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A novel multiplex poliovirus binding inhibition assay applicable for large serosurveillance and vaccine studies, without the use of live poliovirus

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A B S T R A C T

Large-scale serosurveillance or vaccine studies for poliovirus using the "gold standard" WHO neutralisation test (NT) are very laborious and time consuming. With the polio eradication at hand and with the removal of live attenuated Sabin strains from the oral poliovirus vaccine (OPV), starting with type 2 (as of April 2016), laboratories will need to conform to much more stringent laboratory biosafety regulations when handling live poliovirus strains. In this study, a poliovirus binding inhibition multiplex immunoassay (polio MIA) using inactivated poliovirus vaccine (IPV-Salk) was developed for simultaneous quantification of serum antibodies directed to all three poliovirus types. Our assay shows a good correlation with the NT and an excellent correlation with the ELISA-based binding inhibition assay (POBI). The assay is highly type-specific and reproducible. Additionally, serum sample throughput increases about fivefold relative to NT and POBI and the amount of serum needed is reduced by more than 90%. In conclusion, the polio MIA can be used as a safe and high throughput application, especially for large-scale surveillance and vaccine studies, reducing laboratory time and serum amounts needed.

1. Introduction

Functional, neutralizing antibodies that prevent infection are a well-known and accepted correlate of protection after vaccination or infection (Plotkin, 2010; Plotkin, 2013). The in vitro neutralization test (NT) to detect and quantify neutralizing serum antibodies directed against polioviruses of types 1, 2 and 3 is the standard assay for poliovirus (PV) serology. However, the PV NT, which is recommended by the World Health Organization (WHO, 1993), is a very laborious, low throughput assay that requires various laboratory technical skills and has an incubation time of five days. In addition, it requires appropriate techniques to guarantee the viability, purity and stability of the used PV strains. A particular problem of this assay is that different virus stocks can vary in their ratio of infectious to non-infectious poliovirus intermediates (Fricks and Hogle, 1990; Curry et al., 1996; Belnap et al., 2000). Because binding of neutralizing antibodies to PV does not always distinguish between infectious and noninfectious virions (Fricks and Hogle, 1990; Lin et al., 2013), this might lead to variation in the titers. In the light of the Polio Eradication & Endgame Strategic Plan *2013–2018*, developed by the Global Polio Eradication Initiative (WHO, 2013) setting the goal of a polio-free world by 2020, the use of live poliovirus will be restricted in the near future. This means that higher biosafety requirements are to be implemented for OPV/Sabin strains, making the NT even more cumbersome. Previously a poliovirus binding inhibition ELISA (POBI), has been developed showing good correlations with the NT (Edevag et al., 1995; Hashido et al., 1999; Herremans et al., 1997; Herremans et al., 2000). Although this assay has the advantage of not having to use live virus, cell culture and subjective visual CPE scoring, the POBI is still low throughput, demands relatively high amounts of serum and the antibodies against all three types have to be determined separately. A capture (binding inhibition) assay reduces nonspecific cross-reactivity with other enterovirus antibodies that can occur in direct ELISA (Reigel et al., 1985; Swanink et al., 1993) increasing the specificity of the POBI assay.

In the present study we have developed a fast, high throughput microsphere-based binding inhibition multiplex assay for simultaneous quantification of poliovirus antibodies (polio MIA) against

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all three types. High biosafety requirements are not required since inactivated poliovirus vaccine (IPV) is used, making the polio MIA a potential alternative for NT and POBI for large-scale monitoring, especially when laboratories are not able to handle live/infectious poliovirus under the new guidelines.

2. Materials and methods

2.1. Serum samples

A total of 228 serum samples were selected from the second cross-sectional population-based serosurveillance study in the Netherlands (van der Klis et al., 2009) and used for development and validation of the poliovirus binding inhibition multiplex immune assay (polio MIA). At time of sampling (2006), the age of these donors (99 male, 129 female) was evenly distributed ranging from 0 to 79 years. Donors aged >50 (n = 82) are considered not to be IPV vaccinated but naturally infected. NT titers ranged from $\log_2 = 2–17$ (type 1), $\log_2 = 1–17$ (type 2) and $\log_2 = 2–17$ (type 3) for the whole panel. From these 228 serum samples, randomly selected subsets were used to asses intra- and inter-assay variation (n = 80), cross-reactive antibodies in a monoplex vs triplex setup (n = 40), differences in incubation time (n = 40) and in the comparison with a polio binding inhibition ELISA (n = 56).

2.2. Polio MIA/POBI reference standard and control sera

A high titered antipoliovirus serum, from two IPV vaccinees (*RIVM MIA reference standard serum*, Bilthoven, the Netherlands) was calibrated against the 3rd International Standard anti-Poliovirus serum Types 1, 2 and 3 (NIBSC code: 82/585 assigned potency 11/32/3 IU/ml) and used as the reference standard in the polio MIA and POBI assays. After calibration, the international standard was used as a control serum.

2.3. Inactivated poliovirus vaccine (IPV)

Monovalent IPV type 1, 2, 3 (National Vaccine Institute NVI/Bilthoven Biologicals, the Netherlands) was used in POBI and polio MIA. The IPV was the final fraction of a vaccine production consisting of monovalent formalin inactivated poliovirus type 1 (Mahoney PV03-114-6.1; 1888 D-antigen units/ml [DU/ml]), type 2 (Mef-1 PV04-209-6.1; 1132 DU/ml) and type 3 (Saukett PV05-3454-6.1A; 1177 DU/ml).

