Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Wei Dong Date

Approval Sheet

Vaccination of HIV-Infected Pregnant Women with Trivalent Influenza Vaccine in the Prevention of *Streptococcus pneumoniae* and *Staphylococcus aureus* Colonization during Early Infancy in South Africa

By

Wei Dong

MPH

Epidemiology

_____ [Chair's Signature]

Keith P. Klugman, MD, PhD.

Committee Chair

_____ [Member's Signature]

Jorge E. Vidal, PhD, MSc.

Committee Member

Vaccination of HIV-Infected Pregnant Women with Trivalent Influenza Vaccine in the Prevention of *Streptococcus pneumoniae* and *Staphylococcus aureus* Colonization during Early Infancy in South Africa

By Wei Dong

M.S., Indiana University Bloomington, 2011

Thesis Committee Chair: Keith P. Klugman, MD, PhD.

An abstract of

A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University

in partial fulfillment of the requirements for the degree of
Master of Public Health in Department of Epidemiology, 2013

Abstract

Vaccination of HIV-Infected Pregnant Women with Trivalent Influenza Vaccine in the Prevention of *Streptococcus pneumoniae* and *Staphylococcus aureus* Colonization during Early Infancy in South Africa

By Wei Dong

Background: Synergistic lethality of influenza and bacterial pathogens such as *S. pneumoniae* is greatly attributable to the influenza virus associated morbidity and mortality. Data on the efficacy of maternal vaccination of trivalent, inactivated influenza vaccine (TIV) to protect children from influenza and pneumococcal pneumonia, particularly in Africa, is limited. This study evaluated the efficacy of TIV in reducing the risk of *S. pneumoniae* and *S. aureus* acquisition among HIV seropositive children at early infancy.

Method: Nasopharyngeal (NP) specimens were collected and DNA was extracted utilizing the easyMAG 2.0.1 system. Real-time PCR was performed to detect and quantify *Streptococcus pneumoniae* and *Staphylococcus aureus* in the specimens. Association between treatment group and early bacteria acquisition is assessed by cross-tabulation; Kaplan-Meir curves and Cox-proportional model were employed to compare hazards in two groups and a mixed model was used to evaluate the change of colonization density over the three schedule visits.

Results: Laboratory immunogenicity test revealed that the flu vaccination failed to yield immunogenicity against *S. pneumoniae*. The odds ratio between TIV group and placebo group in early *S. pneumoniae* acquisition was 1.01 (95% CI: 0.50-2.04) and was 1.03 (95% CI: 0.51-2.10) in early *S. aureus* acquisition. Taking the placebo group as the reference group, the hazard ratio in *S. pneumoniae* acquisition was 1.2 (95% CI: 0.72-1.87) and the hazard ratio in *S. aureus* acquisition was 1.62 (95% CI: 0.89-2.94). There was therefore no significant percent change in density of *S. pneumoniae* and *S. aureus* in either TIV group or placebo group over the three schedule visits.

Conclusion: Maternal TIV immunization is not efficacious in either delaying incidence of first *S. pneumoniae* or *S. aureus* colonization, or decreasing the density of colonization from both bacteria in HIV seropositive infant younger than 6 months of age. Further evaluation of effectiveness of maternal TIV immunization should be implemented among HIV uninfected infants.

Key Words: Maternal immunization, Trivalent Influenza Vaccine, *Streptococcus pneumoniae, Staphylococcus aureus,* HIV

Vaccination of HIV-Infected Pregnant Women with Trivalent Influenza Vaccine in the Prevention of *Streptococcus pneumoniae* and *Staphylococcus aureus* Colonization during Early Infancy in South Africa

By Wei Dong

M.S., Indiana University Bloomington, 2011

Thesis Committee Chair: Keith P. Klugman, MD, PhD.

A thesis submitted to the Faculty of the

Rollins School of Public Health of Emory University

in partial fulfillment of the requirements for the degree of

Master of Public Health in Department of Epidemiology

2013

Table of Contents

Background

Acute respiratory infection (ARI) is the leading cause of morbidity and mortality worldwide in which influenza is a major cause that can be prevented with vaccination (1). It is also a significant cause of morbidity and mortality particularly during childhood, especially in children less than five years of age (2). The potential way to prevent influenza disease through vaccination may contribute to reducing childhood deaths in developing countries such as sub-Saharan Africa. Since influenza illness is a vaccine preventable disease, vaccines are developed, licensed and available at a reasonable cost. Unfortunately, infants under 6 months of age are at the highest risk for severe disease associated with influenza, for whom trivalent inactivated influenza vaccine (TIV) is poorly immunogenic and not licensed (3). As pregnant women also have an increased risk of serious illness from influenza infection, maternal TIV vaccination/immunization is a critically important path to prevent influenza related morbidity and mortality in the developing world and to prevent complications of influenza in pregnant women and young infants. Growing evidence indicates TIV induced antibodies can be passed on to the developing fetus due to transplacental transfer from the mother (4). Meanwhile, lack of information on effectiveness and concerns about safety contributed toward the virtual non-existent use of TIV vaccination in pregnant women from low or middle income countries, including South Africa.

There was a national campaign for influenza vaccination of pregnant women in South Africa, due to the concern of continued circulation of the H1N1-2009pdm influenza virus

during 2010. However, in spite of all the efforts taken, the uptake of the vaccine remained poor. TIV immunization of pregnant women is still not provided as standard of care to pregnant women attending antenatal-clinics in sub-Saharan Africa, partially because of the absence of data from African countries with regard to its risk-benefit ratio. Recently, data has become available from Bangladesh in which maternal TIV vaccination was associated with a 63% (95% CI: 5 to 85%) reduction in laboratory-confirmed influenza illness in infants under 24 weeks of age and also a 36% reduction in clinical illness among TIV-vaccinated mothers (5). However, there has not been any study on the effectiveness of maternal immunization with TIV on influenza- associated morbidity and mortality either in the mothers or infants in sub-Saharan Africa. Following the 1918 pandemic, the association between influenza and pneumonia came into focus during which an estimated 40 to 50 million people died; most of the deaths were caused by secondary bacterial pneumonia infection. Influenza was shown to predispose to bacterial infections, pathogens that colonize the nasopharynx *S. pneumoniae* and *S. aureus* were the most commonly isolated organisms (1).

