Serotypes and antibiotic susceptibility of *Streptococcus pneumoniae* in the Jigme Dorji Wangchuck National Referral Hospital, Thimphu, Bhutan: a preliminary finding

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ABSTRACT

Introduction: *Streptococcus pneumoniae* cause life-threatening infections, mostly affecting children under five years in developing countries. Several countries in South Asia have incorporated a pneumococcal vaccine in their immunization schedule. Bhutan is currently considering the introduction of a vaccine, although no studies in the country to date have characterized the serotypes of *S. pneumoniae* to gauge the potential vaccine coverage. We, therefore, characterized pneumococcal serotypes isolated from patients in the Jigme Dorji Wangchuck National Referral Hospital (JDWNRH), Thimphu. **Methods:** Four hospitals attempted to study *S. pneumoniae* from various clinical specimens from January 2014 to December 2015, although only the JDWNRH was able to isolate pneumococci. Isolates were tested for antibiotic susceptibility and serotyped at a reference laboratory in India. **Results:** Thirty-seven isolates were preliminarily identified as *S. pneumoniae* at the JDWNRH. At the reference laboratory, two isolates could not be revived and 14 were identified as non-pneumococcal streptococci. From the remaining 21 isolates, 15 different serotypes were identified. The Bhutanese serotypes were more comparable to those of India than Bangladesh or Nepal. Current vaccines would cover 26.7% (Prevnar, 7-valent), 40.0% (Synflorix, 10-valent), and 53.3% (Prevnar, 13-valent and Pneumovax23, 23-valent) of these serotypes. All isolates were sensitive to penicillin, chloramphenicol, and ceftriaxone; 9.5% were resistant to erythromycin and 38.1% to cotrimoxazole. **Conclusions:** For the first time, we characterized serotypes of *S. pneumoniae* in Bhutan. However, the findings need to be interpreted cautiously due to small numbers of isolates from one referral hospital. A larger study is needed to validate the findings and guide selection of an appropriate vaccine.

Keywords: Antibiotic susceptibility; Pneumococcal vaccine; Serotypes; Streptococcus pneumoniae.

INTRODUCTION

Streptococcus pneumoniae is a Gram-positive diplococci, commonly colonising the human nasopharynx. It is a common cause of pneumonia, meningitis, otitis media, and septicaemia, often with a high case fatality rate[1]. These infections are a serious problem worldwide[1,2]. In 2005, the World Health Organization (WHO) estimated that 1.6 million people die from pneumococcal diseases every year, including one million children under five years, mostly in developing countries[3]. Currently, there are 94 different serotypes of *S. pneumoniae* identified based on properties of the bacterial capsule[4]. A wide variety of serotypes can cause non-invasive diseases such as otitis media and sinusitis, but serotypes 1, 5, 6A, 6B, 14, 19F, and 23F are common causes of invasive pneumococcal diseases (IPDs) in children under five years[5]. Serotype distribution varies over time and geographical location, requiring periodic surveillance to determine antibiotic resistance and the regional spread of subtypes to assess potential vaccine coverage[6].

Pneumococcal vaccines are designed to cover the bacterial serotypes commonly associated with IPDs. Currently, three pneumococcal conjugate vaccines (PCV) covering 7, 10, and 13 serotypes (PCV-7, 10 and 13) and an unconjugated pneumococcal polysaccharide vaccine (PPSV) covering 23 serotypes (PPSV-23) are in use. Another conjugate vaccine covering 15 serotypes (PCV-15) is in clinical trials. Existing vaccines target the pneumococcal capsule and are specific to the serotypes included in the particular vaccine. The PCV are used in primary immunization of newborns and the 23-valent PPSV is designed for older children and adults at high risk for pneumococcal diseases.

There is no standard requirement of serotype coverage in a vaccine but ideally, a vaccine should cover as many as possible of the serotypes causing disease in the particular region. There is substantial variation in the proportions of diseases caused by the serotypes covered by PCV-7 in young children worldwide: the highest serogroup coverage is in the USA, Canada, and Australia (approximately 80–90%), followed by Europe and Africa (70–75%), Latin America (65%), and Asia (50%)[7]. There has been a dramatic reduction in the incidence of IPDs in Australia since the introduction of PCV-7, with the greatest benefit in children under two years of age and for IPDs caused by the serotypes covered by PCV-78. A recent review of pneumococcal vaccines in Latin America found significant impact of PCV-10 and PCV-13 in reducing hospitalization due to pneumonia, meningitis, and other IPDs in children under five years[9]. A similar effect was also reported in Taiwan[10].

