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Penicillin and vancomycin tolerance among clinical isolates of *Streptococcus pneumoniae* in Hong Kong

香港肺炎鏈球菌種的臨床分離菌株對青黴素及萬古黴素的耐受性

Objective. To determine the prevalence of penicillin and vancomycin tolerance in clinical isolates of *Streptococcus pneumoniae*.
Design. Laboratory testing of 50 consecutive clinical isolates.
Setting. District hospital, Hong Kong.
Patients. Fifty patients with pneumonia or meningitis who were admitted to a district hospital in Hong Kong between August and October 2002.
Main outcome measures. Lysis, as assessed by loss of optical density at 4 hours at 540 nm, and reduction in viable count expressed as the log of the number killed ('log kill') after exposure of cultures to 10 times the minimum inhibitory concentration of penicillin and vancomycin.
Results. Of the 50 isolates, five (10%) were tolerant to penicillin and one (2%) was tolerant to vancomycin.
Conclusion. This is the first report of vancomycin tolerance in *Streptococcus pneumoniae* in Hong Kong. Because vancomycin tolerance is associated with clinical failure in the treatment of pneumococcal meningitis, increased monitoring for resistant strains of organisms is suggested.

目的：確定青黴素及萬古黴素在肺炎鏈球菌種臨床分離菌株的耐受性的比率。
設計：為連續 50 個臨床分離菌株進行測試。
安排：區域醫院，香港。
患者：於 2002 年 8 月至 10 月期間， 50 名因肺炎或腦膜炎而入院的病人。
主要結果測量：溶解情況；即培養物暴露在青黴素及萬古黴素的最低抑菌濃度的10倍情況下，量度 4 小時後在光密度 540 納米的下降率，及以被殺菌株數目的對數('log kill')所得出活菌數目的下降率。
結果：在 50 個臨床分離菌株當中， 5 個(10%)對青黴素具耐受性， 1 個(2%)對萬古黴素具耐受性。
結論：這是香港首個有關肺炎鏈球菌種對萬古黴素耐受性的報告。因為萬古黴素的耐受性與治療肺炎球菌腦膜炎的臨床失敗有關，建議加強對生物體抗藥性的監察。

Key words:
Streptococcus pneumoniae;
Drug tolerance;
Vancomycin

關鍵詞：
肺炎鏈球菌種；
藥物耐受性；
萬古黴素

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Introduction

Antibiotic tolerance is the ability of bacteria to survive in the presence of antibiotic at a level above the determined minimum inhibitory concentration (MIC). Tolerant bacteria are not lysed or killed in the presence of effective antibiotic therapy.[1] Consequently, the infection relapses once treatment is discontinued—particularly in deep-site infections such as meningitis.[2-5] Antibiotic-tolerant bacteria cannot be detected using conventional susceptibility tests, because the organisms appear to be sensitive when affected by the antibiotic's bacteriostatic activity.

The inability of antibiotics to eradicate tolerant strains may be due to the bacteria's decreased response to the killing signals triggered by antibiotics. For example, in the mechanism of action of penicillin, a non-lytic killing system initiates the death process, followed by activation of lytic enzymes, which lyse the organism.[6] Tolerance can occur as a result of nutritional deprivation, because

non-growing organisms are not affected by antibiotics that inhibit cell wall growth. This phenomenon is known as phenotypic tolerance.[7] On the other hand, genotypic tolerance results from mutations in specific genes. Early studies in pneumococci focused on changes in the murein hydrolases, LytA, LytB, and LytC.[8] LytA is associated with penicillin tolerance: a laboratory-produced *lytA* mutant was non-lytic after treatment with both benzylpenicillin and vancomycin. However, even though these mutants were not lysed by either drug, both agents killed the organism, albeit at a much slower rate. This effect implies that these antibiotics can trigger a death process that is independent of bacterial lysis. Truly tolerant strains may have changes in both the lytic and the non-lytic death processes.[1] It has been suggested that a two-component system VncS/VncR is involved in vancomycin tolerance in *Streptococcus pneumoniae*, because inactivation of the membrane-bound histidine kinase sensor VncS results in complete tolerance.[9] Involvement of ABC transporters (Psa A/B/C/D transports), a zinc metalloprotease (ZmpB), and a heat shock protein (ClpC chaperone) have also been suggested.[10-13]

