of SAD samples and also in the distinct autoimmune disease groups. The concomitance of pathogen- or vaccine-associated and natural antibodies is also verified by the literature. A recent study reported the enhancement of natural antibody repertoire by immunization [28] in laboratory rats. The study suggested that immunization-induced natural antibodies may also contribute to wound repair and tumor surveillance [28]. Another animal study performed on cod juveniles reports an increased natural antibody response of vaccinated compared to unvaccinated fish (against *Vibrio anguillarum*) [29]. Scientific data also describe a significant correlation between levels of natural autoantibodies and response to vaccination in elderly, physically active individuals [30]. Based on our serological findings, supported by cumulative scientific data, it can be hypothesized that natural autoantibodies may play a role in efficient vaccination and the subsequent formation of long-lasting immunological memory.

The connection between disease-specific anti-dsDNA IgG and the IgG isotypes of antibodies of natural origin in SLE samples may support the notion regarding the disease-associated nature of the IgG isotype nAAbs. According to our previous findings, titers of anti-CS antibodies with IgG isotype fluctuate over time; consequently, the presence of these antibodies can be a result of an adaptive-like immune response [3,16]. Natural IgG autoantibodies are reported to be abundant and ubiquitous in human sera, and their number is influenced by age, gender and disease [2].

Anti-topoisomerase-I (anti-Scl-70) autoantibodies are considered highly specific for SSc, although they have been found occasionally in a small number of patients with SLE [31,32]. In our previous study [19], fragment F4 of topo I was recognized by all SSc and SLE patients' sera which were positive for anti-topo I IgG antibodies by a conventional ELISA kit used for the detection of anti-Scl-70 autoantibodies. The serum level of anti-F4 IgG antibodies was significantly higher in SLE and SSc patients than in healthy individuals. Anti-F4 IgM was found in both SAD and healthy controls [19].

Recent publications suggest that IgM autoantibodies may also play a protective role in SLE [7,8,33–35]. According to literature data, secretory IgM deficiency in mice showed a connection with increased susceptibility to autoimmunity [36]; in human studies, a reduction in IgM levels was also associated with SLE [27]. The impaired function of natural IgM in clearance of dying cells can result in the accumulation of apoptotic remnants and fragments of necrotic cells, which aids their pathological elimination and thus contributes to the maintenance of autoinflammation [5]. Protective IgM autoantibodies in SLE are of particular interest. It has been reported that increased levels of IgM are negatively associated with the prevalence of atherosclerotic plaques in patients with SLE [37]. Beneficial clinical associations between natural IgM and autoimmunity, as well as opportunities for potential therapeutic implications, are widely studied [4]. IgM antibodies against dsDNA are frequent in SLE [35]. Additionally, highly significant negative correlation between IgM anti-dsDNA antibodies and glomerulonephritis was observed in mice and in humans [7]. The clearance of pathogenic immune complexes may be improved by IgM, therefore IgM antibodies against dsDNA certainly appear to be protective, and may be a new treatment modality of lupus nephritis in humans [8]. As expected, IgM isotypes of natural anti-F4 and anti-CS autoantibody levels showed correlation with anti-dsDNA IgM titers, supporting the hypothesis that these IgM autoantibodies are part of the natural immune repertoire in SLE.

The current immunoserological data – supported by recent scientific literature [38] – shows associations between IgG isotype natural antibodies (nAbs) and specific humoral antibodies. This finding may corroborate the notion that nAbS not only play an important role in the first line of defense of the immune system, but also contribute to an effective adaptive response through the maintenance of immune homeostasis [38–40] and priming of the adaptive immune functions. Consequently, nAbs can act as a mediator between the innate-like and the adaptive arms of the immune system [3,12,38]. The dogma that high-affinity IgG response is the major goal of immunization and low-affinity antibodies should be avoided has positively contributed to the dearth of information regarding the role of nAbs in vaccination [38]. However, it has been recently proposed that nAbs may serve as potential screening targets to predict the strength

of antigen-induced immune response [41]. The serological investigations presented herein should lead to more focus on nAbs as a first-line component of the adaptive immune response [38], and may promote further research in the potential use of nAbs as predictive tools for vaccine development. Furthermore, measuring the levels of nAAbs may have clinical relevance in SAD, especially in SLE. Therapeutic applications could harness the potency of immune-regulatory nAAbs either by boosting *in-vivo* natural IgM production or via therapeutic infusions of monoclonal or polyclonal IgM preparations [4,42].

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Disclosures

None declared.

Data availability statement

The data that support the findings of this study are available from the corresponding author [Simon D], upon reasonable request.

References

1 Avrameas S, Alexopoulos H, Moutsopoulos HM. Natural autoantibodies: An undersugn hero of the immune system and autoimmune disorders – a point of view. Front Immunol 2018; **9**:1320.

2 Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG. Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. PLOS ONE 2013; **8**:e60726.

3 Czömpöly T, Olasz K, Simon D *et al*. A possible new bridge between innate and adaptive immunity: are the anti-mitochondrial citrate synthase autoantibodies components of the natural antibody network? Mol Immunol 2006; **43**:1761–8.

4 Grönwall C, Silverman GJ. Natural IgM: beneficial antibodies protect against atherosclerosis in SLE? Nature Rev Rheum 2016; **12**:442–4.

5 Mũoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. Nat Rev Rheum 2010; **6**:280–9.

6 McMahon M, Skaggs B. Autoimmunity: do IgM antibodies protect against atherosclerosis in SLE? Nat Rev Rheum 2016; **12**:442–4.

7 Jiang C, Zhao ML, Scearce RM, Diaz M. Activation-induced deaminase-deficient MRL/LPR mice secrete high levels of protective antibodies against lupus nephritis. Arthritis Rheum 2011; **63**:1086–96.

8 Witte T. IgM antibodies against dsDNA in SLE. Clin Rev Allergy Immunol 2008; **34**:345–7.

9 Tarlinton DM, McLean M, Nossal GJV. B1 and B2 cells differ in their potential to switch immunoglobulin isotype. Eur J Immunol 1995; **25**:3388–93.

10 Panda S, Zhang J, Tan NS, Ho B, Ding JL. Natural IgG antibodies provide innate protection against ficolin-opsonized bacteria. EMBO J 2013; **32**:2905–19.

11 Palma J, Tokarz-Deptuła B, Deptuła J, Deptuła W. Natural antibodies – facts known and unknown. Cent Eur J Immunol 2018; **43**:466–75.

12 Panda S, Ding JL. Natural antibodies bridge innate and adaptive immunity. J Immunol 2015; **194**:13–20.

13 Sokolove J, Bromberg R, Deane KD *et al*. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLOS ONE 2012; **7**:e35296.

14 Pisetsky DS. Antinuclear antibodies in rheumatic disease: a proposal for a function-based classification. Scand J Immunol 2012; **76**:223–8.

15 Bournia VK, Vlachoyiannopoulos PG. Subgroups of Sjögren syndrome patients according to serological profiles. J Autoimmun 2012; **39**:15–26.

16 Czömpöly T, Olasz K, Nyárády Z, Simon D, Bovári J, Németh P. Detailed analyses of antibodies recognizing mitochondrial antigens suggest similar or identical mechanism for production of natural antibodies and natural autoantibodies. Autoimmun Rev 2008; **7**:463–7.

17 Simon D, Gilicze O, Farkas N, Najbauer J. Correlation of natural autoantibodies and cardiovascular disease-related anti-bacterial antibodies in pericardial fluid of cardiac surgery patients. Clin Exp Immunol 2018; **193**:55–63.

18 Zorn E, See SB. Polyreactive natural antibodies in transplantation. Curr Opin Organ Transplant 2017; **22**:8–13.

19 Simon D, Czömpöly T, Berki T *et al*. Naturally occurring and disease-associated auto-antibodies against topoisomerase I: a fine epitope mapping study in systemic sclerosis and systemic lupus erythematosus. Int Immunol 2009; **21**:415–22.

20 Böröcz K, Csizmadia Z, Markovics Á *et al*. Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human sera. J Immunol Methods 2019; **464**:1–8.

21 Böröcz K, Csizmadia Z, Markovics Á *et al*. Application of a fast and cost-effective 'three-in-one' MMR ELISA as a tool for

surveying anti-MMR humoral immunity: the Hungarian experience. Epidemiol Infect 2020; **148**:e17.
22 Agócs MM, Markowitz LE, Straub I, Dömök I. The 1988–1989 measles epidemic in Hungary: assessment of vaccine failure. Int J Epidemiol 1992; **21**:1007–13.
23 Lengyel G, Ánosi N, Marossy A, Mátyus M, Bosnyákovits T, Orosz L. Az európai és a magyarországi kanyaróhelyzet összefoglalása és tanulságai [Summary and lessons from the measles situation in Europe and Hungary]. Orv Hetil 2019; **160**:767–73.
24 International Notes Measles – Hungary [internet] [cited 2020 May 3]. Available at: https://www.cdc.gov/mmwr/preview/mmwrhtml/00001472.htm.
25 Lengyel G, Marossy A, Ánosi N *et al*. Screening of more than 2000 Hungarian healthcare workers' anti-measles antibody level: results and possible population-level consequences. Epidemiol Infect 2019; **147**:e7.
26 MMWR Weekly. MMWR Publications. [cited 2020 May 20]. Available at: https://www.cdc.gov/mmwr/publications/index.html.
27 Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. Nat Rev Immunol 2010; **10**:778–86.
28 Beinart D, Ren D, Pi C *et al*. Immunization enhances the natural antibody repertoire. Exp Clin Sci 2017; **16**:1018–30.
29 Gudmundsdó S, Gudmundsdó BK. Specific and natural antibody response of cod juveniles vaccinated against *Vibrio anguillarum*. Fish Shellfish Immunol 2009; **26**:619–24.
30 Bachi ALL, Suguri VM, Ramos LR, Mariano M, Vaisberg M, Lopes JD. Increased production of autoantibodies and specific antibodies in response to influenza virus vaccination in physically active older individuals. Results Immunol 2013; **3**:10–6.
31 Whyte J, Earnshaw WC, Champouxt JJ *et al*. Detection of anti-topoisomerase I antibodies using a full length human topoisomerase I recombinant protein purified from a baculovirus expression system. Clin Exp Immunol 1995; **100**:214–8.
32 Stojanov L, Satoh M, Dooley MA, Kuwana M, Jennette JC, Reeves WH. Autoantibodies to topoisomerase I in a patient with systemic lupus erythematosus without features of scleroderma. Lupus. 1995; **4**:314–7.
33 Grönwall C, Vas J, Silverman GJ. Protective roles of natural IgM antibodies. Front Immunol 2012; **3**:66.
34 Shoenfeld Y, Toubi E. Protective autoantibodies: Role in homeostasis, clinical importance, and therapeutic potential. Arthritis Rheum 2005; **52**:2599–606.
35 Hahn BH. Antibodies to DNA. N Engl J Med 1998; **338**:1359–68.
36 Nguyen TTT, Baumgarth N. Natural IgM and the development of B cell-mediated autoimmune diseases. Crit Rev Immunol 2016; **36**:163–17.
37 McMahon M, Skaggs B. Autoimmunity: Do IgM antibodies protect against atherosclerosis in SLE? Nat Rev Rheumatol 2016; **12**:442–4.
38 Maddur MS, Lacroix-Desmazes S, Dimitrov JD, Kazatchkine MD, Bayry J, Kaveri SV. Natural antibodies: from first-line defense against pathogens to perpetual immune homeostasis. Clin Rev Allergy Immunol 2020; **58**:213–28.
39 Notley CA, Brown MA, Wright GP, Ehrenstein MR. Natural IgM is required for suppression of inflammatory arthritis by apoptotic cells. J Immunol 2011; **186**:4967–72.
40 Maddur MS, Kaveri SV, Bayry J. Circulating normal IgG as stimulator of regulatory T cells: lessons from intravenous immunoglobulin. Trends Immunol 2017; **38**:789–92.
41 Kohler H, Bayry J, Nicoletti A, Kaveri SV. Natural autoantibodies as tools to predict the outcome of immune response? Scand J Immunol 2003; **58**:285–9.
42 Kaveri SV, Silverman GJ, Bayry J. Natural IgM in immune equilibrium and harnessing their therapeutic potential. J Immunol 2012; **188**:939–45.

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Application of a fast and cost-effective 'three-in-one' MMR ELISA as a tool for surveying anti-MMR humoral immunity: the Hungarian experience

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Abstract

In Hungary, between February 2017 and July 2019, 70 confirmed measles cases were reported, raising questions about the adequacy of population-level immunity. Although the assumed vaccination coverage is ≥99%, in a recent study, we detected potential gaps in the anti-measles humoral immunity. In Hungary, according to a decree by the Ministry of Public Welfare, beginning from 2021, the healthcare provider should conduct a serosurvey of anti-measles protection levels of healthcare professionals. To facilitate the compliance with this requirement, we developed a quick 'three-in-one' or 'triple' MMR (measles, mumps and rubella) indirect ELISA (IgG); an assay format that is currently not available commercially. High throughput applicability of the 'three-in-one' ELISA was verified using 1736 sera from routine laboratory residual samples, using an automated platform (Siemens BEP 2000 Advance). Assay verification was performed by comparing the full antigen repertoire-based 'target' assay with in-house 'control' assays using recombinant viral antigen coatings, and by validated commercially available kits. Indirect immunofluorescence was used as an independent reference method. Data were analysed using OriginLab, IBM SPSS, RStudio and MedCalc. In case of measles, we combined our current results with previously published data ($N_{\text{total measles}}$ = 3523). Evaluation of anti-mumps and anti-rubella humoral antibody levels was based on the measurement of 1736 samples. The lowest anti-measles seropositivity (79.3%) was detected in sera of individuals vaccinated between 1978 and 1987. Considering the antigen-specific seropositivity ratios of all samples measured, anti-measles, -mumps and -rubella IgG antibody titres were adequate in 89.84%, 91.82% and 92.28%, respectively. Based on the virus-specific herd immunity threshold (HIT) values ($\text{HIT}_{\text{Measles}}$ = 92–95%, $\text{HIT}_{\text{Mumps}}$ = 75–86%, $\text{HIT}_{\text{Rubella}}$ = 83–86), it can be stated that regarding anti-measles immunity, certain age clusters of the population may have inadequate levels of humoral immunity. Despite the potential gaps in herd immunity, the use of MMR vaccine remains an effective and low-cost approach for the prevention of measles, mumps and rubella infections.

Introduction

Despite the existence of effective measles (M) and measles-containing vaccines (MCV), resurgence of measles cases in the USA and across Europe has occurred, including individuals vaccinated with two doses of the vaccine [1]. In Europe, a safe and effective two-dose vaccination schedule has been made available since the 1960s. The introduction of the trivalent measles, mumps, rubella (MMR) vaccines started in the 1970s [2] (in Hungary in 1991, Fig.1), and it is still in practice, in the form of modern and safe tri- and tetravalent (measles, mumps, rubella and varicella; MMRV) vaccines. However, the risk of continued widespread circulation of measles in EU/EEA still exists, since significant immunity gaps persist, due to suboptimal historical and current vaccination coverage [3]. Despite regional outbreaks of measles infections, in 2016, globally fewer than 1 00 000 individuals died from measles, as a result of recent improvements of national immunisation programmes. In the WHO European Region (WHO EUR), between 2009 and 2017, the estimated regional coverage was 93–95% for the first dose of measles-containing vaccines (MCV1), and increased from 73% to 90% for the second dose (MCV2) [4]. In spite of the improving vaccine coverage tendencies, measles incidence increased again to 89.5 per 1 million population in 2018 in the EU region [4]. From 1 July 2018 to 30 June 2019, 30 EU/EEA Member States reported 13 102 cases of measles, also including fatalities [5]. According to WHO reports, the largest outbreaks occur in countries with low

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measles vaccination coverage. However, outbreaks occurred even in countries with high national vaccination rates [6]. Lately, an alarming surge of measles cases was experienced in countries neighbouring Hungary. From 2017 to 2018, Ukraine had the largest increase in measles cases worldwide [7, 8]. In 2018, Ukraine reported >54 000 measles cases; more than the entire EU. The total estimated number of measles cases for the first 5 months of 2019 was 52 034, including 17 deaths [9, 10]. Romania also bears a high burden of the disease; between the first outbreak (late 2016) and May 2019, Romania has reported 16 627 cases and 63 deaths. Ninety-four per cent of the reported cases were unvaccinated individuals, and 4% received only one of the two-shot vaccination series. Regarding parotitis epidemica (mumps), the last accessible ECDC surveillance report is from 2016; 28 EU/EEA countries reported 14 795 cases of mumps, of which the Czech Republic, Poland, Spain and the UK were responsible for 77% of these cases. The mumps childhood vaccination coverage in Hungary is ≥99% (MCV1 and MCV2 are equivalent to MMR1 and MMR2 in Supplementary Fig. S1. Supplementary materials are available on the Cambridge Core website), consequently, the risk of infection is predominantly by virus importation [11]. In Hungary, the rubella vaccine was introduced in 1990 in the form of measles–rubella (MR) bivalent vaccine. A year later, in 1991, it was replaced by the MMR trivalent vaccine that is still in use today. From 1 July 2018 to 30 June 2019, EU/EEA Member States reported 483 cases of rubella. The highest number of cases was reported by Poland (372), Germany (57), Italy (24), Spain (12) and Romania (4) [5]. For Hungary, between 2007 and 2018, WHO reports only 10 cases [12]. Measles, mumps and rubella statistics (cases per year) based on WHO measles and rubella '*elimination country profile for Hungary*' data (i.e. the number of reported infections of the last decades) are shown in Supplementary Figure S2. WHO-UNICEF estimates of national immunisation coverage show that only four EU/EEA countries, including Hungary, Portugal, Slovakia and Sweden, reported at least 95% coverage for both doses of MCV in 2017 [13]. Despite the estimated 99% measles vaccination coverage in Hungary [12, 14] (Supplementary Fig. S1), from February 2017 to July 2019, 70 measles cases were laboratory confirmed according to the European Centre for Disease Prevention and Control (ECDC) reports.

These data raise the question concerning the reliability of the Hungarian population's herd immunity. Because of recent outbreaks worldwide, not only of measles, but also mumps and rubella (MMR) infections [15–19], and because of waning of immunity over time after vaccination [20–23], there is an urgent need for reliable and affordable laboratory tests for monitoring anti-MMR antibody (IgG) titres. For this purpose, we developed a new, 'three-in-one' immunoassay for quick measurement of all three anti-viral antibodies within a single run. To our knowledge, this triple format of MMR ELISA is currently not available on the market. The ELISA protocol described herein incorporates our previous method [24] that has been further improved to enable the use of the same assay conditions for all three anti-viral antibodies. We demonstrate the high-throughput applicability of this assay using 1736 serum samples from patients of diverse age groups, and provide an estimation of the population-level MMR seropositivity. We present and discuss our results in the context of both assay development and immunosurvey evaluation in relation to the history of M/MMR vaccination in Hungary from 1969 to present.

Materials and methods

Samples

A serum bank consisting of anonymous patient sera was established ($N_{\text{total measles}}$ =3523 measles, N_{mumps} = 1736 mumps and N_{rubella} = 1736) from routine laboratory samples at the University of Pécs, Clinical Centre (Ethical License number 2015/5726). The samples are considered representative, as clinical residual samples were randomly selected (with the exclusion of seriously immunocompromised patients) from the Department of Laboratory Medicine, University of Pécs, Medical School, which serves three counties (Baranya, Somogy and Tolna, with a population of ~8 87 000), and receives laboratory examination requests from all over Hungary. In case of measles serosurvey, in order to give a more accurate estimate at population level, we combined our recently published data with the results of current measurements (previously we tested 1985 serum samples for measles [24], of which the data of 1787 samples have been pooled together with the current data; 'cumulative' data for measles, $N_{\text{total measles}}$ = 3523). Serum samples were from all age groups (beginning from the era before the implementation of measles vaccine, through several different vaccine types, manufacturers and vaccination schedules, up to present), and were categorised based on past changes introduced in measles and MMR immunisation schedules (Table 1). The age group determination in our current study has been based on the landmarks in the history of measles and MMR vaccination schedules in Hungary, as detailed in Table 1.

Given the anonymous nature of samples, the only known data were the date of birth of the patients. Considering that we were interested in the differences between the various vaccination periods, dates of vaccination (instead of dates of birth) were chosen to define age group boundaries. By knowing the dates of birth and the important milestones of the Hungarian vaccination history (e.g. the first measles vaccine was introduced in Hungary in 1969; in 1990, the MR bivalent vaccines were introduced; and in 1991, the MMR trivalent vaccine was introduced; for further details, see Table 1), establishment of the vaccination-based age group matrix became feasible. Neonates and children under the age of vaccination were excluded from our study. As mentioned above, seriously immunocompromised patients were also excluded; however, patients with mild immunocompromised conditions may have been included.

Antigen coating

For our 'target' assay, we used purified, inactivated native virus preparations, derived from disrupted cells; measles Edmonston strain cultured in Vero cells (PIP013 Bio-Rad), mumps Enders strain cultured in BSC-1 cells (PIP014 Bio-Rad), rubella HPV-77 strain cultured in Vero cells (PIP044 Bio-Rad). Antigen preparations were sonicated before use, as per manufacturer's instruction. ELISA 96 well MicroWell™ MaxiSorp™flat-bottom 44-2404 plates (Nunc) were divided vertically into three equal parts and each third was incubated overnight at 4–6 °C with measles, mumps and rubella antigens (100 µl/well), respectively (Fig. 2, Table 2). Testing of blocking reagents was performed using bovine gelatine, milk powder, Block ACE (Bio-Rad) and our in-house polyvinyl alcohol (PVA)-based purely synthetic blocking buffer. Details of sample pre-treatment and assay preparation steps have been described earlier [24].

