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Bordetella holmesii Contamination of Platelet Concentrates: Revisiting the Definition of a Positive Culture

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ABSTRACT Bacterial contamination remains the most important infectious risk of platelet transfusion. After an initially positive result, a second test is performed on the blood products and the initial culture bottle to confirm the contamination. Based on the blood center's decision algorithm used, results can be either confirmed negative, positive, or indeterminate, or be unconfirmed or discordant. Here, we report the first cases of platelet concentrates contaminated with *Bordetella holmesii*. The *in vitro* growth characteristics of this unusual contaminant in platelet concentrate were investigated. Two *B. holmesii* strains isolated from platelet concentrates, as well as a control strain (*Serratia marcescens*), were spiked into platelet concentrates (PCs) at 1 and 10 CFU/ml. PCs were stored at 20 to 24°C under agitation. Samples were collected on days 2, 3, 4, and 7 for colony count and for bacterial screening using the BacT/Alert 3D system. Two PCs were detected as being positive for *B. holmesii*. However, recultures were negative. *In vitro*, *B. holmesii* did not grow but remained detectable in PCs. Its viability diminished rapidly in contact with human plasma. Upon screening using the BacT/Alert 3D system, the majority of products spiked with *B. holmesii* were negative. This is the first description of PCs contaminated with *B. holmesii*. This bacterium survives in blood products and remains dormant at low concentrations in blood products stored at room temperature, thus making difficult its detection with the BacT/Alert 3D system. The present definition of a true-positive culture of PCs may be overly restrictive for certain bacterial strains.

KEYWORDS platelet transfusion, *Bordetella*, infection risk, blood preservation, blood contamination, blood culture, blood product safety, platelets

Since the advent of exquisitely sensitive nucleic acid amplification technologies aimed at detecting hematogenous viruses in donated blood, bacterial contamination has become the dominant risk of transfusion-transmitted infection. This is particularly true for platelet concentrates (PCs) stored at room temperature with agitation in gas-permeable storage bags, conditions that are conducive to bacterial growth (1–4). The recognition of this persistent threat has led blood component suppliers to implement a series of measures aimed at mitigating the risk of transfusion-transmitted bacterial infection in the last 20 years. These measures include optimized skin disinfection, diversion of the first milliliter of drawn blood, and the systematic screening of PCs for bacterial contamination on blood product samples between 12 and 48 h after phlebotomy (1, 5–9). While these mitigation strategies have collectively improved transfusion safety, there have been reports showing evidence of very low levels of bacteria in PCs that initially come out negative upon screening (10, 11). These observations highlight the limitations of the screening of PCs for bacterial contamination.

Effective detection of contaminated products is dependent upon a variety of circumstances. The volume of the initial inoculum and the species of the contaminating bacterium are critical factors. Furthermore, the concentration of the contaminating

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bacterium in blood products is unlikely to remain constant, which may impede its detection, especially if it is in the lag phase of growth (12). Bacterial load may decline or even be eliminated in blood or plasma during transformation or storage (13, 14). The resulting low bacterial concentrations in PCs underscore the need for a reliable and sensitive screening method. Automated bacterial culture systems, such as the BacT/Alert 3D system, are commonly used for the routine surveillance of PCs by blood product manufacturers. In this system, sampling volume and time of sampling are important factors in the accurate assessment of PC contamination (8, 15). Some inconsistency still exists with the nomenclature used to categorize the results of contamination tests. Indeed, blood centers where AABB accreditation is applicable interpret test results according to the AABB standard definitions of test results, while many centers from other countries use different terminologies to design contamination, machine errors, or discordant results (8, 16).

