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The Performance of Multiplex Immunoassays for Antibody Determination to Diphtheria, Tetanus and Pertussis: A Need for Standardisation

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Abstract

Serological surveillance is a powerful method to estimate the risk of infectious diseases and their vaccination coverage among populations. However, enzyme-linked immunosorbent assays commonly used for antibody screening are time consuming when testing multiple analytes on large sample numbers. As multiplex immunoassays for detection of antibodies against tetanus, diphtheria and pertussis (or other vaccine-preventable diseases for that matter) are not commercially available, a number of groups have validated multiplex bead-based immunoassays for simultaneously detection of antibodies against these three infectious diseases. However it is difficult to compare the assays described in the different studies, as various investigators used different materials, reagents and reference sera and reports varied considerably in the amount of methodological details provided. In our recent study, we developed a Luminex xMAP based assay using for the first time magnetic beads and commercially available purified antigens and commercial ELISA kits for assay comparison, parameters which we think are important for future standardisation among different laboratories.

Keywords: Immunoassay; Multiplex detection; ELISA; Luminex xMAP® technology; Antibody; Assay standardisation

Introduction

Despite good vaccination coverage, even in the industrialized world pertussis is still not under control and sporadic cases of tetanus and diphtheria are reported regularly in Europe (http://ecdc.europa.eu). Serological surveillance is an easy manner to estimate the real risk of these vaccine-preventable diseases among populations [1,2]. Paediatric vaccines against tetanus, diphtheria and pertussis, as well as vaccines for use in older children, adolescents and adults, are now mostly combination products [3]. These vaccines induce antibodies which in the past have generally been measured by enzyme-linked immunosorbent assay (ELISA), allowing analysis of only a single vaccine antigen at a time. These ELISAs may be rather expensive and time consuming. Also, the assessment of multiple markers may require a considerable volume of serum. This may be a limiting factor when only small sample volumes (as in children) are available.

The Luminex xMAP® platform, based on detection by multiplexed microsphere immunoassays allows the detection of a large number of analytes, ranging from 50 to 500 according to the Luminex analyzer used, in one sample [2]. Simultaneous analysis greatly reduces the amount of time and labour required for large scale analysis and allows to quantify all analytes of interest under the same conditions [1,2,4]. The successful application of Luminex multiplex immunoassays (MIAs) has resulted in a growing array of commercially available multiplex kits, particularly for cytokine and chemokine detection [5]. So far, there are no commercial multiplex assays for the screening of vaccine-induced antibodies. Several groups have developed and validated in-house multiplex assays to measure IgG antibodies to diphtheria, tetanus and pertussis vaccine antigens [1,3,6-8]. Validation and highly sensitive and precise methods for such quantification are critical, especially when applied to precious sample collections [5]. Even if a similar protocol was used for the assay development, the antigens and reference sera used by the different laboratories differed from each other, making data from different countries difficult to compare.

MIA Development for Antibody Screening: The Principle

An indirect format is used for serological assays to measure the amount of antigen-specific antibody present in blood samples. The surface of Luminex® microspheres contains 100 million carboxyl groups on each bead, which facilitates covalent attachment of "capture" molecules during a two-step carbodiimide reaction. After activation of the carboxyl groups with the aid of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS), the protein antigens are covalently linked through an amide bond with the activated carboxylated beads under defined pH/ionic strength conditions. Antigen-specific antibodies bound to the beads via the "capture" protein are subsequently detected by the reporter molecule phycoerythrin (PE) conjugated to a secondary detection antibody [2]. The washing method between steps as well as the type of beads used in the different laboratories (non-magnetic or magnetic) can influence the results. In our study, we used magnetic beads allowing the washing with a strong magnet to separate the beads from the reaction fluids. In contrast, studies using non-magnetic beads are performed in filter-bottom microplates and liquids are drawn out from the microplate bottom using a vacuum manifold, which can lead to the loss of beads and unreliable results. Using magnetic beads, bead loss is minimized resulting in more reproducible data generation (http://www.luminexcorp.com).

