RAPID DIAGNOSIS OF BACTERIURIA BY MEASURING BACTERIAL ADENOSINE TRIPHOSPHATE USING A BIOLUMINESCENCE ASSAY

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A rapid method for quantifying bacteriuria was evaluated in 114 clinical specimens using a bacterial adenosine triphosphate (ATP) assay. The procedure allows for removal and destruction of non-bacterial ATP and subsequent analysis of bacterial ATP by firefly (*Photinus pyralis*) luciferin-luciferase bioluminescence and requires 30 minutes as a whole. The correlation coefficient between the number of bacteria in urine samples and the ATP concentration was 0.889. Sensitivity and specificity representing more than 10,000 CFU/ml of bacteriuria were 90.0% and 96.8%, respectively. Those representing more than 100,000 CFU/ml were 88.1% and 97.2%.

Key words : Bioluminescence, ATP, Luciferin, Bacteriuria

Quantitative urine cultures are routinely performed to detect bacteriuria. The most widely used technique is the streak or pour plate method for overnight incubation. This method is, however, time-consuming. In response to interest in decreased turnaround time, several techniques have been advocated for rapid detection of bacteriuria[1].

The adenosine triphosphate (ATP) assay with firefly (*Photinus pyralis*) luciferin bioluminescence has been developed for this purpose. ATP is an essential component of all living organisms and its measurement is therefore a good parameter of biomass and cell viability. Several attempts have been made to utilize this ATP assay for the clinical detection of bacteria.

We report the results of our evaluation of the ATP bioluminescence assay for quantifying bacteriuria in clinical specimens.

MATERIALS AND METHODS

Urine specimens. We tested 114 clean voided or catheterized urine specimens from in- and out-patients at Gifu University Hospital. Specimens obtained during or immediately after antibiotic treatment were excluded. The urine specimens were kept at 4℃ immediately after collection and tested within 4 h.

Reference procedure. A conventional quantitative procedure was used as a reference method. The urine specimen (0.1 ml) and those diluted 1/100, 1/10,000 and 1/1,000,000 with sterile saline were inoculated onto a brain heart infusion agar plate for quantification. Cultures were subjected to a colony-count and Gram-stain after overnight aerobic incubation at 37℃.

Bioluminescence assay. Bioluminescent determination of bacteriuria was performed using the following procedure: One milliliter of specimen was mixed with both of 0.1 ml of 1% polyoxyethylene (10) octylphenyl ether (Wako Pure Chemical Industries, Osaka, Japan) and 0.1 ml of 0.04% apyrase (grade 1, Sigma, St. Louis, Missouri, USA) in 0.05 M $CaCl_2$, then allowed to stand at 37℃ for 10 min to destroy ATP released from non-bacterial cells, i.e., red blood cells, white blood cells, epithelial cells and so on. The mixture

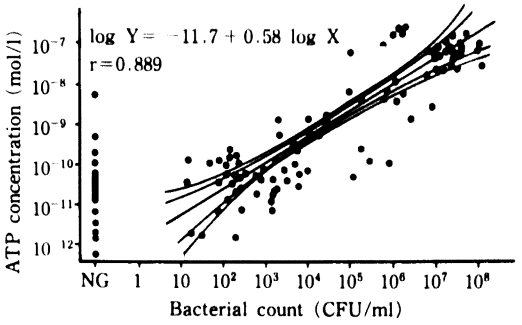


Fig. 1. Correlation between bacterial counts and ATP concentration determined for urine samples

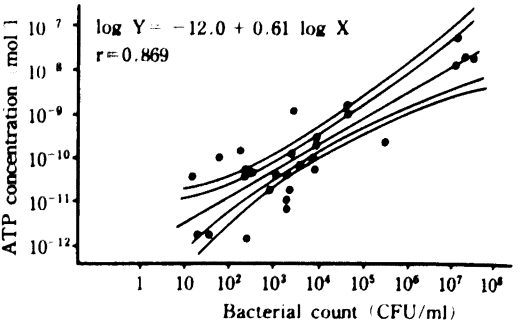


Fig. 2 A. Correlation between bacterial counts and ATP concentration
A : determined for Gram-positive cocci

