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On-line cell lysis of bacteria and its spores using a microfluidic biochip

Research Article

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Abstract: Optimal detection of pathogens by molecular methods in water samples depends on the ability to extract DNA rapidly and efficiently. In this study, an innovative method was developed using a microfluidic biochip, produced by microelectrochemical system technology, and capable of performing online cell lysis and DNA extraction during a continuous flow process. On-chip cell lysis based on chemical/physical methods was performed by employing a sufficient blend of water with the lysing buffer. The efficiency of lysis with microfluidic biochip was compared with thermal lysis in Eppendorf tubes and with two commercial DNA extraction kits: Power Water DNA isolation kit and ForensicGEM Saliva isolation kit in parallel tests. Two lysing buffers containing 1% Triton X-100 or 5% Chelex were assessed for their lysis effectiveness on a microfluidic biochip. SYBR Green real-time PCR analysis revealed that cell lysis on a microfluidic biochip using 5% Chelex buffer provided better or comparable recovery of DNA than commercial isolation kits. The system yielded better results for Gram-positive bacteria than for Gram-negative bacteria and spores of Gram-positive bacteria, within the limits of detection at 10^3 CFU/ml. During the continuous flow process in the system, rapid cells lysis with PCR-amplifiable genomic DNA were achieved within 20 minutes.

Keywords: *Cell lysis • Chelex 100 • Microfluidic biochip • SYBR Green real-time PCR • Waterborne pathogens*

1. Introduction

Waterborne diseases are one of the major world-wide threats to public health, despite significant advances in water and wastewater treatment technology. Waterborne disease is estimated to be responsible for 4% of all deaths and 5.7% for the total of diseases worldwide [1]. Drinking water in industrialized countries is generally very safe for consumption. Although water treatment plants can effectively kill most bacteria, treatment processes with using chlorine or chloramines disinfection and filtration are ineffective against toxins, chemicals and some parasites [2]. The occurrences of natural waterborne disease outbreaks as a result of failures with conventional water treatment barriers have been documented. Outbreaks have occurred in such developed countries as the United States (*Cryptosporidium*, Milwaukee, 1993), Canada (*Escherichia coli* O157: H7, Walkerton, Ontario, 2000), the United Kingdom and Europe (several outbreaks of *Cryptosporidium*).

The most significant pathogens causing infections or epidemics through drinking water include the Gram-negative bacteria *Campylobacter* spp., *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *Yersinia enterocolitica*, the Gram-positive bacteria *Enterococcus* species, the spore-forming Gram-positive bacteria *Clostridium* spp., viruses and protozoa [3]. Current monitoring of drinking water quality includes detection methods for such fecal microorganisms as total and fecal coliform bacteria, *E. coli,* enterococci and then indirectly estimate the number of pathogens in water. The evaluation of the risk associated with water pathogens has traditionally been performed using

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culture-based methods. However, traditional cultivation methods are often labour-intensive, time-consuming with many drawbacks that hamper reliable identification [4]. Moreover, under certain conditions (*i.e.*, low-nutrient environments, oxidative or osmotic stress, *etc.*), numerous bacterial species assume a viable but non-culturable (VBNC) state, and the actual number of viable bacterial cells could be underestimated [5]. These problems make cultivation methods unsuitable for preventive actions and rapid response in emergency situations.

Over the past decade, new microbiological culture-independent detection techniques have emerged in order to track specific pathogens both for the routine monitoring of water and for rapid investigation of disease outbreaks. Molecular detection methods with high sensitivity and accuracy, particularly based on PCR, RT-PCR and recently real-time PCR, have been increasingly developed. With the development of microelectromechanical system (MEMS) technology, a complex molecular analytical system which has the potential for integrating sample pretreatment with DNA extraction, amplification and detection, has been introduced. Micromachined analytical systems have several advantages over their counterparts, including low cost, low reagent and sample consumption, disposability, portability, low power consumption, and the potential for automation and integration. Many such devices have been reported in the literature, including micro-PCR chips [6], micro-DNA chips [7], micro-DNA biosensor [8], *etc.* Most of these analytical systems need an effective and simple method of DNA isolation. Hence, miniature devices for rapid and simple sample pretreatment of DNA, including cell lysis and genomic DNA extraction are crucial for genetic application.

DNA isolation from initial samples requires disruptive cells to liberate the nucleic acids before amplification process. Standard methods for isolating bacterial DNA rely on cell lysis using combinations of heat, enzymes, chemical lytical agents (detergents) or mechanical forces (sonication, bead milling) [9,10]. However, many such lysis techniques are not amenable for implementation in a microfluidic platform.

The purpose of the present study was to develop a simple, effective, and rapid method for cell/spore lysis and DNA extraction which could be integrated into lab-on-a-chip application of water pathogen detection on a microfluidic platform. Several studies have demonstrated using different cell lysis methods in lab-on-a-chip devices such as ultrasonic disruption [11], mechanical pressing [12], electrical methods [13,14], chemical methods, [15-17] or methods utilizing laser-irradiated magnetic bead [18]. The method developed in this study includes a microfluidic incubation chamber where bacteria/spores have been incubated with chemical lysis buffers as well as heating elements to thermally lysed bacteria/spores. The effectiveness of lysis method was assessed using SYBR Green real-time PCR assay.

