

Disseminated Infection with *Mycobacterium genavense*: a Challenge to Physicians and Mycobacteriologists

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In the present study we compared the clinical presentations of patients with a clinical diagnosis of AIDS and disseminated *Mycobacterium genavense* infection ($n = 12$) with those of patients with AIDS and disseminated *M. avium* complex (MAC) infection ($n = 24$). Abdominal pain was seen more frequently in the group of patients infected with *M. genavense* than in patients infected with MAC ($P = 0.003$). Analysis of microbiological data revealed that stool specimens from patients infected with *M. genavense* were more often smear positive than stool specimens from patients infected with MAC ($P = 0.00002$). However, *M. genavense* could be cultured on solid media from only 15.4% of the stool specimens, whereas MAC could be cultured from 71.4% of the specimens. Bone marrow and liver biopsy specimens yielded growth of *M. genavense* within a reasonably short time, allowing species identification by DNA technology. Microbiological data clearly demonstrated the importance of acidic liquid medium for primary culture, the avoidance of pretreatment and the use of additives in culture, and the necessity for prolonged incubation if *M. genavense* is suspected. Susceptibility testing showed that *M. genavense* is sensitive to rifamycins, fluoroquinolones, and macrolides, whereas it is resistant to isoniazid. Susceptibility to ethambutol and clofazimine could not be evaluated. The mean survival times of patients in the two groups were similar.

The human immunodeficiency virus (HIV) pandemic has resulted in an increase in the incidence of disseminated infections caused by nontuberculous mycobacteria (NTM) (12, 27). Infections with NTM in AIDS patients are associated with increased morbidity and high rates of mortality. In one study, the mean survival time for untreated patients was found to be only 5.6 months after a diagnosis of disseminated *Mycobacterium avium* complex (MAC) infection (13). Although the most commonly isolated species belong to MAC, several other mycobacterial species have been described as pathogens for HIV-infected patients. In 1990 Hirschel and coworkers (10) described a fatal infection, characterized by fever, massive weight loss, anemia, and diarrhea, caused by an unidentified mycobacterium. This novel mycobacterium was described in 1992 by Böttger et al. (3), who also proposed the name *M. genavense*. Since then, *M. genavense* has been isolated from patients throughout Europe (3, 26), Australia (14), and North America (1, 17, 18, 23). In the Swiss HIV cohort study, *M. genavense* accounted for 12.8% of the disseminated NTM infections diagnosed between 1990 and 1992 (19).

In this study, we compared the clinical presentations of patients with disseminated infection caused by *M. genavense* to those of patients infected with MAC, which is the most common cause of disseminated NTM disease in Denmark. Results of microbiological analyses performed with clinical specimens from *M. genavense*- and MAC-infected patients were analyzed, and recommendations for specimen collection and laboratory methodologies for the detection of *M. genavense* are proposed.

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MATERIALS AND METHODS

Specimens. All specimens that were received between January 1991 and April 1995 and that grew *M. genavense* on culture were identified retrospectively from the laboratory records at the Department of Mycobacteriology at the Statens Serum Institut. That laboratory serves as a central laboratory for diagnostic mycobacteriology in Denmark, receiving roughly 25,000 specimens each year.

Patients and ethics. For evaluation of symptoms and clinical findings, each patient with disseminated *M. genavense* infection was matched by nationality (Danish versus foreign subjects) and age (within 5 years) with two patients with disseminated MAC infection who had attended the Department of Infectious Diseases at Hvidovre University Hospital within the study period. For all patients and controls, information regarding symptoms, clinical findings, survival, and results of laboratory investigations were obtained from the patients' medical records and were used to fill in a standard questionnaire. Filing of results was approved by the Danish Data Protection Agency (file no. 1997-1200-346).

