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Non-toxigenic *Clostridium sordellii*: clinical and microbiological features of a case of cholangitis-associated bacteremia

Seth T. Walk[1,2], **Ruchika Jain**[1,2], **Itishree Trivedi**[1], **Sylvia Grossman**[4], **Duane W. Newton**[4], **Tennille Thelen**[1,3], **Yibai Hao**[1,3], **J. Glenn Songer**[5], **Glen P. Carter**[6], **Dena Lyras**[6], **Vincent B. Young**[1,2,3], and **David M. Aronoff**[1,2,3,*]

[1]The Department of Internal Medicine, University of Michigan Health System, Ann Arbor, MI, 48109, USA

[2]Division of Infectious Diseases, University of Michigan Health System, Ann Arbor, MI, 48109, USA

[3]The Department of Microbiology and Immunology, University of Michigan Health System, Ann Arbor, MI, 48109, USA

[4]Clinical Microbiology Laboratories, Department of Pathology, University of Michigan Health System, Ann Arbor, MI, 48109, USA

[5]Veterinary Microbiology and Preventive Medicine, Iowa State University College of Veterinary Medicine, Monash University, Victoria 3800, Australia

[6]Department of Microbiology, Monash University, Victoria 3800, Australia

Abstract

Toxigenic *C. sordellii* strains are increasingly recognized to cause highly lethal infections in humans that are typified by a toxic shock syndrome (TSS). Two glucosylating toxins, lethal toxin (TcsL) and hemorrhagic toxin (TcsH) are believed to be important in the pathogenesis of TSS. While non-toxigenic strains of *C. sordellii* demonstrate reduced cytotoxicity *in vitro* and lower virulence in animal models of infection, there are few data regarding their behavior in humans. Here we report a non-TSS *C. sordellii* infection in the context of a polymicrobial bacterial cholangitis. The *C. sordellii* strain associated with this infection did not carry either the TcsL-encoding *tcsL* gene or the *tcsH* gene for TcsH. In addition, the strain was neither cytotoxic *in vitro* nor lethal in a murine sepsis model. These results provide additional correlative evidence that TcsL and TcsH increase the risk of mortality during *C. sordellii* infections.

Introduction

Clostridial species have long been identified as causative agents of human disease, but the importance of these toxigenic bacteria has gained recent attention due to the emergence and spread of pathogens like *Clostridium difficile* [1] and its close relative *C. sordellii* [2, 3]. *C. sordellii* is a sporulating, anaerobic, gram positive bacterium that is often isolated from soil

*Corresponding author address: David M. Aronoff, M.D., 4618-C Medical Sciences Building II, 1150 West Medical Center Drive, Ann Arbor, MI 48109-5623, Phone: 734-615-3604, Fax: 734-763-4168, daronoff@umich.edu.

samples and sometimes causes highly lethal human infections [4]. This bacterium is known to cause gynecological infections following childbirth or abortion [5, 6], necrotizing soft tissue infections associated with the injection use of contaminated heroin [7–9], and postoperative infections complicating musculoskeletal transplants performed with contaminated graft material [10]. The incidence of *C. sordellii* infections is unclear, but an increasing number of cases has been reported over the past 10 years [11–15]. A recent report found that 1 in 200 deaths in women of reproductive age were associated with clostridial toxic shock, due to either *C. sordellii* or another clostridium, *C. perfringens* [3].

Perhaps two thirds of *C. sordellii* infections are associated with a clinically unique toxic shock syndrome (TSS), with mortalities exceeding 70% [4, 16]. However, this percentage may be overestimated, since it is likely that the most dramatic clinical cases (especially those associated with TSS) are reported, while infections that run a more benign course or have a positive outcome remain unpublished.

