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NO_2^- and NO_3^- Measurements for Detection of Escherichia coli in Blood Samples

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Abstract Escherichia (E.) coli, a common causative microorganism in bacteremia, is a member of facultatively anaerobic bacilli that are able to reduce NO_3^- to NO_2^-. To determine whether NO_2^- production or NO_3^- reduction occurs during the growth of E. coli in blood cultures, NO_2^- and NO_3^- were directly and simultaneously measured by capillary zone electrophoresis. The BACTEC 9240 (Becton Dickinson Diagnostic Instrument Systems, USA) was used to culture E. coli cells in blood samples. The NO_2^- concentration in the medium ($6.0 \pm 2.6\ \mu M$ at 0 h) significantly increased at 8 h ($p < 0.0001$) and reached a peak value, $64.1 \pm 22.2\ \mu M$ at 9 h. The NO_3^- concentration in the medium ($215.9 \pm 29.7\ \mu M$ at 0 h) significantly decreased at 8 h ($p < 0.0001$) and lowered to $65.0 \pm 37.6\ \mu M$ at 10 h. The peak of NO_2^- production (9.2 ± 0.7 h) appeared earlier than the CO_2 production (11.1 ± 0.9 h). There is no correlation between the peak time of NO_2^- production and the CO_2 detection time ($p > 0.05$). These data give us an essential potentiality to use the NO_2^- and NO_3^- monitoring system for detecting the presence of E. coli cells.

Key Words: Escherichia coli, capillary zone electrophoresis, bacteremia, CO_2 production

Introduction

Rapid and reliable detection of bacterial growth in blood samples is useful for diagnosis and treatment of patients with sepsis. Recently, automatically and continuously monitoring blood culture systems have been developed for this purpose[1). The BACTEC 9240 (Becton Dickinson Diagnostic Instrument Systems, USA) is one of such instruments, which uses a pH-sensitive fluorescence sensor attached to the bottom of each bottle. When the sensor detects CO_2 increase as a result of bacterial growth in the medium, the cultures are recognized as positive by computer algorithms or delta values.

NO_2^- detection method, when NO_2^- is reduced from NO_3^- by some bacteria such as E. coli, has been utilized to diagnose bacteriuria by means of Griess' nitrite reaction[2). Recently, Ueda et al established the measuring method of NO_2^- and NO_3^- in human blood plasma by capillary zone electrophoresis[3). This method is able to measure the concentrations of NO_2^- and NO_3^- directly and simultaneously, whereas the previous method reported by Green et al[4) needs to reduce NO_3^- to NO_2^- prior to analysis. The production of NO_2^- from NO_3^- or the reduction of NO_3^- to NO_2^- may be relevant to recognize bacterial growth in some species under anaerobic atomosphere.

In this study, we measured the NO_2^- and NO_3^- concentrations in the blood cultures by the method using capillary zone electrophoresis and simultaneously detected the CO_2 production in the Bactec 9240 apparatus.

Materials and Methods

The BACTEC anaerobic Plus/F bottles contain 25 ml of supplemented 2.75% soybean casein broth, 0.05% sodium polyanetholesulfonate, 16% nonionic absorbing resins, and 1% cationic exchange resins. Three mililiters of blood collected from healthy subjects and 150μM KNO_3 were added to each bottle. The bacterial suspension of E. coli ATCC 25922 or E. coli strain isolated from 8 patients was adjusted to 1.5×10^8 cells/ml by ATB 1550 (bioMérieux, France) and diluted to 1,500 cells/ml with sterile saline. Final cell count in the culture bottle was 50/ml. Culture was duplicated. The bottles were incubated at 35.5°C in the BACTEC 9240 instrument until the cultures showed a positive signal of the CO_2 production. At 0, 3, 6, 7, 8, 9 and 10 h, 1 ml of the contents in the bottles was removed by the syringe after the top of the bottles was disinfected by alcohol. The samples were centrifuged at 3,500×g at 4°C for 3 min. The supernatants were filtered using the filter (MILLEX-HV, 0.45μm, Millipore, USA) and were stored at -80°C until analysis. Before analysis the supernatant was deproteinized by centrifugal ultrafiltration (cut-off molecular weight 10 kDa, UFC3, Millipore, USA), and the NO_2^- and NO_3^- concentrations were measured by a capillary ion analyzer connected to Model 805 data station (Waters, USA) described by Ueda at al[3].

