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Reliability of a Bioluminescence ATP Assay for Detection of Bacteria

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The reliability of bioluminescence assays which employ the luciferin-luciferase ATP-dependent reaction to evaluate bacterial counts was studied, both in vitro and on urine specimens. Bioluminescence and cultural results for the most common urinary tract pathogens were analyzed. Furthermore, the influence of the culture medium, of the assaying method, and of the phase of growth on bioluminescence readings was studied. Results show that *Proteus*, *Providencia*, and *Morganella* strains are not correctly detected, neither in vitro nor in urine samples, by the standard assaying method. The analysis of assaying parameters demonstrated that some modifications to the extraction procedure of bacterial ATP could improve the reliability of this technique.

Rapid methods to evaluate bacterial counts in biological specimens have been continuously investigated in the last 20 years (1, 4, 6, 14). The calibrated loop or the serial dilution technique is used in most cases, though they are time-consuming (7).

Methods employing the measurement of bacterial ATP by the luciferin-luciferase, ATP-dependent reaction have been developed and marketed. They were shown to be sufficiently sensitive to detect bacteria in urine specimens containing 10^5 CFU/ml (2, 7, 9, 11, 12). Studies performed on clinical specimens suggested different relative light unit (RLU) values (ranging from 146 to 700) as cutoff values to separate infected from noninfected urine samples. Some authors (1, 4, 7) reported 2 to 4% false-negative tests among specimens infected by *Proteus mirabilis* or mixed flora, but these authors did not investigate the causes of such false-negative results or evaluate the correlation between CFU-per-milliliter and RLU values on pure cultures of different bacterial species.

This paper presents the results of an investigation of the causes of false-negative bioluminescence assays and the correlation between CFU-per-milliliter and RLU values for the most common urinary tract pathogens.

MATERIALS AND METHODS

In vitro studies. (i) Strains. One hundred bacterial strains were used: 10 *Escherichia coli* strains, 5 *Serratia marcescens* strains, 5 *Serratia liquefaciens* strains, 10 *Klebsiella pneumoniae* strains, 10 *Enterobacter cloacae* strains, 10 *Pseudomonas aeruginosa* strains, 4 *Providencia stuartii* strains, 4 *Providencia rettgeri* strains, 2 *Providencia alcalifaciens* strains, 6 *P. mirabilis* strains, 4 *Proteus vulgaris* strains, 10 *Morganella morganii* strains, 10 *Staphylococcus aureus* strains, and 10 *Enterococcus faecalis* strains. All strains were freshly isolated from infected human urine samples.

(ii) Media. Brain heart infusion broth (BHI), nutrient broth (NB) and urine were used to grow strains for bioluminescence assays. Urine pools were obtained from 10 healthy volunteers and were sterilized by sequential filtration through 0.4- and 0.2-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.).

CFU-per-milliliter values were obtained by streaking appropriate dilutions of bacterial cultures on agar plates (McConkey agar for *Proteus* spp. strains and brain heart infusion agar for all the other strains). Bacterial counts were all performed in triplicate, and CFU-per-milliliter mean values were calculated.

All media were purchased from Difco Laboratories, Detroit, Mich.

(iii) Procedures. Bacterial strains were grown in different cultural conditions: (i) in BHI at 37°C both statically and with shaking, (ii) in NB at 37°C both statically and with shaking, and (iii) in urine at 37°C with shaking.

Bioluminescence readings were performed on BHI and NB cultures both in the logarithmic phase of growth and after overnight incubation and on urine cultures only after overnight incubation.

All bacterial cultures were diluted from 10^1 to 10^5 times. Bioluminescence assays were performed in triplicate on 0.3-ml volumes of each dilution. A 25-μl volume was also taken from dilutions 10^{-1} to 10^{-3} of bacterial cultures in urine and processed for bioluminescence as specified below.

Samples were processed by using a Lumac Biocounter M2500 (Lumac bv, Landgraaf, The Netherlands).

The 0.3-ml samples were counted by using NRB/Lumit PM Kit (Lumac), with four different ATP extraction times (10, 20, 30, and 40 s) and after 4 cycles of sonication for 30 s at 26-μm amplitude at 0°C. The 25-μl samples were counted by using the Bacteriuria Screening Kit (Lumac), following the instructions of the manufacturer.

