Identification and management of an outbreak of *Flavobacterium meningosepticum* infection in a colony of South African clawed frogs (*Xenopus laevis*)

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Flavobacteria are gram-negative, aerobic bacteria that inhabit soil, water, raw meat, milk, and various foodstuffs. Recently reclassified under the new genus *Chryseobacterium*,[1] flavobacteria may be commensal flora of many species.[2-7] These bacteria are opportunistic zoonotic pathogens and may cause disease in birds,[7] fish,[6,8,9] turtles,[10] frogs,[2,3,11-13] cats,[14] dogs,[15] and humans.[16] *Flavobacterium meningosepticum* causes nosocomial infections, sepsis, meningitis, and pneumonia in adults and infants, particularly those who are immunocompromised or hospitalized in intensive care facilities.[17-29] *Flavobacterium* spp, including *F meningosepticum*, are resistant to chlorine and other disinfectants.[30-32] Optimal growth conditions include moist, cool environments, or standing water at approximately 21 C.[30-32] As reviewed by Bloch et al,[16] nosocomial isolates from human hospitals may be traced to potable water sources, ice, ventilator tubing, disinfectant bottles, anesthetic and antibiotic solutions, water pipes, sink drains, medical implant devices and indwelling catheters, parenteral nutrition solutions, hands of personnel, and dialysis, bypass, and endoscopic equipment. These isolates generally have intrinsic and acquired multidrug resistance.[16,33-36] Once the organism is established in a particular environment, it is difficult to eradicate and has been associated with mortality in humans and animals. In an animal research facility, an outbreak of *F oderans* in a colony of South African clawed frogs (*Xenopus laevis*) necessitated depopulation and temporary closure of aquaculture housing for sanitization to halt the outbreak.[a] Approximately 2,000 animals died or were euthanatized. In this report, we describe the clinical signs, epidemiology, bacterial biochemical characteristics, molecular strain typing, and management procedures associated with an outbreak of *F meningosepticum* in a colony of South African clawed frogs.

Procedures

Frog colony—Frogs had been purchased from a single commercial supply house and had been in the Stanford University Research Animal Facility for periods ranging from 3 weeks to 3 years. Animal care and housing were conducted according to recommendations in the National Institutes of Health Guide for General Animal Housing and the local, state, and federal fish and game regulations. Four or 5 frogs/cage were housed in polycarbonate rodent cages (dimensions, 50 × 40 × 20 cm) with stainless steel lids, in 3L of conditioned and dechlorinated tap water that was changed every 3 days. Cage water was obtained from one of three 30-gallon barrels that were filled with conditioned tap water and maintained in the animal-housing room. Frogs were fed trout chow 3 times/wk, 3 hours prior to the water change. Barrel and cage water temperature was maintained at approximately 21 C; room temperature was between 22 and 23 C. Approximately 300 additional fish and amphibians were also housed in this room, including leopard frogs (*Rana pipiens*), giant toads (*Bufo marinus*), red bellied newts (*Cynops pyrrhogastor*), spotted salamanders (*Ambystoma maculatum*), and goldfish (*Carassius auratus*). Prior to the outbreak reported here, *F meningosepticum* had not been isolated from any species in the animal facility, and the primary causes of death in the frog colony were infection with *Aeromonas hydrophila* ("red leg" syndrome) or fungal infection with *Saprolegnia* sp.

Data collection—Mean daily census records, daily mortalities, weekly shipment records, results of microbiologic and antimicrobial susceptibility testing, and clinical and postmortem findings were monitored during the outbreak and for 7 months thereafter. Because isolated deaths caused by *F meningosepticum* may have gone unrecognized early in the outbreak, census and shipment records, microbiology reports, mortality records, and necropsy reports for the period from January 1996 to May 1996 were reviewed.

Necropsy procedure—Gross necropsy was performed on 61 of 93 frogs that died or were euthanatized during the outbreak. Histologic examination of tissues was performed on 5 frogs.