The pathogenesis of *C. sordellii* TSS has been a focus of recent studies [16, 17]. Though incompletely understood, the occurrence of TSS depends on the expression of one or both of the large glucosylating cytotoxins (TcsH and TcsL) of *C. sordellii*, which share structural and functional similarity to the large glucosylating cytotoxins of *C. difficile* (TcdA and TcdB, respectively). Both TcsH and TcsL intoxicate epithelial and endothelial cells by inactivating small GTPase proteins that are involved in maintaining cytoskeletal integrity [18–20]. Recent data suggest that *C. sordellii* TcsL is important for the development of TSS [17, 19], though almost nothing is known about the participation of TcsH.

Herein we present a case of invasive *C. sordellii* infection that was associated with neither TSS nor death. Molecular analyses demonstrated that this clinical strain lacked the *tcsH* and *tcsL* genes encoding TcsH and TcsL, respectively. This strain also lacked virulence in a mouse model of peritonitis. These data provide correlative support for the hypothesis that hemorrhagic and lethal toxins are important virulence determinants of the highly lethal and treatment-refractory TSS caused by *C. sordellii*. Knowledge gained from non-lethal *C. sordellii* infections such as this one provides new information regarding the pathogenesis of severe infections caused by this organism.

Materials and Methods

Institutional approval

This case report was reviewed and approved by the University of Michigan Institutional Review Board. Animals were treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

Animals

Eight-to-ten week old, female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Bacterial strains

C. sordellii strain HH-310 was isolated from anaerobic culture of a patient's blood sample (see below). The comparator strain ATCC9714 (*tcsL*$^+$, *tcsH*$^-$) was obtained from the American Type Culture Collection (Manassas, VA) and strain JGS6382 (*tcsL*$^+$, *tcsH*$^+$) was provided by Dr. J. Glenn Songer (Iowa State University).

PCR

DNA was extracted from *C. sordellii* strains HH-310, ATCC9714, and JGS6382 using an Easy-DNA™ extraction kit (Invitrogen, Carlsbad, CA). The taxonomic identity of HH-310 was verified using primers specific for a region of the *C. sordellii* 16S rRNA encoding gene [17]. Subsequent PCR for the *tcsL* gene encoding lethal toxin (TcsL) was performed using a previously reported primer pair (*tcsL* primer pair #3) and conditions [17]. New primers were designed to amplify an internal fragment of the *tcsH* gene encoding hemorrhagic toxin (TcsH) based on the genome sequence of the ATCC9714 strain which was obtained through Roche 454 Titanium genome sequencing (data not shown). Although this strain does not produce an active TcsH, it contains fragments of the *tcsH* gene at the appropriate genomic location downstream of (5') the *tcsL* gene. These remnants were used to design PCR primers. Specifically, these primers were: *tcsH*_F1 (DLP37): GTAAATAAAACACATTTAAGAGCTTTGG and *tcsH*_R1 (DLP38): GGAATTTATATATGATAGGCAAAATAGG). Amplification conditions (94° C denaturation for 10 min, then 35 cycles of 94° C for 30 sec, 55° C for 30 sec, and 72° C for 1 min 30 sec, followed by a final extension at 72° C for 5 min) were validated and optimized using DNA extracted from ATCC9714 and JGS6382 prior to assaying HH-310. All PCR amplicons were visualized using electrophoresis (0.8% agarose gels) and amplicon sizes were estimated using a 1 kb DNA ladder (Invitrogen, Carlsbad, CA).

Cytotoxicity assay

To assay for the production of toxins by strains JGS6382 and HH-310, the TechLab *C. difficile* Toxin/Antitoxin Kit (REF T5000, TechLab®, Blacksburg,VA) was used in conjunction with Vero cell monolayers according to the manufacturer's instructions (see package insert at http://www.techlab.com/product_details/t5000.shtml). This kit contains a toxin control reagent (positive control) and antitoxins that neutralize *C. sordellii* hemorrhagic and lethal toxins.. Strains of *C. sordellii* were grown anaerobically in 10 ml of sterile Reinforced Clostridial Medium (RCM, BD, Franklin Lakes, NJ) for 20 hr at 37° C. Cultures were filter sterilized using 0.2 μm nylon syringe filters (Fisher, Waltham, MA) and the resulting supernatants were assayed for the presence of toxins. Vero cells were cultured as reported elsewhere [21], with the single exception of Dulbecco's Minimal Essential Medium (DMEM) with High Glucose (Invitrogen, Carlsbad, CA) in place of minimal essential media (MEM alpha medium). All assays were performed in triplicate using a final *C. sordellii* culture supernatant dilution of 1:10. Treated Vero cells were fixed with formalin and stained with Wright-Giemsa Stain Mixture (Ricca Chemical Co.). Cell morphology was observed by microscopy on an Olympus 1X71 inverted microscope (20X magnification). A positive cytotoxic reaction was noted by rounding of the Vero cells compared to wells containing toxin antibodies.

