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:

_____ _____

Ida Sahlu Date

THE IMPACT OF MASS AZITHROMYCIN TREATMENTS ON ANTIBIOTIC RESISTANCE AND THE DISTRIBUTION OF *S. PNEUMONIAE* SEROTYPES IN ETHIOPIA

By

Ida Sahlu

Master of Public Health

Global Epidemiology

_____

Keith Klugman, MD, PhD
Faculty Thesis Advisor

_____

Jorge E. Vidal, PhD
Thesis Field Advisor

THE IMPACT OF MASS AZITHROMYCIN TREATMENTS ON ANTIBIOTIC RESISTANCE AND THE DISTRIBUTION OF *S. PNEUMONIAE* SEROTYPES IN ETHIOPIA

By

Ida Sahlu

Bachelor of Arts
Bowdoin College
2008

Faculty Thesis Advisor: Keith 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 Global Epidemiology
2011

ABSTRACT

THE IMPACT OF MASS AZITHROMYCIN TREATMENTS ON ANTIBIOTIC RESISTANCE AND THE DISTRIBUTION OF *S. PNEUMONIAE* SEROTYPES IN ETHIOPIA

By Ida Sahlu

Background: Trachoma is one of the leading preventable causes of blindness in the developing world. The World Health Organization recommends repeated annual mass oral azithromycin treatments for entire districts where the prevalence exceeds 10% among children aged 1-9 years old. *S. pneumoniae*, with more than 93 serotypes, is one of the leading causes of child mortality in the developing world. Repeated annual mass antibiotic treatment has raised concerns in both the development of antibiotic resistant strains of *S. pneumoniae* and the change in serotype distribution. This research was conducted to determine whether treatment with oral azithromycin and increased resistant strains changed the distribution of seven-valent vaccine serotypes.

Methods: The study was a population-based, cluster-randomized clinical trial of quarterly mass oral azithromycin treatments (20 mg/kg) for trachoma. Nasopharyngeal swabs were collected from 10 children of each sentinel community. *S. pneumoniae* strains were isolated and serotypes were identified. Resistance to the following antibiotics: azithromycin, penicillin, clindamycin and tetracycline, was evaluated by MICs and included in the analysis. Chi-square tests were performed to compare serotype distribution. Logistic regression was used to determine whether treatment and population characteristics were associated with seven-valent vaccine serotypes.

Results: The difference in proportions for the most prevalent serotypes in the study (19F, 6B, 6A, 7F and 9L) at baseline and at one-year follow-up was not statistically significant (p-value=0.58). The association between treatment and vaccine serotypes was not statistically significant for the univariate and multivariate analyses (OR=1.02, 95% CI=0.49-2.15, OR=1.14, 95% CI=0.42-3.08, respectively). Azithromycin resistance increased from 5.8% at baseline to 60.7% at one-year follow up (p= <0.0001). The multivariate analysis included gender, trachoma, age, azithromycin-, penicillin-, clindamycin- and tetracycline-resistance. Azithromycin resistance was associated with non-vaccine serotypes (OR=29.59, 95% CI=3.01-291.17), while penicillin-, clindamycin- and tetracycline-resistance were associated with vaccine serotypes after adjusting for other covariates (OR=4.98, 95% CI=0.996-24.87; OR=57.91, 95% CI=5.13-653.83; OR=5.07, 95% CI=1.51-16.98, respectively).

Conclusion: Mass treatments with oral azithromycin did not change the overall distribution of serotypes but did increase antibiotic resistance. Azithromycin resistance selects for non-vaccine serotypes, while resistance to penicillin, clindamycin and tetracycline were associated with vaccine serotypes.

THE IMPACT OF MASS AZITHROMYCIN TREATMENTS ON ANTIBIOTIC RESISTANCE AND THE DISTRIBUTION OF *S. PNEUMONIAE* SEROTYPES IN ETHIOPIA

By

Ida Sahlu

Bachelor of Arts
Bowdoin College
2008

Faculty Thesis Advisor: Keith 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 Global Epidemiology
2011

ACKNOWLEDGEMENTS

I would like to thank Dr. Klugman, Dr. Jorge Vidal and Dr. Herbert Ludewick for their guidance and insight throughout the year. This thesis could not have been possible without their support. They provided me with wonderful feedback for both the laboratory and epidemiological analysis portion of my thesis, and I cannot thank them enough. I want to especially thank Dr. Jorge Vidal for being so optimistic and for believing in me.

I would like to thank Pamela Mink for always having her door open. Thank you for willing to provide me with epidemiological and professional advice during my time at RSPH, and for being my mentor.

I would also like to thank my friends for their constant support and encouragement, especially during times of difficulty.

There are not enough words to express how grateful I am for the unconditional love and support from my parents. I would not have been able to do this without them.

TABLE OF CONTENTS

LIST OF TABLES

BACKGROUND

Streptococcus pneumoniae is a Gram-positive bacterium and one of the leading causes of invasive bacterial infections among high risk groups, such as the elderly and young children (1). The pathogen can cause severe diseases such as meningitis, pneumonia, acute respiratory infections and sepsis, especially in the developing world (2). The World Health Organization (WHO) has recently estimated that yearly there are 700 000 to 1 million deaths among children worldwide due to *S. pneumoniae* (3). The top ten countries with the highest number and proportion of pneumococcal cases are located in Asia and Africa, which account for 66% of all cases worldwide (3).

More than 93 distinct serotypes have been identified in *S. pneumoniae* strains, however, only around 15 serotypes cause the majority of the invasive pneumococcal disease worldwide (1). The seven-valent pneumococcal conjugate vaccine (PCV7) protects against seven serotypes that cause the most invasive disease in the USA, which include serotypes 4, 6B, 9V, 14, 18C, 19F and 23F (1). However, the vaccine does not include other serotypes, such as 1 and 5, which are prevalent in developing countries. The distribution of *S. pneumoniae* serotypes differs by age, geography and time which is a challenge for the development of vaccines (1).

Epidemiology of *Streptococcus pneumoniae*

Humans are carriers of *S. pneumoniae,* where the bacteria reside on the mucosal surface of the respiratory tract (4). Carriage of *S. pneumoniae* in the nasopharynx of sickly and healthy individuals is the means through which the bacterium is spread from one person to another, mainly in highly populated areas (2). An individual can be

colonized with *S. pneumoniae* for weeks to months before its clearance from his or her nasopharynx (4). Colonization is mostly common among younger children, and infants who may carry one or more strains at one time (4). For example, in Gambia, there are over 80% of infants that are carriers of *S. pneumoniae* in the nasopharynx, however, the rates of colonization decrease over the years to <10% among adults (2, 4). The reasons for the variation of distribution over time and geography are still unknown.

Pneumonia, meningitis and other pneumococcal diseases in individuals are acquired when *S. pneumoniae* moves to the sterile parts of the airway system (4, 5). In other words, pneumococcal disease is prefaced by colonization of *S. pneumoniae*, also called the carrier state (4). Therefore, determining the distribution of strains of *S. pneumoniae* is important for public health programs that aim to reduce the spread of pneumococcal disease. By identifying the prevalent serotypes in highly dense populations, the spread of invasive *S. pneumoniae* serotypes can be reduced with vaccines directly targeting transmission of those serotypes, which is important in the fight against pneumonia, meningitis and other respiratory infections due to *S. pneumoniae.*

Elimination of *S. pneumoniae* is complicated due to the adaptability of the pathogen (5). Since the introduction of PCV7 in 2000, populations vaccinated by the pneumococcal vaccine have had a significant decrease in pneumonia cases due to vaccine serotypes, such as in the United States. Invasive vaccine-serotypes of *S. pneumoniae* have been replaced by non-vaccine type serotypes (4, 6). The 10- and 13-valent conjugate vaccines have been developed to cover more invasive serotypes of *S. pneumonia* (7). A study was conducted by Yao et al. in China to determine the distribution of *S. pneumoniae* serotypes among children aged ≤5 years old hospitalized for pneumococcal

pneumonia (7). Yao et al. found that the 10- and 13- valent conjugate vaccines covered 76.9% and 92.3% of isolates (7). Therefore, other vaccines such as the 9-, 11- or 13-valent vaccines may be more appropriate on the global scale (8).

Emergence of antibiotic resistant strains of *S. pneumoniae*

In community-acquired pneumoniae (CAP), the most common antibiotics used for treatment are betalactams and macrolides (9). As a result of antibiotic treatment, there has been an increase in *S. pneumoniae* strains resistant to penicillin and macrolides, which studies state could be due to nasopharyngeal colonization with resistant invasive strains (10, 11). Furthermore, a decrease in macrolide use was found to lead to a decrease in macrolide-resistant *S. pneumoniae*(12).

Macrolide resistance is due to two types of mechanisms: target-site modification and active efflux of the drug from the cell (13). The first mechanism, target-site modification, is caused by the presence of the *erm*B or *erm*A gene. In many countries, these resistance genes are the most common causes of macrolide-resistance(13). The second mechanism, efflux-mediated resistance and encoded as Mef, is caused by the presence of *mef*(A) or *mef*(A) subtypes, *mef*(C) or *mef*(I) (13). This gene has been reported as the main resistance factor for the macrolide erythromycin in the USA (13). The development of antimicrobial resistant strains of *S. pneumoniae* in communities is not simple. Indeed, antibiotic use is believed to eliminate susceptible strains and allows for the replacement of pre-existing resistant strains (14). The emergence of resistant strains may also be due to the introduction from outside a community, or from a gene mutation which is believed to be rare (15).

Trachoma

Trachoma, one of the top three causes of blindness worldwide, is a chronic keratoconjunctivitis caused by recurrent infections with the serotypes A, B, Ba and C of the bacterium *Chlamydia trachomatis* (16). *C. trachomatis* is an obligate intracellular bacterium transmitted between human beings by intimate social or sexual contact (16). Acquisition methods include, direct contact with infectious ocular or nasopharyngeal discharges on fingers, or indirect contact with contaminated fomites from infected people, such as clothes and towels (17). The active form of trachoma is most prevalent among children, equally distributed among boys and girls, due to their close physical contact resulting in frequent re-infection and healing (18). Flies are also contributors to the spread of the disease, especially *Muscasorbens* flies in Africa and the Middle East (17). The incubation period, once infected with *C. trachomatis*, is 5-12 days and an individual remains infectious as long as active lesions are present in the conjunctivae and adenexal mucous membranes, which at times can last a few years (17).

The bacterium generally remains localized on the epithelial surfaces of the eye and does not replicate outside eukaryotic host cells (16). However, inflammatory response may extend into the deeper subepithelial layers (19). The tissue damage, which leads to decreased vision and blindness, is primarily due to an immune response rather than direct damage by *C. trachomatis* (19).

