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Original article

Prevalence and serotypes of *Streptococcus pneumoniae* among under five children attending Toro General Hospital, Bauchi State, Nigeria

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ABSTRACT

Background: *Streptococcus pneumoniae* (*S. pneumoniae*) is an important pathogen responsible for numerous infections in humans particularly children under 5 years causing unparalleled fatality. **Aim:** This study set out to determine the prevalence and circulating serotypes of *Streptococcus pneumoniae* in under 5 years old children attending Toro General Hospital, Bauchi State, Nigeria. **Methods:** The study was cross-sectional, and hospital based. A total of 114 nasopharyngeal swabs were collected from children < 5 years old. *Streptococcus pneumoniae* was isolated, biochemically identified, and molecularly confirmed using PCR via the gene marker *CpsA*. Latex agglutination was used to serotype the confirmed isolates. **Results:** a prevalence of 22.6% was established in this study for *S. pneumoniae*. The age group 0 – 11 months had the highest prevalence of 28.1% while 36 – 47 months had the least prevalence (11.8%). Males had relatively higher prevalence of 29.0% compared to females (20.0%). There was no significant association between the carriage of *S. pneumoniae*, age, and sex ($p > 0.05$). Though there was no significant association between vaccination status and prevalence of *S. pneumoniae* ($p = 0.937$), non-vaccinated group had lower prevalence of 22.4% as opposed to 23.1% in the vaccinated subjects. Six circulating serotypes [1 (23.1%), 6 (34.6%), 9 (3.8%), 11 (11.5%), 19 (3.8%), and 23 (3.8%)] were detected. Serotypes 1 and 6 were found in both vaccinated and non-vaccinated children but serotypes 9, 11, 19, and 23 were detected in vaccinated children only. **Conclusion:** PCV vaccine and non-vaccine *S. pneumoniae* serotypes were found to be prevalent and circulating among under 5 children in the current study.

Introduction

Streptococcus pneumoniae (*S. pneumoniae*) belongs to the *Streptococcus* genus and is a Gram-positive alpha hemolytic diplococcus bacterium. *Streptococcus pneumoniae* is a major cause of pneumonia in the especially among the very young and old population. Other infections caused by the bacterium include acute sinusitis, otitis media, meningitis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess [1].

Nasopharyngeal carriage of pneumococci is believed to be an important step that precedes invasive disease and has been used as a measure for efficacy of vaccines [2], since the vaccines are serotype-specific. Consequently, of the two types of Pneumococcal polysaccharide vaccines: 23-valent pneumococcal polysaccharide vaccine (PPV23), and pneumococcal conjugate vaccine (10-valent PCV10 and 13-valent PCV13) now available on the global market [3, 4], the PCVs have been recommended for children under the age of two

years and older children. But there are concerns that protection is confined only to vaccine serotypes, with the danger of non-vaccine serotypes replacing vaccine serotypes in the population in carriage and disease [5]. It also is important to continuously monitor circulating *S. pneumoniae* serotypes so as to detect any changes in serotypes responsible for invasive disease over time.

Although studies on the phenotypic and antigenic patterns of *S. pneumoniae* have been well reported from parts of African subregion, there is dearth of information from Northern Nigeria, and evidence-based data is very essential in helping to guide the right immunization approach. Consequently, our study aimed to assess the nasopharyngeal (NP) carriage of vaccine-type (VT) *S. pneumoniae*, which can be used to assess the effectiveness of the pneumococcal conjugate vaccine (PCV10). It also sought to detect non-vaccine and emerging serotypes of *S. pneumoniae* among both vaccinated and non-vaccinated children in the study area, which may need to be considered while developing new vaccines in the future.

Materials and Methods

Study area

The study was carried out at General Hospital in Toro Ward of Toro LGA, Bauchi State, North-eastern Nigeria. The hospital has a bed space of 120. Apart from the people within Toro L.G.A, the Hospital also services the neighboring communities from Plateau State (Jos-North and East), North-Central, and Kano State from the North-Western parts of the country. It offer various services based on the units as follows- HIV unit, antenatal care(ANC), family planning, tuberculosis DOT unit, theater unit, out-patient unit for women, men, and pediatrics, maternity unit (in and out), laboratory services, pharmacy unit, specialist unit, immunization, surveillance, health education, sanitation, isolation center for COVID 19, social welfare and record units respectively.

