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Final report

Assessment of vaccination induced pathogen specific antibody level and complement activation on microarray

Vaccination is a very efficient way to induce pathogen-specific immune response and in this way, to prevent outbreaks of epidemics. The levels of pathogen-specific antibodies and the effector functions they induce will determine if the individual is protected against a given pathogen. The effectiveness of immunization is diverse against various pathogens and the protection is fading with time. While traveling long distances becomes easier, individuals may encounter more often even with those pathogens that have been already eradicated in one's living area. So there is a need for simultaneous testing the presence of efficient immunization against several pathogens. Multiplex detection systems are favorable to fulfill this need, like microarray technology in combination with fluorescent detection; we published a topical review in this field for summarizing applicability of microarray in immunoassays (Herbath et al. 2014).

The aim of the project was to develop a multiplex array-based method for determination of complex immune profile of vaccinated individuals. This method allows the parallel measurement of pathogen-specific IgM, IgG and IgA antibodies and, in the same time, the determination of their complement activating properties as well. In this way, not only the level of antigen-specific antibodies but also one of their effector function, the complement activation is measurable.

Technical development

Two parallel platforms of array technology exist: planar microarray - that is applied mainly in this project - and bead-based arrays. The latter one is a more automatized technique with higher sample throughput and can be a promising alternative of the planar microarray. As complement activation measurements were not well defined on this platform, we made effort to find the optimal setup of the technique. We determined the optimal serum dilution and buffers. We also found that rheumatoid factors of IgM isotype altered C3 fragment deposition and introduced false-positive reactivity against viral EBNA-1 antigen (Ayoglu et al. 2014). We further proved that this technique can be used to determine the complex immune profile that contains antigen-specific antibody level and their complement activation as well.

Establishment of serum bank

Beyond technical development of the multiplex antigen specific complement activation measurement, the major aim was to compare pathogen-specific antibody levels and their complement activating capacity. A well characterized serum bank was assembled in the first year of the project, and these serum samples were used in the next two years for microarray development. Based on the 35910-1/2012/EKU (569/PI/12.) ethical permission, serum samples were collected from 95 healthy volunteers. Dr. Zoltán Prohászka (Semmelweis

University, 3rd Department of Internal Medicine, Research Laboratory) helped me in the serum collecting procedure. Serum samples were aliquoted and stored at -70°C, as this storing condition enables subsequent complement measurement. The vaccination protocol has changed many times in Hungary during the last 30 years. In the course of serum collection, individuals born in a narrow time frame (between 1986 and1994) were chosen, as their vaccination had been carried out based on similar protocols. It would be very interesting to compare immune profiles of individuals derived from different age groups, but these measurements would be beyond the financial limit of the current project.

Characterization of the serum bank

Seven pathogens were selected from those which are included in the Hungarian childhood immunization protocol. Bacterial (pertussis, tetanus, diphtheria) and viral (mumps, measles, rubella and hepatitis B virus) pathogens were also included. Commercially available diagnostic ELISA kits were applied for measurement of pathogen-specific IgG level. Antigen-specific IgM level was determined as well for viral pathogens. Since IgM antibodies are effective activators of the complement system, determination of pathogen-specific IgM level was important in the later phase of the project. IgG and IgA seropositivity against Bordetella pertussis was tested by two different ELISA kits, one was coated with filamentous haemagglutinin (FHA) and specific for Bordetella pertussis and also Bordetella parapertussis species, while the other used pertussis toxin (PT) as antigen and specific only for the Bordetella pertussis.