Table 1. Age group categorization

Age groups	Explanation, rationale
	Vaccination groups were defined by adding the number of months indicated for the first childhood vaccine (e.g. 15 months of age) to the dates of birth. For example, a person born in February 1990 was assigned to age-group '*Patients vaccinated between 1991–1995*', since this individual received the first measles (MMR) vaccine in May 1991
Patients born before 1969	Unvaccinated patients, wild-type infections. 1969: introduction of measles vaccine in Hungary (live, attenuated Leningrad-16 strain produced in the Soviet Union)
Patients vaccinated between 1969 and 1977	From 1969 to 1974, a single dose of measles vaccine was administered in mass campaigns to persons 9–27 months of age. The recommended age for vaccination was 10 months until 1978, when it was changed to 14 months. After the 1980–1981 epidemics, persons born between 1973 and 1977, who would have received vaccine when the recommended age was 10 months, were revaccinated. After 1989, children were re-vaccinated at the age of 11 years with monovalent measles vaccine in a scheduled manner. Consequently, the first individuals who received a reminder vaccine at the age of 11 were born in 1978. Thus, the cluster of 1969–1977 was the last that did not receive a reminder vaccine at the age of 11 as a part of the official vaccine schedule
Patients vaccinated between 1978 and 1987	These are the first individuals who benefited from the reminder monovalent measles vaccine at the age of 11. In 1999 the administration of trivalent vaccine was started in Hungary, consequently who received the first trivalent vaccine in 1999 were born in 1988
Patients vaccinated between 1988 and 1990	In 1989 the rubella vaccine was introduced, and the monovalent measles reminder vaccine at age 11 was started 1990: Introduction of measles–rubella bivalent vaccines
Patients vaccinated between 1991 and 1995	The administration of the first vaccine at age 14 months lasted from 1978 to 1991 1991: Measles–mumps–rubella trivalent vaccine 1992: MMR vaccine at age 15 months 1996: Introduction of MERCK MMR II – Enders' Edmonston strain (live, attenuated)
Patients born between 1996 and 1998	1996: Introduction of MERCK MMR II – Enders' Edmonston strain (live, attenuated) 1999: Measles–mumps–rubella re-vaccination (reminder shot) instead of monovalent measles vaccine 1999: Introduction of GSK PLUSERIX – Measles Schwarz Strain
Patients vaccinated between 1999 and 2002	1999: Introduction of GSK PLUSERIX – Measles Schwarz Strain 2003: Introduction of the GSK PRIORIX vaccine
Patients vaccinated in 2003	2003: Introduction of the GSK PRIORIX vaccine – attenuated Schwarz Measles
Patients vaccinated in 2004–2005	2004–2005: Administration of the MERCK MMR II
Patients vaccinated between 2006 and 2010	2006–2010 (5-year tender): GSK PRIORIX – attenuated Schwarz Measles
Patients vaccinated after 2011	Beginning from 2011 we use a Sanofi-MSD product; MMRvaxPro (Measles virus Enders' Edmonston strain, live, attenuated) for vaccination and re-vaccination of children; GSK PRIORIX is still on the market, commonly used for vaccination in adulthood
Epidemics: 1973–74: Large epidemics, affecting primarily unvaccinated 6–9 years old children 1980–81: Another significant epidemic, affecting primarily 7–10 years old children 1988–89: Epidemic with high age-specific attack rates of 17–21 years old individuals, who had been vaccinated during the first years of the vaccination programme in Hungary 2017–18: Smaller epidemics with few connected and sporadic cases, derived mainly from virus importation	

To demonstrate the lack of interference when using cell culture-derived antigen coatings, we compared our 'target' assay to purified recombinant viral capsid protein antigen-based assays. Purity of cell culture-derived, native virus-based coatings was verified by plate-to-plate comparisons to recombinant antigen-based coatings, as described below: 'control' microplates were coated with a series of doubling, four-point dilutions of recombinant antigens; measles virus Priorix, Schwarz strain nucleocapsid protein (Abcam ab74559, source: *Saccharomyces cerevisiae*) 1.66–0.207 µg/ml, mumps virus wild-type, Gloucester strain, nucleocapsid protein (Abcam ab74560, source: *S. cerevisiae*) 0.832–0.104 µg/ml, recombinant rubella virus capsid protein (Abcam ab43034, source: *Escherichia coli*) 2–0.25 µg/ml. To confirm the lack of interference by off-target molecules in whole virus-based assays, results of negative and low positive sample pools, international measles and rubella standards (3rd WHO International Standard for Anti-Measles, NIBSC code 97/648, Anti Rubella Immunoglobulin 1st WHO International Standard Human, NIBSC code RUBI-1-94), and the mumps quality control reagent (Anti-Mumps Quality Control Reagent Sample1) obtained for native virus-derived coatings were compared to the results obtained for different coating concentrations of recombinant antigens. Parallelism was tested to ascertain that the binding characteristic of the analyte (high and low antigen-titred sample pools) was the same, independent of the type of coating. For graphical representation, optical density (OD) values were linearised; dilution series of analytes were depicted as a function of common logarithm of both relative dilutions and OD values. Coating combinations with sufficiently high R^2 values of the linear fittings (with the same slope) were selected for further analysis of correlation between 'target' and 'control' assays, using Bland–Altman plots.

Cut-off

Determination of cut-off values was based on (a) Cohen's κ statistics, as an index of agreement between our assay and commercially available kits, (b) Area Under the Curve Receiver Operating

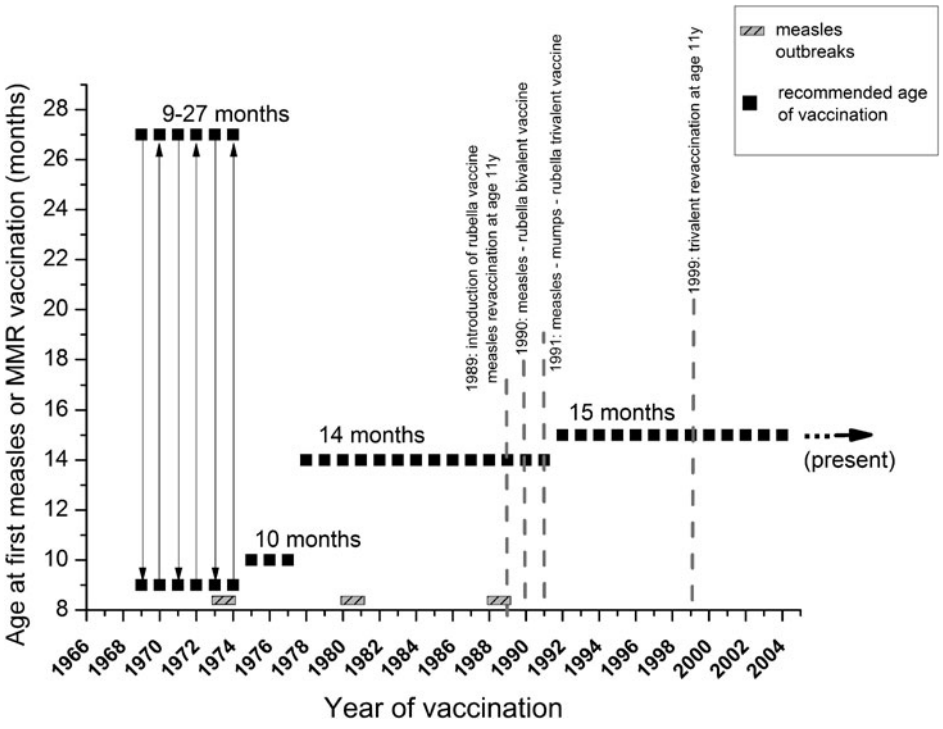

Fig. 1. Measles and MMR vaccination schedules in Hungary. Serum samples were collected from all age groups (excluding neonates), and were categorised based on changes introduced in measles and MMR immunization schedules. Grey, shaded rectangles indicate measles outbreaks, black squares show the recommended age of the first dose of vaccine. Grey dotted lines mark the most important milestones of the vaccination schedule (introduction of reminder vaccines, changes between mono-, bi- and trivalent inoculum). Further details are described in Table 1.

Characteristics (AUROC) analysis (combined with Youden's *J* equation) – which in this case was used for comparing the performance of diagnostic tests [25] and (c) the '*experimental approach*'. The latter one was based on the mean OD and IU-transformed values yielded by our tests, belonging to selected serum samples that had been previously found negative by validated commercial kits. For assay testing, optimisation and comparisons, the following validated kits were used: measles IgG: Novalisa, Immunolab, Euroimmun, Sekisui-Virotech, Serion, Siemens Enzygnost; mumps IgG: Novalisa, Immunolab, Euroimmun, Sekisui-Virotech, Vircell; rubella IgG: Novatec, Immunolab, Euroimmun, DiaPro, Vircell.

Because our samples were anonymous (and consequently lacked clinical background), for the generation of AUROC curves, the establishment of the binary classifier system was based on averaged qualitative (positive, negative) results of commercial ELISAs.

In equivocal cases (and also to periodically check the assay performance), borderline and negative samples were measured using indirect immunofluorescence assays, using measles, mumps and rubella virus-infected cells, IIF (IgG) (Euroimmun). In case of commercial assays, calculation of qualitative results was performed according to default thresholds specified by the manufacturers. AUROC results were analysed using Youden's formula (J = sensitivity + specificity − 1), and the highest OD values were selected and transformed into units based on the standards (3rd WHO International Standard for Anti-Measles, Anti-Mumps Quality Control Reagent Sample 1, 1st WHO International Standard Human). For these transformations, sigmoid dose–response curves were fitted onto the dilution points of the standards.

Analytical values, assay precision and specific assay characteristics

Analytical values such as lower limit of detection (LOD) and limit of quantification (LOQ) were determined by the mean and standard deviation of blank sample absorbance values; LOD was defined as mean + 3 S.D. and LOQ as mean + 10 S.D. (absorbance values), as suggested by the IUPAC Compendium of Chemical Terminology Gold Book. Sensitivity, specificity, positive and negative predictive values were also evaluated using validated commercial kits (Table 3).

Statistical analysis

AUROC analysis, Youden's *J* equation, confidence interval comparison at 95% confidence level (prop test) and Bland–Altman plot were used as statistical methods.

Results

Testing of antigen coating

To check whether the entire virus-based coatings (derived from cell cultures) contain off-target molecules, we compared our assays to purified recombinant viral capsid protein antigen-based (in-house) assays. Based on the linearity tests, the following recombinant viral nucleocapsid antigen coatings were selected: measles 0.83 µg/ml, mumps 0.416 µg/ml and rubella 1.0 µg/ml (R^2 standards ≥0.97, R^2 samples ≥0.93) (Supplementary Fig. S3). Bland–Altman plots were then generated; ratios of the results from the two techniques ('target' *vs.* 'control' assay) were plotted against the averages. As shown in Figure 3, we obtained data points that fell within the range ±1.96 S.D. (confidence interval 95%), with no observable trends, suggesting that the two methods are in agreement, thus demonstrating the adequate purity of the entire virus-based coating system used in the 'target' assay.

Cut-off determination and assay precision

Cohen's κ analysis was performed; plate-to-plate κ statistics (using tests described in the Materials and methods section)

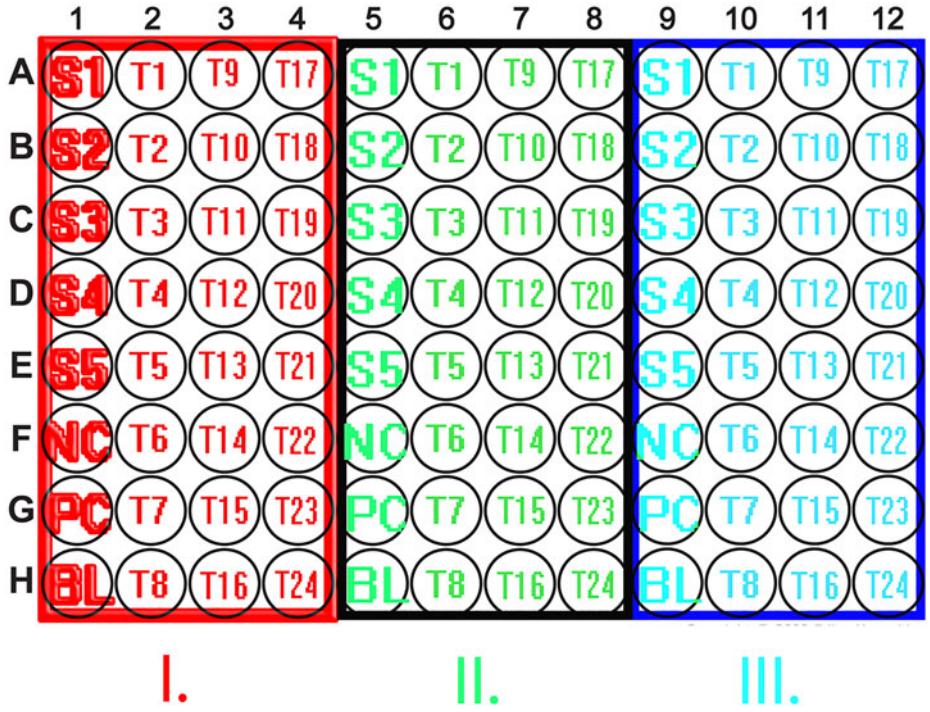

Fig. 2. Schematic representation of the plate layout used for the 'three-in-one' ELISA. ELISA 96-well plates were divided lengthwise into three equal parts and each third was coated with the appropriate antigen. Assay parameters were optimised to enable equal conditions and common reagents for each antigen type. Abbreviations: (S1-S5) Standards; (PC) positive control;(NC) negative control; (BL) blank. (I) Measles antigen coating (measles virus, Edmonston strain); (II) Mumps antigen coating (mumps virus, Enders strain); (III) Rubella antigen coating (rubella virus, HPV-77 strain).

Table 2. Summary of major steps of the MMR indirect ELISA protocol

Coating antigen	Bio-Rad PIP013 Measles virus, Edmonston strain	Bio-Rad PIP014 Mumps virus, Enders strain	Bio-Rad PIP044 Rubella virus, HPV-77 strain
Concentration of the coating antigen used on microplates	2.8 µg/ml	3 µg/ml	0.4 µg/ml
Antigens are dissolved in ELISA Coating Buffer (Bio-Rad BUF030), overnight at 4–6 °C. Blocking ≥2 h, RT with our in-house purely synthetic blocking buffer			
Standard/quality control reagent (S1–S5)	3rd WHO International Standard for Anti-Measles (NIBSC code: 97/648)	Anti-Mumps Quality Control Reagent Sample 1 (NIBSC code: 15/B664)	Anti-Rubella Immunoglobulin 1st WHO International Standard Human (NIBSC code: RUBI-1-94)
Starting concentration of the standard/quality control reagent	~5000 mIU/ml	~1000 'Mumps Assay Unit'/ml, arbitrarily assigned	1600 International Units per ampoule
Negative control (NC)	A sample found to be negative in a previous run		
Positive control (PC)	A sample found to be positive in a previous run		
Incubation	3 × 15 min, 37 °C		
Colour detection	Polyclonal anti-human IgG HRP-conjugated (Dako polyclonal rabbit anti-human IgG or equivalent) + TMB		
Additional reagents	Washing Buffer (WB), used also for sample dilution in combination with the IgM Reducing Assay Diluent (Bio-Rad BUF038), as previously described (Böröcz *et al.* [24])		
Automation and reading	Siemens BEP 2000 Advance System, $\lambda = 450/620$ nm		

gave 'substantial' to 'near-perfect' agreement; $0.64 \leqslant \kappa \leqslant 0.92$ (Fig. 4). AUROC areas were ≥0.92, for all three antigens (Supplementary Fig. S4). Based on the AUROC analysis, with the help of Youden's equation, the following sensitivity–specificity pairs were selected 0.985–0.975, 0.935–0.911, 0.989–0.946 for measles, mumps and rubella, respectively. According to the 'experimental approach', cut-off values were set for all antigen types (measles, mumps, rubella) based on mean observed OD values belonging to diagnostically seronegative samples (3×15 samples, $OD_{\text{negative sample}} \leqslant 0.28$, 0.37, 0.34 for measles, mumps and rubella, respectively; data not shown). Cut-off values calculated based on empirical results were concordant with the statistically computed values. Typical dose–response curves obtained for measles, mumps and rubella standards are shown in Figure 5. Analytical values, such as lower LOD and LOQ are also represented in Figure 5. Sensitivity, specificity, positive and negative predictive values are shown in Table 3. We selected randomly chosen negative samples from the measles, mumps and rubella groups (30 each) that were verified using indirect immunofluorescent microscopy. We

Table 3. Assay precision and specific assay characteristics[a]

Specific assay characteristics ($N = 474$ from diverse age groups)	Measles	Mumps	Rubella
TPF = True Positive Fraction (sensitivity) = TP/(TP + FN)	0.99	0.99	0.99
FNF = False Negative Fraction (1–sensitivity) = FN/(TP + FN)	0.01	0.01	0.01
TNF = True Negative Fraction (specificity) = TN/(TN + FP)	0.93	0.94	0.88
FPF = False Positive Fraction (1–specificity) = FP/(TN + FP)	0.07	0.06	0.12
PPV = Positive Predicted Value = TP/(TP + FP)	0.99	0.99	0.99
NPV = Negative Predicted Value = TN/(TN + FN)	0.87	0.89	0.88
Intra-assay variability (CV%)[b]			
Positive sample 1	0.37	2.68	0.30
Positive sample 2	1.51	3.20	3.00
Positive sample 3	0.89	1.07	2.19
Negative sample 1	3.68	6.06	7.49
Negative sample 2	7.50	3.75	8.27
Negative sample 3	8.52	7.50	8.39
Inter-assay variability (CV%)[b]			
Positive sample 1	5.52	2.83	6.32
Positive sample 2	8.63	4.75	8.81
Positive sample 3	7.26	8.05	9.90
Negative sample 1	3.88	7.65	10.68
Negative sample 2	7.50	9.31	10.54
Negative sample 3	6.53	7.76	9.14

[a]Specific assay characteristics have been determined by comparing our assay to commercially available validated assays.
[b]Reproducibility, assay precision: intra-assay precision (coefficient of variation, CV%) was calculated for each of the three samples from the results of 12 determinations in a single run. Results for precision-within-assay are shown in the table above. Inter-assay precision (coefficient of variation, CV%) was calculated for each of the three samples from the results of three determinations in five different runs. Results for run-to-run precision are shown in the table above.

found 93%, 90% and 96% correspondence for measles, mumps and rubella, respectively (data not shown).

Assay characteristics: cost, ease and time requirement

An important feature of our three-in-one MMR ELISA assay is affordability; it costs only a fraction of the commercially available assays (Fig. 6a, b). An important component for improving the signal-to-noise ratio (background reduction) is a self-developed, low-cost reagent, a protein-free PVA-based blocking buffer (Supplementary Fig. S5). Another important feature is the reduced assay duration time; compared to the ~1.5/2.5 h of time-frame of commercially available tests (used for parallel and justificatory measurements), our test can be performed within 1 h (Fig. 7).

Determination of age groups with highest frequencies of seronegativity

Considering the antigen-specific seropositivity ratios of all samples measured, anti-measles, -mumps and -rubella IgG antibody titres were adequate in 89.84%, 91.82% and 92.28%, respectively (Fig. 8). Taking the following herd immunity threshold (HIT) values as a base; $HIT_{Measles} = 92–95\%$, $HIT_{Mumps} = 75–86\%$, $HIT_{Rubella} = 83–86$, it can be stated that regarding measles, levels of humoral immunity may be inadequate in certain age clusters of the population. Regarding anti-measles antibodies, cumulative data ($N_{total\ measles} = 3523$ serum samples) show that the lowest seropositivity (79.3%) was detected in individuals vaccinated between 1978 and 1987 (Figs 9 and 10), with significant differences from the flanking age groups vaccinated between 1969–1977 and 1988–1990 ($P = 0.00004$ and $P = 0.0015$, respectively) (Fig. 10). For mumps ($N = 1736$ serum samples), the least protected groups were vaccinated during 1978–1987 (11.9%) and 1988–1990 (10.1%); however, these were not statistically different from the adjacent age groups. In the case of rubella ($N = 1736$ serum samples), the least protected groups were vaccinated during 1969–1977 (14.4%) and 1978–1987 (14.5%). Significant differences were observed between the group born before 1969 (not vaccinated) and vaccinated during 1969–1977 ($P = 0.00008$), and between groups 1988–1990 and 1991–1995 ($P = 0.009$).

Discussion

Regarding assay optimisation, an important requirement was the equalisation of incubation times used in the three-in-one MMR ELISA. The establishment of a combined test system using identical serum dilutions, reagent volumes and incubation times that enable the measurement of 24 samples for all three antigens within a single run was only feasible with the maximal reduction of potentially interfering factors. An important step was the reduction of non-specific signal by using an IgM reducing assay diluent (Bio-Rad). The second important step was the use of our in-house PVA-based synthetic blocking buffer that enabled an optimal signal-to-noise ratio at a minimal cost. These steps made it possible to use a high concentration of antigen coatings, which in turn allowed relatively short incubation times and high performance of our assay.

As mentioned in the Introduction section, in Hungary, between February 2017 and July 2019, 70 measles cases were laboratory confirmed according to ECDC reports [5]. During the epidemics of 2017, there have been 36 measles cases in Hungary (five imported, 26 import-related, four unknown/not reported and one endemic). Regarding the infections by age group and vaccination status for 2017, according to WHO data, among the individuals 20–29 years of age, ≈35% had not been vaccinated, and ≈65% received two or more doses of vaccine. Of individuals older than 30 years, ≈18% had not been vaccinated, ≈24% received one dose, ≈26% received two or more doses of vaccine and ≈32% were of unknown vaccination status. Based on these data, it can be hypothesised that in the case of vaccinated adults (≥20 years of age), who had received two or more doses of vaccine, vaccine insufficiency may have underlaid the infections [12]. The last case of parotitis epidemica reported in Hungary was a non-vaccinated 35-year-old man, who became infected during the summer of 2018. Between 2012 and 2016, Hungary reported 21 mumps cases [12, 26]. In the 2007 local mumps outbreak, the epidemic started from an individual who

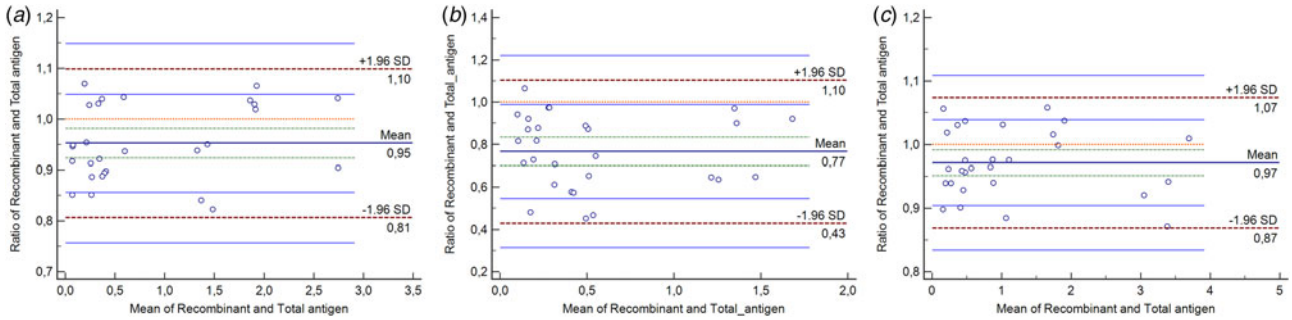

Fig. 3. Comparison of whole virus *vs.* recombinant viral antigen-based ELISA coatings. Bland–Altman graphs display scatter diagrams of the ratios plotted against the averages of the two types of measurements. Sample number = 28 (duplicates of the dilution series of positive and negative sample pools and quadruplicates of the dilution series of standards). Limits of agreement (LoA) are defined as the mean difference ± 1.96 s.d. (95% confidence interval). Since data points do not exceed the maximum allowed difference between methods (dotted brown lines), and no pronounced trend is observable, the two methods (target: total antigen repertoire-based coating *vs.* control: recombinant antigen-based coating) are in agreement and can be used interchangeably. Recombinant antigen coatings: Measles virus Priorix, Schwarz strain nucleocapsid protein, Mumps virus wild-type, Gloucester strain nucleocapsid protein, Recombinant Rubella virus nucleocapsid protein. Optimal recombinant antigen- based concentrations: 0.83 µg/mL, 0.416 µg/mL, 1.0 µg/mL for measles, mumps and rubella, respectively. Optimal inactivated pathogen-based coating concentrations: 2.8 µg/mL, 3.0 µg/mL, 0.4 µg/mL for measles, mumps and rubella, respectively. Sample number (n): N=28 (Samples were used in duplicates, standards were used in quadruplicates).

