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The prevalence and clonal diversity of penicillin-resistant *Streptococcus pneumoniae* in Kuwait

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SUMMARY

Penicillin-resistant *Streptococcus pneumoniae* (PRSP) is widespread all over the world, including countries previously free of PRSP. This study was undertaken to determine the prevalence, the common serotypes and the clonality of PRSP isolated over a period of 1 year, from various clinical samples from three major hospitals in Kuwait. Strains were identified by standard methods and their antibiotic susceptibility was determined by the agar dilution method. The clonality of the isolates was determined by repetitive extragenic palindromic sequence–polymerase chain reaction (REP–PCR) genomic profiling and pulsed field gel electrophoresis (PFGE). Serotyping was done by Quellung reaction using specific antisera. We found that 55% of the *S. pnuemoniae* were resistant to penicillin (46% and 9% exhibited intermediate and full resistance, respectively). Nearly 41% were resistant to sulfamethoxazole-trimethoprim, 9% to cefotaxime and ceftriaxone, 15% to amoxycillin-clavulanate, 17% to cefuroxime, 77% to cefaclor, and 14% to clindamycin. The commonest serotypes among the PRSPs were 6A, 6B, 14, 19F, 23F and nontypable. PFGE and REP–PCR patterns showed a large diversity of genetic clones of the PRSP. Serotypes 6B, 14, 19F and 23F were more clonally related than the others. Our data showed that the prevalence of PRSP was high, the serotypes were diversified and different genetic clones make up the population of circulating PRSP in Kuwait.

INTRODUCTION

Streptococcus pneumoniae is a leading bacterial cause of community-acquired bronchopulmonary disease as well as acute otitis media, sinusitis and meningitis [1]. Resistance to penicillin among strains of *S. pneumoniae*, the so-called penicillin-resistant *S. pneumoniae* (PRSP), is no longer a laboratory phenomenon but of a serious clinical concern with notable impact of intermediate or resistant status on diseases of varying severity. PRSP strains are now widespread in all the continents of the world and its prevalence in these places is on the increase with attendant serious therapeutic problems [2]. A previous study in Kuwait, about 7 years ago, showed that 20·6% of the *S. pneumoniae* isolated from clinical samples, in one of the teaching hospitals, were intermediately resistant to penicillin [3]. Since then higher prevalence rates of PRSP have been observed (personal observations). However, these resistant strains were not serotyped nor examined for their clonal relatedness using

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contemporary molecular techniques. As a consequence, no consensus policy on empirical therapeutic regimen exists nor is there information on the adequacy of the serotypes in the 23-valent pneumococcal vaccine now available in the country.

Kuwait is a small country, where the expatriate workers outnumber the indigenes by a ratio of 2:1. Thus, Kuwait has a diversified population representing approximately 140 different nationals, most of whom come from areas where PRSP is endemic. In addition, Kuwaitis travel abroad frequently and visit many of the countries known to have high prevalence of PRSP. Extended wider surveillance of PRSP, including their serotypes and molecular characterization, is therefore essential in Kuwait in order to understand the epidemiology of these strains and to be able to formulate evidence-based preventive and therapeutic strategies. The present study was, therefore, undertaken to study the *S. pneumoniae* isolates from three major teaching hospitals in Kuwait, serving more than 90% of the population, in order to determine the trend in the prevalence and establish the serotypes of PRSP isolates as well as to determine their genetic relatedness.

MATERIALS AND METHODS

A total of 173 strains were collected over a 1 year period but due to technical reasons, only 128 were evaluable. The clinical sources of the strains were as follows: 25 (14·5%) from blood, 43 (24·8%) from ear, 42 (24·2%) from conjunctiva, 13 (7·5%) from endotracheal tube (ET), 7 (4·0%) from cerebrospinal fluid (CSF), 9 (5·2%) from nasal cavity, 8 (4·6%) from sputum, 1 (0·6%) from peritoneal dialysis catheter, 2 (1·2%) from bronchoalveolar lavage, 1 (0·6%) each from appendicular abscess, postmortem lung tissue and throat; the origins of 20 (11·6%) were not stated.