Study design

The research was a cross-sectional hospital-based study. Participants were strictly allocated to one of two groups: (1) The group of less than five (5) years and vaccinated with the PCV10; (2) The group of those less than five (5) years but not vaccinated with PCV10.

Inclusion and exclusion criteria

All children less than five (5) years attending the hospital for different services like routine immunization, out-patients, in-patients etc. whose parents consented to the study, were enrolled using consent forms. Parents of children above the age of five years, as well as those of less than five (5) years children who declined consent, were excluded for this study.

Ethical clearance and consent

Ethical approval was obtained from the committee on Health Research ethics, of Bingham University Teaching Hospital Jos Plateau State (reference number NHREC/21/05/2005/00644) and Ministry of Health Bauchi State (reference number NHREC/12/05/2013/2019/52).

Data collection

Structured questionnaire was administered to the caregivers to abstract information on demographics and vaccination status of the children.

Determination of sample size

The sample size was determined using a prevalence (p) of 92% from a recent study carried out by Ifedayo, (2018) using the Cochran's formula as stated below:

$$N = Z^2 pq / e^2$$

Where:

e is the desired level of precision (i.e., margin of error). p is the (estimated) proportion of the population which has the attribute in question

q is 1-p. Z is our confidence value which is found in a Z-table. In this study, Z=95% confidence value give us 1.96. P =92%=0.92 e=5% our level of precision. q=1-0.92=0.08. Therefore, substituting into the formula. $N = \{(1.96)^2(0.92)(0.08)\} / (0.05)^2 = 0.2827/0.0025 = 114$.

Sample collection and processing

A total of 114 nasopharyngeal swabs were collected from children younger than 5 (< 5) years old. Each sample was labeled with the appropriate information. Swabs were collected and processed according to the current WHO pneumococcal carriage detection protocol [6]. Using sterile scissors, the tip of the swab was cut off into a 2mL Cryovials containing 1mL STGG transport medium [7]. The NPS-STGG specimens were transported to the laboratory in a cool box, frozen in a laboratory bio-cooler on the day of collection, and subsequently cultured in batches [8].

(i) Isolation of *Streptococcus pneumoniae*

Using a swab of the specimen, the inoculum was applied to a small area of blood agar plate supplemented with 5 µg/mL of gentamycin (to prevent the growth of non-pneumococcal isolates) following manufacturer's instructions. The inoculating loop was then sterilized with flame and used to spread the inoculum [9].

The plates were then incubated overnight at 37°C. After incubation for 24 hours, the plates were checked for bacterial growth. Bacterial colonies were then subcultured onto nutrient agar and incubated for 24 hours to get a pure culture. *Streptococcus pneumoniae* were identified by Gram staining the isolates according to the procedure described by Cheesebrough [9]. *Streptococcus pneumoniae*, or *pneumococcus*, was identified as a Gram-positive, alpha-hemolytic diplococcus bacterium. Confirmation was done using catalase and optochin sensitivity tests as described by Cheesebrough [9] as follows;

(ii) Preservation of *Streptococcus pneumoniae*- All confirmed *S. pneumoniae* were stored in STGG and Dorset egg medium at -7 °C in line with the standard protocols [6,10], until retrieved for further processing.

(iii) Molecular identification of *S. pneumoniae* - *CpsA* was used as a novel genetic marker specific for identification of *S. pneumoniae* and to differentiate it from the closely related *viridans* group streptococci as well as other pneumococcus-like streptococci such as *S. pseudopneumoniae* [11]. The gene marker *CpsA* had a molecular weight of 296bp.

(iv) Molecular characterization of isolates for virulent gene

DNA extraction

BIONEER AccuPowerHotStart PCR PreMix (v1/2016-02-15) was used for the DNA extraction and PCR amplification. Approximately 1×10^9 of the bacteria cells was collected after centrifuging at 8000 rpm for 5 minutes. Then the supernatant was discarded. Then 180 µl of lysis buffer [20mM Tris-HCL (PH 8.0), 2mM EDTA and 1.2% Triton x-100] was added to the collected cell pellet and completely re-suspended by vortexing or pipetting. Next, 20 µl of lysosome and 10 µl of RNase A were added and mixed thoroughly by vortexing. Twenty microliter (20 µl) of proteinase K was added with 200 µl of GB buffer and mixed thoroughly by vortexing. The mixture was then incubated in the tube at 60°C for

30minutes or until cells were completely lysed. Then 400 µl of absolute ethanol was added and mixed well by pipetting. The lysate was carefully transferred into the reservoir of the binding column tube without wetting the rim. It was then closed and centrifuged at 8,000rpm for 1 minute. Next, 500 µl of W2 buffer was then added. The tube was capped and centrifuged at 8,000rpm for 1 minute. The solution was then discarded from the collection tube, centrifuged at 13,000rpm for 1 minute to completely remove the ethanol. The binding column tube was then transferred to a new tube for elution. Lastly, 50-200 µl of EA buffer was added to the binding column tube and allowed to stand for 1 minute at 15-25°C, and it was centrifuged at 8,000rpm for 1 minute to elute.

