Selection of rifampicin-resistant Staphylococcus aureus during tuberculosis therapy: concurrent bacterial eradication and acquisition of resistance

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Background and objectives: Acquired antimicrobial resistance is commonly attributed to regimens that expose bacteria to subinhibitory concentrations; consequently, eradication of susceptible cells is advocated. The mutant selection window hypothesis predicts that resistance can be acquired even when inhibitory concentrations are exceeded and susceptible bacteria are eradicated. The objective was to test that prediction clinically.

Methods: Tuberculosis patients (n = 372) were sampled for nasal colonization by Staphylococcus aureus at the beginning of anti-tuberculosis therapy with rifampicin-containing regimens and again after 2 and 4 weeks. Rifampicin susceptibility of S. aureus was determined, and S. aureus isolates from patients developing acquired resistance were examined by molecular strain typing. Diabetes patients (n = 200) served as untreated controls.

Results: Nasal colonization was 17% and 20% for the tuberculosis and diabetes patients, respectively. Four patients were initially colonized with rifampicin-resistant S. aureus and were excluded from further sampling. Initiation of anti-tuberculosis therapy eradicated S. aureus nasal colonization in 53/58 tuberculosis patients while allowing acquisition of rifampicin resistance in 5/58. Pulsed-field gel electrophoresis (PFGE) band patterns and protein A repeat sequence determination differed in S. aureus isolated from different patients but was identical in isolates obtained from the same patient before and after acquisition of resistance. No resistance was acquired in untreated control patients, which differed statistically from treated patients (P = 0.025).

Conclusions: Acquired resistance and eradication of susceptible bacteria can occur concurrently; restricting acquired resistance may require direct suppression of mutant growth and viability in addition to elimination of susceptible bacteria.

Keywords: mutant selection window, antimicrobial resistance, S. aureus, TB

Introduction

Overuse and misuse of antimicrobials are widely accepted as major causes of acquired resistance, especially when doses are low and bacteria are exposed to subinhibitory concentrations. Dosing to eradicate infecting bacteria (‘dead bugs don’t mutate’) and to avoid subinhibitory drug exposure are advocated.¹ We recently postulated that resistant mutants are also selectively enriched and amplified in a drug concentration range (mutant selection window) that is above MIC but below a concentration that inhibits growth of the least-susceptible, single-step resistant mutant subpopulation.² Traditional treatments that are generally effective at eradicating susceptible bacteria are often expected to place drug concentrations inside the selection window.³ If the selection window hypothesis is accurate, treatments directed mainly at removing susceptible bacteria may be insufficient to restrict acquisition of resistance. To our knowledge, no clinical evidence for the existence of the selection window has been reported.

One prediction of the selection window hypothesis is that eradication of susceptible cells and acquisition of resistance can occur concurrently when antimicrobial concentrations fall inside the selection window. Such concurrence is most likely to be observed when the antimicrobial is very potent against susceptible bacteria and ineffective with single-step resistant mutants. Treatment of
S. aureus eradication and acquisition of resistance

Staphylococcus aureus with rifampicin may be particularly suitable for observing concurrence, since MIC is low with susceptible cells and high with mutants. A direct experiment is difficult because rifampicin is generally not used as monotherapy for S. aureus infections. However, nasal colonization by S. aureus is common, tuberculosis patients are often treated with rifampicin as the only agent having activity against S. aureus, and rifampicin resistance is observed in nasal isolates of S. aureus. To document acquired resistance, it is necessary to follow patients whose isolates are known to be rifampicin-susceptible at the beginning of therapy. From nasal colonization, a procedure not previously performed with nasal colonization.

Below we describe rifampicin susceptibility and genetic features of S. aureus at the beginning and after 4 weeks of anti-tuberculosis therapy to determine whether bacterial eradication and acquisition of resistance can occur concurrently.

