NOVEL FOOD PATHOGEN TESTING TECHNOLOGIES: MOLECULAR BIOLOGY METHODS

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SUMMARY

Food borne pathogens and spoilage bacteria are influencing the safety and quality of foods and can cause serious adverse effects to human and animal health as well as to the food quality. Consequently, microbiological quality control in the food industry has become the priority of the food producers and it aims towards minimizing the risks connected to food pathogens and spoilage bacteria. Conventional methods, used solely until recently for food testing, have had many obstacles in the terms of the time needed for their application as well as their accuracy. They often involve utilization of suitable media for the pre-enrichment and enrichment, isolation of the pathogens on selective media and their confirmation by the employment of morphological, biochemical and/or serological testing. These methods require intensive work, longer time and, however, obtained results often can be false considering the presence of viable but not cultivable microorganisms. Development of biotechnology and bioinformatics has resulted in the development of novel testing technologies that enable tracking, more reliable and faster detection of food pathogens. Furthermore, molecular-biology methods, although still not applied routinely in everyday practice, are the promising alternative that can replace or be auditioned to current reference methods in this area.

Key words**:** *food borne pathogens, molecular biology methods, rapid tests, biosensors*.

INTRODUCTION

Illnesses caused by food poisoning are endangering public health worldwide and represent one of the major health problems (*Wallace et al.,* 2000). The incidence of foodborne illness is highly increased with intensified trade globalization, increased transport and significant changes in food consuming habits (*Käferstein et al.*, 1997). Nowadays there is over 200 different diseases occurring as a consequence of consumption of the microbiologically unsafe foods and their symptoms varying from mild gastroenteritis to the life threatening syndromes that endanger the life of the consumers with the possibility of chronic complications *(Mead et al.*, 1999*).*

Detection of food spoilage and pathogenic bacteria in food represents a challenge due to the fact that these microorganisms are present often in low numbers in the food matrix and are outnumbered by indigenous bacteria. Conventional/ classical microbiology methods employ the utilization of nonselective pre-enrichment media, selective enrichment media, and consecutive confirmation via morphological, biochemical and/or serological tests. Therefore, these methods are laborious, time

consuming and are not always completely reliable (eg. in detection of some viable but not culturable- VBNC bacterial species). In the aim of overcoming of these obstacles a number of alternative, fast detection methods have been developed for identification and quantification of the food pathogens. This is of high importance for the food production industry since it requires faster methods for obtaining of the adequate information on possible presence of food borne pathogens in the purpose of production control and monitoring of hygienic practice in the facilities. These fast methods provide early detection and enumeration of microorganisms and can be divided into modified and automated conventional methods, bioluminescent, immunological and molecular methods (*Fung,* 2002; *Scheu et al.,* 1998).

ESSAYS BASED ON NUCLEIC ACID DETECTION

Molecular methods, currently applied for the detection of foodborne microorganisms are, in most cases, based on hybridization of nucleic acids or Polymerase Chain Reaction- PCR. These methods can detect specific parts of DNA or RNA molecules. DNA isolation and detection techniques are simpler in comparison to those dealing with RNA. However, there is a high possibility that DNA-based techniques can give positive results also when detecting dead and/or inactive bacterial cells. Therefore, the detection of dead and/or inactive foodborne pathogens or spoilage bacteria represents the main problem in direct application of the PCR techniques for food analysis, especially in the assays that don't involve pre-enrichment steps. On the other hand mRNA, although is less stabile in comparison to DNA, offers a bigger potential for more specific detection of live cells of foodborne pathogens.

Depending on desired specificity of detection (genus, species or strains specificity) different parts of the genome can be used as a target sequences *(Scheu et al.,* 1998).