Amplification of DNA by polymerase chain reactions (PCR)

The primer solution was thawed, and the DNA template was prepared. An appropriate volume of the diluted primer was distributed into each AccuPowerHotStartPreMix. The template DNA (≤ 100 ng/reaction) was then added to the individual PCR tubes. Distilled water was added to the tubes until the total volume of mixture becomes 20µl. The thermal cycler was then programmed according to the manufacturer's instructions. PCR tubes were then placed in the thermal cycler and cycling program started. Finally, the sample was loaded on agarose gel without adding dye mixture and electrophoresis performed.

PCR conditions-Conventional PCR was done in 1 volume with x1PCR buffer (10 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.1% Triton X-100, 0.01% [wt/vol] gelatin), 250µM concentrations of each deoxynucleoside triphosphate (dNTP), 0.2µM of DNA polymerase, 1.5mM MgCl₂. The forward (*CpsA*) 5'-AGTGGTAACTGCGTTAGTCC-3' and reverse primer 3'-CTGCCAAGTAAGACGAACTC-5' were used. Thermo-cycling was done in a Hybrid PCR RS232 USA with the following conditions: Pre-denaturing 94°C for 5 minutes; denaturing 35 amplification cycles of 94°C for 30 seconds, annealing 54°C for 30 seconds, and extension 72°C for 60 seconds; and a final extension step at 72°C for 5 minutes. The expected amplicon size was 296bp.

Gel electrophoresis

The amplicon was analyzed by electrophoresis on 2% Seakem LE agarose gel (BMA, Rockland, Maine) in 0.5-TBE buffer at 5.2 V/cm for 2 hours. Gels were stained in a 0.1-g/ml-1 ethidium bromide solution and photographed by standard procedures, and the amplification results were visually analyzed.

The sizes of the PCR products were estimated by comparison with a 100 bp molecular ladder.

(v) Sero-typing of isolates by latex agglutination

A drop of polyvalent serum and physiological saline (30µl) on different sections of a partitioned slide. Next, a small but visible number of bacteria cells on the area above the drop of serum or normal saline is placed and then it was mixed well using a loop. The glass slide was tilted back and forth for 1-minute and observed for agglutination. It was checked for spontaneous agglutination.

When a positive result is observed for the polyvalent serum, a corresponding monovalent antiserum is placed on a glass slide and the procedure is repeated

Polyvalent	Monovalent
1	1, 2, 3, 4, 5
2	6, 8, 9, 10
3	11, 12, 14, 15
4	17, 18, 22
5	20, 33
6	23
8	7, 19

Results

Overall prevalence of 22.6% of *Streptococcus pneumoniae* was obtained among children (<5years) attending Toro General Hospital, Bauchi State. Children within age group 0-11 months had the highest prevalence of 28.1 % (9/32), while the infection was least prevalent [11.8% (2/17)] among children within 36-47 months. Although the males recorded the highest prevalence of 29.0% (14/54) compared to their female counterparts, who had a prevalence of 20.0% (12/60), there was no statistically significant association in the carriage rate with respect to age and sex of the study population [(Age group: $\chi^2=1.446$, $p=0.836$; Sex: $\chi^2=0.020$, $p=0.888$)] as shown in **table (1)**.

The prevalence of *S.pneumoniae* based on vaccination status of the subjects is as shown on **table (2)**. A prevalence of 23.1% (15/65) was observed among the 65 study subjects who were vaccinated. Eleven subjects (22.4%) had the

to determine the result. The pneumococcal serotype was determined with the aid of a chart provided by manufacturer's guide. The antisera used was for the one with 23 serotypes that covers all the serotypes used in vaccines i.e., the pneumococcal polysaccharide vaccine (4 6B 9V 14 18C 19F 23F 1 5 7F 3 33F 19A2 8 9N 10A 11A 12F 15B 17F 20 22F).

