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Real time detection of pathogenic bacteria in veterinary microbiology using isothermal microcalorimetry – A different approach

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ABSTRACT

With today's challenges regarding antibiotic resistance and the importance of the implementation of prudent use of antibiotics, fast and reliable diagnostic tools for bacterial infections and subsequent antimicrobial susceptibility testing are of utmost relevance. Isothermal microcalorimetry (IMC) is a broadly applicable method, with which metabolic heat flow in reproducing bacteria can be measured in real time. To the best of the authors' knowledge, this is the first report on examination of 124 urine samples from feline and canine urinary tract infection with an IMC-based prototype instrument. A concentration-dependent time of peak heat flow by dilution series with *Escherichia coli* and *Enterococcus faecalis* reference strains demonstrated the general good performance of the prototype for detection of these bacteria. With diagnostic culture being set as a gold standard, the diagnostic sensitivity of IMC compared to bacteriological culture was 80 %, the diagnostic specificity was 97 %. With a Cohens' kappa value (κ) of 0.80, the two methods show good concordance. The results from our study demonstrate that the IMC technology is suitable to allow reliable, but much faster detection of bacteria than conventional culture, especially for *Escherichia coli.* Thus, implementing IMC technology could markedly speed up the bacteriological diagnostic process in veterinary medicine.

1. Introduction

Bacterial urinary tract infections (UTIs) belong to the leading diagnosis for antimicrobial prescription in companion animals (Dorsch et al., 2019; Weese et al., 2019; Wong et al., 2015). Reliable and fast detection of the responsible pathogen is a key factor for successful treatment at the beginning of the diagnostic process. The conventional bacteriological analysis of urine samples in veterinary microbiology is based on bacterial culture on non-selective and selective solid media and subsequent species identification of grown colonies. This method is considered gold standard (Lulich and Osborne, 2004), but it takes at least 24 hours (h) as an over-night incubation is needed for culture. If antimicrobial susceptibility testing (AST) is performed after identification of a pathogen, another 24–48 h pass until a result form the laboratory is available for the veterinarian. Although it is best practice to avoid empirical treatment of bacterial infections whenever possible, an antimicrobial therapy with broad-spectrum antibiotics is often started whilst waiting for pathogen detection and identification and AST results. This is unsatisfactory regarding increased antimicrobial resistance (AMR) rates in both, human and veterinary medicine (Fonseca et al., 2021; McCowan et al., 2022). Targeted antimicrobial therapy based on a fast and accurate diagnosis is of utmost importance in order to preserve effective antimicrobial agents.

Isothermal microcalorimetry (IMC) is a non-specific and broadly applicable method based on the measurement of heat emitted or absorbed by any reaction regardless of its nature (e.g. physical, chemical, biological) in real-time (Wadsö, 2001). The calorimetric measurement technique was first described by Lavoisier and Laplace in the 19th century in "Lavoisier & Laplace PS (1780) Mémoire sur la chaleur. Académie des Sciences, Paris" (Lodwig and Smeaton, 1974). Nowadays, a Peltier or thermoelectric element is used, as described by Suurkuusk et al. (2018). IMC devices measure heat, which is produced in reaction vessels at a microwatt range under essentially isothermal conditions (Wadsö, 2001). The technique has been applied in many different settings such as environmental sciences (Rong et al., 2007), pharmaceutical sciences (Phipps and Mackin, 2000) and food sciences (Haman et al., 2017). Braissant et al. (2010) reviewed the general use of IMC for measurement of microbial activities (Braissant et al., 2010). Among the recent studies in the bacteriological field, taking advantage of IMC high sensitivity for early detection of bacterial contamination has become an

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important focus. For example, detection of pathogens in tap water was shown to be practical and cost effective (Maskow et al., 2012). Similarly, diagnosis of bacterial infection of orthopaedic devices by analysing sonication fluid using microcalorimetry was successfully demonstrated (Borens et al., 2013). In animal model as well, early detection of *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Listeria monocytogenes* by microcalorimetry in cerebrospinal fluid of experimental meningitis in rats was shown to be promising as well (Trampuz et al., 2007). Finally, with respect to human urinary tract pathogens, such as *Escherichia coli, Enterococcus faecalis, Proteus mirabilis* and *Staphylococcus aureus*, IMC was able to rapidly detect their presence in urine and artificial urine within a few hours (Bonkat et al., 2013; Bonkat et al., 2012). In addition to the studies focusing on early detection, the use of IMC technology to rapidly assess drug susceptibility has been increasing drastically. For example, IMC has been used to shorten the time period to seven hours for detection of antimicrobial resistance directly from urine samples (Braissant et al., 2014). Similarly, other studies shows that IMC has a high potential in drug susceptibility testing (Antonelli et al., 2022).

Although most of the studies currently performed are made with rather sophisticated isothermal calorimeters intended for research, the field is evolving rapidly and new designs of isothermal calorimeters are considered. Such designs extend from calorimeter on a chip (Maskow et al., 2010) to inexpensive (less expensive) calorimeters with limited number of features (Farto-Vaamonde et al., 2022; Wadsö et al., 2011). In the present study, we used a prototype calorimeter using plastic bags as sample container. To ensure maximum heat transfer between the sample and the measuring sensor, the sample bags are placed between two Peltier elements (Göpfert B., von Tscharner V., 2018,EP 370748444, European Patent Office). Because of biosafety considerations, those plastic single use bags are more compatible with veterinary and clinical routine (Laboratory biosafety manual, fourth edition, World Health Organization, 2020), than their glass or metal counterparts commonly used in other setups.

Hence, the aims of this study are i) to characterize measurement kinetics of detection of bacteria prepared in serial dilutions in a prototype IMC device: ii) to compare results of bacteria detection in canine and feline urine samples with this IMC based device in parallel to conventional bacteriological culture.

