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Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and β-lactam resistance, and to detect common macrolide resistance determinants

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Pneumococcal isolates (*n* = 148) from various countries (mostly from the USA) were tested by a primer set for PCR. Thirty-eight (86.4%) of the 44 penicillin G-susceptible isolates (MIC ⩽ 0.06 mg/L) had unaltered *pbp*s, while six isolates (13.6%) had either one or two alterations in *pbp*s. Of 47 penicillin G-resistant strains (MIC ⩾ 2 mg/L), 41 isolates (87.2%) had all three *pbp*s altered, six isolates (12.8%) had altered *pbp1a* + *2x*. Various combinations of altered *pbp* were seen in penicillin G-intermediate isolates. Prevalence of macrolide resistance genes *mef*(A) and *erm*(B) in isolates was clearly reflected by their MICs. All isolates were positive for *lytA*. The primers were useful for screening for *Streptococcus pneumoniae* and β-lactam resistance, and for detection of common macrolide resistance determinants.

Introduction

A primer set for PCR, which was designed by one of the authors (K.U.), detects the following: unaltered *pbp* genes based on the *pbp* sequences of the penicillin G-susceptible *Streptococcus pneumoniae* R6 strain for PBP1A, 2X, 2B;[1–3] macrolide-resistant genes *mef*(A) and *erm*(B);[4,5] autolysin gene (*lytA*)[6] as screening of *S. pneumoniae*. It is marketed in Japan only for research use. PCR results using the primers correctly reflected antimicrobial susceptibilities of *S. pneumoniae* clinically isolated in Japan.[7] Since those studies were published, however, primers for *pbp1a*, *pbp2x* and *pbp2b* were changed to those used in the current study. We therefore evaluated the efficacy of the new primers for pneumococci isolated in countries other than Japan.

Materials and methods

Bacteria

We tested 148 clinical isolates of *S. pneumoniae* screened by optochin susceptibility and bile solubility collected during the past 5 years from the following countries: Bulgaria (*n* = 3), Canada (*n* = 9), Greece (*n* = 16), Poland (*n* = 1), Romania (*n* = 6), Slovenia (*n* = 8), South Africa (*n* = 2), Spain (*n* = 4) and the USA (*n* = 99).

Susceptibility testing

Agar dilution MICs of penicillin G (Sigma, St Louis, MO, USA), ceftriaxone (Sigma), erythromycin (Abbott Laboratories, Chicago, IL, USA), azithromycin (Pfizer, Groton, CT, USA) and clindamycin (Pharmacia & Upjohn, Kalamazoo, MI, USA) were determined.[8] Each compound was obtained from its manufacturer. Standard quality control strains, including *S. pneumoniae* ATCC 49619, were included in each run. NCCLS susceptibility breakpoints[9] were used to interpret MICs.

PCR primers

The sequences of the primers used for PCR are as follows: *lytA*: 5′-$_{681}$CAACCGTACAGAATGAAGCGG$_{701}$-3′, 5′-$_{999}$TTATTCGTGCAATACTCGTGCG$_{978}$-3′; *pbp1a*: 5′-$_{2256}$AAACAAGGTCGGACTCAACC$_{2275}$-3′, 5′-$_{2450}$ATATACATTGGTTTATAGTAAGTT$_{2427}$-3′; *pbp2x*: 5′-

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$_{1255}$CCAGGTTCCACTATGAAAGTG$_{1275}$-3′, 5′-$_{1451}$ATC CCAACGTTACTTGAGTGT$_{1431}$-3′; *pbp2b*: 5′-$_{1566}$CCTA TATGGTCCAAACAGCCT$_{1586}$-3′, 5′-$_{1693}$GGTCAATTC CTGTCGCAGTA$_{1712}$-3′; *mef*(A): 5′-$_{180}$CTGTATGGAG CTACCTGTCTGG$_{199}$-3′, 5′-$_{581}$CCCAGCTTAGGTAT-ACGTAC$_{562}$-3′; *erm*(B): 5′-$_{721}$CGTACCTTGGATATT CACCG$_{740}$-3′, 5′-$_{944}$GTAAACAGTTGACGATATTCT CG$_{922}$-3′.

The oligonucleotide primers for detection of three *pbp* genes were designed to amplify parts of the *pbp1a*, *2x* and *2b* genes only in susceptible strains. These parts were positioned in blocks of highly diverged sequences identified in the mosaic *pbp* genes of penicillin non-susceptible *S. pneumoniae*. Primer mixture A contained the primers for detecting *lytA* and *pbp1a* genes, primer mixture B contained the primers for detecting *pbp2x* and *2b* genes, and primer mixture C contained the primers for detectin *mef*(A) and *erm*(B). Each primer mixture (100 μL), which contained 0.1 μM of each primer and 8 mM dNTPs, is available commercially in Japan (Wakunaga Pharmaceutical, Co., Ltd, Hiroshima, Japan). Before testing, 50 μL of 10× PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8.4, 1.5 mM $MgCl_2$), 20 U of *Tth* DNA polymerase (Toyobo, Co., Ltd, Osaka, Japan) and 348 μL of dH_2O, were added to each primer mixture. Then, it was divided into 30 μL aliquots and stored at −30°C.

