Contemporary Methods and Novel Approaches in the Identification and Quantification of Foodborne Pathogens – a Review

T. Mikuš, Ž. Cvrtila and L. Kozačinski*

Abstract

Foodborne pathogens are a continuous problem attracting the attention of public health institutions, microbiologists and food producers globally. The scientific community has therefore been focused for years on finding "new" and "better" methods, all in an effort to detect foodborne pathogens in the timeliest manner possible. It should be emphasized that the development of molecular genetics – the use of genetic information of biological macromolecules in routine testing in particular – has led to a revolutionary turn in biological science research. Almost all procedures detect different bacterial genetic footprint or biomarkers, which makes bacterial isolation unnecessary. However, these recent studies of "fast microbiological methods", which require only a few hours, rather than a few days, have not supplanted the classic way of microbiological food testing, *i.e.* the classic microbiological methods and the gold standard. The new methods are therefore faced with high requirements such as great detection limits, speed of analysis, relatively low costs and high sensitivity to identify various foodborne pathogens. Some of them provide the possibility of monitoring the pathogens, which is an important part of establishing a pathogen control network in the agri-food production chain at the international level. In this comprehensive literature review the authors present and review identification and quantification methods with emphasis on the most important EU foodborne pathogens (*Salmonella, Campylobacter*, *Escherichia coli* – STEC, *Yersinia* and *Listeria).* Methods have been divided into two large groups – Contemporary methods (Immunoassays and Polymerase Chain Reaction) and Novel approach methods (Biosensors, Bacterial typing, and Omics), and assessed by their functionality, advantages and disadvantages.

Key words: *immunoassays; PCR; biosensors; PFGE; WGS; omics; food safety; review*

Introduction

Food is a basic need for all humans, regardless of their place of residence or occupation. Today, modern society is depending on agricultural and food science in particular, to secure safe, quality food every day. In the European Union (EU) alone, approximately 5,000 foodborne outbreak incidents occur *per annum*. Some 40,000 people are involved in these yearly incidents, most commonly referred to as "food poisoning" (EFSA, 2019). Based on the last available EFSA, (2019) report, the most significant foodborne pathogens in the EU during

Tomislav MIKUŠ, DVM, PhD, Assistant Professor, Željka CVRTILA*, (Corresponding author, e-mail: zcvrtila@vef.unizg.hr), BSc, PhD, Full Professor, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia; Lidija KOZAČINSKI, DVM, PhD, Full Professor, Zagreb, Croatia

the last five statistically available years are: *Salmonella*, *Campylobacter*, *Escherichia coli* – STEC, *Yersinia* and *Listeria*. These five listed pathogens during the 2014 – 2018 period are responsible for over 67,000 human infections.

To satisfy the abovementioned food postulate on securing food safety and bringing down the aforementioned numbers of incidents, food science is in constant search of new, faster, more specific microbiological methods for identification and quantification of foodborne pathogens.

In general, food is sampled and microbiologically tested in order to either confirm the presence of pathogen bacteria or their metabolites, define the efficiency of production system and hygienic processing, or both. Traditional cultural and microscopy methods for identification and quantification of viable bacteria have very high success rate and still have been considered the gold standard, but at the same time they are tiresome and labour-intensive. Even putting aside these negativities, the biggest drawback of traditional methods is the slow growth of bacteria, requiring at least 18-24 h to reach first results. Throughout time, with scientific advancement, traditional methods have been upgraded with enrichment procedures, selective agars and fluoroscopy to become more reliable, but these upgrades raised challenges such as cultivation of damaged cells, false positive reactions and too low sensitivity, but time management still remained a major issue (Priyanka et al., 2016; Zeng et al., 2016; Umesha and Manukumar, 2018). Another important issue encountered while using traditional methods is the fact that some food pathogens (*Campylobacter jejuni*, *E. coli*, *L. monocytogenes*, and several *Salmonella* species) may enter a viable but non-culturable physiological state, in which they are alive but cannot be grown outside of their natural habitat (Oliver, 2005; Ayrapetyan and Oliver, 2016).

During the last couple of decades, with the development of molecular methods, there has been a strong step forward in rapid identification and quantification of microorganisms. Nowadays, food science is recognising that it is not enough only to identify the presence of a microorganism or its genes/metabolites, but also to be able to assess if that microorganism is viable, and quantify how much of that microorganism is present in the sample.

A good deal of review papers focused on foodborne pathogens have been published in recent years (Park et al., 2014; Zhao et al., 2014; Law et al., 2015; Jannat et al., 2016; Priyanka et al., 2016; Malvano et al., 2020) but due to the constant risk represented by these pathogens, the authors decided to investigate and present contemporary and novel approaches and potential new practices regarding the identification and quantification of most important foodborne pathogens in the EU.