Nucleic acid hybridization

Nucleic acid hybridization represents a relatively fast screening technique for detection of foodborne pathogens. This method can also be used for detection of target pathogens in the pre-enrichment medium. The principle of the reaction is based on the hybridization of the DNA or RNA molecules in the target organism with DNA probe that has the complementary sequence. DNA probes usually contain 15-30 nucleotides (*de Boer & Beumer,* 1999). Specificity of the hybridization assay is completely controlled by the nucleotide sequence of the probe. First step encompass cell lysis and purification in order to obtain free nucleic acid that can hybridize with the probe. Product of the hybridization can be detected with various techniques. Direct hybridization employs radioactive and fluorescent probes for the hybridization of the nucleic acids in the sample. Indirect detection is done by enzyme reporters on solid media-membranes (nitrocellulose or nylon) and polymer particles. In this purpose the most often used formats are Southern blot and Dot blot in which the target nucleic acid is immobilized on the membrane, after the separation on electrophoresis gel (Southern blot) or directly from the solution (Dot blot) (*Barbour & Tice,* 1997).

Today, there are several commercial systems for pathogen detection available on the market such is Gene-Track that utilizes pathogen-specific probes for annealing to the

bacterial rRNA and colorimetric system for detection of the specific probe-rRNA hybrids (*de Boer & Beumer,* 1999). RNA molecules are often used as a target for hybridization due to the fact that they offer high sensitivity since there is a significant number of target sequence copies (>1000) in a single bacterial cell. Lack of this rRNA based method lies in its limited specificity. Closely related species (e.g. *Listeria monocytogenes* and *Listeria innocua*) share highly similar rRNA sequences and therefore their discrimination is not possible *(de Boer & Beumer,* 1999).

Amplification methods

Molecular methods, involving amplification step, are becoming more popular due to their higher sensitivity. The most applicable method is Polymerase Chain Reaction-PCR. During eighties and nineties in the last century PCR has become widespread method for food pathogen detection (*Chen,* 2003). At the beginning PCR assays were used only in the research laboratories but in the last years many companies have developed commercial PCR systems for food pathogen detection such are those for *Listeria monocytogenes, E.coli O157: H7* and *Salmonella* sp. (*Scheu et al.,* 1998). These methods are more specific in comparison to the conventional, biochemical ones.

In the everyday practice we are often facing the fact that different food components, growth media and reagents used for the isolation of nucleic acids can have negative impact or even block the amplification reaction. These components are generally known as amplification inhibitors (*Rossen et al.,* 1992). Known inhibitors are food constituents (organic and phenol components, glycogen, fat and calcium ions), environmental inhibitors (phenol components, heavy metals), bacterial cells constituents, non-target nucleic acids as well as inhibitory components originating form the laboratory environment. Therefore, it is necessary to perform sample preparation- pre-amplification- to conduct the characterization and removal of the inhibitory components. This represents an important step in sample preparation in order to ensure the preciseness of the reaction (*Wilson,* 1997). The purpose of the pre-amplification is the increasing of the concentration of target organism up to the acceptable level for given method and to reduce or eliminate inhibitory substances. Pre-amplification procedures can be biochemical, immunological, physical or physiological (*Rådström et al.,* 2003*).* The most often used amplification methods are PCR, RTi-PCR, MPCR and NASBA.

PCR is simple, adjustable, sensitive, specific and reproducible assy. Every PCR cycle has three phases (denaturation, elongation and termination) and there is, in average, around 30 of such cycles in one PCR reaction *(Figure 1)*. This assay employs the utilization of DNA polymerase, enzyme that amplifies specific fragments of the DNA molecule, short and sequence-specific oligonucleotide added to the reaction *(Powledge,* 2004*)*. These nucleotides are named as primers and contain the sequences complementary to the target sequences of the DNA molecule (*Table 1*). First and most often used enzyme is Taq DNK polymerase (isolated from the bacterial species *Thermus aquaitcus*) but the Pfu DNA polymerase is also often used (isolated from *Pyrococcus furiosus*) due to its high reliability of copying of the DNA sequence. Although these two enzymes are different they possess some mutual features that make them applicable in the PCR reaction: they can generate new chains of DNA, by using the information in the template

DNA sequence and primers, and are, which is of high importance, thermo stabile (*Valasek & Repa,* 2005).

