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Effect of Sodium Citrate on Growth of Bacteria in Blood Culture

Dong-Hyun Lee[1], Eun-Ha Koh[1], Sae-Rom Choi[1], Sunjoo Kim[1], Dong Hoon Kim[2], Nam Yong Lee[3]

Departments of [1]Laboratory Medicine, [2]Emergency Medicine, Gyeongsang Institute of Health Sciences, Gyeongsang National University School of Medicine, Jinju, [3]Department of Laboratory Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Background: This study compared the growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* in blood culture bottles containing anticoagulants, sodium polyanethol sulfonate (SPS) and sodium citrate.
Methods: One hundred and fifty colony forming units of five different bacterial species were inoculated into standard aerobic (SA) and standard anaerobic (SN) bottles and were combined with 5 mL of human blood in solution with SPS or sodium citrate. Time to detection (TTD) was then monitored using the BacT/Alert 3D system (bioMerieux Inc.).
Results: Compared to the bacteria-only controls, cultures containing *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. pneumoniae* plus SPS blood or citrated blood trended toward reduced TTD in both SA and SN bottles; however, there was no significant difference in TTD between SPS and sodium citrate anticoagulant. Although *H. influenzae* showed a remarkable difference in TTD between SPS (SA 14.8 h, SN 15.0 h) and sodium citrate (SA 23.5 h, SN 18.3 h), this difference was not statistically significant (P=0.10).
Conclusion: Addition of blood enhanced growth of bacteria. All experimental bacteria except *H. influenzae* showed similar TTD in SPS blood and citrated blood. These results support the usefulness of sodium citrate anticoagulant for artificial inoculation in blood culture bottles. **(Ann Clin Microbiol 2013;16: 168-173)**

Key Words: Anticolagulants, Bacteremia, Blood, Culture, Sodium citrate

INTRODUCTION

Despite its widespread use as an anticoagulant for blood culture [1,2], sodium polyanethol sulfonate (SPS) is not commonly used in other laboratory analyses. The establishment of an artificial blood culture model is complicated by the difficulties associated with large-scale SPS anticoagulant preparation for phlebotomy. During blood donation, collection bags usually contain sodium citrate as an anticoagulant. Given the many phlebotomies of polycythemia patients in large hospital settings, it would be preferable to use sodium citrated blood in bacteremia analyses. Citrate, heparin, and EDTA are known to be toxic to microorganisms in blood culture [1,3]. However, if the inhibitory effect of citrate on bacterial growth is minimal compared to that of SPS, sodium citrate might be an acceptable alternative for *in vitro* bacteremia detection. We compared the growth of bacteria in the absence of blood to bacterial growth following spiking with SPS or citrate-treated whole blood samples. To our knowledge, no previous studies have directly compared the effects of SPS and citrate on bacterial growth in blood culture. *Staphylococcus aureus* and *Eschericia coli* which are the most common pathogens causing sepsis in Korea [4] were used for the experiment. *Pseudomonas aeruginosa*, an absolute aerobe, as well as *Streptococcus pneumoniae* and *Haemophilus influenzae*, two fastidious organisms, were also included. As the blood culture bottles could be entered to the automatic blood culture machine several hours after inoculated with blood, we contrived preincubation of these bottles either at room temperature or 37°C to see the effect of delayed entry on the growth of bacteria.

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Correspondence: Sunjoo Kim, Department of Laboratory Medicine, Gyeongsang National University School of Medicine, 79 Gangnam-ro, Jinju 660-702, Korea. (Tel) 82-55-750-8239, (Fax) 82-55-762-2696, (E-mail) sjkim8239@hanmail.net

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MATERIALS AND METHODS

1. Bacterial preparation

Standard aerobic (SA) and standard anaerobic (SN) bottles (bioMerieux Inc., Marcy l'Etoile, France) were inoculated with 150 colony forming units (CFU) of *S. aureus* (ATCC 29213) and *E. coli* (ATCC 25922); *S. pneumoniae* and *H. influenzae* (clinical isolates) prepared in brain heart infusion broth. For *P. aeruginosa* (clinical isolate), an absolute aerobe, only SA bottles were used for the experiment. The same experiment was repeated three times on separate days. *S. aureus* grew on blood agar plates (BAP) to a mean±SD CFU of 170±45; *E. coli* on MacConkey agar plates to a mean CFU of 150±25; *P. aeruginosa* on MacConkey agar plates to a mean CFU of 200±90; *S. pneumoniae* on BAP to a mean CFU of 160±77; *H. influenzae* on chocolate agar plates to a mean CFU of 150±67.