The mean system noise value was determined by performing the assaying procedures on each sterile culture medium.

Reagents included in both the NRB/Lumit PM kit and the Bacteriuria Screening kit were used according to the instructions of the manufacturer; reagents purchased in lyophylized form were discarded after reconstitution.

Clinical specimens. (i) Collection. A total of 1,430 independent urine specimens, submitted to our laboratory from inpatient and outpatient populations, was studied. All of them were collected with the midstream clean-catch technique. Of these, 932 were collected from suspected acute urinary tract infections (AUTI) and the remaining 498 spec-

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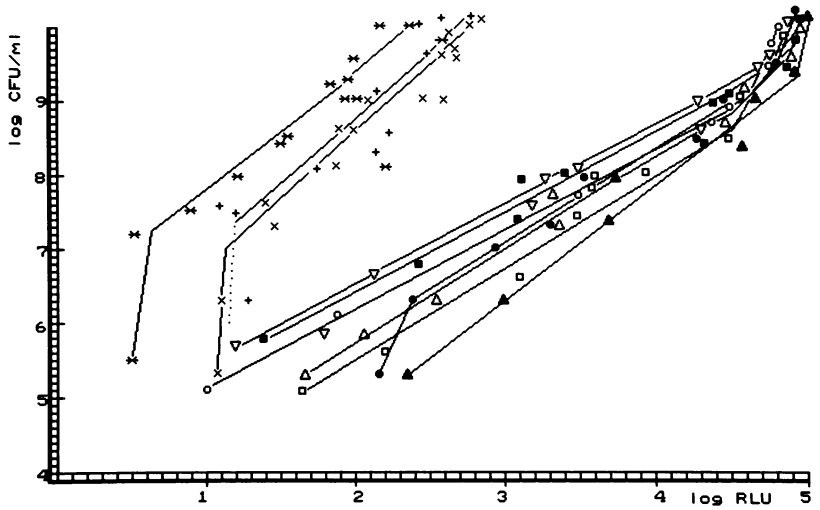

FIG. 1. Correspondence curves between log CFU-per-milliliter and log relative light unit (RLU) mean values obtained from overnight cultures in BHI at 37°C, processed for bioluminescence by using NRB/Lumit PM Kit with an ATP extraction time of 30 s. Each curve was drawn by plotting mean values obtained with 10 strains of the following microorganisms: *, *Morganella morganii*; +, *Proteus* spp.; ×, *Providencia* spp.; ○, *Escherichia coli*; □, *Klebsiella pneumoniae*; ■, *Enterobacter cloacae*; ▽, *Serratia* spp.; ▲, *Pseudomonas aeruginosa*; ●, *Enterococcus faecalis*; △, *Staphylococcus aureus*.

imens were collected from suspected chronic urinary tract infections (CUTI). The specimens arrived in the laboratory within 2 h of collection. After arrival and accessioning, the samples were refrigerated until processing started (no more than 2 h).

(ii) Specimen processing. A 0.01-ml volume of the well-mixed unspun urine samples and of a 100-fold dilution was streaked onto blood agar, mannitol salt agar, and T-mod agar plates (13). After an overnight incubation at 37°C, the plates were observed for growth, and if it was not present, they were reincubated and examined at 48 h. Blood agar plates were used for CFU-per-milliliter counts.

Gram-negative isolates were identified by following the T-mod pathway (3, 13); gram-positive isolates were identified by using Api Strep and ATB 32 Staph (Api System, La Balme Les Grottes, Montalieu Vercieau, France); yeasts were not identified.

A 25-μl volume of the unspun urine samples was also processed for bioluminescence by using the Bacteriuria Screening kit as described above.

RESULTS

Noise value. The mean noise value for all the assaying procedures and media was equal to 8 (with a standard deviation of 2).

Standard correlation curves. Log_{10} mean RLU values obtained from BHI cultures of each tested strain that had been incubated with overnight shaking and processed by using the NRB/Lumit PM Kit with an ATP extraction time of 30 s were plotted versus the corresponding log_{10} mean values. Correlation curves were then traced for each tested species (Fig. 1). The standard deviation of RLU values was equal to 5% for all of the tested strains except those of *Proteus* spp., *Providencia* spp., and *M. morganii* (standard deviation about 100%).