2. Experimental Procedures

2.1 Lysis chamber fabrication

A microfluidic channel structure was designed with 2 inlets (IN1, IN2) and 1 outlet (OU) and 5 ml internal volume. The 3D CAD (Three-Dimensional Computer-Aided) design of lysis chamber is shown in Figure 1. The final structure was bonded together from an upper and a bottom half after 5 min of corona discharge treatment; the halves were fabricated by PDMS (PolyDiMethylSiloxane) casting into 3D rapid prototyped (printed) molds. PDMS was purchased from Dow Corning Corp. (USA). FullCure 720 base material and FullCure 705 support material were purchased from Varinex Inc. (Hungary). For the 3D RPT (Three-Dimensional Rapid Prototyping Technology) printing, we applied an Objet Geometries (Israel) Eden 250 printer with FullCure 720 base material and FullCure 705 support material. All the fabricated structures were immersed in 7% NaOH solution for 30 min after printing to remove all the remaining support material. Autodesk Inventor 2010 software was used for designing the objects. Raw PDMS was prepared by adding Sylgard 184 curing agent to Sylgard 184 silicone elastomer in

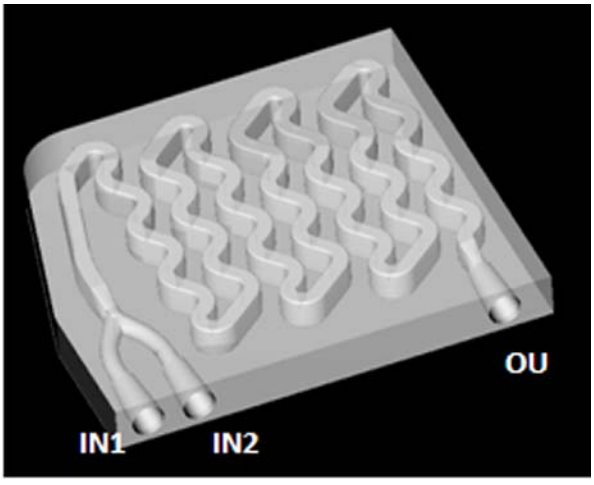

Figure 1. Three-dimensional CAD design of lysis chamber with 2 inlets (IN1, IN2) and 1 outlet (OU) made of PDMS (the maximal outer dimensions are height: 6 mm, width: 72 mm, length: 65 mm). The total process of cell lysis and DNA extraction was performed successfully in one lysis chamber of a microfluidic biochip within 20 min and with using one type buffer.

1:10 m/m ratio. The freshly prepared raw PDMS was cast into the 3D RPT fabricated mold forms in a homemade casting workstation consisting of a vacuum desiccator, a water stream based vacuum pump, and a tubing or in a vacuum chamber with oil based vacuum pump. During the 10 min vacuum exposition all the visible air bubbles left the PDMS body (pressure below 5 kPa). For binding two separately casted PDMS parts together we applied the corona treatment surface activation using an Electro-Technic Products Inc. BD-20AC instrument. This laboratory corona apparatus works with three different shapes of electrodes containing an output voltage between the range of 10–48 kV and 4–5 MHz frequency.

2.2 Microfluidic biochip system

An in-house designed electrical circuitry incorporating a microcontroller communicating with the user personal computer (PC) *via* a USB link was manufactured and assembled in-house on Circuit Board technology and was linked with two MultiPhaser™ NE-501 programmable syringe pumps (OEM product of ProSense, Netherlands). This also contained a lysis chamber holder with a lysis chamber and a temperature actuator unit (Figure 2). In experiments, the ratio of lysis buffers (five times concentrated) and the sample volume were calibrated to 1:5; namely with 10 ml h^{-1} and 50 ml h^{-1} flow, respectively. Thus, the residence time of any part of the liquid column subjected to the continuous lysis treatment was 5 min in the temperature controlled zone. Considering the entire process, the lower and upper surfaces of the single use PDMS lysis chambers were heated and kept on 95±2°C. An additional pressure of 40 kPa ± 2% was applied to decrease bubble/foam formation in the sample and buffer mix due to the heating.

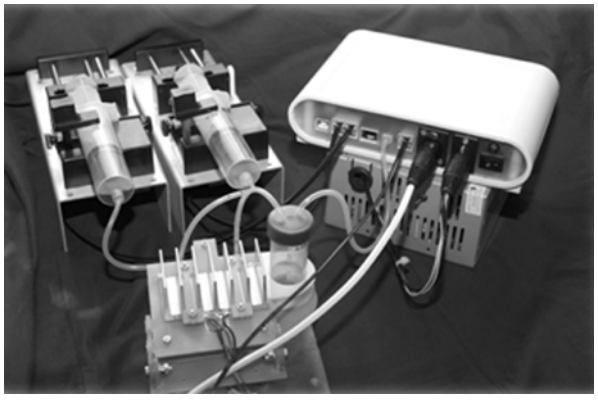

Figure 2. Photograph of module for the continuous flow microchip system. A designed electrical circuitry in-house, incorporating a microcontroller and communicating with the PC was linked with 2 MultiPhaser™ NE-501 programmable syringe pumps and with a microfludic biochip designed and manufactured in-house.

