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ULTRASONIC IMMUNO-AGGLUTINATION TESTS FOR FOOD-BORNE PATHOGENIC BACTERIA

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The effective testing for bacteria requires methods of analysis that meet a number of challenging criteria. Time and sensitivity of analysis are the most important limitations related to the usefulness of microbiological testing. Bacterial detection methods have to be rapid and very sensitive. It is in this context that we developed a quantitative, simple and sensitive, particle agglutination assay for detection of *E.coli O157:H7* and *Listeria.monocytogenes (L. monocytogenes)*.

The ultrasound particle agglutination method (UPA) is based on the use of special multifrequency regimes of ultrasonic exposure of the samples for acceleration of the particles agglutination, and separation of specific and nonspecific aggregates. The use of such regimes as well as quantitative assessment of immunochemically formed specific aggregates using optical means allows one to increase the sensitivity and accuracy of the conventional particle agglutination methods significantly.

Using commercially available particle agglutination tests, we showed that UPA can successfully be employed for detection of *E.coli O157:H7* and *L. monocytogenes* in environmental samples (water), food and clinical samples. In particular, we demonstrated that the analytical sensitivity of detection of the pathogenic bacteria in water and clinical samples can be increased more than 100-fold, and in food approximately 30-fold as compared to conventional particle agglutination tests.

INTRODUCTION

The Center for Disease Control (CDC) calculates that every year in the United States, there are approximately 76 million cases of food-borne illness, with 325,000 hospitalizations and 5,000 deaths. Food-borne pathogen testing is performed in clinical, food industry and environmental control laboratories.

In the food industry, food control for pathogens is an essential part of any existing production line. At present, testing for pathogens is performed in a variety of locations including governmental, centralized corporate and on-site laboratories. Over the past few years, there has been a shift toward performing more pathogen testing on-site in food-processing plants because testing on-site provides results quickly, shortens the time products must be retained in inventory and, hence, lowers the products' storage cost.

The conventional FDA and USDA methods of bacteria detection in food include enrichment steps followed by plate count on selective media. It could take 5 to 7 days to complete the test. Therefore, testing food with short shelf life (seafood, diary etc.) creates a challenge for the food industry. Elimination of the last step of plating on selective media and the development of rapid detection on-site tests would provide important benefits for the food industry, especially with regard to the cost and time of the analysis.

At present, tests that are used for detection of pathogenic bacteria in food within 50 hours following enrichment are either immunoassays such as enzyme-linked immunosorbent assay (ELISA) and particle agglutination assay, or molecular techniques (PCR, hybridization, electrophoresis) [1-8]. Among these methods, ELISA is the most popular assay because of its accuracy and sensitivity. However, ELISA consists of several incubation and washing steps, and, therefore, is time-consuming (3-5 hours) and labor-intensive to perform and requires special equipment such as a plate-reader. As a result, ELISA is a relatively expensive method. Because of the aforementioned limitations of the ELISA method, there is a demand for alternatives to ELISA for highly sensitive, cost–effective, automated, simple and rapid detection of pathogenic bacteria.

A conventional particle agglutination method could be a good alternative to ELISA. However, rapid routine detection of *Listeria* and *E.coli* by traditional agglutination methods is limited in use because these tests are subjectively read and, therefore, qualitative and have a low sensitivity. In order to circumvent the aforementioned serious drawbacks of agglutination assays, a number of approaches have been developed. One of them is the use of non-cavitating standing wave ultrasound for enhancement of agglutination. This approach has been developed by Dr. Coakley's group [9-17]. In this method, a sample, mixed with a coated microparticle suspension, is exposed to an ultrasonic standing wave for several minutes. After sonication the sample is expelled on to a non-adsorbing surface and stirred manually (two to ten stirs) before microscopic inspection for agglutination [18,19]. Stirring of the reagent mixture is a necessary step to disrupt non-specific complexes. The formation of non-specific aggregates is the usual "side effect" of the ultrasonic-enhanced methodology [16,18]. The final results are registered by the naked eye or microscope.