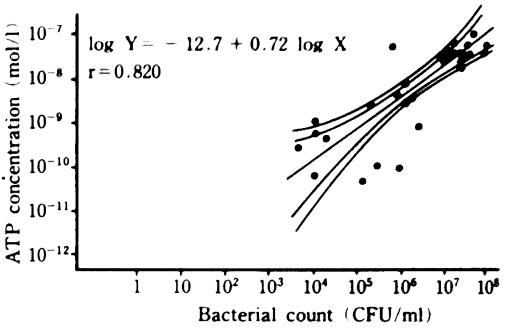


Fig. 2 B. Correlation between bacterial counts and ATP concentration
B : determined for Gram-negative rods

was centrifuged for 10 min at a force of 3,000 g, then 1 ml of 0.025 M HEPES buffer pH 7.75 was added to the sediment. A 0.1 ml aliquot of the mixture was pipetted into another cuvette containing an equal amount of ATP-releasing reagent (Labo Science, Tokyo, Japan). The cuvette was shaken for a few seconds and placed into the counting chamber of the Lumiphotometer TD-4000 (Labo Science, Tokyo, Japan). The measurement of bacterial ATP was started by adding 0.1 ml of luciferin-luciferase reagent (ATP bioluminescence HS, Boehringer Mannheim Biochemica, West Germany) into the cuvette through the dispenser of the Lumiphotometer. The light emission was measured after a 5-second delay and the luminescence parallel to bacterial ATP was expressed as relative light units (RLU) accumulated over a 15-sec integration. ATP concentration in the sample was calculated by using the standard curve of ATP as a reference after correcting for background light emission. This whole procedure required only 30 min.

RESULTS

The correlation between the number of bacteria in urine and the ATP concentration was found to be almost linear with a correlation coefficient of 0.889 (Fig. 1). When the samples were classified into two groups with Gram-positive cocci and Gram-negative rods, the linear correlations of each group were still observed, resulting in no significant difference between the two bacterial groups (Figs. 2 A, 2 B).

The test indices of this assay for screening bacteriuria varied as the cut-off level of ATP concentration and/or the significant level of bacterial count changed. The following formulae were used to calculate sensitivity, specificity, predictive values and efficiency : sensitivity = (true positives)/(true positives + false negatives) × 100 ; specificity = (true negatives)/(true negatives + false positives) × 100 ; positive predictive value = (true positives)/(true positives + false positives) × 100 ; negative predictive value = (true negatives)/(true negatives + false negatives) × 100 ; efficiency = (true positives + true negatives)/(true positives + true negatives + false negatives + false positives) × 100.

As the ATP cut-off level was set higher, the sensitivity decreased and the specificity increased. Fig. 3 illustrates these relationships. Fig. 4 shows the highest efficiencies obtained under the optimal ATP cut-off level for detecting each level of bacteriuria. This assay offered approximately 80

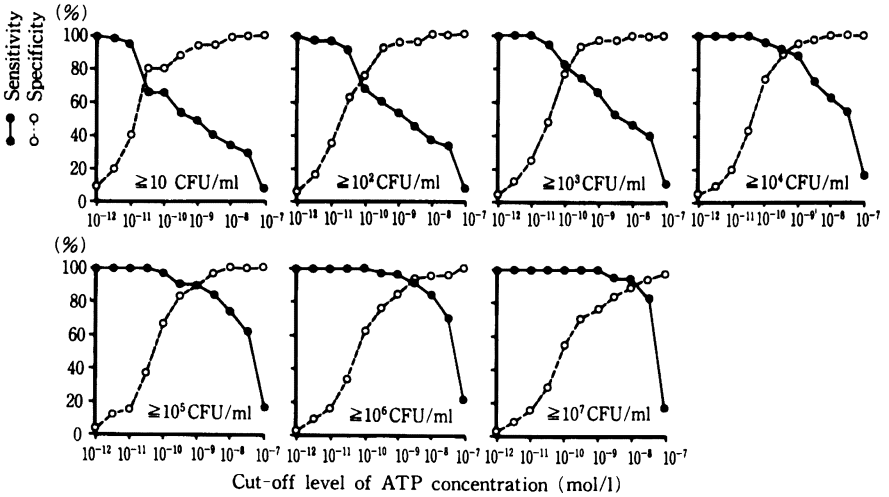


Fig. 3. Sensitivity and specificity (%) of bioluminescence assay compared to quantitative plate culture of each level of bacterial count