2.3 Bacterial strains

All strains used were acquired from the Czech Collection of Microorganisms (Brno, Czech Republic). *Salmonella enterica* serovar *Enteritidis* CCM 4420 was used as Gram-negative test organism, *Enterococcus faecalis* CCM 4224 as Gram positive test organism and *Bacillus subtilis* ssp. *spizizenii* CCM 1999 as spore-forming test organism. *S. enteritidis* was cultured in 20 ml Nutrient Broth (Merck, Darmstadt, Germany) at 37°C for 16-18 hours at 160 rpm and *E. faecalis* was grown in 20 ml. Brain-Heart-Infusion Broth (Merck, Darmstadt, Germany) at 37°C for 16-18 hours at 160 rpm. Bacterial cultures were grown until its mid-late exponential phase in liquid media. The concentration of the bacterial liquid cultures were determined by plate-count technique on solid media of Nutrient Agar (Merck, Darmstadt, Germany) for *S. enteritidis* and Slanetz-Bartley Agar (Merck, Darmstadt, Germany) for *E. faecalis* incubated at 37°C for 24 h. *S. enteritidis, E. faecalis* counts were on average $2x10^9$, $1.5x10^9$ colony forming units (CFUs) per milliliter of the overnight culture. *Bacillus subtilis* ssp. *spizizenii* CCM 1999 was grown overnight in 20 ml Brain-Heart-Infusion Broth (Merck, Darmstadt, Germany) at 37°C. 200-µl aliquots of the vegetative cells were spread onto sporulation agar consisting of 13 g l^{-1} nutrient broth, 15 g l^{-1} agar, 0.51 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.97 g l^{-1} KCl, 0.2 g l^{-1} $CaCl_2 \cdot 2H_2O$, 3 mg l^{-1} $MnSO_4 \cdot H_2O$ and 0.5 mg l^{-1} $FeSO_4 \cdot 7H_2O$ [19]. The plates were incubated at 37°C for 3 to 5 days until more than 95% of cells had formed spores, as determined by phase-contrast microscope (Zeiss, Germany). The spores were harvested by centrifugation at 13000x*g* and washed repeatedly three times in sterile deionized water. Spore counting was carried out with a cell-counting chamber (Thoma chamber, Brand, Wertheim, Germany). Spores of *Bacillus subtilis* counts were on average $1x10^9$ spores per milliliter of deionized water.

2.4 Methods of DNA extraction using lysis buffers

Samples were prepared by tenfold serial dilutions of *Salmonella enteritidis* with final concentrations from 10^7 CFU ml^{-1} to 10^3 CFU $ml^{-1}$and were subjected to five extraction DNA methods with different buffers as stated below. Diluted samples were centrifuged at 13000x*g* for 10 minutes. The supernatants were discarded, and the pellets in Eppendorf tubes were processed for each procedure as follows:

2.4.1 TE method (TE)

A 1 ml aliquot of TE buffer (10 mM Tris-HCl [pH 7.5], 1 mmol l^{-1} EDTA) were added to the pellet, and the contents were briefly mixed on a vortex mixer. The suspension was boiled in a boiling water bath for 5 min.

2.4.2 Triton X-100 method (TX)

The pellet was re-suspended in 1 ml of 1% Triton X-100. It was briefly mixed and boiled as described for TE buffer [20].

2.4.3 SDS-Triton X method (SDS-TX)

The pellet was treated in the same manner as the TE buffer, except 1 ml of the nonionic detergent mix 2% SDS-10% Triton X-100 was substituted for the TE buffer [21].

2.4.4 Drinking water method (DW)

Treated drinking water from municipal distribution system was collected immediately before the testing. A 1 ml aliquot of collected drinking water was used for mixing with bacterial pellet. The bacterial suspension was boiled in a water bath for 5 minutes.

2.4.5 Chelex method (Chelex)

This method is a modification of bacterial DNA extraction protocol described by Suenaga *et al*. [22]. A 1ml aliquot of 5% of Chelex-100 (Bio-Rad Laboratoires, USA) was added to the pellet and samples were incubated at 55°C for 1 h.

All extraction procedures were repeated three times. The supernatants were transferred to sterile tubes and stored at 4°C until testing with PCR. A 10 µl aliquot of DNA extract from each extraction method was used as the DNA template in the real-time PCR.

2.5 Methods of DNA extraction

Overnight cultures of *S. enteritidis, E. faecalis* and spores suspension of *B. subtilis* were used for the preparation of samples for DNA extraction, and the dilution series of samples were prepared in drinking water without disinfectant. For three tested model organisms we prepared five dilutions with concentration from 10^7 bacteria/spores per ml to 10^3 bacteria/spores per ml.

2.5.1 DNA extraction in Eppendorf tube (Epp)

A 1ml. aliquot of sample was centrifuged at 13000x*g* for 10 min. The supernatant was discarded and the pellet in Eppendorf 1.2 ml tubes was mixed with 1 ml tested buffers (1x concentrated). Lysis was achieved by heating at 95°C for 5 min.

2.5.2 ZyGEM method (ZyGEM)

A 100 µl aliquot of samples were processed using *ForensicGEM*™Saliva isolation kit (ZyGEM) protocol, with the following modifications. This extraction kit has been specifically developed for extracting DNA from buccal swabs but it is also applicable for liquid samples. 100 µl of sample was centrifuged at 13000x*g* for 10 minutes, and the pellet was suspended in 89 µl of TE buffer, 10 µl of 10x buffer BLUE and 1 µl enzyme of *Forensic*GEM™ (proteinase K). The DNA was isolated by manufacturer´s recommendations in a final volume of 100 µl.