2. Preparation of anticoagulated blood

A solution of SPS was prepared by adding 1,320 mg of SPS (Sigma-Aldrich, St. Louis, MO, USA) to 200 mL of 0.9% sterile saline. Ten milliliters of SPS solution then was added to each 100-mL aliquot of blood obtained from volunteers [5]. To obtain citrated blood, we used commercial blood collection bags designed for blood donation. Blood was collected from patients who visited our hospital for phlebotomy. Each bag contained 56 mL of 3% sodium citrate solution for 400 mL of phlebotomy. Five milliliters of the anticoagulated blood samples was added to bacteria-inoculated bottles. Final concentration of SPS in the experimental bottle (0.037%) revealed the same with the uninoculated bottle (0.035%).

A negative control was prepared containing only bacteria except *H. influenzae* which requires blood to grow. The experimental protocol was approved by IRB and blood was collected after receiving written consent from the participants.

3. Time to detection

The bottles were preincubated at 25°C or 37°C for 0, 6, or 12 h. Bottles then were inserted to the BacT/Alert 3D system (bioMerieux Inc.) to measure the time to detection (TTD), defined as the period from the insertion of the bottle to the detection of microorganisms [1]. Mean±SD TTDs for the control, SPS blood, and citrated blood were analyzed for each bacterium.

4. Statistical analyses

The Mann-Whitney U test was used for the non-parametric analysis of mean TTD differences. The Kruskal-Wallis test was applied to detect differences in the mean TTDs across all preincubation periods. The Jonckheere-Terpstra test was used to measure a decrease in the TTD with preincubation time. All statistical analyses were two-sided and were performed using IBM SPSS v.20.0 software (SPSS Inc., Chicago, IL, USA) at the statistical significance of P value <0.05.

RESULTS

1. Non-fastidious organisms: *S. aureus* and *E. coli*

Compared to the control (SA 15.0±0.6 h, SN 15.0±1.2 h), the cultures containing *S. aureus* plus SPS blood or citrated blood trended toward reduced TTDs under both SA (SPS 13.3±1.0 h, citrate 13.1±1.0 h) and SN (SPS 13.1±0.9 h, citrate 13.6±1.2 h) bottles (Fig. 1) (P=0.10). However, no significant differences in the TTDs were identified between these two anticoagulants (P=0.70).

The mean TTDs were much shorter for *E. coli* compared with *S. aureus*. Spiking with SPS blood or citrated blood affected less of a reduction in TTDs for *E. coli*. Mean TTDs in the SA bottles were 12.7±0.8 h for the control, 12.0±0.6 h for the SPS blood, and 11.9 ±0.6 h for the citrated blood (data not shown, P=0.40). Mean TTDs were equally 11.4 h in the SN bottles for the control, SPS blood, and citrated blood.

2. Absolute aerobe: *P. aeruginosa*

TTDs of SA in the control, SPS blood, and citrated blood were similar: 17.6±0.3 h, 16.4 ±0.5 h, and 16.5±0.8 h, respectively (P=0.10) for *P. aeruginosa*.

3. Fastidious organisms: *S. pneumoniae* and *H. influenzae*

TTDs of SA in the control, SPS blood, and sodium citrate blood were not significantly different: 14.9±0.5 h, 12.9±0.5 h, and 13.1±0.5 h (P=0.10) in *S. pneumoniae*. TTDs of SN in the control, SPS blood, and sodium citrate blood were similar: 13.9±0.2 h, 13.0±0.3 h, and 13.1±0.4 h, respectively for *S. pneumoniae*. Although TTDs of *H. influenzae* showed a remarkable difference in the SPS blood (14.8±0.1 h) vs. citrated blood (23.5±1.0 h) in the SA bottles, it was not statistically significant (P=0.10, Fig. 2); which showed a similar pattern (15.0±1.4 h vs. 18.3±0.9 h) in the SN bottles (P=0.10).