All of the tested strains except those of *Proteus* spp., *Providencia* spp., and *M. morganii* showed comparable correlation curves in the range of 10^8 to 10^{10} CFU/ml. Curves were linear from about 1×10^5 to 9×10^9 CFU/ml, with RLU values ranging from 10^1 to 10^5; 10^5 CFU/ml corresponded to mean RLU values ranging from 10 to 500, 9×10^9 CFU/ml corresponded to mean RLU values of 87,135 and >100,000 for *E. coli* and *K. pneumoniae*, respectively, and to the RLU mean values 584, 268, and 194 for *Proteus* spp., *Providencia* spp., and *Morganella morganii*, respectively.

Influence of assaying parameters. Preliminary results obtained by performing bioluminescence assays on one strain for each tested species showed that bioluminescence readings were not influenced by the medium of growth (BHI, NB, or urine), the conditions of growth (static or shaking) and the use of different assaying kits (NRB/Lumit PM or Bacteriuria Screening kit). Consequently, all further experiments were run only on BHI cultures, incubated with shaking.

Changes in ATP extraction time with NRB greatly influenced RLU mean values obtained with all tested strains except those of *Proteus* spp., *Providencia* spp., and *M. morganii*. For example, 7.6×10^8 CFU of *P. aeruginosa* per ml corresponded to 7,597 RLU after 10 s of extraction and to 52,347 RLU after 30 s of extraction, while 1.3×10^9 CFU of *P. mirabilis* per ml corresponded to 223 RLU after 10 s of extraction and to 298 RLU after 30 s of extraction. Extensive sonication of samples, resulting in a 99.9% reduction of viable cells for all the tested species, caused a drastic increase in RLU values for samples infected by *Proteus*, *Providencia*, and *Morganella* strains (about 50 to 100 times). RLU values of all the other tested species were increased only about two times. For example, 1.1×10^9 CFU of *E. cloacae* per ml corresponded to 51,063 RLU after 30 s of extraction and to 75,990 RLU after sonication, while 1.4×10^9 CFU of *Proteus* spp. per ml, 1.3×10^9 CFU of *Providencia* spp. per ml, 8.9×10^8 CFU of *M. morganii* per ml corresponded respectively to 340, 599, and 35 RLU after 30 s of extraction and to 15,609, 16,077, and 5,770 RLU after sonication.

RLU values at comparable bacterial concentrations were higher in the logarithmic phase of growth than after overnight incubation. Differences were not comparable for all the

tested species, being maximal for *Proteus* spp. and *Providencia* spp. and minimal for the other tested strains. For example, 8.8×10^7 CFU of *E. faecalis* per ml corresponded to 6,437 RLU after overnight incubation and 3.7×10^7 CFU of the same species per ml corresponded to 11,135 RLU in the logarithmic phase of growth, while 1.4×10^8 of *Proteus* spp., 1.3×10^8 of *Providencia* spp., and 8.9×10^7 of *M. morganii* corresponded respectively to 81, 157, and 10 RLU after overnight incubation, and 1.9×10^8 CFU of *Proteus* spp. per ml, 9.1×10^7 CFU of *Providencia* spp. per ml, and 3.2×10^8 CFU of *M. morganii* per ml corresponded respectively to 12,930, 10,943, and 174 RLU in the logarithmic phase of growth.

Clinical study. Of the 1,430 urine samples examined, 1,047 (73.2%) had $>10^5$ CFU/ml on blood agar plates; of these infected samples, 705 (67.3%) were collected from AUTI and 342 (32.7%) were collected from CUTI. Of the infected samples, 570 (54.4%) gave rise to the growth of a single gram-negative strain, 91 (8.7%) had a gram-negative and a gram-positive strain, 94 (8.9%) had two different gram-negative strains, 136 (12.9%) had only a gram-positive strain, 116 (11%) had three different strains, and 40 (3.8%) had only a yeast strain.