2.5.3 MoBio method (MoBio)

A 100 µl aliquot containing samples, were processed using the Power Water® DNA isolation kit (MoBio Laboratories, Inc.) protocol, with the following modifications: 100 µl of the sample was added directly without the step of membrane filtration into special PowerWater® bead tube, containing bead mix and 1 ml lysis buffer. Tubes were horizontally mixed for 10 minutes at a maximum speed of a Vortex Genie® 2 vortex (Scientific Industries, Inc., Bohemia). The DNA was purified with a spin column according to manufacturer´s recommendations in a final volume of 100 µl of elution buffer.

2.5.4 DNA extraction in lysis chamber of a microfluidic biochip (LCH)

The lysis chamber was placed on temperature actuator unit in a lysis chamber holder with a outlet tubing connected to collecting tube.The final sample volume of 20 ml was loaded into a 25 ml calibrated syringe and connected to the first inlet tubing of lysis chamber. The buffer volume of 4 ml was loaded into a 5 ml calibrated syringe and connected to a second inlet tubing of the lysis chamber. Loaded syringes were placed into programmable syringe pumps. Water sample and buffer were then continuously injected into the microfluidic chip to perform the on-chip extraction of DNA by heating at 95±2°C for 20 minutes.

All extraction procedures were repeated three times. A 10 µl aliquot of DNA extract from each extraction method was used as the DNA template in the real-time PCR.

2.6 SYBR Green real-time PCR

Real-time PCR and data analysis were performed in the Stratagene Mx3005P real-time PCR detection system (Agilent), using 2x Brilliant II SYBR Green QPCR master mix (Stratagene). Previously described *Salmonella* specific primers were chosen to amplify a 291-base pair fragment of the *fim*C gene [23]. For *E. faecalis*, the genus-specific *tuf*-gene [24] was targeted to amplify 112-base pair fragment, and for *B. subtilis* it used published primers for the 16S rRNA [17]. Specific oligonucleotide primers were synthesized by Metabion (Germany). Primer set sequences are shown in Table 1.

The PCR mixture contained 12.5 µl of 2× Brilliant II SYBR Green QPCR master mix, 0.5 µl of each primer

with 200 nM final concentration, 0.375 µl of diluted reference dye (passive reference dye ROX), 10 µl of DNA template and nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl. Each sample was analyzed by PCR in triplicates. In each PCR analysis, a negative control without target DNA (water was used as a no-template control) and a positive control, (genomic DNA purified by Power Water® DNA isolation kit, MoBio), was included in this analysis. Thermal cycling conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 60 seconds and 72°C for 60 seconds.

The Mx3005P real-time PCR detection system and software were used for data analysis. Mx3005P system monitors fluorescence of reaction mixture just before the denaturation step of each amplification cycle, and the cycle number at which fluorescence crosses a specific threshold value in the exponential phase of amplification (the threshold cycle or *CT*). The *CT* is inversely proportional to the logarithm of the initial number of template molecule. The identity of the PCR product in sample was confirmed by performing a melting curve analysis comparing its melting temperature (*Tm*) with *Tm* of the product from the positive control.

2.7 Statistical analysis

One-way analysis of variance with Bonferroni *post hoc* comparisons was used for the evaluation of the results for extraction buffer selection. The null hypothesis was rejected if the significant difference between values was determined to have a probability (*P* value) of less than 0.05. For pairwise comparisons of the procedures, the two sample, one-sided *t* test was used to determine if one procedure gave significantly higher *CT* values than another method.

3. Results

3.1 Comparison of DNA extraction procedures for selection extraction buffer

Several published extraction methods based on thermal lysis for selection of an optimal extraction buffer were tested, with the aim of extracting DNA on a microfluidic biochip. The performance of DNA extraction procedures was evaluated using Gram-negative bacterium *S. enteritidis.* The effectiveness of the DNA extraction method was determined by comparing the *CT*s obtained in a SYBR Green real-time PCR assay with extracted DNA as a template. The extraction methods were examined separately for each of the five concentration levels tested and compared. Statistical evaluation for the different values of *CT*s obtained is shown in Table 2.

With the exception of the SDS-TX method, DNA extracted by the remaining methods was amplified for all concentration levels. Of the five tested methods, samples treated by TX (1% Triton X buffer) and Chelex (5% Chelex buffer) methods displayed similar *CT*

Bacteria	Primer set	Primer sequence (5´→3´)	Target DNA
S. enteritidis	S212f	5´AAA CGT TTA TCG TTA CCG CG 3´	*fim*C
	S500r	5´ATC TTG AGA TGG TTG CCG AC 3´	
E. faecalis	Ent1f	5´TAC TGA CAA ACC ATT CAT GAT G 3´	*tuf*
	Ent2r	5´AAC TTC GTC ACC AAC GCG AAC 3´	
B. subtilis	L-bsubf	5´CCT ACG GGA GGA AGC AG 3´	16S rRNA
	R-bsubr	5´CCA GTT TCC AAT GAC CCT CCC C 3´	

Table 1. Primer sequences for *S. enteritidis, E. faecalis* and *B. subtilis*.

