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Standardization of isothermal microcalorimetry in urinary tract infection detection by using artificial urine

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Abstract

Purpose Isothermal microcalorimetry (IMC) has recently been reported as a new method to rapidly detect urinary tract pathogens (UTP). However, further application of microcalorimetry in the clinical setting requires a standardized procedure. An important step toward such standardization is to use a reproducible growth medium. In this study, we investigated the potential of artificial urine in combination with microcalorimetry for detection of common UTP.

Methods A microcalorimeter equipped with 48 channels was used. Detection was accomplished, and growth was monitored for four bacterial strains in artificial urine at 37 °C by measuring metabolic heat flow ($\mu W = \mu J/s$) as a function of time. The strains were *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis*, and *Staphylococcus aureus*.

Result Bacterial growth was detected after 3–32 h with decreasing inoculums down to 1 CFU. The gram-negative strains grew and were detected faster than their gram-positive counterparts. The growth rates the different strains were 0.75 ± 0.11 for *E. coli*, 0.74 ± 0.10 for *E. faecalis*, 1.31 ± 0.04 for *P. mirabilis*, and 0.56 ± 0.20 for *S. aureus*. The shape of individual heat flow curves was characteristic for each species independent of its initial concentration.

Conclusions IMC allows rapid detection of UTP in artificial urine. Clearly, different heat flow patterns enable accurate pathogen differentiation. UTP detection after only 4 h is realistic. The rapid detection of UTP tested in standardized artificial urine proves the diagnostic potential of IMC and warrants further microcalorimetric studies in the clinical setting of urinary tract infections.

Keywords Artificial urine · Diagnosis of urinary tract infection · Isothermal microcalorimetry · Heat flow curves · Urinary tract infection

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Introduction

Urinary tract infections (UTI) are among the most common bacterial infections in humans and account for significant morbidity and health care expenditures worldwide [1–3]. Pretreatment urine cultures are performed to confirm the diagnosis of UTI and to provide antimicrobial susceptibility patterns of pathogens involved. Although generally not required in cases of acute, uncomplicated cystitis [4], urine culture is still considered as standard of care in

pyelonephritis, pregnant women, and men. Not surprisingly, urine cultures are among the most frequent culture analyses performed in the clinical microbiology laboratory [5]. However, up to 80 % of urine samples are negative, resulting in considerable workload and consumption of resources [6]. Therefore, a rapid and cost-efficient assessment of UTI is still needed. In this context, isothermal microcalorimetry (IMC) has recently been proven to offer the potential to rapidly detect urinary tract pathogens (UTP) in urine [7]. In this proof-of-concept study, detection of bacterial growth was monitored in filtered sterilized urine from a healthy donor. However, further clinical application of microcalorimetry in UTI diagnosis requires a standardized procedure as urine composition could vary between individuals and also fluctuates in time within the same person [8, 9]. Pathologic conditions also might strongly modify urine composition, respectively [10]. An important step in such a standardization is the use of a reproducible growth medium dedicated to UTP. Artificial urine formulations might be such a valuable medium, since they have a reproducible composition and allow growth of the most common UTP [11]. In this study, we combined the use of artificial urine with microcalorimetry to improve and standardize detection of UTP by IMC.

Materials and methods

Microorganisms

Escherichia coli (DSM 10142), *Enterococcus faecalis* (JH2-2), *Proteus mirabilis* (DSM 4479), and *Staphylococcus aureus* (ATCC 25923) overnight cultures prepared in brain heart infusion (BHI—beef heart (infusion from 250 g) 5.0 g L^{-1}, calf brains (infusion from 200 g) 12.5 g L^{-1}, Na_2HPO_4 2.5 g L^{-1}, D(+)-glucose 2.0 g L^{-1}, peptone 10.0 g L^{-1}, NaCl 5.0 g L^{-1}) were diluted in sterile saline (0.85 % NaCl) and adjusted to an optical density (OD_{600}) of ca. 0.132 ($\sim$0.5 McFarland). From this suspension, serial tenfold dilutions were prepared in sterile saline to attain concentrations of ca. 10^5, 10^3, 10^1, and 1 CFU/mL. The inoculums concentrations were confirmed by cultures on BHI agar plates.