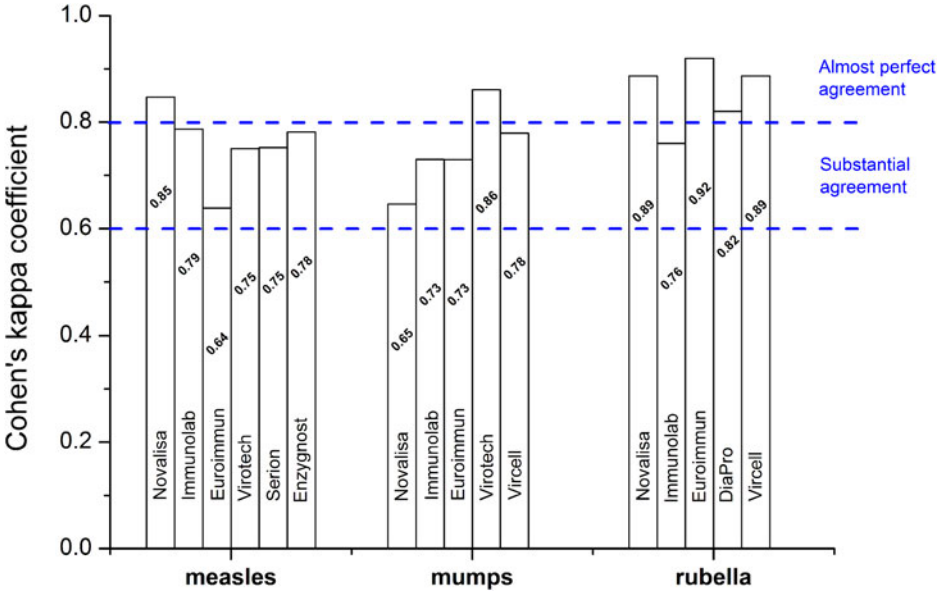

Fig. 4. Cohen's κ analysis of plate-to-plate measurements ($N_{\text{Novalisa, Immunolab, Vircell}} = 84$, $N_{\text{Virotech, DiaPro}} = 80$, $N_{\text{Euroimmun}} = 88$, $N_{\text{Serion, Enzygnost}} = 90$ samples). The measures of agreement describing the inter-rater reliability varied between 'substantial' and 'almost perfect'.

returned home from Ukraine in December 2007. Soon after his case, individuals from his personal connections became affected (later all nine cases were laboratory confirmed). Previously, in 2003, comparably small outbreaks were reported in close communities of unvaccinated people (student houses, schools) [11]. Considering rubella in Hungary, between 2007 and 2018, WHO reported only 10 known cases [12]. This is a significant development compared to the end of 1990s and beginning of 2000s, when ~100 rubella cases per year were reported (WHO statistics). By 2006, this number decreased by 80% (22 reported cases in year 2006). Measles, mumps and rubella statistics (cases per year) are shown in Supplementary Figure S2.

In 1969, the measles vaccine was introduced in Hungary in the form of live, attenuated Leningrad-16 strain vaccine, produced in the former Soviet Union. Between 1969 and 1974, a single dose of vaccine was administered in campaigns to individuals of 9–27 months [5]. After vaccination was implemented, the incidence rate decreased until 1973–1974, when large epidemics occurred primarily in unvaccinated 6–9 years old [5], questioning the effectiveness of the early vaccination programme. Regarding post-vaccination humoral immune response, heterogeneous data are available in the literature. It is generally accepted that the success of vaccination in children is dependent on the presence (or absence) of inhibitory maternal antibodies and the immunologic maturity of the recipient, as well as on the dose and vaccine strain. It is also recognised that the age of ≥12 months is a milestone in the development of an efficient immune response. A 2015 meta-analysis based on WHO study published the following seroconversion rates: 50% (95% CI 29–71%) at age 4 months, 67% (95% CI 51–81%) at 5 months, 76% (95% CI 71–82%) at 6 months, 72% (95% CI 56–87%) at 7 months and 85% (69–97%) at 8 months. Interestingly, the likelihood of seroconversion in children depends not only on the child's age, but also on the age of the mother; older children generally respond better than younger children, and children of younger mothers have the tendency to respond better than children of older mothers.

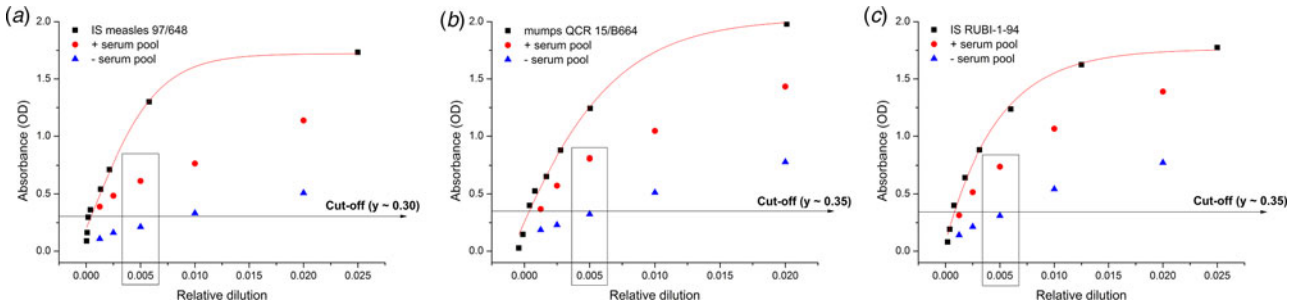

Fig. 5. Typical standard curves of MMR assay. Sigmoid dose–response curves of the dilution series of the standards were generated with optimal data fitting ($R^2 \geq$ 0.97). Absorbance values are plotted in function of relative dilution (1/dilution). These curves serve as the base for the conversion of OD values to units/ml. Rectangles show the optimal serum dilutions (200-fold) used in the final assay format. Model and equation used for calibration curve: sigmoid dose-response curve; y = A1 + (A2-A1)/ (1 + 10^ ((LOGx0-x)*p)). Adjusted R^2 values: 0.97, 0.97, 0.99 for measles, mumps and rubella, respectively. Measurement ranges: 0.025–12.5 mIU/mL, 0.02–10.0 arbitrary U/mL, 2.0 – 265 mU/mL for measles, mumps and rubella, respectively. Cut-off values: 0.15 mIU/mL, 0.15 arbitrary U/mL, 9.5 mIU/mL. LOD (mean + 3SD) extinction (OD) values: 0.08, 0.10, 0.08 for measles, mumps and rubella, respectively. LOQ (mean + 10SD) extinction (OD) values: 0.20, 0.23, 0.20 for measles, mumps and rubella, respectively.

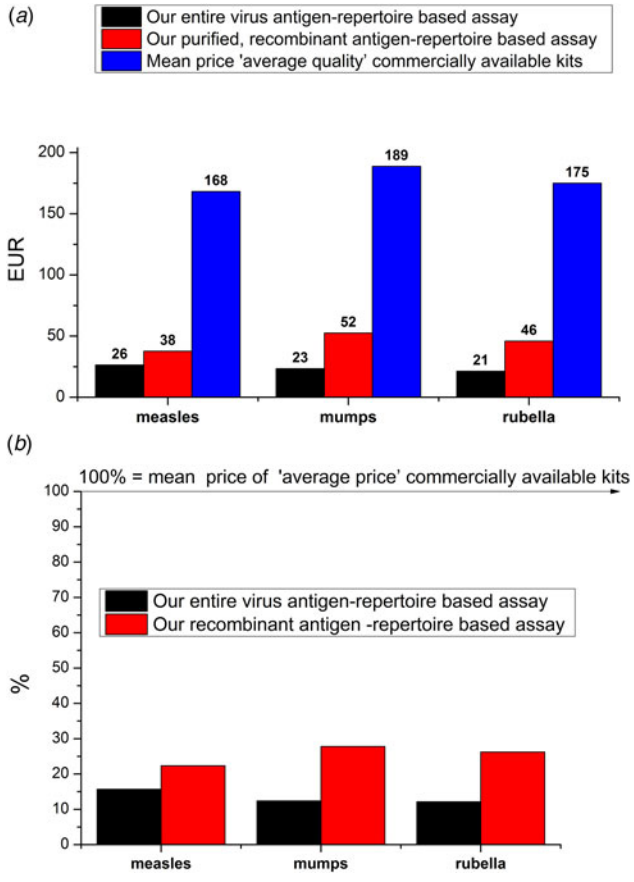

Fig. 6. (a) Comparison of assay prices (commercial kits) and costs (our test) expressed in Euros. (b) Ratios of assay prices: 'average price' commercial kits *vs.* our test expressed in percentages. The average price of commercial kits was calculated based on the Hungarian distributor prices (VAT included), and included only those assays that we applied during the optimization and the test-to-test comparisons (Materials and methods section). Siemens Enzygnost assays – belonging to a higher price-range – were excluded from the calculation.

Moreover, the 'source' of the mother's immunity (disease- or vaccination-induced) also plays a role as a surrogate factor [27]. The current Advisory Committee on Immunization Practices (ACIP 2012) also recommends age ≥12 months for the first MMR vaccination. As a general rule, the optimal vaccination age should be defined by the dynamics of the age-dependent progress in seroconversion, balanced by the level of the epidemiological risk [28, 29]. According to the Hungarian vaccination practice, the MMR vaccine is given twice; at 15 months and 11 years of age.

Regarding immunocompromised individuals and children with contraindications, we would like to note that in Hungary, immunocompromised persons also complete the recommended immunisation series against vaccine preventable diseases, whenever possible. The vaccination practice follows international guidelines (2013 IDSA), and an individualised patient approach is applied. This implies the involvement of a vaccination expert who performs case-to-case risk evaluation. As a general rule, live viral vaccines (e.g. polio, MMR, varicella) that may induce severe systemic reactions in immunocompromised individuals should not be administered to patients with severe immunosuppression and/or immune deficiency. Nevertheless, important exceptions exist: certain live vaccines can be administered in some immune system disorders or when the benefit of the vaccine outweighs the side effects, or major risk arising from the epidemiological environment [30, 31].

Our current serological data are in agreement with our previous report [24] where the estimated seropositivity for cluster '1978–1987' was ~74.6%, followed by cluster '1969–1977' with ~84.6%. A recent publication by Hungarian colleagues has reported 86.2% seropositivity for the 41–45 years old individuals [32], a cluster partially overlapping with the two abovementioned age groups of our classification. The potential gap detected in herd immunity is also supported by the already known insufficiencies during the initial vaccination era [33]. These individuals were vaccinated during the early 1970s, when the optimal age of vaccination was not well defined, and the thermolability of the reconstituted vaccine was not fully characterised [5]. These relatively high measles seronegativity ratios may have been a consequence of vaccine inefficiency, which seems to be supported by historical data: after the starting of the immunisation campaign in 1969, a decade later, in 1978, the recommended age for vaccination was changed from 10 to 15 months. The 1988–1989 epidemics affected individuals (16–22 years old) who were vaccinated in the early era with a singular measles vaccine. Following the 1988–1989 epidemics, persons born between 1973 and 1977 were revaccinated [33]. Even though a significant portion of the age groups indicated with ** in Figure 9 later were

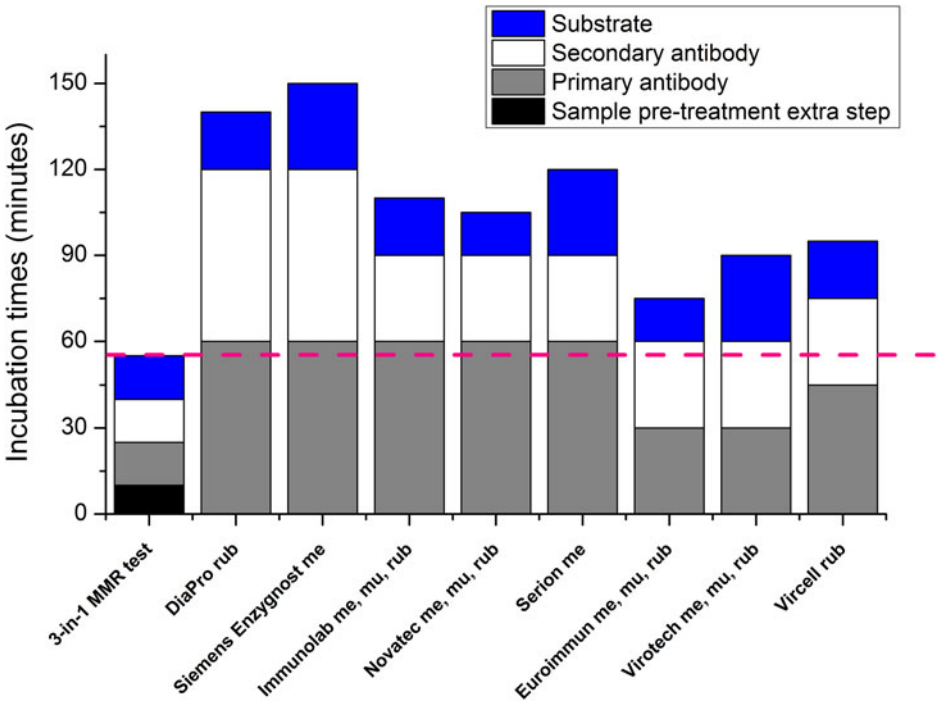

Fig. 7. Comparison of incubation times of our test (three-in-one MMR) to different commercial kits (me = measles, mu = mumps, rub = rubella).

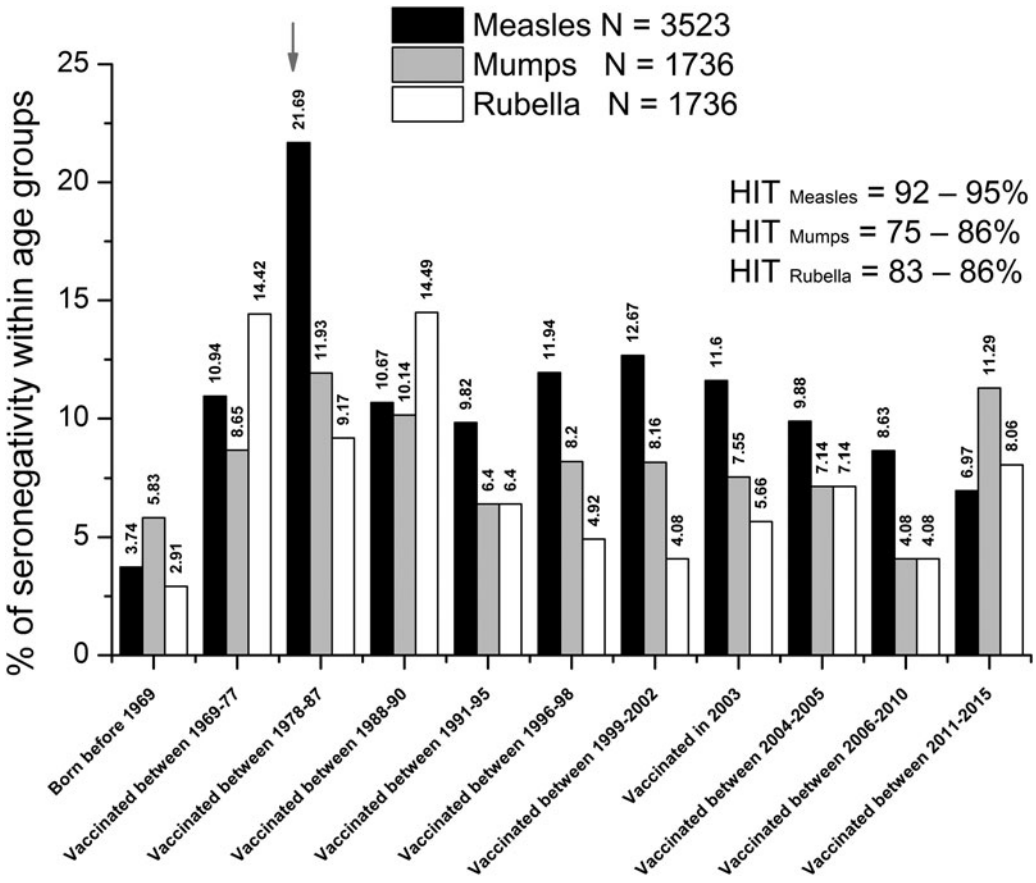

Fig. 8. Vaccination period-independent summary of results. Considering the age-independent totality of samples, the anti-measles, mumps and rubella IgG antibody titres were inadequate in 10.16%, 8.18% and 7.72%, respectively. Considering HIT values, population-level seropositivity ratio of anti-measles antibodies failed to reach the criteria for herd immunity (seropositivity ≥95%). The red arrow shows the vaccination group with highest seronegativity in terms of anti-measles antibody titers.

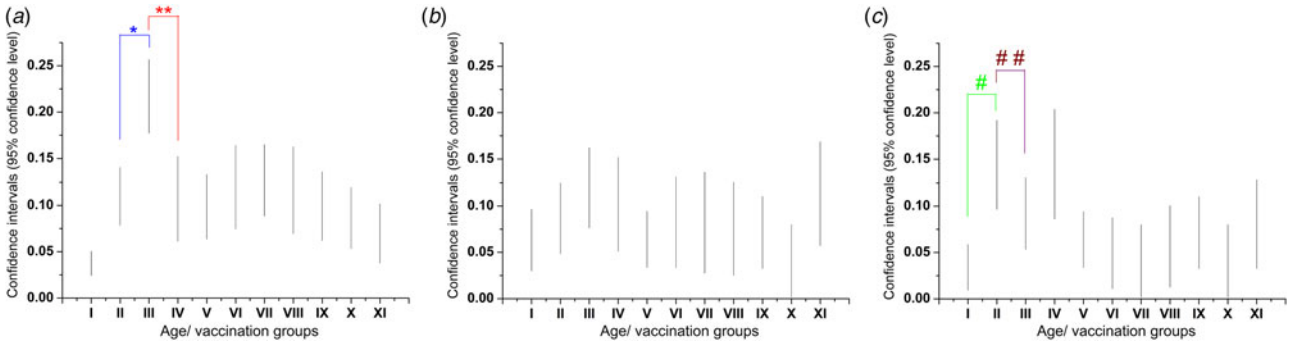

Fig. 9. Summary of seronegativity ratios within different age groups. Age or vaccination groups (X-axis): (I) Born before 1969 (not vaccinated; high probability of wild-type infection), (II) vaccinated between 1969-77, (III) vaccinated between 1978-87, (IV) vaccinated between 1988-90, (V) vaccinated between 1991-95, (VI) vaccinated between 1996-98, (VII) vaccinated between 1999-2002, (VIII) vaccinated in 2003, (IX) vaccinated between 2004-2005, (X) vaccinated between 2006-2010, (XI) Vaccinated between 2011-2015. P-values indicating statistically significant differences between adjacent age groups: (*) vaccinated between 1969-77 and 1978-87 p=0.00003841; (* *) vaccinated between 1978-1987 and 1988-90 p=0.0015; (#) vaccinated between 1969-77 and 1978-87 p=0.00008437; (##) vaccinated between 1988-90 and 1991-95 p=0.008532. We identified samples in the cluster '*Vaccinated between 1978–1987*' as the lowest seropositivity group for measles.

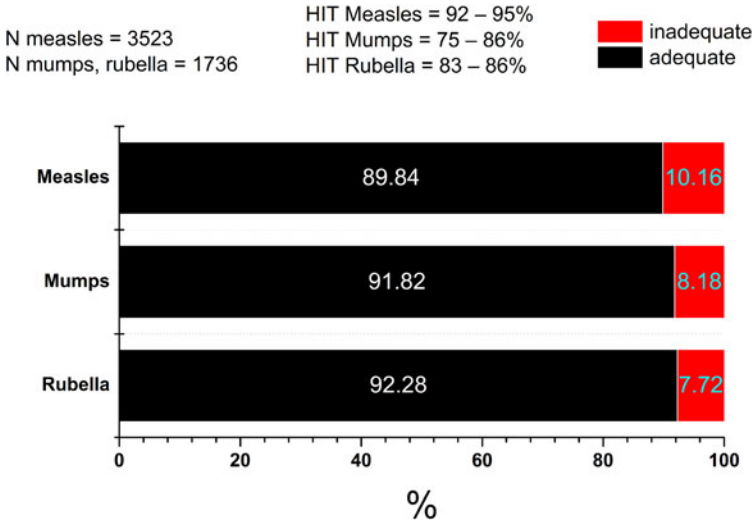

Fig. 10. Vaccination period-dependent confidence intervals of seronegativity. Relative frequencies of measles-, mumps- and rubella-specific seronegativity dependent on the period of vaccination. Vertical lines indicate 95% confidence intervals. Significant differences between the antibody levels of the critical age groups and their flanking age groups are marked with asterisks.

re-vaccinated or contracted wild-type measles infection (and thus mounted high IgG antibody response), in this cluster, we found the lowest cumulative anti-measles antibody titres (i.e. high ratio of seronegativity), which suggests the ineffectiveness of the early vaccination system. Additional support for this hypothesis is the high age-specific attack rates during the 1988–1989 outbreak that affected the population with ≥93% vaccine coverage. After the introduction of the trivalent MMR vaccine (1991), we detected a statistically significant improvement in the anti-measles antibody titres (Fig. 10). The group '*Vaccinated between 1988–1990*' has significantly better humoral response compared to the group '*Vaccinated between 1978–1987*', reflecting the effectiveness of the trivalent reminder vaccine at age 11.

Population-level result evaluation was performed in relation to the concept of herd immunity. The term 'herd immunity' is widely used, but diversely interpreted. We used it in the sense of 'a threshold proportion of immune individuals' [34], strictly limited to humoral antibody titres. This threshold denoting the arrest of disease spread is different for every disease and is affected by many factors; key epidemiological parameters, such as the age-specific force of infection and the basic reproduction number (R_0) are estimated from case notification or serological data [35]. Imperfect immunity (due to individual differences of responders), heterogeneous populations with potential non-random mixing and non-random vaccination schedules may also need to be considered [34]. R_0 is defined as the average number of secondary cases that result from an individual infection in a susceptible population [36]. Estimates of R_0 depend on underlying mixing assumptions. For the virus-specific R_0 values shown below, the model of 'likely mixing patterns' was used [35]. The R_0 estimates are highest for measles, intermediate for mumps and generally lowest for rubella [35]. For measles, R_0 is often cited as 12–18, which implies the need for ~95% herd immunity. This means that each person with measles can infect 12–18 other individuals in a completely susceptible population. For this reason, the achievement of ≥95% of immunity across all age groups (optimal immune response followed by efficient seroconversion on population level) is the official target for measles elimination. In the literature, R_0 and HIT values are generally estimated as follows: $R_{0\ \mathrm{Measles}} = 12–18$, $\mathrm{HIT}_{\mathrm{Measles}} = 92–95\%$, $R_{0\mathrm{Mumps}} = 4–12$, $\mathrm{HIT}_{\mathrm{Mumps}} = 75–86\%$, $R_{0\mathrm{Rubella}} = 5–7$, $\mathrm{HIT}_{\mathrm{Rubella}} = 83–86\%$ [35]. Often used models for population-level estimation are the HIT (I_c); $I_c = 1 - (1/R_0)$, and

the critical vaccination coverage (V_c); $V_c = I_c/E$, where E is vaccine effectiveness [34–37]. Despite the remarkable theoretical knowledge, public health practice aims at 100% coverage, with all the doses recommended, bearing in mind that – because of the diversity of individual immune responses – 100% is never achievable.