Collection of isolates

From 5 November 1995 to 4 November 1996, all consecutive strains of *S. pneumoniae* isolated from clinical samples of patients seen at the Mubarak Al-Kabeer Hospital, Adan Hospital and Ibn Sina Hospital (the three largest health care facilities in Kuwait), were collected for the study. All strains were confirmed by colonial morphology (α-haemolytic colonies), susceptibility to optochin and solubility in 2% bile salt. They were then kept at -70 °C, in Mueller–Hinton broth (BBL, Microbiology Systems, Becton Dickinson and Co., Cockeysville, USA) supplemented with 5% rabbit blood, until needed for further tests. A strain of *S. pneumoniae*, serotype 23F, originally isolated from a patient in Spain and thought to be responsible for the spread of penicillin-resistance in different countries of the world, was also included in this study (kindly provided by Dr M. R. Jacobs, Institute of Pathology, Case Western Reserve University, Ohio, USA).

Determination of minimum inhibitory concentrations (MICs)

The susceptibility of the isolates to penicillin, amoxycillin-clavulanate, cefaclor, cefuroxime, cefotaxime, sulfamethoxazole-trimethoprim, and clindamycin was determined by performing the MICs using the agar dilution method on Mueller–Hinton agar (BBL, Microbiology Systems, Becton Dickinson and Co., Cockeysville, USA) supplemented with 5% horse blood [4]. Test strains were suspended in trypticase soy broth to match a 0·5 MacFarland turbidity standard and inoculated evenly onto Mueller–Hinton blood agar plates to which had been added varying doubling dilution concentrations of the antibiotics in the range of 0·03–64 μg/ml. The inoculated plates were then incubated overnight at 37 °C. The National Committee for Clinical Laboratory Standards (NCCLS) [4] interpretive standard for MICs were used for all antibiotics except sulfamethoxazole-trimethoprim; susceptibility testing of sulfamethoxazole-trimethoprim as well as the interpretative criteria were according to a previous report of Hall and colleagues [5]. In each test system, *S. pneumoniae* ATCC 6305 was used to control the susceptibility tests and the media performance.

Serotyping

The serotyping of the isolates was done by Quellung reaction with a complete set of specific rabbit pneumococcal antisera in the Danish chequerboard typing system according to the instructions of the manufacturer (Serum Institute, Copenhagen, Denmark). The complete set of antisera contained pool, type, group and factor sera. We used the pool sera of pool A to I. The type sera were 1–5, 8, 13, 14, 20, 21, 27, 29, 31, 34, 36–40, 42–46 and 48. The group sera were 6, 7, 9–12, 15–19, 22–25, 28, 32, 33, 35, 41 and 47.

The factor sera were 6a, 6b, 6c, 7b, 7c, 7e, 7f, 7h, 9b, 9d, 9e, 9g, 10b, 10d, 10f, 11b, 11c, 11f, 11g, 12b, 12c, 12e, 15b, 15c, 15e, 15h, 16b, 16c, 17b, 17c, 18c, 18d, 18e, 18f, 19b, 19c, 19f, 20b, 22b, 22c, 23b, 23c, 23d, 24c, 24d, 24e, 25b, 25c, 28b, 28c, 29b, 32a, 32b, 33b, 33e, 33f, 35a, 35b, 35c, 41a, 41b, 42a, 43b and 47a.

Repetitive extragenic palindromic sequence polymerase chain reaction (REP–PCR) genomic profiling

REP–PCR profiling was done by a modified previously described method of Versalovic and colleagues [6]. Briefly, the test bacterial strains were grown in 3 ml of Todd Hewitt broth (Difco Laboratories, Detroit, MI, USA) and harvested in the late exponential phase. The genomic DNA was prepared by the method of Ezaki and colleagues [7] and quantitated by spectrophotometer. Each 50 μl PCR reaction contained 50 pmol of each of the two primers, 100 ng of template DNA, 1·25 mM of each of four dNTPs, 2·5 U Gene Taq DNA polymerase (Wako Pure Chemical Industries Ltd., Osaka, and Nippon Gene, Toyama, Japan). Tubes containing all the above, except the template, was used as a control. PCR amplification reactions were done with a GeneAmp PCR System 2400 (Perkin–Elmer Cetus, Norwalk, CT, USA) DNA thermal cycler with an initial denature step (95 °C, 7 min) followed by 32 cycles of denaturation (94 °C, 30 s), annealing (40 °C, 1 min), extension (65 °C, 8 min), and a final extension step (65 °C, 16 min). REP1R-Dt [8] and REP2-Dt were used as the primers. The sequence of REP1R-Dt and REP2-Dt primer were 5′-CGGNCTACNGCNGCN-III-3′ and 5′-NCGNCTTATCNGGCCTAC-3′ (N = A, G, T and C), respectively. An aliquot (8 μl) of the REP–PCR reaction was electrophoresed in a 1·8% agarose gel containing 1 × Tris-acetate-EDTA and 0·5 μg/ml ethidium bromide, and the banding pattern was photographed with a Polaroid camera.