2. Material and methods

2.1. Isothermal microcalorimetry device

An isothermal microcalorimeter ("Fast4U" Calorimeter, Calbact AG, Kaiseraugst, Switzerland) (Fig. 1) was used for the measurements. The schematic of the loading, equilibration and measurement stage is illustrated in Fig. 2. This device contains seven channels (channel 1 – channel 7). Channel 4, which is located in the middle of the sample bag holder and in the middle of the calorimeter measurement unit, thus withholds the most stable position, serves as a reference channel. Measurements of samples were performed in the remaining six channels. The samples were pipetted into sterile one-mL polyethylene (PE) bags (Calbact AG, Kaiseraugst, Switzerland). Working temperature of the instrument was 37 °C ± 0.1 °C. The detection limit was 1 µW, with a precision of 3 sigma, the short-term noise was +/- 0.2 µW over 1 h, long-term baseline stability (drift over 24 h) was 5 µW or less. Fluctuations due to thermic regulation and thermic equilibrium occurred occasionally as short and long term fluctuations caused by air turbulence and temperature instabilities in the room. These fluctuations did not exceed 1 µW over 12 h. Optimal time for equilibration after insertion of the

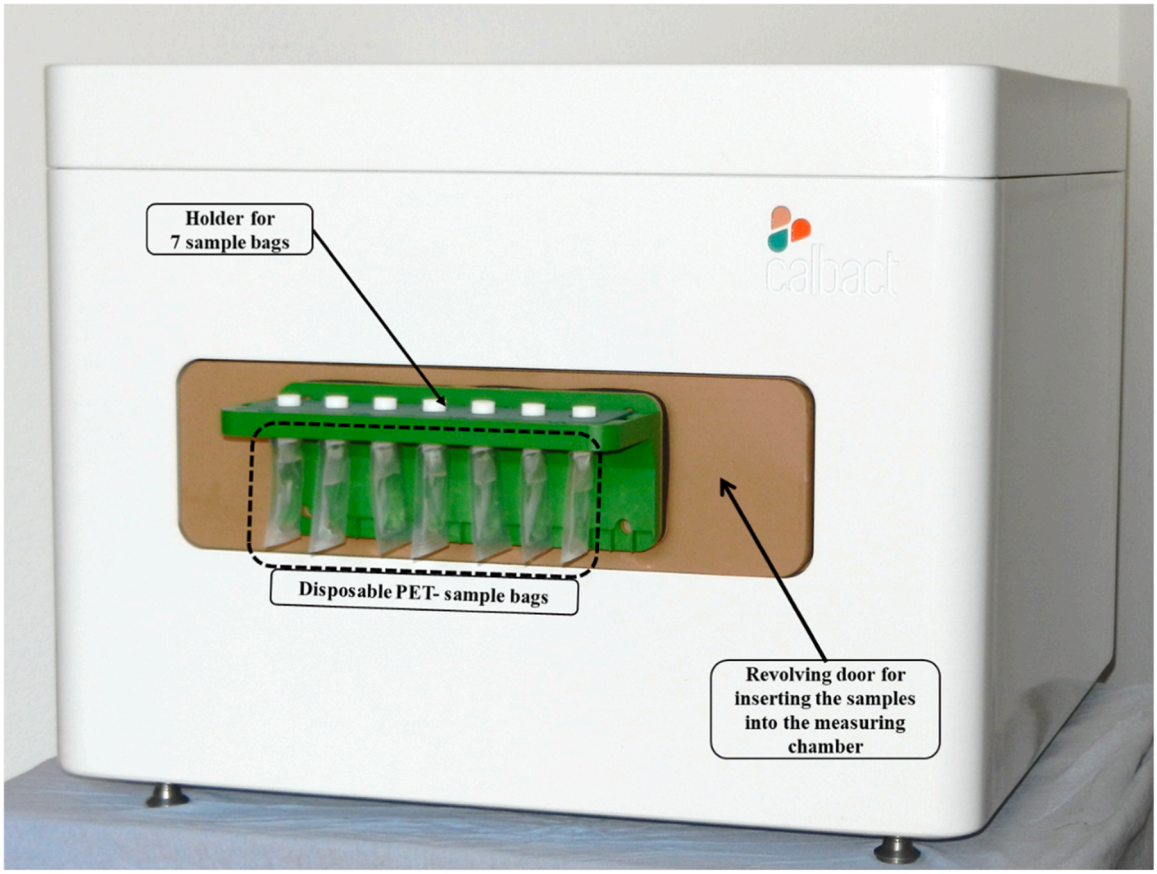

Fig. 1. "Fast4U" Calorimeter - open state with seven filled bags inserted in the rack before loading the instrument. Instrument dimensions (width*height*depth): 520 * 380 * 560 mm.