Clinical manifestations are subdivided into ‘active’ disease and cicatricial or scarring complications (20). Infection triggers a florid chronic inflammatory response, identified as active trachoma, and characterized by papillary hypertrophy and follicular conjunctivitis (19, 20). Subepithelial collections of lymphoid cells develop follicules,

which appear as small, yellow-white elevations of the conjunctiva of the everted upper lid (20). Papillary hypertrophy is another symptom of active trachoma, where there is obstruction of the deep tarsal vessels if severe enough (20). In some cases of active disease, vascular infiltration of the upper cornea may also develop, however, this rarely affects vision (20). Most cases are asymptomatic or have mild symptoms even if clear signs of inflammation are evident. The mild symptoms include, redness, discomfort, tearing, photophobia and scant muco-purulent discharge (20). A particular symptom that allows differentiation of trachoma from any other chronic conjunctivitis is the shallow depression at the upper margin of the cornea after the conjunctival follicles resolve. This depression is known as 'Herbert's pits', which is a pathognomonic sign of trachoma (20).

Scar tissue begins to accumulate later in life within the conjunctiva, particularly the upper eyelid, after repeated infection and inflammation (19). Contraction of the scar tissue causes distortion of the eyelid leading to entropion (inturned eyelashes) and trichiasis (eyelashes touching the eyeball), which is usually painful (19, 20). The blinding end-stage of the disease is corneal opacification as a result of multiple stresses to the cornea (20).

Disease Burden of Trachoma

The World Health Organization (WHO) estimates that 80% of global blindness is avoidable, either through prevention or by controlling infections if timely interventions and education are put in place. This is particularly the case for trachoma and river blindness (21). Indeed, in 2002, it was estimated that 1.4 million people were blind from trachoma, and currently 40 million people are considered to have active disease (20).

Further investigation has shown that possibly 1.8 million were suffering from low vision (20).

Trachoma can be traced back to China in the 27th century BC (16). In the past, the disease was present in all parts of the world, including parts of Europe and North America (20). However, trachoma has disappeared from developed countries, with the exception of Aboriginal communities in Australia (20). Today, trachoma is endemic in 56 countries, present in Africa, Asia and some parts of Latin America, the Middle East and the Western Pacific (22, 23). There is especially a high prevalence of active trachoma in the Sahel region, East Africa, Ethiopia and India (24). Studies have found the prevalence of active trachoma to be 23%-47% in Mali (25), in 36.5% of children aged 2-6 years in Menofiya, Egypt (24), and in 40.1% of children aged less than 10 years in Ethiopia(26).

The highest risk factors for trachoma are poverty, lack of personal and community hygiene, limited access to healthcare and water (23). Ethiopia, one of the poorest nations in the world, has a high disease burden due to trachoma(26). A national survey on blindness, low vision and trachoma was conducted from December 2005 to September 2006. The study found that active trachoma was present in 40.1% of children aged 1-9 years, and 11.5% of blindness and 7.7% of low vision were due to trachomatous corneal opacity (22, 26). Also, 550 of the 611 districts in the country were found to be endemic for trachoma (22). Furthermore, the overall prevalence of active trachoma in rural areas was found to be 4 times the prevalence in urban areas (26). As a result, the Ethiopian Ministry of Health identified trachoma as a priority disease needing urgent attention and intervention in 2001 (26).

Treatment of Trachoma

The WHO established in 1997 the Alliance for Global Elimination of Trachoma (GET) by the year 2020 (23). The Alliance promotes the SAFE strategy for the control of trachoma, which consists of Surgery for trichiasis, antibiotics to treat infections, facial cleanliness and environmental changes (clean water and latrines to interrupt transmission of *C. trachomatis*) (23, 27).

Currently, the WHO recommends a goal of 80% antibiotic coverage for trachoma programs (28). Based on WHO guidelines, districts are characterized for intervention based on the prevalence of clinical signs of disease (29). Oral azithromycin is the antibiotic recommended to control blinding trachoma (31). The recommendation is repeated annual mass azithromycin treatments for entire districts where the prevalence of active trachoma exceeds 10% among children aged 1-9 years (27, 30). However, treatment is only recommended in select communities where the prevalence of trachoma is greater or equal to 10% (27). Yet, there is debate about the level of coverage necessary (28).

The WHO recommended frequency of antibiotic treatment is three annual mass treatments to trachoma-endemic areas, with re-evaluation after the third treatment (28). However, there is debate over the frequency of mass azithromycin treatment in communities needed to eliminate infection. In Niger, the SAFE strategy was implemented from 2002 to 2005 and three mass treatments using oral azithromycin were performed in 2 districts. Follow-up results showed that the prevalence of trachomatous inflammation (TF) in the 2 districts decreased significantly from 62.3% and 49.5% to 7.6% and 6.7% in three years (31). Furthermore, a study in Nepal found that treatment

with three rounds of oral azithromycin directed at children age 1 to 10 years reduced clinically active trachoma from nearly 40% at baseline to 13% one year after the first treatment and to 4% one year after the second one (31). Yet, studies have suggested that a single mass antibiotic treatment to a sufficiently large proportion of the community may prevent the reoccurrence of infection (28). In 2000, a study was conducted in the Rombo district of Tanzania, where active trachoma is endemic. All non-pregnant individuals above the age of 12 months were provided with oral azithromycin treatment and were monitored at 2, 6, 12, 18, and 24 months (32). After mass treatment with azithromycin, the study found a dramatic decrease in the prevalence *of C. trachomatis* and which continued to decline after 2 years of follow-up (32). Also, a five-year study conducted in The Gambia found that a single mass drug administration was effective in reducing infection, although re-infection did occur in two villages (33). The other components of the SAFE strategy were introduced in the communities, resulting in environment conditions and/or reduced susceptibility of the population to re-infection and transmission of *C. trachomatis*(33). In this instance, re-emergence of chlamydial infection could not be explained by antibiotic coverage, which averaged 83% among the villages (28). Indeed, a clinical trial conducted by Lakew et al. in the southern region of Ethiopia found that chlamydial infection depends more on endemic infection than antibiotic coverage. Communities were provided with regular mass antibiotic treatments and after treatment, the amount of endemic infection appeared to be a stronger determinant of chlamydial infection than antibiotic coverage (28). A possible explanation may be that re-infection may occur in areas with severe trachoma due to underlying poor hygiene and sanitation practices (28). Therefore, Lakew et al.'s results do not support the WHO guidelines of

80% antibiotic coverage for hyper-endemic areas, as in the study in Gambia (28, 33). Yet, the WHO's antibiotic treatment strategy has been successful in nearly eliminating infection in areas with a modest to moderate amount of trachoma cases (28).

Indeed, Lakew et al. specify that their results may only be generalizable to severely affected areas with relatively high coverage (28). A possible treatment solution would be to stratify villages by pre-treatment chlamydial prevalence and offer tailored mass antibiotic treatment regimens (28). However, prolonging antimicrobial drug treatment brings the issue of antimicrobial drug resistance.

Impact of trachoma treatment on resistance

The rising concern from repeated annual mass antibiotic treatment is the development of antibiotic resistance, both in *C. trachomatis* and in other bacterial pathogens. Although a study conducted in Tanzania did not find a significant increase in azithromycin resistance in *C. trachomatis* strains after mass treatment (34), studies have found that other bacteria are showing antibiotic resistance following community-based azithromycin treatment (35). Tellis et al. demonstrated that 27% of *S. pneumoniae* strains isolated from Aboriginal communities in Australia, after azithromycin treatment for trachoma, had intermediate to high level of resistance to erythromycin (35). Because identifying antibiotic resistance to erythromycin can serve as a predictor of *S. pneumoniae* resistance to azithromycin, Tellis et al.'s study demonstrated an increase in *S. pneumoniae* bacteria resistant to azithromycin after community-based treatment with azithromycin (35). Indeed, studies have found a correlation between increasing macrolide use in the community and increasing macrolide resistance among streptococci (36). In

addition, studies have found macrolide resistance among *S. pneumoniae* in communities that recommend antimicrobials, such as sulfa and penicillin, for the treatment of childhood infections in countries where trachoma is endemic (36). A longitudinal study conducted among an Aboriginal community in Australia found a high acquisition of azithromycin-resistant strains following treatment for trachoma (37). Furthermore, a study also found that the discontinued use of azithromycin showed a decrease in the prevalence of resistant *S. pneumoniae* in their subjects, which confirms the association between azithromycin antimicrobial use and resistant strains of *S. pneumoniae*(30). Therefore, mass azithromycin treatments for trachoma are ideal to study the role of antibiotic selection pressure on the community spread of pneumococcal macrolide resistance in communities.

Antibiotic resistance in Ethiopia

Ethiopia is an ideal location to study the spread of antimicrobial resistant strains of *S. pneumoniae* because not only does it have a high number of pneumococcal disease cases, but the proportion of cases is among the top ten of the world (3). Ethiopia's population in 2010 was estimated to be 88,013,491 with 46% of the population less than or equal to 14 years of age (38). Because young populations have a higher colonization of *S. pneumoniae* and Ethiopia has a large young population, it is important to understand the distribution of *S. pneumoniae* strains in the country.

Since the Ethiopian Ministry of Health identified trachoma as a priority disease needing urgent attention and intervention in 2001, SAFE-based interventions with mass antibiotic distribution have been expanding in the country (39). From the clinical trials of

mass azithromycin treatments for trachoma conducted in Ethiopia among populations with limited macrolide antibiotic use, the results are useful in understanding the impact of antibiotic use on the spread of antimicrobial resistant strains of *S. pneumoniae* in populations (30). In addition, information may also be gained as to the distribution of *S. pneumoniae* and whether the current seven conjugate vaccine for *S. pneumoniae* is appropriate for Ethiopia's large population.

METHODS

Research Objective: The purpose of this study was to determine whether mass azithromycin treatments for trachoma have an impact on the distribution of *S. pneumoniae* serotypes in communities treated in rural Ethiopia. Furthermore, to determine whether treatment with azithromycin and antibiotic resistance would change the probability of having 7-valent vaccine serotypes.

Null Hypotheses, H_0:

1) There is no association between serotype distribution and treatment for azithromycin from 2006-2007 in the Goncha Woreda (district of Ethiopia), Amhara region, Ethiopia.

2) There is no association between treatment with azithromycin and antibiotic resistance, and serotypes covered by the 7-valent vaccine from 2006-2007 in the Goncha Woreda (district of Ethiopia), Amhara region, Ethiopia.

Study Design

The data used for the analysis is from a completed trachoma clinical trial led by a research group from the University of California in San Francisco. The study conducted was a cluster-randomized controlled clinical trial of trachoma control from 2006-2007 in the Goncha Woreda (district of Ethiopia), Amhara region, Ethiopia (40).