Virulence experiments

Virulence experiments in mice were performed as previously described [17]. Briefly, *C. sordellii* strains HH-310 or JGS6382 were grown overnight in RCM broth and washed with PBS. Five mice each were then injected intraperitoneally with 100 μl PBS containing approximately 1×10^{10} CFU and 1×10^{8} CFU of HH-310 or JGS6382, respectively. Infection was allowed to proceed for 7 d and survival was recorded daily.

Results

Case report

An 81 year-old female presented with the acute onset of stabbing abdominal pain that emanated from her epigastrium and radiated to her right upper abdominal quadrant and

back. This was initially intermittent but became continuous and was exacerbated with inspiration. It was associated with fever and chills. The patient had a history of hypertension, diabetes mellitus, coronary artery disease, portal vein thrombosis and a congenital disorder causing non-obstructive dilation of intrahepatic bile ducts (Caroli disease).

As a result of the Caroli disease, the patient suffered repeated episodes of choledocholithiasis and cholangitis requiring multiple endoscopic retrograde cholangio-pancreaticogram (ERCP) procedures and stent placements. Ten months previously the patient was hospitalized with sepsis and cholangitis associated with *Klebsiella pneumoniae* and *Escherichia coli* bacteremia. Two recent episodes of right upper quadrant abdominal pain radiating to her back associated with fever were managed successfully as an outpatient with empirical, 10-day courses of levofloxacin.

Physical examination revealed a temperature of 37.9 °C, heart rate 137 beats per min, blood pressure 135/92 mm Hg and transcutaneous oxygen saturation of 92–94%. She had dry mucous membranes, and was tender to palpation in the epigastrium and right upper abdominal quadrant. Initial laboratory investigations showed a white blood cell count of 4.0 thou/μL (normal range 4.0–10.0 thou/μL) with 89.2% neutrophils (normal 36.0–75%), and a platelet count of 112 thou/μL (normal 150–450 thou/μL). Her serum creatinine was 1.0 mg/dL (normal 0.5–1.0 mg/dL), amylase 118 IU/L (normal 30–100 IU/L), lipase 62 U/L (normal 5–50 U/L), aspartate aminotransferase was 40 IU/L (normal 8–30 IU/L), alanine aminotransferase 17 IU/l (normal 7–35 IU/L), alkaline phosphatase 115 IU/L (normal 30–130 IU/L), total bilirubin of 0.8 mg/dL (normal 0.2–1.2 mg/dL) and serum lactate was 4.9 mmol/L (normal 0.5–2.2 mmol/L). The patient's blood coagulation studies were normal. An ultrasound revealed marked intrahepatic pneumobilia with a complex cystic region in the porta hepatis that contained shadowing echogenic foci, consistent with gas or stones.