Published papers	Pickering et al. 2002	Prince et al. 2006	Reder et al. 2008	Van Gageldonk et al.2008	Stenger et al. 2011	Cabore et al.2016
Platform Analysis software Number of plex Antibody tested	Luminex 100 3-plex DT/TT/Hib IgG	Luminex 100 / 4-plex PT/FHA IgG and IgA	Flow cytometer / 3-plex PT/DT/TT IgG	Microplex BioPlex Manager 4.1.1 5-plex PT/FHA/prn/DT/TT IgG	Bio-plex 100 BioPlex Manager 6-plex PT/FHA/prn/ Fim2,3/DT/TT IgG	MagPix xPONENT 4.2 5-plex PT/FHA/Prn/DT/TT IgG
Antigen origin	University of Massachusetts Biologic Laboratories (Jamaica Plain, Mass) for DT and TT	List Biological Laboratories, Campbell, CA	/	PT Nederland Vaccine Institute, FHA Kaketsuken, Prn expressed and purified from E.coli DT/TT from Sigma Aldrich	PT/FHA/Fim2,3 purified from B.pertussis Prn expressed in E.coli and purified DT/TT from Sigma Aldrich	PT SSI, FHA Kaketsuken, Prn/DT NIBSC, TT Sigma Aldrich
Antigen/bead ratio	/	10 ug/12.5 × 10^6 beads each	/	10 ug/12.5 × 10^6 for PT/FHA/Prn 100 ug/ 12.5 × 10^6 DT/TT	10 ug/12.5 × 10^6 for PT/FHA/Prn 100 ug/12.5 × 10^6 DT/TT	10 ug/12.5 × 10^6 beads each
Bead nature	Polystyrene	Polystyrene	Polystyrene	Polystyrene	Polystyrene	Magplex-beads
Beads activation	20 min in PBS pH 6.1 containing EDC 5 mg and sulfo-NHS 5mg	20 min in 0.1 M NaH_2PO_4 pH 6.2 buffer containing EDC 5mg and sulfo-NHS 5mg	EDC/sulfo-NHS	20 min in PBS containing EDC/sulfo-NHS 5mg/ml each	EDC/sulfo-NHS 2.5 mg/ml each in PBS	20 min in 0.1M NaH_2PO_4 pH 6.2 buffer containing EDC/ sulfo-NHS 2.5 mg/ml
Couling Antigen to beads	1 h in PBS 7.3	1 h in 50 Mm MES pH 5.0	/	2 h in PBS	2 h PBS pH 7.2	50 Mm MES, pH 5.0
Coupled bead storage buffer	PBS, 0.1% BSA, sodium azide	PBS,1% BSA, 0.05% sodium azide	/	PBS,1% BSA, 0.05% sodium azide	PBS,1% BSA, 0.05% sodium azide	PBS,1% BSA, 0.05% sodium azide
Assay plate	Filtration plate	Filtration plate	Filtration plate	Filtration plate	Filtration plate	Greiner 96 w flat bottom black
Dilution buffer	PBS, 0.05% Tween20	PBS-1%BSA	/	PBS, 3% BSA, 0.1%Tween20	PBS, 3% BSA, 0.1%Tween20	PBS, 3% BSA, 0.1%Tween20
Wash buffer	PBS	PBS-0.05% Tween20	/	PBS	PBS	PBS, 1% BSA
Sample dilution	1/100	1/50	/	1/200 and 1/4000 in dilution buffer	1/1250 and 1/12500 in dilution buffer	1/100 Sample dillution buffer
Standard curve	In-house calibrated against the U.S. human anti-Hib standard reference serum, CBERlot 1983 and NIBSC code 00/496 and 76/589 for DT and TT	In-house calibrate against CBER lot3 for IgG PT and IgG FHA, lot 5 for IgA pT and IgA FHA	/	In-house standard calibrated against US anti-serum Human lot3 for PT/ FHA, and lot4 for Prn, against NIBSc code Di-03 for DT and TE-03 for TT	NIBSC	In-house standard calibrated against NIBSC 06/140 for pertussis antibodies, 10.262 for DT and TE-3 for TT
Dilution series standard curve:	8 steps of 4-fold (1/20-1/81920) in dilution buffer	/	/	6 steps of 4-fold (1/100-1/204800) for pertussis standard and 8 steps of 4-fold (1/50-1/819200) for DT/TT in dilution buffer	8 steps of 3-fold (1/100-1/218700) in dilution buffer	7 steps of 4-fold (1/100-1/409600) in dilution buffer
Bead working conc/well	/	2500/bead set		4000 beads/bead set	4000 beads/bead set	2500 beads/bead set
Assay volume	/	/	/	25 ul beads, 25 ul serum	25 ul beads, 25 ul serum	50 ul beads, 50 ul serum
Detection antibody	Anti-human IgG-RPE	Anti-human IgG-RPE and anti-human IgA-RPE	Anti-human IgG-RPE	Anti-human IgG-RPE	NIBSC anti-mouse IgG-RPE	Anti-human IgG-RPE