Specimen processing. Specimens were processed by a standard methodology, although the decontamination procedure for peritoneal fluid, bronchoalveolar lavage, sputum, stool, skin biopsy, lymph node, pus, synovial joint fluid, and urine specimens was changed from the *N*-acetyl-L-cysteine-sodium dodecyl sulfate method to the NaOH method in August 1993. After decontamination, the bacteria were washed with phosphate-buffered saline (pH 6.8) and concentrated by centrifugation ($3,000 \times g$, 20 min). The pellets were resuspended in phosphate-buffered saline and were used for preparation of smears and for inoculation into culture media. Smears were stained with auramine-rhodamine and were examined for the presence of acid-fast bacilli. The specimens were inoculated into one or more culture media. Blood and bone marrow were inoculated into BACTEC 13A (13A) vials, and the remaining specimens were inoculated into BACTEC 12B (12B) vials and onto a solid medium (Löwenstein-Jensen medium with sodium pyruvate or Middlebrook 7H10 medium). Specimens expected to contain large numbers of contaminating microorganisms, like stool and pus specimens, were inoculated onto solid medium only. The inoculated culture media were incubated for a maximum of 8 weeks (smear-negative specimens) or 16 weeks (smear-positive specimens). Species identification was first performed by hybridization to probes specific for the *M. tuberculosis* complex and MAC to rule out these species (AccuProbe; GenProbe, Sparks, Md.) (7, 8, 15). If these reactions were negative, PCR-restriction enzyme pattern analysis (25) or automated cycle sequencing of hypervariable region A of the gene encoding 16S rRNA (16) was applied. Identification of the species of second or later mycobacterial isolates from individual patients was performed as described above, or identification to the species level was made according to the growth characteristics, pigmentation, and/or morphology identified from the first specimen.

Optimum pH. The optimum pH was investigated with *M. genavense* isolates from six patients. Two hundred microliters from drug-free BACTEC PZA Test Medium vials in which a growth index (GI) of 999 had been reached was

transferred to 12B vials in which the pH had been adjusted to 6.8, 6.4, 6.0, 5.5, or 5.0 with phosphoric acid. The daily GI was recorded, and the generation times were calculated from the slope of the growth curve on the basis of daily GI determinations.

Susceptibility testing. Susceptibility testing was performed by the BACTEC Indirect Qualitative Test as recommended for *M. tuberculosis* (9) yet modified with the use of BACTEC PZA Test Medium vials to enhance the growth of *M. genavense*. One hundred microliters of a BACTEC PZA Test Medium vial in which the GI had just reached 999 was used as the inoculum. The isolates were investigated with the following drugs at the indicated concentrations: ethambutol, 2.5 or 7.5 µg/ml; rifampin, 2 µg/ml; isoniazid, 1 µg/ml; amikacin, 5 µg/ml; clofazimine, 1 µg/ml; rifabutin, 0.25 or 1 µg/ml; ciprofloxacin, 2 µg/ml; ofloxacin, 4 µg/ml; roxithromycin, 5 µg/ml; clarithromycin, 2 µg/ml; azithromycin, 2 µg/ml; and fusidic acid, 25 µg/ml. Isolates were regarded as resistant if the daily change in the GI of the drug-containing vial exceeded that for the 1% control vial. Isolates were regarded as sensitive if the GI of the drug-containing vial decreased, whereas isolates were regarded as borderline if the GI increased less than that for the 1% control vial.

Statistics. The chi-square test with Fisher's exact test (when appropriate) was used to identify possible significant differences in symptoms and clinical findings in patients and controls. Student's *t* test was used to determine whether differences between continuous data (survival, CD4⁺ counts, detection times) were significant.

RESULTS

Patients. Twelve patients with at least one blood, bone marrow, or biopsy specimen that grew *M. genavense* on culture and 24 patients with disseminated MAC infection were identified. Demographic data and information on mycobacterial prophylaxis and antiretroviral treatment for patients and controls are presented in Table 1. No significant differences were observed between the two groups. The male-to-female ratio was 3:1 for patients infected with *M. genavense*, whereas the male-to-female ratio was 11:1 for the MAC-infected controls. The general Danish AIDS population had a male-to-female ratio of 10:1 (1,589 males and 155 females) in the second quarter of 1995 (5).

