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Activities of Antimicrobial Agents against Clinical Isolates of *Mycobacterium haemophilum*

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Mycobacterium haemophilum*, first described in 1978, can cause severe infections of skin, respiratory tract, bone, and other organs of immunocompromised patients. There is no standardized antimicrobial susceptibility test, and for the 27 reported cases, a variety of test methods have been used. This paper reports the in vitro test results for 17 isolates of *M. haemophilum* recovered from 12 patients in the New York City area. MICs of 16 antimicrobial agents were determined in microtiter trays containing Middlebrook 7H9 broth plus 60 μM hemin, inoculated with 10^6 CFU of the organism per ml and incubated at 30°C for 10 days. Ethambutol, ethionamide, tetracycline, cefoxitin, and trimethoprim-sulfamethoxazole were inactive against initial isolates from the 12 patients. Isoniazid was weakly active with a MIC for 50% of strains tested (MIC_{50}) of 8 μg/ml and a MIC_{90} of >32 μg/ml. Three quinolones, ciprofloxacin, ofloxacin, and sparfloxacin, were moderately active with MIC_{50}s of 2 to 4 μg/ml and MIC_{90}s of 4 to 8 μg/ml. Amikacin and clofazamine were active with MIC_{90}s of 4 and 2 μg/ml, respectively. Clarithromycin was the most active macrolide with a MIC_{90} of ≤0.25 μg/ml. The MIC_{90} of azithromycin was 8 μg/ml, and the MIC_{90} of erythromycin was 4 μg/ml. The rifamycins were active with a MIC_{90} of 1 μg/ml for rifampin and one of ≤0.03 μg/ml for rifabutin. For a second isolate from the skin of one patient and an isolate from an autopsy culture of the spleen of a second patient, MICs of rifampin and rifabutin were >16 μg/ml, whereas initial isolates were inactivated by low concentrations of the rifamycins. Both patients had been treated for several months with several antimicrobial agents, including a rifamycin.

Mycobacterium haemophilum was first reported as a human pathogen in 1978 (19). Since then, 26 cases of infection have been described (1, 2, 4–7, 9–18, 20, 21, 23). Patients were usually adults who were immunosuppressed either after renal or bone marrow transplantation or with AIDS or lymphoma, or were children with apparently normal immune function. One patient had coronary bypass surgery (14). Clinical presentation usually included either cutaneous lesions or abscesses, septic arthritis, adenitis, pneumonia, and/or bacteremia. Cases have been reported from the United States, Australia, Canada, France, Israel, and the United Kingdom. *M. haemophilum* is a slowly growing organism in vitro, and media for culture should contain an iron source such as hemin or ferric ammonium citrate, and agar or broth should be incubated at 30°C for 6 weeks.

A cluster of 13 cases of *M. haemophilum* infection that occurred in the New York City metropolitan area in 1990 to 1991 was reported by the Centers for Disease Control (3). Four of the 13 cases (11) and one additional case in 1992 were seen at Memorial Sloan-Kettering Cancer Center (MSKCC) in two patients with AIDS, two patients who had either aplastic anemia or leukemia and had received bone marrow transplants, and one patient with non-Hodgkins lymphoma. No source of the cluster of cases in the New York City area was found; additional cases from the United States, including more from New York City, have been reported to the Centers for Disease Control; and the natural habitat of *M. haemophilum* is still not known.

There is no standardized antimicrobial susceptibility test for *M. haemophilum*. A variety of test methods using both agar and broth media have been described in the case reports. The purpose of the present study was to test a larger number of isolates by one method and to identify agents that might merit clinical evaluation. A modification of a microdilution broth method developed for testing slow-growing mycobacteria (22) was used to test 17 strains of *M. haemophilum* recovered from 12 patients with AIDS or neoplastic disease in the New York City area. Successive isolates from two patients with recurrent or progressive infection despite aggressive therapy were tested for the possibility of developing resistance.