It has been observed that much of the influenza virus associated morbidity and mortality may be due to the synergistic lethality of influenza with bacterial pathogens leading to pneumonia as well as other viral co-infections. Superimposed bacterial infections, especially *S. pneumoniae* and *S. aureus* contribute to a large proportion (28-65%) of pneumonia deaths associated with influenza illness during pandemics(6, 7). Synergistic lethality or pathogenesis between influenza and bacterial co-infection has been observed in animal models since after influenza virus was isolated from the early 1930's (8).

It has been demonstrated that interactions between influenza virus infection and subsequent bacterial infection leads to a synergistic susceptibility to bacterial pneumonia within the week or two following exposure to influenza (1, 9-11). Such idea agrees with the phenomenon that the majority of the influenza related deaths during flu pandemics are due to bacterial co-infections in addition to the viral infection itself (6, 12, 13). The mechanism of synergism between influenza virus and pneumococcus remains unclear. One hypothesis is that the innate immune response is altered in the host after viral infection, which leads to exacerbations of various innate components in the post-influenza state compared to the pre-influenza state. The possibility exists that the host is predisposed to invasion and attachment of the bacteria leading to pneumococcal disease which reduces the ability of the host to clear the pneumococcus due to alteration of the immune response, leading to severity in inflammation and disease. It is evident that the entire process involves several factors and is complex. (1, 14, 15) Additionally, recent research reveals that influenza infection increases susceptibility to pneumococcal acquisition. In a model of ferret transmission, Mc Culler *et al.* demonstrated pneumococcal transmission and disease was promoted with prior influenza infection and the promotion is manifested by an increase in susceptibility to pneumococcal acquisition in terms of percentage of infection and distance over which they could acquire infection, though this effect is pneumococcal strain dependent.(16)

Hence, we proposed to look at the colonization of *S. pneumoniae* and *S. aureus* since colonization could be a precursor to disease (14), to determine and compare the efficacy

of TIV against first acquisition of *S. pneumoniae* and *S.aureus* between infants of TIV-vaccinated and -unvaccinated HIV-infected pregnant women from time of first visit of the infant in the influenza season. It has also been demonstrated that HIV-infected children are at greater risk for severe influenza illness (17), leading to subsequent bacterial infection.

Our exploratory objectives in the colonization study include: 1) To determine and compare the efficacy of TIV against first acquisition of *S. pneumoniae* and *S.aureus* between infants of TIV-vaccinated and -unvaccinated HIV-infected pregnant women from time of first visit of the infant in the influenza season 2) To compare *S. pneumoniae and S.aureus* carriage acquisition in maternal and infant nasopharyngeal samples between subjects infected and non-infected with influenza by vaccination status in the influenza season.

Methods

Study Design

The study design of the primary project conducted in South Africa is a randomized clinical trial. Our study is a secondary analysis and data on treatment group was retrieved from the primary project, which is the exposure of interest. The primary outcome variable is early acquisition, which is defined as time to acquisition earlier than the median of time to acquisition in the study population. The secondary outcome variable is bacterial density and the outcome was log-transformed due to the right skewness of density. Our research hypothesis is that maternal immunization of the influenza vaccine will delay the acquisition and decrease the density of *S. pneumoniae* and *S.aureus* colonization among HIV seropositive children at early infancy during the 2011 flu season.

This study is a secondary epidemiological study with de-identified subject I.D. information extracted from the primary study and therefore is considered as "non-human subjects research", which is exempted from IRB submission.

Sample size

To detect a 25% reduction in early pneumococcal acquisition with a power of 0.8 and alpha level of 0.05, a total sample size of 116 is needed and equally distributed in two groups, which was calculated by SAS Power and Sample Size software. In our study, the total sample size of our study population is 124, among which 67 infants were assigned to vaccine group and 57 infants were from placebo group. The study population consists of 10 cases of Laboratory confirmed illness (LCI) and 114 cases of Influenza like illness

(ILI). The criteria for diagnosis of ILI were based on the detection of at least 3 of 4 symptom-groups of less than 7 days symptom duration. These symptom groups were 1) cough; 2) history of fever, chills, or rigor; 3) sore throat, pharyngitis, or laryngitis; and 4) myalgia or headache.

The study also included 26 maternal participants who received either placebo or TIV between 29 weeks to 53 weeks of gestational age prior to the onset of the influenza season. Mothers and infants were followed until the infants reached 24 weeks of age or if they discontinued the study for any reason. Mothers had 6 schedule visits, samples for analysis were collected at visit 1, 2, 3 and 6 from the mother and at visits 3, 4, 5 and 6 from the baby. First visit was defined as before or during the flu season including follow-up visits before the end of the flu season. HIV-infected pregnant women were identified at antenatal clinics at Chris Hani Baragwanath Academic hospital and primary health care clinics in Soweto including Lillian Ngoyi, Diepkloof and Mofolo clinics.

Laboratory test

Nasopharyngeal specimens

Nasopharyngeal (NP) specimens were collected using a Dacron swab that was placed in 1ml of skim milk tryptone-glucose-glycerin (STGG) transport medium (18) at 4°C and transported to our laboratory on dry ice where they were stored at -80°C. NP specimens were collected from babies at 8 weeks (60 days), 16 weeks (120 days) and 24 weeks (180 days) scheduled visits. NP was also collected from babies when they were present at the clinic for illness visits.