Bacteriologic culture and antimicrobial susceptibility testing—Heart blood and liver from 26 of 61 frogs, brain and coelomic fluid from 4 of 26 frogs, and all environmental specimens were incubated at 29 to 30 C for 18 to 24 hours on various agar[b] plates, including 5% sheep blood agar, chocolate agar, MacConkey agar, phenylethanol agar with 5% sheep blood, and

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Rimler-Shotts agar (selective for *Aeromonas hydrophila*). Biochemical reactions and identification of isolates were tested by use of a carbon source utilization test[c] that yielded test results (carbon substrate utilization profiles) in 24 to 48 hours. The test yielded an 80 to 96% index of similarity for *F meningosepticum*, dependent on final recording time. For confirmation of results, isolates of *F meningosepticum* were selected randomly and tested in parallel using a standardized micromethod combining 8 biochemical tests and 12 assimilation tests for the identification of gram-negative, nonfermentative bacteria (eg, *Pseudomonas* sp, *Acinetobacter* sp, *Flavobacterum* sp, *Moraxella* sp) and some fermentative bacteria not belonging to the *Enterobacteriaceae* (eg, *Vibrio* sp and *Aeromonas* sp).[d]

Antimicrobial sensitivity patterns were determined with Kirby Bauer disk diffusion techniques[e] by inoculating Mueller-Hinton agar plates with a bacterial suspension (MacFarland density, 0.50) from brain-heart infusion broth[b] tubes that had been incubated for 18 hours.

Environmental testing—Because *Flavobacterium* spp have been detected in tap water,[37] all water sources in the frog housing area were screened for *F meningosepticum*. Samples for bacteriologic culture were collected with sterile cotton swabs from water and from the biofilm[38-41] at the waterline in the conditioned water barrels, from equipment and cages in the housing area, and from all equipment and supplies used in conjunction with frog studies that were performed in a separate laboratory room. Because *Flavobacterium* spp are commensal inhabitants of the intestinal tract of crickets and other insects and are found in various foodstuffs,[30-32,42] the trout chow, crickets, and mealworms stored in the housing area were sampled for bacteriologic culture. Because moss used for bedding during shipment of commercially supplied frogs has been suspected as a bacterial source,[11] we also performed bacteriologic culture on bedding moss samples. Wild-caught, healthy, 50-g frogs (6 males, 6 females) were ordered from the supplier, and bacteriologic culture of their tissues was performed upon arrival. Two clinically healthy frogs from the colony were also euthanatized for bacteriologic culture of intestinal contents.

Molecular strain typing—Deoxyribonucleic acid from *F meningosepticum* isolates cultured from 5 frogs was extracted, and macrorestriction analysis of purified chromosomal DNA was performed as described.[43] As a procedural control, the DNA restriction patterns of *Enterococcus fecalis* (ATCC 47077) and an endemic, phenotypically different, unidentified *Flavobacterium* sp isolate from 1 of the water barrels were determined. Restriction endonuclease digestion was performed with *Spe*I and *Sma*I, and restriction fragments were resolved by a pulsed field gel electrophoresis system[f] on a 1% agarose gel. Electrophoresis was performed at 6 V/cm, pulse time was ramped from 5 to 15 seconds over 17 hours at 13 C, and DNA fragments were detected by ethidium bromide staining. Electrophoretic patterns were considered identical if they shared every band, similar if they differed by ≤ 2 bands, and different if they differed by ≥ 3 bands.

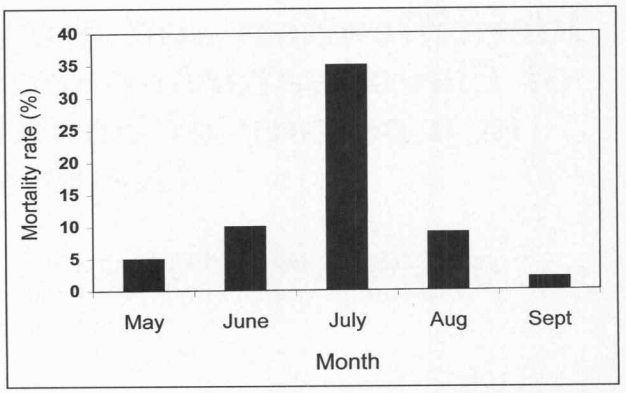

Figure 1—Graph of mortality caused by *Flavobacterium meningosepticum* infection in a colony of South African clawed frogs (*Xenopus laevis*).