In this study, we present a new ultrasound particle agglutination (UPA) method for detection of food-borne pathogenic bacteria. In UPA, a sample ultrasonic treatment is used for both enhancement of specific agglutination and disintegration of non-specific aggregation of latex microparticles in a one-step procedure. In addition, the quantitative assessment of precipitated aggregates using simple optical means is performed while samples are in the resonator cell. These innovations allowed us to develop a one-step sensitive, simple and rapid method for detection of pathogenic bacteria. Since UPA offers crucial advantages over other rapid tests, we believe that our ultrasound particle agglutination technology has great potential to meet the need for sensitive microbial detection in food, environmental and clinical samples.

MATERIALS AND METHODS

Latex agglutination methods. Qualitative latex agglutination tests, Microgen *Listeria* (Microgen Bioproducts Limited, cat # F48) and *E.coli* 0157 (Oxoid Ltd, catalog # 411279) for the presumptive identification of *Listeria* spp. and *E.coli* serogroup O157, respectively, were used in our studies. (It should be noted that the Microgen *Listeria* kit is a test for presumptive identification of *Listeria spp*. including human pathogen *Listeria monocytogenes)*. Conventional agglutination procedures were performed as described by the manufacturers. Briefly, one drop of latex reagent and one drop of test sample were mixed with a clean mixing stick on the slide. The mixtures were gently rocked and the agglutination reaction was read visually. A smooth milky white appearance was considered a non-reactive result, whereas a change from the "milky" solution to a "sour milk" appearance (agglutination) was considered a positive result.

ELISA. Tecra *E.coli* O157 and *Listeria* visual immunoassays were performed as described by the manufacturer (TECRA International Pty Ltd, Australia).

The UPA method. Serial dilutions of positive bacteria controls were mixed with latex beads and immediately 2 µl (*E.coli* test) or 4 µl (*Listeria* test) of the reaction mixtures were transferred to a resonator cell of the UPA system (see below). Then, the sample was exposed to the ultrasonic field, which included the sequential use of various higher harmonics of resonance oscillations. The ultrasound treatment of the sample results in both formation of specific and disintegration of nonspecific aggregates. The ultrasound intensity was adjusted so that no measurable heating of the sample was produced. Original image analysis software was used to quantify the agglutination. The final results are presented as an Average Agglutination Quantity (**AAQ**) Index.

The UPA system. The experiments were conducted using an UPA device, which included an electronic unit, Olympus CX41 microscope and Moticam 350 video camera. The ultrasonic cell was built as a cylindrical chamber using piezoceramic tube PZT 5H with an internal diameter of 2.0 mm, a height of 1.5 mm and a thickness of 0.5 mm. Samples were tested in triplicate. An average of the three measurements was calculated using Microsoft Excel software.

Preparation of food samples. 25 g of French soft cheese *"Delice d'Argental"* was blended into 100 ml of sterile Fraser Broth and incubated at 25^{o}C (primary enrichment). After 24 hr of incubation, 1 ml of primary enrichment was transferred to 100 ml of sterile Fraser broth (secondary enrichment) and incubated 72 hr at 15^{o}C. Morphological diversity of the secondary enrichment was analyzed by direct microscopy after Gram staining. The amount of microorganisms was determined by plating on Nutrient agar and Tecra *Listeria* agar medium. Inactivation of the enriched microbial cells was performed by heating of a 50 ml sample at 100^{o}C for 20 min. Enriched meat samples were provided by Dr. Brewster's laboratory (US

Department of Agriculture). Cell density in the killed commercial samples was assessed by direct microscopy with a UV microscope after staining with DTAF [20].