Patients and methods

Between February and December of 2004, 372 patients were enrolled after diagnosis of pulmonary tuberculosis and before treatment with standard, combination anti-tuberculosis regimens containing rifampicin. As untreated controls, 200 diabetes patients were also enrolled. Anterior nares of patients were swabbed using sterile cotton, swabs were applied to Mannitol Salt Agar for selection of staphylococci, and bacterial colonies were obtained after incubation at 37°C for 24–48 h. Morphological comparison and coagulase testing were used to distinguish S. aureus from Staphylococcus epidermidis following regrowth on Mueller–Hinton Sheep Blood Agar. S. aureus isolates were tested for susceptibility to rifampicin by agar dilution (NCCLS protocol). Patients lacking S. aureus colonization or colonized with rifampicin-resistant isolates at the beginning of therapy were not sampled further. Patients from whom rifampicin-susceptible S. aureus was recovered were re-swabbed 2 and 4 weeks after initial screening, and samples were processed as above. PFGE and protein A repeat sequence determination (spa typing), standard procedures for molecular strain typing of S. aureus, were used to establish that resistant isolates were derived from cells present at initiation of therapy. The study protocol was approved by the ethics committee for clinical studies at PLA General Hospital of Beijing, China (protocol #04012).

Tuberculosis patients (253 males, 119 females) had an average age of 40.2 years (range 18–86 years); control diabetes patients (119 males, 81 females) had an average age of 54.6 years (range 18–85 years). All tuberculosis patients were treated with rifampicin and isoniazid plus additional compounds in heterogeneous protocols established independent of this study. Additional compounds used included pyrazinamide, ethambutol, para-aminosalicylic acid, streptomycin and levofloxacin in various combinations. The control group was untreated with antimicrobial. Patients in both study and control groups were hospitalized throughout the study period, which minimized non-compliance to treatment.

Results

Of the 372 patients in the treated study arm, 63 were positive for S. aureus colonization at the beginning of therapy. Two were colonized with rifampicin-resistant strains, and three withdrew from the study after initial sampling. The average carriage frequency, 17% (63/372) and 20% (41/200) for the tuberculosis and diabetes groups, respectively, was within the range of nasal carriage frequency reported for Chinese populations. Carriage rate for males was 18% and 22% for tuberculosis and diabetes patients, respectively; for females, it was 15% and 18.5%, respectively.

No rifampicin-susceptible S. aureus was recovered after 2 and 4 weeks of treatment; by 2 weeks of treatment, four patients tested positive for rifampicin-resistant S. aureus. One additional resistant isolate was recovered in week 4. At that point, the trial was stopped. Four cases of acquired rifampicin resistance arose from 34 patients receiving rifampicin as the only agent to which S. aureus was susceptible (patients #1, #2, #4, #5, Figure 1).

An additional

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<th>Isolate</th>
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<td>NA</td>
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Figure 1. Genotyping of S. aureus recovered from tuberculosis patients at the beginning and after 4 weeks of tuberculosis therapy containing rifampicin. DNA was prepared from isolates, PFGE was performed (left-hand panel) and spa repeat profiles were determined by nucleotide sequence analysis (right-hand panel, letters correspond to sequence groups). Numbers correspond to patients from whom rifampicin-resistant S. aureus was recovered. ‘Before’ indicates sample was obtained at the beginning of therapy; ‘After’ indicates sample was obtained after 4 weeks of therapy. Patient characteristics were as follows: #1 (male, 18 years, rifampicin isoniazid pyrazinamide); #2 (male, 59 years, rifampicin isoniazid pyrazinamide); #3 (male, 24 years, rifampicin isoniazid pyrazinamide streptomycin), #4 (female, 65 years, rifampicin isoniazid pyrazinamide ethambutol); #5 (male, 22 years, rifampicin isoniazid pyrazinamide ethambutol para-aminosalicylic acid).
19 cases were also treated with streptomycin. Among these, streptomycin-susceptible *S. aureus* (zone of inhibition > 15 mm with a paper disc containing 10 μg streptomycin) was recovered from 15 patients at the beginning of therapy; rifampicin resistance was not observed with these patients. Four other cases treated with streptomycin had streptomycin-resistant *S. aureus* (zone of inhibition < 12 mm) at initiation of therapy. They were treated with rifampicin, isoniazid, pyrazinamide and streptomycin. In one of these four cases, rifampicin resistance was acquired (patient #3, Figure 1). No rifampicin-resistant case was found among the eight patients treated with levofloxacin-containing regimens.