DNA Extraction

To prepare the standards for qPCR, the bacterial strains TIGR4 and ATCC 25923 (19, 20) were grown overnight on blood agar plates where cell suspensions were made in 200 µl of ATL buffer and TE buffer containing 0.04g/ml of lysozyme + 75 U/ml of mutanolysin respectively for TIGR4 and ATCC 25923 strains from the Qiagen QIAamp Mini kit, DNA was extracted and eluted in 100 µl of sterile DNA grade water or AE buffer for *S. pneumoniae* and *S. aureus* respectively according to manufacturer's recommendations and stored at -80°C until use. DNA was extracted from the NP specimens either using the Qiagen QIAamp Mini kit according to manufacturer's recommendations or using the automated BioMerieux NucliSENS easyMAG 2.0.1 instrument (BioMerieux, Durham, NC). The reason for switching from manual extraction to automated extraction was comparable performance of manual vs. automated extraction with additional advantage of conserving both time and labor during the extraction procedure.

Preparation of Standards for qPCR

Purified DNA prepared from TIGR4 and ATCC 25923 strains were used to prepare the standards. The DNA from TIGR4 strain was diluted with TE buffer to prepare the standards at the following concentrations: 1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg and 0.05 pg. These standards represent the following copy numbers of the genome equivalents: 4.29×10^5, 4.29×10^4, 4.29×10^3, 4.29×10^2, 4.29×10^1 and 2.14×10^1. The DNA from ATCC 25923 strain was diluted with TE buffer to prepare the standards at the following concentrations 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg. These standards represent the

following copy numbers of genome equivalents 3.29×10^6, 3.29×10^5, 3.29×10^4, 3.29×10^3, 3.29×10^2, 3.29×10^1.

Quantitative Real-time qPCR amplification of lytA and nuc gene from NP specimens

Acquisition and carriage is interpreted using the quantitative molecular analysis methodology to measure *S. pneumoniae* and *S. aureus* density (CFU's) using real time qPCR for the lytA gene and for the nuc gene. We devised the experimental stage into four different categories 1) lytA(-) and nuc(-) 2) lytA(-) and nuc(+) 3) lytA(+) and nuc(-) 4) lytA(+) and nuc(+). We tested the follow-up samples until we observed acquisition of both the organisms *S. pneumonia* and *S. aureus* – lytA(+) and nuc(+). The DNA extracted from the NP specimens was tested in duplicate for quantitative real-time qPCR amplification of lytA and nuc gene.

Quantitative PCR reactions

PCR reactions were made to a final volume of 25 µl volume using Invitrogen Platinum qPCR master mix UDG according to manufacturer's recommendations with 2.5 µl of purified DNA and 200 nm of lytA primes, 400 nm of nuc primers and 200 nm each of the lytA and nuc probe. All qPCR reactions were processed in a CFX96 real-time system thermal cycler (Bio-Rad, Hercules, CA) with the following cycling parameters, initial denaturation at 95°C for 2 minutes, followed by 39 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 min.

Statistical Analysis

Age at visiting date is calculated from the date of sample collection and date of birth of each infant. Positive qPCR result is determined by average Ct value from duplicate results with the cut-off Ct of 37 cycles for both lytA and nuc targets. Colonization is calculated from average SQ from duplicate qPCR results using the formula (SQ×20) CFU/mL. In regards to the analysis of association and survival analysis, only one infant visiting entry is included and it is either the first visit that colonization is identified or the censored visit. In longitudinal analysis, all the infant visits are included to allow the effects from repeated measures.

Association analysis

The univariate analysis is conducted for time to acquisition and early acquisition is defined as an individual's age at visiting day younger than the median age at visiting day. Odds ratios (OR) were used as measures of associations and the Chi-square value and associated p-value is retrieved to assess the association between treatment group and early acquisition.

Survival analysis

Kaplan-Meir curves were constructed to distinguish the time to acquisition of *S. pneumoniae* and *S. aureus* between the TIV group and the placebo group. Log-rank and Wilcoxon Chi-square p-values were evaluated to differentiate the time to acquisition. A Cox-proportional hazard was used to determine the magnitude of associations in terms of hazard ratio and 95% confidence interval.

Longitudinal analysis for bacterial density

The colonization levels were log-transformed due to the right skewness of the distributions and the presence of outliers. The statistical analysis was conducted separately for *S. pneumoniae* and *S. aureus*. Profile plots of each subject were plotted to determine the changing trend of density over the six months. To investigate the significance of the linear trend of log-transformed bacteria density, a mixed-effects model with exchangeable correlation structure was fit, which took into account the within-subject correlational structure of the repeated measurements. The model then provided an estimate of the density level at 8 weeks after birth (baseline) and an estimate of the percentage change in every 8 weeks following the baseline measure SAS 9.3 (SAS Institute, Cary, NC) was used in the data analysis and all tests were two-sided, conducted with a 5% level of significance.

Results

Efficacy of the Vaccination Among Mothers:

Laboratory immunogenicity tests reveal that flu vaccination failed to yield immunogenicity against *S. pneumoniae* as primary endpoint of immunogenicity was not met in the HIV infected mothers. A revised vaccination schedule with higher dose and double doses are under evaluation in the primary study.

Association between maternal vaccination status and early bacteria acquisition

33 infants (49.25%) in the maternal TIV group have early *S.pneumoniae* acquisition while 28 infants (49.12%) in the placebo group have early acquisition. The odds ratio is 1.01 (95% CI: 0.50-2.04) (Table 1). Similarly, 34 infants (50.75%) in the maternal TIV group have early *S. aureus* acquisition while 28 infants (50%) in the placebo group have early acquisition, with an odds ratio of 1.03 (95% CI: 0.51-2.10) (Table 2). When breaking the acquisition into three time-points (8,16 and 24 weeks), 33% versus 34% of *S.pneumoniae* acquisition occurred at around 8 weeks between TIV group and placebo group when the comparison is 24% versus 21% in the same timepoint for *S.aureus* acquisition (Figure 1).

Difference in time to first acquisition

Survival plot of first acquisition of *S.pneumoniae* suggests no difference in the rate of acquisition with a log-rank p-value of 0.55. In comparison, survival plot of first acquisition of *S.aureus* suggests infants in TIV group suffered a faster rate of acquisition to *S.aureus.* However, this difference is not statistically significant, evidenced by a log-rank p-value of 0.11 (Figure 2 and Figure 3). Applying a cox PH model, the hazard ratio in *S. pneumoniae* acquisition comparing infants in TIV group and placebo group is 1.2 (95% CI: 0.72-1.87) and the hazard ratio in *S. aureus* acquisition comparing infants in TIV group and placebo group is 1.62 (95% CI: 0.89-2.94) (Table 3).

