

Low incidence of antibiotic resistance among invasive and nasopharyngeal isolates of *Streptococcus pneumoniae* from children in rural Philippines between 1994 and 2000

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Abstract The purpose of this study was to determine the prevalence of acquired antimicrobial resistance in *Streptococcus pneumoniae* isolated from nasopharyngeal swabs and blood and cerebrospinal fluid (CSF) specimens of 3,028 children hospitalized with signs or symptoms of pneumonia, sepsis, or meningitis in rural Philippines between 1994 and 2000. Pneumococci were identified using standard methods, serotyped, and their susceptibility to oxacillin, erythromycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole was determined using the disk diffusion method. Penicillin minimum inhibitory concentrations (MICs) of the oxacillin-resistant isolates

were further tested. The clonality of the penicillin-non-susceptible (PNSP) isolates was analyzed using pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Altogether 1,048 isolates were analyzed, of which 35 were invasive and 1,013 nasopharyngeal isolates. None was resistant, but 22 (2.1%) were intermediately resistant to penicillin, 4 (0.2%) were resistant to chloramphenicol, 3 (0.2%) to erythromycin, 39 (3.7%) to tetracycline, and 4 (0.2%) to trimethoprim/sulfamethoxazole. Twelve of the 22 PNSP isolates were of serotype 14 and of sequence type 63. These included the two invasive PNSP isolates. PFGE profiling further identified three separate clusters among the sequence of type 63, serotype 14 (ST63¹⁴) isolates. Antimicrobial resistance in both invasive and nasopharyngeal pneumococcal pediatric isolates in rural Philippines is rare. In spite of this remote setting, the PNSP isolates of the serotype 14 clusters were of ST63 type, which has been described previously on other continents.

The members of the ARIVAC consortium contributing to this study are listed in the Acknowledgements

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Introduction

Streptococcus pneumoniae, the pneumococcus, is among the most important human bacterial pathogens as well as a common component of the normal nasopharyngeal microflora. It causes both mild upper respiratory infections like otitis media or sinusitis, and severe diseases like pneumonia, meningitis, and sepsis, especially in children. Pneumonia is the most common cause of death in children under 5 years of age in developing countries, and the pneumococcus its most important causative agent [1]. Resistance of pneumococcus to penicillin and to other antibiotics has been increasing worldwide [2, 3] and threatens the successful management of pneumococcal disease using

early antimicrobial therapy, the main preventive strategy recommended by the World Health Organization (WHO) [4].

A generally accepted cause of the increasing frequency of antibiotic resistance is the profuse and inappropriate use of antimicrobial agents [5, 6]. In the Philippines, antibiotics are usually purchased over the counter without prescription and the choices and doses are often inappropriate [7]. The free over-the-counter availability of antibiotics has been shown to be reflected in common pre-admission antibiotic treatment in hospitalized patients [8, 9]. Against this background, the results of our present study in the Philippines are surprising. We studied the antimicrobial susceptibility among a large set of pneumococcal isolates from children under 5 years of age hospitalized for pneumonia, meningitis, and sepsis, and found an unexpectedly low frequency of strains with decreased susceptibility to penicillin, and no fully penicillin-resistant strains at all. However, a slightly increasing trend in the prevalence of penicillin-nonsusceptible pneumococci (PNSP) was observed, most likely due to the spreading of one internationally known pneumococcal clone that seems to express various serotypes and penicillin susceptibility patterns on other continents [10].