The clinical trial was conducted at the sub-kebele level. A sub-kebele is defined as a small administrative Ethiopian district, which is comprised of four to five small villages with 1500 individuals (40). Twenty-four sub-kebeles were randomized to either quarterly

mass azithromycin treatment of children, or untreated control groups. Because the treatment protocol was the same within all sub-kebeles to avoid contamination between groups and villages, only one randomly selected sentinel community was monitored. Clinically active trachoma and ocular chlamydial infection was monitored for individuals randomly selected from the sentinel community. Nasopharygeal samples were also collected and *S. Pneumoniae* strains isolated to assess for antibiotic resistance.

Intervention

The treatment group, which consisted of children aged 0 to 9 years, received one dose of directly observed oral azithromycin (20 mg/kg) at months 0, 3, 6 and 9 for one year. The control group was enrolled at baseline and only monitored one year later, where all individuals above age 1 were provided with a single dose of oral azithromycin (20 mg/kg). For ethical reasons, individuals allergic to macrolides and pregnant women in both groups were given a six-week course of topical 1% tetracycline ointment twice daily instead (41).

Source of samples

Nasopharyngeal samples were collected from 10 randomly selected children ages 0 to 9 years from every sentinel community as follows: in the treated group at baseline and one year after the intervention, and for the control group, samples were collected at one year. Samples were not collected from the control group at baseline. Nasopharygeal swabs from each of the randomly selected children were stored and transported in skim milk typtone-glucose-glycerin medium (STGG) (41). A total of 349 samples were collected. The samples were kept on ice in the field, stored in a -20°C freezer in Ethiopia,

and shipped to San Francisco on dry ice, where they were finally kept at -80°C.

Isolation of *S. pneumoniae* strains

Nasopharygeal swabs were inoculated onto blood agar plates and grown overnight in a 5% CO_2 atmosphere. *S. pneumoniae* colonies were identified and confirmed with an optochin test and bile solubility test after incubation at 37°C in 5% CO_2. Colonies of *S. pneumoniae* test positive with the optochin disc if there is a zone of inhibition greater or equal to 14 mm after incubation. While for bile solubility testing, *S. pneumoniae* is positive with the disappearance or flattening of colonies once in contact with a drop of 3% sodium deoxycholate directly placed onto suspected colonies (42).

From 349 nasopharygeal samples collected, *S. pneumoniae* strains were isolated in 76 of 110 samples collected from the children-treated group at baseline, 93 of 119 samples from the children-treated group at one year, and 98 of 120 samples from the control group at one year. Therefore, a total of 267 *S. pneumoniae* isolates were obtained from the azithromycin clinical trial. All isolates were tested by the investigators for susceptibility to azithromycin, clindamycin, penicillin, and tetracycline with Etest (AB Biodisk, Solna, Sweden). Instructions on minimum inhibitory concentrations were deducted from the package insert. *S. pneumoniae* isolates were then grown on blood agar plates, after overnight incubation at 37°C in a 5% CO_2 atmosphere, and the culture was swabbed and introduced in silica gel packets for shipment to the Rollins School of Public Health (RSPH).

IRB approval

The nasopharygeal swabs and data regarding the subjects were de-identified, and

therefore exempt from IRB.

Molecular analysis

Storage of samples

The isolates were packaged in silica gels from San Francisco, as previous research had demonstrated that the strains can survive for at least 25 days at room temperature in silica gel packages (43). At RSPH, we immediately inoculated those swabs onto blood agar plates and plates were grown over night at 37°C in a 5% CO_2 atmosphere. Isolates were then transferred from the agar plate to previously-sterilized 2 ml of 10% skim milk medium for storage at -80°C.

A side study was conducted in the RSPH laboratory to test the viability of samples in silica gel packets at different temperatures. Four randomly selected silica gel packets were stored at: room temperature, 4°C, -20°C and -80°C. Swabs were inoculated every 14 days onto blood agar plates and grown over night at 37°C in a 5% CO_2 atmosphere. The growth of the *S. pneumoniae* colonies were evaluated visually and rated semiquantitatively as 0 to 3, with 3 being the maximum growth. Most of the isolates survived ~10 months at 4°C. Results of this study are shown in the appendix.

DNA extraction from S. pneumoniae Ethiopian isolates

S. pneumoniae frozen stocks were inoculated onto blood agar plates and incubated overnight at 37°C in a 5% CO_2 atmosphere. A cell suspension was made with 200 µl 5% Chelex and 2 µl proteinase-K (20 mg/ml) solution. This suspension was incubated at 56°C for 30-60 minutes, followed by incubation at 95°C for 10 minutes to inactivate proteinase K. The solution was then vortexed and centrifuged at 13,000 rpm for 5 min.

The DNA-containing supernatant was obtained and stored at -80°C.

DNA extraction from S. pneumoniae control strains

S. pneumoniae control strains, belonging to our laboratory collection that had been previously serotyped using Quellung Reaction were used. The strains were inoculated on blood agar plates and incubated overnight at 37°C in a 5% CO_2 atmosphere. DNA was extracted using the Qiagen Kit (Valencia, CA) for isolation of DNA. The colonies were suspended in 20 µl of proteinase K, mixed by vortexing and incubated at 56°C for 30-60 minutes. 4 µl of RNase (100mg/ml) was added and incubated for 2 min at room temperature, followed by the addition of 200 µl of Buffer AL. This solution was incubated at 70°C for 10 min. Thereafter, 200 µl of ethanol (96-100%) was added to the mixture, vortexed and applied to the QIAamp Spin Column. The solution was centrifuged at 6,000 x g (8000 rpm) for 1 min. The collection tube containing the filtrate was discarded, leaving a clean column. 500 µl of Buffer AW2 was added to the solution and centrifuged at 20,000 x g; 14,000 rpm for 3 min. An additional 200 µl of Buffer AE was added to the solution, incubated at room temperature for 5 min and then centrifuged at 6,000 x g (8,000 rpm) for 1 min. The resulting DNA samples were stored at -20°C until use for multiplex PCR.

Molecular serotyping using a Multiplex Polymerase Chain Reaction (PCR) approach

A previously published multiplex PCR approach was used to determine the molecular serotype of the isolates (44). The reactions were performed in 25 µl volumes, with each reaction containing: 1X PCR MasterMix (Qiagen Multiplex PCR kit), PCR

grade water, the appropriate concentration of each primer (Table 1a and Figure 1) and 2.5µl sample DNA. A total of thirty-nine pairs of primers were used to identify the molecular serotype of the isolates. These target serotypes were grouped into 8 reactions (Table 1). Each reaction included primers that amplified the housekeeping gene *cps* (cpsA-f and cpsA-r), found in all *S. pneumoniae strains* (1), to serve as an internal control. Reactions were conducted sequentially; for example, all 267 samples were tested for serotypes in reaction 1 and for *cps*A. Isolates that tested negative for reaction 1 serotypes but positive for *cps* were further analyzed in reaction 2. Therefore, if a serotype is identified then the sample does not require reaction 2. The same process of elimination was used for the following reactions (Figure 1).

Thermal cycling was performed on a BioRad MyCycler thermocycler apparatus (BioRad Laboratories, Hercules, CA) under the following conditions: 95°C for 15 min; 35 amplification cycles of 94°C for 30 sec, 54°C for 90 sec, and 72°C for 90 s; and a final extension step at 72°C for 10 min.

PCR products (20 µl) were analyzed with gel electrophoresis on 2% agarose gels (BioRad Laboratories, Hercules, CA) stained with 5 µl of 0.5-µg/ml ethydium bromide solution and in 1X TBE Buffer (108g TRIS Base, 55g Boric acid, 9.3g EDTA, pH 8.0) at 100V/cm for 1.5 hours. The results were visualized and photographed with a Biorad Universal Hood II equipment and Quantity One software version 4.6.3 (BioRad Laboratories). PCR bands of the isolates were visually compared to PCR bands of controls and with a molecular size standard (100-bp ladder, 500 µg/ml; New England BioLabs, Ipswich, MA).