Change of bacteria density over 6 months since birth

The mean density of *S. pneumoniae* starts from 1.62 x 10^8 (±1.03 x10^9) CFU/ml to 1.91 x 10^6 (±6.44 x 10^6) CFU/ml at week 16, to 5.38 x 10^5 (±1.25 x 10^6) CFU/ml at week 24 in TIV group as compared with 3.49 x 10^5 (±6.17 x 10^5) CFU/ml, 1.08 x 10^6 CFU/ml (±2.37 x 10^6), 6.84 x 10^5 (±1.14 x 10^6) CFU/ml in the corresponding timepoints in placebo group. The mean density of *S. aureus* was 6.15 x 10^5 (±2.78 x 10^6) CFU/ml, 5.73 x 10^3 (±1.28 x 10^4) CFU/ml and 1.49 x 10^5 (±7.20 x 10^5) CFU/ml at 8, 16 and 24 weeks, respectively. The change of density is statistically significant (p=0.002). In contrast, the mean density of *S. aureus* decreased from 2.49 x 10^7 (±1.49 x 10^8) CFU/ml to 4.43 x 10^4 (±2.06 x 10^5) CFU/ml, and to 28 (±98) CFU/ml in placebo group at same timepoints (Table 4).

Profile plots capturing the individual's bacteria density in 8, 16 and 24 weeks show that there is no major departure from linear trend over the 24 weeks among infants in both TIV group and placebo group, which suggests it's valid to implement a mixed model (Figure 4). For *S. pneumonia*, the estimates of density is 3.11 x 10^5 CFU/mL (95% CI: 3.59 x 10^4-2.70 x 10^6) in TIV group and 4.24 x 10^4 (95% CI: 4.21 x 10^3-4.26 x 10^5) in placebo group whereas none of the estimate for percent change over the three scheduled visits is significantly different from 0. As for S. *aureus*, the estimates of density is 4.28 x 10^4 CFU/mL (95% CI: 9.28 x 10^3-1.97 x 10^5) in TIV group and 4.70 x 10^4 (95% CI: 6.98 x 10^3-3.17 x 10^5) in placebo group and likewise, there is no significant percent change over the three scheduled visits in both groups.

Discussion

Annual TIV immunization of HIV-infected adults has been recommended since 1988 (21) in South Africa due to case reports (22, 23) showing duration of increased illness and severe risk of influenza-related complications in HIV-infected individuals. In HIV-infected individuals, TIV vaccination is less immunogenic and the immunogenicity is directly related to CD4+ cell count and inversely to viral load (24-27). There are limited studies on the immunogenicity and efficacy of TIV in HIV-infected adults especially in pregnant women in African countries. One study reported 100% effectiveness of influenza vaccine against symptomatic influenza as well as significant difference between placebo and influenza vaccine regarding laboratory confirmed influenza illness (28). Another study was a community-based randomized, placebo controlled trial conducted at Themba Lethu HIV clinic, Helen Joseph hospital and it reported that TIV was associated with a 75% reduction in influenza-confirmed illness without increase in frequency of adverse events in HIV-infected adults. There was no difference in either CD4+ cell count changes or HIV viral control in those on antiretroviral treatment between TIV recipients compared to placebo recipients, and there were no serious adverse event rates recorded (29). Previous concerns were alleviated regarding the potential negative effects of TIV around the observed transient increase in HIV-1 viral load, especially in HIV infected individuals on ART and those who were virologically suppressed (viral load <400 copies/ml). In HIV-infected individuals after TIV vaccination, decreased CD4+ lymphocyte counts have also been observed. In previous studies these changes were

infrequent (4-18%) and resolved at later time points and were considered to be clinically insignificant.

In spite of the encouraging maternal immunization data from Bangladesh(5) and the data showing that TIV is efficacious in HIV-infected, non-pregnant adults, further data are needed to promote routine use of TIV vaccination during pregnancy in the population with high prevalence of HIV. The primary focus of this proposal and the major public health benefit is targeted at the protection of young infants showing the impact of maternal HIV on the dynamics of TIV induced transplacental antibody transfer, which subsequently protects mothers and infants from secondary bacterial co-infections. Transfer of maternal antibody, which is gestational age dependent, may be more affected by maternal immunization in sub-Saharan Africa. The results of this trial will be used to justify the decision to proceed or not to proceed with a larger trial to assess the efficacy of TIV in HIV infected pregnant women and their infants.

Limitations of our study include some demographic and clinical variables such as birth weights, delivery mode and CD4 counts are not available and they might be potential confounders causing bias to the estimates if not controlled in analyses. Another limitation is our evaluable sample size (n=124) which only allows us detect a difference in early bacterial acquisition greater than 25%. A further limitation of our study is that it was conducted over a single influenza season and the variation of the flu season period and circulating flu strain may cause variability in outcome that our study cannot address for(17).

Despite these limitations, our study embraces several strengths. To our knowledge, it is the first study to evaluate the efficacy of TIV to prevent pneumococcal colonization in African HIV-infected infants younger than 6 months of age. The randomization of treatment groups can guarantee the equal distribution of confounding variables and eliminate the bias introduced from unknown confounding factors. The novel idea of reducing the risks of *S.pneumoniae* and *S. aureus* colonization through vaccine-based intervention against the precursor virus infection may bring us new implications on designing vaccine strategies and policies in in sub-Saharan Africa. Besides, while most of the current studies pay attention to the risk of pneumococcal infection among children above 6 months of age, our study uniquely focused on subjects younger than 6 months of age to evaluate the efficacy of immunization strategies for children at early infancy in South Africa, which was a less studied gap area in this field.