Materials and methods

Study site and preparation

Bohol in the Central Philippines is chiefly a rural area with agriculture as its principal source of livelihood. In the coastal areas, fishing is the main source of living. Of the total of one million inhabitants, about 66,000 live in the provincial capital Tagbilaran City, and the rest in small towns or rural villages. The infant mortality rate in the area is 28 per 1,000 live births, and pneumonia is considered the main health problem of children [11]. The Governor Celestino Gallares Memorial Hospital (Gallares Hospital) is a 250-bed tertiary care government hospital located in Tagbilaran City. In February 1994, preparations were started to establish a study site for acute respiratory infections (ARI) in Bohol, as a joint ARIVAC project of the Gallares Hospital, of the Research Institute for Tropical Medicine (RITM), Metro-Manila, the Philippines, the National Public Health Institute (KTL), Helsinki and Oulu, Finland, and the University of Queensland, Brisbane, Australia. This included the establishment of the ARIVAC microbiological laboratory and training of the pediatric staff at the Gallares hospital [12, 13]. The study protocol was approved by the Institutional and Ethical Review Board of the RITM. Parents/guardians of enrolled patients were requested to sign before admission the Gallares Hospital

Form for Authorization for Medical and/or Surgical Treatment.

Patients

From April 1994 to June 2000, 3,028 children under 5 years of age with severe or very severe pneumonia [4], suspected meningitis, or suspected sepsis [12] were enrolled. Blood and nasopharyngeal swab samples were obtained from all the patients and cerebrospinal fluid (CSF) specimens when indicated.

Bacteriological procedures

Blood, CSF, and nasopharyngeal swab samples were cultured in the ARIVAC laboratory as described earlier [12]. In brief, all invasive isolates from blood and CSF were identified using generally accepted methods. The isolates were stored in STGG [14] at -20°C for a maximum of 1 month, after which they were sent in dry ice to the RITM for confirmation, further tests, and long-term storage at -80°C . From nasopharyngeal swab cultures only pneumococci were sought using gentamicin blood agar [12], identified, and sent to the RITM as described for invasive isolates.

Pneumococcal isolates were serotyped using the capsular Quellung test using serum pools and type-/group-specific antisera obtained from the Statens Seruminstitut, Copenhagen, with quality assurance of all invasive isolates and 10% of the nasopharyngeal isolates at the KTL, Oulu [15, 16]. Antimicrobial susceptibility was tested in the ARIVAC laboratory using the disk diffusion method according to the former National Committee for Clinical Laboratory Standards [17]. Mueller–Hinton agar was supplemented with 5% sheep's blood. To test susceptibility to penicillin, oxacillin disks (1 μg) were used. Other antibiotic disks used were chloramphenicol (15 μg), tetracycline (30 μg), erythromycin (15 μg), and trimethoprim/sulfamethoxazole (1.25/23.75 μg ; BBL, Cockeysville, MD, USA; Oxoid, Basingstoke, Hampshire, England; and Difco, Franklin Lakes, NJ, USA).

For all isolates showing decreased susceptibility in the disk diffusion test, the minimum inhibitory concentration (MIC) was determined using the E-test according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). The susceptibility results were analyzed using the WHO NET computer program version 5.3 [18].

All PNSP isolates were analyzed using pulsed-field gel electrophoresis (PFGE) to assess their genetic relatedness [19]. A modification of the method of Matushek et al. was used as a standard procedure for the preparation of bacterial genomic DNA for PFGE analysis [20], which was performed as described by de Lencastre and Tomasz [21].

Restriction endonuclease *Sma*I (New England BioLabs, Ipswich, MA, USA) was used for digestion and the DNA fragments were separated in a 1.2% PFGE-agarose gel with a CHEF DR^R III system (Bio-Rad, Hercules, CA, USA). Gels were stained in 1 mcg/ml ethidium bromide solution and photographed under UV light. Cluster analysis was performed using the BioNumerics software package (Applied Maths, Austin, TX, USA). In addition, all PNSP isolates were analyzed using multi-locus sequence typing (MLST), slightly modified from previously described protocols ([22–24] Bruno Pichon Health Protection Agency, London, UK, personal communication, 4 April 2006). Briefly, bacterial genomic DNA was isolated with the DNeasy Tissue kit (Qiagen, Hilden, Germany) and seven housekeeping genes were partially amplified using PCR. The PCR products were purified using the QiaQuick PCR Purification kit (Qiagen) or GeneClean Turbo kit (Q-BioGene, Aurora, OH, USA) and sequenced bidirectionally. The sequences obtained were compared with the material in the MLST database [10], according to which the sequence types (STs) were assigned.