The five resistant cases included one female and four male patients aged 18 to 65 years (Figure 1, legend). For each case, PFGE band pattern and spa repeat profile of *S. aureus* DNA were identical before and after acquisition of rifampicin resistance (Figure 1). However, band patterns and spa profiles differed among isolates from different patients (Figure 1). These data are consistent with the five cases of rifampicin resistance arising from de novo selection of resistant *S. aureus*.

Of the 200 patients in the untreated control group, 41 were colonized by *S. aureus*; two carried rifampicin-resistant *S. aureus* at the start of the study and were excluded from further sampling. No rifampicin resistance was observed among the 39 remaining cases after 4 weeks of observation. Using Fisher’s exact test, \( P = 0.025 \) for the acquisition of rifampicin resistance when study and control groups were compared, indicating that study size was sufficient for statistical significance.

### Discussion

When nasal carriage of *S. aureus* was eliminated in 92% of the tuberculosis patients, resistance still arose in the remaining 8% (5/58); when rifampicin was the only compound with anti-staphylococcal activity in the treatment regimen, resistance was recovered in 13% (5/38) of the cases. No susceptible colonization was observed after 2 and 4 weeks of therapy. The high frequency of acquired resistance fits with earlier observations in which rifampicin was observed after 2 and 4 weeks of therapy. The high frequency of acquired resistance fits with earlier observations in which rifampicin resistance was readily observed with *S. aureus* infections, and nasal colonization. However, earlier studies did not examine samples at the beginning of therapy and therefore could not distinguish between acquired resistance and resistance present prior to therapy.

Eradication of susceptible bacteria is explained by drug concentrations in plasma and nasal secretions being maintained above MIC and by drug exposure (i.e. plasma AUC/MIC) for susceptible *S. aureus* (MIC ≤ 0.01 mg/L) being high (AUC/MIC > 7000 h, 700 h when corrected for protein binding). The five resistant cases are most readily explained by assuming that resistant subpopulations were present in the initial colonizing population, since no population expansion of susceptible cells was expected and since even extremely high rifampicin concentrations allow growth of mutant subpopulations. If so, bacterial load in at least some of the colonized patients must have reached 10³ cells or the spontaneous mutation frequency in patients must have been higher than 1 in 10⁸, the value observed in *in vitro*.

An alternative that cannot be ruled out by the present work is rifampicin-induced mutation during treatment. Regardless of how resistant mutant subpopulations were generated, they can be selectively enriched to where they dominate nasal populations at the same time that susceptible bacteria are eradicated. Concurrent eradication of susceptible bacteria and acquisition of resistance are predicted by the mutant selection window hypothesis.

*In vitro* data suggest that acquired resistance will be observed more readily with the *S. aureus*–rifampicin combination than with many other combinations, in part because rifampicin resistance occurs in an all-or-none, rather than a stepwise, fashion. Nevertheless, the distinction between eradicating susceptible bacteria and restricting resistance shown with this model system is likely to be general, since pre-existing mutant subpopulations are not expected to be eliminated by antimicrobial concentrations used to kill susceptible cells. For example, with *Streptococcus pneumoniae* the concentration of levofloxacin must be eight times higher to kill mutants than wild-type cells, and examples of acquired resistance have been reported. However, determining whether eradication of susceptible pathogens and acquisition of resistance occur concurrently for a particular combination may require examining large numbers of patients when mutants tend to be eliminated by the combination of host defence and antimicrobial treatment.

The present work is limited in three ways. First, we were unable to identify tuberculosis patients to serve as untreated controls, since rifampicin is a first-line anti-tuberculosis agent and since most cases of tuberculosis in China are treated with rifampicin. Second, we could not treat tuberculosis patients with very low rifampicin concentrations to determine whether even more resistant mutants would be selected below MIC than above it. Third, rifampicin-resistance mutations reduce the susceptibility of *S. aureus* so much that rifampicin concentrations could not be attained that would be high enough to prevent the amplification of resistant mutants and define an upper boundary of the selection window. Studies are in progress to address these limitations.

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### Transparency declarations

None to declare.

### References

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