4. Effect of preincubation

All experimental bacteria showed a significantly rapid de-

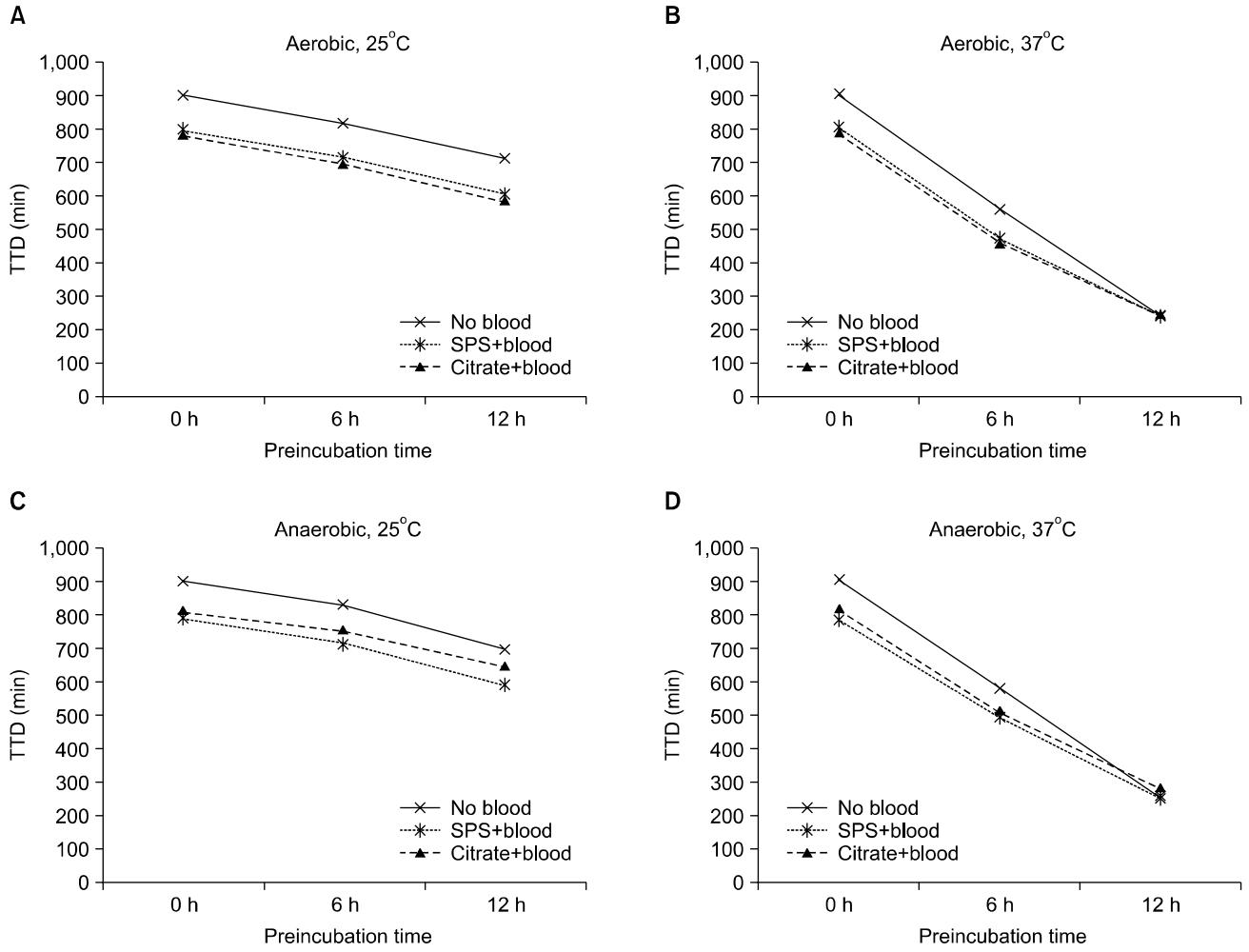

Fig. 1. Effects of blood and the anticoagulants SPS or sodium citrate on the growth (time to detection, TTD) of *Staphylococcus aureus* in the aerobic and anaerobic bottles by preincubation time. (A) Aerobic bottle, 25°C. (B) Aerobic bottle, 37°C. (C) Anaerobic bottle, 25°C. (D) Anaerobic bottle, 37°C.

tection by preincubation at 37°C for 6 h or 12 h ($P<0.05$). All bacteria showed also significantly shorter TTDs even by preincubated at 25°C; except *S. pneumoniae* for citrated blood in SA and SN bottles, and SPS blood in SN bottle; *H. influenzae* for citrated blood in SA and SN bottles, and for SPS blood in SN bottles (data not shown, $P>0.05$).

