Changing the 30-min Rule in Canada: The Effect of Room Temperature on Bacterial Growth in Red Blood Cells

Sandra Ramirez-Arcos^a Yuntong Kou^a Éric Ducas^b Louis Thibault^b

^aCentre for Innovation, Canadian Blood Services, Ottawa, ON, Canada;

^bHéma-Québec, Research and Development, Operational Research, Québec, QC, Canada

Keywords

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Summary

Background: To maintain product quality and safety, the '30-min rule' requires the discard of red blood cells (RBCs) that are exposed to uncontrolled temperatures for more than 30 min. Recent studies suggest this rule may safely be extended to a 60-min rule. **Methods:** A pool-and-split design study (N = 4) was run in parallel at Canadian Blood Services (SAGM RBCs) and Héma-Québec (AS-3 RBCs). RBCs were spiked with ~1 colony-forming unit/ml of mesophilic and psychophilic bacteria. Control units remained in storage at 1–6 °C for 42 days. Test 30 (T30) and T60 units were exposed to room temperature (RT) six times during storage, each time for 30 and 60 min, respectively. Bacterial proliferation was monitored. **Results:** Mesophilic bacteria do not proliferate in RBCs. The growth of psychophilic bacteria is not significantly different in RBCs exposed for 30 or 60 min to RT ($p < 0.05$). **Conclusion:** The study findings were the final evidence to support extension from a 30-min rule to a 60-min rule in Canada.

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Introduction

To maintain product safety and quality, red blood cell (RBC) units for transfusion are stored at 1–6 °C in North America for a maximum of 42 days. In their journey from blood supplier to hos-

pital to patient, excursions outside of refrigerated storage can occur. To maintain quality and safety, the '30-min rule' requires the discard of any RBCs exposed to uncontrolled temperatures for more than 30 min. This rule is based on studies conducted in the early 1970s [1]. The Canadian blood manufacturers Héma-Québec, which serves the province of Québec, and Canadian Blood Services, which serves the rest of Canada, both abide by the Canadian Standards Association (CSA) guidelines and regulations, which stipulate a 30-min rule for RBCs [2].

Over the past 5 years, several studies have investigated bacterial growth and RBC quality in RBC units exposed to uncontrolled temperatures for various time periods. These results challenge the validity of the 30-min rule. In a Canadian Blood Services study, four bacterial species (the Gram-negative *Serratia marcescens*, *Escherichia coli* and *Yersinia enterocolitica* and the Gram-positive *Staphylococcus epidermidis*) were inoculated at realistic concentrations (~1 colony-forming unit(CFU)/ml [3]) in RBC units exposed multiple times to room temperature (RT) for 30 or 60 min [4]. The results demonstrated that neither *E. coli* nor *S. epidermidis* were able to grow in RBCs. While *S. marcescens* and *Y. enterocolitica* reached clinically relevant concentrations (i.e., $>10^5$ CFU/ml) [4, 5], the growth and endotoxin production of the two species was not significantly different in units exposed for 30 or 60 min to RT. These results were echoed by a study conducted at Héma-Québec that showed the quality and safety of contaminated RBC units was not different between units exposed multiple times to RT for 30 or 60 min [6].

These studies were compelling enough to result in an appeal to the CSA to change the 30-min rule to a 60-min rule. Héma-Québec and Canadian Blood Services were asked to increase the power of previous studies, investigate the influence of RBC-additive solutions, and examine more bacterial species. Results of this complementary study are shown herein.

Material and Methods

RBC Production at Canadian Blood Services

The study design was approved by the Canadian Blood Services Research Ethics Board. Blood was collected into citrate-phosphate-dextrose anticoagulant (CPD: citric acid 3.27 g/l, sodium citrate 26.3 g/l, sodium acid phosphate 2.51 g/l, dextrose 25.5 g/l) in B1/‘top-and-bottom’ bags (Macopharma, Tourcoing, France). Whole blood units were stored for up to 24 h at 20–24 °C prior to processing. Leukoreduced (by filtration) RBC units were stored in saline-adenine-glucose-mannitol nutrient solution (SAGM: sodium chloride 8.77 g/l, dextrose 9.00 g/l, adenine 0.169 g/l, mannitol 5.25 g/l) at 1–6 °C.