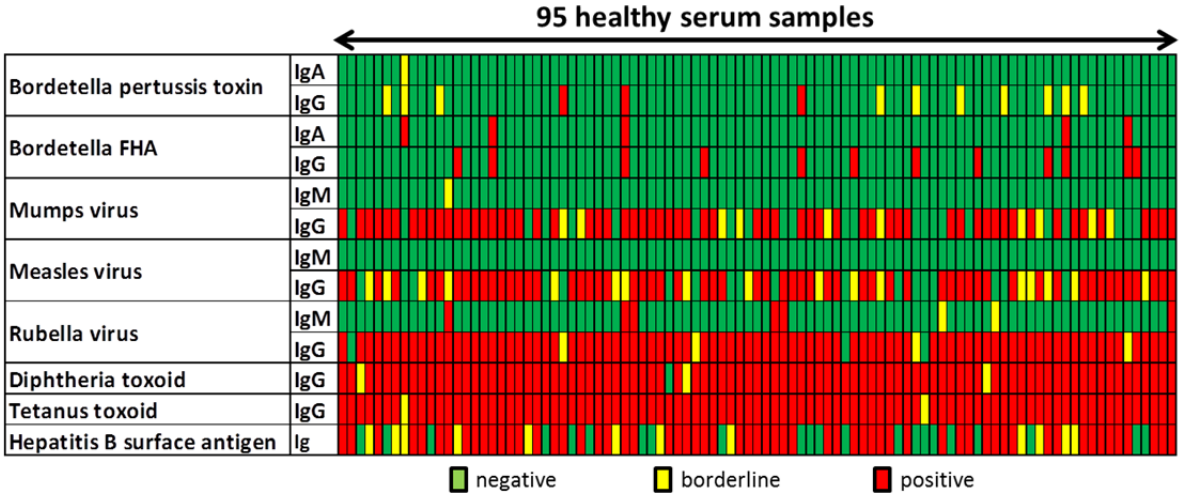

Figure 1. Levels of pathogen-derived antigen-specific antibodies in 95 serum samples from immunized individuals, tested by ELISA kits

Vaccination efficiency against the tested pathogens can be sorted in three groups based on percentage of IgG seropositivity: high percentage of positive individuals (above 90%) for rubella, diphtheria, tetanus; medium percentage (around 60%) for mumps, measles, hepatitis B virus; low percentage for pertussis FHA (13%) and pertussis PT (3%). The cut off limit was applied in each case as the given diagnostic test suggested. Results derived from this limited number of serum samples collected from a narrow age group of the population are of course

not suitable to draw population scale conclusions about the effect of the vaccination, but are useable to validate the microarray measurements in further steps of the project.

Setup of microarray measurements

Optimization of reaction condition and selection of suitable antigens for microarray experiments was carried out on test slides printed by a Calligrapher miniarrayer (BioRad) contact printer. This printer is not a suitable device for printing many slides but a robust technique and readily available for testing various conditions. 1-4 various antigens of each selected pathogens (pertussis, tetanus, diphtheria, mumps, measles, rubella and hepatitis B) were purchased from different sources and were printed in serial dilution onto nitrocellulose coated slides. Whole killed microbes and pathogen-derived purified proteins or protein fragments were also used as antigen on the array. Fifteen other microbes from various sources were also printed next to the selected pathogens (Staphylococcus aureus, Candia albicans, Aspergillus brasiliensis, Escherichia coli etc). Printed purified human proteins (IgG, IgM, IgA, C3, C4) served as technical controls of the experiment. Slides were incubated with 10 times diluted serum samples as earlier results showed that this serum concentration gave the highest signal to noise values. Following serum treatment, the amount of printed antigen-bound IgM, IgG, IgA antibodies and C3, C4 fragments were determined by fluorescently labeled detecting antibodies in two distinct slides. The serum diluting buffer was supplemented with EDTA for antibody and with Ca^{2+}-Mg^{2+} ions in physiological concentration for complement fragment measurements. 11 tested serum samples were chosen based on their reactivity against the seven pathogens that was determined by conventional ELISA tests in the 1st year of the project. Serum samples with low and high pathogen-specific IgG levels were tested. Based on these experiments the optimal concentrations of antigens were determined. Some of the tested antigens were not suitable for microarray experiment as their concentrations were not high enough or they contained some kind of additives or contamination that interfered with microarray studies. I could find at least one proper antigen from each of the tested seven pathogens. These microarray results were comparable with values derived from conventional ELISA tests. There was discrepancy between microarray and ELISA results only in case of the hepatitis B virus antigens, which might be caused by the application of non-suitable antigen or the non-conventional ELISA setup of the commercially available test.