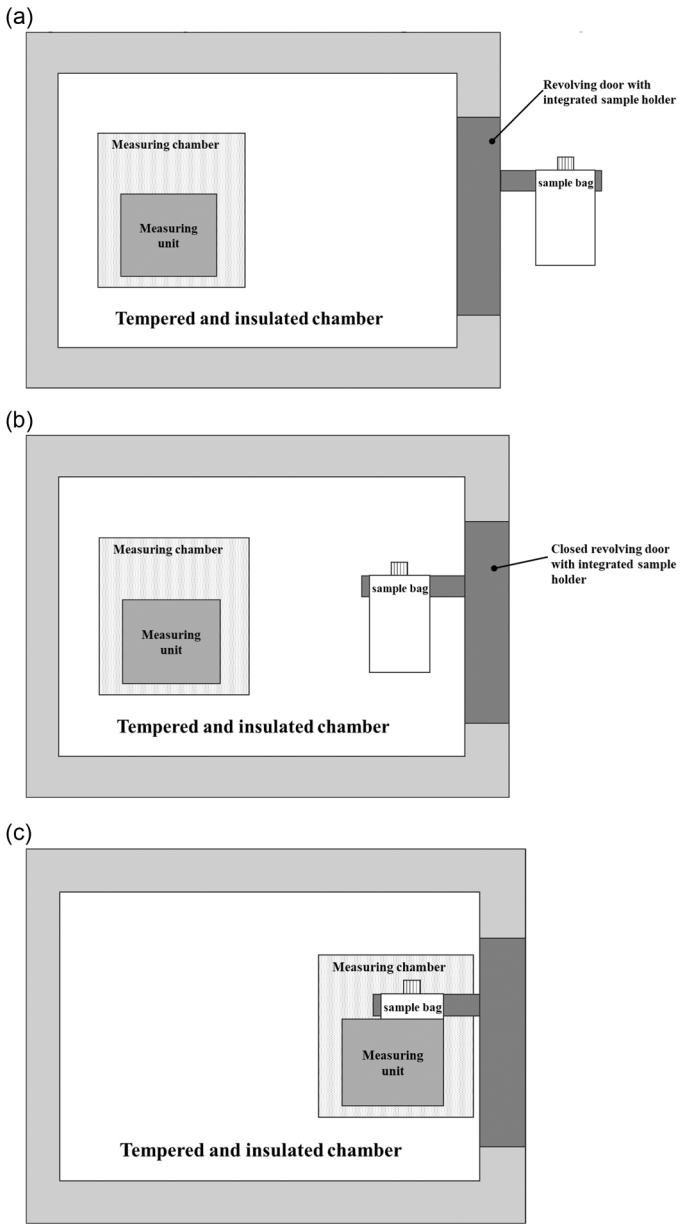

Fig. 2. Schematic side view illustrations of the "Fast4U" Calorimeter construction. a Loading the device with the sample and reference bags. b Device during the equilibration stage before the measurement chamber moves into the measurement position. c Device during the measurement stage.

samples was two hours. Start of measurement was possible after one hour. The raw data of every measurement were stored by the internal data logger for 20 s (s) intervals and transferred to the recording software. For immediate monitoring purpose, the monitor of the data logger displayed the data as averages of 1 min (min). The analysis software first aggregated the data to 2 min intervals ($\Delta t = 2$ min) and subsequently filtered the data by a Gauss filter ($\sigma = 2.2 * \Delta t$). The sampling rate in the final data set was 2 min

2.2. Environmental conditions

Optimal operating temperatures for the "Fast4U" microcalorimeter was between 15 °C and 25 °C. A maximal operating temperature of 33 °C should not be exceeded. Great variations in room temperature can lead to minor temperature fluctuations in the device in the realm of 0.1 °C. Although these fluctuations are compensated for by the reference channel, the room temperature was nevertheless monitored to avoid interference with the measurement.

2.3. Calibration of the device

To perform sensor calibration, the sample bags were filled with one mL of water and a 48-hour-measurement was started. The instrument thermostat temperature was set at 37 °C. During the 48-hour measurement period, four calibration cycles of six hours were performed. For each cycle the temperature was raised by 0.3 °C for 3 h and then lowered again by 0.3 °C. During the first period heat flows through the peltier sensors into the bags until a new equilibrium is reached. During the second period the heat flows out again. Individual conversion factors were calculated according to the following formula:

$$\boldsymbol{convertionfactor} = \frac{\boldsymbol{C \cdot v \cdot \gamma \cdot \Delta T}}{\left(\frac{\boldsymbol{\Phi}}{10^6}\right)}$$

C represent the heat capacity of water (4.184 Joule/g/°C), v is the volume (1 mL), γ is the specific weight of water (0.997 g/mL) (Haynes et al., 2017), ΔT is the temperature difference (0.3 °C) and the thermal power Φ is calculated as the maximum of the integrated signal-squared in μW (baseline was subtracted before the integration) divided by the mean of the initial and final value of the cycle as follows:

$$\boldsymbol{\Phi} = \boldsymbol{IS_{max}} - \frac{\left(\boldsymbol{IS_{initial}} + \boldsymbol{IS_{final}}\right)}{\mathbf{2}}$$

The individual conversion factors for each channel for the calorimeter used in this study were the following: 1.65 for C1, 0.78 for C2, 0.81 for C3, 0.86 for C4, 0.91 for C5, 1.04 for C6, 0.86 for C7. The heat capacity of the PE-bag amounted to 0.003648 J/°C (0.0019 J/(g °C) * 1.92 g = 0.003648 J/°C) and was neglected in the calculation of the heat capacity.

2.4. Measurements of bacterial suspensions

2.4.1. Assessing the reproducibility of microbial measurements over the six channels

To ensure that measurements from all channels were reproducible, 56 measurements (at least nine per channel) of a 10^4 colony forming units per milliliter (cfu/mL) *Escherichia coli* American Type Culture Collection (ATCC) 25922 suspension were analyzed. Time point of maximum heat flow (time to peak, t_{peak}), amplitude of maximal heat flow (hf_{peak}) as well as the respective mean values (Mean) and standard deviations (SD) were calculated for each channel.

2.5. Dilution series

In order to assess the performance of the IMC device with different concentrations of bacteria, dilution series from suspensions of *Escherichia coli* ATCC 25922 as well as *Enterococcus faecalis* ATCC 29212 were measured. One colony of the respective species from a fresh 24 h old culture on tryptone soy agar plate containing 5 % sheep blood (TSA-SB; BD Becton Dickinson, Franklin Lakes, NJ, US) was suspended in 2 mL cation-adjusted Mueller-Hinton broth (CAMBH). The suspension was adjusted to McFarland 0.5 (approx. 1.5×10^8 cfu/mL) using a DensiCHEK plus device (bioMérieux SA, F-69280, Marcy l'Etoile, France). Further dilution steps in CAMBH were performed for final concentrations of 10^1 to 10^7 cfu/mL. The sample bags were filled with 1 mL of the bacterial suspension. For *Escherichia coli* a total of 133 measurements and for *Enterococcus faecalis* a total of 71 measurements were performed with a minimal number of eight measurements per bacterial species and concentration (10^1-10^8 cfu/mL). Mean value and standard deviation for time point and amplitude of maximum heat flow were calculated for every concentration.