S. enteritidis CFUml^{-1}	Mean** *CT* ± SD				
	TE[a]	TX[b]	DW[c]	Chelex[d]	P value
10^7	18.77 ± 0.10	17.79 ± 0.33	20.,71 ± 0.06	17.96 ± 1.41	0.013
10^6	22.20 ± 0.54	20.70 ± 0.34	25.46 ± 0.52	18.92 ± 0.31	<0.001
10^5	26.09 ± 0.79	22.64 ± 0.37	31.40 ± 0.20	23.39± 0.58	<0.001
10^4	29.7 0± 0.25	27.87 ± 0.46	32.42 ± 0.42	27.33 ± 0.41	<0.001
10^3	29.57 ± 0.32	27.16 ± 0.85	34.91 ± 0.47	27.12 ±0.92	0.033

Table 2. Comparison of the statistical difference* in *CT* values for four DNA extraction methods.

** Differences were analyzed by one-way analysis of variance ** Values are means of three determinations*
[a] TE method, [b] Triton X-100 method, [c] Drinking water method, [d] Chelex method

values, which were lower than *CT*s obtained by TE and DW methods. PCR amplification was inhibited for all concentration levels for samples extracted with the SDS-TX method (2% SDS-10% Triton X buffer), and thus *CT* (No *CT*) was not included in the statistical analysis. One-way analysis of variance of the mean differences in *CT* values among the four DNA extraction methods showed a statistically significant difference in all concentration levels (Table 2). The Bonferroni *post hoc* comparison showed a significant difference for all comparisons ($P<0.05$, data not shown) except for the methods of TX and Chelex (methods with the lowest *CT*s). Comparison of extraction methods on different bacteria concentration levels provided the same results.

3.2 Evaluation of DNA extraction in the microfluidic biochip

For the five tested buffers used in five DNA extraction procedures, two types of lysis buffers, 1% Triton X-100 and 5% Chelex 100 were used for testing on a microfluidic biochip. The observed lysis on a microfluidic biochip was measured by assaying the lysate utilizing PCR quality DNA from Gram-negative, Gram-positive bacteria, and from spores of Gram-positive bacteria. For the bacteria and spores tested, the PCR amplification of the extracted DNA from three lysis chambers of a microfluidic biochip was compared regarding bacterial/spores concentration (10^3 to 10^7 CFU ml^{-1}). Lysis chambers loaded with sterile lysis buffers of both types without bacteria and spores were included as negative controls.

To test the efficiency of DNA extraction conducted by using a microfluidic biochip, bacterial/spores samples were processed in parallel tests for each concentration of samples by the other three methods: DNA extraction in Eppendorf tube and DNA extraction by two commercial DNA extraction kits. Commercial Power Water® DNA isolation kit (MoBio Laboratories, Inc.) and *ForensicGEM*™Saliva kit (ZyGEM) served as positive controls.

The efficiency of lysis was compared according to values of *CT* obtained by using SYBR Green real-time PCR. The specific PCR products were identified by melting curve analysis and a reproducible melting point *Tm* of 85.6°C for *Salmonella enteritidis,* 81.2°C for *E. faecalis* and 83°C for *B. subtilis. Tm* values consistently proved to be specific for the different amplicons. The negative controls of PCR and lysis chamber did not give detectable *CT* values (data not shown). In order to compare the different methods used for the paired tests, two samples were analyzed, by employing a one-sided *t* test to determine if one procedure gave significantly higher *CT* values than the other method.

Testing for lysis on a microfluidic biochip using 1% Triton X and 5% Chelex buffers, varied in terms of tested model organisms. Lysis on a microfluidic biochip with 1% Triton X buffer yielded *S. enteritidis* DNA, but with *CT* values greater than those obtained by lysis in Eppendorf tube and also by ZyGEM kit (Figure 3). Two-samples, one-sided *t* test confirmed that extraction with ZyGEM kit produced statistically greater efficiency than extraction on a microfluidic biochip in the presence of 1% Triton X buffer. Although extraction in Eppendorf tubes resulted in greater efficiency than extraction with biochip in the presence of 1% Triton X buffers, the effect was not statistically significant. The results using 5% Chelex buffer were comparable or better than lysis in