MATERIALS AND METHODS

Test organisms. Isolates of *M. haemophilum* were collected from patients at MSKCC, Lenox Hill Hospital, St. Vincent's Hospital, and New York Hospital in New York City; St. John's Hospital in Yonkers, New York; and North Shore Hospital in Manhasset, New York. Isolates were identified as *M. haemophilum* when they were strongly acid fast, had a growth requirement for hemin, grew at 30°C but not at 37°C, and had a positive conventional biochemical test only for the production of pyrazinamidase. The identity of several of the isolates was confirmed at the Centers for Disease Control, Atlanta, Ga.

Antimycobacterial agents. Analytical-grade samples of ethambutol (Lederle Laboratories, Pearl River, N.Y.), ciprofloxacin (Miles Inc., New Haven, Conn.), ofloxacin (R. W. Johnson Pharmaceutical Research, New Brunswick, N.J.),

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sparfloxacin (Parke-Davis Pharmaceutical Research Division, Ann Arbor, Mich.), clofazamine (CIBA-GEIGY, Basel, Switzerland), rifabutin (Adria Laboratories, Dublin, Ohio), azithromycin (Pfizer Central Research, Groton, Conn.), and clarithromycin (Abbott Laboratories, North Chicago, Ill.) were provided by the manufacturers. Isoniazid and ethionamide were purchased from Sigma Chemical Co., St. Louis, Mo. Rifampin (Marion Merrell Dow, Kansas City, Mo.), amikacin and erythromycin (Elkins-Sinn, Cherry Hill, N.J.), tetracycline (Lederle Laboratories), cefoxitin (Merck Sharp & Dohme, West Point, Pa.), and trimethoprim-sulfamethoxazole (Roche Laboratories, Nutley, N.J.) were purchased from the MSKCC pharmacy.

Stock solutions of azithromycin and clarithromycin were prepared in buffered saline (pH 7.4). Stock solutions of rifabutin and clofazamine were prepared in absolute ethanol. Ethionamide was dissolved in ethylene glycol. Sterile distilled water was used as the solvent for preparing stock solutions of the other agents. Stock solutions were stored at −20°C.

Susceptibility testing procedure. Susceptibility tests were performed with a microdilution broth method for slowly growing mycobacteria (22); hemin was added to the broth for the growth of *M. haemophilum*. Inocula were harvested from 2-week-old subcultures of the organism from chocolate agar plates (Becton-Dickinson, Cockeysville, Md.). A loopful of the organism was suspended in sterile saline, the tube was vortexed and centrifuged, and the supernatant was discarded. Suspensions were diluted with saline to the turbidity of a 0.5 McFarland nephelometry standard. A further 100-fold dilution in sterile 2× broth (see below) gave a final concentration of 2×10^6 CFU/ml as determined by quantitative cultures. This concentration facilitated microtiter endpoint determinations. Lower inocula of the organism delayed reading of the results by several days, and antimicrobial degradation became a concern.

Doubling dilutions of the antimycobacterial agents in sterile water were placed in wells of microtiter trays (0.1 ml of drug solution per well), and the wells were inoculated with equal volumes of the test strains suspended in 2× strength sterile broth. The broth, at 1× strength, consisted of complete Middlebrook 7H9 broth supplemented with glycerol and Middlebrook ADC enrichment per manufacturer's instructions (Difco Laboratories, Detroit, Mich.) plus 60 μM hemin (Eastman Kodak, Rochester, N.Y.). The broth was sterilized and cleared of precipitates by filtration through a 0.45-μm-pore-size filter, and wells were inoculated with 10^6 CFU of organism per ml. The final pH was approximately 7.0 for wells containing azithromycin and clarithromycin and 6.8 for the other wells. Plates were placed in a moist chamber to limit evaporation and were incubated for 10 days at 30°C. The minimum concentration of the drug that prevented visible growth was designated the MIC. *Mycobacterium tuberculosis* ATCC 27294 was used as a control organism. *M. haemophilum* MSKCC 1A (Table 1) was tested in all experiments as an additional control for reproducibility. All isolates were tested at least twice.

RESULTS

Table 1 notes the sources of the isolates of *M. haemophilum*. Seventeen strains were recovered from 12 immunocompromised patients. The organisms were recovered antemortum from skin, lung, sputum, synovial fluid, and bone and at autopsy from a lymph node, adrenals, and the spleen of one patient. Strain numbers with the suffix "A" were the first isolates and were recovered before or shortly after therapy was initiated. Additional clinical and laboratory information regarding patients 1 to 4, 6 to 8, 11, and 12 has been published (3, 11, 23).