Artificial urine

Artificial urine was prepared according to the formulation provided in Brooks and Keevil [11]: peptone 1.0 g L^{-1}, yeast extract 5.0 mg L^{-1}, lactic acid 0.1 g L^{-1}, citric acid 0.4 g L^{-1}, $NaHCO_3$ 2.1 g L^{-1}, urea 10.0 g L^{-1}, uric acid 0.1 g L^{-1}, creatinine 70.0 mg L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.37 g L^{-1}, NaCl 5.2 g L^{-1}, $FeSO_4 \cdot 7H_2O$ 10.0 mg L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g L^{-1}, $NaSO_4 \cdot 10H_2O$ 3.2 g L^{-1}, K_2HPO_4 1.2 g L^{-1},

KH_2PO_4 1.0 g L^{-1}, NH_4Cl 1.3 g L^{-1}. To enhance pathogens growth and thus detection, we added 20 mg L^{-1} of glucose, 20 mg L^{-1} of lactose, and 20 mg L^{-1} of saccharose to the artificial urine. This range corresponds to what can be detected in healthy patient urine [12].

Microcalorimetry

A microcalorimetry instrument (TAM48, TA Instruments, New Castle, DE) was used to measure the heat flow during the growth of the different strains in artificial urine at 37 °C. Four-milliliter ampoules were filed with 2.9 ml of artificial urine, and 100 μl of inoculums was added. The microcalorimetric ampoules were then inserted into the measuring channels, and heat flows were recorded until the signal returned to baseline or up to a maximum of 5 days.

Calorimetric detection of microorganisms and estimation of growth rates

Detection time was defined as the time to reach a rising heat flow rate signal of 10 μW (40 times the sensitivity of the calorimeters). Growth rate was calculated using an exponential model ($Qt = Q_0 \cdot e^{\mu t}$, where Q represents the heat—i.e., the integral of the heat flow curve) over the exponential part of the curve. Generation time was obtained using the relation $tg = \ln 2/\mu$, where μ is the growth rate obtained previously. Data analysis was accomplished with the manufacturer's software (TAM Assistant) and the R Statistical package [R Development Core Team 2008].

Results

Detection times are summarized in Table 1, and growth parameters in artificial urine are summarized in Table 2. Detection was rapidly achieved for all different bacterial species tested. The gram-negative bacteria grew and were detected faster than their gram-positive counterparts. *P. mirabilis* and *E. coli* were detected in less than 4 h for highest inoculum concentration and less than 10 h for concentration of 1 CFU/mL. The growth rates for *P. mirabilis* and *E. coli* were 1.31 ± 0.04 and 0.75 ± 0.11, respectively. Similarly, *S. aureus* was also detected in less than 4 h for highest inoculum concentration but required ca. 20 h to be detected when a concentration of 10 CFU/mL was used, and was not detected for concentration down to 1 CFU/ml. This can be easily explained because *S. aureus* has the slowest growth rate observed in urine (0.56 ± 0.20). Finally, *E. faecalis* was detected within 10 h for the highest inoculums and 32 h when a concentration of 1 CFU/mL was used. The growth rate for

Table 1 Detection time in hours achieved for the different pathogens tested at various inoculum concentrations

Microorganism	Inoculum concentration (CFU/mL)			
	10^5	10^3	10^1	10^0
E. coli	3.66 ± 0.09	6.30 ± 0.05	9.38 ± 0.77	9.33[a]
E. faecalis	9.16 ± 0.98	12.25 ± 0.74	16.22 ± 0.24	32.25[a]
P. mirabilis	3.33 ± 0.01	6.39 ± 0.17	9.75 ± 0.41	ND
S. aureus	3.94 ± 0.34	9.11 ± 0.82	20.45 ± 1.00	ND

Measured microbial concentrations at targeted concentrations of 10^5 CFU/mL were 1.12×10^5 CFU/mL for *E. coli*, 1.71×10^5 CFU/mL for *E. faecalis*, 1.41×10^5 CFU/mL for *P. mirabilis*, and 0.81×10^5 CFU/mL for *S. aureus*

ND not detectable

[a] Less than three replicate heat flow curves were obtained; no standard deviation is indicated

Table 2 Growth parameter [growth rate (μ) and generation time (tg)] determined using microcalorimetry data for all dilutions of a microorganism and comparison with available literature data