RESULTS

I. DETECTION OF LISTERIA IN AQUEOUS SOLUTIONS

Increasing of the sensitivity of the particle agglutination test for Listeria detection in phosphate buffer solution using UPA

The Microgen *Listeria* (cat # F48) kit was used for comparison of sensitivities of conventional and ultrasound particle agglutination methods. Serial dilutions of the positive control sample containing 4.7×10^9 cell/ml were prepared in phosphate buffered saline (PBS) and used for both assays. The detection limit of the standard agglutination test (SAT) was expressed as the maximal "positive control" dilution at which agglutination is detected by visual inspection of the test card. We showed that a 1:2-diluted positive *Listeria* sample (~2.4×10^9 cell/m) was the detection limit of the *Listeria* card-test (see Figure 1)).

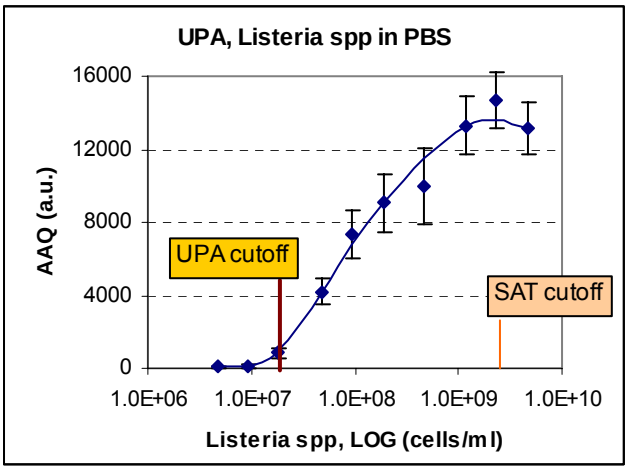

***Figure 1**. Dilution curve for detection of Listeria spp in PBS.*

It should be noted that the performance of the UPA method depends on many factors such as resonant frequency, time of ultrasonic treatment, sample-latex ratio and image analysis parameters. Therefore, in order to reach a maximal sensitivity of the UPA test, these parameters must be optimised. Below we present the most promising results obtained so far using aforementioned optimised parameters.

The UPA "signal-cell density" plot (dilution or calibration curve) for detection of *Listeria spp* in PBS is shown in Figure 1. Using the UPA data presented in Figure 1, and being conservative we estimated the detection limit (sensitivity) as the background + 5SD, which is equal to 1.84×10^7 cell/ml (1:256) dilution of *Listeria* positive control sample. Thus, the sensitivity of determination of *Listeria spp* in aqueous solutions can be increased by more than 100-fold using UPA as compared to that of conventional particle agglutination method.

Detection of Listeria in environmental samples

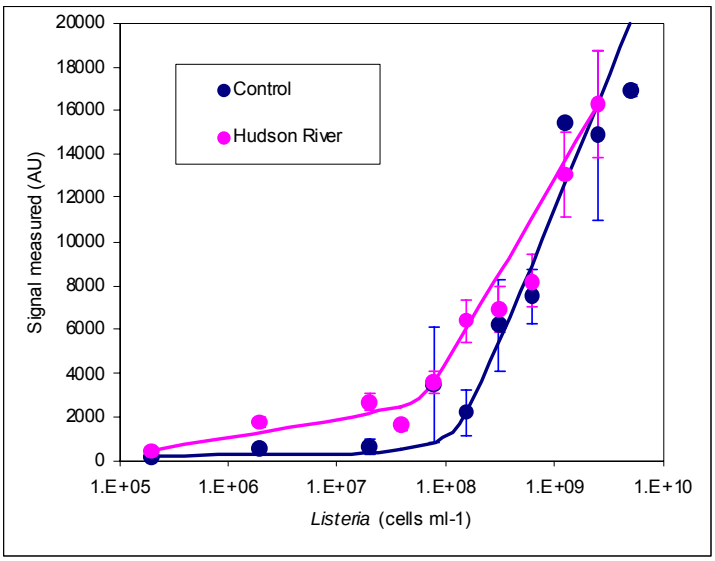

***Figure 2**. Dilutions curves for detection of Listeria spp in deionized water (control) and water collected from Hudson River.*