In 2007, the WHO recommended pneumococcal conjugate vaccines for all countries[11] and urged the highest priority

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for countries with high pneumonia and under-five mortality. As of December 2012, 86 WHO member states (44%) have introduced PCVs in their immunization programs[12]. In South Asia, Pakistan was the first country to incorporate PCV in their immunization program from October 2012[13], followed by Nepal in January 2015[14], and Bangladesh in March 2015[15]. India and Sri Lanka are in the final stages of epidemiological studies and considerations to introduce the vaccine. Pneumococcal diseases remain a public health concern in many countries despite the implementation of pneumococcal vaccines in infant immunization programs. This may be attributed to increasing incidence of IPDs caused by non-vaccine serotypes[4] and to incomplete vaccine implementation due to high cost. Increasing antibiotic resistance in pneumococcus has added to the problem. Antibiotic resistance was more likely to occur in serotypes 6B, 9V, 14, 19A, 19F, and 23F than others in the USA[16].

Bhutan introduced the Haemophilus influenzae type b (Hib) vaccine in 2009 as a pentavalent vaccine. There were no studies specific to pneumococcal pneumonia or other IPDs. However, under-five mortality remains high despite a drastic drop from 97/1000 live births in 1994 to 37.3/1000 live births in 2013[17]. In addition, the incidence of pneumonia in children under five years remained constant; it was 1,031 per 10,000 under-five children in 2009 and 1,080 per 10,000 under-five children in 2013[17]. Bhutan is in the preliminary stages of discussions for the introduction of the pneumococcal vaccine. As the first study in the country, our aim was to provide preliminary insight on pneumococcal serotypes and antibiotic susceptibility in Bhutan.

METHODS

With the initiative of the Vaccine Preventable Diseases Control Program (VPDCP), Department of Public Health, Ministry of Health (MoH), Bhutan, a pilot study on pneumococcus was undertaken in four hospitals with microbiology culture facilities; the Jigme Dorji Wangchuck National Referral Hospital (JDWNRH), Eastern Regional Referral Hospital (ERRH), Central Regional Referral Hospital (CRRH) and Phuntsholing General Hospital (PGH), from January 2014 to December 2015. The hospitals attempted to collect *S. pneumoniae* isolates from any clinical specimen received in their respective laboratories for culture. Unfortunately, only the JDWNRH in the capital city, Thimphu was successful in isolating *S. pneumoniae*. The isolates were sent for serotyping at the Department of Microbiology, Christian Medical College, Vellore, India, a WHO collaborating centre on *S. pneumoniae* studies.

Preliminary identification at the JDWNRH

Microbiological specimens were cultured on sheep blood agar, chocolate agar, and MacConkey agar following routine laboratory procedures. The alpha haemolytic, gram-positive, lancet-shaped cocci in pairs, single or in short chains with a zone of inhibition of ≥14mm in optochin sensitivity were preliminarily identified as *S. pneumoniae*. Antibiotic susceptibility testing was performed using the latest Clinical and Laboratory Standards Institute (CLSI) guideline.

Storage, shipping and serotyping

The preliminarily identified *S. pneumoniae* isolates were stored at -70°C in tryptic soy broth with 5% glycerol until shipment. After collection of a batch of isolates, they were revived by subculturing on sheep blood agar and incubated at 37°C, 5% CO atmosphere for at least 18 hours or until abundant growth was seen. Isolates were collected on cotton swabs, packed in silica gel packs supplied by the reference laboratory and shipped at room temperature. Upon arrival at the reference laboratory, the isolates were revived, identifications confirmed and serotyped using the co-agglutination technique, and reconfirmed with a multiplex polymerase chain reaction (PCR).

Ethical approval

Since this study was carried out as a departmental activity of the VPDP, MoH and used only secondary laboratory data without patient identifiers, ethical approval was waived by the Research Ethics Board of Health (REBH), Ministry of Health, Bhutan.

Data analysis

Data analysis involved a simple description of the number of isolates and serotypes, comparison of serotypes present in the current available pneumococcal vaccines and regional circulating serotypes, and the number and types of antibiotic resistance detected.