Limitations

We would like to note that our '*three-in-one*' assay and the results described in our paper may have certain limitations. As specified in the WHO *Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome*, EIA/ELISA testing may be used for the detection of the presence (or absence) of anti-viral IgG antibodies of individuals, as well as to perform population-level immunity estimations. In case of population-based seroprevalence studies, ELISA/EIA results can help characterise the immune profile of target populations; however, there are important limitations. When applying commercial assays, we used cut-offs and calculation methods as per kit manual, without changing or reinterpreting default thresholds. Each commercially available kit (listed in Materials and methods section) specified one particular method for quantitative (and qualitative) result calculation, with no distinction between periods with or without epidemics, or samples collected with the purpose of clinical diagnosis or population-level survey. However, according to the literature, thresholds for commercial IgG ELISAs/EIAs were determined with the purpose of individual patient management, rather than with population-level antibody prevalence calculations [38, 39]. A positive result of virus-specific IgG clearly demonstrates an immune response, in contrast, given that commercial immunoassays are capable only of humoral antibody detection, a negative or equivocal result cannot be considered as an absolute proof for lack of immunity [40, 41]. The functional characteristics and the 'quantity' of antibodies may be highly correlated with protection or synergistic with other functions (e.g. with cellular immunity). The correlates of vaccine-induced immunity are often a matter of debates; for some vaccines, we have no true correlates, but only useful surrogates [42, 43]. As far as Plotkin's definitions are considered normative [44], entire antigen repertoire-based ELISA/EIA methods of measles, mumps and rubella IgG antibody detection are considered rather than a good surrogate marker for immunity. This is especially true for our test, since our cut-off calibration was based on multiple measurements with independent, commercially available assays, and with indirect immunofluorescent microscopy. The diagnostic ability of our test is calculated based on the results obtained by kits capable of humoral antibody detection, and not on neutralising antibody titres that could serve as an absolute correlate of protection.

Additionally, considering age-specific susceptibility estimates at population level, the phenomenon of vaccination-induced lower antibody levels, compared to those elicited by natural infection, is also to be taken into account [45, 46]. Consequently, low (negative or equivocal) IgG results are to be interpreted with caution, when assessing immunity in populations with effective immunisation programmes [38]. The evaluation of immune status may require additional testing of specimens with results in the equivocal range (we used IIF for this purpose). We also would like to note that the actual level of any immunological marker is a snapshot in time, which needs to be interpreted in the light of the kinetics of the marker. Although the half-lives of antibodies against measles, mumps and rubella are relatively long, unexpected responses cannot be excluded, whereby vaccinees can mount sufficient responses rapidly from a low (even close to zero) background of humoral antibody level [44].

Conclusions

Here we describe the development of a time-saving, cost-effective and standardised immunoserological assay for simultaneous detection of anti-measles, -mumps and -rubella IgG antibodies in human sera. The importance of the 'three-in-one' assay is highlighted by recent outbreaks of measles, mumps and rubella infections in several countries. This triple assay is based on an operation protocol that uses the same reagent load with uniform, short incubation times and equally pre-treated samples, enabling the three-parametric screening of 24 samples per plate within 1 h. In high-throughput automated settings, separate testing of the three antigen types is also feasible, thus allowing the measurement of 80 samples per run. Considering the HIT values, anti-measles seropositivity (79.3%) of the '1978–1987' vaccination group suggests the existence of potential gaps in anti-measles immunity of the population. For mumps and rubella, our preliminary data suggest satisfactory immunity levels. The potential gaps in anti-measles immunity warrant further large-scale serological screening.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0950268819002280.

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Conflict of interest. None.

References

1. **Haralambieva IH *et al.*** (2015) Variability in humoral immunity to measles vaccine: new developments. *Trends in Molecular Medicine* **21**, 789–801.
2. **Tabacchi G *et al.*** (2016) Determinants of European parents' decision on the vaccination of their children against measles, mumps and rubella: a systematic review and meta-analysis. *Vaccines & Immunotherapeutics* **12**, 1909–1923.
3. **European Centre for Disease Control** (2019) Insufficient vaccination coverage in EU/EEA fuels continued measles circulation. Available at https://ecdc.europa.eu/en/news-events/ecdc-insufficient-vaccination-coverage-eueea-fuels-continued-measles-circulation. Accessed 24 July 2019.
4. **Zimmerman LA *et al.*** (2019) Progress toward measles elimination – European region, 2009–2018. *MMWR. Morbidity and Mortality Weekly Report Centers for Disease Control and Prevention* **68**, 396–401.
5. **Centers for Disease Control and Prevention** (1989) International Notes Measles – Hungary. *MMWR Weekly* **38**, 665–668. Available at https://www.cdc.gov/mmwr/preview/mmwrhtml/00001472.htm
6. **World Health Organization** (2019) New measles surveillance data from WHO. World Health Organization. Published online: 2019. Available at https://www.who.int/news-room/fact-sheets/detail/measles
7. **Wadman M** (2019) Measles epidemic in Ukraine drove troubling European year. *Science* **363**, 677–678.

8. **The Lancet Editorial** (2018) Measles, war, and health-care reforms in Ukraine. *The Lancet* **392**, 711. doi: 10.1016/S0140-6736(18)31984-6.
9. **World Health Organization** (2018) A report on the epidemiology of selected vaccine-preventable diseases in the European Region. WHO EpiBrief No. 1/2018.
10. **Angelo KM *et al.*** (2019) Spread of Measles in Europe and Implications for US Travelers. *Pediatrics* **144**, e20190414. doi: 10.1542/peds.2019-0414.
11. **Molnár Z *et al.*** (2007) Local mumps outbreak in Hungary, 2007. *Weekly Releases (1997–2007) European Centre for Disease Prevention and Control* **12**, 3167.
12. **World Health Organization** (2017) Measles and rubella elimination country profile Hungary. Available at http://www.euro.who.int/__data/assets/pdf_file/0013/401116/HUN.pdf?ua=1
13. **European Centre for Disease Control** (2019). Monthly measles and rubella monitoring report, April 2019.
14. **World Health Organization** (2018). Hungary: WHO and UNICEF estimates of immunization coverage: 2018 revision.
15. **Lambert N *et al.*** (2015) Rubella. *The Lancet* **385**, 2297–2307.
16. **Moss WJ** (2017) Measles. *The Lancet* **390**, 2490–2502.
17. **Lewnard JA and Grad YH** (2018) Vaccine waning and mumps re-emergence in the United States. *Science Translational Medicine* **10**, eaao5945.
18. **Hviid A, Rubin S and Mühlemann K** (2008) Mumps. *The Lancet* **371**, 932–944.
19. **MMWR Weekly**. MMWR publications | MMWR. Available at https://www.cdc.gov/mmwr/publications/index.html
20. **Liu Y *et al.*** (2018) Waning immunity of one-dose measles-mumps-rubella vaccine to mumps in children from kindergarten to early school age: a prospective study. *Expert Review of Vaccines* **17**, 445–452.
21. **Bankamp B *et al.*** (2019) Successes and challenges for preventing measles, mumps and rubella by vaccination. *Current Opinion in Virology* **34**, 110–116.
22. **Kontio M *et al.*** (2012) Waning antibody levels and avidity: implications for MMR vaccine-induced protection. *Journal of Infectious Diseases* **206**, 1542–1548.
23. **Gibney KB *et al.*** (2019) Emergence of attenuated measles illness among IgG positive/IgM negative measles cases, Victoria, Australia 2008–2017. *Clinical Infectious Diseases*. Published online: 6 May 2019. doi: 10.1093/cid/ciz363.
24. **Böröcz K *et al.*** (2019) Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human sera. *Journal of Immunological Methods* **464**, 1–8.
25. **Griner PF, Mayewski RJ and Mushlin AIGP** (1981) Selection and interpretation of diagnostic tests and procedures. Principles and applications. – PubMed – NCBI. *Annals of Internal Medicine* **94**, 557–592.
26. **European Centre for Disease Control** (2018) Surveillance Report Annual Epidemiological Report for 2016 Mumps. Available at https://www.ecdc.europa.eu/sites/default/files/documents/AER_for_2016-mumps-rev.pdf
27. **Redd SC *et al.*** (2004) Comparison of vaccination with measles-mumps-rubella vaccine at 9, 12, and 15 months of age. Hinman AR, ed. *The Journal of Infectious Diseases* **189**, S116–S122.
28. **Orenstein WA *et al.*** (1986) Appropriate age for measles vaccination in the United States. *Developments in Biological Standardization* **65**, 13–21.
29. **World Health Organization** (2009) The Immunological Basis for Immunization Series. Module 7: Measles Update 2009.
30. **Health Service Executive Ireland** (2019) Chapter 3 Immunisation of Immunocompromised Persons. Available at https://www.hse.ie/eng/health/immunisation/hcpinfo/guidelines/chapter3.pdf
31. **Arvas A** (2014) Vaccination in patients with immunosuppression. *Turk pediatri arsivi* **49**, 181–185.
32. **Lengyel G *et al.*** (2019) Screening of more than 2000 Hungarian healthcare workers' anti-measles antibody level: results and possible population-level consequences. *Epidemiology and Infection* **147**, e7.
33. **Agócs MM *et al.*** (1992) The 1988–1989 measles epidemic in Hungary: assessment of vaccine failure. *International Journal of Epidemiology* **21**, 1007–1013.
34. **Fine P, Eames K and Heymann DL** (2011) 'Herd immunity': a rough guide. *Clinical Infectious Diseases* **52**, 911–916.
35. **Edmunds WJ *et al.*** (2000) The pre-vaccination epidemiology of measles, mumps and rubella in Europe: implications for modelling studies. *Epidemiology and Infection* **125**, 635–650.
36. **Guerra FM *et al.*** (2017) The basic reproduction number (R0) of measles: a systematic review. *The Lancet. Infectious Diseases* **17**, e420–e428.
37. **Masterson SG *et al.*** (2018) Herd immunity to ebolaviruses is not a realistic target for current vaccination strategies. *Frontiers in Immunology* **9**, 1025.
38. **World Health Organization** (2018) Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome. Third edition, June 2018.
39. **Vyse AJ *et al.*** (2006) Interpreting serological surveys using mixture models: the seroepidemiology of measles, mumps and rubella in England and wales at the beginning of the 21st century. *Epidemiology and Infection* **134**, 1303–1312.
40. **Skendzel LP** (1996) Rubella immunity: defining the level of protective antibody. *American Journal of Clinical Pathology* **106**, 170–174.
41. **Ward BJ *et al.*** (1995) Cellular immunity in measles vaccine failure: demonstration of measles antigen-specific lymphoproliferative responses despite limited serum antibody production after revaccination. *Journal of Infectious Diseases* **172**, 1591–1595.
42. **Plotkin SA** (2010) Correlates of protection induced by vaccination. *Clinical and Vaccine Immunology : CVI* **17**, 1055–1065.
43. **Plotkin SA and Gilbert PB** (2012) Nomenclature for immune correlates of protection after vaccination. *Clinical Infectious Diseases* **54**, 1615–1617.
44. **World Health Organization** (2013) Correlates of vaccine-induced protection: methods and implications. WHO reference number: WHO/IVB/13.01.
45. **Dorigo-Zetsma JW *et al.*** (2015) Immune status of health care workers to measles virus: evaluation of protective titers in four measles IgG EIAs. *Journal of Clinical Virology* **69**, 214–218.
46. **Tischer A *et al.*** (2007) Vaccinated students with negative enzyme immunoassay results show positive measles virus-specific antibody levels by immunofluorescence and plaque neutralisation tests. *Journal of Clinical Virology* **38**, 204–209.

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Research paper

Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human sera

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ABSTRACT

Because of measles outbreaks there is a need for continuous monitoring of immunological protection against infection at population level. For such monitoring to be feasible, a cost-effective, reliable and high-throughput assay is necessary. Herein we describe an ELISA protocol for assessment of anti-measles antibody levels in human serum samples that fulfills the above criteria and is easily adaptable by various laboratories. A serum bank of anonymous patient sera was established (N > 3000 samples). Sera were grouped based on measles immunization schedules and/or changes in vaccine components since the introduction of the first measles vaccine in Hungary in 1969. Newly designed ELISA was performed by using Siemens BEP 2000 Advance System and data were confirmed using commercially available kits. Our indirect ELISA was compared to indirect immunfluoresence and to anti-measles nucleocapsid (N) monoclonal antibody-based sandwich ELISA. The results obtained are in high agreement with the confirmatory methods, and reflect measles vaccination history in Hungary ranging from pre-vaccination era, through the initial period of measles vaccination, to present. Based on measurement of 1985 sera, the highest ratio of low/questionable antibody level samples was detected in cluster '1978–1987' (~25.4%), followed by cluster '1969–1977' (~15.4%).Our assay is suitable for assessment of anti-measles immunity in a large cohort of subjects. The assay is cost-effective, allows high-throughput screening and has superior signal-to-noise ratio. This assay can serve as a first step in assessment of the effectiveness of all three components of the MMR vaccine.

1. Introduction

There is an urgent need for revision of immunological protection against measles infection at population level. This is underscored by the spread of measles virus that has emerged as a new public health risk in several European countries (Ahmed and Lambert, 2014; ECDC Report, 2018; Grammens et al., 2017; Haralambieva et al., 2015; Moss, 2017; Zachariah and Stockwell, 2016). In this paper we report the development of a straightforward, standardized and automatable anti-measles IgG indirect ELISA protocol. The assay is adaptable by research, diagnostics and public health laboratories, and allows performance of large-scale, high-throughput and cost-effective measurements. We would like to note that our current assay focuses on the first level screening of the general population, and in case of certain chronic diseases additional methods, e.g. cytokine-based cellular assays might be required. We describe key steps of assay optimization, together with practical testing of the newly assembled anti-measles antibody detection assay using 1985 human sera. Additional impetus for our current research has been that vaccine-induced protection against measles may not provide life-long protection (Kang et al., 2017; Kontio et al., 2012; Zachariah and Stockwell, 2016). For this reason the actual protection status of the population needs to be closely monitored to ensure flock immunity (for which > 95% vaccination coverage is required), and our assay may be an effective tool in this effort.

2. Materials and methods

2.1. Samples

A serum bank consisting of anonymous patient sera was established

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(N > 3000 serum samples) from routine laboratory residual samples at University of Pécs, Clinical Centre (Ethical License number 2015/5726). As the Clinical Centre is responsible for serving patients from three counties in southern Hungary, we had a random sampling from approximately 1,000,000 inhabitants. Sera were collected from all age groups, and were categorized based on changes introduced in measles immunization schedules and/or in vaccine components since the introduction of the first measles vaccine in Hungary in 1969. Patients with known serious immunocompromised state (e.g. acquired and inherited immunodeficiency, transplant patients, chronic immunosuppressive treatment) were excluded from the study. Samples were distributed between age groups weighted by the number of years covering a specific age group.

2.2. Overall experimental design

The newly designed, parameter-optimized enzyme-linked immunosorbent assay (ELISA) was performed by using Siemens BEP 2000 Advance System (Siemens/Dade Behring, Marburg, Germany). Assay protocol files for the robotics were created using Siemens BEP 2000 software, version 1.23. Results obtained by self-developed tests were compared with multiple commercially available assays, such as Virotech Measles IgG ELISA test (Sekisui Virotech GmbH), Immunolab Measles IgG ELISA test (Immunolab GmbH), Enzygnost® Anti-measles Virus/IgG (Siemens Laboratories), EUROIMMUN Anti-Measles Virus ELISA (EUROIMMUN Schweiz AG), Anti-Measles Virus IgG 24 Alegria® Test Strips (ORGENTEC Diagnostika). Indirect immunofluorescence (IIF) EUROIMMUN Measles virus IgG (EUROIMMUN Schweiz AG) was used as a reference. Results obtained by our new indirect ELISA were compared to those obtained by anti-measles nucleocapsid (N) monoclonal antibody-based sandwich ELISA, which correlates well with the presence of anti-measles protective antibodies. Commercial tests were performed according to manufacturer's instructions.

2.3. Anti-measles IgG Indirect ELISA

ELISA 96-well Maxisorp plates (Nunc) were coated overnight at 4–6 °C with 100 µL/well of 2 µg/mL of native measles virus antigen (Bio-Rad PIP013), sonicated according to manufacturer's instruction, and dissolved in appropriately diluted coating buffer (5× ELISA Coating Buffer, Bio-Rad BUF030). After coating and subsequent three washes with 350 µl/well of washing buffer (WB) ($NaH_2PO_4 \times H_2O$ 0.345 g + $Na_2HPO_4 \times 12$ H_2O 2.68 g + NaCl 28.675 g + Tween-20 1 mL for 1 L made in distilled water) saturation was done using our self-developed, purely synthetic blocking buffer containing polyvinyl alcohol (PVA) dissolved in PBS. Blocking buffer was applied to plates at 37 °C for 4 h.

Sample pretreatment steps can be performed in deep-well dilution plates (Nunc™ 1.0 and 2.0 mL DeepWell™ plates or equivalent) or in tubes (5 mL 75 × 12 mm, Polypropylene, Sarstedt or equivalent). We detail the dilution-tube based method: IgM reduction (IgM Reducing Assay Diluent, Bio-Rad BUF038) starts with the incubation of samples in IgM reducing buffer (IgM RB) (50-fold dilution of samples in undiluted IgM reducing buffer) at room temperature (RT) for 15 minutes. After centrifugation at 2330 x*g* for 5 min at RT, 1 unit from each supernatant was transferred to a new set of dilution tubes containing 3 units of WB, resulting in final 4-fold dilution of IgM RB and 200-fold diluted samples.

Blank (WB), high and low controls (positive and negative samples identified in a previous run, and processed similarly as the patient sera), WHO International Standard (Anti-Measles Serum, Human and Anti-Poliovirus serum Types 1, 2 and 3 NIBSC code: 66/202, 5 IU anti-measles activity) in seven-point serial dilution, and patient sera were applied in duplicates. Primary and secondary antibodies were incubated at 37 °C for 30 min. As secondary antibody we used Dako polyclonal rabbit anti-human IgG horseradish peroxidase (HRP)-conjugated, diluted 6000-fold, according to manufacturer's instructions (five washes between each relevant step were done using 350 µL/well of WB in aspiration mode). The color reaction using 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma, USA) was performed at 37 °C for 15 min in dark. The reaction was stopped by adding 100 µL/well of 4 M H_2SO_4. Result quantification took place based on absorbance measurements at 450 nm (620 nm reference) using a 7-point calibration curve.

2.4. Anti-measles nucleocapsid monoclonal antibody-based sandwich ELISA

ELISA 96-well Maxisorp plates (Nunc) were coated overnight at 4–6 °C with 100 µL/well of 5000-fold diluted anti-measles nucleoprotein antibody (mouse monoclonal to measles nucleoprotein, [2F3] (ab106292) Abcam) dissolved in appropriately diluted coating buffer (5× ELISA Coating Buffer Bio-Rad BUF030). The first saturation step (after 5 washes as described above for the indirect ELISA) was performed using 350 µL/well of our self-developed synthetic blocking buffer (detailed above) at 37 °C for 3 h. After 5 washes, the antigen coating was performed at 37 °C for 2.5 h using 3 µg/mL (100 µL/well) of native measles virus antigen (PIP013 Bio-Rad) dissolved in diluted coating buffer. After incubation and 5 washes, a second saturation step was done using the self-developed purely synthetic PVA-based blocking buffer for 90 min at 37 °C. After final 5 washes, the standard indirect ELISA operational protocol was followed starting from the IgM reducing pretreatment of sera to reading the absorbance at 450 nm (using 620 nm as reference). The only change compared to the standard method was that the secondary antibody (Dako polyclonal rabbit anti-human HRP-conjugated IgG), was diluted 6000-fold, in 0.5% (v/v) naive mouse serum (sterile, heat inactivated, lot number: SM30-25991HI, Gentaur Europe). Nonspecific immunological complexes were removed by centrifugation (2330 x*g*, 5 min at RT), then the supernatant was applied onto ELISA plate (100 µL/well).

2.5. Blocking and diluent optimization

The following assay diluents were tested: Hispec Assay Diluent (BUF049 Bio-Rad), ELISA Neptune Assay Diluent (BUF039 Bio-Rad), Block ACE (BUF029 Bio-Rad), ELISA General Assay Diluent (BUF037 Bio-Rad), ELISA IgM Reducing Assay Diluent applied without dilution, 2-fold, 4-fold, 8-fold dilutions (BUF038 Bio-Rad), and our own washing buffer ($NaH_2PO_4 \times H_2O$ 0.345 g + $Na_2HPO_4 \times 12$ H_2O 2.68 g + NaCl 28.675 g + Tween-20 1 mL for 1 L, made in distilled water). Various blockers were also tested: Block ACE (BUF029 Bio-Rad), gelatin blocker (made from bovine skin), ELISA SynBlock (BUF034 Bio-Rad) and our purely synthetic PVA solution. Results were analyzed to obtain an optimal signal-to-noise ratio.

2.6. Plate and coating buffer selection

We tested the following plates: Nunc Maxisorp™ ELISA 96-well high-binding plates (442,404 Sigma-Aldrich/Merck), 3D NHS and 3D Epoxy with covalent binding capacity, 705,070 and 762,070 with medium binding capacity, 705,071 and 762,071 with high binding capacity (Greiner Bio-One). Using these plates, we tested different types of coating buffers: diluted 5× ELISA Coating Buffer (BUF030 Bio-Rad), PBS (pH 8.5), and 2-(N-morpholino) ethane-sulfonic acid (MES) buffer, 25 mM, pH 6.0. After coating of plates with antigen, the results for different plates were compared to each other, as well as to Siemens Enzygnost kit (Siemens/Dade Behring, Marburg, Germany), known as the gold standard for measles ELISA assays (Tischer et al., 2007).

2.7. Calibration curve and serum antibody quantification

Milli-International Unit (mIU) content of samples was calculated

based on absorbance measurements at 450 nm (620 nm reference) using a 7-point calibration curve (500 to 15,000-fold dilutions) of the WHO International Standard Anti-Measles Serum, Human and Anti-Poliovirus serum Types 1,2 and 3 (NIBSC code: IS 66/202). The generated calibration curve was of sigmoid type, onto which a 4-parameter logistic function was fitted.

2.8. Determination of cut-off values

For the cut-off determination the arbitrary statistical method based on adding three standard deviations to the mean of negative samples was used in the following way: Low-titer sera (obtained from children 10–15 months of age, who were likely lacking maternal immunity and were not vaccinated (Guerra et al., 2018)) that had been clearly proven to be negative by multiple tests, were tested according to our newly developed protocol and were compared to blanks and high-level (15000–20,000-fold) dilution of IS 66/202.

For identification of negative samples, we performed multiple measurements of ~100 anonymous clinical samples. The criteria of sample negativity were low values obtained by tests using two accepted commercial kits and also the age of infants at which anti-measles antibodies derived from the mother were below detectability and the child had not been vaccinated against measles. Accordingly, the cut-off value was determined as follows: the intersection defined by the constant line (calculated by adding 3 SD to the mean OD values of negative samples), and the 4-parameter logistic curve (fitted to the dilution points of IS 66/202) was projected onto the X axis, denoting the concentration.