In order to examine whether UPA is suitable for detection of *Listeria* in environmental samples, we prepared serial double dilutions of the *Listeria* positive control (attenuated pathogenic *Listeria*) in freshly sampled Hudson River water and in deionized (DI) water used as a control solution. The obtained results are shown in Figure 2. As can be noted in the Figure, UPA signals obtained for DI water and Hudson River samples are practically the same at high concentrations of *Listeria*, whereas at low *Listeria* concentrations the UPA signal is slightly higher for Hudson River water than for DI water. The later effect could be because Hudson

River water is brackish, and contains relatively high concentration of ions such as Na^+, Cl^-, SO_4^-, K^+, and Mg^{2+}, and under these conditions, latex beads form aggregates more easily. In the near future, we will standardize conditions for performing of the UPA for detection of pathogenic bacteria in environmental samples by using high ionic strength buffer solution for conducting UPA experiments.

II. DETECTION OF PATHOGENIC BACTERIA IN GROWTH MEDIA AND FOOD SAMPLES

Comparison of the analytical sensitivities of UPA, ELISA and conventional particle agglutination methods

Determination of pathogenic bacteria in food samples is usually performed after enrichment step, and it is desirable to conduct rapid tests using undiluted growth media samples. Therefore, in order to examine the possibility of using UPA for the detection of bacteria in food samples, we prepared dilutions of the bacteria positive controls in corresponding growth media, and compared the results obtained using ELISA and UPA.

Fraser Broth is a standard selective complex organic medium recommended for enrichment of *Listeria monocytogenes* in food samples. Dilutions of *Listeria* positive control containing 2×10^9 cell/ml were prepared in this broth, and samples were analysed using ELISA and UPA. ELISA and UPA were performed as described above. The calibration (dilution) curves obtained using these methods are presented in Figure 3.

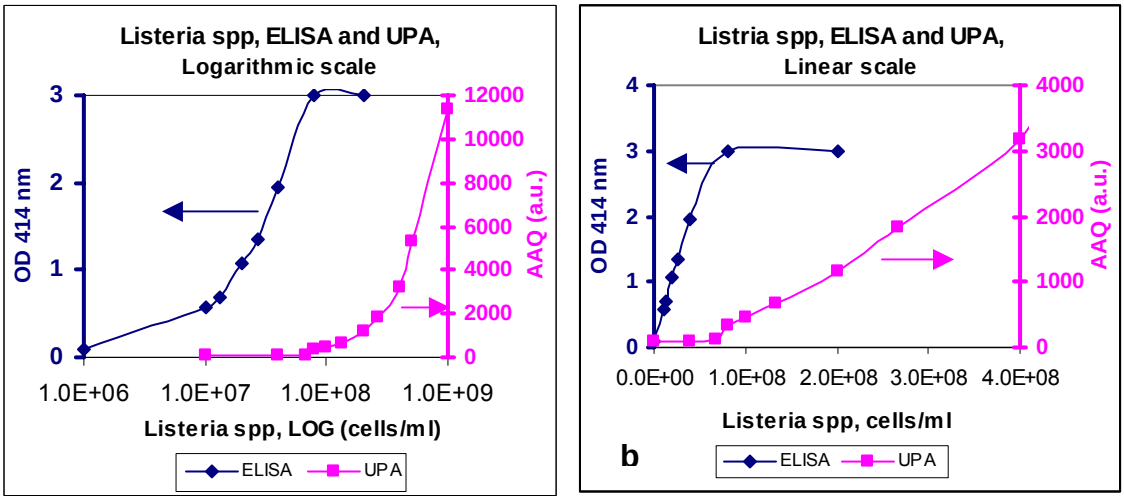

Figure 3. *Dilution curves for detection of Listeria spp in Fraser broth.* ***a*** *– Logarithmic X-axis scale;* ***b*** *– Linear X-axis scale*

In order to compare the analytical sensitivities of ELISA and UPA for detection of *E.coli* O157:H7, we made dilutions of E. Coli positive control in E. Coli Tecra enrichment broth, and prepared dilution curves for both methods. The obtained results are presented in Figure 4.