Our study still clearly show that maternal TIV vaccination is not effective in either delaying incidence of first *S.pneumoniae* or *S. aureus* colonization, or decreasing the density of colonization in both *S.pneumoniae* and *S. aureus* among HIV seropositive infant younger than 6 months of age. The lack of efficacy may be explained by the poor immunogenicity of TIV, especially because the subjects are newborn infants who are at heightened risk of pneumococcal infection and may be also attributed to their undeveloped immune system(17). Therefore, the protective effects of influenza vaccination against other common infectious diseases must be re-evaluated in HIV

seropositive infants and further immunological studies are needed to support such a conclusion.

Future directions

Clearly, studies of maternal influenza immunization are needed among both HIV-seropositive and HIV-seronegative individuals before any guidelines can be made to reduce the risk of *S. pneumoniae* or *S.aureus* infection during early infancy through the approach of maternal influenza immunization in sub-Saharan Africa. In the next step, we will compare the efficacy of TIV against first acquisition of *S. pneumoniae* and *S.aureus* between infant subjects infected and subjects not infected with influenza, controlling for HIV status. If necessary, influenza type-specific analysis will be conducted to investigate the association between the efficacy of maternal TIV immunization and particular influenza subtypes. Furthermore, we will also evaluate the relationship between maternal subjects and infant subjects in time and density of *S. pneumoniae* and *S.aureus* colonization.

References

1. McCullers JA. Insights into the interaction between influenza virus and pneumococcus. *Clin Microbiol Rev* 2006;19(3):571-82.
2. Bryce J, Boschi-Pinto C, Shibuya K, et al. WHO estimates of the causes of death in children. *Lancet* 2005;365(9465):1147-52.
3. Halasa NB, Gerber MA, Chen Q, et al. Safety and immunogenicity of trivalent inactivated influenza vaccine in infants. *J Infect Dis* 2008;197(10):1448-54.
4. Munoz FM, Englund JA. A step ahead. Infant protection through maternal immunization. *Pediatr Clin North Am* 2000;47(2):449-63.
5. Zaman K, Roy E, Arifeen SE, et al. Effectiveness of maternal influenza immunization in mothers and infants. *N Engl J Med* 2008;359(15):1555-64.
6. Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J Infect Dis* 2008;198(7):962-70.
7. Brundage JF. Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. *Lancet Infect Dis* 2006;6(5):303-12.
8. Chertow DS, Memoli MJ. Bacterial co-infection in influenza: a grand rounds review. *JAMA* 2013;309(3):275-82.
9. Ballinger MN, Standiford TJ. Postinfluenza bacterial pneumonia: host defenses gone awry. *J Interferon Cytokine Res* 2010;30(9):643-52.
10. Sun K, Metzger DW. Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection. *Nat Med* 2008;14(5):558-64.

11. Madhi SA, Klugman KP, Vaccine Trialist G. A role for Streptococcus pneumoniae in virus-associated pneumonia. *Nat Med* 2004;10(8):811-3.

12. Morens DM, Fauci AS. The 1918 influenza pandemic: insights for the 21st century. *J Infect Dis* 2007;195(7):1018-28.

13. Chien YW, Klugman KP, Morens DM. Bacterial pathogens and death during the 1918 influenza pandemic. *N Engl J Med* 2009;361(26):2582-3.

14. Simell B, Auranen K, Kayhty H, et al. The fundamental link between pneumococcal carriage and disease. *Expert Rev Vaccines* 2012;11(7):841-55.

15. Diavatopoulos DA, Short KR, Price JT, et al. Influenza A virus facilitates Streptococcus pneumoniae transmission and disease. *FASEB J* 2010;24(6):1789-98.

16. McCullers JA, McAuley JL, Browall S, et al. Influenza enhances susceptibility to natural acquisition of and disease due to Streptococcus pneumoniae in ferrets. *J Infect Dis* 2010;202(8):1287-95.

17. Madhi SA, Dittmer S, Kuwanda L, et al. Efficacy and immunogenicity of influenza vaccine in HIV-infected children: a randomized, double-blind, placebo controlled trial. *AIDS* 2013;27(3):369-79.

18. O'Brien KL, Bronsdon MA, Dagan R, et al. Evaluation of a medium (STGG) for transport and optimal recovery of Streptococcus pneumoniae from nasopharyngeal secretions collected during field studies. *J Clin Microbiol* 2001;39(3):1021-4.

19. Tettelin H, Nelson KE, Paulsen IT, et al. Complete genome sequence of a virulent isolate of Streptococcus pneumoniae. *Science* 2001;293(5529):498-506.

20. Kilic A, Muldrew KL, Tang YW, et al. Triplex real-time polymerase chain reaction assay for simultaneous detection of Staphylococcus aureus and coagulase-negative

staphylococci and determination of methicillin resistance directly from positive blood culture bottles. *Diagn Microbiol Infect Dis* 2010;66(4):349-55.

21. Centers for Disease C. Prevention and control of influenza. *MMWR Morb Mortal Wkly Rep* 1988;37(23):361-4, 9-73.

22. Radwan HM, Cheeseman SH, Lai KK, et al. Influenza in human immunodeficiency virus-infected patients during the 1997-1998 influenza season. *Clin Infect Dis* 2000;31(2):604-6.

23. Nabeshima S, Ariyama I, Chong Y, et al. Influenza in three patients with human immunodeficiency virus infection. *Intern Med* 2000;39(7):592-7.

24. Nelson KE, Clements ML, Miotti P, et al. The influence of human immunodeficiency virus (HIV) infection on antibody responses to influenza vaccines. *Ann Intern Med* 1988;109(5):383-8.

25. Miotti PG, Nelson KE, Dallabetta GA, et al. The influence of HIV infection on antibody responses to a two-dose regimen of influenza vaccine. *JAMA* 1989;262(6):779-83.

26. Fuller JD, Craven DE, Steger KA, et al. Influenza vaccination of human immunodeficiency virus (HIV)-infected adults: impact on plasma levels of HIV type 1 RNA and determinants of antibody response. *Clin Infect Dis* 1999;28(3):541-7.