Results

Description of the outbreak—In the last week of May 1996, 5 frogs were found dead; gross necropsy findings of petechial hemorrhages and abdominal swelling were considered to be consistent with *F meningosepticum* infection. In June, 14 frogs died, and at the peak of the outbreak in late July 58 frogs died (mortality rate, 35%; **Fig 1**), coincident with an increase in the total frog population from 144 frogs in June to 168 frogs in July. Twenty-eight of the frogs that died in July died suddenly over a 4-day period. During this month, the number of personnel working in the laboratory also increased from 7 to 14. By the middle of July, all sanitation and outbreak management procedures were in place. In August and September, 13 frogs and 3 frogs died, respectively. By the end of the outbreak in September 1996, 78 frogs had died and 15 frogs had been euthanatized.

Clinical signs observed throughout the outbreak were consistent with septicemia: ascites, anasarca, dyspnea, inability to dive and stay beneath the water surface, extreme lethargy, congestion of web vessels, and petechial hemorrhage affecting the entire body. Signs developed rapidly, and affected frogs were often found dead, despite having appeared healthy 4 to 6 hours earlier. Results of CBC for 2 frogs were consistent with overwhelming sepsis and characterized by neutropenia (< 4,000 neutrophils/µl; reference range lower limit = 5,000 neutrophils/µl), toxic neutrophils, and gram-negative intracellular bacteria in neutrophils and macrophages.

Oocytes had recently been harvested through a small abdominal incision from 10 of the frogs that died because of *F meningosepticum* infection; oocyte production had been induced in 4 of these frogs by SC administration of 200 units of human chorionic gonadotrophin. Briefly, frogs were anesthetized by immersion in a 0.1% solution of tricaine, and a 3-mm incision was made through the skin and abdominal musculature. The egg sac was exposed, 5 to 10 mg of eggs were collected, and the incision was closed with 3-0 polyglactin[g] suture material. After surgery, frogs were maintained in 1L isotonic saline (0.9% NaCl) solution containing gentamicin (1 mg/ml) for 1 to 2 days before they were returned to regular housing. Two frogs were treated with trimethoprim-sulfadiazine (3 mg/kg [6.6

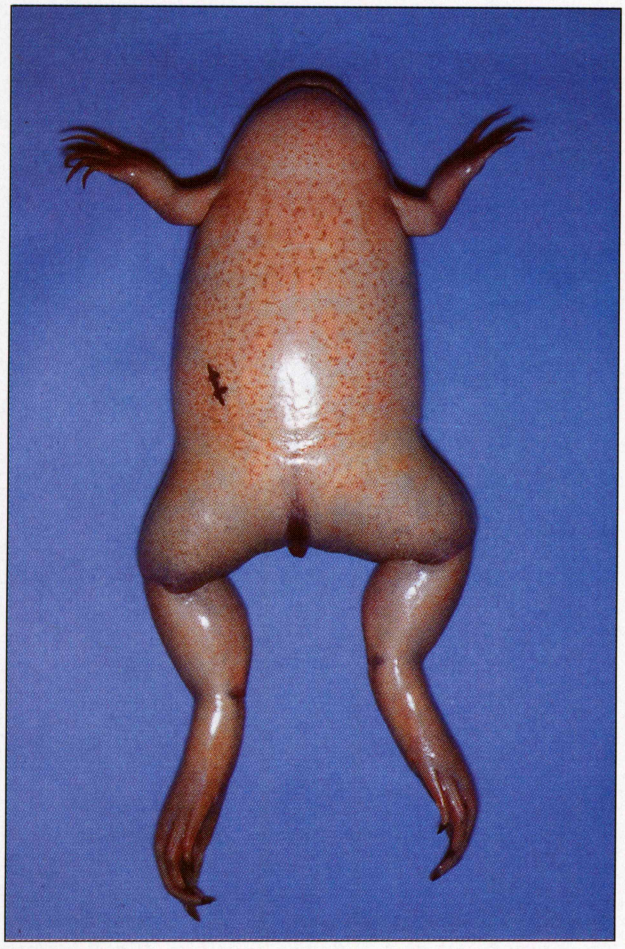

Figure 2—Photograph of a South African Clawed Frog that died of septicemia caused by *F meningosepticum*. Notice abdominal distention caused by ascites, swollen, hyperemic cloaca, and petechial hemorrhages on the ventral aspects of the body. The small skin incision on the abdomen is from surgical harvesting of oocytes.

mg/lb]) of body weight, administered into the dorsal lymph sac, q 24 h).Condition of the frogs continued to decline, and they were subsequently euthanatized by administration of 3 ml of a 10% solution of tricaine delivered intraperitoneally on day 5 of treatment. In late July, *F meningosepticum* was isolated from necropsy specimens from 7 leopard frogs (*Rana pipiens*) that were housed on neighboring racks in the same room.