Table 1. DNA primers used for the detection of food pathogens in PCR reaction

Organism	Target sequence	PCR product	Reference
Listeria monocytogenes	listeriolysine O	520 bp	*Mengaud et al., 1990*
Salmonella.spp.	1.8 kb HIND III	1179 bp	*Tsen et al., 1994*
Campylobacter jejuni	flagelar A gene	450 bp	*Oyfo et al, 1992*
E.coli O157:H7	H7, O157, eaeA, etilA, vt1, vt2 gens	multiplex	*Paton & Paton, 1998*

Thermo stability is necessary due to the fact that at the beginning of every PCR cycle double DNA helix is Denaturated to single strand form (''it is melted'') by the application of high temperature in the reaction tube (93-96^{o}C). Temperature at which half of the DNA molecules become single-stranded is named as melting temperature (Tm). Second phase of the PCR cycle is the primer annealing to the specific complementary sequences of the target single-stranded DNA molecule. Primers suppress the re-annealing of the single DNA strands and enable DNA polymerase to start the synthesis of a new strand. This is the primer annealing phase and it is performed at 65-75^{o}C. Third phase is the elongation phase (at approximately 72^{o}C) which involves binding of the nucleotides from the reaction mixture to the complementary ones of the target sequence. After that, primers are displaced resulting in creation of two copies of target DNA segment.

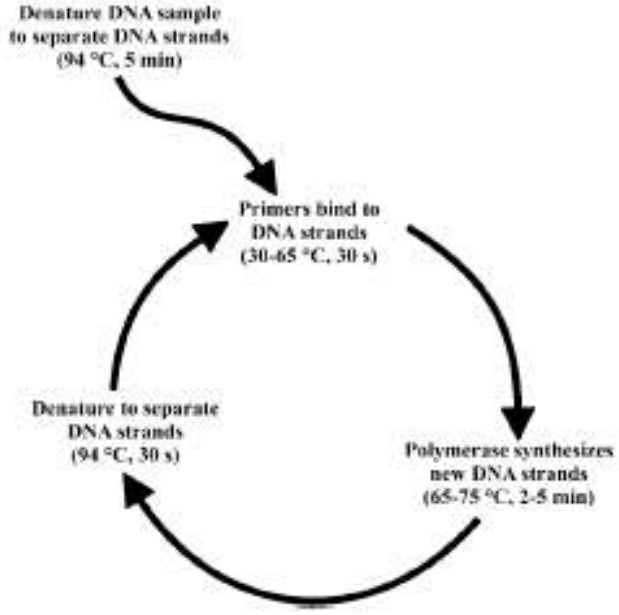

Figure 1. The cycle of the PCR reaction *(taken from Powledge*, 2004)

PCR method enables the amplification of the genes specific for certain taxonomic bacterial group as well genes responsible for virulence of the pathogenic microorganisms

(*Bej et al.,* 1994). Rapid identification of the bacterial strains by PCR doesn't require pure cultures as this is the case with conventional microbiological methods (*Rasmussen et al.,* 1994*)*

Lacks of the PCR method are related to impossibility of differentiation between DNA molecules originating from live or dead cells, impossibility of obtaining the quantitative information (*Rodríguez-Lázaro & Hernández,* 2006), as well as to the time necessary for making the gels to confirm presence/absence of foodborne pathogens (*Fung,* 2002*)*. Furthermore, all of the commercially available PCR assays still require sample pre-enrichment since this decreases the risk from false positive results- detection of dead microorganisms. Another, significant component of these commercial assays is introduction of the internal positive controls that are indicating PCR lacks.