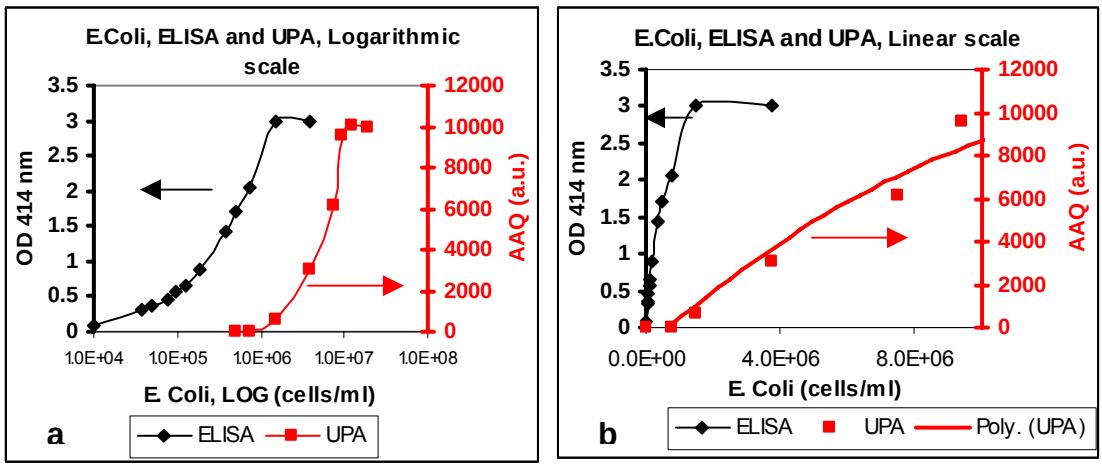

Figure 4. *Dilution curves for detection of E.coli O157:H7 in E. Coli Tecra enrichment broth.* ***a*** *– Logarithmic X-axis scale;* ***b*** *– Linear X-axis scale*

The results shown in Figures 3 and 4 clearly show that the UPA allows one to detect bacteria in growth media used for bacteria enrichment. As can also be seen in these Figures, the "signal-cell density" plots (dilution curves) for detection of *Listeria* spp and *E.coli* have a similar character. Indeed, for both ELISA and UPA at the onset of increasing signal, the plots are almost linear, and at higher bacteria concentrations saturation of the signal occurs.

The detection limit (analytical sensitivity) of the method can be estimated as the background + 5SD (see above). Using regression analysis for the calibration (dilution) curves presented in Figures 3 and 4, we calculated the detection limits of ELISA and UPA for detection of *Listeria spp* and *E.coli*, and summarized our results in Table 1. In this Table, we also included the detection limits of standard agglutination tests (SAT) for *Listeria* and *E.coli*. As we mentioned above, the SAT is subjectively read and qualitative, and, therefore, the detection limit for this test could be estimated only roughly.

Table 1. Sensitivities (detection limits) of ELISA and UPA for detection of Listeria spp and *E.coli* O157 in growth media. The increase of the sensitivity as compared to standard agglutination test (SAT) is also indicated.

Technique	**Listeria spp**		**E.Coli O157**	
	Sensitivity	Increase of the sensitivity	Sensitivity	Increase of the sensitivity
SAT	$\approx 2.4\times10^{9}$ cfu/ml	**1**	$\approx 1.2\times10^{7}$ cfu/ml	**1**
UPA	7.5×10^{7} cfu/ml	32	1.0×10^{6} cfu/ml	12
ELISA	1.9×10^{6} cfu/ml	1263	1.6×10^{4} cfu/ml	750

As can be noted in Table 1, at the present stage of UPA development, the sensitivity of UPA for detection of bacteria in broth is lower than that for UPA performed with water solutions by approximately 4-fold (compare results for *Listeria* presented in Fig. 1 and Table 1). One of the reasons could be the following: Broth, in which all dilutions were prepared, is a more viscous medium than the buffer solutions we used in previous UPA experiments. Therefore, under the currently used ultrasonic treatment, latex beads collide much more rarely in broth than in water. In the near future, we plan to thoroughly optimise conditions for performing UPA in broth using different regimes of ultrasonic exposure of the samples.