Necropsy findings—Gross lesions included swollen abdomen (**Fig 2**), SC edema, hyperemia of the cloacal aperture and epidermis of the ventral portion of the abdomen, and petechial and ecchymotic hemorrhages in the ventral abdominal musculature. Two frogs had corneal opacity. The coelomic cavity contained copious, serosanguineous to opaque, orange-tan fluid. Histologic examination of tissues from 5 frogs revealed infiltration of the liver, spleen, kidney, serosal surfaces, or epicardium by low to moderate numbers of macrophages and neutrophils and few lymphocytes. Many macrophages contained gram-negative, rod-shaped bacteria (**Fig 3**).

Bacteriologic culture and antimicrobial susceptibility results—Numerous (> 300 **colony forming units** [CFU]/ml) *F meningosepticum* were cultured from heart blood and liver of 25 of 26 frogs. Coinfection with other organisms was uncommon: low numbers (1 to 10 CFU/ml) of an unidentified *Flavobacterium* sp were cultured from 2 of 25 frogs, whereas large numbers (> 300 CFU/ml) of *A hydrophila* were cultured from 4 of 25 frogs. *Flavobacterium meningosepticum* was also cultured from 4 samples of coelomic fluid, spleen, and brain. As reported by Webster and Hugh,[44] we observed flagella on a few *F meningosepticum* organisms in wet preparations of fresh blood.

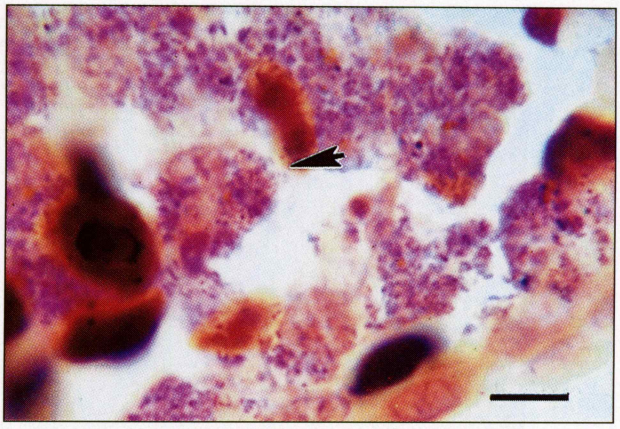

Figure 3—Photomicrograph of a section of kidney from a frog that died of infection with *F meningosepticum*. Notice numerous macrophages in the renal interstitium that contain many gram-negative rod-shaped bacteria (arrowhead) within the cytoplasm. Brown-Brenn stain; bar = 7 µm.

Colonies were pale yellow, caused diffuse, beta-hemolysis on 5% sheep blood agar, and grew abundantly on blood, chocolate, and phenylethanol agars.[44] The organisms were oxidase positive, predominantly nonmotile, gram-negative rods. Growth was not detected at 24 hours on MacConkey or Rimler-Shotts agar (except for *Aeromonas* organisms from blood of 2 frogs with mixed infections). Antimicrobial susceptibility test results indicated that all *F meningosepticum* isolates were susceptible to the fluoroquinolones enrofloxacin and ciprofloxacin, and to trimethoprim-sulfadiazine and chloramphenicol. All isolates were resistant to ampicillin, piperacillin, ticarcillin, ceftazidime, imipenem, tobramycin, ceftiofur, gentamicin, amakacin, tetracycline, and penicillin G. Nine randomly selected isolates were confirmed as *F meningosepticum* by 2 microbiology reference laboratories by means of cellular fatty acid analysis using a gas chromatography system.[h]

Environmental testing—Results of environmental testing indicated that *Flavobacterium* spp were in the animal-housing facility and the separate laboratory room. Low numbers (< 3 CFU/ml) of *F indologenes*, a known opportunistic pathogen in humans,[45,46] were cultured from several environmental sources, but this organism was not cultured in large numbers from affected frogs. *Flavobacterium meningosepticum* was isolated from the biofilm layer at the waterline of a 30-gallon communal conditioned water barrel in the animal-housing facility, the transport buckets used to transfer