Symptoms and clinical findings. Table 1 displays the symptoms and clinical findings recorded in the medical files for patients and controls. Abdominal pain was found significantly more often in *M. genavense*-infected patients than in MAC-infected patients ($P = 0.003$). No significant differences in paraclinical manifestations were observed. The mean survival time after collection of the first smear or culture-positive specimen was 36 weeks (range, 6 to 116 weeks) for *M. genavense*-infected patients and 34 weeks (range, 3 to 163 weeks) for MAC-infected patients, the difference of which is not significant.

Specimens. Eighty-three specimens (range, 1 to 14 specimens per patient) from the *M. genavense*-infected patients and 153 specimens (range, 1 to 15 specimens per patient) from the MAC-infected patients were cultured. The results of microbiological analyses are presented in Table 2. Fifty-seven specimens from the *M. genavense*-infected patients were negative by both smear examination and culture. Acid-fast bacilli were detected by smear examination in 12 (21.1%) specimens, of which 9 (75.0%) were stool specimens. Only 4 (33.3%) of these smear-positive specimens grew *M. genavense* on culture. *M. genavense* was cultured from 14 additional smear-negative specimens. We found, however, that only five (5.7%) specimens from MAC-infected patients were smear positive, of which three were also culture positive. A further 75 (50.0%) specimens were culture positive only for MAC. Detection times differed among culture media and among species. For *M. genavense*, the median detection time was 20 days (range, 7 to 54 days) for 13A, 8 days (range, 6 to 40 days) for 12B, and 41 days (range, 35 to 47 days) for solid media. The corresponding values for MAC were 9 days (range, 6 to 57 days) for 13A, 12.5

TABLE 1. Demographics, symptoms, and clinical findings for HIV-positive patients with disseminated *M. genavense* infection and disseminated MAC infection

Characteristic	<i>M. genavense</i> (<i>n</i> = 12)	MAC (<i>n</i> = 24)
Sex (no. of patients)		
Men	9	22
Women	3	2
Nationality (no. of Danes/no. of foreigners)	11/1	22/2
Age (yr [mean/median])	34.8/34.0	35.2/33.5
Risk group (no. of patients)		
Homosexual	7	18
Heterosexual	2	5
Injection drug abuse	0	1
Blood transfusion	1	0
Other or unknown	2	0
No. of patients with:		
Antimycobacterial prophylaxis	0	0
Antiretroviral treatment	7	11
No. of patients with the following/total no.:		
Fever	9/12	20/22
Weight loss	9/12	16/21
Anorexia	7/12	10/19
Lymphadenopathy	6/12	5/18
Anemia	8/12	11/24
Pancytopenia	6/10	11/24
Abdominal pain ^a	6/10	1/24
Diarrhea	5/12	6/24
Splenomegaly	5/12	8/24
Hepatomegaly	3/12	5/24
Mean CD4 ⁺ count (10 ⁶ /liter)	18 ^b	15 ^c
Mean survival time (wk)	36 ^d	34 ^c

^a Significant ($P = 0.003$).

^b *n* = 11.

^c *n* = 24.

^d *n* = 12.

days (range, 6 to 50 days) for 12B, and 24 days (range, 11 to 49 days) for solid media.

For specimens that grew *M. genavense*, the mean detection times for pretreated and nontreated specimens were 31.8 and 23.8 days, respectively. The corresponding values for the specimens that were culture positive for MAC were 21.4 and 14.4 days. The mean detection time was significantly longer for *M. genavense* than for MAC for both pretreated ($P < 0.025$) and nontreated ($P < 0.010$) specimens.