The data in Figures 3 and 4, and in Table 1 also show that the ELISA sensitivity is much higher than the UPA sensitivity for detection of both bacteria. Nevertheless, we believe, that further development of the UPA technology including optimisation of the resonator cell and regimes of ultrasonic treatment will allow us to increase the UPA sensitivity considerably.

It is also worth mentioning that the sensitivities for detection of E. Coli are much higher than those for *Listeria* for all used methods. This could be because E. Coli is bigger than *Listeria*, and, therefore, smaller amount of enterobacteria is needed to generate the UPA signal (number of bead aggregates) as well as ELISA signal (number of labeled antibody-bacteria complexes). Another reason for increased sensitivity of *E.coli* tests could be that the affinity of antibodies employed for detection of E. Coli is higher than that for antibodies to *Listeria*.

Detection of pathogenic bacteria in food samples

In order to show the possibility of using UPA for detection of pathogenic bacteria in food samples, we performed UPA analysis using an enriched cheese sample inoculated with *Listeria* spp positive control and enriched meat samples inoculated with *E.coli* positive control. In more detail, serial dilutions of pathogenic bacteria positive controls were spiked with growth media used for enrichment of the food samples (initially not containing pathogenic bacteria).

We showed that the UPA dilution curve (AAQ plot vs. cell concentration) prepared using spiked *Listeria* positive control with cheese enrichment growth media (Fraser broth) was similar to that obtained using sterile Frazer broth. We determined the total cell density in cheese enrichment media, which is equal to $1.8\text{x}10^{8}$ cell ml^{-1}. Thus, one can conclude that the presence of microorganisms in the enrichment media in high concentration does not affect the performance of the UPA method. Using meat samples inoculated with E. Coli O157:H7, we also showed the possibility of using UPA for detection of this bacterium in real meat samples.

Because of the lack of space in this manuscript, the results obtained using **clinical samples** will be reported elsewhere.

CONCLUSIONS

The UPA, a new rapid and simple method has been developed for the detection of food-borne pathogenic bacteria, *Listeria spp* and *E.coli* O157:H7, in food, beverage and environmental samples. We showed that UPA allows one to increase the sensitivity of detection of pathogenic bacteria dozens of times as compared with conventional particle agglutination tests. We also demonstrated the feasibility of the UPA assay for the detection of pathogenic bacteria in various growth media that are used for enriching bacteria in food, environmental and clinical samples. The UPA method offers advantages to conventional pathogenic bacteria tests, including a short time of analysis, an increased sensitivity, quantitative results and low cost. We believe that UPA has high commercial and scientific potential in different fields of diagnostics of food-borne pathogenic bacteria.