27. Benne CA, Kroon FP, Harmsen M, et al. Comparison of neutralizing and hemagglutination-inhibiting antibody responses to influenza A virus vaccination of human immunodeficiency virus-infected individuals. *Clin Diagn Lab Immunol* 1998;5(1):114-7.

28. Tasker SA, Treanor JJ, Paxton WB, et al. Efficacy of influenza vaccination in HIV-infected persons. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 1999;131(6):430-3.

29. Madhi SA, Maskew M, Koen A, et al. Trivalent inactivated influenza vaccine in African adults infected with human immunodeficient virus: double blind, randomized clinical trial of efficacy, immunogenicity, and safety. *Clin Infect Dis* 2011;52(1):128-37.

Tables

Table 1. Distribution of early *S.pneumoniae* acquisition in HIV+ Children between vaccine and placebo groups in 2011

Characteristics	Early acqusition* N (%)	Late acquisition N (%)	OR	95%CI		P-value**
Vaccine	33 (49.25)	34 (50.75)	1.01	0.50	2.04	0.99
Placebo	28 (49.12)	29 (50.88)				

* Early Acquisition defined as acqusition earlier than 81 days after birth
****** chi-square p-value

Table 2. Distribution of early *S.aureus* acquisition in HIV+ Children between vaccine and placebo groups in 2011

Characteristics	Early acqusition* N (%)	Late acquisition N (%)	OR	95% CI		P-value**
Vaccine	34 (50.75)	33 (49.25)	1.03	0.51	2.10	0.93
Placebo	28 (50.00)	28 (50.00)				

* Early Acquisition defined as acqusition earlier than 80.5 days after birth
****** chi-square p-value

Table 3. Hazard ratios and confidence interval of colonization-free time in *S.pneumonia* or *S. aureus* with respect to vaccination status using Cox PH Model

Variable	Hazard ratio	95% CI**		P-value
Vaccine (*S.pneumonia*)*	1.155	0.715	1.866	0.5552
Vaccine (*S.aureus*)*	1.62	0.893	2.939	0.1125

* Unadjusted Cox PH model to estimate the Hazard ratio of vaccine group and 95% CI for *S.pneumonia* and *S.aureus* respectively
** 95% Confidence Interval

Table 4. Mean Density of *S. pneumoniae* and *S. aureus* in HIV+ Children between TIV and Placebo group in three schedule visits in 2011

Time		TIV(n=67) Mean (CFU/ml)	SE	P-value#		Placebo (n=57) Mean (CFU/ml)	SE	P-value
S. pneumoniae								
8 weeks*	n=41	162165090	1030120021		n=38	348546	617273	
16 weeks	n=30	1913469	6437509	0.25	n=22	1081812	2369329	0.69
24 weeks	n=25	537918	1246896		n=12	683615	1144251	
S. aureus								
8 weeks	n=41	614717	2779243		n=38	24897972	148945188	
16 weeks	n=30	5725	12795	<0.01	n=22	44318	206270	0.19
24 weeks	n=25	148961	719762		n=12	28	98	

* Three major visits are 8 weeks (visit at infant age= 56 ± 20 days), 16 weeks (visit at infant age= 112 ± 20 days) and 24 weeks (visit at infant age= 168 ± 20 days)
# A small p-value suggests a non-zero slope for CFU level. A mixed-effects model was employed that accounted for repeated measurements. Both *S. pneumoniae* and *S. aureus* densities were log transformed.

Table 5. Estimates of Mean Colonization Level and percent change in HIV+ Children between TI and Placebo group over three schedule visits in 2011 using mixed model

Treatment group	Baseline Estimate* Colonization (CFU/mL)	95% Confidence Interval		Slope Estimate** Percent change	95% Confidence Interval	
S. pneumoniae						
TIV	310705	35872	2691475	-0.51	-0.82	0.37
Placebo	42362	4214	425832	0.10	-0.67	2.73
S. aureus						
TIV	42800	9279	197422	-0.41	-0.69	0.12
Placebo	47019	6982	316602	-0.41	-0.73	0.31

* Baseline colonization refers to colonization level at 8 weeks (visit at infant age= 56 ± 20 days)

** Percent change indicates the change of colonization in percent over three infants' schedule visits. A mixed-effects model was employed that accounted for repeated measurements. Both *S. pneumoniae* and *S. aureus* colonization levels were log transformed.