RBC Production at Héma-Québec

The study was approved by the Héma-Québec Research Ethics committee. Blood was collected into a Leukotrap WB collection system (Haemonetics, Braintree, MA, USA) containing 63 ml of citrate-phosphate-2-dextrose anticoagulant (CP2D: citric acid 3.27 g/l, sodium citrate 26.3 g/l, monobasic sodium phosphate 2.22 g/l, dextrose 51.1 g/l). Whole blood was stored at 1–6 °C for up to 72 h prior to processing. After leukoreduction, blood was centrifuged and plasma expressed. RBC units were suspended in 100 ml additive solution-3 (AS-3: sodium chloride 4.10 g/l, dextrose 11.0 g/l, adenine 0.30 g/l, monobasic sodium phosphate 2.76 g/l, sodium citrate 5.88 g/l, citric acid 0.42 g/l) and stored at 1–6 °C.

Bacterial Strains

Mesophilic (optimal growth at 20–45 °C) species *S. epidermidis* ATCC 700562, *Klebsiella pneumoniae* WHO PEI-P-B-08, *Pseudomonas aeruginosa* ATCC 27853, and *E. coli* ATCC 25922 as well as psychrophilic species *Y. enterocolitica* ATCC 49397, and *S. marcescens* CBS 07–2005 were used. Skin flora *S. epidermidis* was selected as one of the most common blood product contaminants while the 5 Gram-negative species were chosen since they have been shown to either grow in RBCs [7] or been implicated in adverse transfusion reactions involving contaminated RBC units [8–10].

Study Design

The study was run in parallel at Canadian Blood Services (SAGM RBC units) and at Héma-Québec (AS-3 RBC units). At each site, groups of three ABO-Rh-matched units were pooled and split on the day of RBC production from whole blood. After splitting, units were tested for sterility using the BacT/ALERT culture system (bioMérieux – Canada, St Laurent, QC, Canada) as previously described [11]. Units were then spiked with bacteria at ~1 CFU/ml. The split units were divided into 3 groups: Control units remained in storage at 1–6 °C during the 42-day storage time; Test 30 (T30) units were exposed to RT six times during their 42 days of storage, each time for 30 min; and Test 60 (T60) units were exposed to RT six times during their 42-day storage period, each time for 60 min. Specifically, units were exposed to RT on days 14, 16, 21, 23, 28, and 35 of storage. To determine bacterial proliferation during storage, RBC samples of each unit, including control units, were taken 24 h after each RT exposure and at the end of their shelf life (42 days). Samples were serially diluted 10-fold in trypticase soy broth (TSB) and plated in duplicate onto blood agar. Cultures were incubated for 24 h at 37 °C with the exception of plates inoculated with *S. marcescens*, which were incubated at 30 °C. RBC units that yielded negative growth on blood agar during storage were sampled for viability using the BacT/ALERT system at the end of the 42-day storage period [11]. Experiments were independently repeated four times for each bacterium.

Statistical Analyses

Sample size was determined according to an equivalence study design, which assumed that bacteria concentration after a 60-min exposure in RT is not different from a 30-min RT exposure. The equivalence between T30 and T60 units was confirmed for a confidence interval of the overall mean difference ± 1 . The calculated required sample size was determined to be 4 per group. Means of log₁₀-transformed data were calculated from duplicate bacterial counts, for

all the time points from the four repetitions. Mixed-model analysis was performed, with random effects added to address clustering due to repeated measurements from the same split unit or same sample. The overall difference between T30 and T60 was calculated from the model estimate, and its 95% confidence interval was constructed with its standard error. All analyses were done in SAS (SAS/stat 9.1, SAS institute Inc., Cary, NC, USA). A *p* value < 0.05 was considered statistically significant.

Results

Mesophilic Bacteria Do Not Proliferate under RBC Storage Conditions

Viable counts of the bacterial suspensions used for RBC inoculation with *K. pneumoniae*, *P. aeruginosa*, *S. epidermidis*, and *E. coli* ranged from 0.2 to 5.4 CFU/ml. All BacT/ALERT cultures inoculated on the spiking day were positive. For these 4 bacterial species, no increase in bacterial concentration (i.e., proliferation) was observed at all testing times for either control or units exposed to room temperature for 30 or 60 min.

Bacteria growth was verified during RBC storage (by plating on blood agar) and at the end of storage (using BacT/ALERT culture bottles). Results varied for each species depending on the RBC additive solution. While *P. aeruginosa* survived in RBC units prepared in SAGM and AS-3, *E. coli* self-sterilized in both types of RBC units. Interestingly, *K. pneumoniae* and *S. epidermidis* maintained viability in SAGM-RBC units as demonstrated by positive BacT/ALERT cultures at the end of RBC shelf life, but these two species did not survive during storage in AS-3 RBC units.