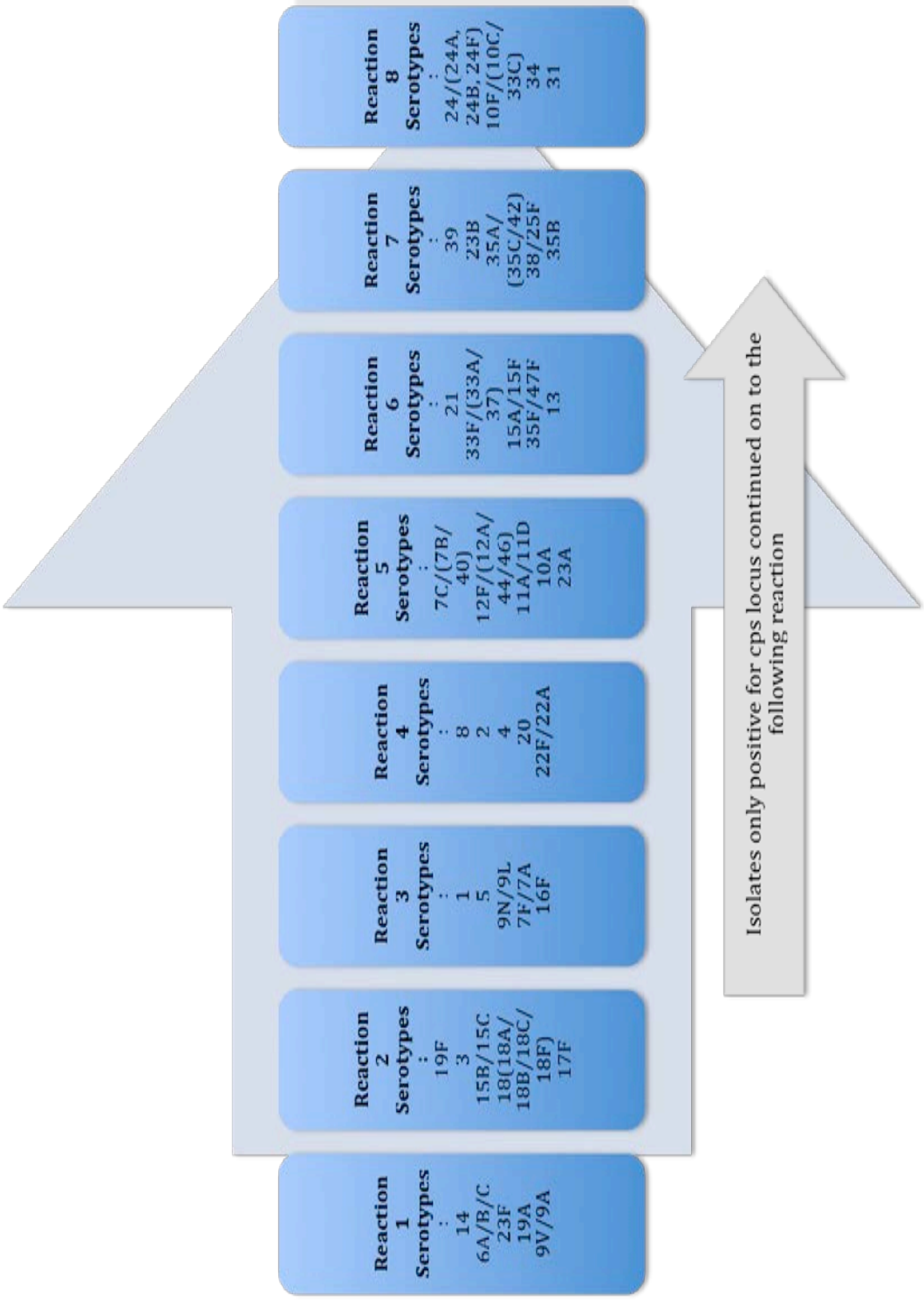
Table 1a: Multiplex PCR reactions

Reaction	Target/Serotype	Primers	Primer Concentration (μM)**	Product Size (bp)
Housekeeping gene*	*cps*A	cpsA-f, cpsA-r	0.1	160
1	14	14-f, 14-r	0.3	189
	6A/B/C	6A/B/C-f, 6A/B/C-r	0.3	250
	23F	23F-f, 23F-r	0.5	384
	19A	19A-f, 19A-r	0.3	566
	9V/9A	9V/9A-f, 9V/9A-r	0.5	816
2	19F	19F-f, 19F-r	0.5	304
	3	3-f, 3-r	0.3	371
	15B/15C	15B/15C-f, 15B/15C-r	0.3	496
	18(18A/18B/18C/18F)	18-f, 18-r	0.3	573
	17F	17F-f, 17F-r	0.5	693
3	1	1-f, 1-r	0.3	280
	5	5-f, 5-r	0.3	362
	9N/9L	9N/9L-f, 9N/9L-r	0.5	516
	7F/7A	7F/7A-f, 7F/7A-r	0.4	599
	16F	16F-f, 16F-r	0.4	717
4	8	8-f, 8-r	0.2	201
	2	2-f, 2-r	0.3	290
	4	4-f, 4-r	0.3	430
	20	20-f, 20-r	0.3	514
	22F/22A	22F/22A-f, 22F/22A-r	0.5	643
5	7C/(7B/40)	7C/(7B/40)-f,	0.3	260
	12F/(12A/44/46)	7C/(7B/40)-r	0.5	376
	11A/11D	12F-f, 12F-r	0.3	463
	10A	11A/11D-f, 11A/11D-r	0.5	628
	23A	10A-f, 10A-r	0.5	722
		23A-f, 23A-r		
6	21	21-f, 21-r	0.2	192
	33F/(33A/37)	33F-f, 33F-r	0.3	338
	15A/15F	15A/15F-f, 15A/15F-r	0.3	434
	35F/47F	35F/47F-f, 35F/47F-r	0.3	517
	13	13-f, 13-r	0.4	655
7	39	39-f, 39-r	0.2	98
	23B	23B-f, 23B-r	0.2	199
	35A/(35C/42)	35A-f, 35A-r	0.3	280
	38/25F	38/25F-f, 38/25F-r	0.3	574
	35B	35B-f, 35B-r	0.5	677
8	24/(24A, 24B, 24F)	24-f, 24-r	0.2	99
	10F/(10C/33C)	10F-f, 10F-r	0.3	248
	34	34-f, 34-r	0.3	408
	31	31-f, 31-r	0.5	701

* cpsA primers are included in all reactions

**Concentration for forward and reverse primers

Figure 1: Multiplex PCR reactions



Statistical Analysis:

The serotyping data from the laboratory analysis were entered into Excel and imported into SAS 9.2 (SAS Institute, Inc., Cary, NC, USA) for all statistical analyses. Although laboratory analysis was performed on 267 samples, statistical analysis was performed for the treatment group. Furthermore, children colonized with several serotypes were excluded from the analysis because associated resistance of multiple serotypes within a sample was not available. As a result, 158 subjects were included in the analysis to evaluate the change in serotype distribution before and after quarterly mass treatment with oral azithromycin.

To assess whether treatment with oral azithromycin is associated with the distribution of serotypes, chi-square tests were conducted to determine whether there was a statistically significant difference between the proportions of the most prevalent serotypes before and after treatment. Prevalent serotypes were defined as being greater or equal to 5 in the study population. When appropriate a Fischer's exact test, two-tailed probability was used. Thereafter, associations with other population characteristics were evaluated. The variables included: age (1-10 years of age), gender (male or female), trachoma status (TF=follicular trachomatous inflammation, TI=intense trachomatous inflammation, N=normal, TS=scarring), azithromycin– resistant strains of *S. pneumoniae* (susceptible or resistant), penicillin–resistant strains of *S. pneumoniae*(susceptible or resistant), clindamycin–resistant strains of *S. pneumoniae*(susceptible or resistant) and tetracycline strains of *S. pneumoniae* (susceptible or resistant). Resistance was defined with the following minimum inhibitory concentrations (MICs) determined by the FDA-approved package insert and withCO_2interpretation values when provided: azithromycin

(CO_2) (≥16 μmg/ml), clindamycin (CO_2) (>2 μmg/ml), benzylpenicillin (≥2 μmg/ml) and tetracycline (≥8 μmg/ml). For further analysis, trachoma status was recorded as infected or ever been infected with trachoma and no disease.

To assess whether treatment with azithromycin was associated with serotypes covered by the 7-valent vaccine, the serotypes were grouped into two groups: PCV7 serotypes or non-PCV7 serotypes. The 7-valent vaccine serotypes comprised of:4, 6B, 9V, 14, 18C, 19F, and 23. Serotypes 23A/B and 23F were included in the 7-valent vaccine serotypes group for serotype 23.

Univariate analysis was performed to assess whether treatment was associated with the 7-valent vaccine serotypes. The population characteristics found to be statistically significant before and after treatment were evaluated also as potential risk factors for vaccine serotypes. The maximum likelihood estimates were used to obtain the crude odds ratios (ORs) for each of the independent variables. The Wald test was done for all models to test for the significance of the independent variables (table 6).

Subsequently, an unconditional multivariable logistic regression analysis was conducted to determine the association between treatment, its resulting characteristics and vaccine serotypes. Collinearity and interaction were assessed prior to performing a backwards elimination to find the best model at determining whether there is an association between treatment and vaccine serotypes. The maximum likelihood estimates were used to obtain the adjusted ORs (after controlling for all of the other independent variables in the model). Furthermore, all statistical analyses were performed at the significance level of α=0.05.

RESULTS

A total of 158 subjects were included in the analysis after the cleaning of data (Table 1 and 2). In the treatment arm, all children aged 1-10 were treated with oral azithromycin (20mg/kg) at months 0, 3, 6 and 9. A total of 69 (44%) children were in the baseline group and 89 (56%) children were in the 1-year follow-up group.

Chi-square tests for all categorical variables and a two-sample t-test for age found that the distribution of covariates was statistically significantly different for all variables except penicillin resistance at the alpha level of 0.05 significant level. The comparison was done between baseline and 1-year follow-up results.

At baseline, the proportion of children with no trachoma was 26% while at 1-year follow-up the proportion was 39% showing that treatment was significantly associated with clearance of the disease (p-value=<0.0001). *S. pneumoniae* strains resistant to azithromycin increased from 5.8% at baseline to 60.7% at one-year follow-up (p-value=<0.0001). Resistance to clindamycin and tetracycline increased from baseline to one-year follow-up (1.5% to 16.9%, p-value=0.002 and 18.8% to 36.0%, p-value=0.02, respectively). The proportion of penicillin resistant strains did not have a statistically significant difference from 5.8% at baseline to 11.2% at one-year follow-up (p-value=0.23).

The distribution of serotypes prevalent in the study population was evaluated (Table 3 and Figure 2). A total of 56 different serotypes were identified by using our multiplex-PCR approach and confirmed by the Quellung reaction. Eight serotypes were not identified and labeled as NT for both serotyping methods.

The overall distribution of serotypes in the study showed that select serotypes were the most prevalent, such as 19F, 6B, 6A, 7F and 9L (table 3). Serotypes that occurred at least five times in the study population were stratified by treatment status to assess whether there was a statistically significant difference in the distribution of prevalent serotypes (table 4 and figure 5). The chi-square statistical test found that when the alpha level was equal to 0.05, the difference in serotype prevalence for baseline and at 1-year follow-up was not statistically significant (p-value=0.58).

Univariate analysis:

The results of the analysis regarding the crude association between treatment and 7-valent vaccine serotypes are shown in table 5. The odds ratio (OR) obtained was not found to be statistically significant at the 0.05 confidence level (OR=1.02, 95% CI=0.49-2.15). To determine whether the additional selected exposures were statistically significant predictors of 7-valent vaccine serotypes, a chi-square analysis was completed at the 0.05 alpha level (tables 6 and 7). The gender of the subject, trachoma status and azithromycin resistance status were not found to be significant at the 0.05 alpha level. The crude associations between penicillin resistance, clindamycin resistance and tetracycline resistance with vaccine serotypes were found to be significant (OR=3.8, p-value=0.02; OR=14.04, p-value=<0.0001; OR=4.5, p-value=0.0002 respectively).

Collinearity assessment:

Collinearity was assessed by running the Collin Macro in SAS and by viewing the condition indices (CIs) and the variance decomposition proportions (VDPs) obtained. A

predetermined cut-off value of 30 or higher for the CI was used to determine whether there is a collinearity problem, meaning two or more variables are correlated and would have trouble estimating the outcome. A VDP of 0.5 or greater for the variable indicates which variables are collinear.

After running the Collin Macro for the full model, the highest CI was 7.9. Thus, no collinearity was found among the exposure variables in the multivariable model because no CIs of 30 or over were obtained.

Interaction and Confounding assessment:

Interaction between treatment and macrolide resistance variables was tested with a likelihood ratio test. The likelihood ratio test was used to statistically compare the fit of the full and reduced models at the 0.05 alpha level. The full model included the interaction terms, while the reduced model did not include the interaction terms being tested.