Within our organization, all platelet products, including whole-blood-derived platelet concentrates, are individually screened for bacterial contamination. Until recently, aliquots of 8 to 10 ml from each platelet product were aseptically collected after 24 h postdonation and cultured in the BacT/Alert 3D system. Aerobic culture bottles were incubated until a positive signal occurs or for at least 7 days postinoculation, whichever came first. Since PCs could only be stored for up to 5 days, they were issued as negative-to-date as soon as samples for bacterial culture were collected and inoculated. In our 10 years of experience with this culture protocol, out of 61 true positives found, 58 were intercepted before being transfused, and the three transfused platelets did not cause any symptoms in the recipients. There are actually no clear recommendations in the literature concerning the holding period of PCs after culture. Since October 2015, we have improved our bacterial detection method by increasing the delay before sampling from 24 to 48 h and doubling the sample volume, with a resulting 7-day shelf life. With this new contamination testing protocol, PCs are now held in quarantine for 12 h after culture before being put into inventory. Upon the detection of initially reactive results by the BacT/Alert 3D system, PCs (and their cocomponents) remaining in inventory were immediately quarantined. For products that had already been distributed to hospitals, a notification was sent to the transfusion service, followed by retrieval of the product that was initially positive (and cocomponents). Initially, positive bottles were subcultured in aerobic bottles and sampled to identify the species of the contaminant. When available, index PCs and/or their cocomponents were also recultured in aerobic culture bottles. Obtaining a positive result for the recultured bottle and the index product or one of the concomitant products with the same species was considered to be confirmed positive or to be discordant positive if the species were different. If the index PCs or the concomitant products were negative or could not be analyzed, the results were considered unconfirmed positive or indeterminate. A positive result for the initial bottle only was considered false positive and could be associated with an instrument-related contamination or with sample handling. All quarantined products were destroyed at the conclusion of the tests. Furthermore, a center's medical director reviewed all reactive culture results to determine whether an investigation of the donor was justified. These guidelines followed essentially those set forth in the AABB bulletin #04-07 and definitions described by Benjamin and McDonald (8, 16, 17).

The proper interpretation of the significance of a positive culture finding is crucial for donor counseling and management. True-positive results with probable bacteremia as the source will trigger donor notification and referral to a physician for appropriate assessment, whereas true-positive results due to contamination by skin bacteria will be disregarded (unless it occurs repeatedly), as will false-positive results.

Here, we report for the first time, the detection of *Bordetella holmesii* in two unrelated PCs. *Bordetella holmesii* is a Gram-negative strictly aerobic slow-growing bacterium that has been associated with a pertussis respiratory syndrome. This bacterium is also a rare cause of sepsis, endocarditis, and respiratory infections, mainly in immunocompromised patients (18–20). While primary cultures were positive for bac-

terial growth, confirmatory tests of the index PCs and the concomitant products were negative, suggesting that the results were false positive according to our decision algorithm. Following isolation of these contaminants from the initial aerobic culture bottle, additional testing was conducted to assess their growth properties under various culture conditions. Spiking experiments in pooled platelet concentrates were also conducted to evaluate the survival of *B. holmesii* in these blood products and its detection by the BacT/Alert 3D system.

MATERIALS AND METHODS

Calibrated frozen bacterial stocks. The two *Bordetella holmesii* strains detected during bacterial aerobic screening were sent to an independent laboratory (Laboratoire de Santé Publique du Québec, Laval, Quebec, Canada) for isolation and identification, and referred to as ID105094 and ID105268. *Serratia marcescens* ATCC 43862 (American Type Culture Collection, VA, USA), a Gram-negative bacterium which has been reported in adverse transfusion reactions associated with contaminated PCs and which is often used in laboratory studies on bacterial contamination of blood components, was chosen as a positive-control strain (21).

Bacteria were cultured at 35 to 39°C. The *Bordetella holmesii* strains were grown in brain heart infusion (BHI) medium (Becton Dickinson, NJ, USA) in 5% CO_2, while *Serratia marcescens* was cultured in Nutrient broth (NB; Becton Dickinson). Concentrations (in CFU) were determined by plating culture aliquots on blood agar, and bacterial stocks were stored at −80°C in vials containing 20% glycerol at 10^5 CFU/ml (% coefficient of variation [%CV], ≤25%).