Blood cultures were obtained and the patient was treated with intravenous fluids and empirical intravenous levofloxacin and metronidazole. These antibiotics were chosen in light of a history of severe allergic reaction to penicillin and sulfa drugs. Although the patient was initially hemodynamically stable apart from a tachycardia, she soon developed a high fever (temperature 39.6 °C), tachycardia (123 beats per min) and hypotension (blood pressure of 77/41 mmHg). The two sets of aerobic and anaerobic blood cultures drawn on admission grew *K. pneumoniae*, vancomycin sensitive *Enterococcus faecium* and *C. sordellii*. Repeat blood cultures were obtained and parenteral antibiotics were changed to vancomycin, aztreonam, and metronidazole. Her *K. pneumoniae* strain was sensitive to aztreonam. Management also included intravascular volume resuscitation. The patient's white blood cell count rose to 18.5 thou/μl and her coagulation parameters became abnormal, with an elevated prothrombin time of 14.3 sec (normal 9.5–11.7 sec) and a partial thromboplastin time of 37.6 sec (normal 21.0–30.0 sec). A repeat pair of aerobic and anaerobic blood cultures again yielded *K. pneumoniae*, *E. faecium*, and *C. sordellii*. An emergent ERCP revealed large amounts of biliary sludge at the hepatic duct bifurcation, and this sludge was removed by balloon extraction but not cultured.

The patient rapidly improved after this procedure and vasopressor agents were never required. Follow up blood cultures obtained three days after presentation were negative. The patient was discharged home and completed two weeks of treatment with parenteral vancomycin combined with oral levofloxacin and metronidazole. Despite ongoing problems with her Caroli disease, the patient remained free of bacteremia eight months after hospital discharge.

Characterization of the *C. sordellii* HH-310 strain

Genomic DNA extracted from the blood borne *C. sordellii* strain of the patient (named HH-310) was used as template for three separate PCR reactions. The first PCR (*C. sordellii*-specific 16S rRNA) was used to verify its correct taxonomic identification and resulted in a strong positive amplicon of the correct size (~944 kb, data not shown), verifying that HH-310 is indeed *C. sordellii*. Two additional PCRs were used to assay for the presence of *tcsL* and *tcsH*. Both PCRs successfully amplified toxin genes from the *tcsL*$^+$ *tcsH*$^+$ JGS6382 control strain, but failed to do so in HH-310, suggesting that these loci are not present in the genome of this strain (Figure 1). Consistent with PCR results, supernatant from an overnight culture of JGS6382 was highly toxic to Vero cell monolayers, while supernatant from overnight culture of HH-310 was not (Figure 2). Furthermore, C57BL/6J mice inoculated intraperitoneally with a large inoculum (1×10^{10} CFU per mouse) of HH-310 did not exhibit typical sickness behaviors (bradykinesia, huddling, ruffled fur, weight loss; not shown) and survived at least 7 days after infection (Figure 3).

Discussion

C. sordellii is an emerging pathogen associated with highly lethal bloodstream and soft tissue infections, particularly in young women following childbirth, abortion, or cervical procedures and also in injection drug users [3, 4]. Herein we describe a non-lethal case of invasive *C. sordellii* infection in the setting of polymicrobial bacterial cholangitis. This case was not associated with a stereotypical *C. sordellii* TSS and the patient had a favorable clinical outcome. Her initial sepsis syndrome was potentially due to, or significantly exacerbated by, the presence in her blood of the gram-negative pathogen, *K. pneumoniae*. Although *C. sordellii* and *E. faecium* were also present, *K. pneumoniae* is a well-known cause of bacteremia and hepatobiliary sepsis and contains the highly inflammatory endotoxin lipopolysaccharide [22]. While the biological basis for the patient's clinical presentation and hospital course is speculative, it is notable that her strain of *C. sordellii* lacked the *tcsH* and *tcsL* genes encoding hemorrhagic and lethal toxins, respectively.

Although asymptomatic bacteremia can occur with several clostridial species [23], most cases of invasive *C. sordellii* reported to date have been fatal and associated with refractory hypotension and/or a stereotypical TSS [4]. The TSS includes the sudden onset of weakness, nausea, and vomiting; progressive and refractory hypotension; local and spreading edema; body cavity effusions, severe hemoconcentration; and a marked leukemoid reaction [24, 25]. These features were largely absent from the patient presented here and no lethality was observed when this clinical isolate was tested in a mouse peritonitis model. Non-fatal cases, or infections presenting without shock, are either less common than fulminant, lethal infections or they are underreported. It is therefore important, for both therapeutic and prognostic reasons, to better define the spectrum of clinical presentations and outcomes of *C. sordellii* infections.

