Head Tilt in Immunodeficient Mice Due to Contamination of Drinking Water by *Burkholderia gladioli*

Chereen Collymore,^{1,2} Frank Giuliano,¹ and E Kate Banks^{1,3,*}

Immunodeficient mice in multiple holding rooms presented with head tilt, circling, spinning when picked up by the tail, dehydration, and lethargy. *Burkholderia gladioli*, a plant pathogen, was identified as the causative agent. Environmental testing revealed the presence of *B. gladioli* within the automatic watering system, water bottles, and sipper tubes. Here we describe steps taken to reduce the presence of this organism within the automatic watering system and water bottles. Facilities housing immunodeficient mice should take measures to minimize the accumulation of biofilm within their water-supply systems.

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Burkholderia gladioli is an aerobic to microaerophilic, motile, nonfermenting, gram-negative rod bacterial species that is primarily known as a plant pathogen.⁸ This organism is found in soil, in environmental water, and on plants and fruits.⁶ *B. gladioli* has emerged as the important cause of pulmonary infection in patients with cystic fibrosis in the United States, where it is now the 3rd most prevalent species associated with infection (comprising 15% of *Burkholderia* spp. infections in these patients).^{7,15} In addition, *B. gladioli* has been associated with fatal infection in cystic fibrosis patients undergoing lung transplantation.^{2,13} This commensal organism can cause pneumonia, bacteremia, chronic granulomatous disease and may ultimately lead to the death of immunosuppressed patients. Nosocomial infections are most often associated with contaminated water sources.⁶

Contemporary laboratory animal facilities house increasing numbers of immunodeficient mice for biomedical research. These mice are particularly susceptible to commensal agents of various origins. A previous outbreak of otitis media caused by *B. gladioli* in immunodeficient mice was reported in 2004 from a facility in Virginia.⁵ A similar outbreak was also described in a facility in France in 2007 involving a related bacteria, *Ralstonia pickettii*.¹ The source of infection could not be determined for these previously reported outbreaks.^{1,5} However, we here demonstrate that *Burkholderia* and *Ralstonia* spp. can be isolated from automatic watering systems, water bottles, and water-bottle sipper tubes.

Case Report

Clinical history. Four female CB17.Cg-Prkdc^{scid}Lyst^{tg}-J/Crl (SCID Beige) mice (Charles River Laboratories, Wilmington, MA) presented with head tilt, circling, rolling, weight loss, hunched posture, dehydration, and spinning when picked up by the tail. Within 2 wk, 14 additional immunodeficient mice

(SCID Beige and NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ [NSG]; The Jackson Laboratory, Bar Harbor, ME) from 3 investigators in 2 separate rooms within the same suite presented with similar clinical signs. A month after the initial 18 cases, additional immunodeficient mice from the same colonies ($n = 11$) and a new colony of C;129S4-Rag2^{tm1.1Flv} Il2rg^{tm1.1Flv}/J mice (Il2rg/Rag2 double-knockouts, bred inhouse; $n = 8$) in a 3rd room presented with similar clinical signs. Some Il2rg/Rag2^{-/-} mice presented with hunched posture, abnormal gait, lethargy and dehydration without ataxia.

Housing. Mice are housed in a SPF facility under barrier conditions consisting of autoclaved filter-top IVC (Allentown Caging, Allentown, NJ), corncob bedding (Anderson's Lab Bedding, Maumee, OH), cotton square nesting material (Nestlets, Ancare, Bellmore, NY), huts (Bio-Serv, Flemington, NJ), and irradiated rodent diet (2018, Envigo, Indianapolis, IN). Rooms are maintained at 20 to 22 °C, 50% to 70% humidity, and a 14:10-h light:dark cycle. All personnel handling mice must wear dedicated gowns, gloves, and face masks. Cages are opened within HEPA-filtered laminar flow biosafety cabinets (Class II, Type A2, Nuaire, Plymouth, MN). Cage changing is performed biweekly, and mice are transferred by using forceps soaked in a high-level hydrogen peroxide disinfectant (PREempt, Virox Technologies, Oakville, Ontario, Canada). The hydrogen peroxide disinfectant is also used by all researchers handling the mice. Housing and care are in accordance with the Canadian Council on Animal Care standards and policies, Ontario Animals for Research Act, and University of Toronto guidelines. Animals within the facility are on protocols approved by the Faculty of Medicine and Pharmacy Animal Care Committee. The University of Toronto is certified for Good Animal Practice in Science from the Canadian Council on Animal Care.