Preparation of platelet concentrates for spiking experiments. Whole-blood donations were collected after obtaining informed consent from 18 healthy volunteer donors who met the Héma-Québec guidelines for platelet donation. The study has been approved by Héma-Québec's ethics committee. Whole blood (450 ml) was drawn in Leukotrap RC-PL blood collection sets (Haemonetics Corp., Braintree, MA) containing 63 ml of citrate-phosphate-double-dextrose (CP2D) anticoagulant solution. Blood bags were kept at 20 to 24°C and processed within 8 h of phlebotomy. Platelet concentrates (PCs) were prepared following the platelet-rich plasma (PRP) method, as previously described (22). After centrifugation (2,024 × *g* for 3 min 50 s), the PRP was transferred into an empty satellite bag, and leukoreduction was done during transfer through the in-line leukoreduction filter. The leukoreduced PRP was centrifuged (4,438 × *g* for 5 min), and plasma was expressed in the remaining empty satellite bag, leaving about 60 ml with the platelet pellet. After a resting time of 60 to 120 min, PCs were finally stored at 20 to 24°C under continuous agitation.

Growth of microorganisms in spiked PCs. To mitigate a possible microbial inhibitory effect by individual donor plasma characteristics, reduce sample size, and allow experiments to be performed for all bacteria in the same PCs, a "pool and split" experimental design was used. A total of 18 fresh (<24-h) PCs were pooled in six groups of three ABO-compatible products. The pools were manually mixed, and samples were drawn from each pool to test for bacterial contamination using an automated microbial detection system (BacT/Alert 3D microbial detection system; bioMérieux, Marcy-l'Étoile, France). These initial aerobic bacterial contamination tests had to be negative with the BacT/Alert 3D system for the next 7 days in order to validate the spiking tests. Each platelet pool was then split back into the three original bags. Each member of the six triplets was inoculated with one of the three bacterial species tested.

Both *Bordetella holmesii* strains and the *Serratia marcescens* strain were inoculated at 1 or 10 CFU/ml into three PCs ($n = 3$ for each concentration for each bacterial species). Spiking was done aseptically using a sterile spike inserted into the storage bag port entry. At 10 to 20 min and 2, 3, 4, and 7 days postspiking, two aliquots (2.5 and 8 ml) were collected for viable bacterial counts on blood agar and for microbial detection in the BacT/Alert 3D system, respectively. Inoculated PCs were placed in quarantine.

Bacterial loads inoculated in PCs were ascertained on blood agar on the basis of the viable bacterial count obtained from the first 2.5-ml aliquot collected at 10 to 20 min postinoculation. Gram staining was performed to confirm the identity of the bacterial species. Bottles containing blood product aerobic (BPA) medium were inoculated with 8-ml aliquots from spiked PCs and tested in the BacT/Alert 3D microbial detection system. Bottles were incubated until a positive signal occurred, or for a maximum of 7 days.

Efficacy of BacT/Alert 3D system to detect *Bordetella* strains in PCs. Calibrated aliquots of the two *Bordetella* strains and the reference bacterium (*S. marcescens*) were thawed, and a postthawing concentration was determined by plating on blood agar to confirm inoculation loads. Aliquots of one PC were inoculated with 1 ml of a bacterial suspension in order to obtain final concentrations of 0.01, 0.1, 1, 10, and 100 CFU per ml of PCs. Immediately after inoculation, samples were drawn from each bacterium-spiked PC, and 8 ml was used to inoculate bottles of BPA medium only, since this organism is strictly aerobic and slow growing. Bottles were incubated in the BacT/Alert 3D system until a positive signal appeared or for a maximum of 7 days, whichever occurred first. For positive cultures, the time span from bottle inoculation to the occurrence of the positive signal was recorded.

Statistics. Averages and standard deviations were determined using the Microsoft Excel software.

RESULTS

Case report. The implicated donors were two females ages 19 and 26 years. Both donors' health statuses revealed no illnesses, and they were eligible for whole-blood