Not all patients infected with *C. sordellii* die from infection or develop TSS [4, 17, 26]. Also, not all infecting strains express TcsH or TcsL [27] and mounting evidence suggests that these toxins in particular determine why some infected persons rapidly die and others do not [17, 19, 28]. Because of the paucity of reports of non-TSS *C. sordellii* infections in the literature, case-report data remain scarce to sufficiently support the link between TcsL-TcsH expression and clinical outcome (TSS vs. non-TSS). However, previous studies demonstrated that immunoglobulin therapy directed against TcsL protected against *C. sordellii*-induced death in rodents [5, 17]. In addition, infection of mice with isogenic *tcsL*$^-$ mutants did not lead to rapid death or cellular toxicity, indicating that TcsL is sufficient to cause disease [21]. The necessary role of TcsH in the development of TSS is less clear. Strains that carry both TcsH and TcsL (JGS6382) and those that carry only TcsL

(ATCC9714 [17]) are highly lethal in our experimental mouse model. In addition, no *tcsH*$^{+}$, *tcsL*$^{-}$ strain, to our knowledge, has been isolated from a case of human TSS, indicating that such strains rarely cause this type of disease. Therefore, the pathogenicity of toxigenic *C. sordellii* and toxigenic *C. difficile* may be similar, in that TcsL is essential for the development of TSS, much like the orthologous TcdB (TcdB and TcsL are orthologs) which is important for the development of fulminant *C. difficile* infections [29].

Similar to the case presented here, Hao *et al*. recently reported a non-TSS case of *C. sordellii* endometritis [17]. The infecting strain (DA-108) was shown to lack the *tcsL* gene and was nonlethal in rodents. Screening of this strain with the *tcsH* primers developed in this study confirmed that DA-108 also lacks *tcsH*. Taken together, these endometritis and cholangitis cases provide independent support for the importance of *C. sordellii* glucosylating toxins in the etiology of TSS. However, it remains unclear how often these genes are present (or absent) in the chromosomes of virulent and avirulent strains and if both are essential for TSS. What is more, the possible advantage afforded to certain pathogenic strains by these toxins is equally unclear.

We speculate that patients presenting without toxic shock or severe hypotension are most likely infected with strains not possessing *tcsL* and/or *tcsH*. In the future, a rapid molecular assessment of *tcsL* and *tcsH* carriage (*e.g.*, by PCR) in clinical isolates might provide therapeutic and diagnostic information at the bedside.

The occurrence of *C. sordellii* bacteremia is uncommon, with fewer than 20 cases identified in the current literature and a mortality of ~75 % in these patients [14, 30, 31]. Although anaerobes such as *Bacteroides* and *Clostridium* have been associated with hepatobiliary infections, *C. sordellii* is an uncommon pathogen in this setting, which might reflect the low prevalence of gastrointestinal carriage of this bacterium, estimated to be < 10% in culture-based stool assays [32, 33]. We were unable to identify previous cases of *C. sordellii* associated with cholangitis. However, liver and brain abscesses have been observed with this anaerobe [14, 26, 30]. A rapidly-fatal case of *C. sordellii* bacteremia and multiple-organ dissemination, including the liver, was reported in the early post-operative period following liver transplantation, in a heavily-immunocompromised patient [34]. Interestingly, experiments conducted with that patient's *C. sordellii* strain suggested that it did not produce TcsL because cell-free culture supernatants did not cause death in mice [34].

In summary, this report describes a rare case of *C. sordellii* bacteremia from a biliary source and correlates the genetic absence of *tcsL* and *tcsH* with a good clinical outcome and the absence of TSS. These data support the need for ongoing studies of clostridial pathogenesis to enhance preventive and therapeutic interventions against these emerging pathogens.