Multiplex PCR (MPCR) represents the method used for simultaneous identification of the several gene sequences belonging to the same pathogen or originating from the mixture of different microorganisms (*Chamberlain et al.,*1988). Main advantage of the multiplex PCR in comparison to the conventional one is its lower price. It is primarily related to decrease of the reagent utilization, such is Taq DNA polymerase. Another advantage of the MPCR in routine diagnostics in comparison to the systems, in which several pathogens are analyzed individually, is also in shorter time required for the sample preparation and obtaining of the results. Reaction mixture in these systems often has more than four primer pairs.

In order to ensure the system specificity it is necessary to design the primers longer than those used in conventional PCR and which are characterized by higher melting temperature (Tm). Magnesium concentration, beside its influence to the reaction specificity, is one of the most significant factors in PCR reaction which determines its efficiency (*McPherson & Møller,* 2000). Generally, in MPCR $MgCl_2$ concentration is higher than that used in conventional PCR reaction. MPCR usually detects 16S rRNA genes (*Rosselló-Mora & Amann,* 2001). This gene is sometimes insufficient for discrimination of closely related species (*Normand et al.,* 1996*; Torrianiet al.,* 2001), and therefore other genes are also detected to ensure specificity of the MPCR reaction.

Real-time PCR is another technique based on the PCR reaction. Essential advantage of the RTi- PCR lies in the fact that it precisely differentiates and measures specific DNA sequences in the sample although these are quantitative very small. At the same time system monitors the synthesis of new molecules during the PCR reaction in the real time by the employment of fluorescent technology (*Heidet al.,* 1996, *Lazcka et al., 2007*). Therefore, data are collected in the course of the PCR reaction-not only at its end. To enable the monitoring of the PCR reaction in the real time the fluorescent probes are used in the PCR reaction. There are several such probes currently available on the market and some of them are DNA binding dyes (EtBr or SYBR green I), sequence-specific fluorescent oligonucleotide probes (TaqMan probe), hybridization probes, etc. (*Valasek & Repa,* 2005).

Every of these probes have its own specific characteristics but their mode of action is quite simple. In principle, they change the fluorescence intensity during the DNA amplification process. SYBR Green I emits thousand times higher fluorescence when it is banded to double-stranded DNA in comparison when it is free in solution. Therefore the SYBR Green fluorescent signal will be higher if the target DNA sequence

amount in the sample is also higher. Main advantages of the RTi-PCR is the fact that it is performed in a closed tube which significantly decreases cross-contamination risk, the analysis is fast and simple, quantification scope is extremely wide and the reliability of the results is significantly higher when compared to the conventional PCR reaction (*Valasek & Repa,* 2005, *Lazcka et al., 2007*).

The most significant achievement of the RTi- PCR technology is its ability of detection of the fluorescent signals and recording of the PCR reaction cycles. It is necessary to provide the excitation energy and detection of the emission wavelength. The excitation energy is provided trough special lamps, light diodes or by laser while detection is done trough different types of photo detectors. Furthermore, to enable PCR reaction it is also necessary to have a thermal cycler that achieves desired temperature by suitable airflow. This is another feature of the RTi-PCR which differentiates it from the conventional PCR where the temperature increase is achieved with thermo-blocks, so the process of heating and cooling also goes slower. For reasons of comparison the termination of 40 PCR cycles in RTi-PCR-a lasts for about 30 minutes, while the same number of cycles in conventional PCR is performed in 1h and 45 minutes (*Edwards et al.,* 2004). Naturally, Real- time instrumentation wouldn't be complete without suitable computer hardware as well as the software for collection and analysis of obtained data.

Results obtained in RTi- PCR are comprised out of amplification curves that can be used for quantification of the initial amounts of sample DNAs with high precision and wide range of concentrations (*Schmittgen et al.,* 2000). Amplification curve typically represents three different phases. (*Figure 2*). First, or initiation phase is done during the first PCR cycle where the emitted energy cannot be differentiated from the base line, followed by the exponential or log phase that follows the fluorescence increase and final, stationary phase in which the regents are exhausted and there is no fluorescence detection (*Rodríguez-Lázaro & Hernández,* 2006*)*.

