Isolation and Characterization of *Burkholderia gladioli* from Orchids in Hawaii

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**ABSTRACT**


Bacterial diseases of orchids continue to be serious problems. Bacterial strains were isolated from orchid plants exhibiting disease symptoms in Hawaii. Small to large leaf spots with or without water-soaking or soft rots were observed on various orchid genera, including *Dendrobium*, *Oncidium*, and *Miltonia* spp. and hybrids. Bacteria isolated and cultured from the lesions were tentatively identified using analytical profile index (API) strips and standard physiological and biochemical tests, and confirmed by species-specific polymerase chain reaction and sequencing of the 16S rRNA gene. The variation in pathogenic, morphological, cultural, and molecular characteristics of the orchid isolates also was evaluated. In our studies, a gram-negative, aerobic, rod-shaped bacterium that produced pale yellow, opaque, round colonies with entire margins on nutrient broth yeast extract agar (NBY) was isolated consistently from diseased orchid plants. On yeast dextrose calcium carbonate agar, the isolates produced brownish-yellow, nonmucoid colonies, with the majority of the strains secreting a diffusible yellow or tan pigment into the media. The bacterium was identified as *Burkholderia gladioli*. Molecular analysis indicated very little diversity in the 16S rDNA gene. Testing *B. gladioli* isolates using media containing copper or streptomycin indicated varying levels of resistance (copper resistant, 

By *Erwinia carotovora* subsp. *carotovora* and *E. chrysanthemi*, brown rot caused by *E. cypripedii*, and corn and basil leaf rot caused by *Acidovorax caviae* (25). In Nongkham, Thailand, the losses of *Dendrobium* orchids due to bacterial diseases were estimated at 50% or more (6), and the primary pathogen was *Burkholderia gladioli* (formerly *Pseudomonas gladioli* and *P. marginata*). *B. gladioli* was described originally as a phytopathogen on *Gladiolus* spp., causing rot of gladiolus corms (18); other hosts include onion, iris, and freesia (9). *B. gladioli* has been reported as a pathogen of *Dendrobium* orchids in Hawaii (30), but the occurrence, the genetic diversity, and the extent of the bacterium’s ability to cause severe rot on various orchid genera remains unknown. The objectives of this research were to determine the extent of *B. gladioli* as a causal agent of orchid disease in Hawaii, to determine the effectiveness of current molecular techniques in identifying and distinguishing *B. gladioli* from other bacteria, and to determine the levels of copper and streptomycin resistance present in the population. To our knowledge, this is the first study conducted to evaluate the importance of *B. gladioli* as a pathogen on orchids in Hawaii and the effectiveness of chemicals used by growers for bacterial disease control.

**MATERIALS AND METHODS**

**Field survey and sampling.** From May 2002 to February 2004, leaves were collected from various orchid genera with leaf spot or rot symptoms from 48 farms located on the windward side of the island of Hawaii. Samples were collected during farm visits and from disease samples submitted by growers. Digital photographs of leaf symptoms were recorded using a Nikon Coolpix 995 model digital camera. Typical symptoms were noted and described.

**Isolation and identification of the pathogen.** Diseased samples were processed within 24 h of the collection. Small pieces of leaf tissue were cut from the edge of the advancing portion of a lesion, surface sterilized in 10% bleach for 2 min, rinsed in sterile distilled water, and macerated in a sterile mortar or petri dish with approximately 2 ml of sterile water. A 20-µl sample was streaked onto plates containing nutrient broth yeast extract agar (NBY; 31) supplemented with a filter-sterilized (0.22-µm pore size, nitrocellulose filter) solution of cycloheximide at 50 µg/ml. Plates were incubated at 28°C. Individual bacterial colonies from plates were purified and stored at −80°C in 15% aqueous glycerol, and working cultures were kept on NBY agar. For all isolates, gram reactions were determined by the lysis of bacteria in 3% KOH (9), cytochrome C oxidase was tested by using filter paper impregnated with NNN′-tetramethyl-p-phenylene diamine dihydrochloride (17), oxidative or fermentation metabolism was evaluated (12), pyocyanin production was tested on King’s Medium B (KMB; 15), and growth on selective media was compared (yeast dextrose calcium carbonate [YDC]; 34) and Miller Schrøth (MS; 20). *Burkholderia* spp. initially were identified using analytical profile index (API) 20NE test strips (bioMérieux, Inc., Durham, NC) incubated at 30°C for 24 to 48 h. The API 20NE test strips combine conventional and assimilation tests for the identification of nonenteric gram-negative rods. *B. gladioli* strains were characterized and compared with the results found in the API (bioMérieux, Inc.). Information from Ber-
were conducted twice.