source	/	Jackson Immunoresearch	Jackson Immunoresearch	Jackson Immunoresearch	Jackson	Jackson Immunoresearch
Beads-serum incubation	20 mins, RT, shaking plate, dark	20 mins, RT, shaking plate, dark	30 mins, RT, shaking plate, dark	45 mins, RT, shaking plate, dark	45 mins, RT, shaking plate, dark	60 mins, RT, shaking plate, dark
IgG-RPE dilution	/	/	/	1/200 in diluent buffer	1/200 in diluent buffer	1/200 in diluent buffer
Beads-IgG-RPE	20 min, dark, shaking, RT.	20 min, dark, shaking, RT.	30 min, dark, shaking, RT.	30 min, dark, shaking, RT.	30 min, dark, shaking, RT.	30 min, dark&shaking, RT
Unit	IU/mL for DT and TT	U/ml	EU/ml for PT and IU/ml for DT/TT	EU/ml for PT/FHA/Prn/Fim 2,3 and IU/ml for DT/TT	U/ml	IU/ml
ELISA (for comparison)	In-house	In-house	In-house and 2 different commercial kits for PT, 3 different commercial kits for DT and TT	In-house	In-house	Commercial kits

Table 1: Comparison of the developed multiplex immunoassay for at diphtheria, tetanus and/or pertussis antibodies.

This was confirmed by Hansenova et al. [9] who reported a higher sensitivity and stronger signals of a magnetic bead-based assay with half the quantity of antigen, as compared to an assay based on non-magnetic beads, likely due to the efficient magnetic separation used in the repetitive washing steps during the coupling procedure.

In our recent study [1], the capture antigen concentration for the three pertussis antigens (pertussis toxin PT, filamentous hemagglutinin FHA and pertactin Prn) used for coupling to magnetic beads was identical to that in previously described studies [7,8,10] but for diphtheria toxoid DT and tetanus toxoid TT, the best results were obtained with less antigen than described by Van Gageldonk et al. [8] and Stenger et al. [10] (Table 1).

Using the protocol of the Luminex Cookbook 2nd edition (http://www.luminexcorp.com), 50 µl of mixed coupled beads (2500 beads/antigen) were incubated during 60 minutes with 50 µl of diluted serum dilution (1:100). Using a smaller assay volume [8,10] could reduce assay sensitivity and shorter incubation times [3,6,7] could miss antibodies with low avidity [11]. Various buffers have been used especially for the coupling step of antigens to the beads. In our study and the one of Prince et al., beads were activated in a monobasic sodium phosphate (NaH_2PO_4 pH 6.2) buffer and coupling buffer contained 2-(N-morpholino) ethane-sulfonic acid (MES pH 5), in contrast with the other studies, which used PBS buffer for both steps [3,6,10,12].

To quantitate the multiplex immunoassay (MIA) results, a standard reference serum is needed for the different antigens of interest, and one reference serum covering all antigens is preferable. We prepared an in-house reference standard for the MIA by pooling human serum samples with high antibody titers to the five vaccine antigens, obtained one month after a Tdap booster vaccine in women during their third trimester of pregnancy [1,13]. Using dilution curves of the international standard preparations, values of diphtheria, tetanus and pertussis-specific antibody concentrations of the in-house reference serum were determined: DT: 3.0 IU/ml; TT: 3.5 IU/ml; PT: 97 IU/ml; FHA: 292 IU/ml and Prn: 1333 IU/ml. With seven points of 4-fold dilution curve of this in-house reference standard, the dynamic range was much wider than in commercial ELISAs, eliminating the need of multiple dilutions of high titred samples. The analytical range was sufficient to detect low pre-vaccination titers. For comparison, Van Gageldonk et al. [8] prepared two in-house reference standards to perform their pentaplex immunoassay and used six points of a 4-fold dilution curve for pertussis antibodies and eight points of 4-fold dilution curve for DT and TT.