2.9. Optimal dilution of samples

High- and low-titer groups of samples were established based on preliminary measurements using two well-established commercial kits. Low-titer sera were diluted 25-fold, while high-titer sera were diluted 50-fold in order to ensure that the OD values of these stock solutions fell within optimal range. These stock solutions were subsequently diluted in two-fold steps (9 times) until the absorbance values became indistinguishable from the background. The main criterion for selecting the dilution level of the sample was the ability to tell the difference between positive and negative samples, while staying in the optimal absorbance range (with acceptable signal-to-noise ratio) and using the lowest amount of standard stock solution for cost effectiveness. On the same experimental setting of samples the linearity and parallelism of dilution were also investigated.

2.10. Laboratory and statistical parameters

SD and CV% values were calculated based on quantitative results based on analysis of 20 samples (applied in triplicates onto three plates with identical sample layouts; antibody levels ranged from low to high). Analytical values such as lower limit of detection (LOD) and limit of quantification (LOQ) were determined by the mean and standard deviation of blank sample absorbance values; LOD was defined as mean + 3 SD and LOQ as mean + 10 SD (absorbance values), as suggested by the IUPAC Compendium of Chemical Terminology Gold Book. Analytical sensitivity was defined as the ratio of optical signal change and concentration change, as suggested in the literature (Iupac, 2014; MacDougall et al., 1980; Ridge and Vizard, 1993) Linearity and parallelism were defined as described by Cambron et al.

2.11. Instrumentation platform

Measurements and the entire assay development were performed on MSZ EN ISO 15189:2013 platform in our laboratory, which is accredited by National Accreditation Agency of Hungary (1-1552/2016). Results were compared for overall vaccine efficacy evaluation and subsequently for age-group based comparison.

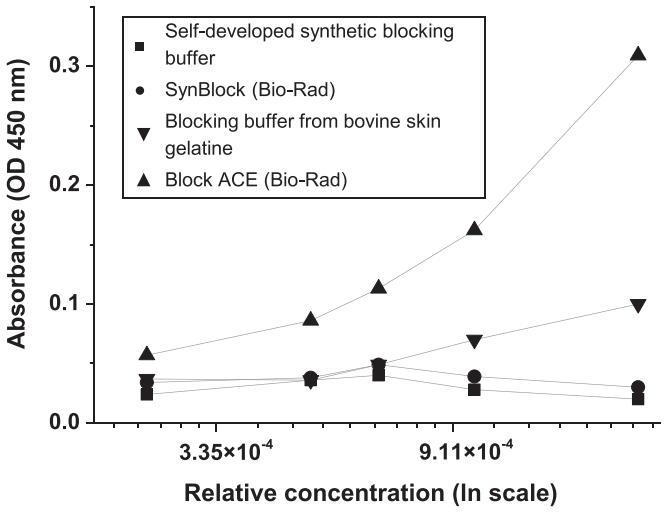


Fig. 1. Effect of protein-free blocking.

2.12. Software used for statistical data evaluation

Microsoft Excel, XLSTAT, MedCalc (MedCalc Software BVBA), Origin Pro (OriginLab), and SPSS were used for data evaluation.

3. Results

3.1. Blocking and diluent optimization

Blocking solutions (protein-containing and protein-free) were tested on plates that had not been coated with antigen; blocking solutions only were applied to plates and incubated overnight at 4–6 °C. Fig. 1 shows the results when the IS 66/202 anti-measles serum was used at five different dilutions (range 10 mIUnit/mL–2.5 mIUnit/mL). Results showed that using Block ACE and bovine skin gelatin saturation the absorbance values reflected the increasing concentration of the standard, which suggests non-specific reactions. Such non-specific reactions were not observed in the case of SynBlock and our PVA-based synthetic blocking solution. Therefore, for our subsequent experiments we used the PVA-based synthetic blocking solution.

3.2. Removal of IgM antibodies from samples

We observed a high background when using undiluted IgM reducing buffer (IgM RB) for diluting the serum samples without centrifugation. Using a two-step dilution process as described in the Materials and methods, a 2-fold and 4-fold final dilution of IgM RB the treatment was effective. Control experiments using 4-fold diluted IgM RB alone (without sera) showed low levels of background (N = 16 wells on 3 separate plates; OD_{mean} +/-SD = 0.0384 +/− 0.0088). IgM RB treatments resulted in little or no change in absorbance of standards, applied at concentrations used for calibration curves. Using 2-fold diluted IgM RB, the absorbance values of patient sera decreased to 30% (70% decrease from the original value), while at 4-fold dilution of IgM RB the absorbance values to 40% of the original value (60% decrease) (Fig. 2). The differences between the means of absorbance of the 2-fold and the 4-fold dilutions of IgM RB were statistically significant ($P = 0.012$, Student's t-test). Standard deviations were equal ($P = 0.305$, Levine's test/ F-test) suggesting that the less concentrated IgM RB was also effective at removing non-specific reactions.

We also verified the effectiveness of IgM RB treatment on 10 randomly selected samples (of varying antibody titers) by adding polyclonal rabbit anti-human HRP-conjugated IgG and IgM (on two separate plates with the same layout) to the samples. The plate with IgM

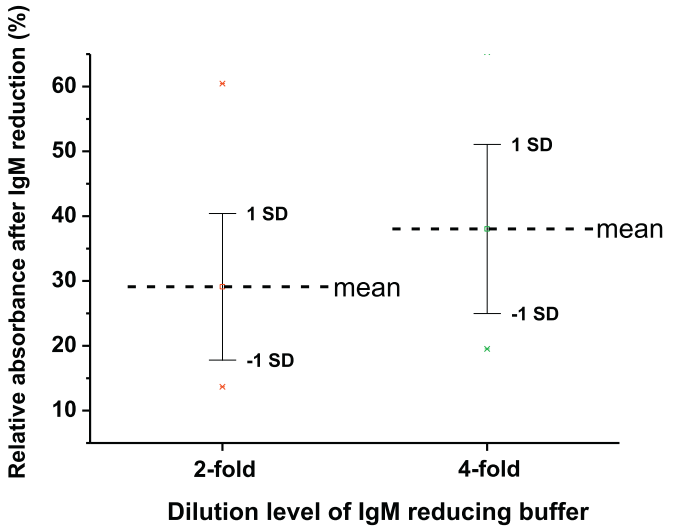


Fig. 2. Effect of IgM reduction expressed as percent decrease in absorbance of samples (N = 25 serum samples).

secondary antibody resulted in close to zero absorbance values. However, when using secondary anti-human IgG, the signal decreased, but it fell well within detectable range (Fig. 3).

3.3. Calibration curve, LOD, LOQ and cut-off determination

4-parameter logistic curve was fitted on the absorbance values given by serial dilution points of the IS 66/202 (Fig. 4). Concentration (as variable x) was expressed from the formula, and this equation was used for determination of analyte concentrations. Cut-off value was set at 0.5 mIU/mL (± 10%) on empirical basis calculated from the mean values and the relative standard deviations of negative samples. As described in Materials and methods, LOD and LOQ values were determined. LOD was 0.298 mIU/mL, while LOQ was 0.473 mIU/mL. A plate-specific representation of the LOD and LOQ values is shown in Fig. 4.

3.4. Optimal dilution of samples

Optimal dilution of samples was 200-fold (0.005 relative concentration), since this yielded an acceptable signal and reproducible difference between positive and negative samples, with minimal use of stock solutions (Fig. 5).

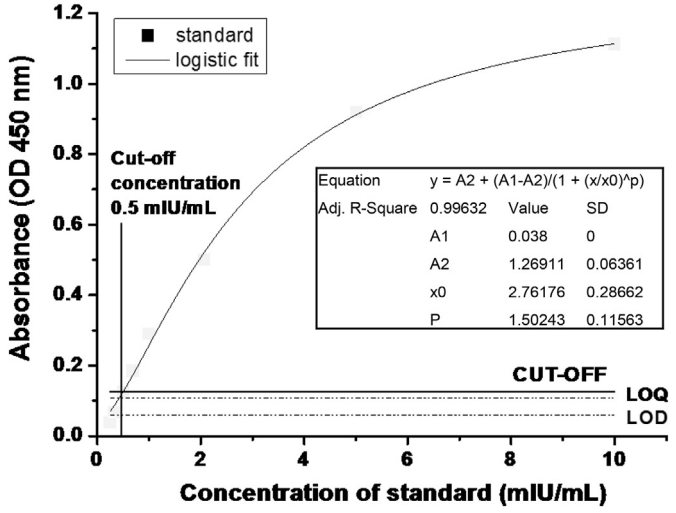


Fig. 4. Calibration curve, fitting algorithm and determination of LOQ (limit of quantification), LOD (limit of detection), CUT-OFF. LOD = mean OD values of blank samples +3 SD, LOQ = mean OD values of blank samples +10 SD, CUT-OFF = mean OD values of low titer samples +3 SD.

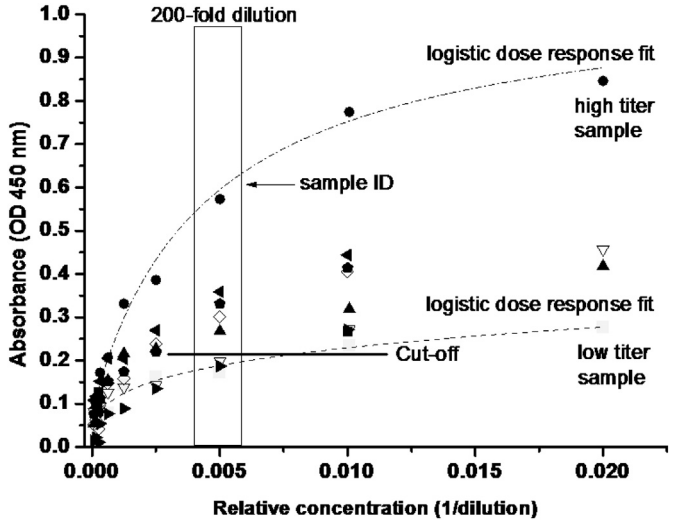


Fig. 5. Determination of optimal dilution of samples. Each symbol denotes a different sample, using consecutive dilutions. CUT-OFF = mean OD values of low titer samples +3 SD. 200-fold dilution was optimal (symbols shown in the box).

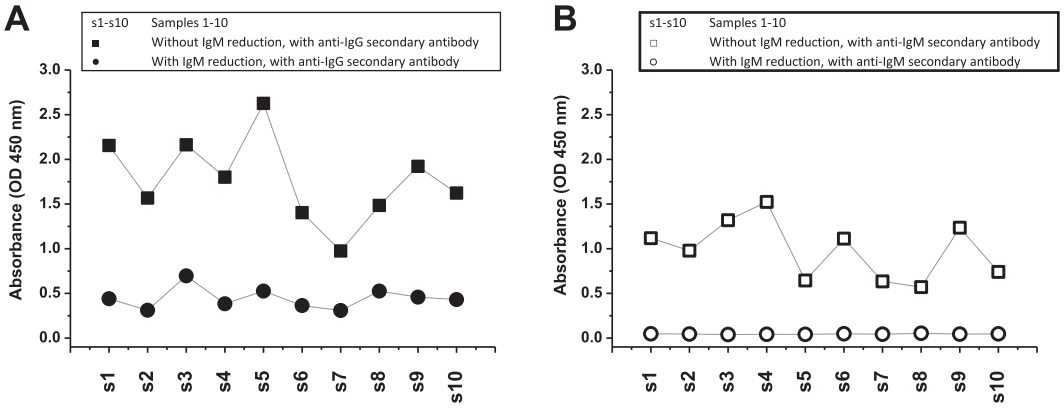


Fig. 3. Effect of IgM reduction on absorbance values of serum samples used for anti-measles IgG detection. (A) Effect of IgM reduction with anti-IgG secondary antibody. (B) Effect of IgM reduction with anti-IgM secondary antibody.

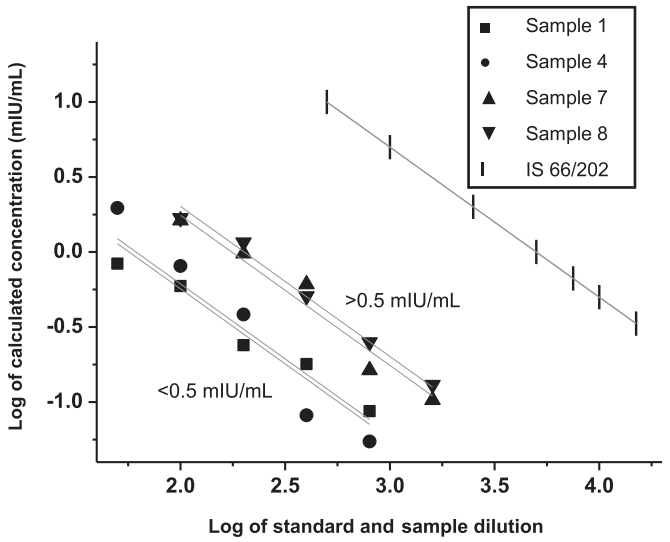


Fig. 6. Linearity and parallelism. Linearized dilutions of high and low titer samples with slope −1.

3.5. Analytical sensitivity

Analytical sensitivity can be defined as the ratio of optical signal change and concentration change (Brian and Eggins, 1996.) In case of a straight calibration curve the slope of the fitted linear function describes the sensitivity of the method (Iupac, 2014). Although the enzymatic assay used in our experiments showed a non-linear response, a linear section was found in the 0.5–2 mIU/mL range, where the sensitivity of the method was 0.162 mIU/mL (not shown).

3.6. Linearity and parallelism

The measured absorbance (OD) of samples plotted versus relative concentration resulted in saturation curves similar to the calibration curve, thus 4-parameter logistic curves were fitted. Dilution curves of two, typical low-titer samples (samples 1 and 4) and two high-titer samples (samples 7 and 8) were linearized by taking the common logarithm of the dilution and the calculated concentration (Fig. 6). Linear fit was performed with slope −1 (determined from previous assays) for each data set. R-square values were close to 1 (0.91–0.99), which suggested that the binding characteristic of the analyte (serum antibodies) to the antigen were co-measurable to the standard. A better linear fit was observed for higher titer samples, because of the better signal-to-noise ratio of the spectrophotometric method in the measured OD range.

3.7. Intra- and inter-plate variation

Intra-plate SD values varied in range 0.01–0.14, and the coefficient of variation was under 13% (Fig. 7). In case of inter-plate variation, the SD values ranged from 0.046 to 0.192 (0.095 mean value), while the coefficient of variation ranged from 4.800% to 18.708% (10.129 mean value).

3.8. Correlation of our indirect ELISA with our anti-measles nucleocapsid-based sandwich ELISA and commercially available IIF slides

Our self-developed indirect ELISA (Reference range; Negative sample < 0.45 mIU/mL, Grey zone/Questionable sample ≥ 0.45 mIU/mL and < 0.55 mIU/mL, Positive sample ≥ 0.55 mIU/mL) was compared to anti-measles nucleocapsid monoclonal antibody-based sandwich ELISA (Reference range; Negative sample < 4.5 mIU/mL, Grey zone/Questionable sample ≥ 4.5 mIU/mL and < 5.5 mIU/mL, Positive sample ≥ 5.5 mIU/mL) and to IIF-based reference assay; i.e. (slides with measles antigen transfected cells). IIF slides were evaluated visually by an independent, experienced investigator. N = 40 sera for each assay type. Correspondence analysis revealed high overlap of data obtained by our indirect ELISA and by both independent verification method. In both comparisons the computed *P*-values were lower than the significance level alpha (0.05), showing that there was a strong link between the compared parameters of the contingency tables (Fig. 8 A, B).

3.9. Population level screening and confidence interval analysis

Based on the measurement of > 2000 sera; the highest ratio of low and questionable antibody level samples was detected in the cluster of '1978–1987' (~25.4%), followed by cluster '1969–1977' (~15.4%) (clusters were defined based on vaccine type and strain, and age at vaccination) (Fig. 9A,B). Confidence interval analysis of ratios of samples with questionable antibody titers revealed that between the initial vaccination period (1969–1988) and the later period (after 1988) significant differences were detected: the relative frequency of the 1978–1987 cluster (95% confidence limit) is disjunctive from other clusters ranging from 1988 to 2010, while between different phases of the modern vaccination era no significant differences were detected (Fig. 9B). We would like to note that for the period 2011–2015 we excluded from the analysis samples from infants who may have lacked maternal anti-measles antibodies, but had not been vaccinated yet.

3.10. Comparison of our assay with commercially available assays

We measured selected samples using different assays (Virotech, Immunolab Siemens Enzygnost, Euroimmun, Orgentec Alegria). Preselected samples (N = 86) were tested, including sera with equivocal or low antibody titers. Preselection was based on our earlier experimental data, which defined the age-groups that contained the highest ratio of samples with questionable/low anti-measles antibody titers. As shown in Suppl. Fig. 1A, the majority of samples (80.23%) showed data that were concordant in our ELISA and five commercially available ELISA tests. Suppl. Fig. 1B depicts discordant results. We found that our assay was comparable to the well-established commercial kits; the number of data points that were standing alone, i.e. discordant with all other assays, was not higher than in the case of other assays.

4. Discussion

Despite the availability of various serological assays for assessment of anti-measles immunity, there is a need for an assay that has superior signal-to-noise ratio, allows high-throughput screening and is cost-effective (Suppl. Fig. 2). Herein we describe the most important steps towards achieving this goal.

4.1. Importance of blocking

In case of ELISA methods blocking plays a key role to prevent non-specific binding antibodies. On high binding polystyrene surfaces using a simple non-ionic detergent, such as Tween-20 or Triton-X, is not enough and a protein blocking step is required. Usually a diluted solution of bovine serum albumin (BSA) or skimmed milk can be applied, but in assays where the antibodies of interest are likely to interact with these agents, use of a synthetic blocker is necessary. BSA cannot be an efficient blocking agent for samples of human sera due to the high background caused by the immunoglobulins present in BSA (Waritani et al., 2017). Polyvinylpyrrolidone (Povidone, PVP), polyethylene glycol (PEG) or polyvinyl alcohol (PVA) are typically recommended synthetic blockers, although it was proven that the latter is preferable. We

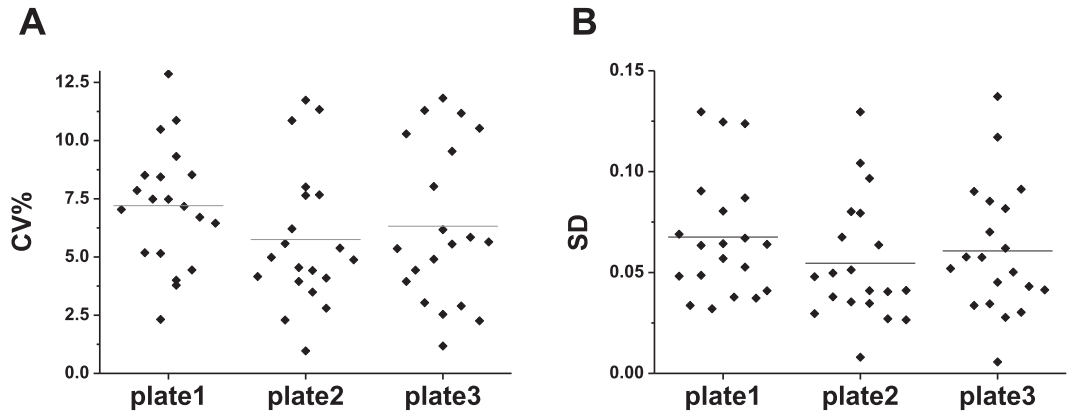
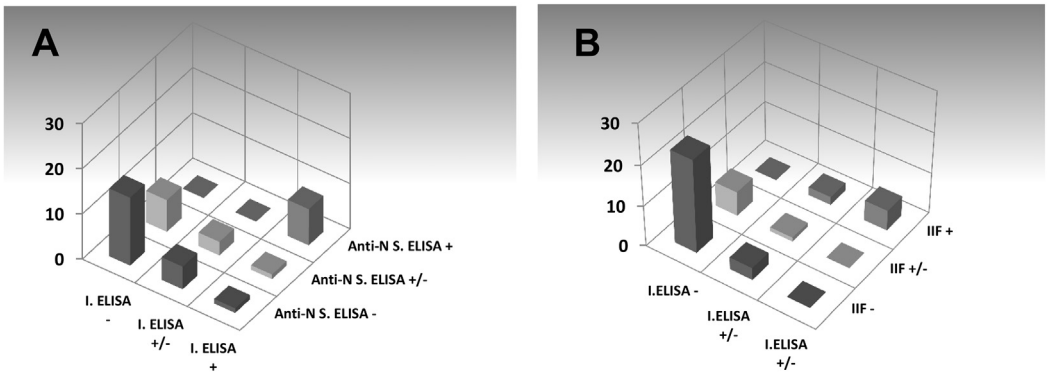


Fig. 7. (A) Intra-assay variability: coefficient of variation. (B) Intra-assay variability: standard deviation. Triplicates of 20 samples were analyzed on each plate.



Chi-square (observed)	30.177
Chi-square (critical)	9.488
DF	4
P-value	< 0.0001
alpha	0.05

Chi-square (observed)	30.798
Chi-square (critical)	9.488
DF	4
P-value	< 0.0001
alpha	0.05

Fig. 8. Correspondence analysis: 3D view of the contingency tables. (A) Indirect ELISA (I. ELISA) compared to anti-nucleocapsid monoclonal antibody based sandwich ELISA (Anti-N S. ELISA). (B) Indirect ELISA (I. ELISA) compared to indirect immunofluorescence (IIF).

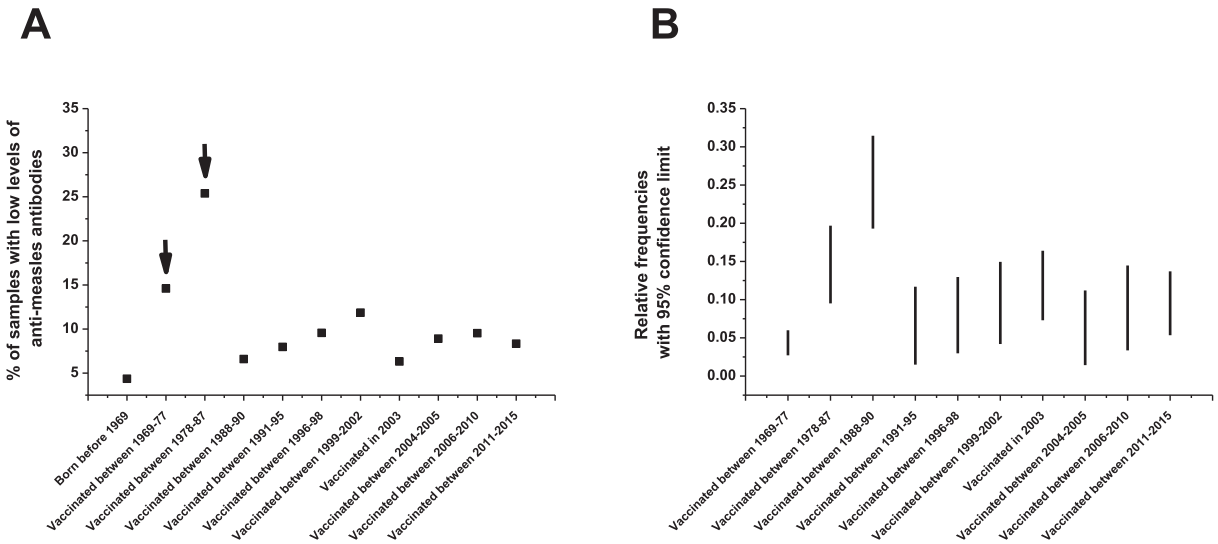


Fig. 9. (A) Cohort-centered distribution of samples with low anti-measles antibody levels. (B) Confidence intervals (N = 1985).

developed our PVA-containing synthetic blocker based on ELISA tests using SynBlock buffer (Bio-Rad) in preliminary experiments. Thompson et al. found that wells blocked with PVA gave comparable results to a commercially available premium synthetic blocker in ELLA (Enzyme-Linked Lectin Assay) experiments (Thompson et al., 2011) Our PVA-based blocker contained 5 g/L PVA dissolved in PBS buffer. PVA of MW 124–186 kDa has been recommended (Rodda and Yamazaki, 1994), although some investigators used PVA-50 (MW 50 kDa) or PEG-360 (MW 360 kDa) (Studentsov et al., 2002). Our synthetic blocker contained PVA of different molecular weight. Conditions of preparation, such as temperature and stirring, may also affect the effectiveness of the blocker as PVA shown poor solubility in water. To prevent degradation of the blocker a preservative was added.