DISCUSSION

It seemed difficult to find out the previous studies on the effect of blood on the growth of organisms. Previous studies involving simulated blood cultures examined only SPS blood [5,6], only citrated blood [7,8], or only bacterial inoculation [9]. Depending on components in blood culture, bacterial growth can be either promoted or inhibited. The addition of anticoagulant-treated blood effected a potential bacterial growth enhancement as suggested by the reduction in TTDs compared with blood-free cultures. Interestingly, this phenomenon was more noticeable in *S. aureus* and *S. pneumoniae* (gram positives) than in *E. coli* and *H. influenzae* (gram negatives). However, it may be plausible to generalize that the addition of blood is somewhat beneficial and/or stimulating to bacterial growth. Theoretically, nutrients and soluble plasma proteins in blood may create a favorable environment for bacteria to proliferate in the absence of certain immune components. The inhibitory effects of cytokines, antibodies, complement molecules, and/or phagocytic cells may be less intense than bacterial growth-promoting factors in the blood. In addition, the anticoagulants may have removed existing immune effectors and thereby allowed for bacterial growth promotion.

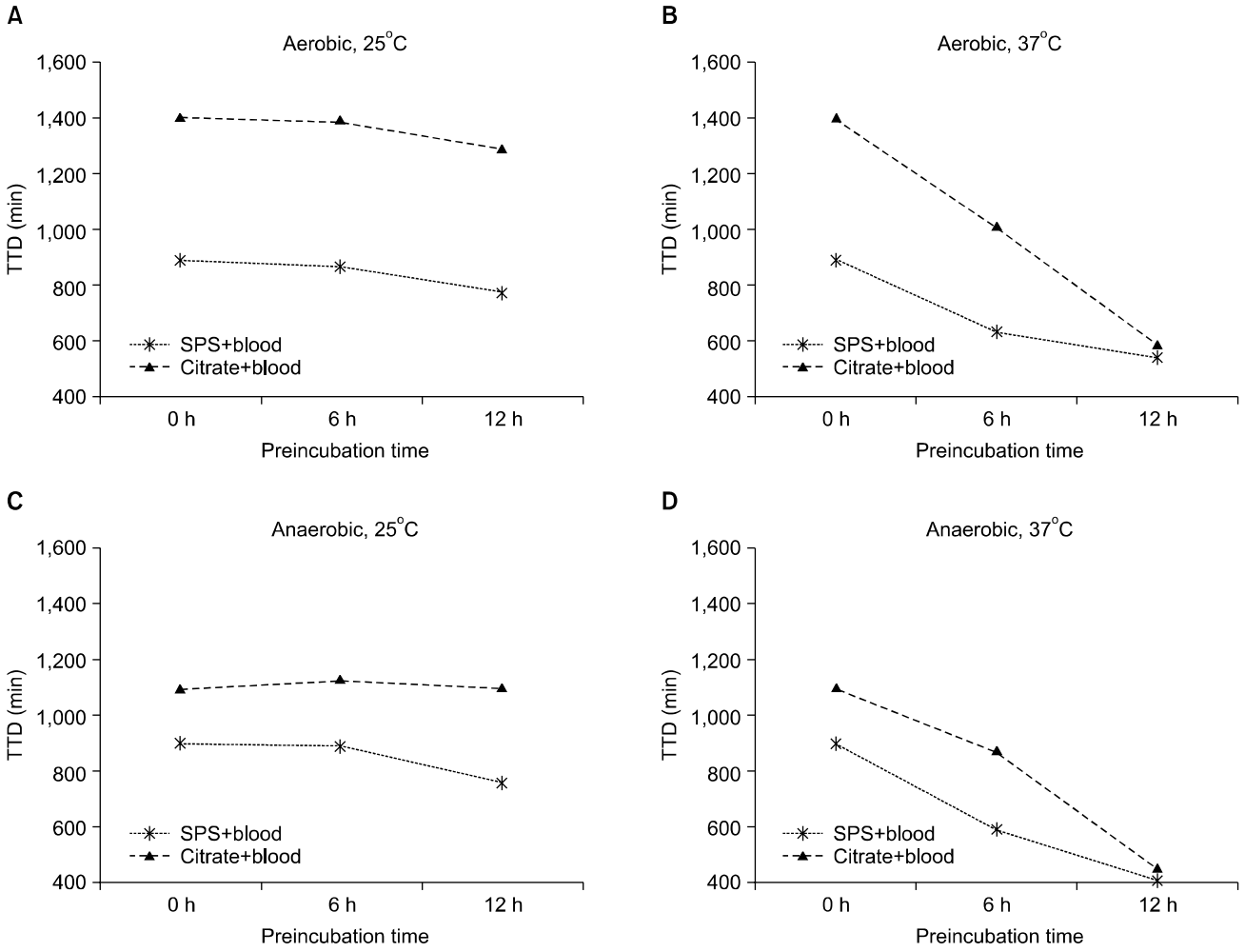

Fig. 2. Effects of blood and the anticoagulants SPS or sodium citrate on the growth (time to detection, TTD) of *Haemophilus influenzae* in the aerobic and anaerobic bottles by preincubation time. (A) Aerobic bottle, 25°C. (B) Aerobic bottle, 37°C. (C) Anaerobic bottle, 25°C. (D) Anaerobic bottle, 37°C.