Species identification. At least one isolate per patient was tested with hybridization probes for *M. tuberculosis* and MAC. All isolates tested were negative with the *M. tuberculosis* complex-specific probe, and all MAC-infected patients had at least one positive result with probes specific for species within MAC. For all *M. genavense*-infected patients, at least one isolate was identified as *M. genavense* by DNA sequencing of hypervariable region A of the 16S rRNA gene (16).

Optimum pH. The bacterial generation times estimated from the time that the daily GI started to increase indicate that growth is enhanced at pH 5.5 (Table 3).

Susceptibility testing. The results of susceptibility testing on BACTEC PZA Test Medium (pH 6.0) are presented in Table 4. All *M. genavense* isolates tested were susceptible to rifampin,

TABLE 2. Microbiological results for various specimen categories^a

Specimen	Culture medium	<i>M. genavense</i>				MAC			
		No. (%) of specimens			Median time (days [range]) to culture positivity	No. (%) of specimens			Median time (days [range]) to culture positivity
		Total	Smear positive	Culture positive		Total	Smear positive	Culture positive	
BAL	12B + solid	1	0 (0)	0 (0)	46 (30-54)	11	0 (0)	4 (36.4)	10 (8-25)
Blood	13A	22	NA	4 (25) ^b		55	NA	31 (58.5) ^c	9 (6-52)
Bone biopsy	12B + solid	1	0 (0)	0 (0)	15 (7-28)	0	NA	8 (72.7)	10 (6-57)
Bone marrow	13A	10	1 (16.7) ^d	7 (70)		11	NA	8 (72.7)	15
CSF	12B + Dubos + solid	6	0 (0)	0 (0)	6 (6-40)	3	0 (0)	1 (33.3)	15
Liver biopsy	12B + solid	4	0 (0)	3 (75)		13	0 (0)	9 (69.2)	12 (6-49)
Lymph node	12B + solid	2	2 (100)	1 (50)	8	1	0 (0)	1 (100)	11
Peritoneal fluid	12B + solid	1	0 (0)	0 (0)	37	0	NA	0 (0)	
Pus	Solid	4	0 (0)	0 (0)		1	1 (100)	0 (0)	
Skin biopsy	12B + solid	1	0 (0)	0 (0)	41 (35-47)	0	NA	0 (0)	
Sputum or gastric lavage	12B + solid	8	0 (0)	1 (12.5)		26	2 (7.7)	8 (30.8)	15 (14-42)
Stool	Solid	14	9 (64.3)	2 (15.4) ^e	22	0 (0)	15 (71.4) ^e	24 (11-35)	
Synovial fluid	12B + solid	3	0 (0)	0 (0)	4	0	NA	0 (0)	
Urine	12B + solid	5	0 (0)	0 (0)		4	1 (25.0)	1 (25.0)	18
Miscellaneous	Variable	1	0 (0)	0 (0)	6	1 (16.7)	0 (0)		
Total	Variable	83	12 (21.1)	18 (23.7)	153	5 (5.7)	78 (52.0)		

^a Abbreviations: BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; solid, a solid medium; NA, not analyzed.
^b Six specimens were contaminated on all culture media.
^c Two specimens were contaminated on all culture media.
^d Four specimens were not analyzed.
^e One specimen was contaminated on all culture media.

rifabutin, roxithromycin, clarithromycin, azithromycin, and fusidic acid. Ten of 12 strains were susceptible to amikacin, and most strains (9 of 12) were susceptible to the fluoroquinolones ciprofloxacin and ofloxacin. Resistance to ethambutol was detected in 9 of 12 isolates, resistance to clofazimine was detected in 4 of 12 isolates, and resistance to isoniazid was detected in 11 of 12 isolates. In order to investigate whether ethambutol, clofazimine, and isoniazid retained their activities at pH 6.0, we inoculated a susceptible strain of *M. tuberculosis* at pH 6.0. We found that *M. tuberculosis* was resistant to both ethambutol and clofazimine and was susceptible to isoniazid at pH 6.0, indicating that ethambutol and clofazimine had decreased activities at pH 6.0.