The REP–PCR fingerprints of strains were compared by visual inspection. The fingerprint of each strain was compared with that of every other strain. The molecular sizes of amplimers were judged by comparison with concurrently run molecular weight standards. Profiles were considered to be highly similar when all visible bands from two isolates had the same apparent migration distance. Variation in the intensity or shape of bands were not taken into account. The absence of up to two bands from a fingerprint was allowed, when all other visible bands in the fingerprints matched, before isolates were considered different by visual inspection [9, 10].

Pulsed field gel electrophoresis (PFGE)

The pneumococcal DNA containing plugs were prepared by the procedure described previously [11]. Restriction of genomic DNA was carried out with equilibration in appropriate restriction buffer for 30 min at room temperature, then 25 U of *Sma*I and *Apa*I (Takara Shuzo Co. Ltd., Shiga, Japan) were added and incubated at 37 °C overnight. The DNA restriction fragments were separated by PFGE in a Gene Navigator System (Pharmacia Biotech AB, Uppsala, Sweden). Plugs were cast in 1·2% agarose gel (Agarose NA, Pharmacia Biotech AB) and the gel was ran in 0·5 × TBE (50 mM Tris, 50 mM boric acid, 0·2 mM EDTA, pH 8·0). The running condition was 200 V for 20 h with pulse time 9·5 s. The lambda DNA ladder (FMC BioProducts, Rocklands, ME, USA) was used as a molecular size marker. Thereafter, the gels were stained with ethidium bromide, rinsed and photographed under UV light.

The banding pattern of each strain was compared with that of every other strain by visual inspection. Strains were considered indistinguishable, closely related, possibly related or different according to the criteria of Tenover and colleagues [12]. Indistinguishable, closely related and possible related patterns were grouped where possible.

RESULTS

Prevalence of PRSP

Of the 128 strains available for susceptibility testing (Table 1), 70 (55%) were resistant to penicillin; 59 (46%) and 11 (9%) of these were intermediately and fully resistant to penicillin, respectively. The prevalence of intermediately and fully resistant strains to the other antibiotics, respectively was as follows: amoxycillin-clavulanate, 1% and 14%; cefotaxime, 4% and 6%; cefuroxime, 5% and 13%; cefaclor 30% and 47% and clindamycin, 5% and 9%. About 41% were resistant to sulfamethoxazole-trimethoprim. Among the PRSP, the frequency of strains isolated from different clinical sources, in a descending order, was as follows; conjunctiva (27%), ear (24%), blood (11%), unknown (11%), ET secretion (9%), sputum (7%), CSF (4%), nasal cavity (3%), throat swab (1%) and appendicular abscess (1%).

Table 1. *Antibiotic susceptibilities of* Streptococcus pneumoniae *against different antibiotics*

Antibiotics	Susceptible	Intermediate	Resistance	Not determined
Penicillin	58 (43·3)*	59 (46·1)	11 (8·6)	
Amoxicillin-clavulanate	109 (85·1)	1 (0·8)	18 (14·1)	
Cefotaxime	115 (90·6)	5 (3·9)	7 (5·5)	1
Cefuroxime	103 (82·4)	6 (4·8)	16 (12·8)	3
Cefaclor	30 (23·4)	38 (29·7)	60 (46·9)	
Clarithromycin	110 (85·9)	6 (4·7)	12 (9·4)	
Sulfamethoxazole-trimethoprim	75 (59·1)		52 (40·9)	1

* Percentages are given within parentheses.

