Detection and Susceptibility Testing of Hypermutable *Pseudomonas aeruginosa* Strains with the Etest and Disk Diffusion

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Resistant development in *Pseudomonas aeruginosa* from chronically colonized cystic fibrosis (CF) patients has been linked to the presence of a high proportion of mismatch repair-deficient hypermutable strains. The detection of hypermutable strains by microbiology laboratories may be useful for establishing adequate antimicrobial therapies. In this work, we find that the Etest and disk diffusion can be used as simple methods for the detection and susceptibility testing of hypermutable *P. aeruginosa* isolates. Strain PAO1 and its hypermutable derivative strain PAOΔmutS were used to standardize the procedure, which was tested with 35 *P. aeruginosa* isolates from 21 CF patients. Mutation frequencies were estimated by standard methods, and 29% of the isolates were found to be hypermutable. MICs and inhibition zone diameters were determined for cefazidime, imipenem, meropenem, ciprofloxacin, and tobramycin by using Etest strips and conventional disks, respectively. The presence (or absence) of resistant mutant subpopulations, as well as their relative numbers and the highest MICs for them (or smallest inhibition zone diameters), was recorded. The presence of resistant mutant subpopulations within the inhibition zones of three or more antibiotics clearly identified the strains as hypermutable (they were present in 10 of 10 hypermutable strains and 0 of 25 nonhypermutable strains) with both methods. Additionally, these methods allowed us to differentiate between dual effects of hypermutation in antibiotic resistance, namely, that (i) hypermutable isolates were substantially more resistant than nonhypermutable isolates and that (ii) the resistance of hypermutable isolates was dramatically increased by the presence of resistant mutant subpopulations. This differentiation may be relevant for the design of adequate treatments, since the second effect, in contrast to the first, may be overcome by antibiotic combinations.

Chronic endobronchial infection by *Pseudomonas aeruginosa* is the major cause of the morbidity and mortality of cystic fibrosis (CF) patients (5, 7, 13). After years of intensive antibiotic chemotherapy used to control the negative outcome of chronic colonization, subsequent development of resistance to most antibiotics frequently occurs. Rates of resistance to *P. aeruginosa* strains isolated from CF patients are in fact substantially higher than rates of resistance to strains found in other settings, including those from patients in intensive care units (8, 12, 17). A link between the high antibiotic resistance rates in CF patients and the presence of a high proportion of hypermutable (or mutator) *P. aeruginosa* strains was found previously (17). Up to 37% of the CF patients were colonized by hypermutable strains in the previous study (17). In the last 2 years, the role of hypermutation in antibiotic resistance development has begun to be acknowledged as a potentially concerning problem (2, 4, 6). Hypermutable strains are those that have an increased spontaneous mutation rate due to defects in genes involved in DNA repair or in error avoidance systems (10, 15). In *P. aeruginosa* isolates from CF patients, as well as in other natural bacterial populations (11, 14), the most frequently involved system is the mismatch repair system, and mutS is the most frequently affected gene (18). Detection of hypermutable *P. aeruginosa* strains from CF patients by clinical microbiology laboratories may be very useful for establishing adequate antimicrobial therapies to avoid resistance development. Recently it has been suggested that hypermutable *P. aeruginosa* strains, because of the immediate selection of resistant mutants, should be considered resistant to most, if not all, antibiotics in monotherapy and that combinations should be used in order to avoid resistance development (A. Oliver, B. R. Levin, C. Juan, F. Baquero, and J. Blazquez, submitted for publication). Mutation frequency estimations require the use of time-consuming procedures that would be difficult to adapt to clinical microbiology laboratory routines. Therefore, simpler techniques are necessary for the routine detection of hypermutable strains. Of the common methods used for routine susceptibility testing in clinical microbiology laboratories, disk diffusion, but not commercial microdilution, is considered appropriate for the testing of *P. aeruginosa* isolates from CF patients (3). Additionally, the Etest has previously been recognized as a useful method for the susceptibility testing of *P. aeruginosa* isolates from CF patients. Compared to disk diffusion, it has the advantage of providing precise MICs but the disadvantage of high cost (3). In this work, we provide evidence that both the Etest and disk diffusion can be used as simple methods for the detection and susceptibility testing of hypermutable strains. Additionally, these methods allowed us to differentiate between dual effects of hypermutation in antibiotic resistance, which may be helpful for the design of adequate treatments.