Pathogenicity tests. Bacteria isolated were grown on NBY agar for 48 h. Healthy orchid plants of the genera Phalaenopsis, Dendrobium, Miltonia, Oncidium (approximately 2.5 years old in 10-cm pots), and Cattleya (approximately 3 years old in 10-cm pots) were used for the inoculation tests. To fulfill Koch’s postulates, individual orchid leaves were inoculated (multiple inoculations on two leaves) by wounding with a sterile toothpick smeared with B. gladioli. Sterile water was used for control inoculation. The inoculated plants were covered with plastic bags or placed in a dew chamber in the laboratory at 24°C with 70% relative humidity for 48 to 72 h. Disease development was evaluated beginning at 2 to 3 days after inoculation by measuring the length of the water-soaked lesion that formed at the inoculation site. Lesion development was monitored for 14 days before bacteria were resolated.

The ability of the B. gladioli orchid strains to cause rot on alternate hosts also was determined. Slices of carrot and onion approximately 1.25 cm thick were surface disinfested by immersion in 10% bleach for 2 min, air dried in a laminar flow hood, and placed in petri dishes containing moistened filter paper. The center, outer surfaces of the carrot and onion slices were inoculated with the B. gladioli strains as indicated above and incubated at 28°C in the dark. A necrotic zone or soft rot lesion at least 2 mm beyond the inoculation site was considered evidence of infection for all pathogenicity tests. Bacteria were reisolated on NBY from tissues near the infected areas to confirm the identity of the pathogen. Additional inoculation experiments were conducted twice.

Cloning and sequencing of 16S rRNA. Molecular methods were used to confirm identification of the strains and allow for phylogenetic analysis. Template DNA was prepared by picking individual bacterial colonies from agar plates using a sterile toothpick and resuspending in 50 µl of sterile distilled water. The suspension was 10-fold diluted filter paper. The center, outer surfaces of the carrot and onion slices were inoculated with the B. gladioli strains as indicated above and incubated at 28°C in the dark. A necrotic zone or soft rot lesion at least 2 mm beyond the inoculation site was considered evidence of infection for all pathogenicity tests. Bacteria were reisolated on NBY from tissues near the infected areas to confirm the identity of the pathogen. Additional inoculation experiments were conducted twice.

Polymerase chain reaction. Bacterial isolates were grown on NBY agar for 48 h. Healthy orchid plants of the genera Phalaenopsis, Dendrobium, Miltonia, Oncidium (approximately 2.5 years old in 10-cm pots), and Cattleya (approximately 3 years old in 10-cm pots) were used for the inoculation tests. To fulfill Koch’s postulates, individual orchid leaves were inoculated (multiple inoculations on two leaves) by wounding with a sterile toothpick smeared with B. gladioli. Sterile water was used for control inoculation. The inoculated plants were covered with plastic bags or placed in a dew chamber in the laboratory at 24°C with 70% relative humidity for 48 to 72 h. Disease development was evaluated beginning at 2 to 3 days after inoculation by measuring the length of the water-soaked lesion that formed at the inoculation site. Lesion development was monitored for 14 days before bacteria were resolated.

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Results from API 20NE strips and descriptions in Bergey’s Manual of Systematic Bacteriology (16) provided further evidence in support of the identification of the isolates.