The serotypes results were recorded as matching or non-typeable using the latex agglutination test kit as shown below:

infection among the non-vaccinated group, but the vaccination status was not significantly associated ($\chi^2=0.106$, $p=0.937$) with prevalence of *S. pneumoniae*.

This study detected 6 circulating serotypes namely serotype 1 (23.1%), 6 (34.6%) 9 (3.8%), 11 (11.5%), 19 (3.8%), and 23 (3.8%). Five (19.2%) of the isolates were non-typeable (NT). The most predominant serotype was 6 (34.6%), while the least occurring serotypes were 9, 19 and 23 at 3.8% (1) each (Fig. 1). The occurrence of circulating serotypes based on *S. pneumoniae* vaccination status of study population (Fig. 2). Whereas serotypes 1 and 6 were found in both vaccinated and non-vaccinated subjects, all the others (9, 11, 19, 23) were not detected among the non-vaccinated population. Both groups also carried the non-typeable strains in varying degrees as depicted in the figure. No statistically significant association ($\chi^2=6.517$, $p=0.368$) was observed in serotype carriage between the two groups.

Table 1. Age and sex-specific prevalence of *Streptococcus pneumoniae* among children (<5years) attending Toro General Hospital, Bauchi State

Age group (months)	Female				Male				Total examined	+ve (%)
	No. examined	+ve (%)	χ^2	p-value	No. examined	+ve (%)	χ^2	p-value		
0 – 11	18	5(27.8)	1.446	0.836	14	4 (28.6)	0.020	0.888	32	9(28.1)
12-23	10	2 (20.0)			11	2(18.2)			21	4(19.0)
24-35	11	1 (9.1)			9	2 (22.2)			20	3(15.0)
36-47	8	1(12.5)			9	1 (11.1)			17	2(11.8)
48-59	13	1 (7.7)			11	5 (45.5)			24	6(26.0)
Total	60	12(20.0)			54	14(29.0)			114	26(22.8)

Table 2.Prevalence of *Streptococcus pneumoniae* according to vaccination status of children (<5years) attending Toro General Hospital, Bauchi State.

Vaccinated	Number examined	Number Positive (%)	Number Negative (%)	χ^2	p-value
Yes	65	15(23.1)	50(76.9)	0.106	0.937
No	49	11(22.4)	38(77.6)		
Total	114	26(22.8)	88(77.2)		