TABLE 1. Sources of clinical isolates of *M. haemophilum*

Patient	Hospital	Underlying disease	Strain	Site
1	MSKCC	AIDS	1A	Skin
			1B	Lymph node (autopsy)
			1C	Adrenals (autopsy)
			1D	Spleen (autopsy)
2	MSKCC	Aplastic anemia, BMT[a]	2A	Lung
			2B	Sputum
3	MSKCC	AIDS	3A	Synovial fluid
4	MSKCC	Leukemia, BMT[a]	4A	Skin
5	MSKCC	Lymphoma	5A	Skin
6	Lenox Hill	AIDS	6A	Bone
7	St. Vincent's	AIDS	7A	Bone
8	New York	AIDS	8A	Skin
9	New York	AIDS	9A	Skin
10	New York	AIDS	10A	Skin
11	New York	AIDS	11A	Skin
12	St. John's	AIDS	12A	Skin
			12B	Skin

[a] BMT, bone marrow transplant.

Final MICs were noted after the microtiter trays had been incubated at 30°C for 10 days. Prior to 10 days, a small amount of precipitate in some wells obscured interpretation of the results. The *M. tuberculosis* control strain was susceptible to all standard antimycobacterial agents. MIC results for the *M. haemophilum* reproducibility control strain did not vary by more than one doubling dilution of the antimicrobial agents in all tests.

Ethambutol, ethionamide, tetracycline, cefoxitin, and trimethoprim-sulfamethoxazole were inactive against the first clinical isolates from the 12 patients. Antimicrobial agents that showed some activity are listed in Table 2. Isoniazid was weakly active with a MIC range of 4 to >32 μg/ml, a MIC for 50% of strains tested (MIC_{50}) of 8 μg/ml, and a MIC_{90} of >32 μg/ml. The three quinolones, sparfloxacin, ofloxacin, and ciprofloxacin, were moderately active, with

TABLE 2. In vitro activity of antimicrobial agents showing some activity against initially isolated strains of M. haemophilum[a]

Antimicrobial agent	MIC (μg/ml)		
	Range	MIC_{50}	MIC_{90}
Rifabutin	≤0.03–0.06	≤0.03	≤0.03
Rifampin	0.5–2	0.5	1
Clarithromycin	≤0.25–0.5	≤0.25	≤0.25
Erythromycin	2–4	2	4
Azithromycin	2–16	4	8
Clofazamine	0.5–4	2	2
Amikacin	2–8	4	4
Ciprofloxacin	1–8	2	8
Ofloxacin	2–8	4	8
Sparfloxacin	0.5–4	2	4
Isoniazid	4–>32	8	>32

[a] Ethambutol, ethionamide, tetracycline, cefoxitin, and trimethoprim-sulfamethoxazole were inactive against all strains.

TABLE 3. MICs for strains of *M. haemophilum* from two patients who developed progressive disease or relapsed despite therapy

Strain	Site	Mo/yr	MIC (μg/ml) for antimicrobial agent:			
			Rifampin	Rifabutin	Clofazimine	Clarithromycin
1A	Skin	9/90	0.5	≤0.03	2	≤0.25
1B	Lymph node (autopsy)	9/91	1	0.125	1	0.5
1C	Adrenals (autopsy)	9/91	1	0.125	1	1
1D	Spleen (autopsy)	9/91	>16	>16	1	1
12A	Skin	1/91	1	≤0.03	2	≤0.25
12B	Skin	7/92	>16	>16	8	1

variable MICs among the three agents. While the MIC_{50}s and MIC_{90}s were similar, the MIC of ciprofloxacin was lower than those of ofloxacin and sparfloxacin for 6 of 12 strains and higher for 2 of 12 strains. Amikacin and clofazamine were active against all strains. Clarithromycin was the most active macrolide, followed by erythromycin and azithromycin. Between the two rifamycins, rifabutin was more active than rifampin. All strains were inhibited by low concentrations of rifampin and very low concentrations of rifabutin.