**MATERIALS AND METHODS**

*P. aeruginosa* strains. Thirty-five *P. aeruginosa* isolates representing different colony morphotypes (with respect to mucoidy, colony morphology or size, and pigment production) were collected from March to October 2003 from a sputum sample from each of 21 CF patients (mean age, 18.8 years; range, 2 to 41 years).
Identification of the isolates was performed with the API 20NE system (bio-Mérieux, Marcy l’Etoile, France) and conventional tests (16). P. aeruginosa reference strain PAO1 (9, 23) and its hypermutable isogenic derivative PAOΔmutS were used as controls and for initial standardization of the procedure (see below). PAOΔmutS contains a deletion of a 1.5-kb NotI-NotI DNA fragment within the PAO1 mutS gene that has been replaced by a kanamycin resistance cassette (Oliver et al., submitted).

### Susceptibility testing and mutant subpopulation quantification.

MICS and inhibition zone diameters were determined for ceftazidime, imipenem, meropenem, ciprofloxacin, and tobramycin in Mueller-Hinton (MH) agar plates, by using Etest strips (AB Biodisk, Solna, Sweden) and conventional disks (Oxoid). A 0.5 or 1 McFarland standard suspension was used for inoculum standardization of regular or mucoid isolates, respectively. MICs were read after 24 h (36 h for slow-growing strains) of incubation at 37°C. The presence (or absence) of resistant mutant subpopulations within the inhibition zones, as well as their relative numbers (<10, 10 to 100, and >100 mutants) and the highest MICs for them (or smallest inhibition zone diameters) was recorded after an extra 12 h of incubation. Five independent replicates of PAO1 and PAOΔmutS were used for the initial standardization of the procedure. Based on results with these control strains, only mutants for which the increase in MIC was at least a twofold dilution (compared to that for the dominant population) or whose inhibition zone diameters were reduced by ≥5 mm were considered for all antibiotics, with the exception of tobramycin, for which the presence of any mutant colony was considered. In the case of ceftazidime, since the number of PAOΔmutS mutants was always >100 (see Results) and sporadic mutant colonies (i.e., those semi-quantitatively numbering <10) were observed in some of the replicates with PAO1, only values of >10 were considered. Three mutant colonies from each of the five replicates for each antibiotic were restested after growing in antibiotic-free medium to confirm the stability of the resistance phenotype and to exclude the possibility that colonies growing within the inhibition zones were a consequence of antibiotic inactivation during the incubation period.

### Estimates of mutation frequencies and complementation studies.

Mutation frequencies were estimated as previously described (17, 18). Briefly, triplicate independent 10-ml MH broth overnight cultures of the P. aeruginosa isolates were collected and resuspended in 1 ml of saline solution. Serial 10-fold dilutions were plated in MH agar with and without 300 μg of rifampin/ml, and after 36 h of incubation (48 h for slow-growing strains), colonies were counted and the mean fraction of mutants was estimated. As previously defined, strains were considered hypermutable when the mutation frequency was at least 20-fold higher than that obtained for the control strain PAO1 (17). To explore the genetic basis for the mutator phenotype of the hypermutable strains, complementation studies using the cloned PAO1 wild-type mutS gene were performed. The pUCPMS plasmid harboring the PAO1 mutS gene (Oliver et al., submitted) was electroporated into the different hypermutable P. aeruginosa isolates as previously described (22). Transformants were selected in 50 or 250 μg of gentamicin/ml on Luria-Bertani agar plates. Complementation of the increased mutation frequencies was studied for three independent transformants.