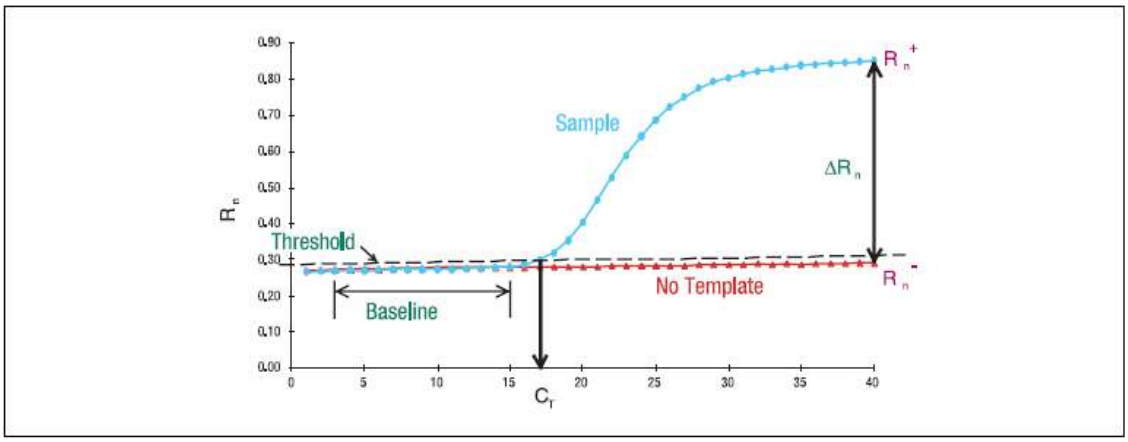

Figure 2. Amplification curve for RTi-PCR (adapted from Higuchi et al., 1993)

Amplification of the nucleic acid sequences - NASBA (Nucleic Acid Sequence Based Amplification) is described for the first time in 1991 (*Compton,* 1991*)*. It is used for the continual amplification of the nucleic acids under isothermal conditions. NASBA represents sensitive system based on transcriptive amplification adjusted for the detection of RNA molecules (*Deiman et al.,* 2002). In such an reaction three different enzymes are used for the amplification of a single stranded RNA molecule (*Rodríguez-Lázaro &*

Hernández, 2006). Reaction also involves two oligonucleotide primers that are complimentary to the target RNA region, deoxiribonucleotide tri-phosphates for the activity of AMV reverse transcriptase and ribonucleotide tri-phosphates for the activity of T7 RNA polymerase. Reaction is performed at 41^{o}C and lasts for 1-2h. At this temperature the genomic DNA stays double stranded and therefore cannot replace the amplification substrate. In the course of NSBA reaction 10-100 copies of target RNA sequence is generated in each of the cycles and after 4-5 cycles approximately 1 million copies of the target sequence is created (*Compton,* 1991). The number of cycles in the NASBA reaction is significantly lower in comparison with conventional PCR methods where it is necessary to have approximately 20 cycles to obtain 1x10^{9} molecules per reaction (*Chan & Fox,* 1999).

Considering the fact that NASBA, same as other molecular techniques, is an instrumental technique it can also give false positive results (*Knowk & Higuchi,* 1989). The most obvious cause of the occurrence of false-positive results is accidental sample or reagents contamination (cross-contamination) in the laboratory. This problem can be overcome by the implementation of internal controls of amplification– IAC (Internal Amplification Controls) (*Hoorfaret al.,* 2004). IAC represent no target sequences of nucleic acids that are simultaneously amplified with target sequence *(Cone et al.,* 1992). When the reaction is also supplemented with IAC it will always give control signal even if there is no target sequence present in the sample (*Rodriguez- Lazzaro & Hernandez*, 2006). If the signal is not registered the reaction failed.