E. coli growth curve

The number of living E. coli ATCC 25922 in anaerobic blood cultures from 0 to 10 h was counted by the pour-plate method. At 0 and 3 to 10 h, 0.2 ml of the content in the bottle was removed and suspended in serial 10-fold dilutions. Each suspension was mixed with molten agar medium in a Petri dish and incubated. The colonies in the plates that were seeded with the dilution giving between 30 to 300 CFU/plate, were counted. To obtain the viable CFU/ml in blood cultures, the CFU/plate was multiplied by the dilution factor.

Statistical analyses

Data are presented as the mean ± SD. ANOVA with repeated measures was used for comparison of more than two variables, to determine significance. When significant differences were observed between the two groups, comparisons were made by Dunnett's test. A value of $p<0.05$ was considered significant.

Results

To confirm the growth of E. coli ATCC 25922 in a BACTEC anaerobic bottle, the number of living bacteria was counted. The logarithmic increase of cell number during 0- to 10-h incubation was observed (Fig. 1).

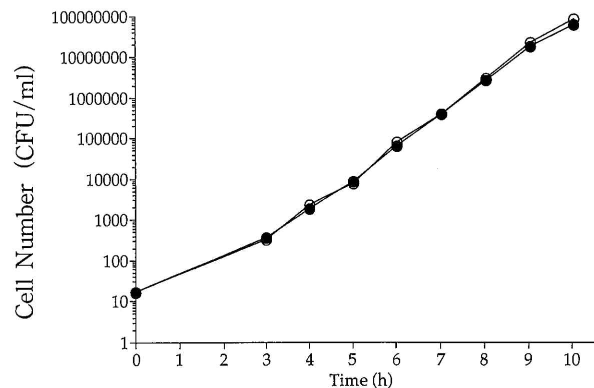

Fig. 1. Changes in the number of E. coli. Data are presented from two experiments.

When E. coli ATCC25922 was incubated in the BACTEC aerobic Plus/F bottles, it did not produce NO_2^- (maximum concentration, 10.0μM at 10 h) although it reduced NO_3^- (176.1 μM at 0 h to 78.9 at 10 h). To examine the changes of NO_2^- and NO_3^-, we inoculated E. coli strain ATCC25922 and 8 clinical isolates in the BACTEC anaerobic culture bottles and incubated at 35.5°C.

The NO_2^- concentration in the medium (6.0±2.6 μM at 0 h) significantly increased at 8 h ($p<0.0001$) and reached a peak value, 64.1±22.2μM at 9 h (Fig. 2). The NO_3^- concentration in the medium (215.9±29.7 M at 0 h) significantly decreased at 8 h ($p<0.0001$) and lowered to 65.0±37.6 at 10 h (Fig. 3).

The peak time of NO_2^- production was 9.2±0.7 h (8.0-10.0 h) and it was earlier

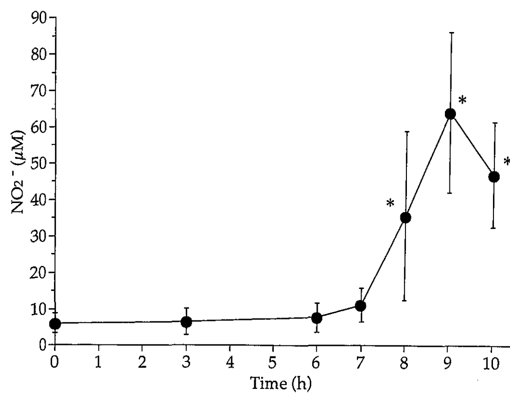

Fig. 2. Changes in NO_2- concentration in the culture medium of E. coli . (mean± SD) *p<0.0001 vs 0 h

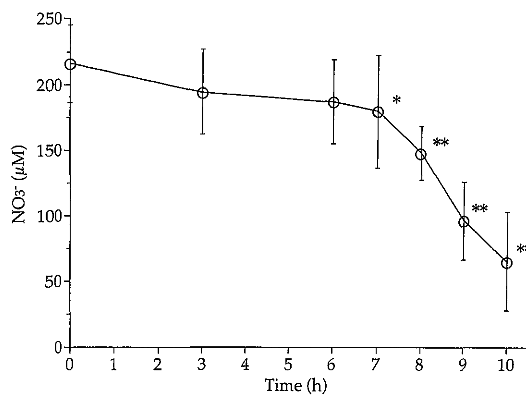

Fig. 3 Changes in NO_3^- concentration in the culture medium of E. coli. (mean± SD) *p<0.05 vs 0h, **p<0.0001 vs 0 h

than that of CO_2 production (11.1±0.9 h, 9.9 -12.3 h) (Table 1). There is no correlation between the peak time of NO_2^- production and the CO_2 detection time ($p>0.05$).