MIA validation

Various investigators used different sources of antigens as well as reagents with different composition, and reports varied considerably in the amount of methodological detail provided (Table 1). Luminex cookbook 2nd edition guidelines for assay validation were used in our study (http://www.luminexcorp.com). The lower limits of detection (LLOD) were 0.31 mIU/ml and 0.35 mIU/ml for DT and TT respectively and 0.012 IU/ml, 0.032 IU/ml and 0.2 IU/ml for PT, FHA and Prn respectively Among the published studies, Reder et al. [3] reported a direct comparison between the developed MIA and 3 different commercial ELISA kits for DT and TT and 2 for PT, but an in-house ELISA for PT was also used (Table 1). Pickering et al. [7] described the measurement of antibodies to diphtheria and tetanus toxin and *Haemophilus influenza* type b capsular polysaccharide using the two step carbodiimide coupling procedure. In the studies of Van Gageldonk et al. [8] and Stenger et al. [10], the performance of the MIA was evaluated by comparing it with an in-house ELISA using the same capture antigens in MIA and in ELISA. If comparisons are made between MIA and ELISA which use identical capture antigens, as well as similar buffers, variability will be minimized, correlations will be good, and similar quantitative values will be achieved. Despite the different origin of antigens used in MIA and in commercially ELISAs in our recent study, we also found good correlation between data obtained by both methods. As it is not easy to purify the respective antigens and as methods may vary between laboratories, the use of commercial ELISA kits and commercial protein antigens for assay development should be preferred for future standardisation of the developed assay and for better data comparison between countries.

For analytical specificity, different methods are used to assess the cross-reaction of antibodies with non-target beads and the reporter

antibody cross-reaction to capture protein antigens coupled to beads. Among the six reported publications (Table1), three [6,8,10] tested the specificity by homologous and heterologous inhibition. In our study, we first performed five separate monoplex assays and compared the data with the pentaplex data. Results showed a good correlation >98% meaning that there was no cross-reaction between coupled beads. Moreover, we obtained very low background levels indicating the absence of cross-reaction between the reporter antibody and the capture antigen [1]. As the protein antigens we used were highly purified, we did not perform inhibition assays. Our coupled beads were stable for over 1 year with an increase of background level with the degradation of the coupling material. For heterophilic and interfering substances, no inappropriate values were observed during the assay validation. Each immunoassay plate contained the three international reference standards as a control and the values obtained after each run were put on a quality control chart. Results of an immunoassay were only validated when the control values were in the ± 2SD range.

Another concern for data comparison between countries is the use of different units in serological studies and how to compare results based on these units [14]. Indeed, the units of measure depend on the origin of serum used for the in-house standard calibration. With standard serum from the Centre for Biologics Evaluation and Research (CBER), results were expressed in ELISA unit (EU/mL) and with the standard from the National Institute of Biological Standards and Control (NIBSC), results can now be expressed in international unit (IU/mL). In contrast with the other studies where CBER reference standard was used for pertussis antibodies and NIBSC sera were used for diphtheria and tetanus, in our developed MIA, all reference standards were from NIBSC and antibodies results were expressed in IU/mL.

MIA limitations and future perspective

Despite the many advantages over ELISA, multiplex immunoassays have some limits. Firstly, uncoupled beads stick to the walls of most tubes and protein coupling should be performed in low binding micro centrifuge tubes as recommended by Luminex (http://www.luminexcorp.com). The beads should be dispersed regularly between the various assay steps to avoid clumping which could lead to a low bead flow in the Luminex device. Secondly, some coupling reagent like EDC and sulfo-NHS are sensible to air exposure and are one-time use solutions. This is a disadvantage when using the one use-coupling kit for assay development. Also, the presence of high salt can interfere with bead classification. In this case, beads tend to spread out on the bead map. To avoid photo bleaching of the Luminex beads, analysis must be performed in obscurity, as photo bleaching can also lead to bead spreading out on the beads gate. Finally, the lack of a commercially certified reference standard for all five antibodies and the limited stability of coupled beads for about one year are crucial points for assay standardisation.