2.6. Measurement of urine samples

One hundred-and-twenty-four (124) urine samples from dogs (n = 76) and cats (n = 48), sent from veterinarians for routine bacteriological diagnostics to the Institute of Veterinary Bacteriology, University of Bern, Switzerland were used for this study. Results of standard cultural bacteriological analysis were available for all the samples. An aliquot of 200 µL per sample was pipetted into an Eppendorf-tube and stored at 4 °C ± 1 °C until microcalorimetric analysis was started. For 94 % of the samples (n = 117) analysis with the Calbact device "Fast4U" took place within seven days after sample receipt at the laboratory. For the remaining seven samples storage took up to 18 days, but cultural results from initial diagnostics did not differ from the results after IMC measurement. Nine hundred (900) µL of CAMHB and 100 µL of urine sample were pipetted into a sample bag. The sample bag in the reference channel was filled with 900 µL CAMHB. An increase of noise was not observed. The rack with the sample bags was placed in the Calbact device and measurement was started immediately. Measurements took at least 21 h. Afterwards, a growth control (referred to as "cultivation after Calbact") was prepared via direct plating onto TSA-SB plates. The plates were incubated at 37 °C ± 1 °C for 24 h ± 4 h. If bacterial growth occurred, identification of colonies was carried out by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI TOF MS) (Biotyper 3.0, Bruker Daltonics GmbH, Bremen, Germany) using the direct transfer method according to the manufacturer's recommendations. In 48 of the 124 samples, an additional growth control (referred to as "cultivation before Calbact") was performed immediately before microcalorimetric measurement by plating out 50 µL of the undiluted sample onto TSA-SB and proceeds as described above. The data were analyzed starting at 2 h up until 20 h of the measurement, complying to the manufacturer's recommendation of implementing an equilibration period of 2 h at the beginning of the measurement. To be considered a positive IMC result in our study, the measurement must show a heat flow >20 µW in the time period >2 h and < 20 h. If no according heat flow within 20 h was registered, the sample was considered negative in IMC.

2.7. Statistics

Mean values, standard deviations and regression analysis were calculated using MS Excel version 2002 and R (version 3.6.3) (development core team, 2013). GraphPad Prism9 and R were used for the illustrations. Conventional culture was considered the gold standard technique for detection of bacterial pathogens. Sensitivity and specificity data for IMC technology are expressed in reference to results from bacterial culture. The performance characteristics, including sensitivity, specificity and the Cohens kappa value (κ), were calculated using standard methods. In total nine outliers that could be related to technical issues were excluded from the analysis. Other outliers (above 2σ) were kept in the analysis.

3. Results

3.1. Reproducibility of microbial growth measurements

To evaluate the performance of the six channels used for measurement, the mean values and standard deviations regarding time point and amplitude of maximum heat flow from 56 measurements with the 10^4 cfu/mL *Escherichia coli* suspension over the six channels were calculated. Under these conditions, maximum heat flows (i.e., heat flow peaks) occurred between 6.0 h and 6.5 h with an average of 6.3 ± 0.2 h. With 10^4 *Escherichia coli* cfu/mL, the average maximum heat flow reached 153.1 ± 43.6 µW.

3.2. Dilution series with Escherichia coli and Enterococcus faecalis

The data from measurements with *Escherichia coli* and *Enterococcus faecalis* suspensions were analyzed per bacterial concentration (cfu/mL) regarding amplitude and time point of maximum heat flow including standard deviations. As expected from previous studies, the heat flow curves from the dilution series with *Escherichia coli* showed a concentration dependency of the time to peak (Fig. 3a). Indeed the whole thermogram and, as a consequence, the time to peak was increasingly delayed with decreasing bacterial concentration. A regression analysis of the time to peak versus concentration regression analysis with *Escherichia coli* shows a strong negative correlation of the two parameters. The slope that represents the additional time to reach the peak per 10 fold dilution was 1.1 ± 0.02 h (r^2 = 0.97, n = 133, $p < 0.05$) (Fig. 3b). The amplitude of maximum heat flow varies considerably between individual measurements. One reason for the variability of the maximum heat flow could be the specific metabolic performance of the isolate measured. In addition, the aggregation of the bacterial cells and their distance from the surface of the Peltier element could lead to a different amplitude of the maximum heat flow. However, no specific trend is observed over all dilutions (r^2 = 0.00, n = 133. p = 0.24). Overall, for all *Escherichia coli* suspensions, the maximal heat flow ranges from 65.0 µW to 317.1 µW with an average of 162.9 ± 55.3 µW that is consistent with the measurements shown above.

Similar results with delayed thermograms and decreasing concentrations were observed for *Enterococcus faecalis* with lower values compared to the measurements with *Escherichia coli.* For *Enterococcus faecalis* the maximal heat flow was comprised between 13.9 µW to 125.4 µW with an average value of 44.8 ± 21.7 µW (Fig. 4a). Time points of maximal heat flow again show marked concentration dependency with an increase of time to peak of 1.5–0.0 h per 10 fold dilution (r^2 = 0.98, n = 71. $p < 0.05$) (Fig. 4b). As with *Escherichia coli*, the amplitude of maximum heat flow varies greatly between measurements. Furthermore a trend showing a decreasing maximum heat flow over decreasing bacterial concentration steps is observed (r^2 = 0.2, n = 71. $p < 0.05$).

3.3. Measurements of urine samples

A total of 124 urine samples were tested. Seventy-three (73) samples were negative (Table 1) and fifty-one (51) samples were positive in the diagnostic culture (Table 2). The initial diagnostic culture result could be confirmed by IMC in 71 negative samples and 41 positive samples.

3.4. Culture negative samples

Seventy-three (73) samples had a negative result from diagnostic culture (Table 1). For 71 samples (97 %), IMC measurement showed within 2–20 h values for the maximum heat flow not higher than 14.4 µW (mean maximum heat flow 2.1 ± 9.3 µW) and complied to the definition of a negative IMC result (threshold set at 20µW). No peaks were visible in the corresponding heat flow curves (Fig. 5). One sample showed a negative growth control, but an amplitude of maximal heat flow of 57.4 µW at 2.0 h was measured (Table 1). The corresponding heat curve exhibits a peak shape incompatible with bacterial growth.

Sixty-nine (69) of the 73 samples showed a negative result from the growth control "cultivation after Calbact", too. Four samples showed bacterial growth in the recultivation "after Calbact". For three samples with growth of gram-positive cocci, no peaks in the corresponding heat flow curves could be detected, which is in line with the definition of a negative result with IMC and the growth onto blood agar plates is most likely a contamination of the samples after the IMC measurement. In one sample, which exhibited *Klebsiella aerogenes* in the culture "after Calbact", an amplitude of maximum heat flow of 77.5 µW at 10.8 h was measured, which is in line with a positive IMC result.