Results

Among the 3,028 study children, pneumococci were isolated in 1,367 (45%) of the nasopharyngeal samples (carriage isolates) and 40 (1.3%) from blood or CSF samples (invasive isolates). All of the pneumococcal bacteremias were monobacterial. One carriage and one invasive, if existent, isolate per patient were included in the study. Due to accidental defrosting of the storage freezer at the RITM in 2000, some of the isolates were lost before testing was complete. Consequently, serotype and antimicrobial susceptibility results are now available for only 35

invasive and 1,013 nasopharyngeal isolates, and these form the material of the present study.

Of the 1,048 pneumococcal isolates, 42 (4.0%) were resistant to oxacillin in the disk diffusion test. These isolates were then tested using the penicillin E-test, and 22 (2.1%) of them were confirmed to be of decreased susceptibility to penicillin (MIC: 0.125–0.25 mg/L). Two of these PNSPs were invasive and 20 were nasopharyngeal isolates (Table 1). In the first 2 years of the study, only 2 PNSPs (0.9%) were found among the 218 isolates tested. In the following 4 years the PNSPs showed a modest increase to 2.4% of the 754 isolates tested, but in the final half-year no PNSPs were found among the 76 isolates.

Among the 1,013 nasopharyngeal isolates, 4 were resistant to chloramphenicol, 3 to erythromycin, 37 (3.6%) to tetracycline, and 4 to trimethoprim/sulfamethoxazole. Two (5.7%) of the 35 invasive isolates were resistant to tetracycline.

Among the 22 PNSP isolates, ten of the nasopharyngeal isolates and both of the invasive isolates were of serotype 14. Five of the remaining 10 PNSP nasopharyngeal isolates were noncapsulated and the serotypes of the remaining 5 were 23F (two isolates), 4, 5, and 28. All of the 12 PNSP serotype 14 isolates were resistant to tetracycline (MIC: 32–64 mg/L). Among the remaining serotype 14 PNSP isolates, this resistance pattern was found in only the one PNSP isolate with serotype 28.

The PFGE profiling of the 12 serotype 14 PNSP isolates revealed three clusters (Fig. 1): cluster A (patterns 994, 1720, and 2118 to 2552b), cluster B (patterns 1969 to 2070), and cluster C (patterns 1867 and 1932). Both of the invasive isolates (2118b and 2552b), as well as the nasopharyngeal isolates from the same patients (2118 and 2552), belonged to the major cluster A. The degree of similarity within the clusters was 100%. The remaining one

Table 1 Annual incidences and serotypes of invasive and carriage isolates of penicillin-nonsusceptible pneumococci (PNSP)

Year of isolation	Number of isolates						
	All	Penicillin-nonsusceptible pneumococcal isolates					
		Oxacillin-resistant according to disk diffusion		Confirmed by MIC		Serotypes (number of isolates)	
		Invasive	Carriage	Invasive	Carriage	Invasive	Carriage
1994	102	0	3	0	1	5	
1995	116	0	6	0	1	NC	
1996	235	0	6	0	4	14 (2), 23F (1), NC (1)	
1997	240	0	5	0	4	14 (2), 23F (1), NC (1)	
1998	186	2	10	1	5	14	14 (5)
1999	93	2	8	1	5	14	4 (1), 14 (1), 28 (1), NC (2)
1–6/2000	76	0	0	0	0		
Total	1,048	4	38	2	20		

MIC, minimum inhibitory concentration; NC, noncapsulated

serotype 14 PNSP isolate (1189) was closely (>85%) related to the major cluster A. In addition to the three PFGE clusters, the ten non-serotype 14 PNSP isolates represented an individual PFGE profile each (data not shown). The MLST results showed that all 12 of the serotype 14 PNSP isolates of the three clusters defined by PFGE profiling shared an identical sequence type, ST63. None of the remaining ten PNSP isolates were alike according to MLST. Among these ten isolates, ST361, ST771, and eight previously undescribed STs were represented.