For future studies, the potential of the pentaplex could be increased by combining with the other vaccine components of the existing hexavalent vaccines (eg. *Haemophilus influenza* type b capsular polysaccharide and Hepatitis B surface antigen) and vaccines directed against viral diseases such as Poliomyelitis, Measles, Mumps, Rubella and Varicella. Despite limits, the MIA has many advantages over the classical ELISA for serological studies but the use of the same protocol and materials for MIA development is needed to enable reliable data comparison between different studies.

Conflicts of Interest

The authors declare no conflict of interest.

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References

1. Cabore RN, Pierard D, Huygen K (2016) A Belgian Serosurveillance/Seroprevalence Study of Diphtheria, Tetanus and Pertussis Using a Luminex xMAP Technology-Based Pentaplex. Vaccines 4.
2. McDonald JU, Ekeruche-Makinde J, Ho MM, Tregoning JS, Ashiru O (2016) Development of a custom pentaplex sandwich immunoassay using Protein-G coupled beads for the Luminex(R) xMAP(R) platform. J Immunol Methods 433: 6-16.
3. Reder S, Riffelmann M, Becker C, Wirsing von Konig CH (2008) Measuring immunoglobulin g antibodies to tetanus toxin, diphtheria toxin, and pertussis toxin with single-antigen enzyme-linked immunosorbent assays and a bead-based multiplex assay. Clin Vaccine Immunol 15: 744-749.
4. Basile AJ, Horiuchi K, Panella AJ, Laven J, Kosoy O, et al. (2013) Multiplex microsphere immunoassays for the detection of IgM and IgG to arboviral diseases. PLoS One 8: e75670.
5. Belabani C, Rajasekharan S, Poupon V, Johnson T, Bar-Or A (2013) A condensed performance-validation strategy for multiplex detection kits used in studies of human clinical samples. J Immunol Methods 387: 1-10.
6. Pickering JW, Martins TB, Schroder MC, Hill HR (2002) Comparison of a multiplex flow cytometric assay with enzyme-linked immunosorbent assay for auantitation of antibodies to tetanus, diphtheria, and Haemophilus influenzae Type b. Clin Diagn Lab Immunol 9: 872-876.
7. Prince HE, Lape-Nixon M, Matud J (2006) Evaluation of a tetraplex microsphere assay for Bordetella pertussis antibodies. Clin Vaccine Immunol 13: 266-270.
8. Gageldonk VPG, Schaijk VFG, Klis VDFR, Berbers GA (2008) Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus. J Immunol Methods 335: 79-89.
9. Hansenova MS, van BA, Endtz HP, Bikker FJ, Veerman EC, et al. (2016) Comparison of non-magnetic and magnetic beads in bead-based assays. J Immunol Methods .
10. Stenger RM, Smits M, Kuipers B, Kessen SF, Boog CJ, et al. (2011) Fast, antigen-saving multiplex immunoassay to determine levels and avidity of mouse serum antibodies to pertussis, diphtheria, and tetanus antigens. Clin Vaccine Immunol 18: 595-603.
11. Ibrahim NM, El-Kady EM, Eissa SA, Wahby AF (2016) Assessment of antibody level and avidity against Bordetella pertussis in a cohort of Egyptian individuals aged 1-18 years. J Adv Res 7: 105-111.
12. van Gageldonk PG, von HC, van der Klis FR, Berbers GA (2011) Improved specificity of a multiplex immunoassay for quantitation of anti-diphtheria toxin antibodies with the use of diphtheria toxoid. Clin Vaccine Immunol 18: 1183-1186.
13. Huygen K, Caboré RN, Maertens K, Van Damme P, Leuridan E (2015) Humoral and cell mediated immune responses to a pertussis containing vaccine in pregnant and nonpregnant women. Vaccine 33:4117-23.
14. Barkoff AM, Grondahl-Yli-Hannuksela K, He Q (2015) Seroprevalence studies of pertussis: what have we learned from different immunized populations. Pathog Dis 73.