Automatic watering system. Municipal water treated by reverse-osmosis deionization, activated charcoal filtration, UV sterilization, and acidification (pH 3.0) is supplied to the ventilated racks through a recirculating automatic watering system (RECIRC, SE Lab Group, Napa, CA). The system (Figure 1) includes a 200-gallon polyethylene reservoir tank and 1/2-in. polyvinylchloride supply and return distribution system equipped with recoil hoses and quick-disconnects to

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¹Division of Comparative Medicine, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada; ²Animal Care and Veterinary Services, University of Ottawa, Ottawa, Ontario, Canada; and ³Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada

*Corresponding author. Email: k.banks@utoronto.ca

Figure 1. Schematic of the automatic watering system.

ventilated rack manifolds. Charcoal-filtered reverse-osmosis-treated municipal water is pumped into the storage reservoir. This processed water is acidified in the reservoir and then gravity-pumped through the UV sterilizer before entering the distribution system. Water is delivered from the rack manifold to the cage level through a drinking valve mounted in a cage grommet (SE Lab Group). All water remaining in the system after consumption by animals or rack flushes is returned to the tank for redistribution. A prefilter is located between the UV sterilizer and the reservoir tank.

Colony health monitoring. Health monitoring is based on a quarterly dirty-bedding sentinel program. Sentinel mice (Crl:CD1(ICR), Charles River Laboratories) are considered free of the following viral, bacterial, and parasitic agents (IDEXX Bioresearch, Columbia, MO): ectromelia virus, epizootic diarrhea of infant mice, lymphocytic choriomeningitis virus, mouse hepatitis virus, mouse parvovirus, minute virus of mice, pneumonia virus of mice, reovirus 3, Sendai virus, Theiler murine encephalomyelitis virus, *Mycoplasma pulmonis*, *Syphacia obvelata*, *Aspicularis tetraptera*, *Myobia musculi*, *Myocoptes musculus*, *Radfordia ensifera*, *Radfordia affinis*, and *Polyplax* spp. Regular screening for bacterial pathogens is not performed. *Helicobacter* spp., *Pasteurella* spp., and mouse norovirus are considered enzootic in the facility. Water-quality testing (Gelda Scientific, Mississauga, Ontario, Canada) is performed quarterly; values lower than 1 cfu/mL are considered acceptable.

Necropsy and microbiologic analyses. Affected mice were euthanized by CO₂ asphyxiation followed by cervical dislocation. Gross lesions observed on necropsy included purulent material within the ear canal and generalized random, multifocal, pinpoint (2 mm) white foci on the liver surface (Figure 2). All other tissues appeared normal. Sterile swabs (FisherFinest Transport Swab, Fisher HealthCare, Pittsburgh, PA) were used to collect ear-canal specimens for microbial analysis and *Mycoplasma* spp. PCR testing at a diagnostic laboratory (IDEXX Bioresearch, Columbia, MO). Bacterial isolates were identified by using MALDI-TOF MS (Microflex LT, Bruker Daltronics, Billerica, MA). Many clinically affected animals often displayed similar gross liver lesions or purulent material within the ear canal (or both). In addition, one mouse had a liver abscess, which was sampled for bacterial culture by using a sterile swab. The head, spleen, liver, kidneys, and lungs of 2 mice were collected, fixed in 10% neutral buffered formalin, and sent to a veterinary pathologist for histopathologic evaluation. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (Animal Health Laboratory, University of Guelph, Guelph, Ontario).