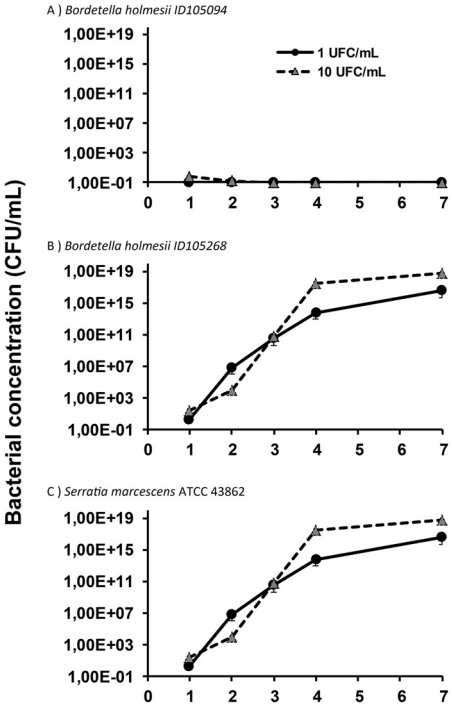

FIG 1 Bacterial growth in spiked platelet concentrates. Platelet concentrates were spiked with one of two *Bordetella holmesii* strains, ID105094 (A) and ID105268 (B), at 1 CFU/ml (●) or 10 CFU/ml (△) or *Serratia marcescens* (ATCC 43862) (C), and growth was assessed by counting CFU on blood agar. Data are from 3 independent experiments.

donations. Whole-blood samples from both donors were collected at two separate collection sites 8 days apart and were processed into PCs, packed red cells, and plasma. BacT/Alert 3D aerobic culture bottles inoculated with PCs (PRP) came out initially positive. The initially positive cultures occurred 3.0 and 3.1 days postinoculation. Both PC units, already delivered to hospitals, were returned to our facility for further analysis, along with the related packed red blood cells and plasma units. Reculture of PCs and related red blood cells and plasma samples after a 3-day storage period failed to detect bacterial growth, suggesting that the initially reactive results were false positive. Initially positive BacT/Alert 3D aerobic bottles were referred to the Laboratoire de Santé Publique du Québec for bacterial species identification using 16S rRNA sequencing.

The bacteria were identified as being *Bordetella holmesii*. The medical director first suspected that the contamination occurred during laboratory culture bottle inoculation. However, further characterization of the two isolated *B. holmesii* strains by pulsed-field gel electrophoresis (PFGE) revealed that the two samples were contaminated by distinct *B. holmesii* strains. As part of a follow-up, both donors were then contacted by telephone. The donors claimed to be in good health, and neither was asplenic or had respiratory or infectious symptoms in the days preceding or following their blood donation, conditions usually associated with an infection with *Bordetella holmesii* (18–20). No blood or oropharyngeal cultures were performed.

The peculiar aspects of these two specific cases were that *B. holmesii* contamination in blood products had never been documented, that these bacteria are not environmental contaminants, and that it took as long as 72 h for culture bottles to turn positive in the BacT/Alert 3D system. These observations led to the hypothesis that these microorganisms probably originated from the donors' blood and might have not remained viable in the platelet products before reculture was done.

Growth of microorganisms in spiked PCs. Regardless of the spiking dose and sampling time (either 2, 3, 4, or 7 days postspiking), the two *B. holmesii* strains were unable to grow in PCs (Fig. 1A and B). As expected, the positive control, i.e., PCs spiked

TABLE 1 *Serratia marcescens* ATCC 43862 and *Bordetella holmesii* ID105094 and ID105268 detection using the BacT/Alert 3D automated culture system in PCs spiked with 1 or 10 CFU/ml

Bacterial concn in inoculate (CFU/ml)	Day of sampling	No. detected/total no. of bacteria (time [h] elapsed before detection) (avg ± SD)		
		S. marcescens ATCC 43862	*B. holmesii* ID105094	*B. holmesii* ID105268
1	2	3/3 (7.2 ± 0.2)	0/3	1/3 (86.4)[a]
	3	3/3 (3.7 ± 0.1)	1/3 (86.4)[a]	0/3
	4	3/3 (3.8 ± 0.1)	0/3	0/3
	7	3/3 (3.7 ± 0.0)	1/3 (67.2)[a]	0/3
10	2	3/3 (5.2 ± 0.8)	3/3 (80.8 ± 2.8)[a]	2/3 (73.2 ± 1.7)[a]
	3	3/3 (3.2 ± 1.0)	0/3	0/3
	4	3/3 (3.8 ± 0.1)	0/3	1/3 (76.8)[a]
	7	3/3 (3.7 ± 0.0)	0/3	0/3

[a]The identity of the bacteria has not been confirmed by an independent laboratory.

with *S. marcescens*, clearly showed rapid growth at the two concentrations tested and as early as 2 days after spiking the PCs (Fig. 1C).