Measurement of serum immune profile by protein microarray

After optimization procedure, as many microarray slides were printed that were suitable for testing all the 95 serum samples against 41 different types of antigens those were printed in triplicates and in some cases also in various dilution. For technical reasons this printing was carried out on a sciFlexarrayer S11 printer that uses non-contact ink-jet technique that enabled to produce enough pieces of slides in a single run. This printer is not available in Hungary and unfortunately for reasons beyond my control this procedure took much more time than I expected, so the final results are not published yet. The single company in Europe that has printing facility with this device did not finished the procedure in promised time but many months later. The change of printer device required minor modification; Table 1 shows the

Pathogen antigens	
	Diphtheria toxoid
	HbsAg
	Bordetella FHA
	Pertussis toxoid
	Pertussis vaccine
	Rubella virus
	Mumps virus
	Measles virus
	Tetanus toxin
Various microbes	
	Herminiimonas saxobsidens
	Sphingobium scionense
	Candida albicans
	Enterobacter aerogenes
	Escherichia coli
	Pseudomonas aeruginosa
	Staphylococcus aureus ssp. Aureus
	Pusillimonas ginsengisoli
	Kocuria varians
	Staphylococcus
Complement proteins	
	C1q
	C3
	C4
	factor H
	factor P
Human antibody	
	IgM
	IgG
	IgG1
	IgG2
	IgG3
	IgG4
	IgA serum
	IgA secreted
	IgE
Other	
	acetilated BSA
	BSA
	LPS
	mannan
	protein G
	protein A
	PBS
	ssDNA

Table 1. *List of printed antigens*

final list of antigens. GenePix Pro software was used to quantify the fluorescent signal on each spots. Microarray data was normalized on printed IgM, IgG and IgA spots for antibodies and on printed protein G spots for deposited C3 and C4 fragment measurements. Protein G spots reflects also the overall activity of the complement system in a given serum sample and not only technical controls like printed C3 and C4 molecules. A large database was constructed from ELISA and microarray derived data. The great size of dataset does not enable to present here all data, therefore I selected only a few representative results.

Validation of protein microarray measurements

In the first step, ELISA and microarray data was compared to see if this multiplex measurement is comparable with ELISA method. We found significant strong positive correlation (r = ~0.8) in case of pertussis toxin-, pertussis FHA-, tetanus toxin- and diphtheria toxin-specific IgG antibody levels measured by ELISA and protein microarray (Figure 2.). This suggests that our protein microarray results are comparable with the officially used ELISA technique. Correlation coefficient was not determined for mumps-, measles-, rubella-specific IgM and pertussis toxin-specific IgA levels, as there were not enough positive serum samples in the tested cohort. High level of pathogen-specific IgM would indicate fresh infection that is not very likely in our case, since individuals were immunized several years before collection of blood. IgG signals on printed mumps and measles virus containing spots were low, not useable to draw reliable conclusions, maybe the change in the printing technique caused the low signal. There was weak (r=0.3) correlation between ELISA and microarray data in case of rubella virus antigen. The discrepancy could be derived from the