DISCUSSION

Between January 1991 and April 1995, we identified 12 patients whose clinical specimens grew *M. genavense* on culture. All patients had at least one blood, bone marrow, or biopsy specimen which was culture positive, suggesting that *M. genavense* was the source of infection and not an opportunistic colonizer. In addition, more than half of the patients had smear-positive specimens that demonstrated numerous bacteria. All patients infected with *M. genavense* were suffering from

AIDS and were severely immunocompromised at the time that specimens containing *M. genavense* were taken. The median age and HIV risk groups were comparable to those for the general Danish AIDS population (5), whereas the male-to-female ratio was lower.

The only significant difference between patients and controls was more frequent abdominal pain in *M. genavense*-infected patients. This could possibly be due to the presence of a larger number of bacteria in the intestines of *M. genavense*-infected patients, as we found that stool specimens from five of six patients who had submitted such specimens were smear positive. Of these, four experienced abdominal pain. No stool specimens from MAC-infected patients were positive. Sex, age, nationality, HIV risk group, and prophylactic treatment were not significantly different between patients and controls. No difference in survival was observed, consistent with the finding of similar CD4⁺ cell counts in the two groups. No significant

TABLE 3. Generation times for *M. genavense* subcultured at various pHs

pH	Generation time (h) for isolates from the following patients:						
	1	2	5	7	8	10	Mean
6.8	NG ^a	86	NG	145	NG	NG	115.5
6.4	NG	57	336	64	187	302	189.5
6.0	146	43	61	40	82	72	74.0
5.5	50	39	49	39	44	40	43.5
5.0	60	56	84	44	69	44	59.5

^a NG, no growth.

TABLE 4. Testing of susceptibility of clinical isolates of *M. genavense* from 12 patients to antimycobacterial drugs evaluated from BACTEC PZA Test Medium vials at pH 6.0^a

Drug	Concn (µg/ml)	No. of strains		
		Sensitive	Borderline	Resistant
Rifampin	2.0	12	0	0
Isoniazid	1.0	0	1	11
Amikacin	5.0	10	2	0
Rifabutin	0.25/1.0 ^b	12	0	0
Ciprofloxacin	2.0	9	2	1
Ofloxacin	4.0	9	2	1
Roxithromycin	5.0	12	0	0
Clarithromycin	2.0	12	0	0
Azithromycin	2.0	6	0	0
Fusidic acid	25.0	12	0	0

^a Susceptibility testing on ethambutol and clofazimine could not be carried out at pH 6.0.
^b Results are for nine/three isolates, respectively.

differences in the frequencies of fever, weight loss, anorexia, lymph adenopathy, anemia, pancytopenia, diarrhea, splenomegaly, and hepatomegaly were observed between the two groups.

We detected *M. genavense* by smear examination in bone marrow, stool, and biopsy specimens only, indicating the presence of numerous bacteria in such specimens. It has been described previously (1, 2, 4, 24) that *M. genavense* grows poorly in vitro. Our data support this observation, as only one-third of the smear-positive specimens became culture positive. Culture-positive specimens comprised blood, bone marrow, liver biopsy, lymph node, sputum, and stool specimens. Several scientists have cultured *M. genavense* in broth media; however, few have been able to culture *M. genavense* on solid media (4). Although scanty growth was detected in the condensed water of Löwenstein-Jensen medium, we also found growth to be superior in broth media. Our study therefore supports the recommendation that primary isolation should include a broth medium incubated for at least 8 weeks (4).

The low culture positivity rate for smear-positive specimens raises questions concerning viability after transportation and decontamination and the suitability of culture conditions. The mean detection time for *M. genavense* was significantly longer than that for MAC in both pretreated and nontreated specimens. Recovery of *M. genavense* from specimens subjected to decontamination was found to be 10.3%, while recovery was 37.8% for nontreated specimens. It is unlikely that these figures were caused by the presence of larger numbers of bacteria in nontreated specimens, as 1 nontreated and 11 pretreated specimens were smear positive. The low rate of recovery from pretreated specimens is believed to be due to a decontamination that was too harsh or the exclusive use of a solid medium for some specimens.