Microorganism	μ (AUM) [h^{-1}]	tg (AUM) [h]	n	tg (literature) [h]	References
E. coli	0.75 ± 0.11	0.94 ± 0.13	11	0.75–1.67	Roos et al. [24]
E. faecalis	0.74 ± 0.10	0.95 ± 0.13	11	0.81 ± 0.05	Vebo et al. [25]
P. mirabilis	1.31 ± 0.04	0.53 ± 0.02	9	0.40–1.02	Sosa et al. [26]
S. aureus	0.56 ± 0.20	1.42 ± 0.51	9	1.41 ± 0.21	Bonkat et al. [7]

AUM artificial urine medium

E. faecalis was 0.74 ± 0.10 and was similar to the growth rate of *E. coli*. However, in urine, *E. faecalis* seems to have a slightly longer lag phase. For *S. aureus* and *P. mirabilis*, the lowest dilution was not detected within the time frame investigated. Finally, the growth-related heat flow pattern of each bacterial species tested was clearly different. Strong differences in the pattern shape and peak intensity potentially allow pathogen identification (Fig. 1) as proposed in other studies [13, 14].

Discussion

Urinary tract infection (UTI) is among the most common infectious diseases diagnosed in the clinical microbiology laboratory. However, the majority of urine samples sent to the laboratory are negative. Screening methods (e.g., dip stick testing, automated urine flow cytometry, microscopy) for UTI are limited [15–17]. In addition, these tests are not being able to replace urine cultures because they neither identify the causal pathogen nor establish antibiotic susceptibility–guided antibiotic treatment. The diagnostic gold standard, urine culture, is limited in itself by the typical delay of 2–3 days to deliver results and drug susceptibility patterns. Therefore, there is still a significant interest in developing rapid diagnostics for UTI. In this context, we could recently demonstrate that isothermal microcalorimetry (IMC) offers to be such a promising approach [7]. IMC measures heat production rates of

physical, chemical, or biological processes and offers several advantages. Its high sensitivity in the order of 0.2 μW allows detection of 10^2 active microorganisms per sample. This is even more important as studies comparing voided urine specimens and bladder aspirate specimens in women with UTI have shown that the traditional criterion for a positive culture of voided urine (10^5 CFU/mL) is insensitive for cystitis, and 30–50 % of women with bladder infection have colony counts of 10^2–10^4 CFU/mL in voided urine [18], a range that is mostly covered by IMC. In our present study conditions (i.e., artificial urine), the high sensitivity of isothermal microcalorimetry (IMC) allowed the rapid detection of all urinary tract pathogens (UTP) investigated. In addition, the use of artificial urine of defined composition allows standardizing the results and thus in turn will also allow comparisons between bacterial strains pathogenicity. For example, Gordon and Riley [19] suggested that *E. coli* strains isolated from the urinary tract have significantly higher in vitro growth rates in urine compared with strains isolated from other areas. Thus, based on their growth rate determined by IMC, it seems possible to assess the pathogenicity of different strains. For this purpose, it must be noted that (1) growth rate measured in this study is consistent with growth rate measured in filter-sterilized urine (Table 2) and (2) very small differences have been observed when comparing microcalorimetric data obtained in real urine and those in artificial urine [7]. In contrast to the previous study performed in filter-sterilized urine from a healthy donor,
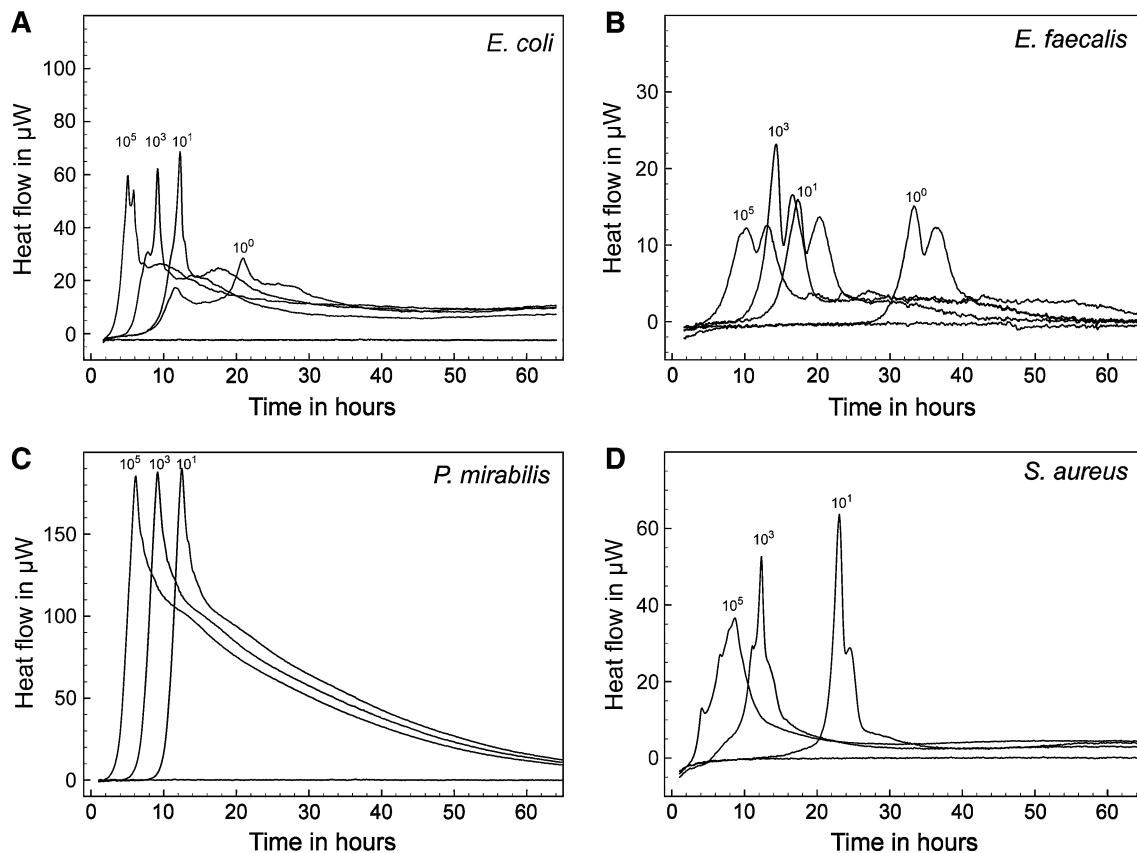
Fig. 1 Representative heat flow curves of urinary tract pathogens in artificial urine. Growth-related heat flow curves of serial dilutions of the four urinary pathogens investigated: **a** *E. coli*; **b** *E. faecalis*; **c** *P. mirabilis*; **d** *S. aureus*