Tolerant strains were initially defined as those in which the minimum bactericidal concentration to MIC ratio exceeded 32, although other ratios were also used, thus causing difficulties in result interpretation.[14] The cell lysis assay measures the loss of turbidity of a culture in the presence of the antibiotic over a 4-hour period. This approach provides a simple screening method for possible tolerant strains.[15,16] Strains that appear to be resistant to lysis are then confirmed as tolerant by a time-kill study, because tolerant strains will have little reduction in viable count.[17] The limits for the reduction in bacterial count that indicates penicillin tolerance were defined by Henriques Normark et al[4] as a 'log kill' of less than or equal to 2.7 (ie a reduction of $10^{2.7}$ or less in viable count) and a reduction in optical density (OD) at 540 nm of less than or equal to 16%. For vancomycin, these limits are a 'log kill' of less than or equal to 2.0 and an OD loss of less than or equal to 43%. These limits were based on mean OD loss and mean 'log kill' of penicillin- and vancomycin-tolerant mutants within two standard deviations.

Penicillin tolerance in *S pneumoniae* has been recognised since 1985[18]; the first report of a vancomycin-tolerant clinical isolate appeared in 1999.[9] Surveys have shown the presence of vancomycin-tolerant strains in clinical isolates from the United States,[19] Sweden,[4] and Italy.[20] This study attempted to detect antibiotic tolerance in clinical isolates of *S pneumoniae* in Hong Kong.

Methods

A total of 50 consecutive isolates of *S pneumoniae* collected from 50 patients with pneumococcal pneumonia or pneumococcal meningitis between August and October 2002 from a district hospital in Hong Kong were investigated for tolerance to penicillin and vancomycin. Only one isolate was obtained from each patient. The MIC to penicillin and vancomycin for each strain was determined by the E-test using the recommended method.[21] To determine likely presence of tolerance by resistance to lysis, 30 mL of Todd-Hewitt broth was inoculated with bacterial culture in logarithmic growth phase (also grown in Todd-Hewitt broth) and incubated at 37°C until an OD_{540} of 0.2 was reached, which corresponded to a cell density of approximately 1×10^8 colony forming units per millilitre. This culture was then divided into three parts. Penicillin at 10 times the MIC was added to the first and vancomycin at 10 times the MIC to the second; the third served as a control. The cultures were re-incubated at 37°C, and the OD_{540} was measured at 1, 2, and 4 hours. Strains that showed resistance to lysis, showing less than a 16% reduction in the OD for penicillin or 43% for vancomycin, were suspected to be tolerant and were further characterised by means of a time-kill assay, in which viable counts were measured hourly for 4 hours following addition of antibiotic. The culture aliquot removed for the viable count was diluted in normal saline and then spread on blood agar prepared with GC (Gonococcus) agar base (Oxoid, Basingstoke, UK) and 5% defibrinated horse blood. Colony counts were determined after overnight incubation in 5% CO_2 at 37°C.

Results

Five (10%) isolates were tolerant to penicillin according to results of the cell lysis assay. One of these strains was also tolerant to vancomycin (Table). The time-kill assay confirmed antibiotic tolerance, with less than 2.7 log decrease in viability in the presence of penicillin and less than 2 log decrease in the presence of vancomycin within 4 hours' exposure to 10 times the MIC (Fig). Lysis and killing rates of the five tolerant strains were much less than those of non-tolerant strains. Of the five penicillin-tolerant strains, susceptibility test results showed that one (isolate S04) was penicillin-sensitive (MIC, <0.016 μg/mL), three (I12, I15, and I45) were moderately penicillin-resistant (MICs, 0.19, 0.25, and 0.190 μg/mL, respectively), and one (R10) was highly penicillin-resistant (MIC, 4.00 μg/mL). The vancomycin tolerant strain (R10) appeared to be susceptible to vancomycin (MIC, 0.273 μg/mL).