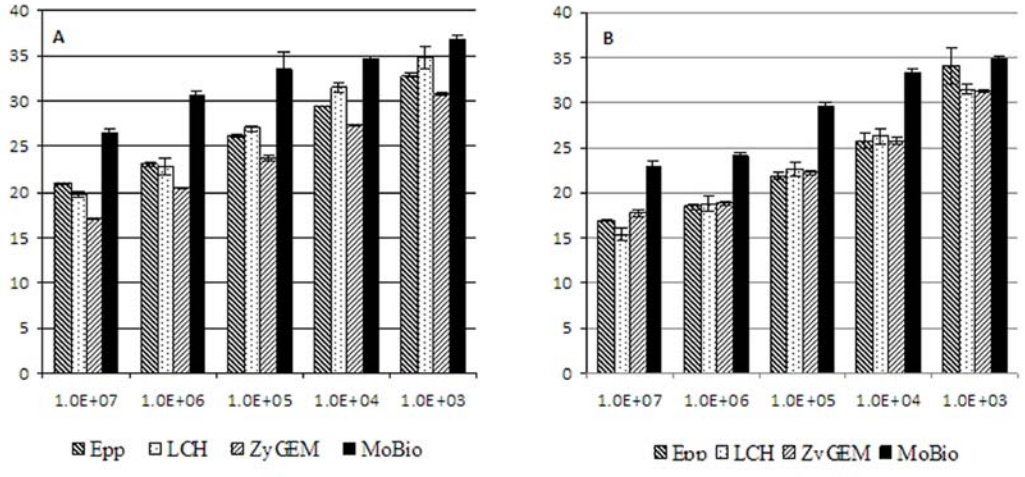

Figure 3. Amplification threshold cycles (Mean *CT* ± SD) for *Salmonella enteritidis* at 10^3 to 10^7 CFU ml^{-1} in sample. DNA extraction in a lysis microfluidic chip (LCH) and off-chip extraction in Eppendorf tube (Epp) were performed using 1% Triton buffer (chart A) and 5% Chelex buffer (chart B). The positive controls were extracted using two commercial DNA extraction kits (MoBio, Zygem) according to manufacturer´s recommendations.

Eppendorf tubes and the ZyGEM kit. The results of lysis on a microfluidic biochip with 5% Chelex buffer were not statistically different from the results of extraction using both lysis in Eppendorf tubes and ZyGEM kit, respectively (Tables 3, 4). The second-tested commercial MoBio kit produced the lowest results from all methods tested.

The best results of DNA extraction on a microfluidic biochip were obtained for the Gram-positive bacterium *E. faecalis*. This was the only organism tested that was detectable at all the concentration levels and for both lysis buffers with the lowest *CT* values in comparison with other tested methods (Figure 4). Statistical analysis confirmed that extraction on a microfluidic biochip in presence of both lysis buffers produced significantly greater efficiency than other extraction methods, although in one case, the effect of extraction on a microfluidic biochip in presence of 1% Triton X buffer was not statistically significant in comparison to ZyGEM kit (Tables 3, 4).

For spores of *B. subtilis* extraction, results from lysis chip with using 1% Triton X buffer were equivalent to or

Extraction method	P value		
	S. enteritidis	*E. faecalis*	Spores of *B. subtilis*
LCH-Triton *vs* Epp	0.337	0.009	0.02
LCH-Triton *vs* ZyGEM	<0.001	0.164	0.118
LCH-Triton *vs* MoBio	<0.001	0.003	0.03

Table 3. Pairwise statistical comparison of the difference in *CT* values for four DNA extraction methods was performed by two sample, one-sided *t* test. Method of DNA extraction in a lysis microfluidic biochip using 1% Triton buffer (LCH-Triton) was compared with off-chip extraction method in Eppendorf tube using 1% Triton buffer (Epp) and with two commercial DNA extraction kits (MoBio, Zygem) performed according to manufacturer´s recommendations.

Extraction method	P value		
	S. enteritidis	*E. faecalis*	Spores of *B. subtilis*
LCH- Chelex *vs* Epp	0.469	<0.001	<0.001
LCH- Chelex *vs* ZyGEM	0.637	<0.001	0.482
LCH- Chelex *vs* MoBio	0.001	<0.002	0.104

Table 4. Pairwise statistical comparison of the difference in *CT* values for four DNA extraction methods was performed by two sample, one-sided *t* test. Method of DNA extraction in a lysis microfluidic biochip using 5% Chelex buffer (LCH-Chelex) was compared with off-chip extraction method in Eppendorf tube using 1% Triton buffer (Epp) and with two commercial DNA extraction kits (MoBio, Zygem) performed according to manufacturer´s recommendations.

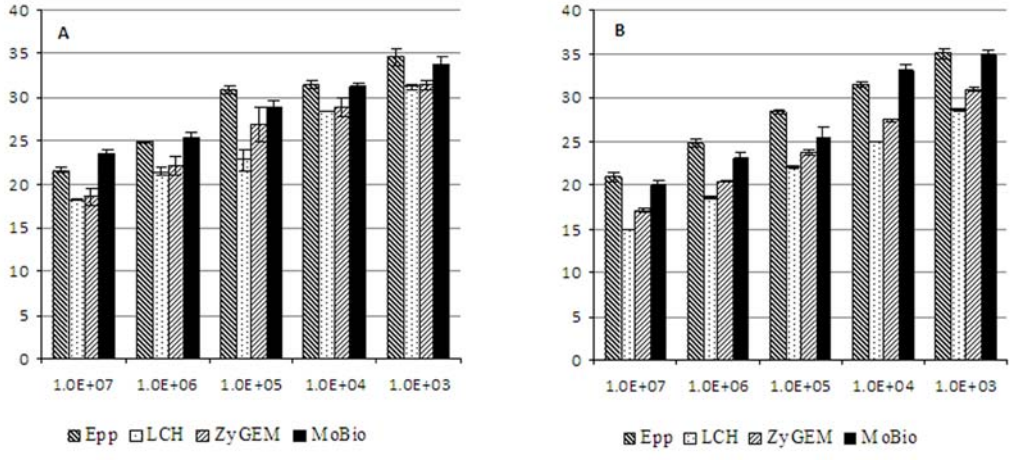

Figure 4. Amplification threshold cycles (Mean *CT* ± SD) for *Enterococcus faecalis* at 10^3 to 10^7 CFU ml^{-1} in sample. DNA extraction in a lysis microfluidic chip (LCH) and off-chip extraction in Eppendorf tube (Epp) were performed using 1% Triton buffer (chart A) and 5% Chelex buffer (chart B). The positive controls were extracted using two commercial DNA extraction kits (MoBio, Zygem) according to the manufacturer´s recommendations.

better than remaining methods. The effect of extraction on a microfluidic biochip in presence of 1% Triton X buffer was not statistically significant in comparison to both commercial kits, but in comparison to lysis in Eppendorf tube, the effect was significantly greater. The same results were detected for lysis on a microfluidic biochip with 5% Chelex buffer (Table 3, 4). The greatest differences in *CT* values were detected in comparing lysis in the Eppendorf tubes and on the microfluidic biochips, using the 5% Chelex buffer. Lysis on microfluidic biochips reduced the *CT* value by 7-8 cycles in comparison with lysis in Eppendorf tubes (Figure 5).