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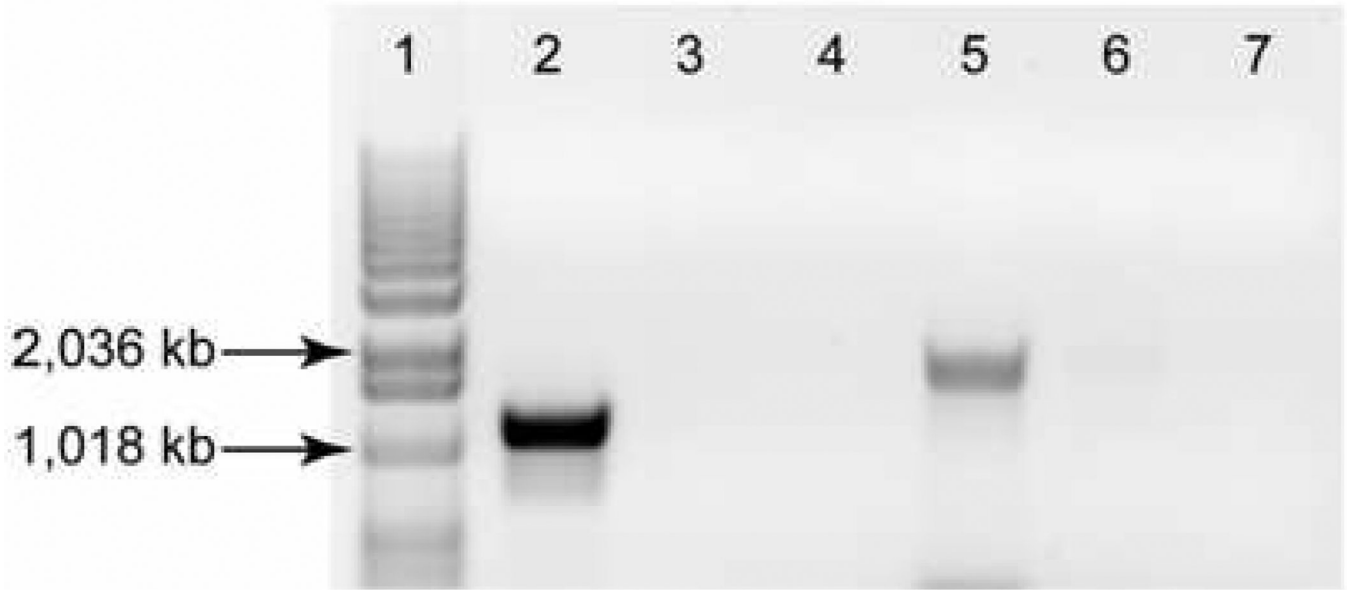

Figure 1. Absence of the *tcsH* and *tcsL* genes in the bloodstream *C. sordellii* strain HH-310

Two independent PCRs were performed for fragments of the *tcsH* (lanes 2–4) and *tcsL* (lanes 5–7) genes using genomic DNA obtained from the *C. sordellii* strain, HH-310, obtained from the patient discussed in the present report or the comparator strain JGS6382. Lanes 1–7 are: 1kb ladder, JGS6382 *tcsH*, HH-310 *tcsH*, water *tcsH* (*tcsH* PCR negative control), JGS6382 *tcsL*, HH-310 *tcsL*, water (*tcsL* PCR negative control). Sizes of pertinent ladder fragments are indicated.