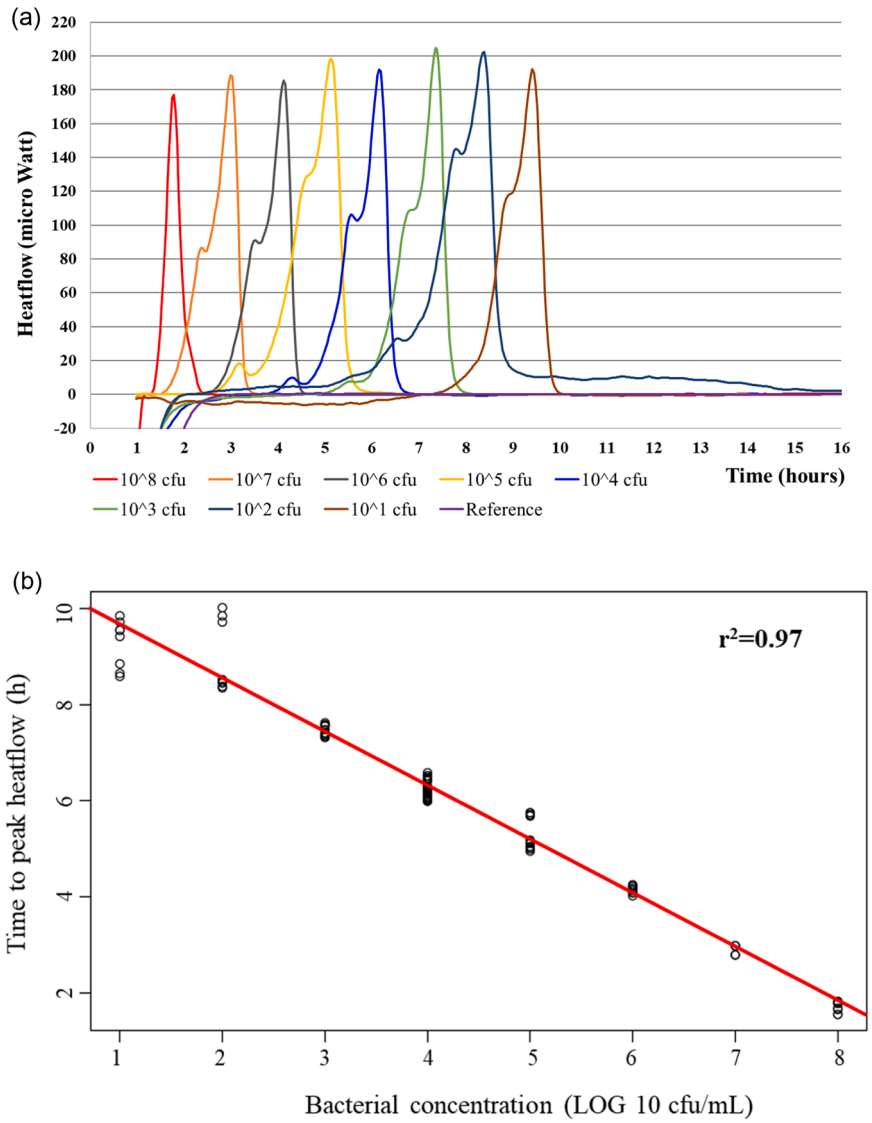

Fig. 3. a. Heat flow curves from *Escherichia coli* ATCC 25922 suspensions of 10^1 to 10^8 colony forming units (cfu) and the baseline signal from the reference channel. b. Time versus concentration regression for *Escherichia coli* ATCC 25922 suspensions of 10^1 to 10^8 colony forming units (cfu).

3.5. Culture positive samples

Fifty-one (51) samples showed a positive initial culture results in diagnostics (Table 2). The most frequently isolated bacteria were *Escherichia coli* in pure culture (61 %, n = 31). Eight samples (16 %) contained three or more different bacteria species (mixed culture). Two samples each contained pure culture of *Enterococcus faecalis, Enterococcus faecium, Staphylococcus felis* and *Proteus mirabilis*, respectively. *Klebsiella aerogenes, Streptococcus gallolyticus* and *Acinetobacter baumanii* where isolated each once in pure culture. Results from semi-quantitative analysis revealed bacterial concentrations ranging between 10^3 and 10^5 cfu/mL, with the majority of the samples containing $>10^5$ cfu/mL. In 41 samples (80 %) a positive signal was measured by IMC, too. Remarkable differences regarding time to peak were observed. Mean time to peak was 3.1 ± 1.5 h in *Escherichia coli* pure culture samples. For the other positive samples, the times to peak reached from 2.0 h for *Staphylococcus felis* to 7.8 h for *Klebsiella aerogenes*. Moreover, the maximum amplitude of heat flow varies in a broad range depending on the bacterial species detected. *Escherichia coli* produced the highest amplitudes with a mean of 166.7 ± 104.2 µW. Other *Enterobacterales,* like *Proteus mirabilis, Klebsiella aerogenes* and mixed cultures with *Escherichia coli* showed a weak positive result with maximum amplitude of heat flow from 81.0 µW to 29.0 µW. Only one of the four samples containing *Enterococcus faecalis* or *Enterococcus faecium* yielded a positive IMC result (maximal heat flow 43.8 µW at 3.8 h). Two samples with either *Staphylococcus felis* (n = 2) or *Streptococcus gallolyticus* (n = 1) showed no peak > 20 µW between 2 h and 20 h (Table 2). Moreover, the same was observed for one *Acinetobacter baumanii* positive sample.