Full model: Logit P (X) = β_0 +β_1 TREAT +β_2AGE + β_3GENDER + β_4TRACHOMA + β_5AZITH + β_6PEN + β_7CLIN + β_8TETRA + β_9TREAT*AZITH + β_{10}TREAT*PEN + β_{11}TREAT*CLIN + β_{12}TREAT*TETRA + β_{13}AZITH*PEN + β_{14}AZITH*CLIN + β_{15}AZITH*TETRA

Reduced model: Logit P (X) = β_0 +β_1 TREAT +β_2AGE + β_3GENDER + β_4TRACHOMA + β_5AZITH + β_6PEN + β_7CLIN + β_8TETRA

Where:

- Treat (1=Treatment with azithromycin, 0=baseline)
- Age=age of child
- Gender (0=male or 1=female)
- Trachoma (1=with or ever had trachoma disease and 0= no disease)
- Azith=azithromycin resistant (1=resistant, 0=sensitive)
- Pen=Penicillin resistant(1=resistant, 0=sensitive)
- Clin=Clindamycin resistant (1=resistant, 0=sensitive)
- Tetra=Tetracycline resistant (1=resistant, 0=sensitive)

The chi-square value obtained for the test was 11.409 with 7 degrees of freedom (DF), resulting in a p-value=0.1217. Thus, the null hypothesis is failed to be rejected and there is no statistically significant interaction between the two-way product terms assessed. Also, all the variables in the analysis were treated as exposures because there are in the causal pathway to the outcome, therefore, confounding was not assessed in this analysis.

Multivariate Analysis:

The final logistic model obtained with the parameter estimates are presented in table 7.

The final model was:

Logit P (X) = $\beta_0 + \beta_1$TREAT + β_2AGE +β_3GENDER + β_4TRACHOMA + β_5AZITH + β_6PEN + β_7CLIN + β_8TETRA

Where:

- Treat (1=Treatment with azithromycin, 0=baseline)
- Age=age of child
- Gender (0=male or 1=female)
- Trachoma (1=with or ever had trachoma disease and 0= no disease)
- Azith=azithromycin resistant (1=resistant, 0=sensitive)
- Pen=Penicillin resistant(1=resistant, 0=sensitive)
- Clin=Clindamycin resistant (1=resistant, 0=sensitive)
- Tetra=Tetracycline resistant (1=resistant, 0=sensitive)

Based on the final model, treatment with azithromycin was not found to be a statistically significant predictor for vaccine serotypes (OR=1.14, 95% CI: 0.42-3.08) after controlling for all variables in the model. Also, gender and age were not found to be associated with acquiring 7-valent vaccine serotypes (OR=1.00, 95% CI=0.41-2.47 and OR=1.04, 95% CI=0.88-1.23 respectively). However, subjects with azithromycin resistant strains were more likely to be colonized by non-vaccine serotypes (OR=29.59, 95% CI=3.01-291.17), and therefore less likely to have 7-valent vaccine serotypes after controlling for treatment, gender, age, penicillin-, clindamycin- and tetracycline-resistance (OR: 0.03, 95% CI: 0.003-0.33). Tetracycline resistance and penicillin resistance were found to positively associated with 7-valent vaccine serotypes, while controlling for the other variables in the model (OR=5.07, 95% CI: 1.51-16.98 and OR: 4.98, 95% CI: 0.996-24.87, respectively). In addition, clindamycin resistance was found

to be a strong predictor of carrying 7-valent vaccine serotypes (OR: 57.91, 95% CI: 5.13-653.83) after controlling for all other variables in the model. Therefore, according to the final model, treatment with oral azithromycin was not found to be statistically significantly associated with the presence of 7-valent vaccine serotypes.

DISCUSSION

Trachoma is a serious disease in the developing world and has been identified by Ethiopia's Ministry of Health as a priority disease in need of urgent attention and intervention. With the inclusion of quarterly mass oral azithromycin treatment in SAFE-based interventions and the increase in antibiotic resistance of *S. pneumoniae*, determining the impact of oral azithromycin treatment on the distribution of serotypes is essential. This study investigated whether quarterly mass treatments with oral azithromycin changed the distribution of serotypes in treated communities after one year.

A statistically significant difference was found for the following baseline population characteristics before and after treatment: gender, age, trachoma status, azithromycin resistance, clindamycin resistance and tetracycline resistance. The study population was randomly selected from the sentinel communities; yet, randomization was not effective as there should not have been a statistically significant difference in the gender and age distributions before and after treatment. To account for this difference, gender and age were included in the model when evaluating risk factors for 7-valent vaccine serotypes. The increase in azithromycin resistance in *S. pneumoniae* strains after mass treatment was consistent with Haug et al.'s findings that treatment with azithromycin increases resistance in *S. pneumoniae* (30). The proportion of children resistant to penicillin was not statistically different before and after treatment. Although azithromycin treatment can drive multiple resistances by reducing susceptible bacterial strains and shifting the competitive balance in favor of existing resistant strains (45), such as penicillin resistance, the treatment with azithromycin in this study did not drive penicillin resistance. However, azithromycin treatment could explain the increased

prevalence of azithromycin-, clindamycin- and tetracycline resistance from baseline to 1-year follow-up.

The most prevalent serotypes in this study were 19F (8.2%), 6B (5.1%), 6A (4.4%), 7F (3.8%), 9L (3.8%), 41F (3.8%), 34 (3.2%) and 23F (3.2%). However, the difference in proportions for the prevalent serotypes from baseline to 1-year were not found to be statistically significant, which could be due to the general low frequencies for each serotype obtained. A study conducted by Muhe et al., from 1993 to 1995 in a hospital in Addis Ababa, Ethiopia, had found the most prevalent *S. pneumoniae* serotypes were 14, 19F, 20, 1, 18 and 5 (46). The difference in prevalent serotypes was most likely due to the population selected for Muhe et al.'s study, where samples from children with pyogenic meningitis with positive cerebrospinal fluid culture growing *S. pneumoniae* or *H. influenzae* were analyzed (46).

The present study found that the azithromycin-treated population carried an increased proportion of resistant strains to azithromycin, tetracycline and clindamycin. Treatment with azithromycin was not found to be significantly associated with 7-valent vaccine serotypes after adjusting for age, gender and resistance to antibiotics (OR=1.14, 95% CI: 0.42-3.08). Interestingly, the carriage of azithromycin resistant strains was found to be associated with non-vaccine serotypes after adjusting for the other covariates (OR=29.59, 95% CI: 3.01-291.17). However, individuals carrying clindamycin– , penicillin -or tetracycline- resistant strains of *S. pneumoniae* were more likely to have vaccine serotypes after adjusting for treatment, gender, azithromycin resistance and age (OR=57.91, 95% CI: 5.13-653.83; OR=4.98, 95% CI: 0.996-24.87 and OR= 5.07, 95% CI: 1.51-16.98, respectively). A possible explanation for why treatment was not

associated with the outcome of changing the serotype distribution was that the opposing effect of the azithromycin resistance (selection of non – vaccine types) with the clindamycin-, penicillin- and tetracycline resistances (selection of vaccine types) made the net effect of the treatment with azithromycin negligible.

A study conducted by Reinert et al. found 6A, 6B, 9V, 12, 19A, 19F and 23F to be prevalent serotypes that often showed antimicrobial resistance worldwide (11). Dagan et al. found serotypes 6A, 6B, 9V, 14, 19A, 19F and 23F carried by children to be associated with resistance, particularly with high-level penicillin resistance (47). Among Reinert et al. and Dagan et al.'s identified serotypes, our study found 19F, 6B and 23F to be among the most prevalent serotypes. These serotypes, present in the 7-valent vaccine, may explain the strong association found in our study between penicillin-, tetracycline- and clindamycin resistance and vaccine serotypes. Dagan et al. state that introducing the 7-valent vaccine should reduce the carriage of antibiotic resistant serotypes (47). In light of our results regarding the strong association between increasing azithromycin resistance and non-vaccine serotypes, the carriage of azithromycin resistant strains may increase with the introduction of the 7-valent vaccine.

Furthermore, a study by McCormick et al., using data from active population-based surveillance from 1996 to 1999 in the United States, found that there are geographical differences in the proportions of *S. pneumoniae* strains resistant to antimicrobials (48). Also, McCormick et al. did not find an impact of antibiotic use on serotype distribution, after adjusting for geographical differences (48). The results from McCormick et al.'s study agree with our analysis that treatment with azithromycin was not associated with 7-valent vaccine serotypes after controlling for the other variables.

Greenberg et al. has found that azithromycin is an efficient selector of macrolide resistance (49). Therefore, treatment with azithromycin may continue to increase resistance to the other antibiotics as well. The prevention of pneumococcal disease caused by antimicrobial resistant strains not included in the vaccine, such as azithromycin resistant strains, will be a challenge for public health interventions coupled with the increase of vaccine specific antimicrobial resistant strains.

Our findings suggest that although quarterly mass treatments with azithromycin are effective in reducing the disease burden due to trachoma, the proportion of strains resistant to azithromycin, clindamycin and tetracycline are increasing. Interestingly, individuals carrying azithromycin resistant strains were less likely to have 7-valent vaccine serotypes. Yet, subjects carrying strains resistant to penicillin, tetracycline and clindamycin were more likely to have vaccine serotypes. Therefore, future research endeavors should emphasize the role of oral azithromycin treatment on selection of resistance in non – vaccine types of pneumococci. Due to limited region specific research on *S. pneumoniae* antibiotic resistance and serotype distribution, region specific identification of serotypes resistant to antibiotics is necessary.

Public Health implications

Streptococcus pneumoniae, also referred to as the pneumococcus, is a major cause of infant mortality globally, accounting for 700 000 to 1 million deaths every year worldwide (3).

Seven-valent pneumococcal conjugate and 23-valent pneumococcal polysaccharide vaccines have been developed and have been effective in reducing the

burden of pneumococcal pneumonia in the United States. However, the characteristics of the population have a large impact on the effectiveness of the vaccine (2). The seven-valent vaccine has been in circulation in the developing world, and the roll-out of the 10-valent vaccine is beginning in Kenya, which may soon be followed by other African nations (50).

Furthermore, there has been a rapid growth in antimicrobial resistance due to the unnecessary use or misuse of antibiotics in communities (14). Among pathogens that have asymptomatic colonization prior to infection, such as *S. pneumoniae, Staphylococcus aureus,* and *Enterococcus* spp., the selective effects of antibiotic use are poorly understood and further research is necessary to understand the relationship between antibiotic use and resistance (14). As one of the countries with the highest rates of morbidity and mortality due to pneumonia for children under 5, identifying whether quarterly mass treatments with oral azithromycin is modifying the distribution of serotypes in the Ethiopian population is necessary. Although treatment with azithromycin was not found to be associated with 7-valent vaccine serotypes, treatment is a driving factor for the increase in antimicrobial resistance in *S. pneumoniae*. Because resistance to a specific antibiotic can either be positively or inversely associated with 7-valent vaccine serotypes, identifying the specific serotypes leading to either azithromycin-, clindamycin-, penicillin- or tetracycline resistance is important for vaccine efforts. Mass treatment with azithromycin may challenge vaccination efforts on resistance as it selects resistance in non-vaccine serotypes.