2.4. Neutralization test (NT)

The $\log_2$ reciprocal NT titers from the 228 sera, previously determined (van der Maas et al., 2014), were converted to IU/ml using the potency of the international standard. To this end, the GMT of the reference standard serum (*RIVM NT reference standard serum*, Bilthoven, the Netherlands) and the GMT of the 3rd international standard, used repeatedly as a control sample, were obtained followed by conversion to IU/ml. The international cut-off in the neutralization assay for PV type 1, 2 and 3 is a titer $\geq$1:8 and after conversion 0.23, 0.29 and 0.12 IU/ml respectively in the NT performed at the RIVM lab.

2.5. Poliovirus binding inhibition assay (POBI)

A subset of the serum panel, consisting of 56 samples with a broad concentration range was tested in the POBI. The POBI test was performed according to the method previously described (Herremans et al., 1997), with some modifications. In short, a twofold serial dilution (1:2–1:4096) of serum samples, in-house reference serum *(RIVM MIA reference standard serum*) and a control serum (WHO 3rd International Standard) was made in the plate (Greiner N655180) by adding 75 μl serum to 75 μl dilution buffer (1xPBS, 1% BSA, 0.5% Tween20, 0.5% M NaCl). After adding 75 μl/well monovalent formalin-inactivated vaccine poliovirus vaccine type 1 (20 DU/ml), type 2 (4 DU/ml) or type 3 (16 DU/ml), mixtures were incubated overnight at 22 °C. Maxisorp ELISA plates (Nunc, Sigma-Aldrich) were coated overnight at 4 °C with the IgG fraction of bovine antipoliovirus hyperimmune serum (RIVM) (Herremans et al., 1997) in dilutions of 1:500 for serotypes 1 and 2 and 1:250 for serotype 3 in 0.05 M carbonate-bicarbonate buffer pH 9.6. After blocking the coated plates with 1% BSA in PBS, 100 μl of the pre-incubated serum-virus mixture was transferred to the ELISA plate and incubated for 2 h at 37 °C. For detection, rabbit antipoliovirus of types 1, 2 or 3 hyperimmune serum (RIVM, 1/10,000 in dilution buffer) was added and incubated 1 h at 37 °C. Subsequently plates were incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma A3687, ≈2.4 mg/ml) in a dilution of 1/2000 in dilution buffer and incubated 1 h 37 °C. Then 1 mg/ml *p*-nitrophenyphosphate (*p*NPP) substrate in diethanolamine Buffer (ThermoFischer scientific) was added and incubated 30–60 min at room temperature. Optical density read at 405 nm was converted to IU/ml by interpolation from a 5-parameter logistic (5PL) standard curve of the in-house reference.

2.6. Binding inhibition multiplex immunoassay (polio MIA) for quantification of poliovirus type 1, 2 and 3 antibodies

2.6.1. Conjugation of type specific antipoliovirus monoclonal antibodies to carboxylated beads

Type specific capture monoclonal antibodies (MAb), respectively antipoliovirus type 1 clone 9B4 (HYB 295-17-02 ThermoFischer scientific, Waltham, MA USA), type 2 clone 24E2 (HYB 294-06-02 ThermoFischer scientific) and type 3 clone 4D5 (HYB 300-06-02 ThermoFischer scientific) were conjugated to three distinct activated carboxylated microspheres. Hereby 800 μl of carboxylated microspheres (12.5 × 106 beads/ml; Bio-Rad Laboratories, Hercules, CA USA) were activated by adding 100 μl 50 mg/ml N-hydroxy-sulfosuccinimide (sulfo-NHS, ThermoFischer scientific) and 100 μl 50 mg/ml 1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, ThermoFischer scientific) in PBS. The microspheres were activated at room temperature for 20 min in the dark under constant rotation (25 rpm), washed once with 1 ml PBS pH 7.2 by centrifugation (12,000 × *g* for 4 min) and resuspended in 1 ml PBS pH7.2 containing 50 μg/ml of antipoliovirus monoclonal antibody. The beads were incubated for 2 h at RT in the dark under constant rotation at 25 rpm. Subsequently, the beads were washed three times with PBS pH 7.2 and stored in the dark in PBS containing 0.05% (wt/vol) sodium azide and 1% (wt/vol) bovine serum albumin at 4 °C.

2.6.2. Quantification of poliovirus type 1, 2 and 3 specific antibodies

For quantification of PV antibodies (assay design depicted in Fig. 1), serum, reference standard and control sample dilutions were pre-incubated with a predetermined optimal concentration of IPV 1, 2 and 3, in 2 ml polypropylene tubes (Micronic, Lelystad, the Netherlands). For this pre-incubation, the reference standard *(RIVM MIA reference standard serum)* was diluted in 10 steps of 1.5-fold dilutions (1/18–1/692). Sera was diluted 1/2 and 1/25 and the control serum diluted 1/20 in dilution buffer (PBS pH 7.2 containing 0.25% (v/v) Tween-20, 1% (w/v) BSA and 0.5 M NaCl). Subsequently all dilutions were mixed 1:1 (v/v) with IPV in dilution buffer containing IPV type 1:56 DU/ml, IPV type 2:16 DU/ml and IPV type 3:80 DU/ml, ergo resulting in a final reference serum dilution of 1/36–1/1384, in serum dilutions of 1/4 and 1/50, in a control serum