A total of 1,424 bacterial strains were isolated from urine samples; of these, 1,081 (75.9%) were gram-negative and 343 (24.1%) were gram-positive strains. Out of the 1,081 gram-negative isolates, 74 (6.8%) were *Proteus*, *Providencia*, or *Morganella* strains (36 *Proteus* spp., 18 *Providencia* spp., and 20 *M. morganii* strains). Of the 74 *Proteus*, *Providencia*, and *Morganella* isolates, 42 were isolated from AUTI (20 *Proteus* spp., 11 *Providencia* spp., and 11 *M. morganii* strains) and 32 were isolated from CUTI (16 *Proteus* spp., 7 *Providencia* spp., and 9 *M. morganii* strains).

Bioluminescence results. Of the 1,430 urine samples, 981 (68.6%) gave RLU values of >500 and, according to the instructions of the manufacturer, were considered infected; 449 (31.4%) gave RLU values in the range 23 to 227 and were considered noninfected. Of these 449 samples, 66 (4.6%) gave rise to values of $>10^5$ CFU/ml on blood agar plates and were thus considered false negatives. All the 66 false negatives were registered with samples infected by *Proteus*, *Providencia*, or *Morganella* strains; they were collected from both AUTI and CUTI.

Eight samples infected by *Proteus*, *Providencia*, or *Morganella* strains were detected as infected by bioluminescence; all of them were collected from AUTI and five of them contained mixed flora (namely, two *E. coli* strains, one *P. aeruginosa* strain, one *S. aureus* strain, and one *E. faecalis* strain, all of them $>10^5$ CFU/ml).

DISCUSSION

Several techniques have been developed to evaluate bacterial counts in clinical specimens rapidly. The measurement of bacterial ATP by the luciferin-luciferase reaction is one of the most interesting (5, 8, 15). Some authors reported a certain incidence of false negatives when tests were performed on specimens containing mixed flora or *P. mirabilis* (7, 10).

This work was performed to evaluate the causes of false negatives and the interpretative criteria of bioluminescence assays in the screening of significative bacteriuria.

None of the *Proteus*, *Providencia*, and *Morganella* strains grown in vitro were correctly detected by bioluminescence assays performed according to standard methods. The present data give no evidence of the causes of these problems, though some suppositions can be advanced. The observation that extensive sonication of bacterial samples (reducing the number of viable cells by about 99.9%) caused a drastic increase in mean RLU values obtained from samples infected by *Proteus*, *Providencia*, and *Morganella* strains (an increase that was not observed for all the other tested strains) supports the hypothesis that NRB is not efficient in exposing the ATP of these strains to the luciferin-luciferase system. Moreover, the observation that mean RLU values obtained from samples taken from cultures of *Proteus*, *Providencia*, and *Morganella* strains were not comparable to those of the other tested strains, even after sonication, could be an indication that these strains may have a minor ATP content or possess some substance interfering with the luciferin-luciferase reaction. This hypothesis should be substantiated by further specific data, which are not available.

Altogether, these data indicate the necessity to revise the correlation curves used to interpret bioluminescence readings and to perform simple tests to exclude the presence of *Providencia*, *Proteus*, and *Morganella* strains in the sample (3, 13). Moreover, since mean RLU values corresponding to 10^5 CFU/ml are frequently comparable to the mean noise value, it could be useful to perform bioluminescence assays after sonication of samples.

The present data, together with other data (2), suggest the necessity to determine a RLU interval to distinguish infected from noninfected urine specimens, instead of using a single breakpoint value, as suggested by other authors (7, 9, 11, 12).

According to our experience, when urine samples are sonicated before bioluminescence readings, RLU values of <100 indicate a noninfected specimen and RLU values of >200 indicate an infected specimen. When RLU values in the range 100 to 200 are obtained, it is always necessary to perform plate counts to obtain reliable results. Otherwise, it would be advisable to study permeabilizing agents showing a better efficiency on all bacterial species. The clinical origin of samples should also be considered; in fact, the clinical data we obtained on 1,430 urine specimens indicated that a higher incidence of false negatives derived from specimens collected from CUTI. Such a difference could be explained by the observation that clear differences were shown in bioluminescence readings of specimens taken from bacterial cultures in different phases of growth. We suppose that specimens collected from AUTI were counted more efficiently than those from CUTI, where bacteria could be in the stationary phase of growth. This aspect could account for differences in the incidence of false negatives reported by different authors and for their relative low incidence (1, 4, 7).

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