Strengths and weaknesses

This clinical trial had many strengths and weaknesses. The goal of the original clinical trial was to evaluate the effectiveness of quarterly mass oral azithromycin treatment in eliminating the prevalence of trachoma. However, this analysis further evaluated the results to determine its effect of *S. pneumoniae* serotype distribution in relation to the 7-valent vaccine.

A major strength of this study is the prospective nature of the data. The study design is a clinical trial, which provides a temporal sequence between exposure with azithromycin treatment and serotype distribution 1 year after treatment. As a result, the analysis did not have to control for certain characteristics that were not available, considering the exposures were the same for all individuals in the village. Furthermore, the issue of seasonality of serotypes was eliminated because the samples were collected exactly one-year after the baseline data.

Furthermore, the duration of the study was short, which reduced selection bias due to loss to follow-up. Children were randomly selected from the villages at baseline and one-year later; therefore, longitudinal and clustered analysis was not necessary.

By administering quarterly azithromycin oral treatments to all children under the age of 10 within a sub-Kebele by health officials, the study reduced possible misclassification of exposure and selection bias. Indeed, a major challenge in clinical trials is assuring the treatment group adheres to the medication. However, in this study, quarterly mass oral azithromycin treatments eliminated this problem.

However, there were some weaknesses to this study. A control population was not available to compare to the baseline data of the treatment arm and at 1-year follow-up.

Although the sample size is sufficient, this study may not be generalizable to the region and Ethiopia as whole because a control population was not available. Therefore, the results are only appropriate for the communities within the clusters analyzed. Lastly, thorough information, including vaccination history, was not available for the children and may have influenced the results.

REFERENCES

1. Pai R, Gertz RE, Beall B. Sequential multiplex PCR approach for determining capsular serotypes of Streptococcus pneumoniae isolates. *J Clin Microbiol* 2006;44(1):124-31.
2. Darboe MK, Fulford AJ, Secka O, et al. The dynamics of nasopharyngeal streptococcus pneumoniae carriage among rural Gambian mother-infant pairs. *BMC Infect Dis* 2010;10:195.
3. O'Brien KL, Wolfson LJ, Watt JP, et al. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet* 2009;374(9693):893-902.
4. Kadioglu A, Weiser JN, Paton JC, et al. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol* 2008;6(4):288-301.
5. Brugger SD, Frey P, Aebi S, et al. Multiple colonization with S. pneumoniae before and after introduction of the seven-valent conjugated pneumococcal polysaccharide vaccine. *PLoS One* 2010;5(7):e11638.
6. Finkelstein JA, Huang SS, Daniel J, et al. Antibiotic-resistant Streptococcus pneumoniae in the heptavalent pneumococcal conjugate vaccine era: predictors of carriage in a multicommunity sample. *Pediatrics* 2003;112(4):862-9.
7. Yao KH, Wang LB, Zhao GM, et al. Pneumococcal serotype distribution and antimicrobial resistance in Chinese children hospitalized for pneumonia. *Vaccine* 2011.

8. Motlova J, Benes C, Kriz P. Incidence of invasive pneumococcal disease in the Czech Republic and serotype coverage by vaccines, 1997-2006. *Epidemiol Infect* 2009;137(4):562-9.

9. Mandell LA, Bartlett JG, Dowell SF, et al. Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. *Clin Infect Dis* 2003;37(11):1405-33.

10. Bogaert D, De Groot R, Hermans PW. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *Lancet Infect Dis* 2004;4(3):144-54.

11. Reinert RR. The antimicrobial resistance profile of Streptococcus pneumoniae. *Clin Microbiol Infect* 2009;15 Suppl 3:7-11.

12. Jenkins SG, Farrell DJ. Increase in pneumococcus macrolide resistance, United States. *Emerg Infect Dis* 2009;15(8):1260-4.

13. Nielsen KL, Hammerum AM, Lambertsen LM, et al. Characterization and transfer studies of macrolide resistance genes in Streptococcus pneumoniae from Denmark. *Scand J Infect Dis* 2010;42(8):586-93.

14. Lipsitch M, Samore MH. Antimicrobial use and antimicrobial resistance: a population perspective. *Emerg Infect Dis* 2002;8(4):347-54.

15. Musher DM, Dowell ME, Shortridge VD, et al. Emergence of macrolide resistance during treatment of pneumococcal pneumonia. *N Engl J Med* 2002;346(8):630-1.

16. Mabey DC, Solomon AW, Foster A. Trachoma. *Lancet* 2003;362(9379):223-9.

17. Heymann D. *Control of Communicable Diseases Manual*. 19th ed. Washington, D.C.: American Public Health Association; 2008.

18. Gyasi ME, Nsiire A, Yayemain D, et al. Trachoma in northern Ghana: a need for further studies. *Ophthalmic Epidemiol* 2010;17(6):343-8.

19. Burton MJ, Rajak SN, Bauer J, et al. Conjunctival transcriptome in scarring trachoma. *Infect Immun* 2011;79(1):499-511.

20. Hu VH, Harding-Esch EM, Burton MJ, et al. Epidemiology and control of trachoma: systematic review. *Trop Med Int Health* 2010;15(6):673-91.

21. WHO. Blindness: Vision 2020 - The Global Initiative for the Elimination of Avoidable Blindness. *Fact Sheet No 213,* 2000.

22. WHO. Report of the Eleventh Meeting of the WHO Alliance for the Global Elimination of Blinding Trachoma. Geneva: WHO, 2007.

23. Mariotti SP, Pascolini D, Rose-Nussbaumer J. Trachoma: global magnitude of a preventable cause of blindness. *Br J Ophthalmol* 2009;93(5):563-8.

24. Cumberland P, Hailu G, Todd J. Active trachoma in children aged three to nine years in rural communities in Ethiopia: prevalence, indicators and risk factors. *Trans R Soc Trop Med Hyg* 2005;99(2):120-7.

25. Hagi M, Schemann JF, Mauny F, et al. Active trachoma among children in Mali: Clustering and environmental risk factors. *PLoS Negl Trop Dis* 2010;4(1):e583.

26. Cumberland P, Edwards T, Hailu G, et al. The impact of community level treatment and preventative interventions on trachoma prevalence in rural Ethiopia. *Int J Epidemiol* 2008;37(3):549-58.

27. Polack S, Brooker S, Kuper H, et al. Mapping the global distribution of trachoma. *Bull World Health Organ* 2005;83(12):913-9.

28. Lakew T, Alemayehu W, Melese M, et al. Importance of coverage and endemicity on the return of infectious trachoma after a single mass antibiotic distribution. *PLoS Negl Trop Dis* 2009;3(8):e507.

29. Bamani S, King JD, Dembele M, et al. Where do we go from here? Prevalence of trachoma three years after stopping mass distribution of antibiotics in the regions of Kayes and Koulikoro, Mali. *PLoS Negl Trop Dis* 2010;4(7):e734.

30. Haug S, Lakew T, Habtemariam G, et al. The decline of pneumococcal resistance after cessation of mass antibiotic distributions for trachoma. *Clin Infect Dis* 2010;51(5):571-4.

31. Amza A, Goldschmidt P, Einterz E, et al. Elimination of active trachoma after two topical mass treatments with azithromycin 1.5% eye drops. *PLoS Negl Trop Dis* 2010;4(11):e895.

32. Solomon AW, Holland MJ, Alexander ND, et al. Mass treatment with single-dose azithromycin for trachoma. *N Engl J Med* 2004;351(19):1962-71.

33. Burton MJ, Holland MJ, Makalo P, et al. Profound and sustained reduction in Chlamydia trachomatis in The Gambia: a five-year longitudinal study of trachoma endemic communities. *PLoS Negl Trop Dis* 2010;4(10).

34. Hong KC, Schachter J, Moncada J, et al. Lack of macrolide resistance in Chlamydia trachomatis after mass azithromycin distributions for trachoma. *Emerg Infect Dis* 2009;15(7):1088-90.

35. Tellis B, Keeffe JE, Taylor HR. Trachoma surveillance annual report, 2007. A report by the National Trachoma Surveillance and Reporting Unit. *Commun Dis Intell* 2008;32(4):388-99.

36. Fry AM, Jha HC, Lietman TM, et al. Adverse and beneficial secondary effects of mass treatment with azithromycin to eliminate blindness due to trachoma in Nepal. *Clin Infect Dis* 2002;35(4):395-402.

37. Leach AJ, Shelby-James TM, Mayo M, et al. A prospective study of the impact of community-based azithromycin treatment of trachoma on carriage and resistance of Streptococcus pneumoniae. *Clin Infect Dis* 1997;24(3):356-62.

38. Ethiopia. The Central Intelligence Agency. (https://www.cia.gov/library/publications/the-world-factbook/geos/et.html). (Accessed November 26, 2010).

39. Roba AA, Wondimu A, Patel D, et al. Effects of intervention with the SAFE strategy on trachoma across Ethiopia. *J Epidemiol Community Health* 2010.

40. Porco TC, Gebre T, Ayele B, et al. Effect of mass distribution of azithromycin for trachoma control on overall mortality in Ethiopian children: a randomized trial. *JAMA* 2009;302(9):962-8.

41. Xiao SK, Zhao CJ, Liu CL, et al. [Resistance and serotype distribution of Streptococcus pneumoniae among adults and children in China.]. *Zhonghua Jie He He Hu Xi Za Zhi* 2010;33(8):601-7.

42. Richter SS, Heilmann KP, Dohrn CL, et al. Accuracy of phenotypic methods for identification of Streptococcus pneumoniae isolates included in surveillance programs. *J Clin Microbiol* 2008;46(7):2184-8.

43. Joshi HH, Gertz RE, Jr., da Gloria Carvalho M, et al. Use of silica desiccant packets for specimen storage and transport to evaluate pneumococcal

nasopharyngeal carriage among Nepalese children. *J Clin Microbiol* 2008;46(9):3175-6.

44. CDC. Multiplex PCR for pneumococcal serotype deduction in clinical specimens. (http://www.cdc.gov/ncidod/biotech/strep/pcr.htm). (Accessed February 28th 2011).

45. Skalet AH, Cevallos V, Ayele B, et al. Antibiotic selection pressure and macrolide resistance in nasopharyngeal Streptococcus pneumoniae: a cluster-randomized clinical trial. *PLoS Med* 2010;7(12):e1000377.

46. Muhe L, Klugman KP. Pneumococcal and Haemophilus influenzae meningitis in a children's hospital in Ethiopia: serotypes and susceptibility patterns. *Trop Med Int Health* 1999;4(6):421-7.

47. Dagan R, Klugman KP. Impact of conjugate pneumococcal vaccines on antibiotic resistance. *Lancet Infect Dis* 2008;8(12):785-95.