Two samples (20/1683 and 20/1696) with a pure culture of *Escherichia coli* showed negative IMC results in the first measurement (Table 2). Very high bacterial load being present in the samples would probably lead to a culture being in stationary phase already. In stationary phases, lower metabolic activity is expected showing only decay or an initial peak during the non-recorded first 2 h of the measurement. We assumed that this was the most likely possible explanation for the negative IMC measurement of these two samples. To test this hypothesis, a 1:100 dilution of the sample 20/1683 in CAMHB was measured. In 1:100 dilution, the sample had a positive IMC result with a maximum amplitude of 139.21 µW at 9.36 h. Retesting of sample 20/1696 was not possible due to the lack of remaining urine. Moreover, another sample in question (20/1656) with a pure culture of *Escherichia coli* showed a low peak heat flow of 48.5 µW at 3.0 h in its first measurement. In the measurement repeated in 1:100 dilution this sample yielded a peak heat flow of 130.9 µW at 3.1 h. The two cultural positive samples with

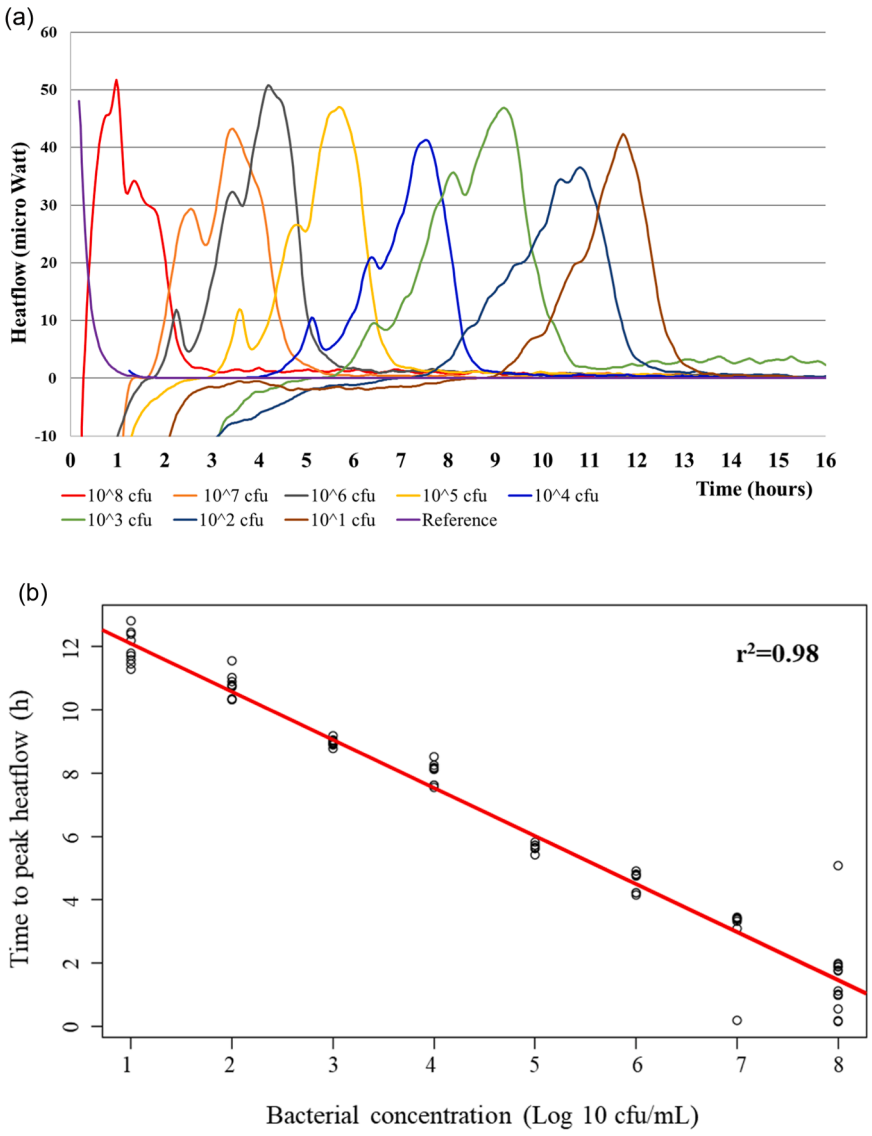

Fig. 4. a. Heat flow curves from *Enterococcus faecalis* ATCC 29212 suspensions of 10^1 to 10^8 colony forming units (cfu) and the baseline signal from the reference channel. b. Time versus concentration regression for *Enterococcus faecalis* ATCC 29212 suspensions of 10^1 to 10^8 colony forming units (cfu).

Table 1
Time to maximum heat flow and Maximum heat flow of 73 culture negative urine samples from dogs and cats measured a prototype isothermal microcalorimeter.

Number of samples	Cultural result diagnostics	Result cultivation "after Calbact"	t_{peak} (h) Mean ± SD*	hf_{peak} (μW) Mean ± SD*
68	negative	negative	n.d.	n.d.
1	negative	negative	2.0	57.4[+]
2	negative	*S. warneri*	n.d.	n.d.
1	negative	*E. faecalis*	n.d.	n.d.
1	negative	*K. aerogenes*	10.8	77.5

tpeak = time to maximum heat flow, hfpeak = Amplitude of maximum heat flow, SD = standard deviation, μW = microwatt, n.d. = heat flow > 20 μW in the time period >2 h and <20 h not detected
[+] 20/1410, unusual peak, *E. faecalis* = *Enterococcus faecalis*, *K. aerogenes* = *Klebsiella aerogenes*, *S. warneri* = *Staphylococcus warneri*

Enterococcus faecium, which turned out to be negative with IMC were also measured again after dilution. The second measurement in 1:100 dilution still yielded a negative result with IMC.

Forty-eight (48) samples with a positive result from diagnostic culture also showed a positive result in the growth control "cultivation after Calbact". In three samples, in which *Escherichia coli* or mixed culture was detected by cultural diagnostics, this was not confirmed in the growth control "cultivation after Calbact" (Table 2). Two samples exhibited negative results in IMC measurement, one sample (20/1893) with initial *Escherichia coli* detection, showed a weak signal with a maximum amplitude of 28.5 μW at 2.0 h.

3.6. Performance parameter of measurement with a prototype isothermal microcalorimeter

Based on the results of 124 urine samples, the diagnostic sensitivity was 80 % compared to bacterial culture, the diagnostic specificity was 97 %, with conventional culture set as gold standard (Table 3). A Cohens' kappa value (κ) of 0.80 indicated a good concordance of the two methods.