Although SPS is widely accepted as an anticoagulant in blood culture [1,2], it is very hard to prepare a large scale of phlebotomy. Citrate is commonly used for blood donation. The inhibitory effect of citrate was compared with SPS in this experiment with commonly encountered bacteria in the sepsis. TTDs were virtually identical between these two anticoagulants for all bacteria except *H. influenzae*. However, a statistical significance was not found for this organism. Reduction of TTDs was observed with respect to the preincubation time (6 h or 12 h) regardless of storage temperature (25°C or 37°C) or bottle type (SA or SN) except *S. pneumoniae* and *H. influenzae*, two fastidious organisms. During preincubation for 6 h or 12 h, non-fastidious microorganisms might grow significantly either at 25°C or 37°C. In contrast, the fastidious organisms seemed to be more likely indolent by storage at 25°C, especially in the citrated blood.

In conclusion, citrated blood showed comparable TTDs with SPS blood for five experimental bacteria including fastidious organisms. As SPS is difficult to prepare and rarely is used in the laboratory, we may consider using sodium citrate in simulated blood culture experiment.

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=국문초록=

혈액배양에서 구연산 항응고제가 세균 증식에 미치는 효과

경상대학교 의과대학 [1]진단검사의학교실, [2]응급의학교실, 건강과학연구원
[3]성균관대학교 의과대학 삼성서울병원 진단검사의학교실

이동현[1], 고은하[1], 최새롬[1], 김선주[1], 김동훈[2], 이남용[3]

배경: 항응고제(sodium polyanethol sulfonate, SPS와 구연산)가 패혈증에서 중요한 5가지 세균의 증식에 미치는 효과에 대해서 관찰하였다.

방법: 세균 150개 집락을 호기성병(SA)과 혐기성병(SN)에 접종하고, SPS 혈액과 구연산 혈액 5 mL를 첨가하였다. 이들 혈액배양 병을 BacT/Alert 3D 장비(bioMerieux Inc.)에 투입하고 배양검출시간을 측정하였다.

결과: 포도알균, 대장균, 녹농균 및 폐렴알균에서는 세균만 넣은 병에 비해 SPS혈액 혹은 구연산 혈액을 넣은 병에서 배양검출시간이 1-2시간 단축되었다. 하지만 SPS혈액과 구연산 혈액 간에 차이는 없었다. *Haemophilus influenzae*는 SPS 혈액(SA 14.8시간, SN 15시간)과 구연산 혈액(SA 23.5시간, SN 18.3시간) 간에 뚜렷한 차이가 있었지만 통계적으로는 유의하지 않았다(P=0.10).

결론: 혈액 주입으로 세균의 증식이 촉진되었고, SPS혈액에 비해 구연산 혈액의 세균 증식 억제 효과는 발견되지 않았다. 따라서 혈액배양 실험에 검사실에서 쉽게 구할 수 있는 구연산 혈액을 사용할 수 있을 것으로 판단된다. [Ann Clin Microbiol 2013;16:168-173]

교신저자 : 김선주, 660-702, 경남 진주시 강남로 79
경상대학교 의과대학 진단검사의학과
Tel: 055-750-8239, Fax: 055-762-2696
E-mail: sjkim8239@hanmail.net