### RESULTS

As described in Materials and Methods, P. aeruginosa strains PAO1 and its isogenic hypermutable derivative PAOΔmutS were used for the initial validation of the procedure. The median MICs for and inhibition zone diameters of both strains were almost identical for the five agents tested (Table 1). Whereas no resistant mutant subpopulations were observed for PAO1 with any of the five antipseudomonal agents tested, in all five different replicates with PAOΔmutS, resistant mutants were observed within the inhibition zones for all the antibiotics, both with the Etest (Fig. 1) and with disk diffusion (Fig. 2). The numbers of mutants and the highest antibiotic concentrations at which mutants grew (or smallest inhibition zone diameters) were relatively stable for the different replicates (i.e., the numbers of mutants varied within 1 order of magnitude, and the highest concentrations were within 1 two-fold dilution of each other) for the five antipseudomonal agents tested. The patterns of resistant mutant subpopulations of PAOΔmutS differed considerably for the five antibiotics tested. The number of mutants and the highest MICs at which the mutants grew were greatest for ceftazidime (Table 1; Fig. 1). With imipenem, the number of mutants was lower but the MICs were higher than those of meropenem (Table 1; Fig. 1). Finally, the number of mutants was relatively high with ciprofloxacin and low with tobramycin, despite the fact that the strain remained quite susceptible (Fig. 1). If the MICs for and inhibition zone diameters of the resistant mutant subpopulations are considered (as they should be) for the interpretation of the susceptibility testing results instead of those of strains from the general population, PAOΔmutS would be considered resistant to ceftazidime and imipenem (intermediate with disk diffusion), whereas the MICs of meropenem, ciprofloxacin, and tobramycin would remain just 1 or 2 dilutions below the NCCLS breakpoints (Table 1).

Based on the results obtained for PAO1 and PAOΔmutS, both the Etest strips and conventional disks seemed to be adequate tools for detecting P. aeruginosa hypermutable strains through the observation of the presence (or absence) of resistant mutant subpopulations. These methods also provide an advantage for the susceptibility testing of hypermutable strains, since information on the MICs (or the inhibition zone diameters when measured with disks) for the dominant and resistant mutants populations cannot be directly obtained with reference agar or broth dilution techniques.

To explore the potential utility of the Etest and disk diffusion for the detection and susceptibility testing of natural hy-

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**TABLE 1.** MICs for and inhibition zone diameters of strains PAO1 and PAOΔmutS and their respective resistant mutant subpopulations.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>PAO1* Etest MIC (μg/ml)</th>
<th>Disk diffusion diam (mm)</th>
<th>PAOΔmutS* Etest MIC (μg/ml)</th>
<th>Disk diffusion diam (mm)</th>
<th>NCCLS breakpoints*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftazidime</td>
<td>1</td>
<td>25.6</td>
<td>48 (+ + +)</td>
<td>25.4</td>
<td>≤8, ≥32</td>
</tr>
<tr>
<td>Imipenem</td>
<td>1.5</td>
<td>21.9</td>
<td>12 (+ to + +)</td>
<td>22.8</td>
<td>≤4, ≥16</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.38</td>
<td>29.4</td>
<td>3 (+ +)</td>
<td>27.2</td>
<td>≤4, ≥16</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.064</td>
<td>32.4</td>
<td>0.064 ( + + +)</td>
<td>34.0</td>
<td>≤1, ≥4</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>1.5</td>
<td>20.0</td>
<td>3 (+)</td>
<td>18.0</td>
<td>≤4, ≥16</td>
</tr>
</tbody>
</table>

* Results shown are the median values of five independent determinations. MICs and inhibition zone diameters were measured at 24 h for the general population and at 36 h for the resistant mutant subpopulation. The highest MICs or the smallest inhibition zone diameters of resistant mutant subpopulations are listed.

b Relative number of resistant mutant colonies (see Materials and Methods). +, < 10 mutants; ++ 10 to 100 mutants; +++ > 100 mutants.

c N, nonsusceptibility breakpoint; R, resistance breakpoint.
FIG. 1. Etest antibiograms of PAO1 and PAO1 mutS with ceftazidime, imipenem, meropenem, ciprofloxacin, and tobramycin.
permutable P. aeruginosa strains obtained from CF patients, 35 isolates recovered from 21 patients were studied. Of the 35 isolates, 10 (29%) were found to be hypermutable. The mean mutation frequency of hypermutable isolates was $3.0 \times 10^{-6}$ (range, $5.0 \times 10^{-7}$ to $8.0 \times 10^{-6}$), and that of nonhypermutable isolates was $2.0 \times 10^{-8}$ (range, $2.6 \times 10^{-9}$ to $6.6 \times 10^{-8}$). Hypermutable strains were found in 5 (24%) of the 21 CF patients. The percentage of hypermutable isolates was slightly higher (29% versus 20%) than that observed in a previous study (17), but the number of patients studied was slightly lower (24% versus 37%), probably due to the long-term follow-up (5 years) of CF patients in that study. Hypermutable strains from two of the five patients were found to be defective in mutS, as demonstrated with complementation studies; this is a proportion similar to that previously found (18).