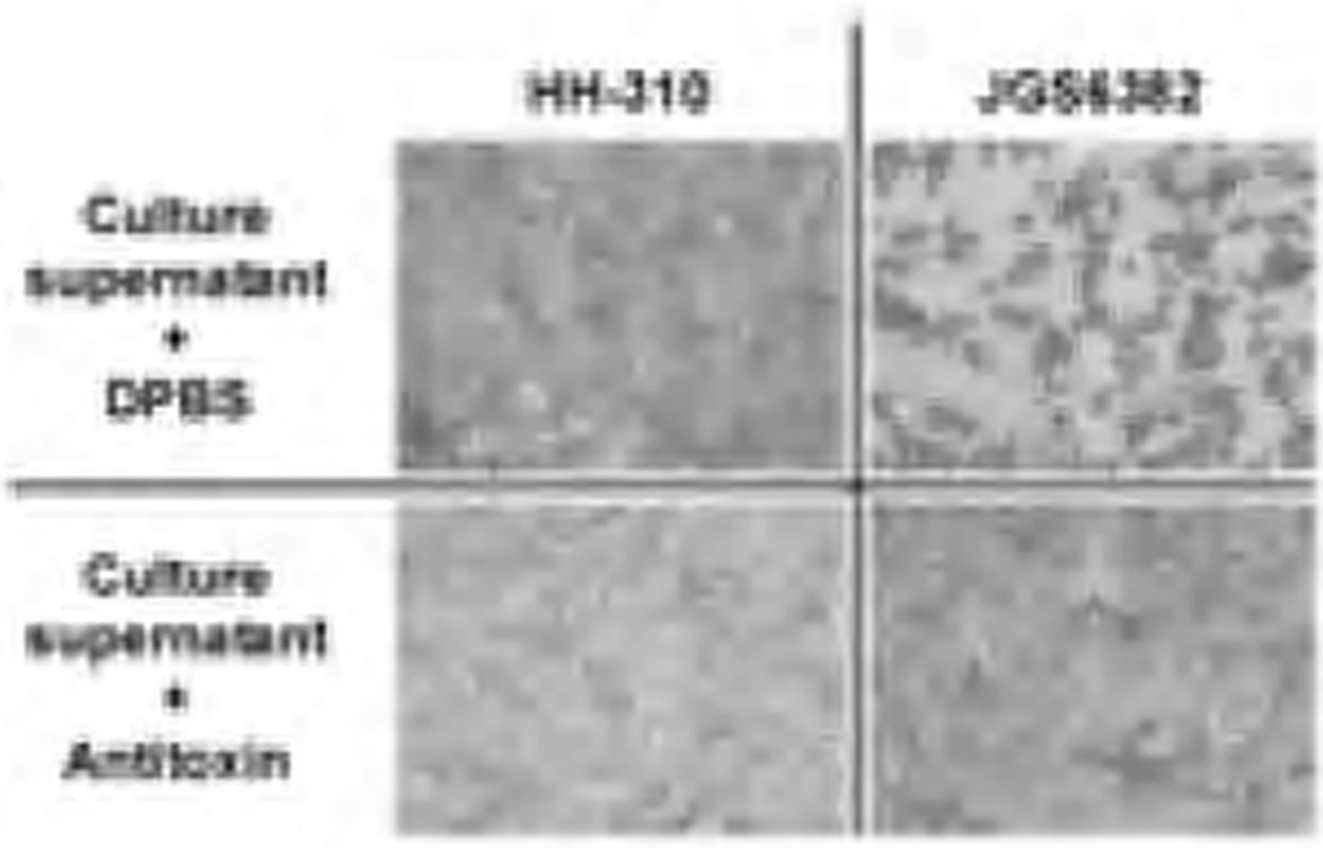

Figure 2. No cytoxicity observed with culture supernatant of *C. sordellii* strain HH-310

The upper-right panel shows the classic cell rounding phenotype caused by *C. sordellii* toxins (JGS6382). The bottom-right panel shows that this effect was ameliorated when toxin antibodies were present. HH-310 culture supernatant was non-toxigenic to Vero cells (upper-left panel) and was indistinguishable from the antibody control (bottom-left panel). All panels represent the same (20X) magnification. DPBS, Dulbecco's phosphate buffered saline (vehicle control for antitoxin).