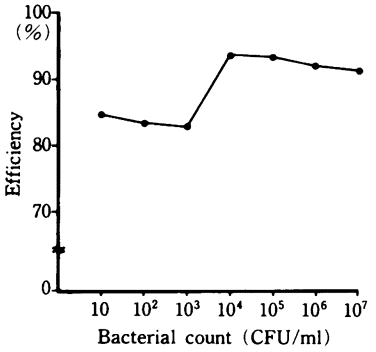


Fig. 4. The highest efficiency (%) obtained under the optimal ATP cut-off level for screening each level of bacteriuria

-85% efficiency in detecting bacteriuria at levels of 10, 100 or 1,000 CFU/ml. But a very high efficiency of over 90% was obtained for screening bacteriuria at levels of 10,000 CFU/ml or more.

The most effective cut-off level of ATP concentration was statistically revealed to be 7.0×10^{-10} mol/l using 10,000 CFU/ml as the criterion of significant bacteriuria. Under this cut-off level, sensitivity, specificity, positive and negative predictive values were 90.0%, 96.8%, 95.7% and 92.5%, respectively. When the criterion of significant bacteriuria was shifted to 100,000 CFU/ml, they were 88.1%, 97.2%, 94.9% and 93.3% with the most effective cut-off level of ATP concentration at 2.5×10^{-9} mol/l.

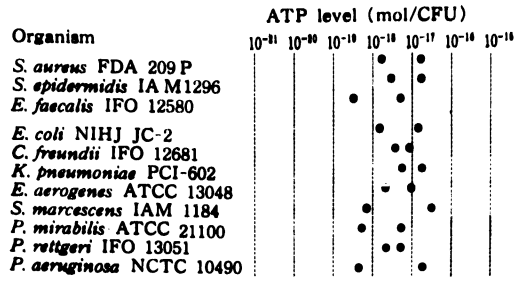
DISCUSSION

Firefly luciferin and luciferase were prepared in a highly purified state from *Photinus pyralis*. In the presence of luciferin, Mg^{++} and ATP, luciferase catalyzes a reaction in which adenylate is transferred from ATP to the carboxyl group of luciferin. The luciferyl adenylate interacts with oxygen through a series of steps leaving the oxyluciferin in the excited state.

$$\text{D-luciferin} + \text{ATP} + O_2 \xrightarrow[Mg^{++}]{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light (560 nm)}$$

If the concentration of luciferin, luciferase and oxygen are held constant, the light intensity is directly proportional to the ATP concentration. For this reason, the firefly luciferin-luciferase system is sensitive for determining ATP concentration over a wide range[2]. Our conditions are arranged to get light intensity proportional to ATP concentration in a range from ten picomoles to one micromole.

The ATP content of 11 different bacterial species was calculated in our previous study (Fig. 5). The ATP levels corresponding to 1 CFU of bacterium ranged approximately from 10^{-18} to 10^{-17} mol. With this bioluminescence assay, ATP concentration can be measured in a range from ten picomoles in a final volume of 0.1 ml. Hence



S. aureus : *Staphylococcus aureus*
S. epidermidis : *Staphylococcus epidermidis*
E. faecalis : *Enterococcus faecalis*
E. coli : *Escherichia coli*
C. freundii : *Citrobacter freundii*
K. pneumoniae : *Klebsiella pneumoniae*
E. aerogenes : *Enterobacter aerogenes*
S. marcescens : *Serratia marcescens*
P. mirabilis : *Proteus mirabilis*
P. rettger : *Providencia rettgeri*
P. aeruginosa : *Pseudomonas aeruginosa*

Fig. 5. ATP levels corresponding to 1 CFU of bacterium

the measurement of an ATP level of 10,000 CFU/ml of bacteria is easily achievable[2].