4.2. Elimination of interfering antibodies

Specificity and sensitivity are basic criteria of immunological assay development. Nonspecific binding of proteins, e.g. serum IgM antibodies can decrease specificity because both pathogen specific and natural (low affinity, multi-specific) IgM antibodies are present in human serum. Autoantibodies present in patients with autoimmune disease can potentially interfere with the results Cross reaction caused by rheumatoid factor and heterophilic antibodies can often occur. (Ahmed and Lambert, 2014; Bolstad et al., 2013; Haller-Kikkatalo et al., 2017; Kaplan and Levinson, 1999; Loeffler and Klaver, 2017; Tate and Ward, 2004) To solve these problems, interference reducing methods have been suggested in the literature, such as pre-incubation of sera with animal serum or immunoglobulin (Kragstrup et al., 2013; Sturgeon and Viljoen, 2011). We used IgM Reducing Assay Diluent, a buffer enriched by mammalian proteins and recommended for matrix equalization to eliminate “sticky” or non-specific IgM from assays (Datasheet: BUF038A Description, 2018).

4.3. Confirmatory experiments

Comparison with anti-nucleocapsid monoclonal antibody-based sandwich ELISA.

In confirmatory experiments we used anti-nucleocapsid monoclonal antibody-based sandwich ELISA, although haemagglutinin (H) and fusion (F) protein could be used for correlate studies, their measurement is difficult and uncommon in high-throughput screening (Moss and Johns Hopkins, 2009) (Brinckmann et al., 1991; Cohen et al., 2006; Sheshberadaran et al., 1985). The most abundant antibodies are formed against the viral nucleoprotein (N), and their absence is the most accurate indicator of the lack of antibodies to measles virus, explaining why these antibodies are most frequent antigen targets used in commercial assays. Therefore, we compared our self-developed whole virus antigen repertoire-based indirect ELISA with the anti-measles nucleocapsid monoclonal antibody-based sandwich ELISA with (Fig. 8A). Since for plate coating we used the entire gamma-irradiated measles virus of the *Edmonston* strain, we wanted to examine how it is related to the specific anti-nucleocapsid antibody measurement. For comparison we selected samples that contained low measles antibody levels (Thompson et al., 2011), when measured by our assay and by commercial kits (we also included positive samples in our measurements). Qualitative results (judged as positive or negative) were obtained from indirect ELISA and anti-N sandwich ELISA tests using (IS 66/202). The comparison between the two ELISA tests showed high correlation (Fig. 8A).

We used IIF as a reference method (de Ory et al., 2015) to confirm our indirect ELISA results (Bayer and Hübl, 2001; Tonutti et al., 2004; Waner et al., 2000). For independent evaluation of IIF slides, we asked the help of an expert from the National Public Health Institute, Budapest, Hungary. Despite difficulties of paralleling two such techniques the correspondence proved to be good, thus supporting the comparability of our assay to an independent method (Fig. 8B).

4.4. Waning of immunity

Fig. 9 shows the percentages of samples with low levels of anti-measles antibodies depending on the time period of vaccination. The samples were grouped according to the years of vaccination and changes introduced in the Hungarian vaccination schedule. Two groups showed the highest percentages where little or no protection was observed (clusters ‘Vaccinated between 1969- 77’ and ‘Vaccinated between 1978- 87’; marked with arrows). Waning or lack of anti-measles protection in these groups might be due to multiple factors (Guerra et al., 2018;Kang et al., 2017;Kontio et al., 2012) including primary vaccine failure, poor vaccine handling, or suboptimal vaccination age.

5. Conclusion

Our findings are in agreement with data from literature: we found low antibody levels in age-groups that included individuals who were immunized during the initial vaccination periods when the age at vaccination, the composition and/or handling of vaccines were poorly defined (Fig. 9) (Contemporary data can be found online at https://www.cdc.gov/mmwr/preview/mmwrhtml/00001472.htm). The group that had the highest level of anti-measles antibodies included persons born before 1969 that were likely exposed wild-type virus and thus acquired life-long protection.

In summary, we have developed a new ELISA assay for assessment of immunological protection status against measles infection. The assay is cost-effective, allows high-throughput screening and has superior signal-to-noise ratio. We believe that our new protocol may be applicable in the population-level surveillance of immunity against measles.

Potential conflict of interest

None declared.

Acknowledgements

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We thank Greiner Bio-One for their generous gift of ELISA plates. The present scientific contribution is dedicated to the 650th anniversary of the foundation of the University of Pécs, Hungary.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.07.009.

References

Ahmed, S.S., Lambert, P.-H., 2014. Autoimmune diseases. In: The Autoimmune Diseases. Elsevier, pp. 275–282. https://doi.org/10.1016/B978-0-12-384929-8.00020-4.

Bayer, B., Fabian, W., Hübl, P.M., 2001. Immunofluorescence assays (IFA) and enzyme-linked immunosorbent assays (ELISA) in autoimmune disease diagnostics - technique, benefits, limitations and applications. Scand. J. Clin. Lab. Invest. 61, 68–76. https://doi.org/10.1080/003655101753352077.

Bolstad, N., Warren, D.J., Nustad, K., 2013. Heterophilic antibody interference in immunometric assays. Best Pract. Res. Clin. Endocrinol. Metab. 27, 647–661. https://doi.org/10.1016/J.BEEM.2013.05.011.

Brian, R., Eggins, 1996. Chapter 7. Performance Factors. In: Biosensors: An Introduction. Wiley and Sons, pp. 133–139. https://doi.org/10.1002/9780470511305.fmatter. (ISBN 0-471-96285-6. John Wiley & Sons, Ltd., pp. i–xxi).

Brinckmann, U.G., Bankamp, B., Reich, A., ter Meulen, V., Liebert, U.G., 1991. Efficacy of individual measles virus structural proteins in the protection of rats from measles encephalitis. J. Gen. Virol. 72, 2491–2500. https://doi.org/10.1099/0022-1317-72-10-2491.

Cohen, B.J., Parry, R.P., Doblas, D., Samuel, D., Warrener, L., Andrews, N., Brown, D., 2006. Measles immunity testing: comparison of two measles IgG ELISAs with plaque reduction neutralisation assay. J. Virol. Methods 131, 209–212. https://doi.org/10.1016/J.JVIROMET.2005.08.001.

Datasheet: BUF038A Description, 2018. ELISA IgM REDUCING ASSAY DILUENT Datasheet BUF038A Description [WWW Document].

de Ory, F., Minguito, T., Balfagón, P., Sanz, J.C., 2015. Comparison of chemiluminescent immunoassay and ELISA for measles IgG and IgM. APMIS 123, 648–651. https://doi.org/10.1111/apm.12413.

Grammens, T., Schirvel, C., Leenen, S., Shodu, N., Hutse, V., Mendes Da Costa, E., Sabbe, M., 2017. Ongoing measles outbreak in Wallonia, Belgium, december 2016 to march 2017: characteristics and challenges. Euro Surveill. 22. https://doi.org/10.2807/1560-7917.ES.2017.22.17.30524.

Guerra, F.M., Crowcroft, N.S., Friedman, L., Deeks, S.L., Halperin, S.A., Severini, A., Hatchette, T.F., Bolotin, S., Immunity of Canadians and Risk of Epidemics (iCARE) Network, 2018. Waning of measles maternal antibody in infants in measles elimination settings – a systematic literature review. Vaccine 36, 1248–1255. https://doi.org/10.1016/j.vaccine.2018.01.002.

Haller-Kikkatalo, K., Alnek, K., Metspalu, A., Mihailov, E., Metsküla, K., Kisand, K., Pisarev, H., Salumets, A., Uibo, R., 2017. Demographic associations for auto-antibodies in disease-free individuals of a European population. Sci. Rep. 7, 44846. https://doi.org/10.1038/srep44846.

Haralambieva, I.H., Kennedy, R.B., Ovsyannikova, I.G., Whitaker, J.A., Poland, G.A., 2015. Variability in humoral immunity to measles vaccine: new developments. Trends Mol. Med. 21, 789–801. https://doi.org/10.1016/j.molmed.2015.10.005.

Iupac, 2014. Compendium of Chemical Terminology Gold Book.

Kang, H.J., Han, Y.W., Kim, S.J., Kim, Y.J., Kim, A.R., Kim, J.A., Jung, H.D., Eom, H.E., Park, O., Kim, S.S., 2017. An increasing, potentially measles-susceptible population over time after vaccination in Korea. Vaccine 35, 4126–4132. https://doi.org/10.1016/j.vaccine.2017.06.058.

Kaplan, I.V., Levinson, S.S., 1999. When is a heterophile antibody not a heterophile antibody? When it is an antibody against a specific immunogen. Clin. Chem. 45, 616–618.

Kontio, M., Jokinen, S., Paunio, M., Peltola, H., Davidkin, I., 2012. Waning antibody levels and avidity: implications for MMR vaccine-induced protection. J. Infect. Dis. 206, 1542–1548. https://doi.org/10.1093/infdis/jis568.

Kragstrup, T.W., Vorup-Jensen, T., Deleuran, B., Hvid, M., 2013. A simple set of validation steps identifies and removes false results in a sandwich enzyme-linked immunosorbent assay caused by anti-animal IgG antibodies in plasma from arthritis patients. Springerplus 2, 263. https://doi.org/10.1186/2193-1801-2-263.

Loeffler, D.A., Klaver, A.C., 2017. Polyvalent immunoglobulin binding is an obstacle to accurate measurement of specific antibodies with ELISA despite inclusion of blocking agents. Int. Immunopharmacol. 52, 227–229. https://doi.org/10.1016/J.INTIMP.2017.09.016.

MacDougall, D., Crummett, W.B., et al., 1980. Guidelines for data acquisition and data quality evaluation in environmental chemistry. Anal. Chem. 52, 2242–2249. https://doi.org/10.1021/ac50064a004.

Moss, W.J., 2017. Measles. Lancet 390, 2490–2502. https://doi.org/10.1016/S0140-6736(17)31463-0.

Moss, William J., Johns Hopkins, S.S., 2009. The Immunological Basis for Immunization Series -Module 7: Measles Update 2009. WHO.

Report, E.C.D.C., 2018. Monthly Measles and Rubella Monitoring Report March 2018.

Ridge, S.E., Vizard, A.L., 1993. Determination of the optimal cutoff value for a serological assay: an example using the Johne's absorbed EIA. J. Clin. Microbiol. 31, 1256–1261.

Rodda, D.J., Yamazaki, H., 1994. Poly(vinyl alcohol) as a blocking agent in enzyme immunoassays. Immunol. Investig. 23, 421–428. https://doi.org/10.3109/08820139409066836.

Sheshberadaran, H., Norrby, E., Rammohan, K.W., 1985. Monoclonal antibodies against five structural components of measles virus. Arch. Virol. 83, 251–268. https://doi.org/10.1007/BF01309921.

Studentsov, Y.Y., Schiffman, M., Strickler, H.D., Ho, G.Y.F., Pang, Y.S., Schiller, J., Herrero, R., Burk, R.D., 2002. Enhanced enzyme-linked immunosorbent assay for detection of antibodies to virus-like particles of human papillomavirus. J. Clin. Microbiol. 40, 1755–1760. https://doi.org/10.1128/JCM.40.5.1755.

Sturgeon, C.M., Viljoen, A., 2011. Analytical error and interference in immunoassay: minimizing risk. Ann. Clin. Biochem. 48, 418–432. https://doi.org/10.1258/acb.2011.011073.

Tate, J., Ward, G., 2004. Interferences in immunoassay. Clin. Biochem. Rev. 25, 105–120.

Thompson, R., Creavin, A., O'Connell, M., O'Connor, B., Clarke, P., 2011. Optimization of the enzyme-linked lectin assay for enhanced glycoprotein and glycoconjugate analysis. Anal. Biochem. 413, 114–122. https://doi.org/10.1016/j.ab.2011.02.013.

Tischer, A., Andrews, N., Kafatos, G., Nardone, A., Berbers, G., Davidkin, I., Aboudy, Y., Backhouse, J., Barbara, C., Bartha, K., Bruckova, B., Duks, A., Griskevicius, A., Hesketh, L., Johansen, K., Jones, L., Kuersteiner, O., Lupulescu, E., Mihneva, Z., Mrazova, M., De Ory, F., Prosenc, K., Schneider, F., Tsakris, A., Smelhausova, M., Vranckx, R., Zarvou, M., Miller, E., 2007. Standardization of measles, mumps and rubella assays to enable comparisons of seroprevalence data across 21 European countries and Australia. Epidemiol. Infect. 135, 787–797. https://doi.org/10.1017/S0950268807008266.

Tonutti, E., Bassetti, D., Piazza, A., Visentini, D., Poletto, M., Bassetto, F., Caciagli, P., Villalta, D., Tozzoli, R., Bizzaro, N., 2004. Diagnostic accuracy of Elisa methods as an alternative screening test to indirect immunofluorescence for the detection of anti-nuclear antibodies. Eval. Five Commer. Kits. Autoimmun. 37, 171–176. https://doi.org/10.1080/08916930310001657010.

Waner, T., Strenger, C., Keysary, A., 2000. Comparison of a clinic-based ELISA test kit with the immunofluorescence test for the assay of Ehrlichia canis antibodies in dogs. J. Vet. Diagn. Investig. 12, 240–244.

Waritani, T., Chang, J., McKinney, B., Terato, K., 2017. An ELISA protocol to improve the accuracy and reliability of serological antibody assays. MethodsX 4, 153–165. https://doi.org/10.1016/j.mex.2017.03.002.

Zachariah, P., Stockwell, M.S., 2016. Measles vaccine: past, present, and future. J. Clin. Pharmacol. 56, 133–140. https://doi.org/10.1002/jcph.606.

Original article

Imported Infections Versus Herd Immunity Gaps; A Didactic Demonstration of Compartment Models Through the Example of a Minor Measles Outbreak in Hungary

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Abstract

Introduction: In Hungary, where MMR vaccine coverage is 99%, in 2017, a minor measles epidemic started from imported cases due to two major factors – latent susceptible cohorts among the domestic population and the vicinity of measles-endemic countries. Suspended immunization activities due to the COVID-19 surge are an ominous precursor to a measles resurgence. This epidemiological demonstration is aimed at promoting a better public understanding of epidemiological data.

Materials and Methods: Our previous MMR sero-epidemiological measurements (N of total measles cases = 3919, N of mumps cases = 2132, and N of rubella cases = 2132) were analyzed using open-source epidemiological data (ANTSZ) of a small-scale measles epidemic outbreak (2017, Hungary). A simplified SEIR model was applied in the analysis.

Results: In case of measles, due to a cluster-specific inadequacy of IgG levels, the cumulative seropositivity ratios (measles = 89.97%) failed to reach the herd immunity threshold (HIT Measles = 92–95%). Despite the fact that 90% of overall vaccination coverage is just slightly below the HIT, unprotected individuals may pose an elevated epidemiological risk. According to the SEIR model, ≥74% of susceptible individuals are expected to get infected. Estimations based on the input data of a local epidemic may suggest an even lower effective coverage rate (80%) in certain clusters of the population.

Conclusion: Serological survey-based, historical and model-computed results are in agreement. A practical demonstration of epidemiological events of the past and present may promote a higher awareness of infectious diseases. Because of the high R0 value of measles, continuous large-scale monitoring of humoral immunity levels is important.

(Böröcz K, Markovics Á, Csizmadia Z, Najbauer J, Berki T, Németh P. Imported Infections Versus Herd Immunity Gaps; A Didactic Demonstration of Compartment Models Through the Example of a Minor Measles Outbreak in Hungary. SEEMEDJ 2021; 5(1); 1-16)

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Introduction

Testing of acquired immunity and effectiveness of vaccination against infectious diseases has been increasingly important in the design of preventive public health strategies. Resurgence in measles cases in the United States and across Europe has occurred, including in individuals vaccinated with two doses of the vaccine (1). According to the Centers for Disease Control and Prevention (CDC), World Health Organization (WHO) and United Nations International Children's Emergency Fund (UNICEF), measles has already been a global issue and now it has been aggravated by disrupted immunization protocols due to the COVID-19 pandemic (2–5). All six WHO regions have reported disrupted immunization activities, with major adverse effects on routine immunization and mass vaccination campaigns (4). According to CDC reports, in 2020, more than 117 million children were at the risk of missing out on measles vaccines as a consequence of the COVID-19 surge (2). Measles immunity gaps resulting from suspended immunization activities are an ominous precursor to a measles resurgence (4). In Ukraine, one of Hungary's neighboring countries that was already endemic for measles, vaccination has been interrupted in many regions (3). Regarding Europe, ECDC surveillance data have indicated an exceptionally high number of measles cases in 2018, 2019 and 2020 in EU/EEA countries.

Vaccination remains one of the safest and most effective interventions available in public health for the primary prevention of infectious diseases, resulting in both direct and indirect immunity in individuals vaccinated (herd immunity) (6–8). Even though a safe and effective two-dose measles/MMR vaccination schedule has been available in Europe since the 1960s, maintaining high vaccine coverage is still difficult, despite the fact that in Hungary, the MMR vaccine is mandatory and consequently the vaccine coverage is estimated to be at 98-99%. According to our previous publications (9,10) and in agreement with the results obtained by our colleagues (11), there are latent immunization gaps in certain age (or immunization) clusters of the Hungarian population, with predominance of the ~35-45-year-old adults. These are individuals who form a significant portion of the active labor force of the country, for instance health care workers (HCWs).

Between January 2017 and May 2019, there were 76 reported measles cases in Hungary (12), 54 of which were reported between 21 February and 22 March 2017 (13). Because of the recent outbreaks worldwide, not only of measles, but also mumps and rubella (MMR) infections, and because of waning of immunity over time after vaccination (14–17), the importance of continuous MMR seroepidemiological screening is evident.

Suboptimal vaccine effectiveness in certain clusters of the population has a negative impact on overall vaccination coverage. Small-scale outbreaks suggest that certain measles vaccines – applied during the early phases of the Hungarian vaccination history – failed to elicit the desired immunological response. The resulting immunization gap(s) raise the concern of potential further outbreaks (9,11). The 2020 COVID-19 outbreak called attention to the importance of mathematical modelling of epidemics (18). Based on a reliable model, the timescale and economic impact of the disease can be predicted and preventive countermeasures can be taken (19). Through the example of the measles epidemic in Makó (2017, southeast Hungary), we demonstrated that, in possession of key epidemiological data (e.g. R0 value, estimated vaccination coverage of a given population, number of infected and recovered individuals and duration of the epidemic), a simple open-source mathematical model can give a good approximation of the course of an infection and may provide better general compliance with protective measures.

Materials and Methods

Experimental work

In this seroepidemiological survey, we combined the data from our previous findings with recent measurements, including anti-

measles, -mumps and -rubella antibody level (IgG) determination. Measurements were performed on the automated Siemens BEP 2000 Advance® platform (Siemens AG, Germany), using our self-developed ELISA assays validated by well-established commercial kits, as previously described (9,10). Indirect immunofluorescent microscopy was used a reference (Euroimmun, Germany).

In case of large-scale seroepidemiological measurements, a serum bank consisting of anonymous patient sera was used (N of total measles cases = 3919, N of mumps cases = 2132, and N of rubella cases = 2132) (Ethical License number 2015/5726). Nationally representative samples included randomly selected clinical residual samples, with the exclusion criteria of neonates, children under the vaccination age and severely immunocompromised patients. Samples were collected from the Department of Laboratory Medicine (University of Pécs, Clinical Centre). Serum samples were from all listed age groups participating in this study and they were categorized based on past changes introduced in measles and MMR immunization schedules. Age group determination was based on the landmarks in the history of measles and MMR vaccination schedules in Hungary (Figure 1). Human sera were stored in the accredited laboratory of the Department of Immunology and Biotechnology (University of Pécs, Medical School, Pécs, Hungary) according to quality assurance criteria (ISO 17025).

Population-level result evaluation and seropositivity ratio assessment was performed in relation to the concept of herd immunity threshold (HIT) values (HIT Measles = 92–95%, HIT Mumps = 85–90%, HIT Rubella = 83–86). The study relies on the full virus antigen repertoire-based indirect ELISA method. Therefore, it must be considered a good surrogate, rather than an absolute correlate marker for immunity – as far as Plotkin's nomenclature is considered normative (20–22). We examined vaccination group-related infection- and vaccine-induced antibody titres using the following software: SPSS, Origin Pro, Excel.

SEIR model example and input data

A small-scale measles outbreak in Hungary in 2017 raised questions about the vaccination coverage rate in the country. Experimental results supported the theory of ineffective vaccines, as previously mentioned (9). In spite of its limitations, it seemed reasonable to set up a SEIR model calculation in order to see whether a few percent decrease in effective vaccination could result in a local epidemic. To demonstrate the disease spread in a well-immunized population where latent immunity gaps may be present, input data were based on the data of the 2017 measles outbreak in Makó, southeast Hungary. The following parameters were used to perform the calculations:

Population (N): The epidemic was linked to the small-town hospital. During that year, 65 physicians were responsible for medical attendance of the estimated 30,000 inhabitants of Makó and the surrounding villages. In our model, a population of N = 400-1,000 people was assumed, including patients, health-care workers and family members.

Number of infected individuals (I): A total of 29 cases were reported.

Incubation time and contagious period: The incubation time for measles ranges from 10 to 12 days on average, an infected person can be contagious even 1-2 days before the first characteristic symptoms are visible, up to 4 days after the rashes appear. In our model, the incubation time (Tinc) was assumed to be 10 days, whereas the contagious period (Tcont) was 6 days. Based on these values, and parameters were determined by equations (5) and (6).

Reproduction rate ranging from 12 to 18 can be found in the literature and both values were tested. The higher value is applicable to communities where no social distancing is present and the ratio of vaccinated or immunized inhabitants is low. In Central Europe, the use of the lower value seems more rational, although this specific epidemic was kept mainly in a hospital, where circumstances promote the spread of the infection. In this case, the start of

the outbreak was defined as the possible first day of the first patient's infection, while the model was set to stop after the recovery of the last infected person. When it comes to large-scale epidemics, a different approach is used. If no new cases are found after a certain period, the outbreak is over. This time period is usually determined by the incubation time, with a calculation method suggested by the WHO.