Discussion

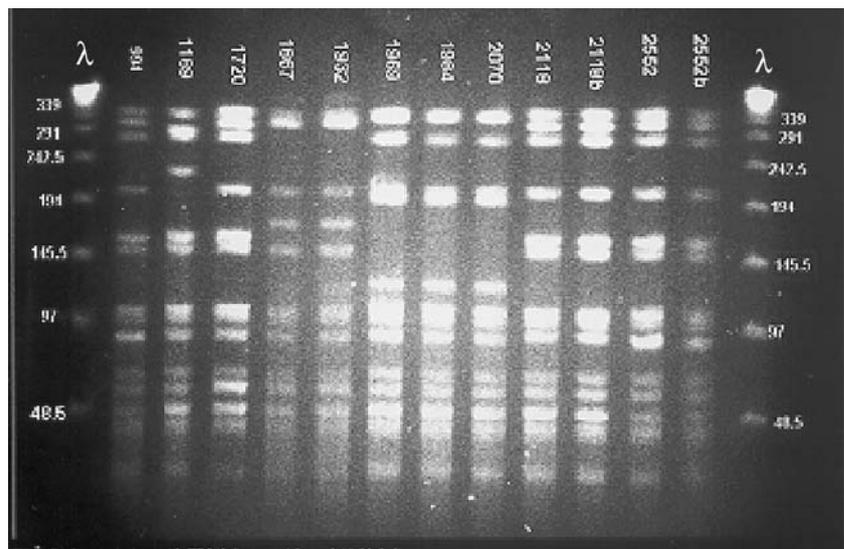
This study is part of prospective surveillance of invasive bacterial infections in children under 5 years of age in the rural province of Bohol in the Central Philippines [12]. This is the first large-scale study of pneumococcal antimicrobial resistance in the Philippines. The patients came from the small city of Tagbilaran and from the surrounding rural municipalities, therefore representing a population different from the urban populations in Metropolitan Manila, where pneumococcal antibiotic resistance has been studied previously [25–27]. In these studies, 65 pediatric blood culture isolates [24], 100 nasopharyngeal isolates from nonhospitalized children under 5 years of age [26], and 22 invasive isolates [27] collected mainly in the 1990s were studied, 3, 2, and 6 of which were reported to be penicillin-nonsusceptible respectively.

The results of the present study show that resistance to penicillin and other clinically important drugs is uncommon in both invasive and nasopharyngeal isolates of pneumococci in the rural Filipino children. This low prevalence of PNSP observed is in contrast to other reports from the

South East Asia. In Taiwan, Korea, Sri Lanka, Vietnam, Singapore, and Thailand, 37–92% of pneumococcal nasopharyngeal strains from children under 5 years of age attending daycare centers or outpatient clinics or hospitalized with acute respiratory infection, were penicillin-nonsusceptible [26, 28]. A lower prevalence of PNSP (13%) was reported from China, Malaysia, and India, where the number of pneumococcal isolates varied from 44 to 204 per country [26]. Similar figures from these countries have also been reported by others [29, 30]. A recent report of the ANSORP indicates even higher resistance rates in invasive pneumococci in most of the countries in the area of South East Asia [27].

