Unexplained agglutination of stored red blood cells in Alsever’s solution caused by the gram-negative bacterium
Serratia liquefaciens

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Alsever’s solution has been used for decades as a preservative solution for storage of RBCs. From October 2005 to January 2006, unexplained hemagglutination of approximately 10 to 20 percent of RBCs stored for several days in a modified version of Alsever’s solution was noticed in quality control testing at the Canadian Blood Services Serology Laboratory. An investigation, including microbial testing, was initiated to determine the cause of the unexplained hemagglutination. The gram-negative bacterium Serratia liquefaciens was isolated from supernatant solutions of agglutinated RBCs. Further characterization of this strain revealed that it has the ability to form biofilms; presents high levels of resistance to chloramphenicol, neomycin, and gentamicin; and causes mannose-sensitive hemagglutination. The source of S. liquefaciens contamination in RBC supernatants was not found. However, this bacterium has not been isolated since January 2006 after enhanced cleaning practices were implemented in the serology laboratory where the RBCs are stored. This biofilm-forming, antibiotic-resistant S. liquefaciens strain could be directly linked to the unexplained hemagglutination observed in stored RBCs. Immunohematology 2008;24:39–44.

Key Words: hemagglutination, red blood cells, Alsever’s solution, Serratia liquefaciens

Alsever’s solution was first described by J.B. Alsever in 1941. It is an isotonic solution (2.05% glucose, 0.42% sodium chloride, 0.8% trisodium citrate, 0.055% citric acid in double-distilled water [ddH2O]) routinely used as an anticoagulant and preservative, which permits the storage of whole blood and RBCs at refrigeration temperatures. The National Serology Laboratory at Canadian Blood Services (CBS) used to prepare a modified version of Alsever’s solution (supplemented with 0.03% adenine, 0.2% inosine, 0.03% chloramphenicol, and 0.07% neomycin), which was sent to the hospitals for detecting antibodies of relevance in transfusion medicine. Each new lot of modified Alsever’s solution underwent quality control testing for stability every 8 months for a period of 24 months, which is the shelf life of this product. The quality control testing consisted of measuring pH, presence of hemolysis, and serologic reactivity for D, Fy, and M. From October 2005 to January 2006, unexplained hemagglutination of approximately 10 to 20 percent of different types of RBCs stored for several days in modified Alsever’s solution was noted during quality control testing. The CBS Serology Laboratory initiated an investigation, including microbial testing, to determine the cause of the unexplained hemagglutination. The gram-negative bacterium Serratia liquefaciens was isolated from supernatant solutions of agglutinated RBCs.

Serratia spp are opportunistic gram-negative bacteria that belong to the Enterobacteriaceae family and include several species, such as Serratia marcescens and S. liquefaciens. Once considered harmless, S. liquefaciens has emerged as an opportunistic pathogen in recent years and has been implicated in nosocomial infections and also in severe and in some cases fatal reactions associated with transfusion of contaminated RBCs or platelets. The virulence of S. liquefaciens is related not only to the production of endotoxin but also to the organism’s high antibiotic resistance and ability to form surface-attached cell aggregates embedded in a matrix, known as biofilms. Adhesion, which is the first step of biofilm formation, is directly related to the presence of short, thin, hairlike projections called fimbriae, also known as “filamentous
hemagglutinins. mannose-sensitive hemagglutinin is associated with type 1 fimbriae whereas mannose-resistant hemagglutinins are linked with different types of fimbriae including type 3 fimbriae. This report describes a case of initially unexplained agglutination of stored RBCs that was subsequently determined to be caused by a hemagglutinin-positive, biofilm-forming, antibiotic-resistant \textit{S. liquefaciens} strain.

**Materials and Methods**

Microbial studies were performed on the following samples from CBS Serology Laboratory: supernatants of different lots of stored RBCs, samples of PBS used to wash RBCs, and water from the water bath used to thaw frozen RBCs. Samples were plated on 5 percent sheep blood agar, Luria Bertani (LB) agar, and trypticase soy agar. After identification of \textit{S. liquefaciens} in the stored RBCs, the frozen RBCs, glassware, environmental samples (air and surfaces), and personnel were also tested. Different lots of modified Alsever’s solution prepared by the Serology Laboratory, including those used to store the agglutinated RBCs, underwent sterility testing at an external microbiology laboratory (Nucro-Technics, Scarborough, ON, Canada).