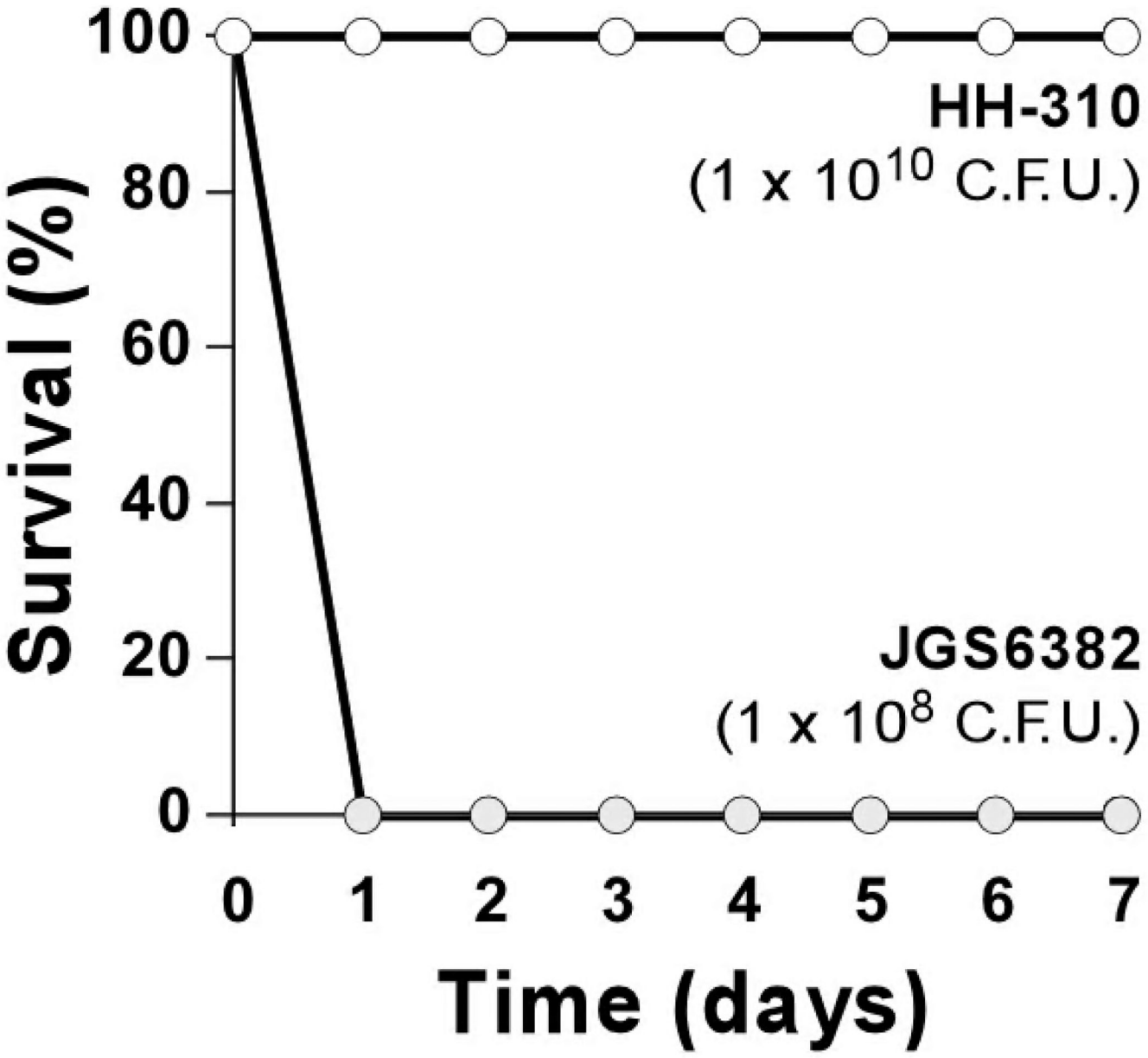

Figure 3. Failure of *C. sordellii* strain HH-310 to cause death in a peritonitis model

Female C57BL/6 mice (n = 5 per group) were inoculated by intraperitoneal injection with *C. sordellii* strains HH-310 (white circles; 1×10^{10} CFU per mouse) or JGS6382 (gray circles; 1×10^{8} CFU per mouse) at time 0 and survival was followed daily for 7 additional days.