4. Discussion

Until now, mainly in research studies, IMC has been shown to be a

Table 2
Time to maximum heat flow and Maximum heat flow of 51 culture positive urine samples from dogs and cats measured a prototype isothermal microcalorimeter.

Number of samples	Cultural result diagnostics	Result cultivation "after Calbact"	t_{peak} (h) Mean ± SD*	hf_{peak} (μW) Mean ± SD*
27	*E. coli*	*E. coli*	3.1 ± 1.5	166.7 ± 104.2
2	*E. coli*	*E. coli*	n.d.+	n.d.+
2	*E. facealis*	*E. facealis*	n.d.; 3.8	n.d.; 43.8
2	*E. faecium*	*E. faecium*	n.d.	n.d.
1	*K. aerogenes*	*K. aerogenes*	7.8	81.0
2	*S. felis*	*S. felis*	n.d.; 2.0	n.d.; 24.4
1	*S. gallolyticus*	*S. gallolyticus*	n.d.	n.d.
2	*P. mirabilis*	*P. mirabilis*	2.8; 8.3	83.1; 29.0
1	*A. baumanii*	*A. baumanii*	n.d.	n.d.
8	mixed culture	mixed culture	4.9 ± 2.7	69.4 ± 72.8
2	*Escherichia coli*	negative	2.0; n.d.	28.5; n.d.
1	mixed culture	negative	n.d.	n.d.

tpeak = time to maximum heat flow, hfpeak = Amplitude of maximum heat flow, SD = standard deviation, μW = microwatt, *mean for *E. coli* and mixed culture results only; n.d. = heat flow > 20 μW in the time period > 2 h and < 20 h not detected, + one sample could be retested at a 1:100 dilution *E. coli* = *Escherichia coli*, *E. faecalis* = *Enterococcus faecalis*, *E. faecium* = *Enterococcus faecium*, *K. aerogenes* = *Klebsiella aerogenes*, *S. felis* = *Staphylococcus felis*, *S. gallolyticus* = *Streptococcus gallolyticus*, *P. mirabilis* = *Proteus mirabilis*, *A. baumanii* = *Acinetobacter baumanii*

promising alternative technique for real time detection of bacterial pathogens. But broad introduction into veterinary routine diagnostic laboratories currently lacks on isothermal microcalorimeters, which are robust, easy to use and comply with biosafety regulations. In this study, to the best of the authors' knowledge, a prototype microcalorimeter that meets the above requirements was used to evaluate its general performance and diagnostic suitability for the first time on a larger set of clinical samples.

With respect to the overall performance of this IMC prototype, which uses plastic bags instead of glass vials as sample containers, we focused on the time to peak, which depends on the concentration of bacteria to be detected, as this is one of the most important factors in detection. In contrast, the amplitude of peak heat flow is influenced by other factors (biological, e.g. strain specific metabolic differences as well as device specific factors such as contact between sampling bag and sensor). Repeated measurements with a 10^4 cfu/mL *Escherichia coli* suspension over the six channels showed a very good reproducibility of the time to peak (6.3 ± 0.2 h). Thus, we included data from all channels. With this prototype microcalorimeter, start of measurement was possible after two hours without misinterpretation of detectable heat flow from thermal equilibration processes as metabolic heat stemming from bacteria. For future generations of the microcalorimeter, this transient phase should be shortened to approximately one hour, which was shown to be possible for IMC measurements (Braissant et al., 2010). Moreover, preheating of the samples to 37 °C before starting the measurement might also allow quicker equilibration.

With *Escherichia coli* and *Enterococcus faecalis* serial dilutions the prototype instrument shows a strong negative correlation for the time to peak versus concentration, which demonstrates that reliable detection of bacteria is possible within a broad range of clinically important concentrations. In case of bacterial concentrations of at least 10^6 cfu/mL very rapid detection within four hours is possible. Moreover, even low amounts of at least 10^1 cfu/mL were detectable within 12 h of measurement. In general, microcalorimeters are capable of measuring metabolic heat production of 10^4 to 10^5 bacteria (Braissant et al., 2015b), demonstrating the high sensitivity of this prototype calorimeter. Our findings are in accordance with a study from Bonkat et al. (2012), where detection limits of one cfu for *Escherichia coli*, 10 cfu for *Enterococcus faecalis* and *Proteus mirabilis* and of 30 cfu for *Staphylococcus aureus* were reached with a different microcalorimeter (Bonkat et al.,

Table 3
Sensitivity (Se) and specificity (Sp) for measurement with a prototype isothermal microcalorimeter with conventional culture as gold standard based on results for 124 urine samples.

		Cultural result	
	total (n = 124)	positive (n = 51)	negative (n = 73)
IMC result*	positive+	41	2[a]
	negative+	10	71

$$\text{Sensitivity (Se)} = \frac{41}{51} = 0.80 = 80\ \%$$

$$\text{Specificity (Sp)} = \frac{71}{73} = 0.97 = 97\ \%$$

* Isothermal micro calorimetry; + positive defined as tpeak (time to maximum heat flow) between 2–20 h and hfpeak (amplitude of maximum heat flow) > 20 microwatt (μW)

[a] 20/1410 (2.0 h, 57.4 μW, no growth in growth control after Calbact, unusual heat curve shape); 20/1724 (10.8 h, 77.5 μW, *Klebsiella aerogenes* in growth control after Calbact)