The ATP bioluminescence assay for detection of bacteriuria was first used by THORE[3]. Currently this assay is commercially available and is both accurate and simple for screening for significant bacteriuria[4~7]. Although there have been several reports on the efficiency of this assay, this was evaluated only by semi-quantifying bacteriuria and by a single ATP cut-off level. In our present study, we determined the optimal ATP cut-off level at each level of bacteriuria from 10 to 10,000,000 CFU/ml.

This assay offered high efficiency for screening bacteriuria at levels of 10,000, 100,000 CFU/ml or more. The setting of an optimal ATP cut-off level which corresponds to each level of bacteriuria improved the efficiency, in comparison with most previous reports[4~6]. The efficiency is, however, somewhat too low to detect bacteriuria at levels of 10, 100 or 1,000 CFU/ml, which is theoretically attributed to the lower limit of ATP measurement.

As already noted in other reports, erroneous results in the ATP bioluminescence assay can arise for the following reasons: 1. Contaminating anaerobes or yeasts contribute false-positive results. 2. The variation in ATP content among bacterial species, as shown in Fig. 5, or the metabolic rate is a source of error[8]. 3. Previous antibiotic treatment is a cause of false-positive results by yielding negative cultures with several ATP values[4,6]. 4. Possible false-negative results stem from excessive acidity or basicity or from substances such as chloride, and urea which inhibit luciferase activity[6]. Hence centrifugation of samples and suspension in HEPES buffer are indispensable.

Although misleading interpretations must be avoided, this assay is highly effective for screening significant bacteriuria and also offers a considerable reduction in the time required for quantifying bacteriuria. Although it costs as much as ¥300 (equivalent to $2.3) or more per test for materials alone, the advantage of a speedy result is significant.

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REFERENCES

1) SMITH T K, HUDSON A J, SPENCER R C: Evaluation of six screening methods for detecting significant bacteriuria. J Clin Pathol 41: 904 ~ 909, 1988
2) MCELROY W D, DELUCA M A: Firefly and bacterial luminescence: Basic science and applications. J Appl Biochem 5: 197 ~ 209, 1983
3) THORE A, ANSEHN S, LUNDIN A, BERGMAN S: Detection of bacteriuria by luciferase assay of adenosine triphosphate. J Clin Microbiol 1: 1 ~ 8, 1975
4) RUOKONEN A, KOSKINEN M, LEINONEN M, TIILIKAINEN A, VIHKO R: Screening of bacteria in urine using luciferin-luciferase assay of microbial ATP: a comparative study. Ann Clin Biochem 19: 416 ~ 420, 1982
5) PARK C H, HIXON D L, MC CLINTOCK C C, FERGUSON C B, LAWLESS C C, RISHEIM C C, COOK C B: Rapid detection of insignificant bacteriuria by

concomitant use of Lumac system and Gram's stain. Am J Clin Pathol 82 : 593 ~ 596, 1984
6) Welch W D, Thompson L, Layman M, Southern Jr P M : Evaluation of two bioluminescence-measuring instruments, the Turner design and Lumac systems, for the rapid screening of urine specimens. J Clin Microbiol 20 : 1165 ~ 1170, 1984
7) Martin E T, Cote J A, Perry L K, Martin W J : Clinical evaluation of the Lumac bioluminescence method for screening urine specimens. J Clin Microbiol 22 : 19 ~ 22, 1985
8) Conn R B, Charache P, Chappelle E W : Limits of applicability of the firefly luminescence ATP assay for the detection of bacteria in clinical specimens. Am J Clin Pathol 63 : 493 ~ 501, 1975

生物発光を利用した細菌内ATP量の測定による細菌尿の迅速診断方法

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細菌内ATP量の測定による細菌尿の迅速な定量方法を114検体に対し検討した。この方法は細菌以外から由来するATPを除いた上で，細菌内のATPを抽出し，ホタル（*Photinus pyralis*）より精製されたルシフェリン＋ルシフェレースによる生物発光現象により計測するものであり，測定までに30分を要するのみである。尿中細菌数とATP濃度との間には相関係数0.889の高い相関関係が認められた。10^4 CFU/mlの細菌尿が感度90.0%，特異度96.8%で，10^5 CFU/mlの細菌尿が各々88.1%，97.2%で示された。

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