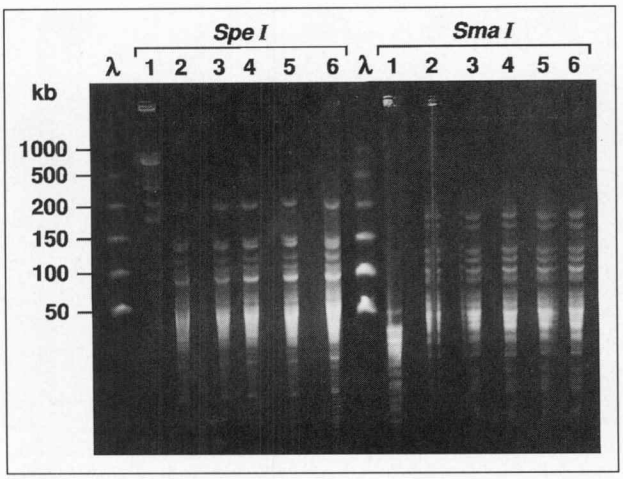

Figure 4—Pulsed field gel electrophoretogram revealing macrorestriction patterns of chromosomal DNA after digestion with *Spe*I or *Sma*I. λ = Molecular weight marker (lambda DNA ladder). Size of bands given in kilobases (kb) to the left. Lane 1, unidentified *Flavobacterium* sp; lane 2 to 6, *F meningosepticum*. The unidentified *Flavobacterium* isolate was cultured from a water barrel. The *F meningosepticum* isolates were cultured from 5 frogs that died of septicemia. Notice that the polymorphic restriction patterns for the *F meningosepticum* isolates are identical and differ from that of the unidentified isolate.

frogs between the animal-housing facility and laboratory, and an ice bucket, surgical procedure tray, and frog-holding tank in the laboratory. Importantly, we were unable to isolate the organism from other locations or materials in the environment, from the intestinal tracts of healthy frogs (even those housed in cages where frogs had died), cage water, barrel water, foodstuffs, wild-caught frogs, or from moss used as shipment bedding.

Molecular strain typing—Molecular strain typing results indicated that all *F meningosepticum* isolates from frogs, including 1 from the initial outbreak in May and 4 from frogs that died 4 months later, had identical restriction fragment length patterns and were related to the same strain (**Fig 4**). An unidentified *Flavobacterium* sp isolated from the conditioned water barrel at the onset of the outbreak had a unique restriction pattern and, therefore, differed from isolates from affected frogs.

Sanitation and management—Sanitation and management changes were fully implemented in July. Replacement shipments of frogs were halted for 1 month. All equipment was scrubbed with detergents and autoclaved, and bacteriologic cultures were performed to ensure freedom from contamination with *Flavobacterium* spp. Each conditioned water barrel was scrubbed and autoclaved once a week. Sink hoses were replaced with new hoses that were mounted so that the end of the hose did not rest in the sink drain. Investigators and animal care personnel were counseled on the importance of sanitation and hand washing and were advised of the zoonotic potential of this pathogen. Frogs that were lethargic and floating on top of the water were immediately examined, euthanatized, and submitted for necropsy. Cage-stocking density was decreased from 4 or 5 frogs/cage to ≤ 3 frogs/cage.

Discussion

To our knowledge, this is the first report of *F meningosepticum* as a pathogen in *X laevis*. Infection was invariably fatal, and treatment was unsuccessful, even when the appropriate antibiotic (trimethoprim-sulfadiazine), chosen on the basis of antimicrobial susceptibility testing, was used. Treatment failure may have been the result of advanced disease by the time clinical signs appeared or inappropriate drug dosage. Antibiotic dosage regimens based on pharmacokinetic studies are lacking for frogs. In addition, disk diffusion antimicrobial susceptibility tests can be unreliable for *Flavobacterium* spp.[16,36] Although isolates in this study appeared to be sensitive to trimethoprim-sulfamethoxazole by the disk diffusion test, evaluation by **minimum inhibitory concentration (MIC)** testing at a reference laboratory indicated that several isolates were resistant. Furthermore, MIC breakpoints for *Flavobacterium* spp have not been established by disk diffusion.

Consistent with other reports,[11-13,16] we were unable to identify the point source of the organism in our facility; however, we were able to document that *Flavobacterium* spp were ubiquitous in the aquaculture. Factors such as increased colony population density, increased number of people handling the frogs, and increased number of frogs undergoing experimental manipulation appear to have played a role in the outbreak.

