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Cefamandole-Susceptible Strains of *Legionella pneumophila* Serogroup 1: Implications for Diagnosis and Utility as an Epidemiological Marker

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The standard selective *Legionella* medium that contains cefamandole failed to grow *Legionella pneumophila* serogroup 1, subtype Bellingham, from a sputum sample from a patient with nosocomial Legionnaires' disease; the isolate did grow on a similar selective medium that substitutes vancomycin for cefamandole. Two Bellingham isolates from this patient's hospital environment also failed to grow when tested on the cefamandole medium. We tested 106 additional *L. pneumophila* serogroup 1 isolates that belonged to nine different monoclonal antibody subtypes and demonstrated that susceptibility to cefamandole was rare (10%) and limited to the Bellingham subtype. The diagnosis of Legionnaires' disease may be missed unless the culture protocol includes both a nonselective medium and a selective medium that does not contain cefamandole. In vitro susceptibility to cefamandole also provided an epidemiologic marker that linked a water source for a patient to nosocomial Legionnaires' disease.

Isolation of *Legionella pneumophila* from sputum samples cultured onto antimicrobial agent-containing media is the definitive means of diagnosing Legionnaires' disease. Commercial *Legionella* selective media used for clinical specimens contain the antimicrobial agents polymyxin B, anisomycin, and vancomycin or cefamandole to inhibit competing microorganisms.

An unusual incident prompted evaluation of our selective medium containing cefamandole. *L. pneumophila* serogroup 1 was isolated from a patient's sputum on the nonselective *Legionella* medium and the *Legionella* selective medium that contained vancomycin; however, this strain failed to grow on the selective medium that contained cefamandole.

The patient had received blood transfusions as an outpatient at the Pittsburgh Veterans Affairs Medical Center (VAMC) and radiation therapy at a local community hospital prior to being admitted to the VAMC with pneumonia. Environmental samples from both hospitals were cultured simultaneously for *Legionella* spp. to determine whether the reservoir was one of these two institutions. The patient's *L. pneumophila* isolate and two *L. pneumophila* serogroup 1 isolates recovered from the community hospital's water distribution system were subsequently discovered to be the same monoclonal antibody subtype (Bellingham). Only the isolates that were recovered from the community hospital failed to grow on the cefamandole media.

We therefore conducted a study to determine (i) the frequency of cefamandole susceptibility among *L. pneumophila* serogroup 1 isolates and (ii) whether cefamandole susceptibility was confined to the Bellingham subtype.

MATERIALS AND METHODS

Strains. We tested 109 *L. pneumophila* serogroup 1 strains (39 patient and 70 environmental strains) from 26 institutions in five states. The monoclonal antibody subtype of each strain had been determined previously by the indirect fluorescent-antibody method (3). The breakdown of the strains by subtype was as follows: Bellingham, 54; Oxford, 13; Allentown, 10; Philadelphia, 10; OLDA, 9; Benidorm, 5; Knoxville, 4; France, 2; and Heysham, 2. The 54 Bellingham strains tested included the strain recovered from the patient's sputum and the 2 strains recovered from the community hospital.

Cefamandole media. A standard commercial selective buffered charcoal-yeast extract (BCYE) agar medium that contains 80 U of polymyxin B, 80 μg of anisomycin, and 4.0 μg of cefamandole (2) per ml was purchased from Remel, Lenexa, Kan. (Remel PAC). A nonselective BCYE medium and a modified selective BCYE plate medium that contains the above antimicrobial agents (except the concentration of anisomycin is reduced to 40 μg/ml) were prepared at the VAMC as described previously (9). The cefamandole MIC for selected strains was determined by using prepared BCYE agar media that contained 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, and 0.063 μg of cefamandole per ml.

Inoculum preparation and MIC procedures. The 109 *L. pneumophila* serogroup 1 strains were transferred from −70°C storage onto nonselective BCYE agar plates, and the plates were incubated at 35 to 37°C in air for 48 h. Growth of each strain from the 48-h subculture was suspended in 2.0 ml of sterile deionized water at a turbidity that matched a 0.5 McFarland standard. A Pepper Laboratory Inocula Replicator (Craft Machine, Inc., Chester, Pa.) was used to inoculate all test media. Sixteen strains were inoculated simultaneously onto the three media that were incubated in air at 35 to 37°C and observed for growth at 48 h. The 109 strains were tested over several days; strains that did not grow on the test media were retested. Strains that demonstrated no growth on the cefamandole media were tested further by inoculating them with the replicator onto the BCYE media containing 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, and 0.063 μg of cefamandole per ml. *L. pneumophila* serogroup 1, subtype Philadelphia (ATCC 33152), was the positive growth control