Differences in antibiotic policies between countries offer an explanation for the varying frequencies of resistant pneumococcal strains. In Europe in the 1990s, high frequencies of penicillin-intermediate or -resistant strains were observed in Spain (41.2%), France (42.3%), and Hungary (36.2%), but considerably lower in the UK (9%), Germany (4%) [31], Sweden (3–5%) [32], and Finland (6%) [33]. In Spain and Hungary, antibiotics are freely available, whereas in the UK, Sweden, and Finland a prescription is required. Thus, rational use of antibiotics seems to prevent or at least hinder the appearance and spread of antibiotic resistance [34]. Similar to elsewhere in the Philippines, antibiotics are also available over the counter in Bohol. According to patient histories, antibiotics have often been given to seriously ill children before admission to hospital [12]. Against this background, our finding of a low prevalence of PNSP in Filipino children is surprising. Without a study including a questionnaire for the parents on pre-admission antimicrobial treatment of the children and testing the urine antibacterial activity of the hospitalized children, reasons for this finding can be only

Fig. 1 *Sma*I fragments of pulsed-field gel electrophoresis (PFGE) of the 12 serotype 14 penicillin-nonsusceptible *Streptococcus pneumoniae* isolates (lanes 994–2552b). Lanes λ , molecular weight (kb) markers



speculated upon. In the South East Asian studies referred to above [25–30], the study population consisted of urban children often attending daycare. Our study population represents mostly a rural lifestyle where small children live at home. It stands to reason that transmission of resistant bacteria in a low-density population, such as in our study, is less frequent than in dense urban populations, and this could contribute to the low rate of resistance in our study population.

Epidemiological analysis of PNSP strains in South East Asia [26, 28, 35], as well as in the Americas [36, 37] and in Europe [38], indicate that the major cause of high prevalence of PNSP is due to the introduction and spread of pandemic multi-resistant clones. According to the nomenclature established by the Pneumococcal Molecular Epidemiology Network (PMEN) [39], the Spain^{23F}-1 and Spain^{9V}-3 multi-resistant clones are among the most common and wide-spread, and often the main cause of high prevalence of PNSP. While MLST is nowadays considered the gold standard for typing pneumococci due to its high resolution and ease of comparison, PFGE is more sensitive in detecting subtle changes in the genome [23]. Thus, the results achieved using the two methods reported here are not contradictory. None of the PFGE or MLST patterns of the PNSP isolates in our study resembled any of the patterns of the international clones published by PMEN [39]. However, 15 strains expressing five different serotypes (14, 15A, 19A, 19F, 23F) in the MLST database [10] are identical to the ST63¹⁴ PNSP isolates in this study. The antibiotic susceptibility data are not available for all the previously reported sequence type 63 isolates, but the majority of them are intermediately resistant to penicillin (MIC between 0.12–1 µg/ml). The earliest of these findings is a serotype 15A strain isolated in Sweden in 1992 [40]. The ST63¹⁴ PNSP isolates were not present in the material of the present study collected in 1994–1995, but were regularly present among the isolates from 1996–1999, consistent with its recent introduction into the population. The question of the further spread of this clone cannot unfortunately be answered because of the discontinuation of the study in June 2000.

A good situation can rapidly turn worse. The introduction of a PNSP clone with the high ability to spread can rapidly increase the proportion of resistant strains [41]. The proportion of penicillin-intermediate or -resistant strains increased in the United States from 6.6% in 1991–1992 to 33.5% in 1996–1997 [42] and to 43.6% in 1997 [43]. During the 1990s, the prevalence of multiply resistant pneumococci increased by approximately 3% in a year in the USA [44]. While resistance in pneumococci is often associated with the pediatric serotypes, i.e., major serotypes causing infections in young children [45] included in the modern pneumococcal vaccines, it has been proposed that

vaccination could “clean the table” by eradicating the pediatric PNSP [46]. However, pneumococcus is a highly variable and versatile organism, and it is quite likely that under selection pressure caused by vaccination, for example, anomalous serotypes of common pediatric PNSP clones can become prevalent and the resistance problem will continue. This has already been reported from the US [47]. In our study, the ST63¹⁴ PNSP isolates were more common among the invasive isolates, in spite of their low numbers, than among the carriage isolates. One reason for this might be their ability to resist pre-hospitalization antimicrobial treatment, which is likely to prevent growth of susceptible pneumococci in blood cultures taken during admission.

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