4. Discussion

The results that were obtained demonstrate that lysis in the lysis chamber of microfluidic chip is able to lyse all tested bacteria and extract genomic DNA that may be assayed by molecular diagnostic methods. The efficiency of lysis methods such as thermal lysis for DNA release have been reported for various bacteria [25-27]. Because Gram-negative bacteria are presumed to be less resistant to lysis, for the initial series of experiment, a Gram-negative bacterium *S. enteritidis* was employed.

With the exception of the SDS-TX method using 2% SDS-10% Triton X buffer, DNA extracted by the remaining methods was amplified for all concentration levels. The presence of SDS in the buffer completely inhibited the real-time PCR reaction. The results obtained in this study were not unexpected, because an excess of SDS above 0.01% has been shown to inhibit PCR, primarily due to denaturation of the DNA polymerase [25].

Although the extraction by boiling with the TE buffer and deionized water is considered the gold standard of DNA extraction from bacteria for PCR application, the results obtained by TE and DW methods gave *CT* values statistically higher than for the other two methods, namely, TX and Chelex. In the DW method, sterile drinking water from the distribution system was used instead of deionized water for discovering whether it is possible to extract DNA directly from drinking water without the effect of a specific buffer. Although extracted DNA was amplified for all concentration levels, this method gave the highest *CT* values among all tested methods.

There have been many reports describing DNA extraction from different samples using Chelex-100. The results vary according to the use of different methods [26-28]. The use of Chelex -100 has been recommended for DNA extraction in some papers, but other reports did not regard it as optimum because of its lowest efficiency for DNA amplification [28,29]. However, the extraction method using Chelex-100 buffer was found to be the best method of extracting DNA from Gram-negative bacteria. The same results with the best DNA release was observed for the method with Triton X buffer. From five tested buffers used in five DNA extraction procedures, two types of lysis buffers, 1% Triton X-100 and 5% Chelex 100, were used for testing lysis on a microfluidic biochip.

To determine whether lysis in microfluidic channels of lysis chamber is advantageous for detecting pathogens,

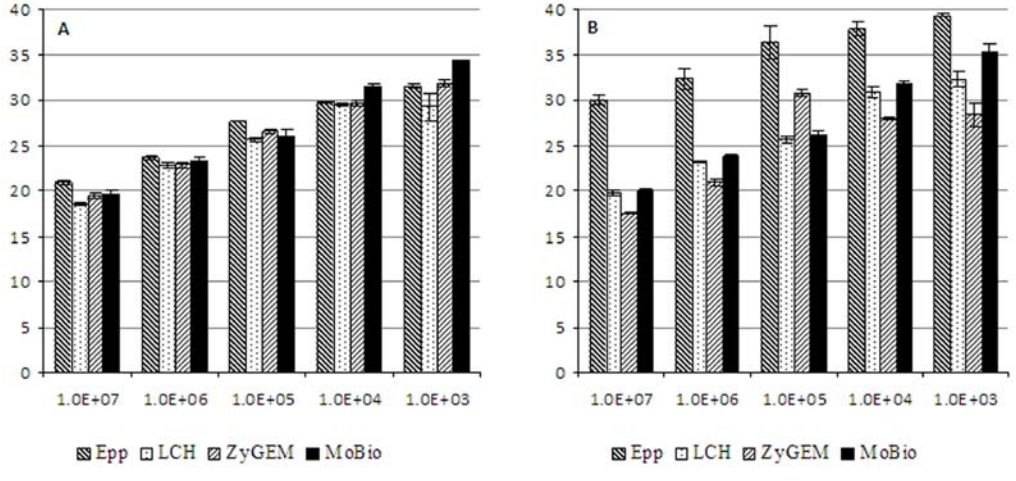

Figure 5. Amplification threshold cycles (Mean *CT* ± SD) for *Bacillus subtilis* at 10^3 to 10^7 CFU ml^{-1} in sample. DNA extraction in a lysis microfluidic chip (LCH) and off-chip extraction in Eppendorf tube (Epp) were performed using 1% Triton buffer (chart A) and 5% Chelex buffer (chart B). The positive controls were extracted using two commercial DNA extraction kits (MoBio, Zygem) according to the manufacturer´s recommendations.

the lysis efficiency was investigated not only for Gram-negative bacteria but also for Gram-positive bacteria and spores of Gram-positive bacteria, which are known to be difficult to disrupt with common extraction methods.