The bacteriostatic or bactericidal effect of modified Alsever’s solution on \textit{S. liquefaciens} was investigated. Ten-fold serial dilutions (10^8–10^2 colony forming units [CFU/mL]) of \textit{S. liquefaciens} isolated from RBC supernatants were prepared in modified Alsever’s solution.

One milliliter of the 10^2 CFU/mL dilution was inoculated into 500 mL of thioglycollate broth immediately after the dilutions were made (day 0) followed by 24-hour incubation at 30°C; this was used as a positive control. The 10^2 CFU/mL dilution was then kept at 2 to 8°C, and after vortexing, 1 mL was taken to inoculate 500 mL of thioglycollate at 8, 15, and 30 days after storage. The 2 to 4°C temperature was chosen for storage as this is the temperature at which RBCs are maintained in modified Alsever’s solution.

Pulsed-field gel electrophoresis (PFGE) genotyping was performed for \textit{S. liquefaciens} isolated from six supernatants of agglutinated RBCs at the Division of Microbiology of the Hospital for Sick Children (Toronto, ON, Canada).

Minimal inhibitory concentration (MIC) for chloramphenicol (Cl), neomycin (Neo), and gentamicin (Gm) was determined for \textit{S. liquefaciens} isolated from RBC supernatants and for control strain \textit{S. liquefaciens} ATCC 27592 according to the Clinical and Laboratory Standards Institute (previously known as National Committee for Clinical Laboratory Standards [NCCLS]).

Ten-fold serial dilutions (10^9–10^0 CFU/mL) of \textit{S. liquefaciens} cultures in PBS were tested in hemagglutination assays using 3 percent suspensions of group O and A RBCs previously stored frozen in liquid nitrogen. The RBCs were suspended in PBS with or without 50 mM D-mannose (Man), in modified Alsever’s solution, and in “RBC storage solution” (Immucor Inc., Norcross, GA) following protocols previously established. \textit{S. liquefaciens} ATCC 27592 and \textit{Escherichia coli} ATCC 25922 were used as negative and positive controls for hemagglutination, respectively.

\textit{S. liquefaciens} isolated from RBC supernatants was assayed for its ability to form biofilms. Cultures were grown in LB broth supplemented with 0.2 percent glucose and 0.5 percent casamino acids (CAA) and incubated at 30°C for 24 hours. Biofilm formation was measured by crystal violet staining as previously described. The presence of type 1 fimbriae was tested by PCR using primer pair SliqFW 5′-CTGGTATTGTCTGCAACTG-3′ and SliqREV 5′-ACCTGCAGCACGTTATCTTG-3′, which anneal at positions 433 and 1359, respectively, of the \textit{S. marcescens} fimA gene (accession number AY730610). Primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). PCR reactions were carried out in a Mastercycler ep gradient S (Eppendorf Corp., Mississauga, ON) as follows: 5 minutes at 95°C; 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 54°C, and extension for 1 minute at 72°C; 5 minutes at 72°C; and hold at 4°C. PCR was carried out in a final volume of 25 μL containing the following reagents: 22.5 μL of sterile ddH2O, 10 × PCR buffer supplemented with 1.5 mM MgCl2 (Roche Diagnostics Corp., Indianapolis, IN), dNTPs (Boehringer Mannheim Corp., Indianapolis, IN), each primer (0.2 μg/μL), Hot Star Taq DNA polymerase (Roche), and 2.5 μL of template DNA. \textit{S. liquefaciens} cell suspensions were prepared by diluting cells from overnight cultures in sterile ddH2O. Cell concentrations were adjusted using a 0.5 McFarland Equivalence Turbidity Standard (Remel, Lenexa, KS) to provide chromosomal DNA templates for PCR.