NASBA technique represents the promising diagnostic tool for the detection of viable microorganisms because it is based on detection and amplification of RNA molecule. Since NASBA has the same speed and accuracy as PCR, and in addition has advantage of detection of live pathogens it represents promising laboratory method in food microbiology.

MOLECULE SUB-TYPING METHODS

Sub-typing methods are used for strain (subtypes) differentiation of spoilage and pathogenic bacteria in food and very often are applied in the laboratory practice more often due to the fact that these methods are fast, precise, efficient and help in the process of monitoring of foodborne disease transmission. Generally, methods of bacterial sub typing can be divided into those based on phenotype and molecular-genetic, DNA based methods *(Wiedmann,* 2002).

Most often used DNA-based sub typing methods include plasmid profiling, Pulsed Field Gel Electrophoresis (PFGE), Ribotyping, Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD) as well as other methods such is e.g. Multi Locus Sequence Typization (MLST). These methods enable sensitive discrimination of the strains and higher standardization and reproducibility levels in comparison to the phenotype-based methods (serotyping, biotyping, multilocus enzyme electrophoresis) (*de Boer & Beumer, 1999, van Belkum et al.,* 2001).

Pulsed Gel Field Electrophoresis- PFGE is characterizing the bacteria into subtypes giving suitable DNA sequences after the digestion of bacterial DNA by restriction enzymes. In the course of this procedure, bacterial DNA is isolated and cut by

enzymes into DNA fragments. These enzymes cut DNA at the places where short, specific sequence is present. Restriction enzymes give 8-25 bands of the DNA molecule containing 40-600 kilo base pairs. (*Weidmann,* 2002). Considering the fact that the fragments of DNA molecule of this size cannot be separated by standard electrophoresis techniques a special electrophoresis technique is used. In the course of electrophoresis the direction of electric field is changed and the separation of DNA fragments is achieved. These are, later on, visualized by ethidiumbromide staining. This like generated fragments are compared with the existing database for determination of similarity degree of examined with already analyzed strains. During the continual electrophoresis, DNA fragments of more than 30-50 kb migrate with the same speed no matter of size and this is observed as one diffuse band on the gel. However, if the DNA fragments are ‘’forced’’ to change the direction during the course of electrophoresis, fragments of different sizes will be separated. With every new re-orientation of the electric filed, smaller DNA fragments will start to move in the new direction faster then those of higher molecular weight. Therefore, bigger DNA fragments will be retained at the beginning of the gel ensuring the separation of smaller DNA fragments

PFGE sub typing exhibits high discrimination level in food pathogen detection and therefore it represents gold standard in laboratory diagnostics (*Swaminathan & Feng,* 1994).

Ribotyping is another DNA-based sub typing method in which bacterial DNA is firstly cut into the fragments with restriction enzymes. Differently from the restriction enzymes used in PFGE reaction, which cut DNA in bigger fragments, in the rybotyping reaction genomic DNA is cut into high number of smaller fragments (more than 300-500) 1-30 kb in size. Obtained fragments are separated according to their size by agarose gel electrophoresis. In the following, Southern blot step DNA probes are specifically bind to DNA fragments that contain genes coding rRNA synthesis. Therefore, target fragments are only those DNA fragments containing r RNA genes (*Bruce,* 1996*).*

Amplified Fragment Length Polymorphism- AFLP represents another genotyping technique based on selective amplification of restriction fragments of the DNA molecule (*Vos et al.,* 1995). Technique involves three steps: cutting of the DNA and binding of oligonucleotide adapters, selective amplification of restriction fragments sets and electrophoresis of amplified fragments. Method enables simultaneous analysis of 50-100 restriction fragments on denaturating ply-acryl amide gel.

