Supplementary material: Study participant selection criteria:

**Study design and site**

This was a retrospective analysis of a prospective cohort on the immunogenicity of vaccines within the Expanded Program of Immunization in South Africa (EPI-SA) in HIV infected, HIV exposed uninfected and HIV unexposed uninfected children as the control group. These children were predominantly from the public sector either born or receiving care at the Chris Hani-Baragwanath Hospital in Johannesburg, and at Tygerberg Hospital in Stellenbosch, South Africa. HIV-uninfected children were recruited from the maternity wards of the respective study sites by a trained health worker in HIV counselling. The mothers must have tested HIV negative based on their participation in the mother to child transmission programme that was conducted at each study site. HIV exposed uninfected children were recruited from a cohort of children originally identified to participate in the “Children with HIV Early Antiretroviral (CHER)” study, but the child tested HIV negative at 4 weeks of age by polymerase chain reaction (PCR). HIV-exposed infected children were identified to be HIV-infected within the CHER study and had to have a baseline CD4+ T-lymphocyte count available. These children were randomized to either early or delayed antiretroviral treatment groups. The vaccination schedule was based on the WHO and South African Department of Health guidelines. Quantitative antibody responses to DTwP-Hib-CV and Hepatitis B vaccines among HIV- uninfected (exposed and non-exposed) and HIV-infected children stratified by antiretroviral therapy status were measured.

**Study population, randomization, inclusion and exclusion criteria**

The study was conducted on archived serum samples from 578 children in whom the effect of HIV exposure and timing of ART initiation was evaluated on the quantitative and qualitative antibody responses to pneumococcal conjugate vaccine as the primary objective. [[1](#_ENREF_1)] Five groups of children, aged at 6-12 weeks, were enrolled from April 2005 to June 2006. This included HIV-uninfected children born to HIV-uninfected mothers (HUU; N=124); and HIV-exposed uninfected children born to HIV-infected mothers (HEU; N=125). In addition, peri-natal HIV-infected children with a CD4+T-lymphocyte cell percentage ≥25% at baseline were randomized to initiate ART when clinically and/or immunologically indicated (ART-Def; N=105) as per then WHO recommendations, or immediately upon enrolment (ART-Immed; N=210). [[2](#_ENREF_2)] Furthermore, a convenience sample of HIV-infected children with CD4+<25% initiated on ART at enrolment (ART-CD4<25%; N=14) were included. HIV-infected children were co-enrolled from the “Children with HIV Early Antiretroviral (CHER)” Study as described. [[2](#_ENREF_2)] Children were included in the study had to be between four and ten weeks of age and have a birth weight of at least 2000 grams. The parent or legal guardian of the child had to have indicated that they would stay in the study area for the study duration. Documentation of the mothers HIV status at 24 weeks had to be available, and breastfeeding was permitted. Inclusion criteria for HIV-infected children were that the child had to be co-enrolled in the CHER study, HIV status had to have been confirmed by a positive DNA or RNA polymerase chain reaction and a second one at four to ten weeks of age. Children born to HIV-infected mothers were allowed into the study if they were ART naïve except for receipt of ART to prevent mother to child transmission. Allowed medications were antipyretics for auxiliary temperatures ≥38°C after vaccination. HIV-infected children were allowed to receive ART as part of the study as well as trimethroprim-sulfamethoxazole prophylaxis as per the South African guidelines. Children were excluded from the study if they had received any blood products, any vaccine with the exception of BCG and oral polio vaccine, prior to study entry. Children who had received immunosuppressant agents for more than 2 weeks and had were unable to tolerate oral medication. Presence of any major congenital anomalies that were life threatening. Children with acute, moderate to severe intercurrent illness or fever ≥38°C within 72 hours before vaccination that resulted in hospitalization, were excluded from the study. The presence of Grade 2 allergic reaction and any Grade 3 clinical toxicity; the presence of any Grade 3 or 4 clinical or laboratory toxicity; were excluded from the study. Parent or guardian unable or unwilling to attend scheduled study visits. The use of ART other than the regimens described within the study, systemic cytotoxic chemotherapy and interleukin or other immune modulators.

**Antiretroviral therapy administered:**

At Chris-Hani Baragwanath Hospital, mothers who were identified as HIV positive received a single dose of nevirapine at the time of delivery, this was also give to their child at the time of delivery to prevent mother-to-child transmission. While atTygerberg Hospital the regimen consisted of zidovudine given to the HIV positive mother from 34 weeks gestation and to the neonates for 7 days and a single-dose nevirapine to both at the time of delivery. ART received during the study was as follows: first-line antiretroviral was zidovudine at a dose of 240mg per square meter of body-surface area twice daily; lamivudine at a dose of 4mg per kilogram of body weight twice daily and lipinavir (300mg)-ritonivar (75mg) given twice daily up-to 6 months of age and thereafter at 230mg and 57.5mg respectively.

Supplementary material: Luminex methods and validation results:

**Methods:**

We had validated the antibody measures of the Luminex assays for DT, TT, PT, FHA and HBsAg in reference to commercially available individual ELISA assays in a separate group of 25 children enrolled in a previous study which involved 4 to 7 year old children who had documented DTwP-Hib and HBV vaccination during infancy.[[3](#_ENREF_3)] Antibodies to DT and TT were measured with the anti-diphtheria toxoid and VaccZyme TM anti-tetanus toxoid IgG EIA (MK014 and MK010, The Binding Site Ltd, Birmingham, England), with concentrations of ≥0.004IU/ml and ≥0.0093IU/ml regarded as positive, respectively.