Growth of Psychrophilic Bacteria Is Not Significantly Different between RBC Units Exposed for 30 or 60 min to RT

Viable counts of the bacterial suspensions used for RBC inoculation with *Y. enterocolitica* and *S. marcescens* ranged from 0.2 to 5.5 CFU/ml, and all BacT/ALERT cultures inoculated on the spiking day were positive. There was no significant difference in the growth of *Y. enterocolitica* between the control and exposed units or between units exposed for 30 or 60 min (*p* > 0.05) (fig. 1A, B). Although significant differences in growth were observed between the control and exposed units of *S. marcescens* (*p* < 0.05), there was no difference in the overall growth between units exposed for 30 or 60 min during RBC storage (*p* > 0.05) (fig. 1C, D).

Discussion

The present findings confirm that there is no difference in bacterial growth in RBCs exposed to RT for 30 or 60 min. Importantly, warming of RBCs does not induce growth of bacteria unable to proliferate under RBC storage conditions in either SAGM or AS-3. Although psychrophilic bacteria are able to grow in RBCs, here we show that for the chosen species, growth is not significantly different in RBC units exposed for 30 or 60 min to RT. This study confirms previous findings showing that there is no additional risk to RBC safety by increasing RT exposures from 30 to 60 min [4, 6,

Fig. 1. Psychrophilic bacterial growth during RBC storage. Growth of *Y. enterocolitica* in SAGM (A) and AS-3 (B) and *S. marcescens* in SAGM (C) and AS-3 (D) during 42 days of RBC storage. Insert shows statistical comparisons between control (CTL) units (remained in storage at 1–6 °C for 42 days), T30 units (exposed 6 times to RT, each time for 30 min during 42 days of storage), and T60 units (exposed 6 times to RT, each time for 60 min during 42 days of storage). Graphs show mean growth $\pm$ SD; n = 4 pooled and split samples. AS-3 = Additive solution 3; SAGM = saline-adenine-glucose-mannitol.

12]. It should be noted that for *S. marcescens* there was a significant increase in bacterial growth between the control (non-exposed) units and units exposed to RT for either 30 or 60 min. It is therefore highly recommended to minimize RT exposure of RBCs, regardless of the exposure time, to decrease the safety risk for transfusion recipients.

An extension of the 30-min rule beyond 60 min is not recommended as we have previously shown that pathogenic bacteria start growing in RBCs after 2 h of RT exposure [12]. Hence, multiple exposures for periods longer than 60 min would likely increase the risk of having high bacterial titers early during RBC storage. The previous study also challenged the 4-hour rule for transfusion, demonstrating significant bacterial growth during a single 5-hour RT exposure of RBCs [12]. For that reason, clinicians should be cautious of this safety risk and have a strict time limit for the transfusion process.

The experimental settings of the present study with bacterial spiking of RBC units were useful to address our question regarding extension of the 30-min rule. These settings do not reflect real-life practice when bacteria enter into the whole blood unit during venipuncture and migrate to the RBC unit during the manufacturing process. However, we have recently shown that bacteria present in whole blood migrate preferentially towards RBCs during buffy coat production of platelet concentrates [13].

The preferential survival of *S. epidermidis* and *K. pneumoniae* in SAGM compared to AS-3 RBC units is an interesting observation. Although both additive solutions have an acidic pH (5.7 or 5.8), they differ greatly in chemical composition. In addition to the additive solutions, the manufacturing processes, anticoagulants, and residual plasma could be factors affecting bacterial growth and survival in RBCs; therefore this aspect of our study warrants further investigation.

A 2012 systematic review of the evidence to support the 30-min rule concluded that to reliably determine whether this ‘rule’ could be extended without increasing the safety risk of RBCs would require ‘robust, modern studies using multiple combinations of blood, anticoagulant, and additive solutions with defined temperatures and times of exposure’ [14]. This collaborative effort between the two Canadian blood manufacturers re-examined the 30-min rule using a variety of bacterial strains spiked into RBCs prepared by standard procedures at each blood supplier and stored in both additive solutions used in Canada. Extension from a 30-min to a 60-min rule has been approved by the CSA. The wording of the new guideline states that a blood component shall not be re-released unless ‘... the blood component has not been outside of a controlled environment for more than 60 min (measured per occurrence, not cumulatively)’. Extending the amount of time permitted outside of controlled temperature may reduce unnecessary

product discards [14, 15], adding efficiencies, and cost savings to the blood system without compromising safety. The approach that led to a change in the Canadian regulations could inform other jurisdictions where the 30-min rule is also under challenge.

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Disclosure Statement

The authors declare no conflicts of interest.

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