**Pathogenicity tests.** Plants artificially inoculated with *B. gladioli* developed symptoms similar to the commonly observed field symptoms, indicating that *B. gladioli* strains are pathogenic to multiple orchid species. All *B. gladioli* strains tested caused lesions around the inoculation sites with necrosis around the wound. On *Cattleya* spp. and hybrids, *Dendrobium* spp., *Miltonia* spp. and hybrids, *Oncidium* spp. and hybrids, the water-soaked lesions appeared 1 to 2 days after inoculation, which expanded rapidly to resemble natural infection within 3 to 4 days after inoculation (Fig. 1E). On *Miltonia* and *Oncidium* spp., the brown lesions expanded rapidly and were surrounded by a water-soaked margin. Certain strains of *B. gladioli* (1BB1, 2, and OW4) were noticeably more virulent on certain orchid genera. For example, deep tissue-penetrating lesions were recorded in inoculated *Cattleya* samples. The inoculated site developed 6-mm dark-brown, sunken lesions that appeared to be superficial; however, when the leaf was dissected crosswise, brown discoloration of the tissue underneath the epidermal layer, measuring up to 29 mm from the inoculation site, was recorded. Reisolations from the artificially infected plants yielded pure cultures of *B. gladioli*. Bacterial identification was confirmed with API 20NE strips or PCR using *B. gladioli* species-specific primers, thus fulfilling Koch’s postulates. Control plants inoculated with a sterile toothpick never showed symptoms. When inoculation experiments were repeated, lesion sizes were virtually identical in size and shape for all *B. gladioli* strains and orchid genera tested. Lesions appeared within 1 to 2 days and continued to expand. Lesions on *Miltonia* and *Oncidium* spp. were surrounded by water soaking. For all experiments, high humidity after bacterial inoculation was critical for symptom development.

**Cross-inoculation studies.** The development of a necrotic zone or lesion of at least 2.0 mm beyond the inoculation site was considered positive for the sliced carrots and onions. The majority of the *B. gladioli* isolates produced a creamy white or light-brown lesion or soft rot when they were administered to the surface of the slices of carrot or onion (Fig. 2). Symptoms usually were visible within 3 days after inoculation and progressed as time elapsed. Some differentiation was observed on the slices between strains of *B. gladioli* from different orchid hosts. For example, on carrot, *B. gladioli* strain OW4 caused a soft rot that covered the width of the slice (data not shown), whereas strain OW5 caused a soft rot with visible necrosis (Fig. 2A). On onion slices, *B. gladioli* strain 5-1 caused a spreading, soft rot area (Fig. 2B), whereas strains OW61 and OW62 caused more localized lesions (data not shown). Control slices inoculated with a sterile toothpick did not show symptoms. When cross-inoculation studies were repeated, all *B. gladioli* strains re-

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**Table 1.** Sources of 18 strains of *Burkholderia gladioli* recovered from diseased orchid plants in Hawaii and used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host</th>
<th>Tissue source*</th>
<th>Location collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1BB1</td>
<td><em>Dendrobium</em> sp.</td>
<td>Leaf, S</td>
<td>Pahoa</td>
</tr>
<tr>
<td>5-1</td>
<td><em>Oncidium</em> sp.</td>
<td>Leaf, S</td>
<td>Pahoa</td>
</tr>
<tr>
<td>6-1</td>
<td><em>Oncidium</em> sp.</td>
<td>Leaf, S</td>
<td>Pahoa</td>
</tr>
<tr>
<td>Dean4-2</td>
<td><em>Oncidium</em> sp.</td>
<td>Leaf, S</td>
<td>Orchidland Estates</td>
</tr>
<tr>
<td>F</td>
<td><em>Miltonia</em> hybrid</td>
<td>Leaf, S</td>
<td>Mountain View</td>
</tr>
<tr>
<td>06023</td>
<td><em>Oncidium</em> sp.</td>
<td>Leaf, S</td>
<td>Kurtistown</td>
</tr>
<tr>
<td>1</td>
<td><em>Oncidium</em> sp.</td>
<td>Leaf, P</td>
<td>Hilo</td>
</tr>
<tr>
<td>2</td>
<td><em>Oncidium</em> sp.</td>
<td>Leaf, P</td>
<td>Hilo</td>
</tr>
<tr>
<td>3</td>
<td><em>Oncidium</em> sp.</td>
<td>Leaf, P</td>
<td>Hilo</td>
</tr>
<tr>
<td>4</td>
<td><em>Oncidium</em> sp.</td>
<td>Leaf, P</td>
<td>Hilo</td>
</tr>
<tr>
<td>OW2</td>
<td><em>Oncidium</em> hybrid</td>
<td>Leaf, O</td>
<td>Hakalau</td>
</tr>
<tr>
<td>OW3</td>
<td><em>Oncidium</em> hybrid</td>
<td>Leaf, O</td>
<td>Hakalau</td>
</tr>
<tr>
<td>OW3A2</td>
<td><em>Oncidium</em> hybrid</td>
<td>Leaf, O</td>
<td>Hakalau</td>
</tr>
<tr>
<td>OW4</td>
<td><em>Oncidium</em> hybrid</td>
<td>Leaf, O</td>
<td>Hakalau</td>
</tr>
<tr>
<td>OW5</td>
<td><em>Oncidium</em> hybrid</td>
<td>Leaf, O</td>
<td>Hakalau</td>
</tr>
<tr>
<td>OW61</td>
<td><em>Oncidium</em> hybrid</td>
<td>Leaf, O</td>
<td>Hakalau</td>
</tr>
<tr>
<td>OW62</td>
<td><em>Oncidium</em> hybrid</td>
<td>Leaf, O</td>
<td>Hakalau</td>
</tr>
<tr>
<td>OW7</td>
<td><em>Oncidium</em> hybrid</td>
<td>Leaf, O</td>
<td>Hakalau</td>
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</tbody>
</table>