The pathogenesis of *F meningosepticum* infection is poorly understood. Most strains of this species produce elastase and protease,[47] several hydrolases,[48] and endotoxins typical of gram-negative pathogens.[49] Some *Flavobacterium* spp are motile,[44] as we observed in wet preparations of our isolates. Together, these factors may enhance this species' ability to invade multiple organ systems, including the CNS. Genetic heterogeneity has been demonstrated by DNA-DNA hybridization studies on 52 strains of *F meningosepticum* from different geographic locations.[4] One genomic subgroup appears to have a predilection for the CNS,[4,50] so subgroups of *Flavobacterium* spp may differ with regard to pathogenicity.

The portal of entry into aquatic housing or hospital environments has been proposed to be city water supplies, as the organism has been recovered in open city water reservoirs.[37] In addition, *Flavobacterium* spp can survive the chlorination process, resisting concentrations of chlorine up to 100 mg/kg.[37] However, the organism has not been identified in the Stanford University Hospital,[i] which shares the city water supply with our facility. We were not able to isolate the organism from water samples taken directly from the tap in the animal-housing room, water barrels, or cage water of healthy or sick frogs.

Other potential sources of contamination included stress-induced shedding of the organism by newly introduced, subclinically infected frogs, or shedding by unstressed healthy frogs, because the organism may be part of the commensal flora of aquatic species.[2-6] However, we were not able to isolate the organism from healthy frogs in our facility or from wild-caught frogs purchased from the vendor. Competition from other commensal organisms may keep *Flavobacterium* spp

populations in healthy frogs too low for detection with the methods used in our study. The organism may have gained entry through surgical wounds, although surgery was not performed on all infected frogs. The 30-gallon conditioned water barrels that were the water source for the *X laevis* and *R pipiens* colonies were contaminated with *F meningosepticum* and other *Flavobacterium* spp and may have been the source of contamination for the buckets, trays, and tanks from which the organism was isolated. The prompt decline in the mortality rate after changes in laboratory management and cleaning procedures is convincing evidence that these sources played an important role in maintaining a suitable reservoir for the organism. The increase in the number of frogs in the colony and the number of laboratory personnel may have exacerbated conditions conducive to an outbreak. Cross-contamination by hand carriage and ineffectual cleaning of communal equipment, water containers, animal workstations, and other materials likely contributed to the high mortality rate at the height of the outbreak. By halting all experiments, delaying replacement shipments of frogs, and instituting intensive sanitation, surveillance, and environmental monitoring, we were able to contain the outbreak without depopulation.

On the basis of results of biochemical and antimicrobial susceptibility testing, phenotypic characteristics indicated that all isolates from infected frogs were *F meningosepticum*. Although antimicrobial susceptibility profiles were identical for all isolates, the usefulness of this information has limitations. For example, antimicrobial susceptibility profiles generally lack discrimination for isolates that do not have unusual resistance patterns, and a fourfold variation in MIC results may be detected.[16,36,51] In addition, susceptibility to antibiotics may change as a result of a single genetic event, and susceptibility patterns of identical isolates may change.[51] For nosocomial outbreaks, phenotypic differentiation among the outbreak strain and other strains recovered from the environment has proven difficult. We used molecular strain typing to make this differentiation and to determine that the frogs were temporally infected by dissemination of a single strain of *F meningosepticum*, presumably from a common, but unidentified, source. Infection was not the result of antibiotic resistance developing in multiple strains. Isolates from infected frogs were apparently phenotypically and genotypically different from *Flavobacterium* spp detected commonly in the environment. This information enhanced our ability to monitor the outbreak, implement changes in sanitation procedures, and resume operations without depopulation.

[a]Warren H, Harvard University, Boston: Personal communication, 1998.
[b]Remel, Lenexa, Kan.
[c]95 test Biolog system, GN MicroPlate panel, Biolog, Inc, Hayward, Calif.
[d]48-hour API NFT, bioMerieux VITEK Inc, Hazelwood, Mo.
[e]DISPENS-O-DISC, DIFCO Laboratories Inc, Detroit, Mich.
[f]CHEF-DR II System, Bio-Rad Laboratories, Philidelphia, Pa.
[g]VICRYL, Ethicon Inc, Somerville, NJ.
[h]MIDI/Sherlock, MIDI, Inc, Newark, Del.
[i]Tompkins L, Stanford University, Stanford, Calif: Personal communication, 1998.

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