RESULTS

Thirty-seven isolates were preliminarily identified as *S. pneumoniae* in the microbiology laboratory at JDWNRH and shipped to the reference laboratory in India. Of the 37 isolates, two could not be revived, 14 were identified as non-pneumococcal streptococcus (alpha streptococcus), and 21 were serotyped. The isolates were from the eye, blood, throat, sputum, ascitic fluid, and pus specimens (Table 1).

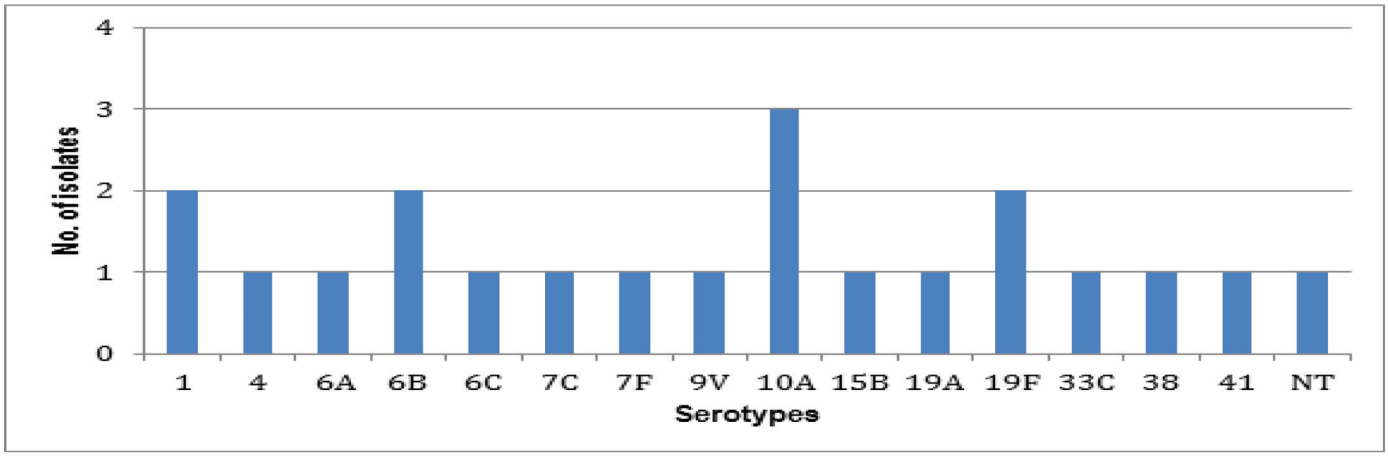
There were 15 different serotypes among the 21 isolates that could be confirmed at the reference laboratory. Serotype 10A was the most common, present in three isolates (Figure.1). Serotype 1, 6B, and 19F were detected in two isolates; all other serotypes detected appeared in one isolate each. One isolate from eye swab was a non-typeable pneumococcus.

As shown in Table 2, of the 15 serotypes from the JDWNRH, the current vaccines PCV-7 and PCV-10 covered 26.7% and 40% while PCV-13 and PPSV-23 covered 53.3% each.

All isolates were susceptible to penicillin, chloramphenicol, and ceftriaxone. Resistance to erythromycin and cotrimoxazole were 9.5% (2/21) and 38.1% (8/21), respectively. Of the three serotypes of 10A, one showed resistance to both erythromycin and cotrimoxazole.

Table 1. Clinical samples and serotypes of Streptococcus pneumoniae isolated from specimens at the Jigme Dorji Wangchuck National Referral Hospital, Thimphu, Bhutan, 2014-2015 (*n*=21)

Sample type	No. of samples	Serotype (number of isolates)
Eye swab	10	6B (1), 7C (1), 7F (1), 6C (1), 15B (1), 4 (1), 33C (1), 19F (1), 19A (1), non-typeable (1)
Blood culture	5	1 (2), 6B (1), 38 (1), 9V (1)
Throat swab	2	10A (2)
Sputum	2	41 (1), 10A (1)
Ascitic fluid	1	6A (1)
Pus	1	19F (1)



NT: Non-typeable

Figure 1. Serotypes of Streptococcus pneumoniae isolated from specimens at the Jigme Dorji Wangchuck National Referral Hospital, Thimphu, Bhutan, 2014-2015 (*n*=21)

Table 2. Currently available vaccines and serotype coverage by the different vaccines