* Tissue source: S = small potted plant, 4-in. square; P = potted plant, 5-in. round; O = orchid plant on an Oasis cube.

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**Fig. 2.** Symptoms of cross-inoculation studies of *Burkholderia gladioli* on alternative hosts. A, *B. gladioli* 5-1 on carrot. B, *B. gladioli* OW5 on onion. Pictures were taken 9 days after inoculation.
acted in a similar manner on carrot and onion slices. Symptoms of soft rot with or without necrosis took about 3 days to become visible. Results indicate that carrot and onion can serve as alternative hosts for more than 90% of the *B. gladioli* orchid strains.

**16S rRNA sequence analysis.** When subjected to PCR with primer pair Y1/Y2, which amplifies the variable region located at the 5′ end of the 16S rRNA gene, all suspected *B. gladioli* isolates yielded an approximately 300-bp product (data not shown). Partial 16S rDNA sequences of the 18 suspected *B. gladioli* strains were obtained and deposited into the GenBank database with accession numbers DQ090070 through DQ090087. Both DNA strands were sequenced for verification. The partial 16S rRNA gene sequences of the *B. gladioli* strains were compared with gene sequences of known strains in GenBank. A BLAST search of the 16S rDNA sequences supported the morphological, physiological, and biochemical results that the isolates were *B. gladioli*. A multiple sequence alignment of the gene products was constructed using CLUSTALX. The 18 strains, except for a few nucleotide differences, were virtually identical. Phylogenetic trees were produced from the analysis of the aligned sequences of the 16S rDNAs generated in this study (Fig. 3). The tree indicated that, although overall genetic diversity is low, diversity does exist even among populations isolated from a single location (i.e., OW strains).

**Species-specific PCR.** The identity of the isolated bacterial strains also was confirmed using *B. gladioli*-specific PCR (32). An approximately 700-bp amplified fragment of the 23S rRNA gene was obtained for all *B. gladioli* strains tested (Fig. 4). No amplified fragment was observed with the other organisms tested. Thus, the species-specific-PCR test with LP1-LP4 could be used to accurately identify the *B. gladioli* isolates.

**Copper and streptomycin sensitivity determination.** A summary of the bactericide-resistant *B. gladioli* strains is presented in Table 2. Growth of bacteria on MGYCu (50 µg/ml) or MGYSm (25 µg/ml) was sufficient to identify strains with resistance to copper or streptomycin because copper- or streptomycin-sensitive strains of *B. gladioli* did not grow on these media. Resistance to streptomycin was widespread among strains of *B. gladioli* isolated from orchids in this study. Of 16 strains tested, 94% grew on MGY amended with streptomycin sulfate at 50 µg/ml. Copper-resistant (Cur) strains were not as prevalent as antibiotic-resistant strains, but a high percentage of resistance was evident (75% of the strains) and resistance to copper occurred over a much greater range. The MIC of cupric sulfate varied among Cu′ strains from 50 to 1,000 µg/ml, indicating a broad range of resistance to the copper salt. The MIC of streptomycin was 50 to 100 µg/ml for all strains tested. Interestingly, those strains that were resistant to copper also were resistant to streptomycin sulfate. The MICs remained constant for all *B. gladioli* strains when bactericide-sensitivity experiments were repeated.