The first group of experiments aimed at determining detectable concentration of bacteria in water by PCR after cell lysis and DNA extraction on a microfluidic biochip. Model samples were prepared by spiking with bacteria in drinking water from its source without employing disinfectant. For the three organisms tested, seven dilutions were prepared with concentrations from 10^7 bacteria/spores per ml to 10^1 bacteria/spores per ml. Model samples were lysed on a microfluidic biochip with real-time PCR analysis of extracted DNA. Before the analysis of the results, it was verified if the *CT* s of the negative control samples were five cycles higher than the *CT*s of any tested samples. Therefore, it is correct to assume that these results are accurate. In the experiments of this study, *CT*s in the negative control samples were from 34 to 36. With only concentration levels higher than 10^2 these requirements were met, so in further trials, the range of tested concentration was from10^3 to 10^7 bacteria/spores per ml.

For evaluation of lysis efficiency in microfluidic channels, comparisons in parallel tests were conducted utilizing commercially available DNA extraction kits, a method that should ensure high DNA yields without the inhibition of PCR amplification. Both *Forensic*GEM and Power Water® DNA isolation kits yielded enough DNA to provide positive results in real-time PCR assays. The *Forensic*GEM commercial kit is the method for DNA extraction, but it is not a purification protocol (recommended protocol). Its purpose is to lyse cells and strip the DNA nucleoproteins. The results obtained in this study indicate that the ZyGEM kit provides comparable results with microfluidic channels for *S. enteritidis* and for spores of *B. subtilis.* Power Water® DNA isolation kit involves filtration of water sample, rapid and thorough lysis with bead particles in lysis buffer, inhibitor removal steps and purification of DNA on silica spin column. Theoretically, column-purified DNA should be the cleanest, containing the least PCR-inhibitory substances. However, Power Water® DNA isolation kits yielded the lowest amount of DNA from all tested methods. Moreover, the time required to complete ZyGEM extraction was 30 minutes without transfer of lysed material to new tubes, compared to less than 1 hour for MoBio with extensive hand-on processing.

The performance of lysis on a microfluidic biochip using 1% Triton X and 5% Chelex buffers differed according the model organisms tested. For *S. enteritidis*, the effect of lysis in lysis chamber was not better in comparison to other methods for both tested buffers; however 5% Chelex buffer in microfluidic channels provided the same results of detection in comparison to lysis in the Eppendorf tube and ZyGEM kit. The performance of lysis of Gram-positive bacterium *E. faecalis* in lysis chamber was achieved for both the buffers tested, and it was found to be the best method for extracting DNA for the various methods studied.

Successful lysis of spores in microfluidic channels in the presence of both buffers, was comparable using both commercial kits. In the case of Chelex lysis, significant reduction of the *CT* values by microfluidic biochip in comparison with lysis in Eppendorf tubes indicated better blending in coiled channels and the mechanical effects of chelex beads. To further demonstrate this, triplicate 10^3 CFU/ml dilutions of *Bacillus* spores were prepared in sample in the same manner as those extracted with microfluidic biochip, but without the Chelex lysis buffer. Of the three samples tested, two were detected at *CT* values of 38.1 and 38.4 and the third sample was undetectable. These higher *CT* values are comparable to previous experiments conducted, suggesting a significant effect of the polar resin, Chelex beads in combination with coiled channels of lysis chamber.

Thus microfluidic biochip capable of destroying cells in real-time and extracting DNA from the cell and spores lysates during continuous flow has been developed. The two inlets (*i.e*. cell inlet and buffer inlet) have been designed to introduce bacterial/spores samples and lysis buffers simultaneously, leading to the rapid lysis of bacterial cells and spores. The model of lysis chamber with coiled channels in a microfluidic biochip was optimized by numerical simulations. Some important factors have been considered in connection with preparatory numerical calculations: sample volume, the temperature field uniformity, continuous flow lysis protocol, the sensitivity of the final lab-on-a-chip type detection method, the reduction of the processing time and the number of steps for final detection. The final microfluidic channel structure of the lysis chamber can be determined with 5 ml of internal volume, where the sample and lysis buffers are mixed and lysed in one step. Despite the fact that the purification process of DNA by washing and elution steps is not integrated in the biochip, successful cell/spores lysis was achieved on a microfluidic chip [30]. Continuous flow lysis protocol allows the experimenter to lyse bacteria in a sample with a volume of 20 ml for 20 minutes in the presence of 40 kPa overpressure.

Carlo *et al.* [31] reported a mechanical cell lysis device with nanostructural barbs which were used to disrupt sheep blood cells. However, the fabrication process of nanostructures is complex. Many other reported miniaturization cell lysis methods including

thermal [32], electrical [33] and chemical [34] treatments have been developed. However, they all depend on the use of an external power supply and the devices may be quite complicated and costly to fabricate. The developed microfluidic biochip is incorporated in in-house designed and manufactured simple system without using of expensive device. Chemical disruption methods with lysing buffers were chosen because it was found to be compatible with SPE on chips and do not require complex process of fabrication either.

It should be noted that this study was not designed to yield truly quantitative data, but to illustrate qualitative differences achieved using different DNA extraction methods. The quality of the extracted DNA in terms of purity, concentration and fragmentation or other PCR performance quality parameters has not been studied. Consequently, an easy and efficient cell and spores lysis method that releases DNA by microfluidic channels thermal lysis using Chelex-100 buffer, provides a fresh extraction method that is well-suited to lab-on-a-chip applications and other applications related to DNA analysis.

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