As can be observed in Table 2, resistant mutant subpopulations were observed (i) with all hypermutable isolates with ceftazidime, imipenem, and meropenem; (ii) with all but 1 and 2 isolates as determined by the Etest and disk diffusion, respectively, with ciprofloxacin; and (iii) with 6 and 7 out of 10 isolates as determined by the Etest and disk diffusion, respectively, with tobramycin. In contrast, resistant mutant subpopulations were seldom observed in the nonhypermutable isolates for any of the antibiotics (Table 2). The observation of resistant mutant subpopulations with individual antibiotics may be suggestive only of the presence of a hypermutable strain due to the occasional presence of resistant mutant subpopulations in nonhypermutable strains. However, the presence of mutant subpopulations for three or more antibiotics clearly identified the strains as hypermutable (Table 3). As shown in Table 3, 10 out of 10 hypermutable and 0 out of 25 nonhypermutable isolates met this criterion both with the Etest and with disk

![Image](FIG. 2. Disk diffusion antibiograms of PAOΔmutS and PAO1 with ceftazidime (CAZ), imipenem (IPM), meropenem (MEM), ciprofloxacin (CIP), and tobramycin (TOB).)

**TABLE 2. Numbers of nonhypermutable or hypermutable isolates for which resistant mutant subpopulations were observed for each antibiotic**

| Antibiotic     | No. of isolates with resistant mutants subpopulations observed/no. tested by
g 
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Etest</td>
</tr>
<tr>
<td></td>
<td>Nonhypermutable isolates ($n = 25$)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>3/25</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2/25</td>
</tr>
<tr>
<td>Meropenem</td>
<td>3/25</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>2/25</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>1/24</td>
</tr>
</tbody>
</table>

* Isolates for which the MIC was higher than the highest concentration on the Etest strips were excluded (see Materials and Methods).
The number of hypermutable isolates for which we documented subpopulations resistant to four of the five antibiotics decreased (Table 3) for two main reasons: (i) no resistant mutant subpopulations could possibly be detected when the MIC of a given antibiotic for the dominant population was higher than the highest concentration in the Etest strips (or when no inhibition zone was documented with disks) and (ii) tobramycin-resistant mutant subpopulations were not observed in an important proportion of the isolates.

As can be observed in Table 4, hypermutable isolates were substantially more resistant to the antibiotics than were non-hypermutable isolates (their percentages or resistance and/or the mean MICs for them were higher), even without considering the resistant mutant subpopulations. Except with tobramycin, the mean MICs for hypermutable strains were from 4 to 6.5 times higher than those for nonhypermutable strains. The percentages of hypermutable strains that were resistant to cefazidime, imipenem, and ciprofloxacin were substantially higher than those of nonhypermutable strains. All strains were susceptible to meropenem (although the mean MICs of this drug for hypermutable strains was fourfold higher than those for nonhypermutable strains), and no significant differences in the MICs of tobramycin were found. The inclusion of the resistant mutant subpopulations for the interpretation of the results had no effect on the resistance rates of or mean MICs for nonhypermutable isolates, due to their minimal presence in these isolates (Table 2). In contrast, the inclusion of resistant mutant subpopulations in hypermutable isolates increased dramatically both the resistance percentages and the mean MICs of all antibiotics (Table 4). The effect of the inclusion of resistant mutant subpopulations on the interpretation of the results was once again smallest for tobramycin. As for disk diffusion, susceptibility test results for nonhypermutable and hypermutable strains, as well as the percentages of resistance of mutant subpopulations, were quite similar to those obtained with the Etest, although the agreement was not 100% (Table 4).

Figure 3 shows the distributions of the MICs for nonhypermutable and hypermutable isolates with and without consideration of the resistant mutant subpopulations. In these distributions, as anticipated from the susceptibility data of Table 4, a dual effect of hypermutation in antibiotic resistance can clearly be observed: (i) hypermutable isolates are substantially more resistant than nonhypermutable isolates and (ii) the resistance of hypermutable isolates is dramatically increased due to the presence of resistant mutant subpopulations. Choosing, for instance, the most active antibiotic, meropenem, it can be observed in Fig. 3 that the MICs for none (0%) of the nonhypermutable strains are greater than 0.5 μg/ml but that they are greater for 40% of the hypermutable strains and 90% of strains when resistant mutant subpopulations are considered. As will be discussed, differentiating between these two reasons for the increased resistance of hypermutable isolates may have important consequences for the design of adequate therapeutic options to treat CF patients.