growth rate was slightly higher (4–16 %), resulting in shorter generation time. This higher growth rate is likely explained by the presence of small amount of sugars in the artificial urine formulation used in our study. In addition, the absence of natural antimicrobial molecules such as antimicrobial peptides [20] commonly found in urine from our artificial urine is also likely to promote a faster growth of microorganisms tested. Another benefit of using artificial urine is the possibility to achieve identification of the pathogens involved. It is commonly admitted that a particular bacterium in a defined medium will produce a specific heat flow pattern. However, although bacterial identifications using microcalorimetry have already been proposed in the 1970s [21], it was also emphasized that environmental conditions influenced the heat flow pattern and might compromise such identification [22]. In this context, artificial urine provides the stable environmental conditions necessary to obtain reproducible heat flow patterns and allows identification. The identification process itself (i.e., the assignation of a genus or species name to an unknown heat flow pattern) can be performed by various means including cross-correlation analysis [14], discriminant analysis [13], and support vector machines

[23]. Note that all these methods focus on the pattern identification and not on parameters such as growth rate, detection time, or any other. Increasing performance of computer since the 1970s has allowed performing such analyses in a few seconds to minutes nowadays. For example, in their paper, Lopez and colleagues could achieve identification with an accuracy of ca. 100 % using single strains only [14]. The major difficulty we have to be concerned with might be polymicrobial growth. However, knowledge of growth-related heat flow patterns in individual species might allow splitting the contribution of several (up to 3) pathogens using deconvolution algorithm. Similarly, slightly different heat flow pattern has been observed for methicillin-resistant *S. aureus* and for methicillin-susceptible *S. aureus* [24]. Therefore, use of pattern recognition algorithm should be used with care, and knowledge of heat flow pattern should be based on a rather large number of strains belonging to a single species and including resistant and susceptible strains to catch the "natural variation" within the considered species. Considering all these elements, we believe that further studies will allow IMC to be used in the field of UTI diagnosis or even exclusion.

Conclusions

IMC allows rapid detection of UTP in artificial urine. Clearly, different heat flow patterns enable accurate pathogen differentiation. The rapid detection of UTP tested in standardized artificial urine proves the potential of IMC and warrants further studies in the setting of urinary tract infections. Especially, clinical strains obtained during routine microbiological investigations should be investigated to further validate this approach.

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