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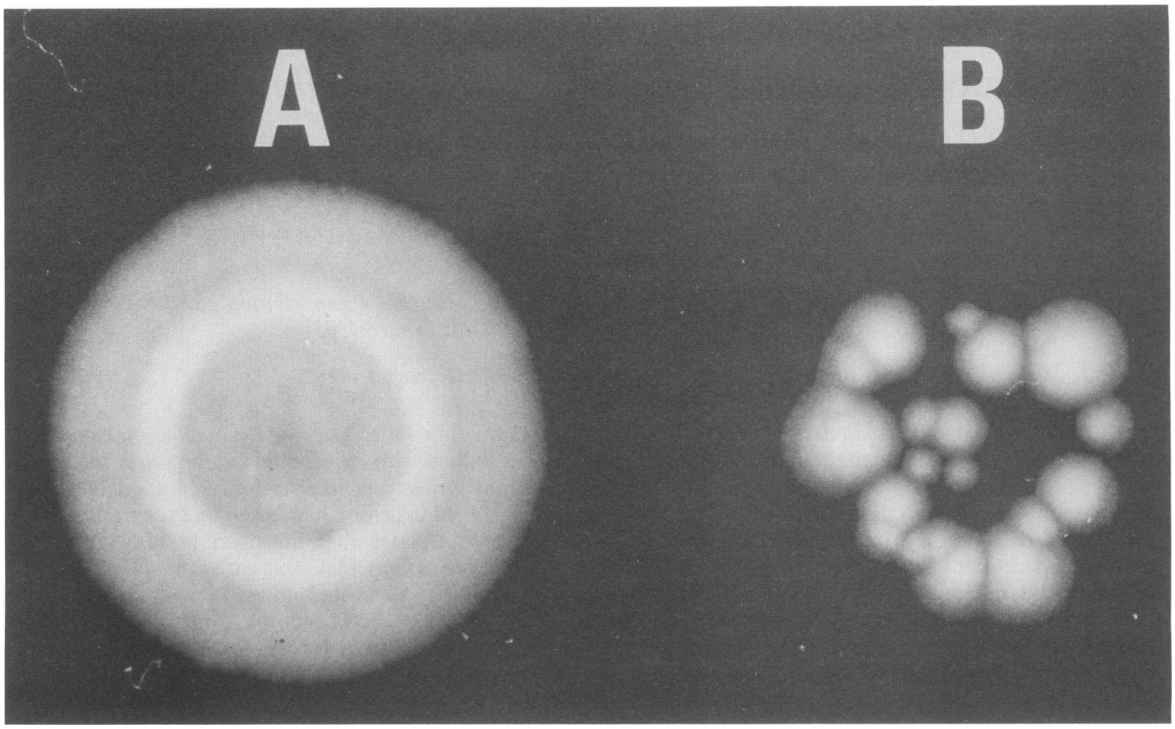

FIG. 1. (A) *L. pneumophila* serogroup 1 strains resistant to 4.0 μg of cefamandole per ml grew on the cefamandole-containing BCYE media as wheels of total or confluent growth at the inoculation site, as demonstrated here. (B) Strain VAMC 879 repeatedly produced residual colonies at the inoculation site estimated to be <0.1% of the inoculum. Retesting of these residual colonies on media containing 4.0 μg of cefamandole per ml resulted in total or confluent growth as pictured in panel A.

for all media, and each daily run included the positive control.

REA. Eleven Bellingham strains (five cefamandole susceptible and six cefamandole resistant) were selected for restriction endonuclease analysis (REA). Purified whole-cell DNA was analyzed with two restriction endonucleases, *Eco*RI and *Hin*dIII, as described previously (7). Strains were each tested at least twice prior to photography under UV light with a Polaroid camera. Lambda DNA restriction fragments were included as molecular weight standards. REA patterns were visually reviewed.

β-Lactamase test. β-Lactamase activity was determined by the chromogenic cephalosporin test (4) for all strains that did not grow on media that contained 4.0 μg of cefamandole per ml. The six cefamandole-resistant strains analyzed by REA were also tested for β-lactamase activity. *L. pneumophila* serogroup 1, subtype Philadelphia (ATCC 33152), and *Haemophilus influenzae* (VAMC) were used as positive controls, and *Neisseria gonorrhoeae* (CDC 98) was used as the negative control.

RESULTS

Of 109 strains of *L. pneumophila* serogroup 1 tested, 11 (10%) failed to grow on media that contained 4.0 μg of cefamandole per ml. All 11 strains belonged to the monoclonal subtype Bellingham. The remaining 43 Bellingham strains and all 55 strains of the other subtypes tested grew well on the media containing 4.0 μg of cefamandole per ml, as did the control strain.

The MICs of cefamandole for the 11 Bellingham strains that did not grow on the 4.0-μg/ml cefamandole media ranged from 0.125 to 0.5 μg/ml. The cefamandole MIC for the control strain was >4.0 μg/ml. One strain (VAMC 879) continuously demonstrated residual colonies when retested on all media that contained 0.5 to 4.0 μg of cefamandole per ml (Fig. 1); the MIC of cefamandole for these residual colonies (designated VAMC 960) was always >4.0 μg/ml.

β-Lactamase as determined with the chromogenic cephalosporin test was negative for 10 of 11 of the Bellingham cefamandole-susceptible strains; VAMC 879 was weakly positive for β-lactamase, and its residual colonies (VAMC 960) were always strongly positive for β-lactamase. Thus, susceptibility to 4.0 μg of cefamandole per ml was attributable to the lack of β-lactamase activity, a lack which is unusual for *L. pneumophila* (11). However, strain VAMC 879 did not appear to be totally susceptible to cefamandole (residual colonies), which accounted for the weak β-lactamase production observed.