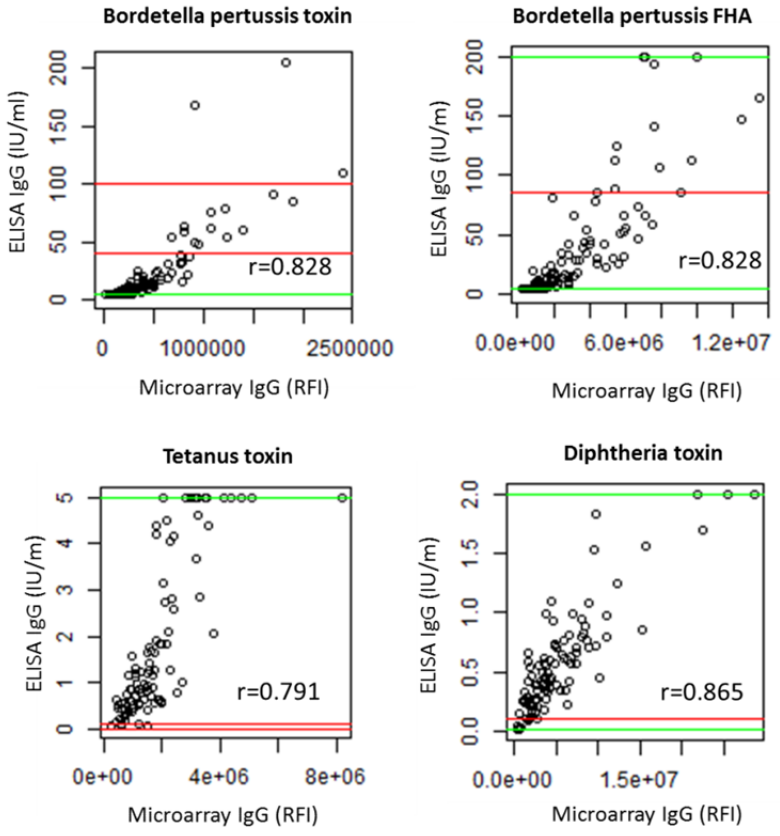

Figure 2. *Correlation between ELISA and microarray measurements. Antigen-specific IgG level, determined by ELISA, was plotted as a function of IgG level determined by protein microarray. Each spot represents a single serum sample. Red lines close the borderline region while green lines indicate the detection limits of ELISA measurements. Only significant ($p<0.05$) Pearson correlation coefficients were indicated on graphs.*
IU-International Unit; RFI-Relative Fluorescent Intensity

different virus strains those were applied for ELISA and microarray measurements, HPV-77 and RA27 strains, respectively. Unfortunately I could not purchase the same rubella virus strains for microarray tests.