![Fig. 3.](image1)

**Fig. 3.** Phylogenetic tree showing the relationship between *Burkholderia gladioli* strains isolated from orchids. The tree was constructed based on the fragment amplified of the 16S rDNA, using the neighbor-joining algorithm (24). A bootstrap analysis was performed with 100 repetitions.

![Fig. 4.](image2)

**Fig. 4.** Ethidium-bromide-stained 0.8% agarose gel displaying the 700-bp amplification product of the 23S rRNA gene from *Burkholderia gladioli* using species-specific polymerase chain reaction primers. Lanes 1 to 5 are different strains of *B. gladioli* isolated from orchids. Lane 1, strain 1; lane 2, strain 2; lane 3, strain 3; lane 4, strain 4; lane 5, 1BB1. Lane 6 is a *B. cepacia* strain which served as a negative control; M = Invitrogen 1-kb DNA ladder.
DISCUSSION

In the United States, more than 130 plant diseases are reported to affect orchids. In Hawaii, orchid diseases caused by bacteria are not well characterized; however, they can be considered as persistent, with the potential to cause large economic losses if not controlled early. The objectives of this study were to identify the common symptoms, determine the predominant disease-causing agent, evaluate the potential host range of the strains, and determine whether bactericide resistance exists in these strains. Throughout the study, B. gladioli was isolated consistently from surface-sterilized plant tissue showing water-soaked lesions, indicating that the pathogen can survive on the leaf surface as an epiphyte. Over a period of 18 months, B. gladioli was detected in more than half of the diseased samples tested using traditional and molecular techniques. Strains of B. gladioli isolated from orchids were similar to each other in most biochemical reactions evaluated (oxidase test, assimilation of glucose, arabinose, man- nose, mannitol, gluconate, caprate, and malate), and were similar to descriptions of the bacterium in Bergey’s Manual of Systematic Bacteriology (16) and the type strain used in the API 20NE test from bioMerieux. All strains of B. gladioli produced similar-looking colonies on NBY, YDC, MS, and KMB; however, one strain (OW61) produced a faint purple pigment on NBY. Whitby et al. (32) successfully developed a procedure for the identification of B. gladioli using species-specific PCR primers. This primer pair was used in this study and was able to differentiate B. gladioli from other bacteria, including the closely related species B. cepacia, which was included as a negative control.

Koch’s postulates were fulfilled for all 18 strains of B. gladioli, and symptoms produced on artificially inoculated samples were virtually identical to those seen in collected field samples (Fig. 1C and E). The host range of the pathogen also was determined and more than 90% of the strains produced symptoms on five different orchid genera (Phalaenopsis, Miltonia, Cattleya, Dendrobium, and Oncidium) as well as on onion and carrot (Fig. 1E). These results indicate the potentially damaging nature of this organism.

In the 16S rRNA gene sequencing, the primer pair Y1/Y2 amplified a hypervariable region of the 16S rRNA gene which allows for phylogenetic comparison of close relatives. This study identified the genotypic diversity of B. gladioli strains that cause disease on orchids in Hawaii. Cluster analysis showed that the B. gladioli groups converged to the same origin and were not derived from each other, suggesting the divergent evolution from a common ancestor. The homogeneity of the strains based on genetic, biochemical, and physiological evidence also strongly supports a clonal origin of the strains tested. In spite of the relatively short length of the sequences, the phylogeny could be constructed to show diversity that was not based on host or location (Table 1; Fig. 3).

Jensen et al. (13) developed universal primers and conditions for amplifying the intergenic transcribed spacer (ITS) regions from all prokaryotes, and these primers have been used to identify both human (11,19) and plant (10) pathogens. The multiplex 16S-23S ITS, which separates the rRNA genes, exhibits a greater sequence and length variation and will be used for additional analysis.