PCR condition

A single colony of *S. pneumoniae* grown on a blood agar plate was suspended in 30 μL of lysis solution. The composition of the lysis solution has been reported previously.[7] The tubes with the lysis solution were set into a thermal cycler (GeneAmp 9700; PE Applied Biosystems, Foster City, CA, USA) and incubated at 60°C for 10 min and 94°C for 5 min. These lysates were then used as template DNA for PCR.

Next, 2 μL of the bacterial lysate was added to each of three tubes containing primer mixtures A, B and C. A positive and negative control was included in each run. PCR was performed with the thermal cycler for 30 cycles at 94°C for 15 s, 30 cycles at 53°C for 15 s and 30 cycles 72°C for 15 s. Following amplification, 10 μL of each of the three PCR products was electrophoresed on a 3% agarose gel (Agarose LE; Promega Co., Madison, WI, USA) for 40 min at 100 V.

For interpretation of PCR results, the three bands were seen on the agarose gel if the isolate did not have altered (abnormal) *pbp*s, while one or more of these bands were not detected for strains with alterations in the *pbp*s. Bands appeared on the gel if the isolate has the *erm*(B) and/or *mef*(A) gene. The positions of DNA fragments amplified from a positive control in this primer mix and three isolates tested are shown in the Figure.

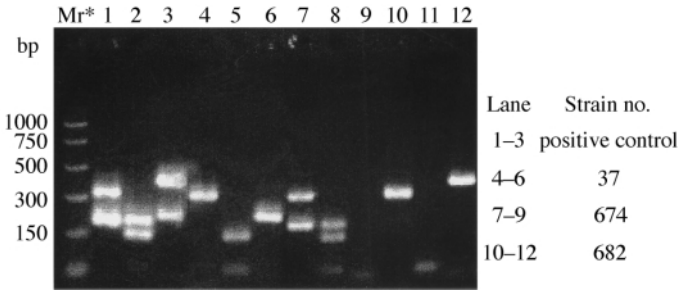

Figure. Results of amplified DNA fragments of positive control included in the kit (lanes 1–3) and three test isolates (lanes 4–12). *PCR marker (Promega) was used as size standard in lane marked 'Mr'. PCR product of *lytA* (319 bp product) and *pbp1a* (195 bp product) are shown in lanes 1, 4, 7 and 10. Products of *pbp2x* (197 bp product) and *pbp2b* (147 bp product) are shown in lanes 2, 5, 8 and 11. *mef*(A) (402 bp product) and *erm*(B) (224 bp product) are shown in lanes 3, 6, 9 and 12.

Results

The MIC_{50}s/MIC_{90}s for the 148 pneumococcal isolates were: penicillin G 0.5/4, ceftriaxone 0.25/1, erythromycin 0.06/>64, azithromycin 0.12/>64 and clindamycin 0.06/>64 mg/L. The percentage distribution of isolates susceptible/intermediate/resistant to each compound was: penicillin G 29.7/37.8/32.4 (44 S, 56 I, 48 R), ceftriaxone 68.9/21.6/9.5, erythromycin 56.8/2.0/41.2, azithromycin 56.8/4.0/39.2 and clindamycin 68.9/0/31.1.

PCR results and the MIC distribution of penicillin G and ceftriaxone are shown in the Table. Thirty-eight (86.4%, 95% CI 72.7–94.8) of the 44 penicillin G-susceptible isolates (MIC ⩽ 0.06 mg/L) had unaltered *pbp*s, while six isolates (13.6%, 95% CI 5.2–27.4) had either one or two alterations in *pbp*s. Twenty-four of the 58 (41.4%, 95% CI 28.6–55.1) penicillin G-intermediate isolates had alterations in all three *pbp*s, 17 isolates (32.1%, 95% CI 20.3–46.0) had two *pbp*s altered and 20 isolates (34.5%, 95% CI 22.5–48.1) had one *pbp* altered. Of 47 penicillin G-resistant isolates (MIC ⩾ 2 mg/L), 41 isolates (87.3%, 95% CI 74.3–95.2) had all three *pbp*s altered, six isolates (12.8%, 95% CI 4.8–25.7) had altered *pbp1a* + *2x*.

The MICs of ceftriaxone for 63 of the 73 isolates that had *pbp1a* + *2x* or all three *pbp*s altered ranged between 0.5 and 8 mg/L (86.3%, 95% CI 76.3–93.2). Thirty-one (49.2%, 95% CI 36.4–62.1) of the 63 isolates with *pbp1a* + *2x* or all three *pbp*s altered were ceftriaxone intermediate (MIC 1 mg/L), and 14 of the 63 (22.2%, 95% CI 12.7–34.5) isolates were ceftriaxone resistant (MIC ⩾ 2 mg/L). Of 102 ceftriaxone-susceptible isolates (MIC range 0.016–0.5 mg/L), alterations in either *pbp1a* or *2x* were found in 59 isolates (57.8%, 95% CI 47.7–67.6).

