Journal of Immunological Methods 464 (2019) 1–8

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

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

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ARTICLE INFO

Keywords:
Measles
Immunity
IgG
ELISA
Assay development

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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https://doi.org/10.1016/j.jim.2018.07.009
Received 7 May 2018; Received in revised form 9 July 2018; Accepted 25 July 2018
Available online 27 July 2018
0022-1759/

(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.

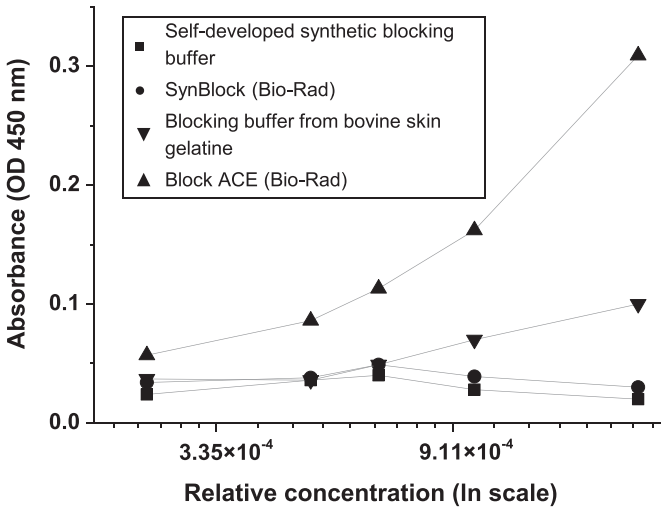


Fig. 1. Effect of protein-free blocking.

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

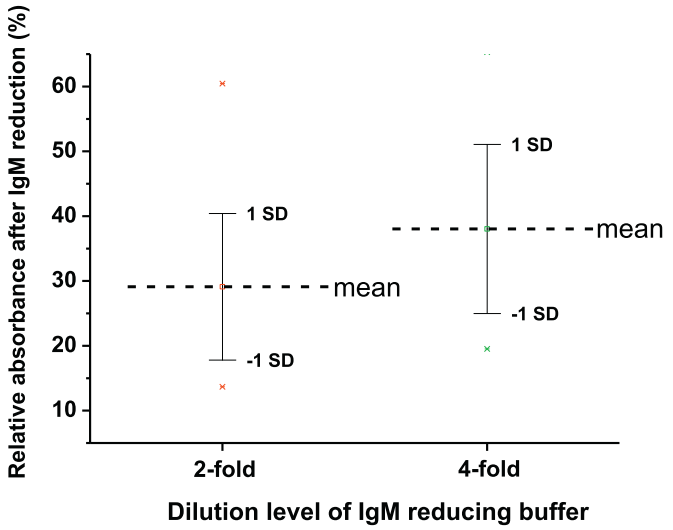


Fig. 2. Effect of IgM reduction expressed as percent decrease in absorbance of samples (N = 25 serum samples).

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).

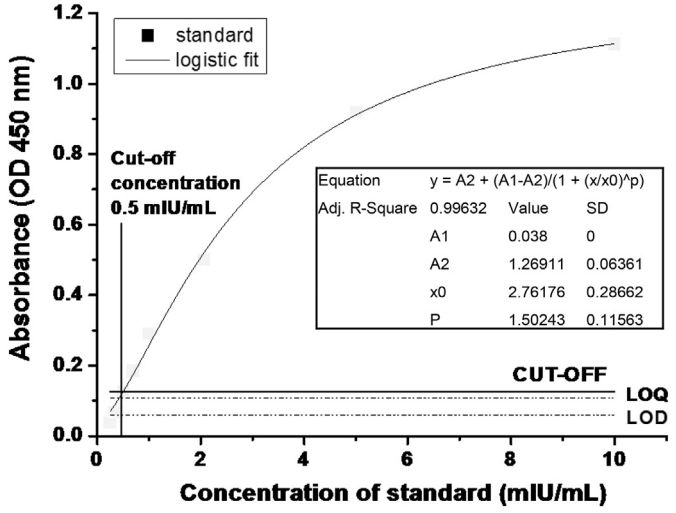


Fig. 4. Calibration curve, fitting algorithm and determination of LOQ (limit of quantification), LOD (limit of detection), CUT-OFF. LOD = mean OD values of blank samples +3 SD, LOQ = mean OD values of blank samples +10 SD, CUT-OFF = mean OD values of low titer samples +3 SD.