Antibodies to PT and FHA were measured with the Serion enzyme-linked immunosorbant assay classic kit (ESR120G, Serion Immundiagnostica GmbH, Würzburg, Germany), with a titer of ≥30FDA-U/ml being considered as sero-positive. Antibodies to HBsAg were measured using ImmunoLISA TM HBsAb (Organics Ltd. Part of the Inverness Medical Innovations Group, Israel), with titers ≥10mIU/ml considered as sero-positive. All reagents including controls were supplied with the commercial kits and ELISA assays were performed according to the manufacturers’ instructions.

Antigens used in the multiplex Luminex assay were sourced as follows: diphtheria- toxoid (DT; batch number D52-2) and TT (batch number T177-1) from Staten Serum Institute, Denmark, pertussis toxin (PT; batch number 181209A1) and filamentous hemagglutinin (FHA; batch number: 17040B1) from List Biological Laboratories, California, USA and hepatitis B surface antigen (HBsAg, Engerix B B59, Batch number AHBVBPA 097) from GlaxoSmithKline, Rixenstadt, Belgium. The multiplex anti-human IgG antibody assays were performed similarly as described,[[4](#_ENREF_4)] but modified by exclusion of anti- polyribosyl-ribitol phosphate (PRP) and inclusion of HBsAg, FHA and PT. Serum samples, reference standards and controls were diluted in phosphate buffered saline with 10% fetal bovine serum and 0.05% sodium azide (PBS-FN) and added to filter plates that were pre-blocked with the same buffer. An in-house reference standard was used for all assays, that consisted of pooled human serum that had been calibrated against International standards (NIBSC code 00/496 (diphtheria antitoxin), 76/589 (tetanus antitoxin), 07/164 (anti-HBsAg immunoglobulin) and 06/140 (Pertussis antiserum (human) 1st IS). Serum antibody values were either assigned in IU/ml for DT, TT, PT, FHA and mIU/ml for HBsAg.

The in-house reference serum was diluted in seven 4-fold dilutions (1/50–1/204800). The R2 obtained was ≥0.99 for the study reference tested for antibodies against DT, TT, PT FHA and HBsAg with percentage of relative error (%RE) being less than 20% for the seven step dilution series. Percentage recovery was set to be between 80 to130% and all antibodies tested fell well within the set criteria (range: 96-105%). For quality control purposes positive and negative controls were included in duplicate in all tests.

Statistical analysis: Correlation between ELISA and multiplex assay was measured with Pearson’s correlation coefficients (R2) and *kappa* statistic used to compare the proportion of samples above or below specified thresholds between ELISA and the multiplex assay. Percentage recovery of specific antibodies was determined by recalculating the concentration of each reference standard obtained by extrapolating its response (MFIs) through the fitted curve. The RE of each standard point was as follows: %RE = [(recalculated value – assigned value)/assigned value] x 100%.

**Results**:

The specificity of the assay was >92% for all tested antibodies and the coefficient of variation for intra- and inter-assay ranged between 5-22% for individual assays. The correlation coefficients of individual ELISAs to multiplex Luminex assay was 0.97, 0.93, 0.97, 0.99 and 0.72 for DT, TT, PT, FHA and HBsAg respectively; supplementary Figure 1. There was greater than 92% agreement in proportion of children with antibody threshold above sero-protective titers for DT, TT and HBsAg and complete concordance for the proportion of samples that were sero-positive for PT and FHA between the Luminex and uniplex assays.

**Discussion:**

We describe for the first time the determination of anti-HBs with a multiplex platform. We also show that a single reference serum can be used to verify all the test antibodies. Commercially available antigens with high purity were utilized to ensure antigen availability for future testing. The major advantage of this multiplex assay was the need for small sample volumes (5µl) compared to ELISAs which ranged from 10µl to 100µl of sample. In addition, reduction of assaying time with the multiplex assay, that required 2.5 hours to test 38 samples for all five vaccine antigens simultaneously; instead of the 10.5 hours required for ELISAs. Studies have demonstrated the ability of ELISAs to accurately determine vaccine responses [[5](#_ENREF_5)], but with advances in the vaccinology fields of new vaccines with increased number of combined antigens the use of ELISA has become increasingly unsuitable.[[6](#_ENREF_6)] The ability to effectively multiplex a range of biological analytes has been shown for cytokines,[[7](#_ENREF_7)] bacterial pathogen nucleic acid [[8](#_ENREF_8)] as well as antigen and antibodies to specific pathogens [[4](#_ENREF_4), [6](#_ENREF_6), [9](#_ENREF_9)].

The optimal bead conjugation concentration and procedure was found to be similar to those reported for elsewhere.[[9](#_ENREF_9)] MFIs generated from all five conjugated microsphere bead regions showed linearity over a seven step 4-fold reference serum dilution range, the consequence of this increase in range was the resultant increase in accuracy, a reduction in the number of sample dilutions required and the ability to detect much lower antibody levels. MFI comparisons between uniplex and pentaplex assays allowed for determination of interference between the microsphere bead regions. Multiplexing did not affect specificity and sensitivity of the assay when compared to monoplexing. The multiplex assay was found to be reproducible, with minimal variability inter- and intra assay, and correlated well with commercially available ELISAs.

**References:**

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Supplementary Figure 1: Correlation between ELISA and Luminex assays.