Figures

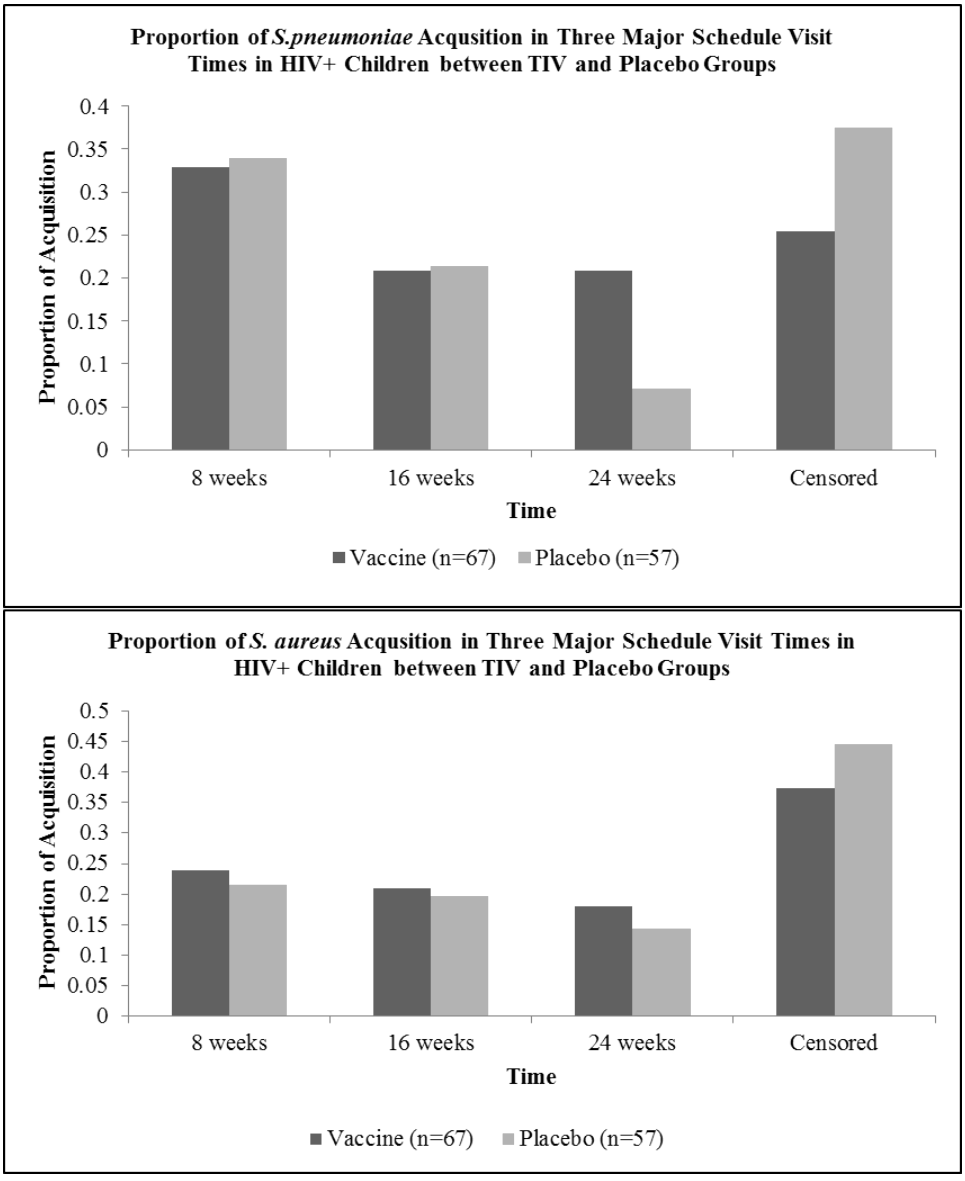
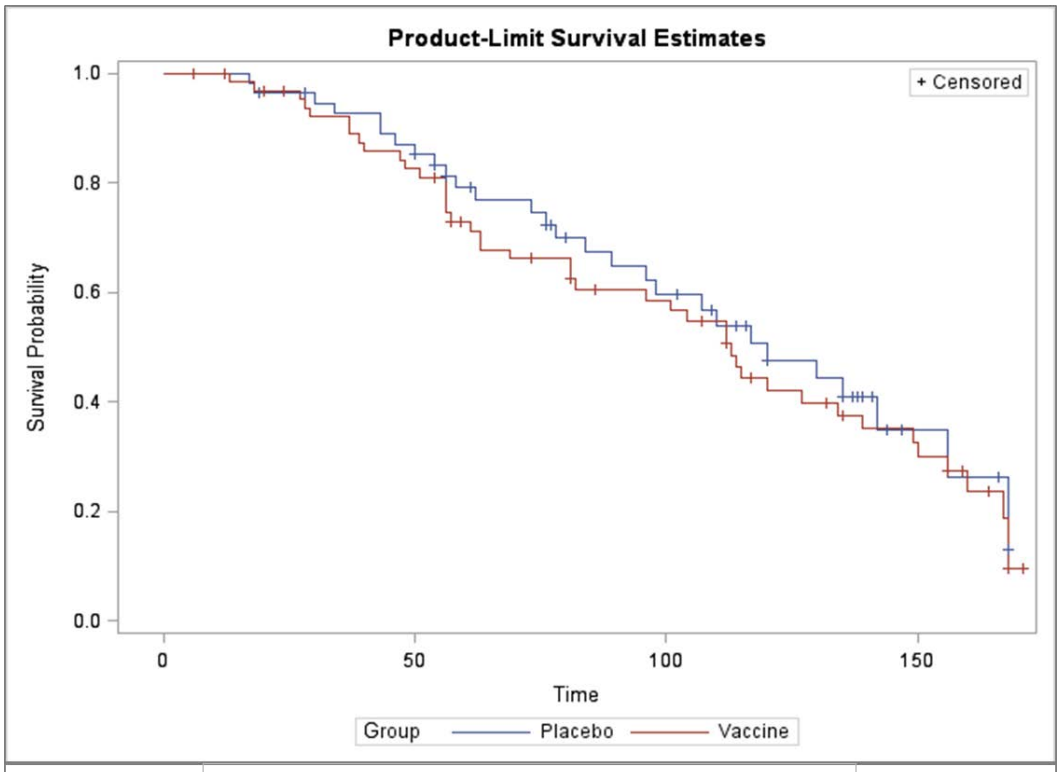
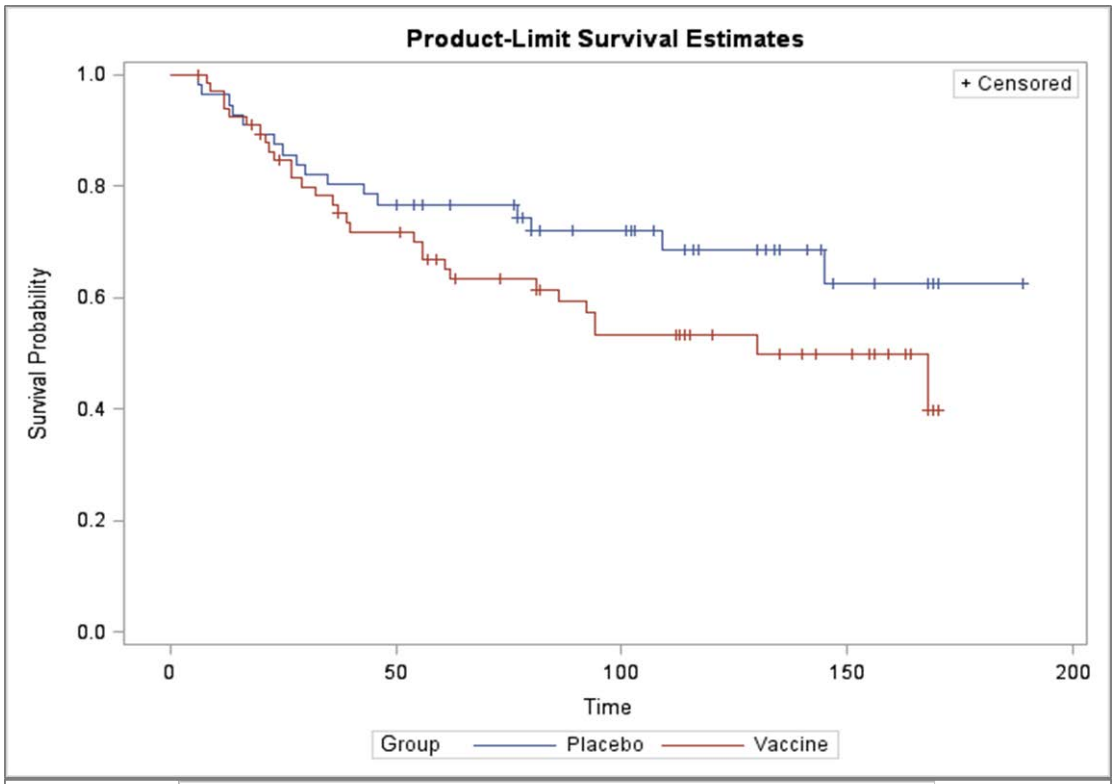