Realini et al. (22) recently described that the addition of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) antibiotic mixture to primary culture impedes the in vitro growth of *M. genavense*. This is substantiated by our observation that no specimens culture positive for *M. genavense* have been identified after an increased amount of PANTA was added to primary cultures in mid-1995 in order to reduce overgrowth by contaminating microorganisms.

Use of BACTEC PZA Test Medium or lowering of the pH of the culture medium might further improve the isolation of *M. genavense*, as optimal growth was found at pH 5.5. The detection time for *M. genavense* was seen to be significantly lower for bone marrow and liver biopsy specimens than for other culture-positive specimens ($P < 0.005$). From the microbiological point of view, microbiological analyses of these specimens are recommended if disseminated *M. genavense* infection is suspected by the clinician. At present, species identification of *M. genavense* relies on nucleic acid-based methods. DNA sequencing, which might be a rather unsuitable technique for many laboratories, and the PCR-restriction enzyme pattern analysis method developed by Telenti and co-workers (25) work equally well in our experience (data not shown). Alternatively, a genus-specific PCR followed by an *M. genavense*-specific hybridization or an *M. genavense*-specific PCR could be used to identify isolates from solid or liquid media to the species level. As *M. genavense* did not produce a sufficient amount of colonies on solid media, traditional biochemical tests for comparison with other species could not be performed.

Regarding drug susceptibility, we found that *M. genavense*, in contrast to MAC, is quite susceptible to common antimycobacterial drugs in vitro. Most isolates were resistant to ethambutol and isoniazid, as has been described previously (24), and

one-third were resistant to clofazimine. The use of acidic pH in susceptibility testing might influence the solubility, chemical structure, and activity of the investigated drug; and it has been shown previously that some drugs, e.g., clarithromycin, are less active at pH 6.0 (21). Results from studies with susceptible isolates of *M. tuberculosis* indicate that ethambutol and clofazimine are not active or are less active at pH 6.0, whereas isoniazid seems to retain its activity at pH 6.0. In conclusion, it is not possible to evaluate the susceptibility of *M. genavense* to ethambutol and clofazimine at pH 6.0. These results emphasize the need for standardization of susceptibility testing with NTM and the need for a correlation between results of in vitro susceptibility testing and clinical outcome.

Attempts for the identification of a reservoir for *M. genavense* have been made during the last few years. Although *M. genavense* was detected in birds in two studies (11, 20), the source of infection for humans and animals remains to be explained. In the Swiss cohort study (19), a reasonably high proportion of disseminated mycobacterial infections were caused by *M. genavense*. In addition, Dumonceau et al. (6) found that 40% of intestinal tissue specimens from patients with inflammatory and noninflammatory bowel disease were positive for *M. genavense* by PCR, suggesting a ubiquitous distribution of *M. genavense*. Subtyping methods like restriction fragment length polymorphism analysis or random amplified polymorphic DNA analysis with PCR might reveal a link between human, animal, and possible environmental strains of *M. genavense* in the future.

We conclude that patients with disseminated MAC and *M. genavense* infections have very similar clinical presentations. From the small number of patients that we studied, it seems that patients infected with *M. genavense* more frequently suffer from abdominal pain. Survival was similar for the two groups. A high rate of smear positivity was found for stool specimens from patients infected with *M. genavense*, although the culture results for these specimens were disappointing. For *M. genavense*, the shortest detection times and highest recovery rates by culture were observed with liver biopsy and bone marrow specimens. The use of liquid medium for primary culture and prolonged incubation is essential for culture of clinical specimens, and the use of an acidic culture medium can be expected to further improve recovery rates and reduce detection times.

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