**Results**

\textit{S. liquefaciens} was only isolated from supernatants of RBCs that had been stored for several days in modified Alsever’s solution and had presented...
unexplained agglutination. Unusual hemolysis was not noticed in the contaminated RBCs. Samples of the same RBCs which had not been stored in modified Alsever’s solution as well as all of the other samples tested, including PBS, water from water baths, glassware, staff, and environmental samples, were negative for the presence of this bacterium. In addition, sterility testing of different lots of modified Alsever’s solution repeatedly showed negative results. Interestingly, it was observed that this strain of S. liquefaciens grew better when incubated at refrigeration (2–8°C) and ambient (20–25°C) temperatures than when grown at higher temperatures (37°C).

The cell concentration of S. liquefaciens was substantially decreased with time when it was stored in modified Alsever’s solution at 2 to 8°C. Weekly sampling of the 10^2 CFU/mL cell dilution was performed by taking 1 mL of the dilution for inoculation into 500 mL of thioglycollate followed by incubation at 30°C for 24 hours. Visual examination showed heavy growth of the sample taken at day 0. Recovery of bacteria decreased proportionally with storage time of the bacterial suspension, being less heavy when the assay was performed after 8 days of storage and very light after 15 days, until no growth was observed after 30 days of storage.

PFGE analysis revealed that the six genotyped S. liquefaciens strains isolated from the RBC supernatants had the same band pattern (Fig. 1). This S. liquefaciens strain was resistant to Cl and Neo at concentrations used to prepare modified Alsever’s and RBC storage solutions with MICs of 1024 and 1400, respectively (Table 1). This strain was also considered to be resistant to Gm based on the Clinical and Laboratory Standards Institute (formerly NCCLS) (MIC > 8 µg/mL). In contrast, S. liquefaciens ATCC 27592, used as a control, was sensitive to all three antibiotics (Table 1).

S. liquefaciens isolated from the RBC supernatants caused mannose-sensitive hemagglutination of RBCs suspended in PBS supplemented with 50 mM of mannose. Hemagglutination was also present when this strain of S. liquefaciens was suspended in modified Alsever’s solution or the commercial RBC storage solution at bacterial concentrations of at least 10^6 CFU/mL (Table 1, Fig. 2). Similar results were obtained for the positive control E. coli ATCC 25922, whereas the negative control S. liquefaciens ATCC 27592 did not cause hemagglutination in any of the conditions tested. The fimbrial hemagglutinin(s) of the S. liquefaciens isolated from the RBC supernatants do not share sequence homology at the DNA level with type 1 fimbriae of S. marcescens as PCR to amplify fimA yielded negative

### Table 1. Minimal inhibitory concentration and hemagglutination of S. liquefaciens isolated from stored RBCs

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (µg/mL)</th>
<th>PBS</th>
<th>Commercial solution</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cl</td>
<td>Neo</td>
<td>Gm</td>
</tr>
<tr>
<td>S. liquefaciens (RBC supernatant)</td>
<td>1024</td>
<td>1400</td>
<td>16</td>
</tr>
<tr>
<td>S. liquefaciens ATCC 27592</td>
<td>8</td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>NT</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

HA = hemagglutination
Man = mannose
NT = not tested

*a A bacterium is resistant to chloramphenicol (Cl), neomycin (Neo), and gentamicin (Gm) if minimal inhibitory concentrations (MICs) are > 32, > 8, and > 8 µg/mL, respectively.

† Modified Alsever’s solution (MAS) contains 350 µg/mL of Cl and 700 µg/mL of Neo, and the commercial RBC storage solution is supplemented with 250 µg/mL of Cl, 100 µg/mL of Neo, and 50 µg/mL of Gm.

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**Fig. 1.** Genotyping of S. liquefaciens isolates. Genomic DNA was extracted from six strains of S. liquefaciens isolated from different RBC supernatants (lanes 1–6) and control strain ATCC 27592 (lane C) followed by digestion with SpeI and pulsed-field gel electrophoresis (PFGE) analysis. M, lambda ladder.
results (data not shown). As expected, positive hemagglutination was associated with biofilm formation of this *S. liquefaciens* isolate (Fig. 3) and with a microscopic filamentous phenotype (Fig. 4).