Discussion

Penicillin tolerance was detected in 10% and vancomycin tolerance in 2% of clinical isolates studied. This is the first report of vancomycin tolerance in *S pneumoniae* in Hong Kong, and compares to 10% penicillin tolerance reported by Tuomanen et al,[16] and 8% penicillin tolerance and 3% vancomycin tolerance reported by Henriques Normark et al.[4] In our study, four strains were tolerant to penicillin but not vancomycin, suggesting that vancomycin tolerance occurs through different or additional genetic mechanisms. As both penicillin-sensitive and penicillin-resistant strains demonstrated tolerance, and isolates that were penicillin-resistant were not tolerant, it seems that a strain may be

Table. Preliminary determination of antibiotic tolerance by cell lysis assay

Isolate No.	Treatment	OD_{540}				% loss of OD at
		0 hr	1 hr	2 hr	4 hr	4 hr
S04	Control	0.215	0.469	0.479	0.485	0
	Penicillin	0.215	0.296	0.276	0.248	0
	Vancomycin	0.215	0.197	0.062	0.022	90
R10	Control	0.196	0.465	0.468	0.479	0
	Penicillin	0.196	0.338	0.326	0.306	0
	Vancomycin	0.196	0.300	0.293	0.270	0
I12	Control	0.215	0.424	0.420	0.413	0
	Penicillin	0.215	0.319	0.281	0.207	0
	Vancomycin	0.215	0.082	0.049	0.030	86
I15	Control	0.208	0.370	0.399	0.413	0
	Penicillin	0.208	0.333	0.308	0.222	0
	Vancomycin	0.208	0.129	0.037	0.021	90
I45	Control	0.197	0.403	0.421	0.461	0
	Penicillin	0.197	0.326	0.267	0.247	0
	Vancomycin	0.197	0.024	0.016	0.018	91
I14	Control	0.205	0.334	0.321	0.313	0
	Penicillin	0.205	0.210	0.045	0.021	90
	Vancomycin	0.205	0.048	0.016	0.015	93