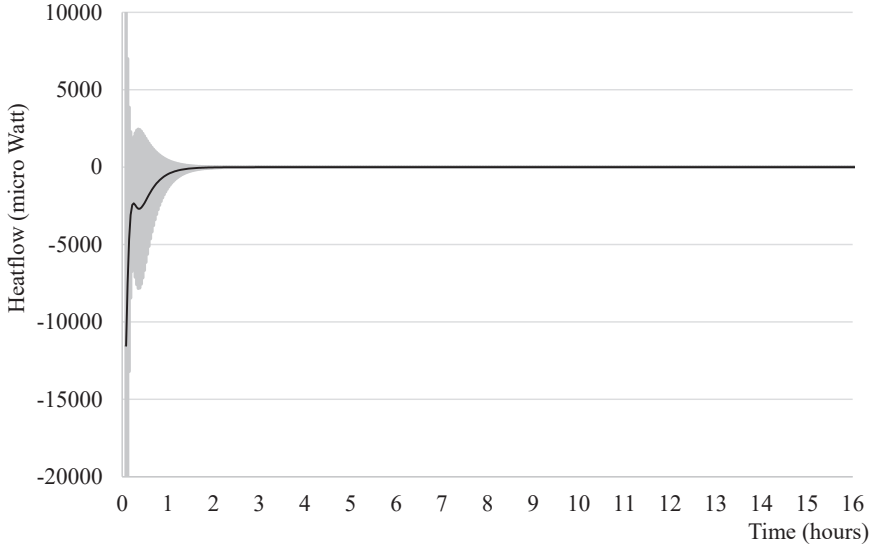

Fig. 5. Heat flow curves of 73 culture negative urine samples The range of the standard deviation (+/- SD) is shown in gray.

2012). In contrast to our microcalorimeter, up to 20 h of measurement were necessary. Moreover, not only detection, but also quantification within a broad range of bacterial concentrations is possible within the same measurement. This was shown in a study by Nykyri et al. (2019), which demonstrated that thermal microbial count determination of microorganisms such as *Pseudomonas* spp. using IMC is faster, more sensitive and more accurate compared to conventional culture (Nykyri et al., 2019).

With diagnostic culture results of 124 urine samples being set as a gold standard, the protoype microcalorimeter used in this study showed a diagnostic sensitivity of 80 % and a diagnostic specificity of 97 %. The Cohens' kappa value (κ) of 0.80 demonstrated a good concordance between the two methods. With our prototype microcalorimeter we could demonstrate that reliable classification of urine samples as bacteriologically negative could be achieved very fast. Taken into account that very low amounts of at least 10^1 cfu/mL were detectable within 12 h, the time to get a reliable negative culture result is feasible by IMC in at least half of the time needed for conventional bacteriological culture (20–24 h) with this prototype microcalorimeter. Moreover, as in human medicine, bacterial urogenital tract infections from dogs and cats are most often caused by mono-infections with high amounts of *Escherichia coli.* In case of bacterial concentrations of at least 10^6 cfu/mL very rapid detection within four hours was possible. Therefore, the IMC can decisively shorten the valuable time span from sampling to reporting the results to the clinician. Although exceptional cases are described, where heat was measured by IMC which were not correlated to bacterial growth, these cases are very rare and therefore seem not to cause problems, at least in urine samples (Braissant et al., 2015a). Moreover, also for bacteriological positive urine samples, reliable detection of bacterial growth, either as pure or mixed culture, could be demonstrated. For *Escherichia coli* pure culture samples, mean time to peak was 3.1 ± 1.5 h, which demonstrated the veritable possibility to speed up pathogen detection with this method in comparison to conventional culture techniques. For other bacterial species being present in our sample set, the mean times to peak reached from 2.0 h for *Staphylococcus felis* to 8.3 h for *Proteus mirabilis*, which is still more rapid than conventional culture. Other authors have described detection of *Escherichia coli* and *Staphylococcus aureus* within three to six hours by IMC measurement (Zaharia et al., 2013). It has to be considered that in our study all analyses were carried out by using a small volume of urine (100 µL), which was diluted into 900 µL cation-adjusted Müller-Hinton broth, which decreased the bacterial amount. The starting volume as well as the broth influenced the growth rate of the pathogens. By optimization of these parameters an even more rapid detection might be feasible with the microcalorimeter. We chose 100 µL urine because it might be difficult to obtain more urine from dogs or cats suffering from urinary tract infection. Cation-adjusted Müller-Hinton broth was chosen because it is directly suitable for AST as well. While reducing the final volume for measurement as well using a more suitable broth for enhancing growth of bacteria, the time to get measurable heat activity could be even faster and more sensitive.

In our study, the visual properties of the heat flow curves were reproducible for *Escherichia coli* and *Enterococcus faecalis*, independent of the inoculum concentration and even directly in the urine samples. This shows the possible capacity not only for detection and quantification, but also identification of different bacterial species. Because metabolic activities differ between bacterial species, the pattern of heat flow varies. Identification of bacterial species by mathematical analysis of whole thermal power curves was described for *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Enterococcus faecalis* and *Staphylococcus aureus* (Braissant et al., 2015a; Fredua-Agyeman et al., 2018). Moreover, even in mixed cultures it was possible to identify at least the dominant bacterial species. The authors showed that the thermal power curves occurred in mixed culture revert to the pattern of the pure culture when density of one species decreased (Fredua-Agyeman et al., 2018). Another advantage of IMC measurement is the fact that the technique does not harm the bacteria during measurement. As we have shown, for almost all samples it was possible to reculture the detected pathogen after IMC measurement. This opens the door for other, complementary diagnostic tests. Of utmost importance is antimicrobial susceptibility testing. This could be done with conventional techniques such as disc diffusion or broth microdilution, but it has already been shown that IMC technology is also suitable for these types of analyses. In addition, identification of the pathogens by means of Matrix -Assisted Laser-Desorption/Ionization-Time of Flight Mass Spectrometry (MADLI TOF MS), but also PCRs or whole genome sequencing after IMC measurement are of course possible, since the bacteria, or their DNA, can be easily isolated from the enriched sample.

5. Conclusion

Even with our prototype we could demonstrate that IMC is a reliable tool for real time detection of bacterial pathogens within a few hours suitable for routine diagnostics. Device specific improvement of the contact between sensor and sampling bag is already initiated for the next generation of the prototype microcalorimeter. In conjunction with optimization of sample measurement thereby an alternative measuring technique for routine veterinary bacteriological diagnostics will be available. Moreover, our data indicate that quantification and identification will be possible, too. Further studies to gain knowledge about the growth characteristics leading to specific thermal curves of different bacterial species are needed. In the longer term, the establishment of an antimicrobial susceptibility testing procedure could become the most important application of IMC technology in the clinical setting.

Declaration of Competing Interest

We declare that we have no competing interests.

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