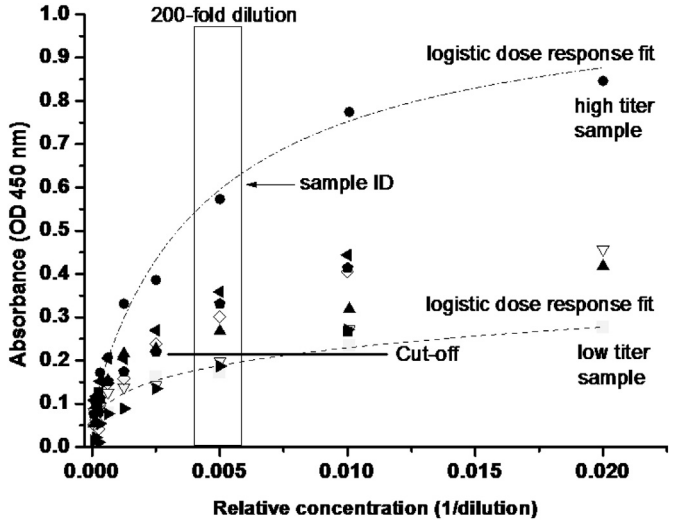


Fig. 5. Determination of optimal dilution of samples. Each symbol denotes a different sample, using consecutive dilutions. CUT-OFF = mean OD values of low titer samples +3 SD. 200-fold dilution was optimal (symbols shown in the box).

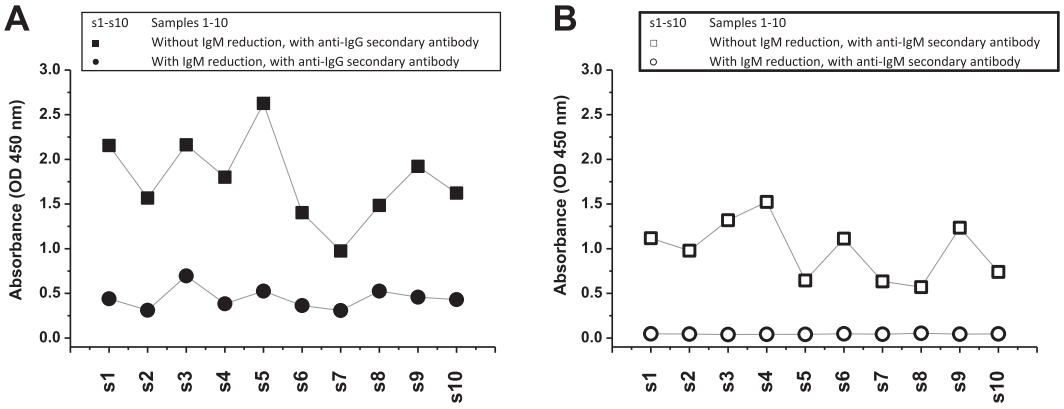


Fig. 3. Effect of IgM reduction on absorbance values of serum samples used for anti-measles IgG detection. (A) Effect of IgM reduction with anti-IgG secondary antibody. (B) Effect of IgM reduction with anti-IgM secondary antibody.

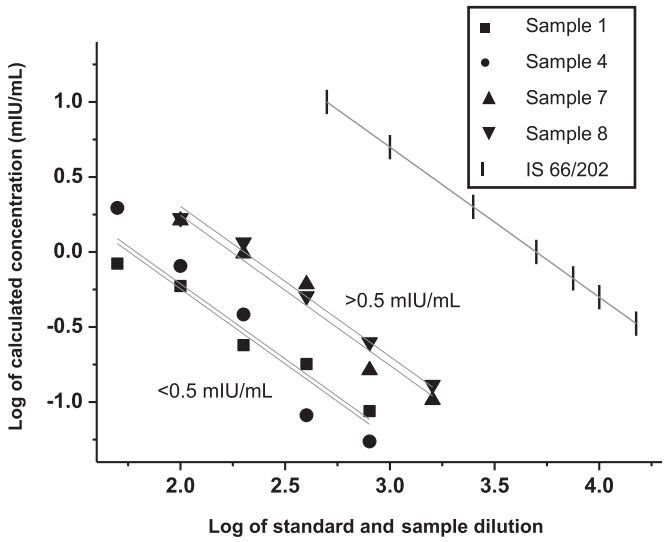


Fig. 6. Linearity and parallelism. Linearized dilutions of high and low titer samples with slope −1.