**Discussion**

This report describes a case of agglutination of test RBCs stored in modified Alsever’s solution caused by contamination with *S. liquefaciens*. With the exception of the supernatants of agglutinated RBCs, none of the other samples taken at the CBS Serology Laboratory during the investigation of this case were positive for the presence of *S. liquefaciens*. There is a recall report for modified Alsever’s solution prepared by Gamma Biologicals, Inc., owing to microbial contamination. However, the sterility testing of the modified Alsever’s solution prepared at the CBS Serology Laboratory was negative, indicating that this was not the source of contamination. Because of the virulent characteristics of the *S. liquefaciens* strain isolated from the supernatants of agglutinated RBCs (i.e., highly resistant to antibiotics and ability for biofilm formation), it was speculated that the source of contamination was a clinical sample received at the CBS Serology Laboratory, although this could not be confirmed.

It is assumed that the level of *S. liquefaciens* contamination of the original source was high, probably with bacteria concentrations greater than 10^6 CFU/mL, because our results demonstrated that lower concentrations of *S. liquefaciens* did not cause hemagglutination.

In addition, we showed that modified Alsever’s solution has a bactericidal effect at lower concentrations of this strain (10^2 CFU/mL). This may be attributable to the synergistic effect of the two antibiotics present in the solution, Cl and Neo, or lack of nutrients. However, this should be further investigated as MICs to Cl or Neo alone revealed that this strain is highly resistant to each of these antimicrobials.

*S. liquefaciens* is an emergent nosocomial pathogen and a cause of severe reactions associated with transfusion of RBCs. This bacterium is also a common contaminant of reagents used in clinical environments. There is a study reporting an *S. liquefaciens* outbreak in a hemodialysis center where the bacterium was isolated from empty vials of epoetin alfa as well as from antibacterial soap and hand lotion. Isolation of *S. liquefaciens* from these sources, and in this case from agglutinated RBCs, indicates that this bacterium has the ability to survive under stressful conditions, likely by forming biofilms. This report confirmed the biofilm formation ability of the *S. liquefaciens* strain isolated at the CBS Serology Laboratory and demonstrated that this ability is linked to a high level of antibiotic resistance as occurs in other bacteria that form biofilms.

The agglutination observed in the stored RBCs was reproduced in in vitro hemagglutination assays. Our results showed that the fimbriae of *S. liquefaciens* responsible for this hemagglutination are functionally homologous to the type 1 fimbriae of *S. marcescens* or *E. coli* inasmuch as the agglutination was sensitive to the presence of mannose. However, our PCR results
demonstrated that the sequence of the gene encoding *S. liquefaciens* fimbriae is different from that of the *S. marcescens* fimA gene.\(^{11}\)

PFGE genotyping showed that all of the RBC supernatants tested contained the same strain of *S. liquefaciens*, demonstrating that there was probably an initial introduction of the strain and subsequent cross-contamination in the laboratory during manipulation of these samples.

After this investigation was carried out, the CBS Serology Laboratory implemented enhanced cleaning practices including constant disinfection of work areas and flushing of Pasteur pipettes used to wash RBCs with 1% hypochlorite solution. The Pasteur pipettes are washed with a vacuum bottle system, and a sterile tip is attached so the pipette tips do not touch the RBCs. In addition, the pipette washing is followed by several rinses with PBS. After implementation of these new measurements, *S. liquefaciens* has not been isolated again, and no other cases of unexplained hemagglutination have been reported by the CBS Serology Laboratory.

**Acknowledgments**

We would like to thank Ms. S. Baldwin and Ms. J. Barnes (Serology Laboratory, Canadian Blood Services) for their technical assistance. We are also grateful to Ms. S. Hamilton (Quality Assurance, Canadian Blood Services) for her assistance with quality control testing and Dr. S. Richardson (The Hospital for Sick Children, Toronto, Ontario, Canada) for bacteria genotyping using pulse-field gel electrophoresis.

**References**


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**Fig. 4.** Microscopic phenotype of *S. liquefaciens* isolated from supernatants of agglutinated RBCs. Contaminant *S. liquefaciens* presents normal morphology (i.e., coccobacilli) when grown on Luria Bertani plates (A) and filamentous phenotype when grown in Luria Bertani broth supplemented with glucose and casamino acids to induce biofilm formation (B).

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