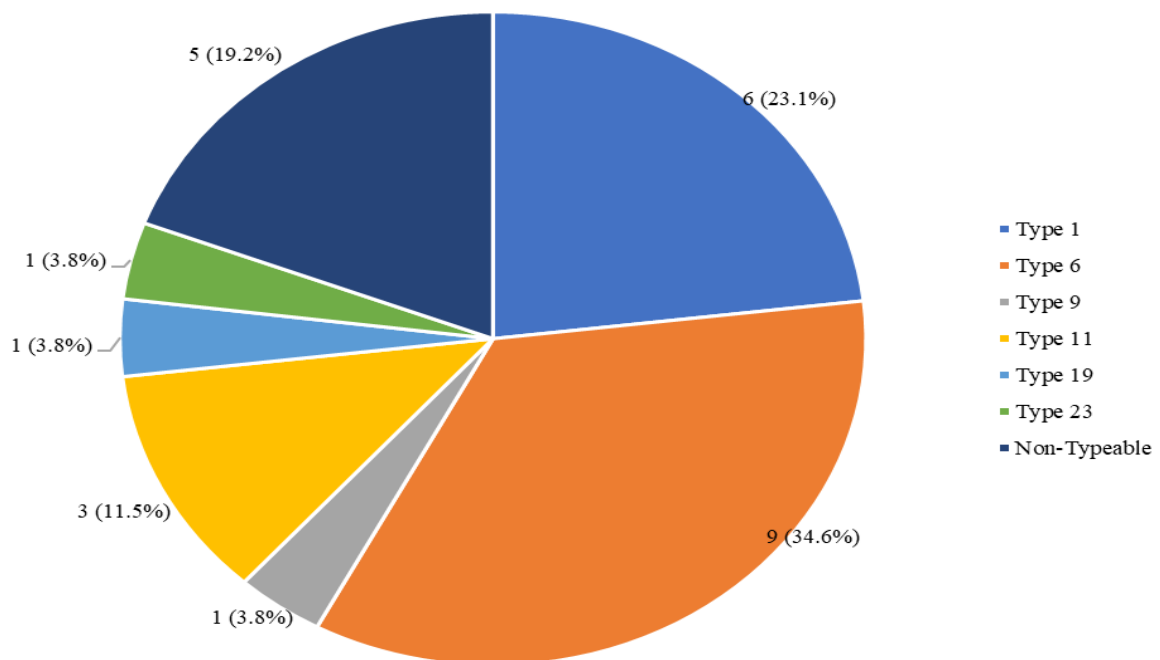


Fig. 1: Prevalence of *S. Pneumoniae* serotypes among under five years children attending Toro General Hospital, Bauchi, Nigeria

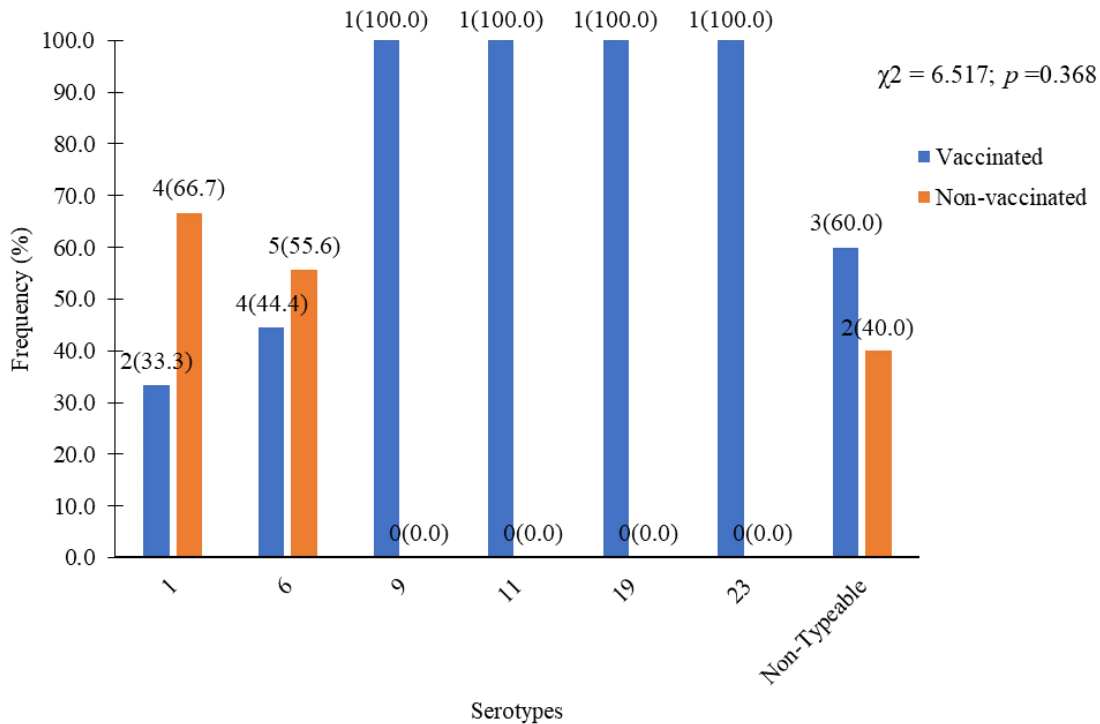


Figure 2. Circulating serotypes of *S. pneumoniae*, based on immunization status of children (<5years) attending Toro General Hospital, Bauchi State.

Discussion

The prevalence of 22.6% recorded in this study is high although shows a reduction in prevalence of *S.pneumoniae* among the study population when compared with the previous 33% reported by **Falade et al.** [12], before the introduction of PCV10 vaccines as part of children's immunization routines in Nigeria. This may suggest some improvement because of the vaccination. A similar observation was reported from the United States where the introduction of the PCV7 vaccines reduced the incidence of both invasive and carriage serotypes of *S. pneumoniae* across all age groups [13].

It was also observed that prevalence was more (28.1%) in the younger age (0-11 months) compared to the older age groups. This agrees with the work by **Eduardo et al.**[14], where the incidence of *S. pneumoniae* in developed countries was reported to be highest among those aged 6 months to 1 year, while in developing countries, the disease was particularly common in children younger than 6 months [14]. The carriage rate is seen to vary across age groups with a decreasing carriage rate observed with increasing age, due to weaker immune system among the younger age [15,

16]. Nevertheless, both age and sex were not significantly associated with carriage in our study. This could also be explained by the fact that generally children under 5 years of age have same or similar risk of being infected by this bacterium. This is because many of the vaccinated population that came positive with the bacteria were those who could not take more than one dose of the vaccine. This underscores the importance of taking at least two or more doses of the vaccine for full protection as stipulated by the WHO [17].