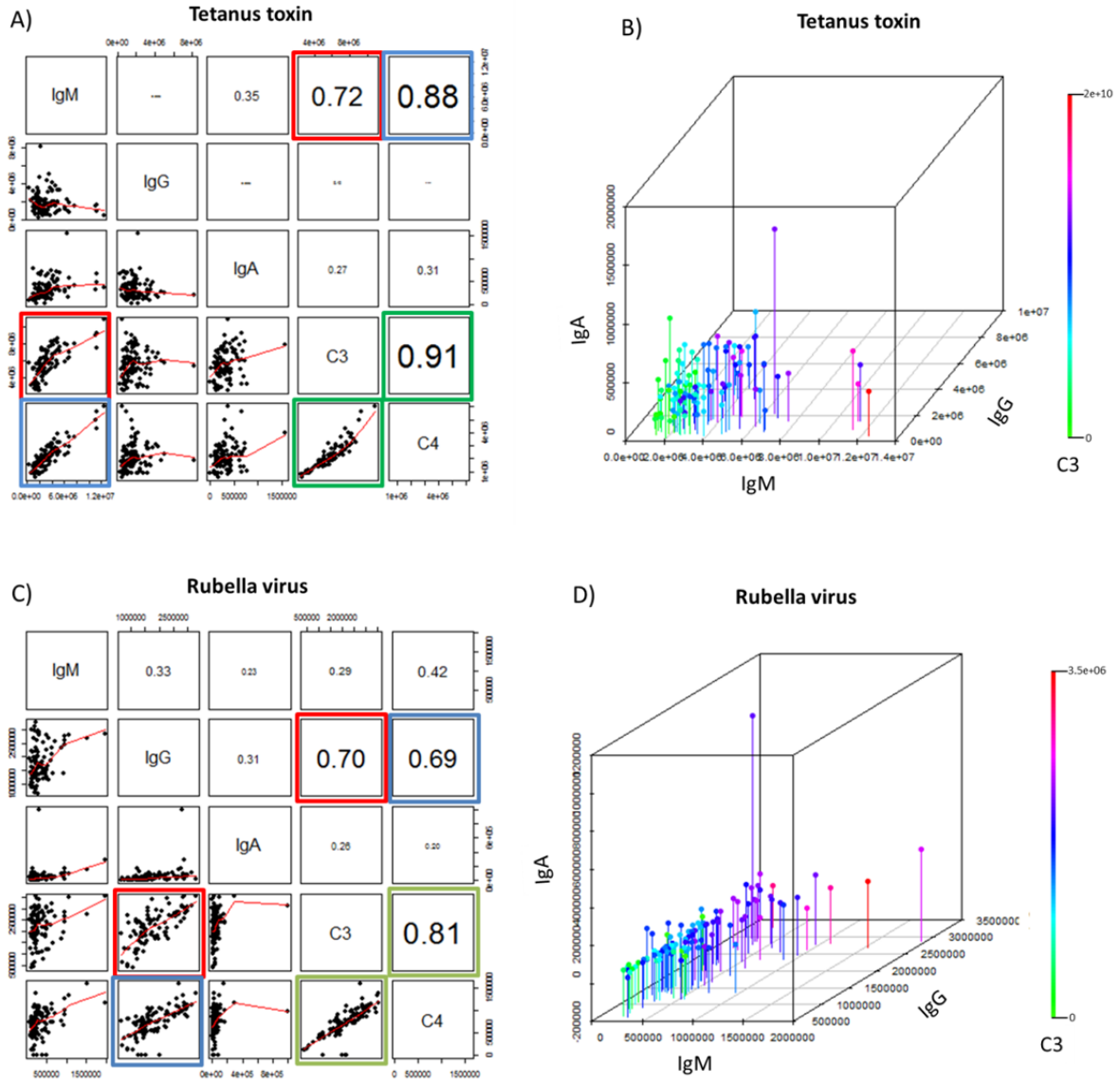

Figure 3. *Correlation of antibody binding and complement activation on tetanus toxin (**A-B**) and rubella virus (**C-D**) printed spots. **A,C**) The five parameters were correlated to each other. The plotted parameters (IgM, IgG, IgA, C3, C4) were written in the diagonal line while plots are below and Pearson correlation coefficients are above the diagonal. Colored squared were used to emphasized the strongest correlations and similar colors were used to easily connect the given plots and correlation coefficient. **B,D**) Antigen specific IgM, IgG and IgM levels were plotted in 3D graph and the amount of deposited C3 fragments are indicated by color codes.*

Comparison of antigen-specific antibody level and complement activation

Correlation between the levels of antigen-specific IgM, IgG and IgA and the amount of deposited C3 and C4 fragments were calculated. We found very strong (r>0.8) correlation between the bound C3 and C4 fragments in all cases. Antibody-antigen complexes induce mainly activation of the classical complement pathway, in this way C4 and in a later step of the cascade C3 fragment deposition occurs, explaining the high correlation coefficient.