Table 2. *Serotype of* Streptococcus pneumoniae *isolated from different specimens in Kuwait and number of PRSPs in each serotype*

Serotype	No. of isolates	No. of PRSPs (intermediate)	No. of PRSPs (full)
1	5 (4·1)*	1 (1·9)	
3	6 (4·9)		
4	1 (0·8)		
5	3 (2·5)	1 (1·9)	
6A	8 (6·6)	5 (9·4)	
6B	11 (9·0)	6 (11·3)	1 (10·0)
7F	1 (0·8)		
8	1 (0·8)		
9A	3 (2·5)	1 (1·9)	1 (10·0)
9N	1 (0·8)		
9V	3 (2·5)	2 (3·9)	
11A	3 (2·5)	1 (1·9)	1 (10·0)
13	1 (0·8)		
14	12 (9·8)	8 (15·1)	1 (10·0)
15B	1 (0·8)	1 (1·9)	
16	2 (1·6)	1 (1·9)	
17F	2 (1·6)	1 (1·9)	
18F	2 (1·6)		
19A	2 (1·6)	2 (3·8)	
19B	1 (0·8)		
19C	4 (3·3)	3 (5·6)	
19F	12 (9·8)	7 (13·2)	
22F	1 (0·8)		
23A	1 (0·8)	1 (1·9)	
23B	1 (0·8)		1 (10·0)
23F	11 (9·0)	2 (3·8)	4 (40·0)
24F	1 (0·8)		
29	4 (3·3)	3 (5·6)	
32A	1 (0·8)		
33F	1 (0·8)		
34	1 (0·8)		
38	1 (0·8)		
Non-typable	14 (11·5)	7 (13·2)	1 (10·0)
Total	122 (100)	53 (100)	10 (100)

* Percentages are given in parentheses.

A total of 24 strains of intermediately, and all the 11 strains of fully penicillin-resistant pneumococci, were resistant to two or more antibiotics, i.e. multi-drug resistant. Two of the penicillin-sensitive *S. pneumoniae* were resistant to amoxycillin-clavulanate and sulfamethoxazole-trimethoprim.

Serotypes

A total 122 strains were serotyped (Table 2). Over one-third, 46 (37·7%) out of 122, of the strains belonged to serotypes 6B, 14, 19F and 23F; 14 (11·5%) were non-typable. Serotype 6A was also relatively common; eight (6·6%) belonged to this serotype. Seventy-seven (63%) of these strains are included in the 23-valent vaccine. Of the strains isolated from blood and CSF, 15 (58%) out of 26 also belonged to the serotypes included in the pneumococcal vaccine.

Amongst the 59 isolates of intermediate resistant *S. pneumoniae*, serotyping was done in 53 isolates, the serotype distribution was in the following order (Table 2); non-typable (13·2%), 14 (15·1%), 19F (13·2%), 6B (11·2%), 6A (9·4%), 19C (5·6%), 29 (5·6%), 9V (3·8%), 19A (3·8%), 23F (3·8%), 1 (1·9%), 5 (1·9%), 9A (1·9%), 11A (1·9%), 15B (1·9%), 16 (1·9%), 17F (1·9%) and 23A (1·9%). Among the fully resistant *S. pneumoniae* (Table 2), four strains were of serotype 23F and each of the rest of the strains belonged to serotypes 23B, 14, 6B, 11A, 9A and non-typable. One strain was not available for serotyping.

PFGE pattern

A total of 40 patterns were recognized from 67 PRSPs with PFGE of *Sma*I digested chromosomal DNA. Ten main types and their corresponding subtypes

Table 3. *Comparison among* Sma*I and* Apa*I restricted patterns and serotypes*

PFGE group	Serotype	Strains of *Sma*I restriction	Serotype	Strains of *Apa*I restriction
1	19F	95-37 (A)*, 96-15 (A), 96-87 (A), 95-16 (A1)	19F	95-3 (A), 95-37 (A), 96-15 (A), 96-87 (A)
	17F	96-4 (A)	6B	95-11 (A1)
	ND	96-62 (A1), 96-41 (A2)		
2	14	95-36 (B), 96-47 (B), 96-51 (B), 96-16 (B1), 96-31 (B1), 95-6 (B2)	14	95-36 (B), 96-51 (B), 96-47 (B1), 96-31 (B2), 95-6 (B3)
			29	96-19 (B4)
3	23F	96-32 (C), 96-121 (C), 96-120 (C2)	23F	96-32 (C2)
	9A	96-10 (C)	9A	96-10 (C)
	14	96-59 (C1)	6B	96-29 (C1)
	NT	96-28 (C)		
4	6B	95-11 (D), 96-29 (D2), 96-69 (D3)	16	95-10 (D)
	6A	96-11 (D1)	23F	96-120 (D1)
			14	96-59 (D2)
5	9A	95-32 (E)	9A	95-32 (E)
	9V	96-66 (E1)	9V	96-66 (E1)
	NT	96-82 (E2)	ND	96-99 (E1)
	29	95-7 (E3)	29	95-7 (E)
6	6A	95-46 (F), 96-48 (F)	6A	96-46 (F), 96-48 (F1)
7	19C	95-20 (G), 96-43 (G1)	19C	95-20 (G), 96-23 (G1)
8	6A	95-47 (H)		
	6B	96-107 (H1)		
9	19F	95-5 (I), 95-19 (I1)		
10	NT	96-81 (J)		
	ND	96-89 (J1)		