Data research and search methodology

The authors examined and collected scientific research and review papers from relevant online international scientific databases (Web of Science, Medline, Google Scholar). Keywords and their combinations were used for the paper search methodology, while focusing on the criterium of reviewing novel practices in addressing the identification and quantification of the most relevant foodborne pathogens in the EU. The data collected with the above-mentioned approach resulted in over 250 papers, out of which over 100 manuscripts have been included in this review, which are arranged into two major categories based on the methods reviewed. If needed, the originally collected list of papers is available upon request from the corresponding author.

Methods reviewed

Modern, rapid methods used for the identification and quantification of foodborne pathogens are present in the field of food science since the last few decades of the 20th century

(Park et al., 2014; Priyanka et al., 2016). Based on the collected data, the authors divided reviewed methods into two large groups – *Contemporary methods* (Immunoassays and Polymerase Chain Reaction) and *Novel approach methods* (Biosensors, Bacterial typing, and Omics).

Contemporary methods

Immunoassays

Enzyme-Linked Immunosorbent Assay (ELISA)

The most well-known and used immunoassay method today is the Enzyme-Linked Immunosorbent Assay (ELISA) which was simultaneously developed by two research teams as early as 1971 (Aydin, 2015). Like all other immunological detection methods, ELISA is based on the antigen-antibody reaction. As is well-known, our body produces specific antibodies as a defence mechanism for foreign substances (antigen). Regardless of whether the antigens are peptides, hormones or food pathogens, antibodies are very specific, which is why there is very low risk of interference with other types of antigens. Several different types of ELISA are in use in today's food science (Direct, Indirect, Sandwich and Competitive), but the fundamental principle is the same for each. The solid phase of the reaction, made up of antigen bound to the microplates, binds with specific antibodies which are then fixed so that the reaction can be read. To prevent possible unwanted impedance, it is essential to apply a washing stage after the binding, so that anything except the antigen-antibody complex is removed from the medium. Furthermore, the microplates must not interfere with the other stages of the reaction to avoid false-positive or false-negative results. Special attention should be paid to the stable expression of target antigens in a pathogen, which can be influenced by temperature, preservatives, acids, salts, or other chemicals found in foods so that antibodies can be successfully used to detect pathogens (Aydin, 2015; Mangal et al., 2016). In their review, Mangal et al. (2016) present the fact that Feng, (1997) determined the detection sensitivity of ELISA at around 10^5 colony-forming units (CFU)/mL for whole bacterial cells, and a few ng/mL for toxins or protein analytes.

The most commonly used ELISA method for food pathogens is Sandwich ELISA. This highly sensitive method is widely used for detection of foodborne pathogens in various foods (St Clair and Klenk 1990; Brigmon et al., 1995; Wu et al., 2015; Alexandre et al., 2018). The method is nicknamed "sandwich" because the main reaction of the antigen-antibody interaction is upgraded with a secondary antibody conjugated with an enzyme (most often horseradish peroxidase or alkaline phosphatase), so that it forms an antibody-antigen-conjugate sandwich. After the sandwich binding, the reaction can be read via spectrophotometer or visually. The positive result of the reaction is coloured, while the lack of coloration indicates a negative result. Sandwich ELISAs have been reported to be 2-5 times more sensitive than all other ELISAs (Aydin, 2015).

Lateral Flow Immunoassay

A relatively novel immunoassay method called Lateral Flow Immunoassay has been in development in recent years. As reviewed by Raeisossadati et al. (2016), this method has an extremely wide application range which can also include foodborne pathogens detection. It is a form of immunoassay in which the test sample flows along a solid substrate via capillary action. Antigen (or antibody) is marked with a colour indicator (colloidal latex or gold particles) which reacts with the sample. After 2-10 minutes the reaction is visible as a test line or zone. This method is fast and reliable for detection, and due to the recently developed upgrades to semi-qualitative and qualitative tests, we can expect lateral flow immunoassay to soon take on a much more significant role

in both detecting and quantifying foodborne pathogens (Zhao et al., 2014).

Immunomagnetic Separation Assay (IMS)

Immunomagnetic separation (IMS) is another technique that can be used for the identification and isolation of microbes. This method binds antibodies to magnetic beads that are placed in suspension and allows them to interact with specific antigen cells. After the isolation, microorganisms can be plated for further growth, or as a pre-step of molecular-based detection and enumeration (Gracias and Mckillip, 2004; Zhao et al., 2014).

Polymerase chain reaction

Gene amplification with the purpose of identification and research called Polymerase Chain Reaction (PCR) was discovered in 1983, and upgraded in 1986 by Kary Banks Mullis. Although there are some discussions regarding the credit for the discovery, Mullis received the Nobel Prize for the PCR. Today, PCR is the most commonly used molecular method for the detection of foodborne bacterial pathogens (Law et al., 2015), and some researchers and scientists compare the significance of its discovery to the significance of the World Wide Web (Bartlett and Stirling, 2003; Priyanka et al., 2016). Main advantages of the molecular methods are rapidity, specificity and high sensitivity, while disadvantages include high costs (referring to culture-based or immunoassay) and the inability to distinguish between dead and viable cells. Nowadays, there are a lot of different variations of PCR developed to fit the needs for gene amplification in different fields – from forensics and toxicology to medicine and research. Further in the paper, the authors will give a review of the most important PCR variations with regards to food pathogens.