References. [1]V.M. Bohaychuk, G.E. Gensler, R.K. King, J.T. Wu, L.M..McMullen: Evaluation of detection methods for screening meat and poultry products for the presence of foodborne pathogens. J Food Prot. **68** (2005) 2637-2647
[2] U. Gasanov, D. Hughes, P.M. Hansbro: Methods for the isolation and identification of Listeria spp. and Listeria monocytogenes: a review. FEMS Microbiol Rev. **29** (2005) 851-875
[3] Beumer RR, Hazeleger WC. Listeria monocytogenes: diagnostic problems. FEMS Immunol Med Microbiol. **35** (2003) 191-197
[4]S.H. Kim, M.K. Park, J.Y. Kim, P.D. Chuong, Y.S. Lee, B.S. Yoon, K.K. Hwang, Y.K. Lim: Development of a sandwich ELISA for the detection of Listeria spp. using specific flagella antibodies. J Vet Sci. **6** (2005) 41-46
[5] L. Cocolin, K. Rantsiou,1 L. Iacumin, C. Cantoni, G. Comi: Direct Identification in Food Samples of *Listeria* spp. and *Listeria monocytogenes* by Molecular Methods . Applied and Environmental Microbiology **68** (2002) 6273–6282
[6] L.T. Nguyen, B.E. Gillespie, H.M. Nam, S.E. Murinda, S.P.Oliver: Detection of Escherichia coli O157:H7 and Listeria monocytogenes in beef products by real-time polymerase chain reaction. Foodborne Pathog Dis. **1** (2004) 231-240
[7] H. Fukushima, K. Katsube, Y. Hata, R. Kishi, S. Fujiwara: Rapid separation and concentration of food-borne pathogens in food samples prior to quantification by viable-cell counting and real-time PCR Appl Environ Microbiol. **73** (2007) 92-100
[8] M. Ikeda, N. Yamaguchi, K. Tani, M. Nasu: Rapid and simple detection of food poisoning bacteria by bead assay with a microfluidic chip-based system. J Microbiol Methods. **67** (2006) 241-247
[9] M. A. Grundy, W. T. Coakley and D. J. Clarke: Rapid detection of Hepatitis B virus using haemagglutination assay in an ultrasonic standing wave field. J. Clin. Immunol. **30** (1989) 93–96
[10] R. I. Jepras, D. J. Clarke, W. T. Coakley: Agglutination of Legionella pneumophilia by antiserum is accelerated in an ultrasonic standing wave. J. Immunol. Methods **120** (1989) 201–205
[11] M. A. Grundy, R. A. Barnes and W. T. Coakley: Highly sensitive detection of fungal antigens by ultrasound-enhanced latex agglutination, J. Med. Vet. Mycol. **33** (1995) 201–203
[12] M. P. Gualano, M. A. Grundy, W. T. Coakley, S. H. Parry and D. J. Stickler: Ultrasound-enhanced latex agglutination for the detection of bacterial antigens in urine, Br. J. Biomed. Sci. **52** (1995) 178–183
[13]. P. Jenkins, R. A. Barnes and W. T. Coakley: Detection of meningitis antigens in buffer solution and body fluids by ultrasound-enhanced particle agglutination, J. Immunol. Methods **205** (1997) 191–200
[14]. R. A. Barnes, P. Jenkins and W. T. Coakley: Preliminary clinical evaluation of meningococcal disease and bacterial meningitis by ultrasonic enhancement, Arch. Dis. Child **78** (1998) 58–60.
[15]. S. J. Gray, M. A. Sobanski, E. B. Kaczmarski, M. Guiver, W. J. Marsh, R. Borrow, R. A. Barnes and W. T. Coakley: Ultrasound-enhanced latex immunoagglutination and PCR as complementary methods for non-culture-based confirmation of meningococcal disease, J. Clin. Microbiol. **37** (1999) 1797–1801
[16]. N. E. Thomas, M. E. Sobanski and W. T. Coakley: Ultrasonic enhancement of coated particle agglutination immunoassays: Influence of particle density and compressibility, Ultrasound Med. Biol. **25** (1999) 443–450
[17]. M. A. Sobanski, R. Vince, G. A. Biagini, C. Cousins, M. Guiver, S. J. Gray, E. B. Kaczmarski and W. T. Coakley: Ultrasound enhanced detection of individual meningococcal serogroups by latex immunoassay, J. Clin. Pathol. **55** (2002) 37–40
[18] R. W. Ellis, M. A. Sobanski: Diagnostic particle agglutination using ultrasound: a new technology to rejuvenate old microbiological methods. J. Med. Microbiol. **49** (2000) 853–859.
[19] M. A. Sobanski, R. Vince, G. A. Biagini, C. Cousins, M. Guiver, S. J. Gray, E. B. Kaczmarski and W. T. Coakley: Ultrasound enhanced detection of individual meningococcal serogroups by latex immunoassay, J. Clin. Pathol. **55** (2002) 37–40.
[20]. Sizova, M.V., Panikov, N.S., Tourova, T.P. and Flanagan, P.W.. Isolation and characterization of oligotrophic acido-tolerant methanogenic consortia from a Sphagnum peat bog. *FEMS Microbiology Ecology* **45**(3) (2003) 301-315.