* Parentheses indicate pulsed field gel electrophoresis (PFGE) type and subtype within each strain.

were recognized in 37 strains; therefore these strains formed 10 groups (Table 3). The rest of the strains, i.e. 30 strains each had one indistinguishable pattern. The number of DNA fragments (Fig. 1) ranged from 9–26 bands (generally 12–15 bands). In the first group there were a total of 7 strains, 4 were designated as type A (3 strains of serotype 19F and 1 of 17F) and closely related strains were designated as subtype A1 (2 strains, serotype 19F and undetermined) and A2 (1 strain, undetermined serotype). In the second group (6 strains), 3 were designated as type B and closely related patterns were designated as subtype B1 (2 strains) and B2 (1 strain). All strains of this group belonged to serotype 14. The third group also had 6 strains, which were type C (4 strains; 2 of serotype 23F, 1 of 9A and 1 non-typable) and 1 strain of closely related and possibly related patterns were subtype C1 (serotype 14) and C2 (serotype 23F). In the fourth group there were 4 strains, designated as type D and subtype D1, D2 and D3 with closely related patterns. All belonged to serotype 6B except D1 that belonged to setotype 6A. In the fifth group again there were 4 strains, designated as type E (serotype 9A), with possibly related strains designated as subtype E1 (serotype 9V), E2 (non-typable) and E3 (serotype 29). Type F in the sixth group had 2 strains of serotype 6A. The seventh group had type G (1 strain) and subtype G1 (1 strain) both belonged to serotype 19C. Each strain of serotype 6A and 6B made the eighth group, designated as type H and possibly related subtype type H1. Two strains belonged to serotype 19F formed the ninth group with type I and closely related subtype I1. The last group was formed by type J (1 strain) and closely related subtype J1 (1 strain). The serotypes in this group were non-typable and undetermined.

A total of 42 main patterns were recognized from 60 PRSP isolates in PFGE of *Apa*I restricted chromosomal DNA. Seven main types with their corresponding subtypes were recognized in 25 strains therefore these strains formed 7 groups (Table 3). The rest of the strains, i.e. 35 strains, each had one indistinguishable pattern. The number of DNA fragments ranged from 8 to 23 bands (generally 12–15

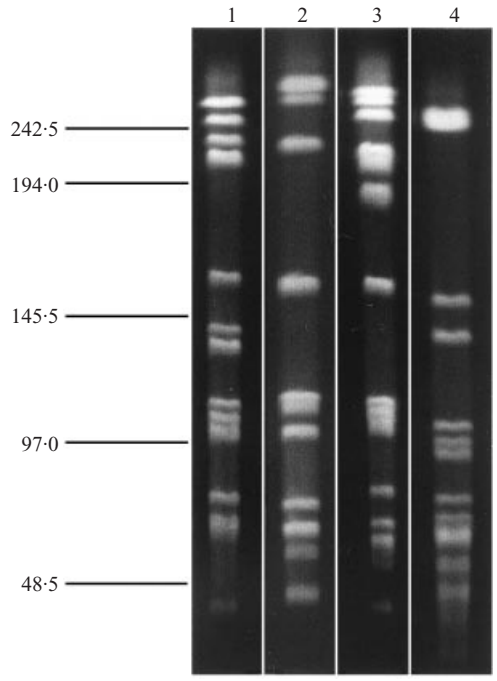

Fig. 1. *Sma*I restricted chromosomal DNA patterns of penicillin resistant *Streptococcus pneumoniae* (PRSP) generated by pulsed field gel electrophoresis. Lanes 1–4 are chromosomal DNA pattern of serotypes 19F, 14, 23F and 6B of PRSP strains. The molecular markers in kilobasepair (kb) are on the left of the first lane.