### DISCUSSION

Hypermutable strains have been described to occur in natural populations of *Escherichia coli*, *Salmonella* spp., *P. aeruginosa*, *Helicobacter* spp., *Neisseria meningitidis*, and *Staphylococcus aureus* (1, 11, 14, 17, 19, 20). The presence of hypermutable strains has been linked to resistance to host immunological defenses, as in mutation-dependent phase variation in *N. meningitidis* (20, 21) or in resistance to antibiotics in *P. aeruginosa* isolates from chronically infected CF patients, in whom the frequency of hypermutable variants is by far the highest ever

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Nonhypermutable isolates (n = 25)a</th>
<th>Hypermutable isolates (n = 10)b</th>
<th>Hypermutable isolates + subpopulations (n = 10)c</th>
<th>NCCLS breakpointsd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% S by Etest/dd</td>
<td>Mean Etest/MIC (μg/ml)</td>
<td>Mean dd diam (mm)</td>
<td>% S by Etest/dd</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>96/96</td>
<td>1.3</td>
<td>28.4</td>
<td>60/70</td>
</tr>
<tr>
<td>Imipenem</td>
<td>100/100</td>
<td>1.1</td>
<td>22.1</td>
<td>50/60</td>
</tr>
<tr>
<td>Meropenem</td>
<td>100/100</td>
<td>0.1</td>
<td>36.5</td>
<td>100/100</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>92/92</td>
<td>0.3</td>
<td>27.4</td>
<td>20/20</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>84/72</td>
<td>2.4</td>
<td>15.9</td>
<td>90/70</td>
</tr>
</tbody>
</table>

a S, percentage of strains susceptible according to NCCLS breakpoints by the Etest and by disk diffusion (dd). Means are geometric means.

b Resistant mutant subpopulations were considered for these values.

c N, nonsusceptibility breakpoint; R, resistance breakpoint.
found in natural populations (17). Recently, a high frequency of mutator variants of \textit{S. aureus} from CF patients has also been found to be linked with resistance to macrolides (19).

Recently, it was proposed that hypermutable \textit{P. aeruginosa} strains should be considered resistant to most, if not all, antibiotics in monotherapy and that combinations should be used in order to avoid resistance development (Oliver et al., submitted). This consideration was based on the observation that minimal bactericidal concentrations and MICs (after 36 h of incubation) of 11 antipseudomonal agents for strain PAO\textit{ΔmutS}, determined by broth microdilution, were substantially higher than those for its parent strain, PAO1, with most antibiotics reaching the breakpoint concentrations for nonsusceptibility. It was also found in this work that the increased resistance was due to the ascent to dominance of resistant mutants during the incubation period and that the susceptibility could be restored with combinations of two antipseudomonal agents (as found with synergy testing).

As found in this work, with just a slight adaptation of the conventional Etest or disk diffusion method used for routine susceptibility testing of \textit{P. aeruginosa} isolates from CF patients, information on whether a strain is hypermutable or nonhypermutable can easily be obtained. The detection of hypermutable strains is based on the observation of the presence of resistant mutant subpopulations within the inhibition zones. As was found, the presence of mutant subpopulations that are resistant to three or more antibiotics clearly identifies a strain as

FIG. 3. Distributions of MICs of ceftazidime, imipenem, meropenem, ciprofloxacin, and tobramycin. White bars, percentages of non-hypermutable isolates; black bars, percentages of hypermutable isolates considering resistant mutant subpopulations; gray bars, percentages of hypermutable isolates without considering mutant subpopulations.
hypermutable. The potential benefits of the detection of hy-
permutable strains from CF patients are multiple. First, from a
basic point of view, the information collected from large
collections of sequential isolates from diverse geographical ori-
gins would certainly be of great value for gaining insights into
the role of hypermutable P. aeruginosa populations in chronic
infections as well as into the factors that determine their pres-
ence and their consequences in the physiopathology of CF. In
addition to this indirect benefit, the detection of hypermutable
strains, and specifically the appropriate approach to their sus-
ceptibility testing, may be a step forward for clinical microbi-
ology practice in the complex field of CF lung infection and, as
discussed below, may have a positive impact on the clinical
management of patients.