Current available Vaccines	Serotypes included in the vaccine	JDWNRH serotypes included in the vaccine	JDWNRH serotype coverage
Prevnar (PCV-7): 7-valent pneumococcal conjugate vaccine	4, 6B, 9V, 14, 18C, 19F, and 23F	4, 6B, 9V, 19F	4/15 (26.7%)
Synflorix(PCV-10): 10 valent Pneumococcal conjugate vaccine	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F	1, 4, 6B, 7F, 9V, 19F	6/15 (40.0%)
Prevnar 13(PCV-13): 13-valent pneumococcal polysaccharide-protein conjugate vaccine	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F	1, 4, 6A, 6B, 7F, 9V, 19A, 19F	8/15 (53.3%)
Pneumovax23(PPSV-23): 23-valent pneumococcal polysaccharide vaccine	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F	1, 4, 6B, 7F, 9V, 10A, 19A, 19F,	8/15 (53.3%)

DISCUSSION

In this first study of the serotypes and antibiotic resistance patterns of *S. pneumoniae* in Bhutan, we detected 15 different serotypes and resistance to two antibiotics. Our small snapshot provides some insights that may help guide prevention and treatment of this agent responsible for considerable mortality worldwide.

The study results suggest that the common serotypes in Thimphu were more comparable to India[4] than to Bangladesh [18] or Nepal[19]. The most common serotype (10A) is not among the most common in other South Asian countries[20], although the small sample size bears consideration. The coverage of 26.7% to 53.3% of the JDWNRH serotypes by current vaccines was similar to other findings in Asia[1,7], including India[21], but higher than that of Bangladeshi serotypes (23-48%)[18]. The antibiotic resistance level was lower than that observed in Bangladesh[18], Nepal[19] and India[21]. This low level of resistance is reassuring since most hospitals in Bhutan have no microbiology laboratory support and manage patients on clinical judgement.

We acknowledge limitations of our study. While we attempted to include four hospitals capable of microbiological cultures to increase representation of the country, only the JDWNRH was able to grow *S. pneumoniae* in their laboratory. The probable reasons for other hospitals failing to get any isolates were the unavailability of sheep blood for the culture of fastidious organisms and limited technical expertise of staff. In order to generate better results for future studies and routine laboratory processes these laboratories should be supplied with sheep blood and the technical expertise of the laboratory staffs need to be improved.

We also acknowledge that of the 37 isolates, 14 (37.8%) were determined as non-pneumococcal streptococcus (alpha Streptococcus) in the reference laboratory in India. This initial misidentification suggests the need for training of staff on technical procedures and updates of on-going quality assurance programs. In addition, we recognize that only isolates from invasive infections should be included in a burden assessment of IPDs. However, due to the limited laboratory capacity and a small number of samples received for suspected pneumococcal diseases in our setting, all clinical samples were included. For a preliminary study, we considered this acceptable since the main aim was to find the circulating serotypes of *S. pneumoniae* under the assumption that the common circulating serotypes are the ones that will be responsible for causing most diseases in the community. We also recognize that the overall sample size is small. In brief, results should be interpreted cautiously.

Despite the small sample size and logistical challenges, this study serves as the only preliminary report, highlighting fifteen different serotypes of *S. pneumoniae* detected in patients of the JDWNRH. Although the JDWNRH is Bhutan's apex hospital in the capital city and caters to patients from all over the country, these results, limited by number and scope, should not be extrapolated to the whole country without verification in a larger, more inclusive study.

CONCLUSIONS

This study describes a preliminary finding on the serotypes and antibiotic resistance of *S. pneumoniae* in clinical specimens from Bhutan's national referral hospital. A detailed study on pneumococci with a substantial number of isolates and IPDs with improved laboratory expertise should authenticate these preliminary findings. It is also crucial to have more regional collaborations in view of limited laboratory capacity in the country.

ACKNOWLEDGEMENTS

We acknowledge the following contributions: Vaccine Preventable Disease Control Program (VPDCP), Ministry of Health, Bhutan for support in surveillance coordination meetings and cost of sample shipment to India. Department of Microbiology, Christian Medical College, Vellore, India, WHO Collaborating centre for their assistance

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AUTHORS CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

TT: Concept, design, literature search, data collection and analysis, manuscript writing and review.

DY: Concept, analysis of data, study design, manuscript drafting and revision.

RS: Concept, analysis of data, study design, manuscript drafting and revision.

NT: Concept, analysis of data, study design, manuscript drafting and revision.

Author agree to be accountable for all respects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

None

GRANT SUPPORT AND FINANCIAL DISCLOSURE

None