bands). The first group consisted of 5 strains, type A (4 strains, serotype 19F) and possibly related subtype A1 (1 strain, serotype 6B). The second group (6 strains) had the following patterns: type B (2 strains) and subtype B1 (1), B2(1), B3(1) and B4(1). Subtype B4 was of serotype 29, the rest were serotype 14. Three strains formed the third group, the patterns were designated as type C (serotype 9A), and closely related subtype C1 (serotype 6B) and C2 (serotype 23F). In the fourth group there were 3 strains designated as type D (serotype 16) and closely related subtype D1 (serotype 23F) and D2 (serotype 14). Type E (2 strains, serotype 9A and 29) and closely related subtype E1 (2 strains, serotype 9V and undetermined) formed the fifth group. Two strains of serotype 6A formed the sixth group was consisted of type F and closely related subtype F1. The last group had 2 strains of serotype 19C designated as type G and closely related subtype G1.

REP–PCR pattern

REP–PCR patterns generated 45 groups in 62 strains. There were 4 strains in each of groups 1, 2 and 3; 3 strains in each of groups 4 and 5; in groups 6, 7, 8, 9 and 10 each had 2 strains; in the rest of the groups there was 1 strain each. The serotype of each strain in a group was different except in group 3, where there were 2 strains of serotype 6B, 1 of serotype 6A and 1 of serotype 29. In group 5, two strains of serotype 6B and undetermined serotype showed similar pattern with the Spanish strain (Fig. 2).

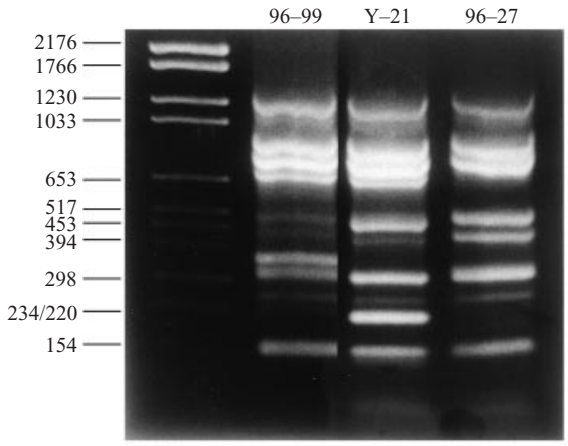

Fig. 2. REP–PCR pattern of two penicillin resistant *Streptococcus pneumoniae* isolated in Kuwait related to the Spanish 23F strain as indicated by Y-21 in the figure.

Comparison among *Sma*I, *Apa*I and REP–PCR patterns

The strains belonging to each group of *Sma*I and *Apa*I restricted patterns were fairly in agreement (Table 3). In each group, strains of one serotype or serogroup was usually predominant with a few strains from other serotypes. However in group 3 and 5, strains were of unrelated serotypes, however, *Sma*I and *Apa*I restriction patterns of these strains were fairly in agreement, therefore indicating that they were genetically related. Strains 96-32 and 96-10 of group 3 of PFGE pattern were in the same group of REP–PCR pattern. Strains 96-11, 96-29 and 96-69 of group 4 of *Sma*I restricted patterns were also in the same group of REP–PCR patterns. All these strains were of serotype 6B.

DISCUSSION

The data generated in this hospital-based surveillance study provide information on the pattern of pneumococcal antimicrobial resistance in Kuwait. This shows that the prevalence of penicillin-resistant *S. pneu-*

moniae isolates was more than half (55%) of the clinical isolates, indicating an almost threefold rise in the resistance rate than previously reported 4 years earlier [3]. It is also pertinent to note the rise in the prevalence of fully resistant strains from 0% in 1992 to 9% in 1996 as shown in this study. The resistance to cefotaxime, cefuroxime, amoxicillin-clavulanate and clindamycin are also at unacceptable levels. About 20% of the PRSP strains are multiple resistant to two or more drugs. Although studies from South Africa and New Mexico [13], Hungary [14] and Spain [15] have all reported PRSP prevalence rates nearing 50%, only the recent report from Palestine of an extremely high rate of 88% [16] has surpassed the high resistance rate found in this study. The current figure indicates that Kuwait has the highest prevalence rate of PRSP among the member states in the Gulf region [17–20]. This high penicillin-resistance rate in Kuwait has serious therapeutic implications, insofar as nearly 20% of the PRSP are multi-resistant. About 40% of the strains were resistant to sulfamethoxazole-trimethoprim, a finding similar to that reported in neighbouring Saudi Arabia [18]. Sulfamethoxazole-trimethoprim has been suggested by World Health Organization for the management of presumptive bacterial respiratory infections in children in developing countries [21] and for the management of non-severe pneumonia [22]. However, in the light of our present finding, using sulfamethoxazole-trimethoprim as suggested for empirical therapy in these clinical settings would be inadvisable and besides, there is evidence that widespread use of this antibiotic could result in the rapid selection of pneumococci with reduced susceptibility [23]. Therefore, caution is recommended for its widespread use.