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

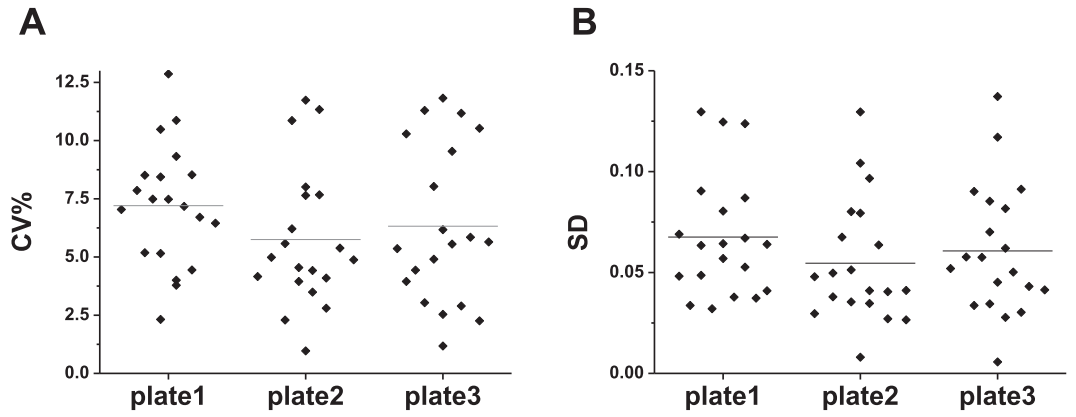
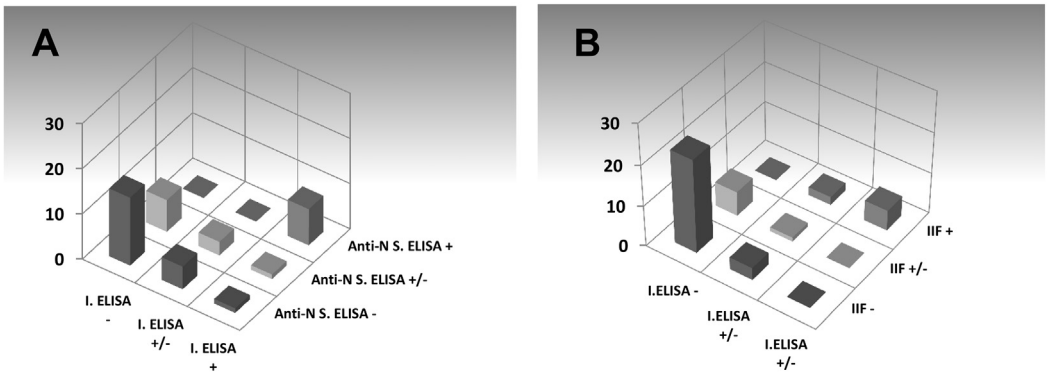


Fig. 7. (A) Intra-assay variability: coefficient of variation. (B) Intra-assay variability: standard deviation. Triplicates of 20 samples were analyzed on each plate.



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).

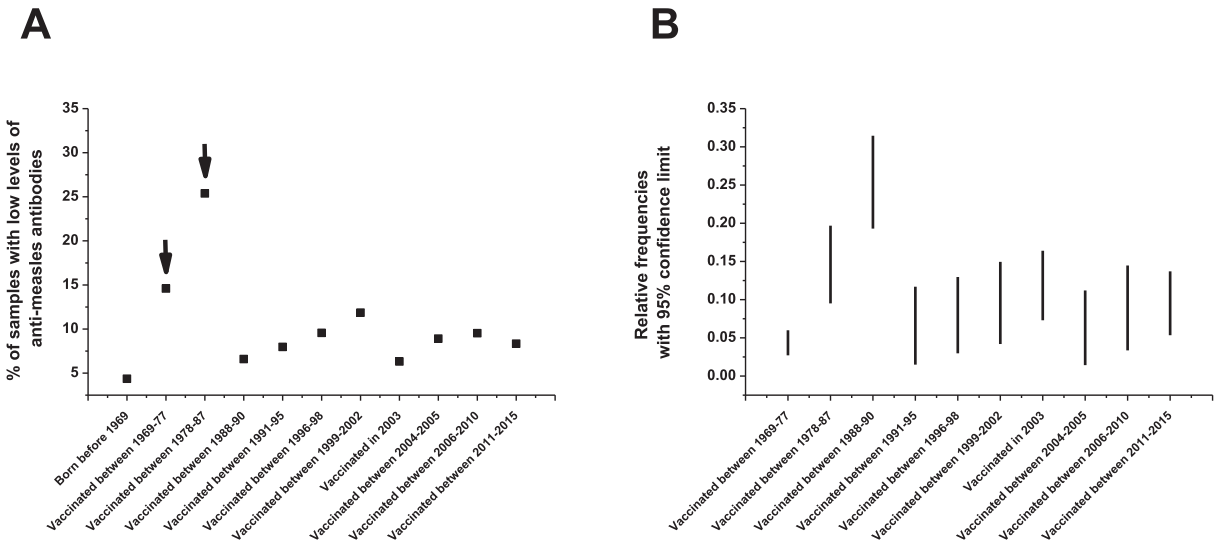


Fig. 9. (A) Cohort-centered distribution of samples with low anti-measles antibody levels. (B) Confidence intervals (N = 1985).

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

This work was supported by the following grants: GINOP-2.3.2-15-2016-00050 and EFOP 3.6.1-16-2016-0004 EU Grant.

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.

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