48. McCormick AW, Whitney CG, Farley MM, et al. Geographic diversity and temporal trends of antimicrobial resistance in Streptococcus pneumoniae in the United States. *Nat Med* 2003;9(4):424-30.

49. Greenberg D, Givon-Lavi N, Sharf AZ, et al. The association between antibiotic use in the community and nasopharyngeal carriage of antibiotic-resistant Streptococcus pneumoniae in Bedouin children. *Pediatr Infect Dis J* 2008;27(9):776-82.

50. WHO. Kenya Launches Ten Valent Pneumococcal Conjugate Vaccine (PCV 10). 02/14/2011. (http://www.afro.who.int/en/kenya/press-materials/2735-kenya-

launches-ten-valent-pneumococcal-conjugate-vaccine-pcv-10.html). (Accessed 04/08/2011).

FIGURES

Figure 2.

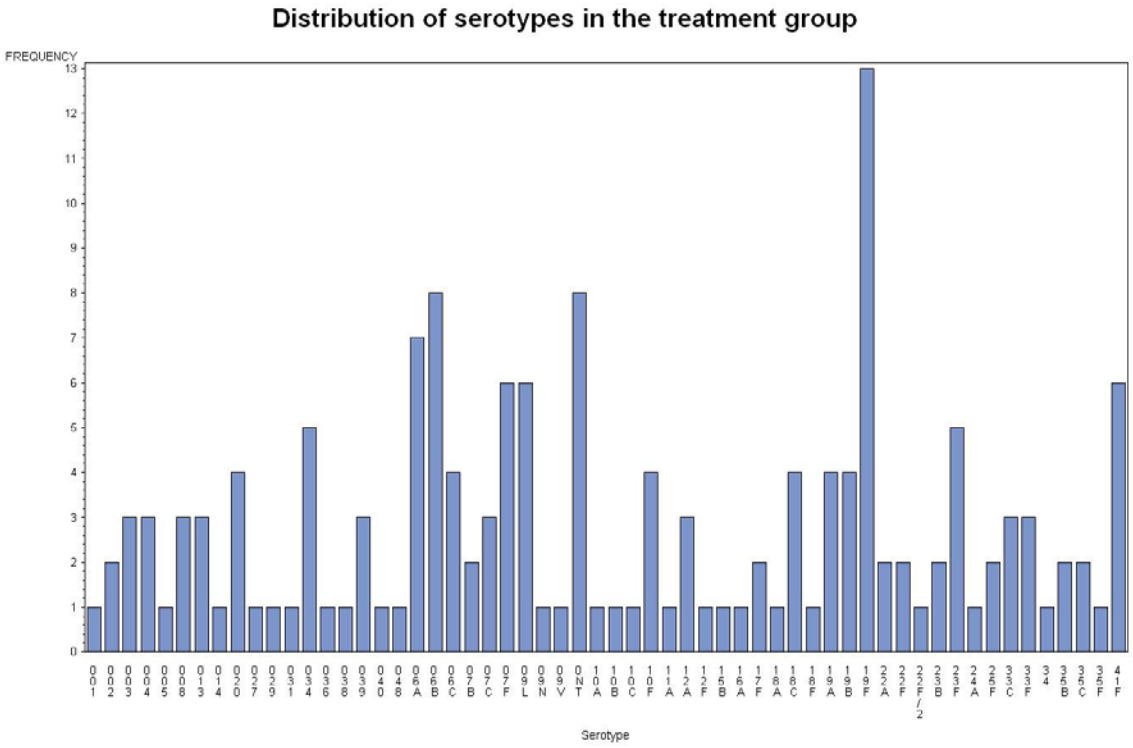


Figure 3.

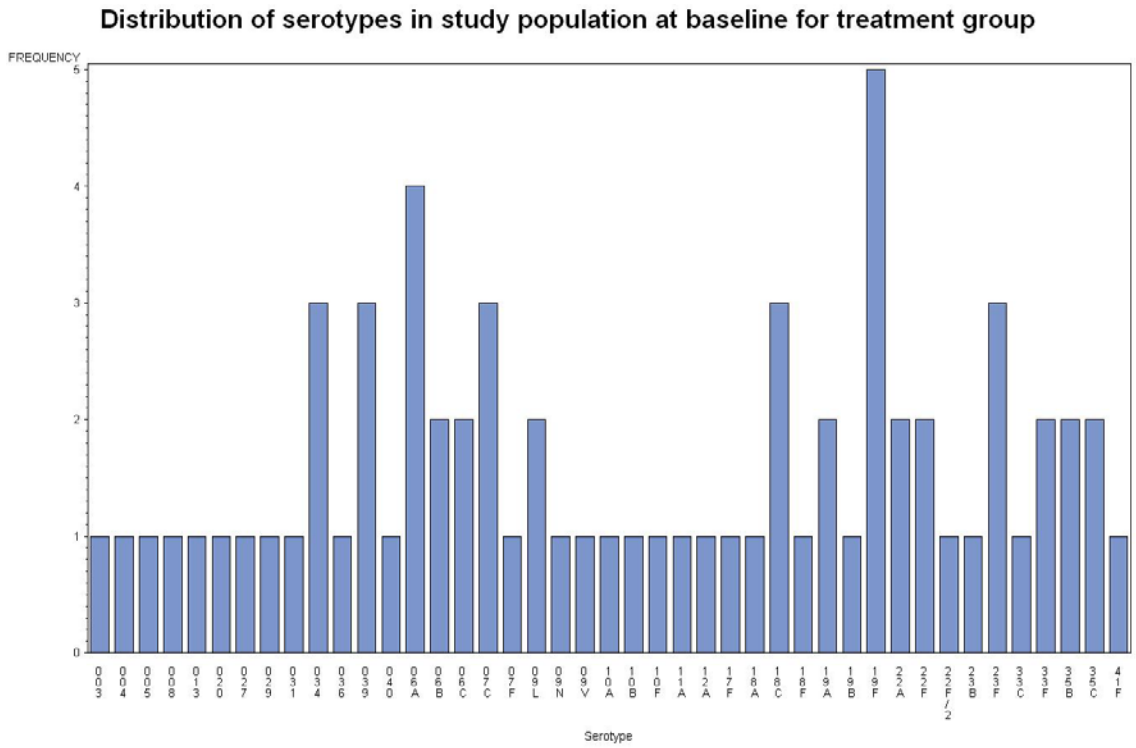

Distribution of serotypes in study population at baseline for treatment group
FREQUENCY
0
1
2
3
4
5
003
004
005
008
013
020
027
029
031
034
036
039
040
06A
06B
06C
07C
07F
09L
09N
09V
10A
10B
10F
11A
12A
17F
18A
18C
18F
19A
19B
19F
22A
22F
23F/2
23B
23F
33C
33F
35B
35C
41F
Serotype

Figure 4.

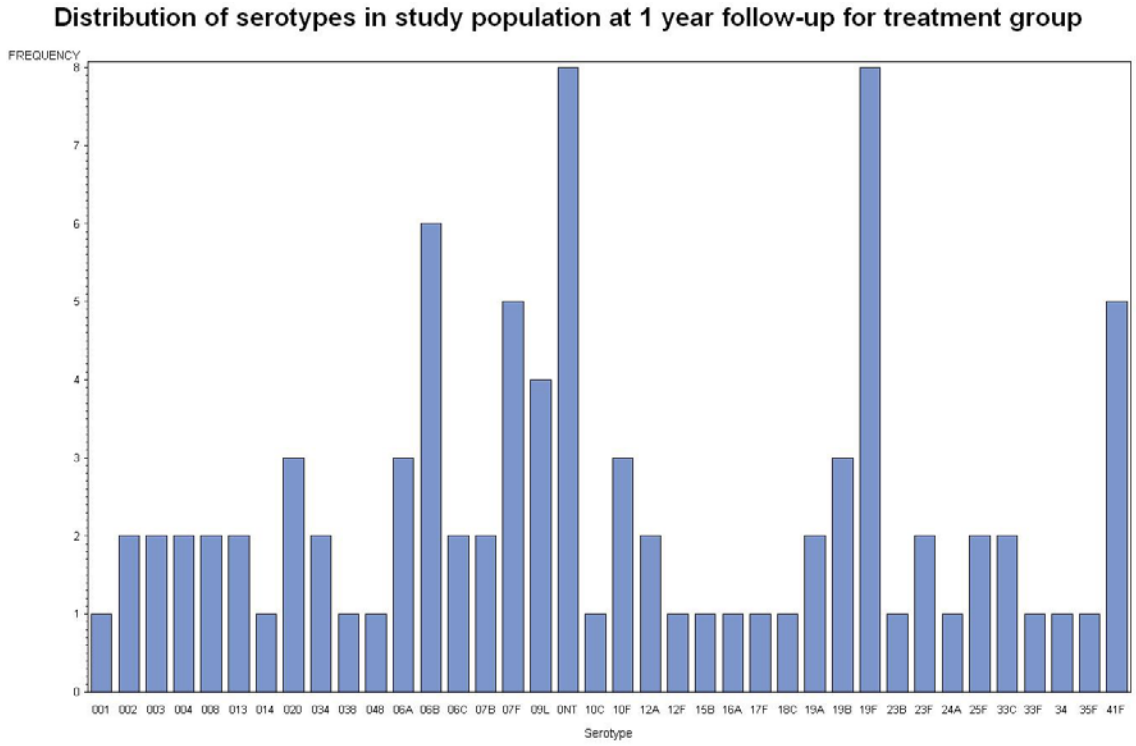
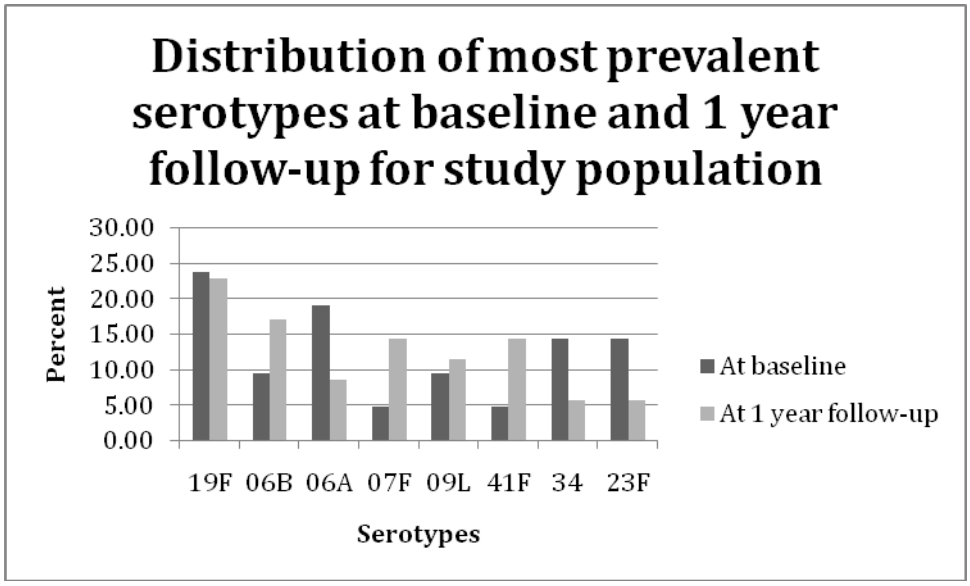

Distribution of serotypes in study population at 1 year follow-up for treatment group
FREQUENCY
0
1
2
3
4
5
6
7
8
001 002 003 004 008 013 014 020 034 038 048 06A 06B 06C 07B 07F 09L 0NT 10C 10F 12A 12F 15B 16A 17F 18C 19A 19B 19F 23B 23F 24A 25F 33C 33F 34 35F 41F
Serotype

Figure 5.