The increasing trends of pneumococcal resistance to the third-generation cephalosporins, particularly cefotaxime and ceftriaxone, are a major problem for therapy of invasive disease, especially meningitis. The consensus among experts is that vancomycin be added to the therapeutic regimen whenever pneumococcal meningitis is suspected in areas with known high prevalence of PRSP. At resistance rate of about 55% PRSP and 10% cephalosporin-resistant strains (CRSP), this recommendation should be strongly endorsed in this area.

The serotyping technique with the Danish antisera enabled us to define the serotypes of 88·5% of our isolates; 11·5% being untypable. Studies have shown that clones of penicillin-resistant pneumococci have emerged from a limited number of serotypes, namely 6B, 9V, 14 and 23F [24, 25]. In this study, PRSP isolates belonged predominantly to serotypes 6A, 6B, 14, 19F and 23F. However, non-typable clones were also encountered in relatively large numbers and as could be seen from their sources, they were also involved in invasive infections. This finding may conceivably pose a dilemma with the vaccination strategies in this area, especially the young and the elderly who constituted the majority of patients from these strains were isolated. Only about 63% of the total isolates and about 57% of the CSF and blood isolates are covered by the 23-valent pneumococcal vaccine. A recent publication from Saudi Arabia reports that most of the PRSP belonged to serotypes 9V, 14 and 23F [20]. Extensive serotyping studies in other Gulf countries are urgently needed since they share common socioeconomic cultures and conditions. A similar serotyping pattern in these countries may then lead to the development of vaccine that could include the non-typable strains for use in the Gulf region.

The discriminatory power of serotyping is considered poor because of the different genotypes that may express the same phenotypic characteristics [26]. This is confirmed by the molecular typing of our strains which demonstrated that strains from most serotypes were genetically heterogenous and many clones were detected during this period. However, PRSP of serotype 6B, 14, 19F and 23F appeared to be more clonally related. An overall genetic diversity observed among the PRSP serotypes indicates that serotypes 6B, 14, 19F and 23F may have a common origin with significant changes occurring in the later stages of circulation in the country. The PFGE pattern of our isolates demonstrated that no Kuwaiti strain was related to the Spanish 23F strain. Nonetheless, with the REP–PCR technique, the Spanish strain showed similar pattern with a strain belonging to serotype 6B and another with undetermined serotype. Further studies is therefore needed to find out the contribution of the Spanish PRSP, serotype 23F, to the emergence of penicillin-resistant *S. pneumoniae* in Kuwait.

These results suggest that the PRSP strains, other than serotypes 6B, 14, 19F and 23F, in Kuwait, did not arise from a single clone, but rather from several independent clones and that some strains may have spread widely in Kuwait more than the other. It is highly possible that the unusually large diversity of resistant genotypes present in this study is due, at least in part, to multiple importation of strains originating

from outside Kuwait. In the Gulf region, large number of working class people are from different geographical areas of the world. The tremendous movement of these population may bring various types of resistant penumococci to Kuwait. We can speculate at this stage, that, as *S. pneumoniae* is naturally transformable, these imported strains may give rise to new genotypes in Kuwait such as the non-typable strains of PRSP and strains of other serotypes which appeared to be unique to Kuwait. This line of thinking is buttressed by the report of a recent study on PFGE of *Moraxella catarrhalis* isolated from respiratory infections from different geographical areas which showed that only the strains from Kuwait had a large diversity of clones [27]. About two-thirds of the population of Kuwait are expatriates, mainly from Egypt and other Arab countries, India, Philippines, Pakistan and Bangladesh. A comparative study, using molecular techniques, of strains isolated from these countries, is needed to find out the divergence of PRSP strains in Kuwait. The rapidly increasing prevalence of PRSP, CRSP and multi-drug resistant strains in Kuwait emphasizes the importance of judicious use of antibiotics and vaccination to prevent serious PRSP infections in persons at greater risk for pneumococcal disease.

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