Other factors may contribute to the onset of bacterial diseases in Hawaii, including insect and snail vectors causing wounds for bacterial entry and colonization (data not shown); a warm, humid environment; low light levels; contaminated water, media, and supplies; and contact between plants (30). Weeds within or near farms also could serve as alternative hosts. In a study conducted by Norman et al. (21) over a period of 1 year, irrigation and storm water runoff (using recycled water) were identified as a potential source of introduction and concentration of soft rot Erwinia populations to tropical foliage crops such as Dieffenbachia, Syngonium, and Aglaonema spp., resulting in rapid spread and extensive crop loss. Burkholderia spp. can occupy diverse ecological niches, from the soil, water, and the rhizosphere of plants, to humans, animals, and even the hospital environment (7).

The results of this study indicate that copper and streptomycin resistance is widespread among strains of B. gladioli causing rot on orchids in Hawaii. The MIC was designated as the concentration of copper sulfate or streptomycin which prevented growth of the culture after a 72-h incubation at 28°C. Levels of resistance were in the normal range commonly recommended for use in disease control. Even though little streptomycin has been used over the past 10 years to control bacterial diseases in ornamentals in Hawaii (K. T. Sewake, unpublished information), resistant strains still are readily isolated. This is similar to findings for Pseudomonas cichorii isolated from celery seedbeds in Florida (22). Even though little streptomycin has been used in the past 25 years to control bacterial blight of celery in the Everglades Agricultural Area of Florida, resistant strains still are readily isolated (22). This phenomenon also was seen in studies by Sundin and Bender (27). Results in their studies indicated that resistance to copper and streptomycin by P. syringae pv. syringae associated with pear trees remained even though the bactericide selection was withdrawn (27).

Copper sometimes is used as a fungicide for disease control on orchids. This practice can contribute to the resistance found in phytopathogenic bacteria. Some of the copper-resistant strains of B. gladioli that were isolated formed bright blue colonies when grown on media containing high concentrations of copper (data not shown). The blue colonies of resistant bacteria suggest that cellular copper sequestration may play a role as a mechanism of copper resistance (8). In Xanthomonas campestris pv. vesicatoria and P. syringae pv. tomato, copper tolerance is encoded on a gene cluster located on a self-transmissible plasmid (3,4,26) that could be transferred to other phytopathogenic bacteria. The plasmids also could harbor other genetic determinants that are beneficial to the bacteria, making them harder to control (27). To our knowledge, this is the first report of copper and streptomycin resistance among populations of B. gladioli in Hawaii. The inadvertent selection of copper-resistant strains from continuous application of copper-based bactericides by growers could be a major reason for the failures in controlling the bacterial disease in many plant-production areas (4). Our results show similarities to the copper-resistant strains of P. syringae isolated from mango orchards after repeated treatments with Bordeaux mixture (5).

The results of this study indicate that B. gladioli is a commonly found pathogen on orchids in Hawaii, with the potential to cause serious damage if not monitored and controlled. Its widespread nature, as indicated by the consistent isolation from different farms over a period of more than a year, may contribute to its ability to cause problems not only on orchids but also on unknown alternate hosts under the right environmental conditions. Because of limited available bactericides and the presence of genetic elements which confer copper and antibiotic resistance, prevention of plant disease caused by B. gladioli using consistent practices such as sanitation and roguing is a much better option than chemical control once the problem has presented itself. The molecular and physiological background and adaptability of Burkholderia spp. are largely unknown (7).

Table 2. Copper- and streptomycin-resistant strains of Burkholderia gladioli recovered from orchid plants in Hawaii

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Cupric sulfate</th>
<th>Streptomycin</th>
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<tr>
<td>0</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>50</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
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<td>500</td>
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<tr>
<td>750</td>
<td>7</td>
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</tr>
<tr>
<td>1,000</td>
<td>1</td>
<td>nd</td>
</tr>
</tbody>
</table>

* nd = Not determined.
The correct microbial identification and the determination of virulence factors of the bacterium will provide essential information for risk assessment and infection control (7).

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