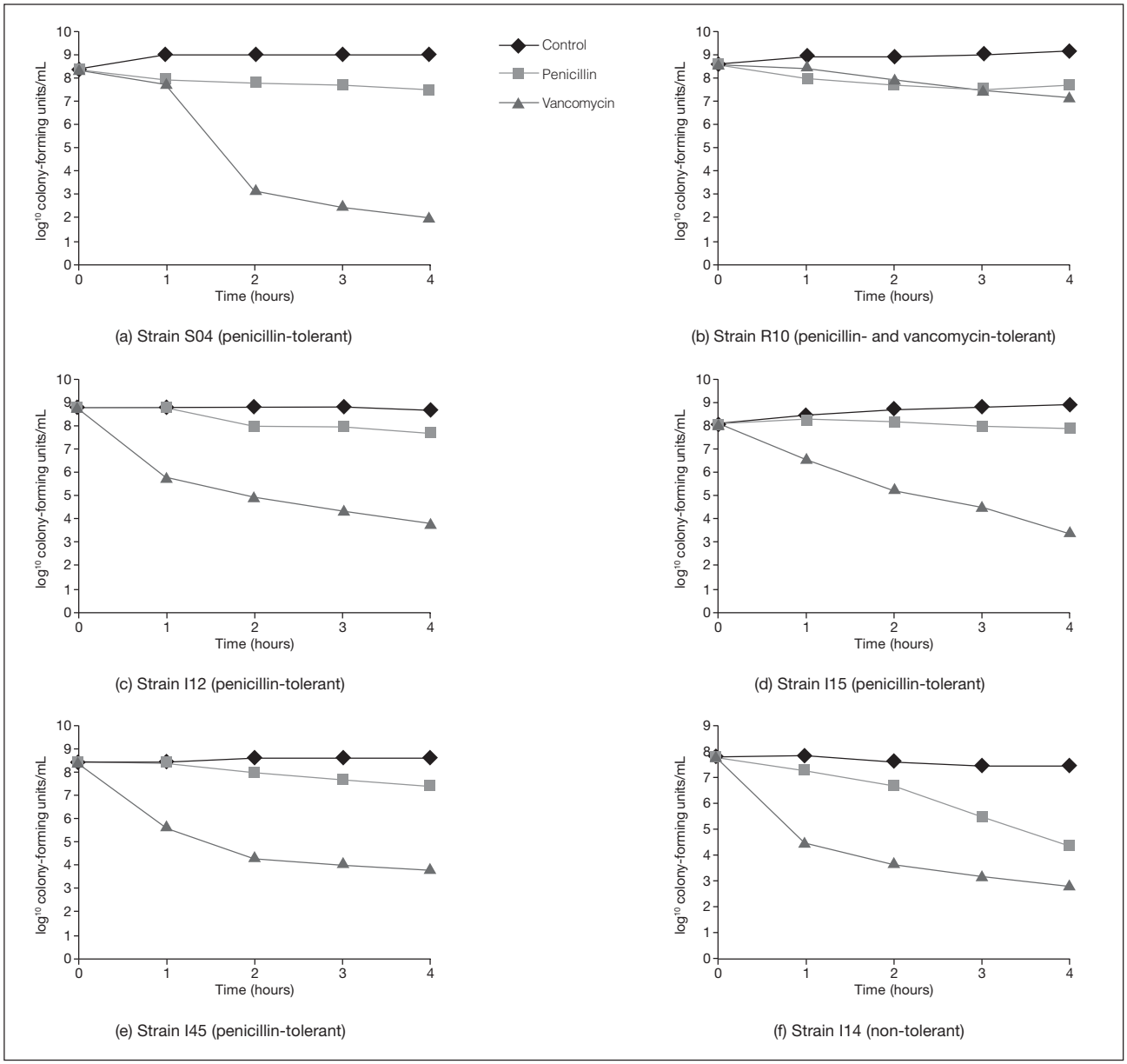

Fig. Death curves of *Streptococcus pneumoniae* strains exposed to penicillin and vancomycin

penicillin-resistant but not tolerant, or penicillin-sensitive and yet become tolerant. These findings demonstrate that the mechanism for tolerance differs from that of resistance in *S pneumoniae*, especially because the binding affinity to penicillin remains unchanged in tolerant strains.[10] Penicillin can thus still reach the target site and inhibit growth.

The presence of vancomycin-tolerant strains is a cause for concern, because penicillin resistance is high in both clinical[22] and community[23,24] isolates of *S pneumoniae* in Hong Kong, and vancomycin is used in the management of meningitis caused by penicillin-resistant strains. Treatment failures, such as those reported in the literature,[5] may occur because vancomycin-tolerant strains appear susceptible by routine methods of susceptibility testing. Laboratory detection of these strains is necessary to avoid recrudescence of infection following antibiotic treatment. The methods used in this study are quite tedious and are a drawback in the recognition of tolerant strains. The increasing knowledge of the mechanisms of cell death in the presence of antibiotics may soon lead to the localisation of the genetic determinant for the components that trigger the lytic cascade in *S pneumoniae*, and hence the development of a molecular diagnostic method for the rapid detection of tolerant strains. Until these methods become available, we recommend that penicillin-resistant pneumococci isolated from patients with meningitis should be examined for the presence of vancomycin tolerance and that the local prevalence of vancomycin-tolerant strains be routinely monitored using the methods described.

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