Our study did not find any significant association in the carriage of *S. pneumoniae* with sex of study population. Our findings are contrary to a recent surveillance data from 1998-2013 which showed that IPD incidence rates in male subjects were as much as 1.5- 2 times higher than rates seen in females, regardless of race or existing co-morbidities [18]. Reports from IPD surveillance across different geographical regions indicate that higher rates of IPD in males have persisted through the pre-PCV era into the post PCV era [19]. This may be because whereas theirs was a general population study, ours concentrated on under five years children only.

Circulating serotype of *Streptococcus pneumoniae* among the study population

Out of the 26 positive isolates of *S. pneumoniae*, 6 serotypes were identified as follows;

serotype 1(23.1%), serotype 6(34.6%), serotype 9(3.8%), serotype 11(11.5%), serotype 19(3.8%) and serotype 23(3.8%), while 5(19.2%) were Non-typeable (NT). This is congruent to work done in Zaria, Northwestern Nigeria by **Ebruke et al.**[5], except that our study detected additional serotypes 1, 11 and 23. The detection of the circulating serotypes of *Streptococcus pneumoniae* in both vaccinated and non-vaccinated among the study population shows diversity in terms of serotypes of *S. pneumoniae* included in PCV10 and those are not.

Furthermore, the only non- PCV10 (Used in Nigeria) serotype found was serotype 11 which agrees with the work done in Ethiopia, where serotype 11, was also detected as a non-vaccine serotype in 3.5% of the population [20]. Although this serotype 11 was earlier reported to be non-invasive, a study found it to contain all the virulence genes used. This could be due to any of the factors associated with evolution of *Pneumococci* which include-; serotype replacement [21] and capsular switching. Through capsular switching, non-vaccine type pneumococcal strains have been able to evade the effects of conjugate vaccines, and sometimes become more virulent in the process [22, 23].

The most prevalent serotype detected is serotype 6, found in 9(34.6%) isolates, while the least in circulation were serotypes 9, 19 and 23 in 1(3.8%) each. The finding of Serotype 6 as the most prevalent in this study is indicative that the study population is still at high risk of the disease since this serotype has been earlier reported as the most invasive strain so far identified in most regions and therefore is a component of all 4-vaccines for pneumonia [24]. The WHO [9] had reported *S. pneumococcal* serotypes 1, 6 &19A as the predominant causes of invasive pneumococcal pneumonia and are therefore targeted in all vaccine types. The presence of both vaccine and non-vaccine serotypes in this study may signal vaccine failure or possible mutation, which are both potential threats to the prevention and subsequent eradication of diseases caused by this organism.

The reason why children continue to be colonized with vaccine types of *Streptococcus pneumoniae* after vaccination schedule is still unknown but could be due to the following reasons- concentration of antibodies produced because of carriage may not be sufficient to prevent colonization. Higher concentration of IgG might be required to avoid colonization than those necessary to prevent IPD [25, 26].

We conclude that there is high prevalence of PCV vaccine and non-vaccine *S.pneumoniae* serotypes in circulation among the under five years children attending Toro General Hospital Bauchi. Many of these serotypes have been reported as invasive (1, 6,9,19,23) except for serotype 11 which is also a non-PCV vaccine serotype. Our study also showed a high risk of pneumococcal disease among the study population in the region since the study site (Toro General Hospital) services people from 3 of the 4 sub-regions of Northern Nigeria.

We recommend further studies to detect virulence genes on the serotypes identified. Also, a full-scale surveillance to detect additional serotypes that may be associated with invasive pneumococcal disease (IPD), should be carried out throughout the country to provide sufficient data that will guide future vaccine policies. There should also be more advocacies on the need for complete vaccination doses for children under five years, to benefit from the full protection offered by the vaccines. This is even as our study found vaccine serotypes of *S. pneumoniae* circulating among children who received incomplete doses of the vaccines, a development that could lead to invasive disease in the future. Increased advocacies can help to sensitize people and avoid this ugly scenerio.

Conflict of interest: No conflict of interest was declared.

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