Table 3 notes the MIC results for serial strains of *M. haemophilum* isolated from two patients (patients 1 and 12 in Table 1) whose infections progressed despite aggressive therapy. Both patients were severely immunosuppressed as a result of CD4 lymphocyte depletion due to human immunodeficiency virus infection, and *M. haemophilum* was their first serious opportunistic infection.

Patient 1 presented with skin lesions and received treatment with multiple agents including isoniazid, ethambutol, rifampin, doxycycline, ciprofloxacin, and amikacin. During the next 11 months, the patient improved but later developed disseminated *M. haemophilum* infection while therapy was stopped because of abnormal liver function tests. Growth of isolates recovered at autopsy from a lymph node (strain 1B) and the adrenals (strain 1C) was inhibited in vitro by low concentrations of the rifamycins, as was that of the initial isolate (strain 1A), while for an isolate from the spleen at autopsy (strain 1D), MICs of both rifampin and rifabutin were >16 μg/ml.

Patient 12 presented with a gluteal abscess that yielded positive acid-fast smears and was culture negative when conventional methods for mycobacterial culture were used. Subsequently, *M. haemophilum* was recovered from cheek lesions (strain 12A). The patient was initially treated with isoniazid, ethambutol, and rifampin; this was followed by several months of treatment with rifabutin, clofazamine, ciprofloxacin, and amikacin. The patient was also briefly treated with clarithromycin. Strain 12B was recovered from a specimen taken 18 months after the specimen that yielded strain 12A. Rifampin and rifabutin were inactive, and clofazamine and clarithromycin were only moderately active against strain 12B, whereas the four antimicrobial agents were very active against the first isolate, 12A.

DISCUSSION

Although there are no standardized methods for antimicrobial susceptibility testing of *M. haemophilum*, several of the case reports have included test results. The most detailed descriptions of test procedures used were noted in six papers reporting the results of 18 isolates. A variety of agar methods were described in five papers (3, 7, 11, 13, 14), and a broth microdilution method was used for the sixth paper (20). Media were supplemented with hemin, and results were noted after 1 to 2 weeks. Not all strains were tested against the same antimicrobial agents; however, a summary of results indicates that amikacin, ciprofloxacin, and rifampin were usually active and doxycycline, ethambutol, ethionamide, isoniazid, and streptomycin were relatively inactive against strains tested.

The results in this paper generally agree with previous test results. The most active antimicrobial agents in the present study were the rifamycins, macrolides, and quinolones, clofazamine, and amikacin. Ethambutol, ethionamide, tetracycline, cefoxitin, and trimethoprim-sulfamethoxazole were inactive, and isoniazid showed some activity. Isoniazid may be more active than the in vitro test results indicate, since hemin, used as a broth supplement, can antagonize the in vitro activity of isoniazid (8). The new macrolides, clarithromycin and azithromycin, may even be more active than indicated. Although the pH of the test broth was approximately 7.0, macrolide activity has been shown to increase at a pH of 7.2 to 7.4. However, increasing the pH may slow the growth of *M. haemophilum*; thus, results may suggest inhibition that is actually not due to antimicrobial action. Trimethoprim-sulfamethoxazole may also be more active than these results indicate. Sulfonamide susceptibility has been defined as ≥80% inhibition of growth. Use of a high inoculum and an endpoint of no visible growth may have obscured some activity of the antimicrobial agent.

This is the first report of the emergence of resistance in *M. haemophilum*. In vitro growth of isolates from two patients was initially inhibited by low concentrations of the rifamycins. After the patients were treated with several antimicrobial agents, including a rifamycin, for prolonged periods, subsequent isolates were not inactivated by low concentrations of the rifamycins. Results indicate the need to culture another specimen from the patient if therapy appears to be failing and to test the new isolates against the appropriate antimicrobial agents.

M. haemophilum has a markedly different pattern of susceptibility to antimicrobial agents than do other mycobacterial species. Patients with *M. haemophilum* infection will receive optimal therapy only if there is prompt recognition of the causative agent. Recovery of the organism from clinical specimens requires the use of media supplemented with hemin or other iron sources and incubation of cultures at 30°C. Awareness of the clinical presentation and good communication with the clinical laboratory are essential for detection of this emerging pathogen.

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