With *S. marcescens*, there was a correlation between the predicted number of CFU spiked into the PCs and the actual number measured after inoculation. However, with both *B. holmesii* strains, the actual number of CFU obtained after spiking was significantly reduced compared to the predicted number of CFU spiked into PCs. Although these experiments do not exactly reproduce the current practice and quarantine delays in our operations, they suggest a rapid decline in the viability of the bacteria when in contact with human plasma and/or platelets.

S. marcescens was detected by the BacT/Alert 3D system, with an average detection time of 4.3 h. However, both strains of *B. holmesii* were clearly more difficult to detect by the BacT/Alert 3D system. Although the time before the detection of *B. holmesii* contamination (average time, 77.8 h) was much longer than that with *S. marcescens*, the overall analysis of the PCs spiked with 10 CFU/ml of either *B. holmesii* strain indicates that contamination was detected in 5 out of 6 PCs when sampling was done 2 days postinoculation. However, for all other sampling times after inoculation with 10 CFU/ml or for any sampling times after inoculation with 1 CFU/ml, the detection of *B. holmesii* contamination was inconsistent, indicating that this bacterium can survive at low concentrations in the PCs (Table 1).

Efficacy of *B. holmesii* detection by the BacT/Alert 3D system. The antimicrobial properties of plasma from blood donors can affect the growth and survival of bacterial contaminants and the ability of the BacT/Alert 3D system to detect their presence in PCs. To mitigate individual donor plasma inhibition, pooled PCs were spiked with one of five concentrations of either one of the two *B. holmesii* strains or with the reference *S. marcescens* strain. The results of these experiments are shown in Table 2. The detection of *S. marcescens* at a concentration of 0.01 CFU/ml in PCs is probably fortuitous, since no bottle was found to be reactive at a concentration of 0.1 CFU/ml. Under routine PC contamination tests, the limit of detection of this bacterium with the BacT/Alert 3D system would rather be 1 CFU/ml with an 8-ml inoculate, with a delay of

TABLE 2 BacT/Alert 3D system detection of *Serratia marcescens* ATCC 43862 and *Bordetella holmesii* ID105094 and ID 105268 in platelet concentrates

Bacterial strain	Detection result (time to detection [h]) by spiking dilution (CFU/ml)[a]				
	0.01	0.1	1	10	100
S. marcescens ATCC 43862	Positive (15.7)	ND	Positive (12.2)	Positive (11.2)	Positive (10.2)
B. holmesii ID105094	ND	ND	ND	Positive (74.4)	Positive (67.2)
B. holmesii ID105268	ND	ND	Positive (81.6)	Positive (76.8)	Positive (64.8)

[a]ND, not detectable.

about 12 h before detection. The BacT/Alert 3D system has detected the presence of *B. holmesii* ID105268 in PCs inoculated with 1 CFU/ml, while a concentration of at least 10 CFU/ml was required for detection of the second strain (ID105094). At a concentration of 10 CFU/ml, the delay before a positive signal by the BacT/Alert 3D system was approximately 6 times longer for both *B. holmesii* strains than for the *S. marcescens* reference strain (76.8 and 74.4 h compared to 11.2 h). The detection time of *B. holmesii* with the BacT/Alert 3D system is otherwise comparable to those reported by Jonckheere et al. (23).

DISCUSSION

The systematic screening of platelet concentrates for microbial contamination, as well as improved methods that mitigate the risk of contamination, has had a significant impact on transfusion safety. However, a residual risk of undetected bacterial contamination remains, and different strategies have been proposed to mitigate this threat (5, 8, 9, 24). Indeed, automated culture systems cannot reliably detect very low bacterial titers or bacteria that become nonviable during storage (25). Herein, we report the investigation conducted following the initially positive result by the BacT/Alert 3D system of aerobic culture bottles from two independent PCs, tested 24 h postcollection, for unrelated strains of *B. holmesii*. Repeat cultures of the implicated PCs and their concomitant products, sampled after a 3-day storage period, failed to confirm the presence of bacteria in the samples. These products were first considered false positive. Upon identification of the contaminant and after thorough investigation of its characteristics, it was felt that the organism most probably came from the donors' blood and that inactivation of *B. holmesii* during blood product storage was the reason for the inconsistent detection of contamination. Spiking experiments were then undertaken to assess the growth characteristics of these two *B. holmesii* strains in PCs.