All 84 pneumococcal isolates that were susceptible to erythromycin, azithromycin and clindamycin, were negative for both *mef*(A) and *erm*(B). MIC_{90}s (MIC ranges) (mg/L) for these isolates were: 0.06 (<0.008–0.12), erythromycin; 0.12 (0.016–0.25), azithromycin; 0.06 (0.016–0.12), clindamycin. *mef*(A) was found in 18 isolates with MIC_{90}s

Table. PCR results and MICs of penicillin G and ceftriaxone for 148 pneumococcal isolates

	MIC (mg/L) distribution											MIC range	MIC_{50}	MIC_{90}
	<0.008	0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8			
PCR results and penicillin G MICs															
no alteration		1	17	18	2	1							0.008–0.12	0.016	0.03
pbp1a, *2x* or *2b*				1	4	4	9						0.03–0.25	0.12	0.25
pbp1a + *2x*							2		1	4	2		0.25–4	4	–
pbp2x + *2b*					1	6	5	5	1				0.06–1	0.25	0.5
pbp1a + *2x* + *2b*							3	9	11	23	13	5	0.25–8	2	4
PCR results and ceftriaxone MICs															
no alteration	2	4	15	16	1	1							<0.008–0.12	0.016	0.03
pbp1a, *2x* or *2b*			1	1	4	6	6						0.016–0.25	0.12	0.25
pbp1a + *2x*						1			4	4			0.12–2	1	–
pbp2x + *2b*					1	6	8	2	1				0.06–1	0.25	0.5
pbp1a + *2x* + *2b*					1	6	3	18	27	8		2	0.06–8	1	2

(MIC ranges) (mg/L) of 8 (0.5–8), erythromycin; 8 (1–16), azithromycin; 0.12 (0.03–0.12), clindamycin. All 18 strains with *mef*(A) were susceptible to clindamycin.

erm(B) was found in 43 isolates for which the MIC_{90}s were >64 mg/L of erythromycin (MIC range 2–>64 mg/L), azithromycin (MIC range 8–>64 mg/L) and clindamycin (MIC range 2–>64 mg/L). Three isolates had both *mef*(A) and *erm*(B), and MICs for these isolates were >64 mg/L of erythromycin, azithromycin and clindamycin.

All 148 isolates were optochin sensitive, bile soluble and were positive for *lytA* by PCR. Positive and negative controls were detected correctly in each run.

Discussion

The primers tested in this study detect *lytA* gene, *pbp1a*, *2b* and *2x* gene alterations, and *erm*(B) and *mef*(A) macrolide resistance genes. Furthermore, the results can be obtained within 3 h of testing one pneumococcal colony growing on a plate.

Optochin susceptibility and bile solubility have been recommended for screening for *S. pneumoniae*; however, the *lytA* gene seemed to have good reliability for screening of pneumococci in this study. The specificity of the *lytA* gene for *S. pneumoniae* had already been tested when the primers were developed. *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus salivarius*, *Staphylococcus aureus* and *Staphylococcus epidermidis* were all *lytA* negative; only *S. pneumoniae* proved *lytA* positive (K. Ubukata, unpublished data).

Determination of the *pbp* genotype seemed to be useful in estimating the degree of penicillin G resistance in our study. Six penicillin G-susceptible isolates (MIC range 0.03–0.06 mg/L) had one or two altered *pbp*(s) by PCR. Similar patterns have been seen in a previous study.[7] Antibiotic treatment of the latter strains, especially in meningitis, should be chosen carefully because these strains may not be detected by conventional oxacillin screening, but may also have altered *pbp*s.

MICs of ceftriaxone for isolates with alteration of *pbp1a* + *2x* or all three *pbp*s were higher (≥0.5 mg/L) and those patterns of altered *pbp*s might be useful in estimating isolates with higher MICs of this agent. However, 10 isolates for which ceftriaxone MICs were 0.06–0.25 mg/L also had these alterations. DNA sequencing of *pbp1a*, *2x* and *2b* for six of the latter isolates (penicillin G MICs 0.25–0.5 mg/L) showed that there was no mutation in the $_{370}$SerThrMetLys$_{373}$ motif, which is the area of conserved amino acids in *pbp1a*. The primers for *pbp1a* were located *c.* 240 bp away from the SerThrMetLys region, and those isolates that had altered *pbp2x* + *2b* had point mutations in the region of the *pbp1a* primers. These results indicate that the results of *pbp1a* PCR for those six isolates were false positive and re-design of *pbp1a* primers is necessary to detect isolates in those categories more accurately.

Macrolide resistance mechanisms, *erm*(B) and *mef*(A), were accurately detected and correlated with MICs of erythromycin, azithromycin and clindamycin. The PCR results in this study matched our previous results (P. C. Appelbaum, unpublished data), which had been been determined already using other primers described by Sutcliffe *et al.*[10]

In summary, the primers that we tested may be helpful for rapid screening of pneumococcal isolates from patients with severe systemic infection such as meningitis or life-threatening pneumonia, where rapid MIC results are necessary. It was also thought to be useful for detection of common macrolide resistance. However, more appropriate primers are required to detect and distinguish β-lactam resistance accurately.

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