In addition to the detection of hypermutable strains, the
Ettest offers the possibility of determining the MICs for both
the dominant and resistant mutant populations. Similarly, al-
though not conferring precise MIC information, disk diffusion
also offers the possibility of determining the resistance levels
(in terms of inhibition zone diameters) of both populations.
This goal could hardly be achieved with reference agar or broth
dilution techniques. The estimation of these two different re-
sistance endpoints is necessary to characterize and differenti-
ate between the dual effects of hypermutation in antibiotic
resistance, namely, that (i) hypermutable isolates are substi-
tially more resistant than nonhypermutable isolates, probably
reflecting their higher efficiency in developing resistance dur-
ing previous antibiotic exposures in CF patients’ lungs, and
that (ii) the resistance of hypermutable isolates is dramatically
increased due to the presence of resistant mutant subpopula-
tions, which are produced during the regular growth of the
strains (in the absence of antibiotics) due to their high sponta-
aneous-mutation rate. Differentiating between these two rea-
sons for the increased resistance of hypermutable isolates may
have important consequences for the design of adequate ther-
apetic options for treating CF patients, because the second
effect, in contrast to the first, may be overcome by the use of
antibiotic combinations (Oliver et al., submitted). In fact, syn-
ergy testing with any of the three β-lactams (ceftazidime, imi-
penem, and meropenem) plus either tobramycin or ciprofloxa-
cin eliminated the presence of resistant mutant subpopula-
tions of strain PAOΔmutS and therefore restored its susceptibility to
PAO1 levels (data not shown). From the perspective of clinical
microbiology practice, the proposed approach could add valu-
able information to the standard “susceptible” or “resistant”
terpretation of the P. aeruginosa antibigrams of CF patients.

The presence of resistant mutant subpopulations for most an-
tibiotics in hypermutable strains raises the question of how
these results should be interpreted by the clinical microbiolo-
 gist. If the standard criteria are used and the resistant mutant
subpopulations are considered (as they should be) for the
reading of MICs or inhibition zone diameters, we would face
the problem of hypermutable strains appearing to be resistant
to most antibiotics by definition, not because they had acquired
resistance during previous antibiotic exposures. A good exa-

ple of this point is the case of the otherwise susceptible hyper-
mutable derivative of PAO1, PAOΔmutS. As shown in Table 1
and Fig. 1 and 2, this strain would be considered resistant by
definition, at least to ceftazidime and imipenem. The problem
with using the standard criteria, and considering a hypermut-
able strain resistant whether it is really resistant or resistant
only as a direct consequence of its high spontaneous-mutation
rate, is that most hypermutable strains would be reported as
resistant to most (in some cases, all) antibiotics (Table 4). In
this sense, differentiating between the aforementioned dual
effects of hypermutation on antibiotic resistance could cer-
tainly be very helpful for the design of potentially useful anti-
biotic combination regimens.

In conclusion, a reasonable and practical approach for clinical
microbiology practice in those laboratories which follow a
specific routine for chronic lung infection in CF patients and
have direct personal contact with the clinicians who treat those
patients is proposed. (i) Read the MICs (or the inhibition zone
diameters) for the dominant bacterial population after 24 or
36 h (for normally growing or slow-growing strains, respect-
ively) of incubation, and apply the defined breakpoints (i.e.,
NCCLS breakpoints or those used in a given country) for the
susceptibility categories (sensitive, intermediate, or resistant).
(ii) Examine the presence of resistant mutant subpopulations
after an extra 12 h of incubation. (iii) Apply the defined criteria
(mutant subpopulations that are resistant to three or more
antibiotics) to identify the strain as hypermutable, read the
MICs for the resistant mutant subpopulation (or the inhibition
zone diameters), and apply the defined breakpoints for sus-
ceptibility categories. Report those antibiotics whose MICs for
the dominant population are in the range indicating suscepti-
bility but whose MICs for resistant mutant subpopulations are
above the breakpoint as “resistant, but potentially usable for
combined therapy.”

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