BIOSENSORS

Biosensors are devices that detect biological or chemical complexes and are based on antigen-antibody, enzyme-substrate or receptor-ligand principle. Most of the biosensors designed for food pathogen detection have been tested solely on the pure bacterial cultures (*Lazcka et al., 2007*). For these applications, pathogens are firstly isolated from the food matrix and then subjected to the analysis by biosensors. There were several attempts of isolating pathogens directly from the food matrix but this still represents a challenge. Furthermore, populations of the target microorganisms are extremely small in comparison to those naturally occurring in foods. Therefore, different strategies for detection of such a small number of pathogens directly from the food are applied.

Surface Plasmon resonant sensor is the optical sensor that has the ability to detect the moment of biomolecule binding in the real time (few seconds or minutes) via detection of the differences in the intensity of the light reflected from the excited surface. When there is binding it conditions changes in the light refraction angle from the medium resulting in creation of the signal. Although this method is mostly used for the detection of live cells of *E. coli* O157:H7, *Salmonella* and *Listeria,* it has the application in the detection of small toxin molecules such are staphylococcal and botulinum toxins *(Pimbley et al., 1998*).

DNA biosensors are being applied after discovering of very interesting chemical and physical features of the DNA molecule. DNA biosensor is the diagnostic device that immobilizes single-stranded DNA at suitable matrix in the aim of detection of the hybridization signal after its exposure to complementary DNA molecule *(Lu et al.,* 2000*; Mandrell & Wachtel,* 1999*).* Target microorganism can be detected by hybridization of the specific sequence at the surface of transducer (*Davis et al.,* 2005). Although there are certain dilemmas on the origins of the conductivity of DNA molecule it has been concluded that DNA can serve as an elegant model for one-dimensional electron transport (*Kavita et al.,* 2006). There is a high number of materials used for making the matrixes serving for DNA molecule binding such are graphite, gold, platinum, carbon electrode, etc. (*Cha et al., 2003, Wang & Zhou,* 2002). This type of detection has several advantages such are: easy DNA immobilization (physical or electro-chemical), decreased response time, higher stability and sensitivity, easy transporting of the device, etc.

DNA MICROARRAYS AND NEXT GENERATION SEQUENCING

Development of DNA microarrays initiated a new phase in the field of pathogen detection. DNA Microarray application provides sensitive and accurate analysis of transcriptome and DNA sequence variations (*Yoo et al., 2004*). Depending on the concept of the method Microarray techniques can be divided in those targeting the rRNA or DNA expression (cDNA microarrays) and those targeting DNA sequence variation (oligonucleotide microarrays) (*Tillib & Mirzabekov, 2001*). *Wilson et al.* (2002) have developed Multi Pathogen Identification (MPID) microarray that can be used for the identification of eighteen different pathogens. The assay is based on oligonucleotide detection and it has very high specificity. DNA Microarray technology (DNA chip) is a promising tool for pathogen detection and can find its application in various areas, among which also food microbiology, detecting either expression of virulence genes or specific diverse individual sequences of the DNA molecule (*Yoo et al., 2004; Mandal et al., 2011*).

Another revolutionary discovery in bioinformatics is next generation sequencing-Pyrosequencing. This is a powerful new technology which enables generation of over one million DNA sequences per run, parallel analysis of multiple samples, detection of unknown pathogens in complex samples and is yet to widen its application in food pathogen detection (*Adams et al., 2009*).

INSTEAD OF CONCLUSION

Advances in the field of immunology, molecular biology, automatisation and bioinformatics continue with their positive effects for the development of fast, sensitive and reliable methods for detection of pathogenic and spoilage food microorganisms. Molecular-biology, DNA or RNA based methods, especially PCR can gradually replace conventional ones. There are still many problems waiting to be solved such are sample preparation, elimination of the effects caused by the unspecific binding and cross-hybridization and achieving biggest sensitivity of the methods. However, the potential of molecular-biology techniques is almost revolutionary.

Furthermore, biosensors are representing a new era in foodborne pathogen detection. It is believed that by their further development and advances in modern biotechnology, microbial biosensors will have promising and light future.

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