Distribution of most prevalent serotypes at baseline and 1 year follow-up for study population
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Percent
19F
06B
06A
07F
09L
41F
34
23F
Serotypes
At baseline
At 1 year follow-up

TABLES

Table 1. Characteristics of study population at baseline and after one year of treatment

	Treatment group (N=158)				
	At baseline (N=69)		At 1-year (N=89)		
	No.	%	No.	%	p-value
Gender of child					
Female	39	56.5	43	48.3	0.01
Male	25	36.2	46	51.7	
Missing	5	7.3	0	0	
Trachoma status of child*, **					
no disease	18	26.1	35	39.3	<0.0001
TF	20	29.0	35	39.3	
TI	10	14.5	3	3.4	
TF/TI	11	15.9	3	3.4	
TS	1	1.5	12	13.5	
TI/TS	2	2.9	0	0	
TI/TF/TS	1	1.5	0	0	
Missing	6	8.7	0	0	
Azithromycin resistant					
Yes	4	5.8	54	60.7	<0.0001
No	65	94.2	35	39.3	
Missing	0	0.00	0	0	
Penicillin resistant					
Yes	4	5.8	10	11.2	0.23
No	65	94.2	79	88.8	
Missing	0	0	0	0	
Clindamycin resistant					
Yes	1	1.5	15	16.9	0.002
No	68	98.6	74	83.2	
Missing	0	0	0	0	
Tetracycline resistant					
Yes	13	18.8	32	36.0	0.02
No	56	81.2	57	64.0	
Missing	0	0	0	0	

*TF=follicular trachomatous inflammation, TI=intense trachomatous inflammation, No disease=normal, TS=scarring

** After dichotomizing trachoma status, the p-value was no longer statistically significant (p-value=0.7238).

Table 2. Age distribution at baseline and at follow-up for study population

	Treatment	
	At baseline	**At 1-year**
n	65	89
mean	4.70	4.91
s.d.	2.71	2.77
median	5	5
25th percentile	3	3
75th percentile	7	7
minimum	0	0
maximum	9	9
Skewness	0.00	-0.10
Kurtosis	-1.14	-1.12

Table 3. Distribution of serotypes in the study population

	Total		Treatment group (N=158)			
			At baseline (N=69)		At 1-year (N=89)	
Serotypes	**No.**	**%**	**No.**	**%**	**No.**	**%**
19F	13	8.2	5	7.3	8	9.0
06B	8	5.1	2	2.9	6	6.7
0NT	8	5.1	0	0.0	8	9.0
06A	7	4.4	4	5.8	3	3.4
07F	6	3.8	1	1.5	5	5.6
09L	6	3.8	2	2.9	4	4.5
41F	6	3.8	1	1.5	5	5.6
34	5	3.2	3	4.4	2	2.3
23F	5	3.2	3	4.4	2	2.3
20	4	2.5	1	1.5	3	3.4
06C	4	2.5	2	2.9	2	2.3
10F	4	2.5	1	1.5	3	3.4
18C	4	2.5	3	4.4	1	1.1
19A	4	2.5	2	2.9	2	2.3
19B	4	2.5	1	1.5	3	3.4
3	3	1.9	1	1.5	2	2.3
4	3	1.9	1	1.5	2	2.3
8	3	1.9	1	1.5	2	2.3
13	3	1.9	1	1.5	2	2.3
39	3	1.9	3	4.4	0	0.0
07C	3	1.9	3	4.4	0	0.0
12A	3	1.9	1	1.5	2	2.3
33C	3	1.9	1	1.5	2	2.3
33F	3	1.9	2	2.9	1	1.1
2	2	1.3	0	0.0	2	2.3
07B	2	1.3	0	0.0	2	2.3
17F	2	1.3	1	1.5	1	1.1
22A	2	1.3	2	2.9	0	0.0
22F	2	1.3	2	2.9	0	0.0
23B	2	1.3	1	1.5	1	1.1
25F	2	1.3	0	0.0	2	2.3
35B	2	1.3	2	2.9	0	0.0
35C	2	1.3	2	2.9	0	0.0
1	1	0.6	0	0.0	1	1.1
5	1	0.6	1	1.5	0	0.0
14	1	0.6	0	0.0	1	1.1
27	1	0.6	1	1.5	0	0.0

Table 3 (continued).

	Total		Treatment group (N=158)			
			At baseline (N=69)		At 1-year (N=89)	
Serotype	**No.**	**%**	**No.**	**%**	**No.**	**%**
29	1	0.6	1	1.5	0	0.0
31	1	0.6	1	1.5	0	0.0
36	1	0.6	1	1.5	0	0.0
38	1	0.6	0	0.0	1	1.1
40	1	0.6	1	1.5	0	0.0
48	1	0.6	0	0.0	1	1.1
09N	1	0.6	1	1.5	0	0.0
09V	1	0.6	1	1.5	0	0.0
10A	1	0.6	1	1.5	0	0.0
10B	1	0.6	1	1.5	0	0.0
10C	1	0.6	0	0.0	1	1.1
11A	1	0.6	1	1.5	0	0.0
12F	1	0.6	0	0.0	1	1.1
15B	1	0.6	0	0.0	1	1.1
16A	1	0.6	0	0.0	1	1.1
18A	1	0.6	1	1.5	0	0.0
18F	1	0.6	1	1.5	0	0.0
22F/22A	1	0.6	1	1.5	0	0.0
24A	1	0.6	0	0.0	1	1.1
34	1	0.6	0	0.0	1	1.1
35F	1	0.6	0	0.0	1	1.1

Table 4. Distribution of the most prevalent serotypes

	Treatment group (N=158)				
	At baseline (N=69)		At 1-year (N=89)		
Serotypes	**No.**	**%**	**No.**	**%**	**Fisher's exact p-value**
19F	5	23.8	8	22.9	0.58
06B	2	9.5	6	17.1	
06A	4	19.1	3	8.6	
07F	1	4.8	5	14.3	
09L	2	9.5	4	11.4	
41F	1	4.8	5	14.3	
34	3	14.3	2	5.7	
23F	3	14.3	2	5.1	

Table 5. Distribution of 7-valent vaccine serotypes in the study population

	Treatment group (N=158)					
	At baseline (N=69)		At 1-year (N=89)			
	No.	**%**	**No.**	**%**	**OR**	**95%CI**
7-valent vaccine serotypes						
Yes	16	23.2	21	23.6	1.02	0.49-2.15
No	53	76.8	68	76.4	--	

Table 6. Crude association between 7-valent vaccine serotypes with subjects characteristics

	With vaccine serotype (N=37)		Non-vaccine serotype (N=121)		
	No.	**%**	**No.**	**%**	**p-value**
Gender of child					0.7534
Female	19	23.2	63	76.8	
Male	18	25.4	53	74.6	
Trachoma status of child					0.8532
no disease	15	24.2	47	75.8	
With or ever had infection	22	22.9	74	77.1	
Azithromycin resistant					0.8205
Yes	13	22.4	45	77.6	
No	24	24.0	76	76.0	
Penicillin resistant					0.0197
Yes	7	50.0	7	50.0	
No	30	20.8	114	79.2	
Clindamycin resistant					<0.0001
Yes	12	75.0	4	25.0	
No	25	17.6	117	82.4	
Tetracycline resistant					0.0002
Yes	20	44.4	25	55.6	
No	17	15.0	96	85.0	

Table 7. Crude association between having 7-valent vaccine serotypes and subject characteristics

	Odds Ratio	95% CI	p-value
Gender of child			
Female	0.88	0.42-1.86	0.7534
Male	Ref.		
Trachoma status of child			
With or ever had infection	0.93	0.44-1.97	0.8532
No disease	Ref.		
Azithromycin resistant			
Yes	0.92	0.42-1.97	0.8205
No	Ref.		
Penicillin resistant			
Yes	3.80	1.24-11.67	0.02
No	Ref.		
Clindamycin resistant			
Yes	14.04	4.18-47.14	<0.0001
No	Ref.		
Tetracycline resistant			
Yes	4.52	2.07-9.88	0.0002
No	Ref.		

Table 8. Multivariate Analysis results for final model

Parameter	Estimate	Standard Error	OR	95% CI		Wald Chi-Square	Pr > ChiSq
Intercept	-1.5777	0.6798				5.3860	0.0203
Treatment with azithromycin	0.1274	0.5094	1.14	0.42	3.08	0.0625	0.8026
Trachoma Status	-0.2817	0.4824	0.75	0.29	1.94	0.3411	0.5592
Gender	-0.00023	0.4611	1.00	0.41	2.47	0.0000	0.9996
Age	0.0394	0.0844	1.04	0.88	1.23	0.2183	0.6403
Azithromycin Resistance	-3.3875	1.1665	0.03	0.003	0.33	8.4324	0.0037
Penicillin resistance	1.6049	0.8207	4.98	0.996	24.87	3.8237	0.0505
Clindamycin resistance	4.0589	1.2367	57.91	5.13	653.83	10.7709	0.0010
Tetracycline resistance	1.6227	0.6171	5.07	1.51	16.98	6.9153	0.0085

APPENDIX

Viability of *S. pneumoniae* strains after storage in silica gel packets at different temperatures*

Temperature	Day 0	Day 7	Day 14	Day 28	Day 48	4 months 3 weeks
RT	3	3	3	2	3	0
	3	1	2	1	0	0
	3	2	3	1	0	0
	3	1	1	1	0	0
4 C	3	2	3	3	3	2
	3	1	3	3	2	2
	3	2	3	3	3	2
	3	3	3	3	3	2
-20 C	3	1	2	2	2	2
	3	2	3	3	2	2
	3	3	3	3	3	2
	3	2	3	3	2	2
-80 C	3	3	3	3	3	2
	3	3	3	3	3	2
	3	3	3	3	3	2
	3	3	3	3	3	2

*Scores= 3 (abundant growth), 2 (regular growth), 1 (low growth) and 0 (non viable)