Figure 1. Proportion of *S. pneumoniae* and *S. aureus* acquisition in three schedule visits among HIV+ children in TIV and placebo groups



Test	Chi-Square	P-value
Log-Rank	0.3573	0.5500
Wilcoxon	0.5083	0.4759

Figure 2. Survival Plot of First acquisition of *S.pneumoniae* in HIV+ children between vaccine group and placebo group in 2011



Test	Chi-Square	Pr >Chi-Square
Log-Rank	2.5758	0.1085
Wilcoxon	1.6671	0.1966

Figure 3. Survival Plot of First acquisition of S.aureus in HIV+ children between vaccine group and placebo group in 2011

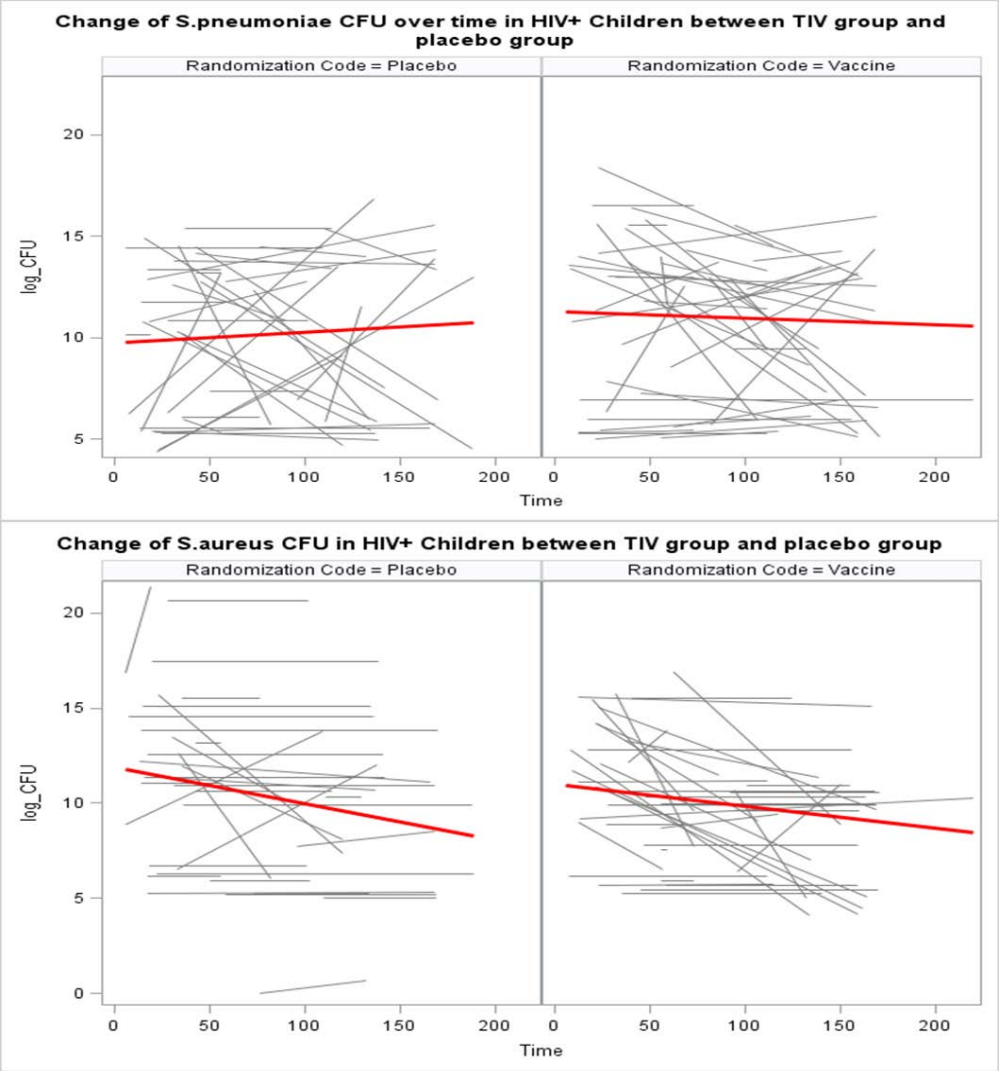


Figure 4. Profile plots of S. pneumoniae and S. aureus colonization over time by infants in TIV and placebo groups. Each subject has 1 to 5 visits and individual fitting lines represent the change of mean colonization for each individual. The red summazring regression line represents the change of mean colonization for the entire category.

Appendix