Results

Microbiology. Samples collected from the ear canals and livers of several mice were positive for *B. gladioli*. Of the total number of cases ($n = 37$) of head tilt, ataxia or wasting observed during

Figure 2. Gross necropsy. (A) Purulent material within the left ear canal. (B) Generalized random, multifocal, pinpoint (2 mm) white foci on the surface of the liver.

the initial 10 wk of the outbreak, 5 of the 9 samples submitted for culture were confirmed positive for *B. gladioli*. *Mycoplasma* spp. was not detected in any sample.

Histology. Histologic sections of filter organs demonstrated the presence of septicemia. Microabscesses with neutrophils and macrophages were present within the spleen, kidneys, and liver. Necrosuppurative otitis was present, with infiltration of numerous neutrophils and some macrophages, which even extended into the brain cavity in one mouse. The lungs were normal in all mice.

Clinical outbreak management. All clinically affected mice were euthanized by CO₂ asphyxiation followed by cervical dislocation. Culture and sensitivity tests indicated *B. gladioli* that was susceptible to trimethoprim-sulfa (TMS) in addition to various other antimicrobial agents. We attempted to treat 6 cages of SCID Beige mice ($n = 9$) with 5 mL TMS (40 mg trimethoprim and 200 mg sulfamethoxazole/5 mL, Novo-Trimel, Teva Canada, Toronto, Ontario, Canada) in 250 mL water for 1 wk; however, treatment was unrewarding. All of the mice progressed to barrel-rolling in the cage without any stimulation or lost body condition (or both); all 6 cages of mice were euthanized. In addition, we attempted to identify subclinically infected cage mates ($n = 5$) by using oropharyngeal swabs, no growth other than scant normal respiratory flora was observed.⁵

Environmental testing. We conducted extensive environmental sampling in an effort to determine the source of the outbreak. To this end, we sampled food, bedding, cage surfaces, biosafety cabinets, and various locations within our automatic watering system, including the manifolds on racks housing clinically affected mice and the system prefilter. We focused on low-flow locations within the automatic watering system, which were presumed to favor biofilm formation. Samples collected were sent to the same commercial diagnostic company that initially identified *B. gladioli* in the ear canal samples.

Environmental testing results. Environmental sampling revealed the presence of *Burkholderia* spp. and *Ralstonia* spp. within various portions of the automatic watering system, including within the region of decreased to absent water flow at the end of the system (that is, the dead leg piping), on a rack manifold, and in and around the prefilter. The antibiotic resistance profile of the *Burkholderia* spp. identified on a rack manifold matched that of the *B. gladioli* isolated from clinically affected mice. No other samples submitted were positive for *B. gladioli*.

Management of automatic watering system. After receiving the environmental testing results, we updated our water quality assurance program (Figure 3). All immunodeficient mice were given autoclaved water bottles (Ancare). Disinfection of the entire water distribution system was performed to reduce biofilm formed by *B. gladioli*.

After these changes were implemented, cases of head tilts fell dramatically over the next 2 mo. However, sporadic cases ($n = 5$) continued to appear. After further investigation, we were able to trace some cases back to mice that were offspring of clinically affected animals. New breeders were purchased and immediately placed on water bottles to prevent possible infection.

Despite these interventions, a few cases ($n = 3$) developed in adult NSG mice that had access only to water bottles appeared. Sterile swabs were collected from inside sipper tubes and water bottles and sent for bacterial culture. Results revealed that *B. gladioli* was present within biofilms inside the sipper tubes and water bottles. In response, the sanitation procedures for our sipper tubes and water bottles were modified (Figure 3).

For nearly 1 y, no cases developed, until sporadic cases ($n = 8$) reoccurred in a colony of NSG mice. This outbreak was traced to the autoclave cycle, when multiple biologic indicators (MagnaAmp, Fisher Scientific, Hampton, NH) were positive for growth (that is, failed validation). Previous quality control had used chemical indicators, which were calibrated to yield a color change in response to 121 °C steam for a 15-min exposure time (Steraffirm Control Tubes, Steris, Mentor, OH). The steam source of the autoclave was validated and found to be within normal limits. Similarly, testing of the air removal during the prevacuum was found to be effective (Steraffirm Bowie-Dick test, Steris, Mentor, OH). Next, the autoclave cycle was validated (Asepsys, Mississauga, Ontario, Canada) through thermocouple testing using 10 thermocouples positioned at various locations within the load estimated to be most difficult for steam to penetrate. Heat penetration testing revealed that the use of microisolation cages to contain water bottles was impeding steam penetration, leading to unacceptably long cycles to achieve adequate lethality. We replaced the microisolation cages with autoclavable bags; bottles in bottle baskets were placed inside the bags. Heat penetration testing was then repeated. The final cycle length (121 °C for 90 min) was chosen to achieve a 12-log reduction in bacteria. No further cases have appeared in the 6 mo since implementing these changes.