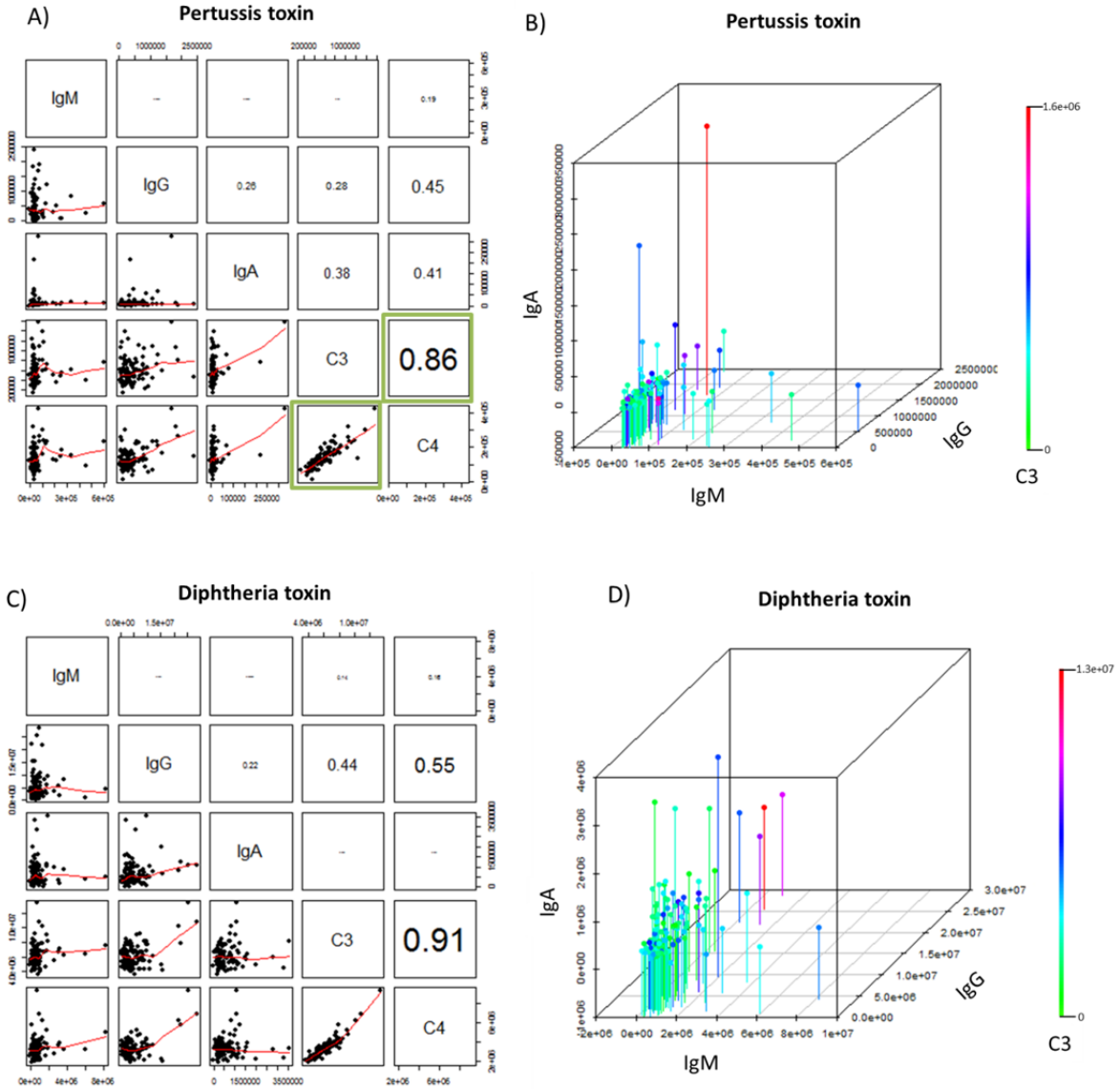

Figure 4. *Correlation of antibody binding and complement activation on pertussis toxin (**A-B**) and diphtheria toxin (**C-D**) printed spots. **A,C**) The five parameters were correlated to each other. The plotted parameters (IgM, IgG, IgA, C3, C4) were written in the diagonal line while plots are below and Pearson correlations coefficients are above the diagonal. Colored squared were used to emphasize the strongest correlations and similar colors were used to easily connect the given plots and correlation coefficient. **B,D**) Antigen-specific IgM, IgG and IgM levels were plotted in 3D graph and the amount of deposited C3 fragments are indicated by color codes.*

Both IgM and some IgG isotypes activate the classical pathway well. The major contributor Ig isotype was different for various antigens. In case of tetanus toxin antigen, for example, the level of toxin-specific IgM antibodies determined the antigen-specific complement activation in this way C3 and C4 fragment binding (r=0.72 and r=0.88) (Figure 3.). The serum samples contained tetanus toxin-specific IgG antibodies that was measured by both ELISA and protein microarray technique. The contribution of IgG to complement activation is minimal here or it increased the complement activation to an elevated level and further complement activation was driven by antibodies with IgM isotype. In contrast to tetanus toxin, the rubella virus specific IgG level showed strong correlation (r=0.7) with complement activation in the tested serum samples. Only a few samples contained rubella specific antibodies with IgM isotype and these serum samples also contained high antigen specific IgG level.

There was not strong correlation between any Ig isotypes and complement activation by pertussis toxin. Based on ELISA, the protectivity against pertussis toxin faded away as there was only a few individual in this cohort with suitable high IgG level. But complement measurement revealed several serum samples those did not give enough high IgG level yet resulted strong complement deposition. Maybe protection against pertussis toxin exists also in these samples.

ELISA measurement showed enough diphtheria toxin-specific IgG for protection almost in each sample. It is interesting that the level of IgG did not correlate well with complement activation. The value of correlation coefficient (r=0.44 and r=0.55) would be zero if the 4 samples with extreme high antigen specific IgG is omitted from calculation.

Summary

In summary we were able to determine complex immune profiles of immunized individuals by multiplex protein array method. These results strongly correlated with the conventional ELISA method that measured pertussis, tetanus and diphtheria pathogen-specific IgG antibody levels. Measurement of pathogen-induced complement activation revealed that complement activation does not necessary correlate strongly in each cases with the antibody levels. We are planning to carry out toxin/virus neutralization assays - that can more precisely predict the protectivity - on these serum samples and we will determine its correlation with antigen-specific complement activation measurement.

References those are already published as results of the project

1) Herbáth M, **Papp K**, Balogh A, Matkó J, Prechl J: **Exploiting fluorescence for multiplex immunoassays on protein microarrays**, METHODS AND APPLICATIONS IN FLUORESCENCE 2:(3) Paper 032001. 26 p. (2014)
2) Ayoglu B, Szarka E, Huber K, Orosz A, Babos F, Magyar A, Hudecz F, Rojkovich B, Gáti T, Nagy G, Schwenk JM, Sármay G, Prechl J, Nilsson P, **Papp K**. **Bead arrays for antibody and complement profiling reveal joint contribution of antibody isotypes to C3 deposition**, PLOS ONE 9:(5) Paper e96403. (2014)