These experiments revealed that the viability of the two *B. holmesii* strains rapidly declines when spiked into PCs. Both strains appear to be strongly and rapidly affected by the plasma contained in blood products, although pooled products were used to mitigate donor-to-donor plasma antimicrobial activity. While growth curves showed that *B. holmesii* does not proliferate in PCs, both strains were detectable when tested in the BacT/Alert 3D system, showing that the bacteria are not self-sterilized but are dormant in the blood product. However, the detection was inconsistent at smaller spiking doses and at sampling times longer than 2 days. These observations provide a ready explanation for the negative results obtained upon confirmatory testing. Indeed, the growth kinetics of some bacteria are known to be variable, thereby making their detection less predictable, especially if bacteria are slow growing (12). Bacteria might be rapidly and transiently inactivated by the plasma present in the product or might form a biofilm. Unfortunately, the possibility that *B. holmesii* develops a biofilm has not been reported and has not been addressed in this study. While PCs were thoroughly mixed before sampling, no specific effort was made to dislodge any bacteria adhering to the surface of the bag. Growth assays for the detection of the two *B. holmesii* strains using the BacT/Alert system revealed that not only the ability of the instrument to detect *B. holmesii* was limited but that it also took longer incubation periods of time to obtain a positive result. The longer incubation period before detection can become a significant operational concern, given the limited shelf life of PCs and the risk of transfusion of a product whose contamination remained undetectable at the time of product release and/or transfusion to a patient. Transfusion safety of contaminated PCs with very low concentrations of viable *B. holmesii* during storage, particularly to immunocompromised patients, remains unknown.

Data from the literature indicate that *B. holmesii* is not an environmental contaminant, and it is usually found in nasopharyngeal swabs in cases of pertussis-like illnesses (19). Over 30 cases of septicemia involving *B. holmesii* have been reported so far (20). They usually follow a benign clinical course, and 85% of the patients are asplenic (anatomical or functional). Cases of endocarditis and severe pneumonia have also been reported. For the two cases described here, blood donors stated being in perfect health,

and no additional blood tests were performed. These donors were not deferred, but an annotation was added to their blood donor record. None of the two donors returned to give blood.

Furthermore, the two cases reported herein highlight the importance of making more thorough investigations whenever an initially positive culture is detected. Two elements have had a major influence on our medical director's decision to investigate further. First, the isolated contaminants had never been reported in blood products, suggesting very unusual contamination by *B. holmesii*. Second, the growth pattern was uncommon. While positive signals usually occur on the first day following bottle inoculation and incubation in the BacT/Alert system, in these two cases, the occurrence of positive signals required 3 days of incubation. The spiking tests, although they do not allow us to conclude that the original products were truly contaminated, still support the hypothesis that the bacteria may have been present in very low concentration in these products. The inability to confirm the initially positive contamination was likely due to the growth kinetics of the contaminant or because it was inactivated during storage.

To our knowledge, this is the first report of probable isolation of *B. holmesii* in blood components. Several factors, such as the concentration, the growth kinetics of the contaminating bacteria, or their neutralization by plasma, can adversely affect the detection of contaminated PCs. *B. holmesii* most likely survives during storage but cannot proliferate in PCs, making its detection difficult. Given that the effective decision algorithm would have normally led to define these cases as false positive, the consensus definition of a true-positive culture obtained during microbial screening of PCs may be overly restrictive for microorganisms such as this one, as previously proposed (8). We believe that the criteria for interpretation on culture results set out by Benjamin and McDonald are adequate for the great majority of situations. However, we would suggest the following modification: in cases where the microorganism can only originate from bacteremia in the donor (as is the case in our 2 case reports), the result should be considered true positive even in the absence of positive results when reculturing the blood component.

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We declare that we have no conflicts of interest relevant to this paper and did not receive support of any kind from the manufacturers or suppliers of any test or process referred to in this article.

A.J., M.J.D.G., É.D., H.D., and M.-È.N. designed the study, performed the experiments, and wrote and approved the article. M.C. analyzed the results and wrote and approved the article. G.D. and L.T. designed the study, analyzed the results, and wrote and approved the article.

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