Discussion

We were able to isolate *Burkholderia* spp. from various components of our automatic watering system and water bottles. *B. gladioli* can survive reverse-osmosis treatment, UV sterilization and acidification to persist in water lines. *B. gladioli* belongs to the rRNA group II pseudomonads, which includes the genera *Ralstonia*, *Cupriavidus*, *Pandoraea*, *Brevundimonas*, *Comamonas*, *Delftia*, and *Acidovorax*;⁶ this group of agents is found in water and soil.¹⁶ They can survive in a variety of environments, particularly when the growth of other bacteria is inhibited. *Burkholderia* spp.

Component of the system	Sanitation measures
Ventilated racks (every 3 mo)	1) Processed in a rack washer (9122 LS, Getinge, Rochester, NY): wash temperature, 60 °C; wash cycle, 2 min; final rinse, 90 °C for 1 min; total cycle, 20 min 2) Acidified detergent (Acid Power, Pharmacal Research Labs, Waterbury, CT) flushed through the watering manifold 3) Drained 4) Autowatering manifold then ozonated and drained 5) Rack autoclaved (autoclave temperature, 132 °C for 4 min; GE152222 ARB2, Getinge)
Coils from distribution system to ventilated racks (every 3 mo)	1) Sanitized in a closed loop (water temperature, 83 °C; Acid Power, Pharmacal Research Labs, Waterbury, CT) in the tunnel washer 2) Ozonated
Automatic watering distribution system (every 6 mo)	1) All ventilated racks disconnected from the water supply (including coil from distribution system to rack) 2) Dilute peracetic acid-hydrogen peroxide solution circulated through system; validated by using 1% Minncare (Cantel Medical, Little Falls, NJ) test strips to have achieved an adequate concentration (between 2% and 3%) of peracetic acid-hydrogen peroxide throughout the system 3) System pressurized to 20 lb for 2h 4) Pressure released and water allowed to circulate for 2 h 5) System flushed with reverse-osmosis-treated water
Sipper tubes	1) Disinfected every 3 mo with a 2-h soak in 2% Minncare 2) Sonicated in 2% Minncare for 20 min at 35 °C between uses 3) Double-rinsed 4) Sanitized in tunnel washer (water temperature, 83 °C; Acid Power [Pharmacal Research Labs, Waterbury, CT]; 3236 LS, Getinge) 5) Autoclaved
Water bottles	1) Manually scrubbed once (at the beginning) to remove biofilm, then soaked in 2% Minncare 2) Passed through the tunnel washer between uses 3) Autoclaved

Figure 3. Updated water sanitation program.

are important opportunistic nosocomial pathogens because they are often resistant to antibiotics and disinfectants.¹⁶ Currently there is no evidence for horizontal transmission in people or animals; most patients are infected by contaminated water sources.^{6,7,16}

Previous authors have identified numerous bacteria within the manifolds of automatic watering systems.^{9,10} These bacteria reside within biofilms on system surfaces. *Burkholderia* spp. can survive harsh environments, due to their metabolic versatility, production of multiple virulence factors and ability to form biofilms.⁸ Biofilms comprise communities of bacteria embedded in a self-generated extracellular matrix made primarily of polysaccharides, proteins, and nucleic acids. This extracellular matrix helps bacteria to survive hostile, low-oxygen environments.⁸ Biofilms are believed to be involved in about 65% of human infections.¹² Biofilms render bacteria resistant to antibiotics as well as to innate immune defenses such as phagocytosis.⁴