Discussion

The Enterobacteriaceae is a family of gram-negative, facultatively anaerobic bacilli that ferment glucose with the production of acid and reduce NO_3^- to NO_2^-. Anaerobic respiration is unique to some bacteria, and an electron acceptor is NO_3^-, SO_4^{2-}, or CO_2, instead of O_2[5]. E. coli has been reported as a causative organism in bacteremia[6]. To examine the relationship between the NO_3^- reduction and bacterial growth, E. coli was selected.

The studies presented here show that a logarithmic growth of E. coli in anaerobic blood cultures induces reduction of NO_3^- to NO_2^- in a time-dependent manner. The peak time of NO_2^- production was 9.2±0.7 h and detectable earlier than that of CO_2 productin (11.1±0.9 h). To measure NO_2^- concentration in the medium is useful to detect positive blood cultures. The decline of NO_2^- concentration after the peak is not due to NO_2^- oxidation to NO_3^-. A possible explanation is that NO_2^- is reduced to NO, N_2O or nitrogen (denitrification), or NO_2^- is reduced to ammonia[7]. The NO_3^- concentration significantly decreased at 8 h and reached less than a third of initial concentration at 10 h. The initial point of NO_3^- decline corresponds to

Table. Comparison of NO_2^- and CO_2 production time

Esherichia coli	Peak time of NO_2^- Production (h)	CO_2 detection time (h)
ATCC25922	9.0	10.5±0.2
# 1	9.0	12.3
# 2	9.0	12.0±0.5
# 3	8.5±0.7	10.2±0.1
# 4	9.5±0.7	11.1±1.7
# 5	9±1.4	10.8±0.7
# 6	10.0	11.0±0.5
# 7	9.5±0.7	11.7±0.5
# 8	9.5±0.7	10.8±0.6

the peak time of NO_2^- production.

The NO_3^- reduction test has been used to determine whether the family Enterobacteriaceae has the ability to reduce NO_3^- to NO_2^- and further reduce NO_2^- to nitrogen[8], and has been useful in detecting bacteria in urine [2]. The test should be performed on a urine sample that has been collected at least 4 hours or more after the last voiding to allow the organisms in the bladder to metabolize the NO_2^-[9]. Our data are in agreement with this and showed 9 hours as a peak time of NO_2^- production. Dougall et al presented subsequent change of salivary NO_2^- concentration after ingestion of KNO_3[10]. Interestingly, 2 days administration of amoxicillin affected NO_3^- reductase-containing bacteria in the mouth and reduced salivary NO_2^- concentration. Because they used a standard Griess reaction to measure NO_2^- concentration [11], NO_2^- alone was measured. In our studies, both of NO_2^- and NO_3^- concentration were measured, and each change followed bacterial growth in the medium.

Compared with CO_2 measurement by the BACTEC 9240, NO_2^- and NO_3^- measurements were 2 hours earlier , which might be more useful for physicians to treat patients with suspected sepsis. But there is two disadvantage on NO_2^- and NO_3^- measurements that 1 ml of contents in the culture bottle is needed for each measurement and that further process such as centrifugation and filtration is needed. However, NO_2^- detection in gas phase is possible even at very low concentration by a chemiluminescence analysis[12], which will solve the disadvantage in future. Furthermore, other limiting aspects remain. Firstly, an anaerobic medium, which has been eliminated from routine use by many institutions, is essential. Secondly, detection of bacteremia would be limited to those organisms that reduce NO_3^-. Although further studies are needed, we speculate that an increase of NO_2^- concentration between 0 h and 9 h is reliable for existence of E. coli. and other bacteria that reduce NO_3^- to NO_2^-.

In conclusion, to measure NO_2^- and NO_3^- concentration in anaerobic blood cultures is useful to detect existence of E. coli.

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