REA analysis for the five cefamandole-susceptible and six cefamandole-resistant Bellingham strains demonstrated seven different REA patterns with both *Eco*RI and *Hin*dIII enzymes (Fig. 2). The cefamandole-susceptible strain from a patient and the two cefamandole-susceptible environmental strains associated with this patient had the same REA pattern (lanes 2 to 4). Five cefamandole-resistant strains demonstrated four different REA patterns (lanes 7 to 11). One cefamandole-susceptible strain was different from all other strains (lane 1). VAMC 879 and its cefamandole-resistant daughter strain VAMC 960 had the same REA pattern (lanes 5 and 6). Overall, the 11 selected Bellingham strains demonstrated seven different REA patterns (Fig. 2).

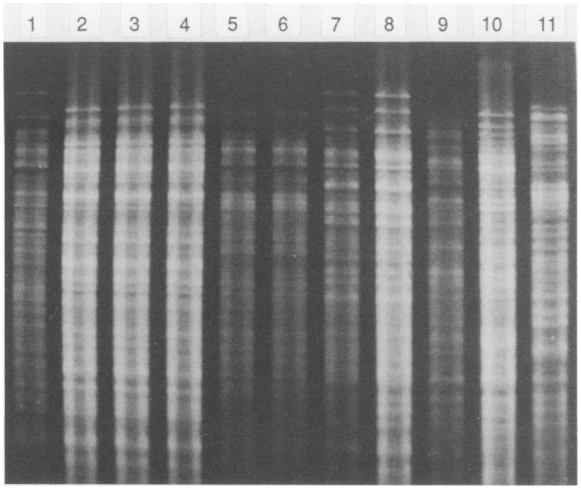

FIG. 2. Restriction endonuclease analysis patterns for five cefamandole-susceptible and six cefamandole-resistant *L. pneumophila* serogroup 1, subtype Bellingham, strains after digestion of whole-cell DNA with *Hin*dIII. Lanes: 1, cefamandole-susceptible environmental strain (VAMC 515); 2, cefamandole-susceptible outpatient strain (VAMC 910); 3 and 4, cefamandole-susceptible environmental strains (VAMC 914 and 916) linked to patient strain VAMC 910 (lane 2); 5, cefamandole-susceptible environmental strain (VAMC 879); 6, cefamandole-resistant residual strain (VAMC 960); 7 and 8, cefamandole-resistant environmental strains (VAMC 871 and VAMC 445); 9, cefamandole-resistant environmental strain (VAMC 707); 10, cefamandole-resistant environmental strain (VAMC 453); 11, cefamandole-resistant patient strain (VAMC 510).

DISCUSSION

The frequency of *L. pneumophila* susceptibility to cefamandole (4.0 μg/ml) was reported to be only 2% (1 of 52 isolates) in one study (2); we found that 10% (11 of 109) of strains were susceptible in our study. The susceptible strains were from three geographic areas in two states. Our patient strain and two strains recovered from a community hospital environment where the patient received radiation therapy failed to grow on the BCYE media containing cefamandole. Given this unusual similarity of the clinical and environmental strains, an epidemiological link was postulated. The link was further strengthened when the strains were shown to be the same monoclonal antibody subtype (Bellingham) and demonstrated an identical restriction endonuclease pattern (Fig. 2, lanes 2 to 4).

Environmental strains of *L. pneumophila* serogroup 1, subtype Bellingham, had been recovered previously from the VAMC; however, these strains were resistant to cefamandole. Further analysis by REA showed that the pattern for two VAMC environmental strains (Fig. 2, lanes 7 and 8) was different from that of the cefamandole-susceptible strain from the patient (lane 2). Thus, the VAMC could be eliminated as a potential source of Legionnaires' disease for this patient on the basis of susceptibility to cefamandole.

The possibility that cefamandole-susceptible strains would have a unique REA pattern compared with resistant strains was tested by chromosomal DNA analysis of 11 selective Bellingham strains. The five cefamandole-susceptible Bellingham strains did not demonstrate a unique pattern that could differentiate them from the six cefamandole-resistant strains by REA (Fig. 2). However, REA may not demonstrate small differences in phenotypically similar *L. pneumophila* strains (8).

We conclude that cefamandole susceptibility among *L. pneumophila* serogroup 1 strains is not widespread on the basis of the following: (i) only 10% of the strains we tested were susceptible; (ii) susceptibility appears to be limited to the Bellingham subtype; and (iii) the Bellingham subtype is an infrequent cause of Legionnaires' disease (1, 6, 10).

We have recommended the routine use of nonselective BCYE and selective BCYE media with and without cefamandole for *Legionella* culture of clinical specimens (9). Since we have demonstrated that cefamandole-containing selective media can inhibit a select population of *L. pneumophila* as well as other *Legionella* species (5), the correct diagnosis of Legionnaires' disease may be missed unless the culture workup also includes a selective medium that does not contain cefamandole.

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