We were able to isolate bacteria within various portions of the automatic watering system. Culture of the prefilter is a novel and economical way to screen for bacteria that may be present in incoming water. Interestingly, samples of the incoming water itself were culture-negative for *Burkholderia* spp. The media that we used to isolate the bacteria from the water and automatic watering system included blood agar, Sabouraud dextrose,

and Tyes. Although both *Ralstonia* and *Burkholderia* spp. were isolated from the prefilter and plastic housing of the filter, we were unable to specifically isolate *B. gladioli*, the agent associated with our clinical cases of head tilt. Of note, both *Ralstonia* and *Burkholderia* spp. have been established as important opportunistic nosocomial pathogens because of their ability to survive in aqueous environments.⁶

Many methods have been proposed to remove biofilm and sanitize automatic watering systems.^{3,9,10} The first is flushing and autoclaving of ventilated racks. We increased the flushing of rack manifolds in animal rooms from once weekly to twice weekly. Racks were put on a quarterly autoclave schedule, as mandated by our standard operating procedure. With this approach, we observed a considerable reduction (from 37 cases over a 10-wk period to 7 cases over the subsequent 10-wk period) in cases of ataxia, head tilt, and similar presentations in our immunodeficient mice. However, we were unable to eliminate these cases with this approach alone.

Another method of sanitizing automatic watering systems includes the use of hyperchlorination and a combination of peracetic acid and hydrogen peroxide (Minncare, Cantel Medical, Little Falls, NJ).¹⁰ We chose 2% Minncare for sanitizing our automatic watering distribution system twice yearly. We were thus able to further reduce cases of ataxia, head tilt, and similar presentations through this approach but were again unable to

completely eliminate the issue. We therefore elected to remove our immunodeficient strains from the automatic watering supply and to place them on autoclaved water bottles.

A year after the initial outbreak, several cases of head tilt were observed in immunodeficient mice on autoclaved water bottles. Culture of sipper tubes and water bottles revealed *B. gladioli*. Since we instituted new procedures to sanitize water bottles and sipper tubes and revalidated our autoclave cycle for water, we have not noted any new cases of ataxia, head tilt or similar presentations. In reviewing various quality-assurance procedures for sterilizing water bottles, we consulted with numerous colleagues, many of whom reported the use of chemical indicators as sole indicators of adequacy of sterilization; our findings caution against this practice. Periodic revalidation using biologic indicators is required to ensure that autoclaves are achieving adequate sterilization.

A few clinical cases occurred in mice that were weaned from dams that succumbed to infection after weaning of the litter. The dams were clinically normal at the time of weaning. The offspring only had access to autoclaved water bottles (prior to autoclave failure). Two possible sources of infection for these weanlings include the cage water bottle or transmission from the dam's milk. We did not confirm which scenario may have been the source of infection. The transmission in humans of *Burkholderia pseudomallei* through contaminated milk has been described, thus indicating that this property may be inherent to the genus.¹⁴ Whether other bacteria in this group can transmit to offspring in rodents is unknown.

Vestibular syndromes due to bacterial infection have been associated with *Mycoplasma pulmonis*, *Pasteurella pneumotropica*, *Pseudomonas aeruginosa*, *Streptococcus* spp., and *Klebsiella oxytoca*.¹¹ These agents are controlled by acidification, chlorination, and UV sterilization. Given that none of these agents were isolated from either the animals themselves or environmental sampling, it is unlikely they were involved in causing the clinical disease observed.

In this case report, we describe a vestibular and wasting syndrome caused by *B. gladioli*. The source of contamination was incoming municipal water. Despite reverse-osmosis treatment, UV sterilization, and acidification, bacteria likely formed a biofilm within the automatic watering system. Regular flushing and autoclaving of ventilated racks reduced the incidence of infections, but they were not eliminated until we achieved complete decontamination of all components of the water supply system and revalidated our autoclave cycles. Regular sanitization and autoclaving of water bottles and sipper tubes were effective in reducing or eliminating the ability of this bacteria to form biofilms and infect immunodeficient mice. The prefilter of the automatic watering system may be an ideal substrate for screening for bacteria present within a water system.

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