Based on the ELISA antibody measurements, it can be assumed that only ~90% of the Hungarian population has effective immunization, which is under the theoretical 92-94% of HIT. In the model, 90% of seroprevalence was assumed, but lower values were also tested subsequently. No additional vaccinated (V) compartment was created and immunized individuals were treated as recovered. Vital dynamics was disregarded due to the short period of the epidemic. Death rate was not taken into consideration either, as no fatalities were observed during the Hungarian outbreak. Calculations were performed using Microsoft Excel Visual Basic Application (VBA), but the graphs were plotted in Origin. VBA is a built-in feature of the Microsoft Office Suite with several limitations, but its prevalence and the user-friendly computer language makes it suitable for educational purposes.

Results

Changes and historical data regarding epidemics in the Hungarian measles/MMR vaccination schedule (23–25) have been plotted on a timeline in order to evaluate seroepidemiological data accordingly. Figure 1 shows changes in measles and MMR vaccination schedules in Hungary since the introduction of the vaccine (1969). High age-specific attack rates characterizing major epidemics (1980-81 and 1988-89) along with 93%-99% of vaccine coverage evidence insufficiencies of the early vaccination program.

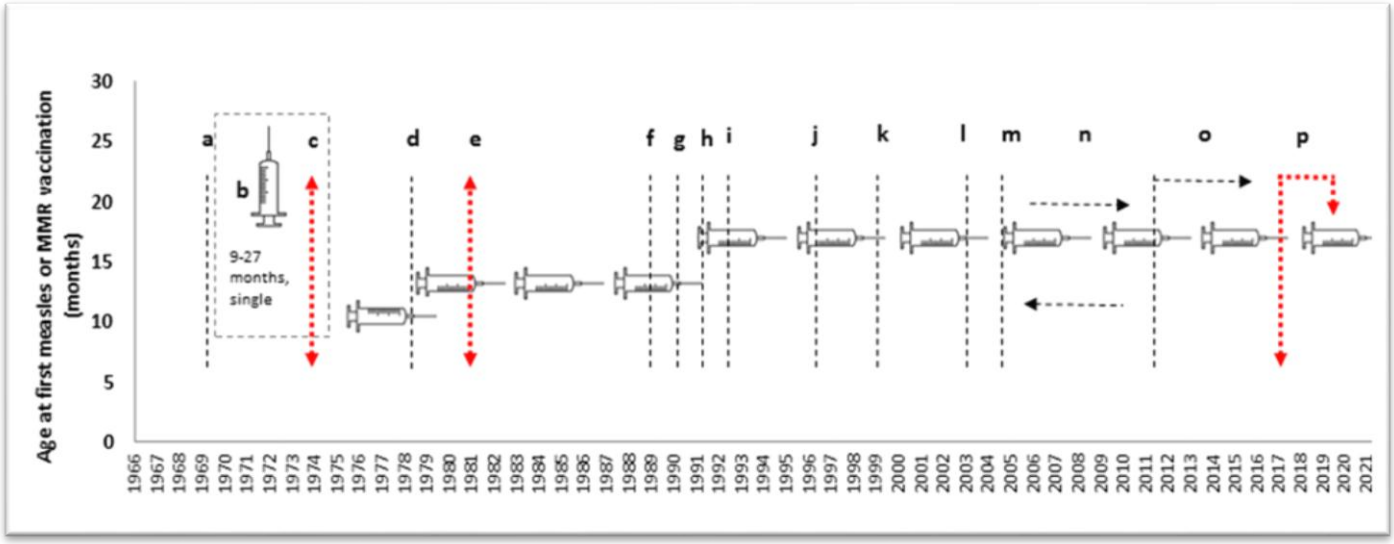
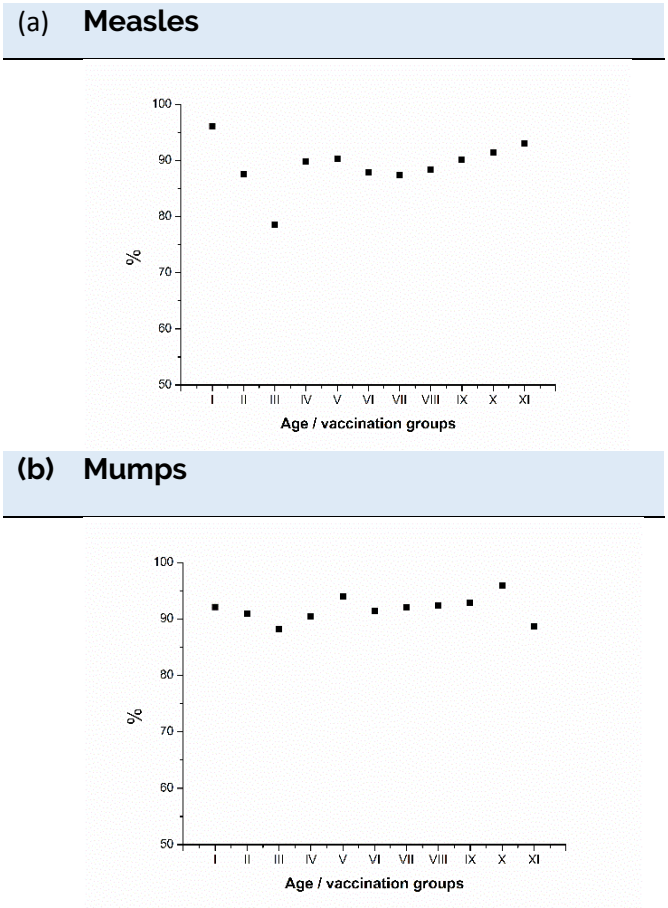


Figure 1. Measles and MMR vaccination schedules in Hungary

(a) Vaccination against measles was introduced in Hungary in 1969. (b) From 1969 to 1974, a single dose of measles vaccine was administered in mass campaigns to persons aged 9-27 months. (c) After vaccination was implemented, the incidence rate decreased until 1973-74, when large epidemics occurred primarily in unvaccinated 6-9-year-olds. (d) The recommended age for vaccination was 10 months until 1978, when it was changed to 14 months. (e) After the 1980-81 epidemic, persons born between 1973 and 1977, who received vaccine when the recommended age was 10 months, were revaccinated. (f) The 1988-89 epidemic mainly affected persons aged 17-21, who had been targeted to receive vaccine during mass campaigns in the first years of the vaccination program in Hungary. After 1989, children were re-vaccinated at the age of 11 with a monovalent measles vaccine in a scheduled manner. Also, in 1989, the rubella vaccine was introduced. (g) In 1990, measles-rubella bivalent vaccines were introduced. (h) The administration of the first vaccine at the age of 14 months lasted from 1978 to 1991. Also, in 1991, the measles-mumps-rubella trivalent vaccine was introduced. (i) In 1992, the administration of the first MMR vaccine was shifted to 15 months of age. (j) In 1996, the MERCK MMR II vaccine (Enders' Edmonston

strain, live attenuated) was introduced. (k) In 1999, measles-mumps-rubella revaccination replaced the monovalent measles vaccine. Also, in 1999, the GSK PLUSERIX vaccine (Measles Schwarz Strain) was introduced. (l) In 2003, the GSK PRIORIX vaccine was introduced. (m) Between 2004 and 2005, the MERCK MMR II vaccine was used. (n) Between 2006 and 2010, the GSK PRIORIX vaccine was in use. (o) Starting from 2011, we have been using a Sanofi-MSD product, MMRvaxPro (Measles virus Enders' Edmonston strain, live, attenuated), for vaccination and revaccination of children. GSK PRIORIX is still on the market, commonly used for vaccination in adulthood. (p) Between January 2017 and December 2019, there were 76 reported measles cases in Hungary (according to ECDC Surveillance reports). (Source of information: MMWR Weekly October 06, 1989 / 38(39); 665-668, International Notes Measles – Hungary, http://www.vacsatc.hu, https://www.ecdc.europa.eu)

Figure 2 shows the age or vaccination group-specific seropositivity and seronegativity ratios for measles, mumps and rubella. The lowest seropositivity ratios in terms of anti-measles antibody titres (IgG) were observed in the groups 'Vaccinated between 1969-1977' (87.56%) and 'Vaccinated between 1978-1987' (78.48%). These results are further confirmed by the abovementioned vaccine insufficiencies of the relative periods, described in Figure 1. Regarding the mumps and rubella seroepidemiological survey, in terms of humoral antibody levels, all vaccination groups satisfied the requirements necessary for the achievement of herd immunity.



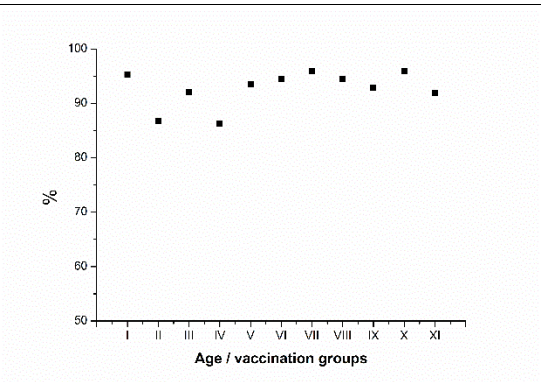


Figure 2. Measles, mumps and rubella seropositivity ratios according to vaccination groups

Age / vaccination groups: (I) Individuals born before 1969. (II) Individuals vaccinated between 1969 and 1977. (III) Individuals vaccinated between 1978 and 1987. (IV) Individuals vaccinated between 1988 and 1990. (V) Individuals vaccinated between 1991 and 1995. (VI) Individuals vaccinated between 1996 and 1998. (VII) Individuals vaccinated between 1999 and 2002. (VIII) Individuals vaccinated in 2003. (IX) Individuals vaccinated between 2004 and 2005. (X) Individuals vaccinated between 2006 and 2010 (XI) Individuals vaccinated after 2011. The lowest seropositivity ratio (78.48%) was observed in the anti-measles antibody titres (IgG) in the group 'Vaccinated between 1978 and 1987'.

In case of measles, mumps and rubella cumulative results, the seropositivity ratios were 89.97%, 91.60% and 92.58%, respectively, as shown in Figure 3. Due to previously detailed

cluster-specific inadequacy of humoral antibody levels, the cumulative anti-measles seropositivity ratios also failed to reach the herd immunity threshold (HIT Measles = 92–95%).

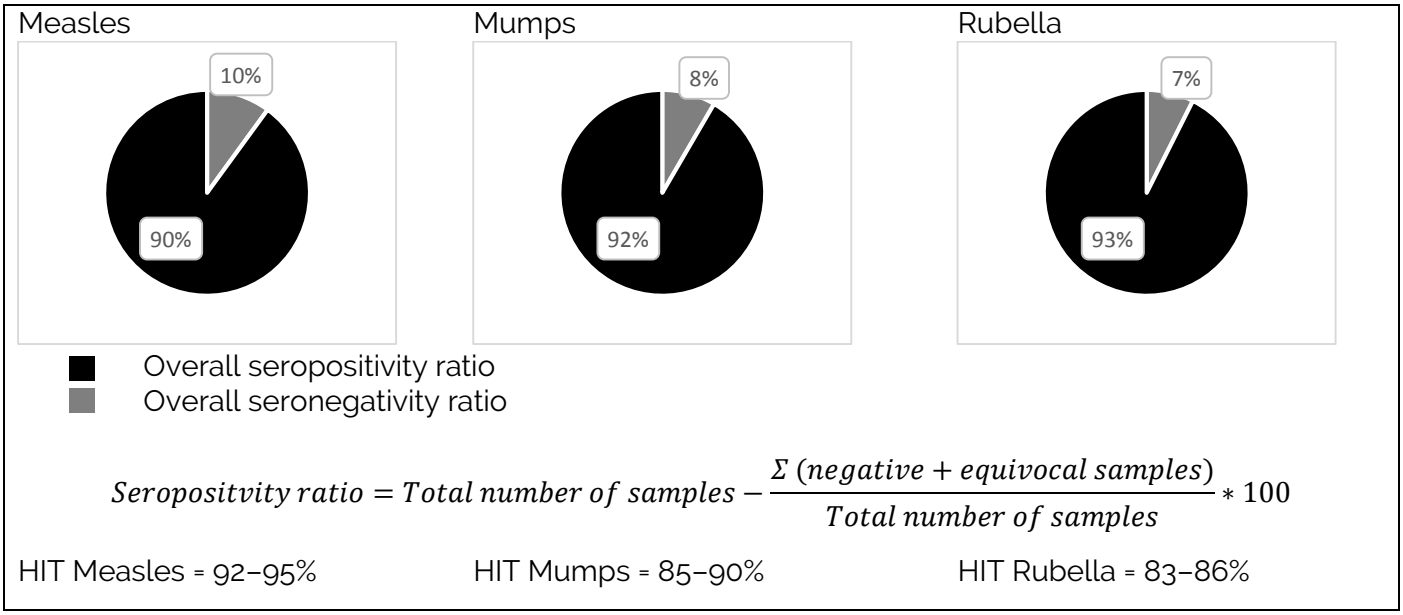


Figure 3. Overall seropositivity and seronegativity ratios

N measles = 3,919; N mumps, rubella = 2,132. In case of measles, mumps and rubella cumulative results, the seropositivity ratios were 89.97%, 91.60% and 92.58%, respectively. The overall ratio of seropositive samples was the lowest in the 'measles' group, where it remained under the threshold value. Seropositivity ratios were calculated as follows:

Using the seronegativity ratio of 89.97% (≈ 90%) obtained by the cumulative data representation of anti-measles (IgG) antibody levels, the model of possible outcomes of a measles outbreak in a hospital as a function of the vaccination coverage rate was investigated. The results of the VBA-based SEIR model of the 2017 epidemic in Hungary are summarized in Table 1. Three parameters – population of the sample, ratio of immunized individuals and reproduction rate of the virus – were set to different values. The effect of these adjustments was investigated and changes in the number of measles cases and timescale of the epidemic were observed.

Table 1. SEIR model results for the 2017 measles epidemic in Makó, Hungary

Population of the sample (N)	Ratio of immunised (%)	Total number of measles cases	Duration of epidemic
		$R_0 = 18$	
1000	90	73	6 months
400	90	29	4 months
400	80	78	3 months
150	80	29	2.5 months
		$R_0 = 12$	
1000	90	2	6 days
400	90	2	6 days
400	80	70	4 months
150	80	26	3 months
Empirical values			
?	90	29	2 months

At R_0=18 and N = 1000, assuming 90% effective vaccination, 100 susceptible individuals can be found in the population. The model estimates a total number of infected persons at 74 and the duration of the epidemic at half a year, which is more than double of the real values. By setting the population at N = 400, 30 infected individuals and 4 months were given by the model. This way the number of infected persons corresponds to the actual clinical data, but the duration is still longer compared to empirical findings.

Timescale of the epidemic can be compressed by increasing the proportion of susceptible people. If the vaccination coverage rate is changed from 90% to 80%, the duration of the epidemic is reduced to 3 months, but the total number of infected individuals becomes higher. Based on this anomaly, it can be presumed that the total number of involved population might be even lower than 400. Unfortunately, the results of the contact tracing procedure were not available for a better approximation.

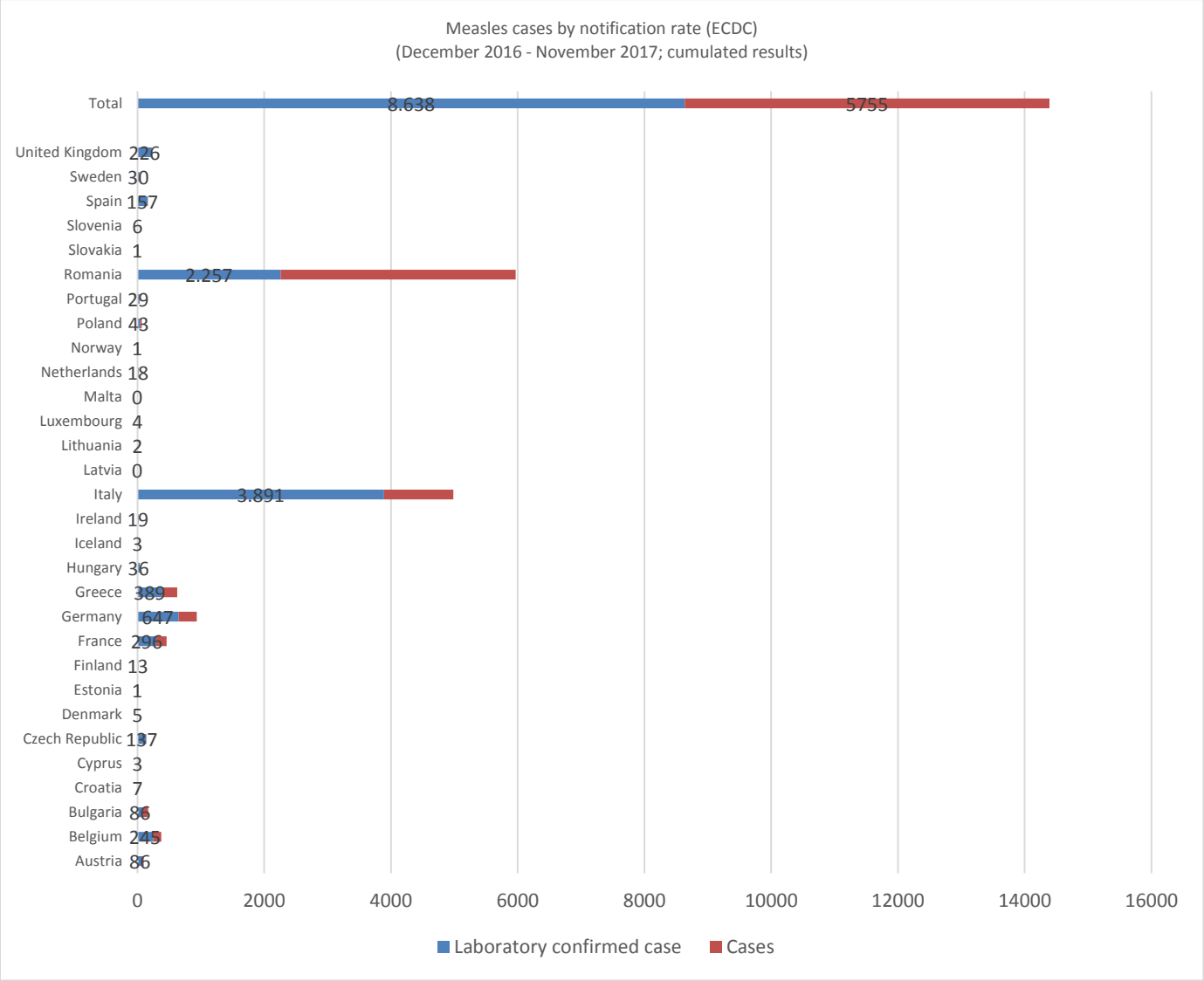
An acceptable correspondence between the model calculations and the clinical data was observed by assuming N = 150 and 80% of vaccination coverage as input parameters.

The results – 30 infections in a two-month period – are close to the official values. For a better comparison, modelling with R_0=12 was also performed. The less contagious the virus, the fewer cases are found. Using this lower reproduction rate, only isolated cases can occur at 90% of vaccination coverage (which is a value that resembles the HIT). By decreasing the vaccination rate, the number of cases increases and the timescale is shortened, similarly to previous test examples.

Discussion

MMR vaccination in Hungary

In Hungary, MMR vaccine is mandatory. A single-dose, live-virus combined measles-mumps-rubella (MMR) vaccine is used to vaccinate infants of ≥15 months of age. A reminder vaccine is given to sixth year primary school students (~11 years of age). PRIORIX (GSK), PRIORIX-TETRA (GSK), ProQuad (MERCK) and the M-M-RVAXPRO (MSD Pharma) vaccines are currently used in Hungary for vaccination of children (at 15 months and 11 years of age) and for adults (62). The vaccines contain live attenuated viruses (26). Regarding insufficient cumulative anti-measles seropositivity levels, we would like to emphasize that potential gaps in the population-level humoral immunity (IgG) are attributable to early vaccination periods and are not a general phenomenon relative to the current immunization practices. The susceptibility of certain cohorts is likely attributable to the thermal instability of the historical Leningrad-16 vaccine, inefficient seroconversion owing to vaccination at a premature age (e.g. 9 months of age) and the questionable efficiency of the inoculum itself (9, 11, 25, 29, 30, 31). The 2017 measles outbreak in Makó was provoked by imported cases. Some of our bordering countries are still endemic for measles (27–30). Supplementary Figure 1 shows the European measles cases in the time period relative to the epidemics in Makó and Szeged. COVID-19 is increasing the risk of measles outbreaks. According to CDC Global Measles Outbreak reports of January 2021, 41 countries may postpone their measles campaigns for 2020 or 2021 due to the COVID-19 pandemic. This increases the risk of bigger outbreaks around the world (31).



Supplementary Figure 1. European measles cases in the time period relative to the epidemics in Makó and Szeged (ecdc.europa.eu)

Between December 2016 and November 2017, numerous measles cases occurred in Europe, most of which were reported by Romania, one of Hungary's neighbouring countries. Source of data: https://www.ecdc.europa.eu/

2017 measles epidemic in Hungary

In 2017, according to the data of the national authorities, a total of 76 persons were infected with measles (corrected to 73 laboratory confirmed cases by ECDC Surveillance reports). The outbreak in the hospital of the small town of Makó involved 29 individuals and lasted from January 2017 to March 2017 (32,33). In order to demonstrate the spread of virus in a well-immunized population, where despite good vaccination coverage, latent immunization gaps (unprotected, seronegative cohorts) are present, we used an open-source epidemiological report of the Hungarian National Public Health and Medical Officer Service (ANTSZ) (17 March 2017): 'At the peak of the Hungarian measles epidemics during the spring of 2017, 52 cases with measles-specific symptoms were reported. Of these, 15 laboratory confirmed cases (National Reference Laboratory for Measles and Rubella, National Public Health Institute, Budapest, Hungary) were registered by 16 March. Of these patients, 12 were health care workers (HCWs) and two were hospitalized patients. One of them was a foreigner, while the

other one was a patient living in the vicinity of a HCW. The epidemic affected two health care institutions, the Hospital of Makó and the clinics of the University of Szeged. The first measles case was imported in mid-February 2017 to the Hospital of Makó. The epidemic affected the hospital staff and their contacts. By 17 March, a measles infection was confirmed in case of a patient who was presumed to be the original importer of the virus, in case of 11 HCWs and in case of one of the HCW's contacts. At the time of this report, additional 11 cases (of which seven HCWs and three patients' contacts) were still under investigation. At the clinics of the University of Szeged, two persons – a HCW and a patient – fell ill with measles. Another 11 persons (six patients and five HCWs) were also suspected at the time of the report. Following the appearance of the abovementioned measles cases, in Csongrád County, a total of 391 people were vaccinated against measles, mumps and rubella (MMR). As the first cases of this period had been revealed, the National Chief Physician ordered strict monitoring and reporting of suspected measles virus infections. Thus, another 15 suspected cases were registered in several other counties. At the time of the report, laboratory testing was still ongoing (12)'.

The second group of imported cases was detected at the end of July 2017 in Nyíregyháza, Szabolcs-Szatmár-Bereg County, Hungary (11). Six unvaccinated Romanian children were hospitalised with clinical symptoms of measles. These cases were later laboratory confirmed (National Reference Laboratory for Measles and Rubella, National Public Health Institute, Budapest, Hungary). The subsequent disease spread among two additional HCWs (also laboratory confirmed) supports the susceptibility of certain clusters in the Hungarian population (11).

Epidemiological model- a didactic representation

In this section, we explain the spreading mechanism of infectious diseases for those who are not familiar with the computational background of modelling. To understand the basics of epidemic models, a simplified mathematical interpretation can be used. The spread of a disease can be described by S-shaped sigmoid mathematical functions, similar to the well-known pH titration curve, or haemoglobin saturation curves. As infectious diseases spread from human to human, the number of susceptible persons is decreasing over time and it influences the propagation of the pathogenic agent. In the beginning of the outbreak, the damping effect of recovered patients is minimal; the curve is very close to exponential and the number of new cases increases rapidly. At a certain time, a kind of equilibrium follows, daily recoveries can balance new infections and the curve reaches its inflection point. Afterwards, in the saturation phase, the epidemic slows down and at the end, no new cases are found and the vast majority of the population has recovered (Figure 4). The curves represented in Figure 4 are a graphic interpretation of a commonly used method for epidemic modelling – the compartment model. In this model, the population is divided into compartments – well-defined categories based on their epidemiological properties. In a compartment, all individuals behave exactly the same, e.g. they are all infected, all vaccinated, all exposed, etc. The simplest among these compartment models is the SIR model, where the letters of the acronym stand for susceptible, infectious and recovered.

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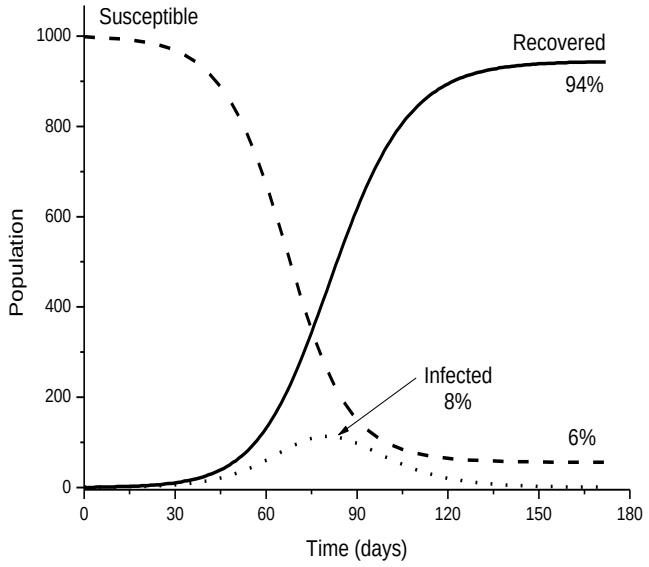

Figure 4. SIR model curves of a hypothetical epidemic

As the disease spreads, the number of susceptible individuals decreases. First they get infected (I), but later on they will progress to the recovered compartment (R). Approximately 6% of the population managed to avoid contact with infected individuals. The peak of infections could be observed almost three months after the first case was recorded, affecting 8% of the population at the same time.

The progression of an individual in this model is easy to follow, each member of the population progresses from susceptible to infectious to recovered.

$$S \overset{\beta}{\Rightarrow} I \overset{\gamma}{\Rightarrow} R \quad (1)$$

Transition between compartments is described by transition rates. Infection rate () represents the probability of transmitting the disease between a susceptible and an infectious person. In other words, the value of shows the number of individuals to whom an infectious person can pass the disease per day (18,39,40) For example, if the infection rate is 0.2, it will take five days on average to infect someone. If we assume that the patient is contagious for 10 days, two new infections are expected in this case.

The overall efficacy of the epidemic can be described by the number of these secondary infections originated from the primary infection, our first patient. This important parameter is the basic reproduction number (R0). Each virus has its own average R0 value – 12-18 for measles and 3.3-5.7 for COVID-19, according to the literature.

The recovery rate (describes the probability of transition into the recovered compartment. For instance, if this rate is 0.1, the contagious period will last for 10 days.

From a mathematical perspective, the transitions can be described by the following differential equations, where S, I and R are the number of individuals in the corresponding compartments, while N is the whole population.

$$\frac{dS}{dt} = -\frac{\beta IS}{N} \quad (2)$$

$$\frac{dI}{dt} = \frac{\beta IS}{N} - \gamma I \quad (3)$$

$$\frac{dR}{dt} = \gamma I \quad (4)$$

Mathematical methods (such as the Runge-Kutta method) are available for solving similar equations, but there is a simpler option. Using the built-in features of Microsoft Excel (or any equivalent spreadsheet application), it is possible to make calculations using an iterative method. Instead of solving the equations, the computer performs calculations that follow the daily changes in different compartments.

For modelling, and R0 have to be defined. Based on the definition of the transition rates, it can be seen that the recovery rate can be determined by the number of contagious days (T_cont).

$$\gamma = \frac{1}{T_{cont}} \quad (5)$$

The basic reproduction number can be given as follows:

$$R_0 = \frac{\beta}{\gamma} \quad (6)$$

Let us assume that in a certain population measles can be transmitted from a single person to 12 others (R_0=12) and they stay contagious for 6 days (T_cont=6). In this case:

$$\gamma = \frac{1}{T_{cont}} = \frac{1}{6} \text{ (7)}$$

$$\beta = R_0\gamma = 2 \text{ (8)}$$

Incubation time plays an important role in the spread of a disease. In a more sophisticated model (SEIR model), this can also be taken into consideration. A new compartment for the exposed part of the population can be generated. The susceptible person first gets exposed and will progress to the infectious state only after a certain time.

$$S \overset{\beta}{\Rightarrow} E \overset{\alpha}{\Rightarrow} I \overset{\gamma}{\Rightarrow} R \text{ (9)}$$

The parameter 'α' is a new transition rate, which can be determined by the incubation time (T_inc) , similarly to γ :

$$\alpha = \frac{1}{T_{inc}}$$

New compartments can be added to the model anytime, such as the compartment M for individuals with maternal immunity or the compartment E for exposed individuals, who are already infected, but not infectious. Based on the characteristics of certain infectious diseases, further models have been developed, such as the SIS, MSIR, SEIR, SEIS, MSEIR and MSEIRS models. The second 'S' in the acronym indicates that after the infection, no permanent immunity can be reached and the individuals step to the S compartment again. In other models, the ratio of hospitalization, the ratio of mild and severe cases and epidemiological interventions can be included, with a more complex mathematical background.

In the examples described above some important parameters are simply disregarded, although it is possible to perform a more detailed computation. Vital dynamics, the natural dynamics of birth and death, can be included by adding two further parameters.

It is necessary to mention that compartment models have their well-known limitations and shortcomings. For instance, all individuals in the population are assumed to have an equal probability of coming in contact with others, although society is inhomogeneous from the perspective of social distancing. Another drawback is that the traditional compartment model cannot handle uncertainty in model parameters. Working with a smaller set of data increases this uncertainty, making predictions unreliable. To overcome this problem, it is usual to calculate the SIR model over a few possible values for each parameter. A more complex solution is to use distribution functions instead of single numbers and if real-time data is available (e.g. we are in the middle of a pandemic), a clinical dataset can be utilized to adjust these parameters (36–38).

Regarding the SEIR model resembling the 2017 measles outbreak in Makó (Figure 4), we would like to note that both the simplified mathematical method and the input data were unreliable. With more sophisticated models, many different parameters can be taken into consideration (37,39). Despite that fact, the calculated values correspond in order of magnitude to the available data on the epidemic and support the experimental results describing the vaccination gap.

Model curves using a lower percentage of the population-level anti-measles protection rate are more fitting. This finding may indicate an even lower percentage of effectively vaccinated population than it was found previously (~90%).

It is concluded that the importance of seroepidemiological surveys is confirmed by the recent outbreaks of measles, mumps and rubella infections in several countries (14,16,17,40–45). Considering the HIT values, suboptimal anti-measles seropositivity ratios were detected in certain clusters of the early vaccination era (78.48% of sufficient anti-measles IgG antibody titres among individuals vaccinated between 1978 and 1987). This finding, which is in accordance with a recent study published by our colleagues (11) and historical literature data (46), suggests the existence of age-specific immunization gaps in the Hungarian population. For mumps and rubella, our preliminary data shows satisfactory immunity levels. Nowadays, in our country, the MMR vaccination coverage is ideal due to the

mandatory administration of safe and modern trivalent vaccines. Nevertheless, dubious immunization practices in some of our neighboring countries, aggravated by the detrimental effect of the COVID-19 pandemic and subsequent suspension of measles vaccination campaigns, may facilitate the occurrence of minor importation-related MeV outbreaks in susceptible cohorts. Using the example of the 2017 measles outbreak in Makó, it has been demonstrated that in possession of key epidemiological parameters (e.g. R0 value, estimated vaccination coverage of a given population, number of infected and recovered individuals, duration, etc.), a simple SEIR model can give a good approximation regarding the course of an infection.

We believe that awareness may significantly reduce the extent of an epidemic (38,47). In the light of current disquieting epidemiological circumstances, we suggest the introduction of open-access mathematical and epidemiological models into modern natural science education of students. Today, online epidemic models are easily available for the public (35,36). Practical introduction to these plain calculation models could help students understand the rationale behind epidemiological data. We believe that a practical demonstration of epidemiological events can promote a better understanding of countermeasures and also allow for an easier adaptation to the current epidemiological regulations.

Limitations of experimental work

The diagnostic ability of our assay was calculated based on the results obtained by well-established kits capable of humoral antibody detection, rather than on neutralizing antibody titres that could serve as an absolute correlate of protection (48–50). It is important to emphasize that immunity to measles is a complex orchestration between the cellular and humoral immunity. For this reason, only antibody-based definitions of vaccine success and failure may be misleading, or at least simplistic and incomplete (51).

Limitations of mathematical modelling

Input data plays a key role in modelling of epidemics. Even when the number of cases is high – like in the 2020 COVID outbreak – the confidence of fitting is poor at the beginning of new cases vs. time graph. The first cases are usually unexpected, quarantine and social distancing protocols are not applied yet and if the disease has a low prevalence in the population, the accuracy of the diagnosis might be low. Besides that, atypical symptoms can be misleading for physicians. Furthermore, statistical values, such as basic reproduction number, incubation and recovery time, depend on other factors, such as social distancing and the health care system.

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References

1. Haralambieva IH, Kennedy RB, Ovsyannikova IG, Whitaker JA, Poland GA. Variability in Humoral Immunity to Measles Vaccine: New Developments. Trends Mol Med 2015;21(12):789-801. doi: 10.1016/j.molmed.2015.10.005.

2. COVID-19's Impact on Measles Vaccination Coverage. Available from: https://www.cdc.gov/globalhealth/measles/n

ews/covid-impact-on-measles-vaccination.html

3. Suspension of vaccination due to COVID-19 increases the risk of infectious diseases outbreaks - UNICEF and WHO. Available from: https://www.unicef.org/ukraine/en/press-releases/suspension-vaccination-due-covid-19-increases-risk-infectious-diseases-outbreaks (press release)

4. Durrheim DN, Andrus JK, Tabassum S, Bashour H, Githanga D, Pfaff G. A dangerous measles future looms beyond the COVID-19 pandemic. Nat Med 2021; 27:360–361. https://doi.org/10.1038/s41591-021-01237-5

5. WHO Western Pacific Region. WHO Western Pacific Region: Countries and areas. Measles-Rubella Bull. 2021;15(1):1–10. https://iris.wpro.who.int/bitstream/handle/10665.1/14713/Measles-Rubella-Bulletin-2021-Vol-15-No-01.pdf

6. Torre G La, Saulle R, Unim B, Meggiolaro A, Barbato A, Mannocci A, Spadea A. The effectiveness of measles-mumps-rubella (MMR) vaccination in the prevention of pediatric hospitalizations for targeted and untargeted infections: A retrospective cohort study. Hum Vaccin Immunother. 2017;13(8):1879- 1883. doi: 10.1080/21645515.2017.1330733

7. Rosian-Schikuta I, Fröschl B, Habl C, Stürzlinger H. The Measels-Mumps-Rubella Vaccination from a health political and economical point of view. GMS Health Technol Assess. 2007 Nov 28;3:Doc12.

8. Plans-Rubió P. Evaluation of the establishment of herd immunity in the population by means of serological surveys and vaccination coverage. Hum Vaccin Immunother. 2012; 8(2):184–8.

9. Böröcz K, Csizmadia Z, Markovics, Farkas N, Najbauer J, Berki T, Nemeth P. Application of a fast and cost-effective "three-in-one" MMR ELISA as a tool for surveying anti-MMR humoral immunity: The Hungarian experience. Epidemiol Infect. 2020; 148: e17. doi: 10.1017/S0950268819002280

10. Böröcz K, Csizmadia Z, Markovics Á, Mészáros V, Farkas K, Telek V, Varga V, Ouma Maloba G, Bodó K, Najbauer J, Berki T, Németh P. Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human sera. J Immunol Methods. 2019 Jan; 464:1-8. doi: 10.1016/j.jim.2018.07.009.

11. Lengyel G, Marossy A, Ánosi N, Farkas SL, Kele B, Nemes-Nikodém É, et al. Screening of more than 2000 Hungarian healthcare workers' anti-measles antibody level: results and possible population-level consequences. Epidemiol Infect. 2019 Sep 11;147:e7.

12. ANTSZ - Tájékoztatás a kanyarós megbetegedésekről. Available from: https://www.antsz.hu/felso_menu/temaink/jarvany/jarvany_archivum/kanyaro/tajekoztatas kanyarosmegbetegedesekrol.html

13. Epidemiological update: Measles - monitoring European outbreaks, 7 July 2017. Available from: https://www.ecdc.europa.eu/en/news-events/epidemiological-update-measles-monitoring-european-outbreaks-7-july-2017

14. Lambert N, Strebel P, Orenstein W, Icenogle J, Poland GA. Rubella. Lancet. 2015; 385(9984):2297–307.

15. Moss WJ. Measles. Lancet. 2017; 390(10111):2490–502.

16. Hviid A, Rubin S, Mühlemann K. Mumps. Lancet. 2008;371(9616):932–44.

17. Lewnard JA, Grad YH. Vaccine waning and mumps re-emergence in the United States. Sci Transl Med. 2018; 10(433):eaao5945.

18. Peng L, Yang W, Zhang D, Zhuge C, Hong L. Epidemic analysis of COVID-19 in China by dynamical modeling. 2020. arXiv:2002.06563 [q-bio.PE]

19. Kretzschmar M. Epidemic Model - an overview | ScienceDirect Topics. International Encyclopedia of Public Health (Second Edition). 2017. p. 571–84. https://www.sciencedirect.com/topics/medicine-and-dentistry/epidemic-model

20. Böröcz K, Csizmadia Z, Markovics Á, Mészáros V, Farkas K, Telek V, Varga V, Maloba GO, Bodó K, Najbauer J, Berki T, Németh P. Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human sera. J Immunol Methods. 2019 Jan;464:1-8. doi: 10.1016/j.jim.2018.07.009.

21. Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol. 2010; 17(7):1055–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20463105

22. Plotkin SA. Complex correlates of protection after vaccination. Clin Infect Dis. 2013;56(10):1458–65.

23. Plotkin SA, Gilbert PB. Nomenclature for immune correlates of protection after vaccination. Clin Infect Dis [Internet]. 2012 Jun [cited 2019 Aug 30];54(11):1615–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22437237

24. Böröcz K, Csizmadia Z, Markovics Á, Farkas N, Najbauer J, Berki T, et al. Application of a fast and cost-effective "three-in-one" MMR ELISA as a tool for surveying anti-MMR humoral immunity: The Hungarian experience. Epidemiol Infect. 2020;

25. Agócs MM, Markowitz LE, Straub I, Dömök I. The 1988-1989 measles epidemic in Hungary: assessment of vaccine failure. Int J Epidemiol [Internet]. 1992 Oct [cited 2019 May 5];21(5):1007–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1468837

26. MMWR Weekly. MMWR Publications | MMWR [Internet]. [cited 2019 May 5]. Available from: https://www.cdc.gov/mmwr/publications/index.html

27. Hungary V. Vacsatc - Oltásbiztonság | Nyitólap [Internet]. [cited 2020 Mar 31]. Available from: http://www.oltasbiztonsag.hu/

28. Vacsatc - Oltásbiztonság | Szakmai oldalak | Virális oltóanyagok [Internet]. [cited 2019 Dec 21]. Available from: http://www.vacsatc.hu/?Vir%E1lis-olt%F3anyagok&pid=37

29. International Notes Measles -- Hungary [Internet]. Available from: https://www.cdc.gov/mmwr/preview/mmwrhtml/00001472.htm

30. Böröcz K, Csizmadia Z, Markovics Á, Mészáros V, Farkas K, Telek V, et al. Development of a robust and standardized immunoserological assay for detection of anti-measles IgG antibodies in human sera. J Immunol Methods. 2019;464.

31. Lengyel G, Ánosi N, Marossy A, Mátyus M, Bosnyákovits T, Orosz L. Az európai és a magyarországi kanyaróhelyzet összefoglalása és tanulságai. Orv Hetil [Internet]. 2019 May 12 [cited 2019 Jul 31];160(20):767–73. Available from: https://www.akademiai.com/doi/10.1556/650.2019.31397

32. Habersaat KB, Pistol A, Stanescu A, Hewitt C, Grbic M, Butu C, et al. Measles outbreak in Romania: understanding factors related to suboptimal vaccination uptake. Eur J Public Health [Internet]. 2020 Oct 1 [cited 2021 Mar 5];30(5):986–92. Available from: https://academic.oup.com/eurpub/article/30/5/986/5847956

33. Dascalu S. Measles Epidemics in Romania: Lessons for Public Health and Future Policy [Internet]. Vol. 7, Frontiers in Public Health. Frontiers Media S.A.; 2019 [cited 2021 Mar 5]. p. 98. Available from: /pmc/articles/PMC6496956/

34. The Lancet. Measles, war, and health-care reforms in Ukraine. Vol. 392, The Lancet. Lancet Publishing Group; 2018. p. 711.

35. Wadman M. Measles epidemic in Ukraine drove troubling European year [Internet]. Vol. 363, Science. American Association for the Advancement of Science; 2019 [cited 2021 Mar 5]. p. 677–8. Available from: http://stm.sciencemag.org/content/scitransmed/10/433/eaao5945.full

36. Global Measles Outbreaks [Internet]. [cited 2021 Jan 31]. Available from: https://www.cdc.gov/globalhealth/measles/data/global-measles-outbreaks.html

37. ECDC. Epidemiological update: Measles - monitoring European outbreaks, 7 July 2017 [Internet]. [cited 2020 Mar 31]. Available from: https://www.ecdc.europa.eu/en/news-events/epidemiological-update-measles-monitoring-european-outbreaks-7-july-2017

38. ANTSZ. ANTSZ - Public information on measles epidemics [Internet]. [cited 2020 Mar 31]. Available from: https://www.antsz.hu/felso_menu/temaink/jarvany/jarvany_archivum/kanyaro/tajekoztatas kanyarosmegbetegedesekrol.html

39. Stella R. Quah WC. International Encyclopedia of Public Health - Second Edition-Google Books [Internet]. Elsevier. 2017 [cited 2020 Apr 4]. Available from: https://books.google.hu/books?hl=hu&lr=&id=WAnpCgAAQBAJ&oi=fnd&pg=PP1&dq=Measurement+and+Modeling:+Infectious+Disease+Modeling+Mirjam+Kretzschmar,+in+International+Encyclopedia+of+Public+Health+(Second+Edition),+2017&ots=k662f-7bGN&sig=CQ-q-rJ9YMRIG9BDwTm

40. Epidemic Calculator [Internet]. [cited 2020 Apr 4]. Available from: https://gabgoh.github.io/COVID/index.html

41. Buckman SR, Glick R, Lansing KJ, Petrosky-Nadeau N, Seitelman LM, Francisco S. Replicating and Projecting the Path of COVID-19 with a Model-Implied Reproduction Number. 2020;2020–44.

42. Röst G, Wu J. SEIR EPIDEMIOLOGICAL MODEL WITH VARYING INFECTIVITY AND INFINITE DELAY [Internet]. Vol. 5, AND ENGINEERING. 2008 [cited 2020 Apr 4]. Available from: http://www.mbejournal.org/

43. Li M, Wang M, Xue S, Ma J. The influence of awareness on epidemic spreading on random networks. J Theor Biol. 2020 Feb 7;486:110090. doi: 10.1016/j.jtbi.2019.110090.

44. Lipsitch M, Cohen T, Cooper B, Robins JM, Ma S, James L, et al. Transmission dynamics and control of severe acute respiratory syndrome. Science (80-). 2003 Jun 20;300(5627):1966–70.

45. Ujiie M. Rubella Resurgence in Japan 2018–2019. J Travel Med [Internet]. 2019 Sep 2 [cited 2021 Feb 1];26(6). Available from: https://academic.oup.com/jtm/article/doi/10.1093/jtm/taz047/5523888

46. WHO | New measles surveillance data from WHO. WHO [Internet]. 2019 [cited 2019 Aug 22]; Available from: https://www.who.int/immunization/newsroom/new-measles-data-august-2019/en/

47. Wadman M. Measles epidemic in Ukraine drove troubling European year. Science (80-) [Internet]. 2019 Feb 15 [cited 2019 May 16];363(6428):677–8. Available from: http://www.sciencemag.org/lookup/doi/10.1126/science.363.6428.677

48. The Lancet. Measles, war, and health-care reforms in Ukraine. Lancet [Internet]. 2018 Sep 1 [cited 2019 May 16];392(10149):711. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30191812

49. Ukraine: Measles Outbreak - Jan 2019 | ReliefWeb [Internet]. [cited 2019 Aug 22]. Available from: https://reliefweb.int/disaster/ep-2019-000017-ukr

50. Balbi AM, Van Sant AA, Bean EW, Jacoby JL. Mumps: Resurgence of a once-dormant disease. J Am Acad Physician Assist [Internet]. 2018 May 1 [cited 2021 Feb 1];31(5):19–22. Available from: https://pubmed.ncbi.nlm.nih.gov/29642091/

51. Takahashi S, Sato K, Kusaka Y, Hagihara A. Public preventive awareness and preventive behaviors during a major influenza epidemic in Fukui, Japan. J Infect Public Health. 2017 Sep 1;10(5):637–43.

52. Liu Y, Liu Z, Deng X, Hu Y, Wang Z, Lu P, et al. Waning immunity of one-dose measles-

mumps-rubella vaccine to mumps in children from kindergarten to early school age: a prospective study. Expert Rev Vaccines [Internet]. 2018 May 4 [cited 2019 May 16];17(5):445–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29478347

53. Bankamp B, Hickman C, Icenogle JP, Rota PA. Successes and challenges for preventing measles, mumps and rubella by vaccination. Curr Opin Virol [Internet]. 2019 Feb [cited 2019 May 15];34:110–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30852425

54. Kontio M, Jokinen S, Paunio M, Peltola H, Davidkin I. Waning antibody levels and avidity: Implications for MMR vaccine-induced protection. J Infect Dis. 2012;206(10):1542–8.

55. Ovsyannikova IG, Dhiman N, Jacobson RM, Vierkant RA, Poland GA. Frequency of measles virus-specific CD4+ and CD8+ T cells in subjects seronegative or highly seropositive for measles vaccine. Clin Diagn Lab Immunol [Internet]. 2003 May [cited 2019 Aug 2];10(3):411–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12738640

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