Polymerase Chain Reaction (PCR) – simple

The simple PCR method is based on the amplification of a specific target DNA sequence in a three-step process: 1) high temperature denaturation of targeted double-stranded DNA into single-stranded DNA; 2) binding of two single-stranded synthetic oligonucleotides (specific primers that are front (F) and reverse (R) primers) to DNA strands; 3) polymerization using deoxyribonucleotides and thermostable DNA polymerase, where complementary single-stranded DNA is amplified. The process cycle is repeated, resulting in the doubling of the initial number of target sequences with each new cycle. Results of the amplification process can be visualized as thinner or thicker lines on an ethidium-bromide-stained electrophoresis gel. When properly implemented and validated, this method is extremely reliable, but it is important to note that in order for PCR to function we need to have a known positive sequence of genes that we seek to confirm in the samples. The most significant advantages of this method are its high specificity (sensitivity), simplicity and speed. Due to the possibility of obtaining amplified products in just 30 minutes, it is faster than immunoassay, but PCR, even in this simple form, still remains a somewhat expensive method (Priyanka et al., 2016).

Multiplex PCR (mPCR)

As an upgrade to simple PCR, mPCR provides faster detection through the simultaneous amplification of multiple gene targets. With this additional ability mPCR is capable of rapidly detecting multiple microorganisms of different or same species in a single reaction (Chen et al., 2012; Ryu et al., 2013). Although the basic methodology of mPCR is very similar to that of simple PCR, the ability to use several sets of specific primers gives it the edge advantage in rapidity. Zhao et al. (2014) points out several important steps of the procedure in order for the mPCR reaction to have a successful outcome. Primers used in the reaction should have similar binding temperature, and their concentration needs to be adjusted in order to generate reliable results of all PCR products. These steps need to be considered due to the

possible interaction between multiple primer sets. Further literature research reveals additional factors for a successful mPCR assay, such as correct concentration of the PCR buffer, balance between the concentration of magnesium chloride and deoxynucleotides, amount of template DNA, Taq DNA polymerase, and the exact temperature cycle (Markoulatos et al., 2002; Cheah et al., 2008; Khoo et al., 2009; Law et al., 2015). Although mPCR was until recently used only to detect two to three pathogens at the same time, literature data today suggest multifunctional possibilities of mPCR. Examples are the simultaneous detection of the same species foodborne Listeria pathogens that were successfully identified by Ryu et al. (2013), and different pathogen species *Salmonella Enteritidis, Staphylococcus aureus, Shigella flexneri, Listeria monocytogenes*, and *Escherichia coli* O157:H7 identified by Chen et al. (2012). With this multifunctionality mPCR enables the possibility to define the structure of certain microbial communities and to evaluate community dynamics, e.g. during fermentation or in response to environmental variations (Zhao et al., 2014; Law et al., 2015).

Gene Expression Profiler PCR (GeXP-PCR)

The next step in the development of mPCR and maybe even more interesting for this review is the new GenomeLab Gene Expression Profiler (GeXP)-PCR which has been presented by ZHOU et al. (2013). GeXP-PCR consists of a genetic analysis system which allows high-throughput and detection of multiple pathogens in a single reaction. The GeXP multiplex PCR amplification involves the use of chimeric primers, universal primers and capillary electrophoretic separation of PCR products instead of agarose gel electrophoresis. The chimeric primers contain a gene-specific sequence with a universal tag at the 5′-end and they are used to produce amplicons with universal tags. Subsequently, the universal primers which contain the same sequence as the universal tags used in the chimeric primers drive the remaining PCR reactions. The forward universal primer is covalently labelled with a fluorescent dye at the 5′-end and it is used for detection during capillary electrophoresis (Zhou et al., 2013). Due to its higher sensitivity, GeXP-PCR was found to be more suitable for high-throughput analysis, while its reported detection limits for the simultaneous detection of *Salmonella, Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Shigella* spp. and *Campylobacter jejuni* were 420, 310, 270, 93, 85, and 66 CFU/mL respectively (Zhao et al., 2014; Law et al., 2015).

Real-time PCR (qPCR)

Real-time PCR has the capability to continuously monitor the formation of PCR product throughout the time of the reaction. It is highly sensitive, specific and enables simultaneous detection of different microorganisms (Priyanka et al., 2016). The method does not require agarose gel electrophoresis, it instead obtains results by measuring the fluorescent signal produced by specific dual-labelled probes or intercalating dyes, while the quantification is based on the analysis of the fluorescent signal at the exponential phase. Reaction intensity is proportional to the amount of PCR amplicons (Omiccioli et al., 2009; Zhao et al., 2014). As reviewed by Law et al. (2015) several fluorescent systems have been developed for qPCR and the most commonly used fluorescent systems are SYBR green and its alternatives – TaqMan probes and molecular beacons. SYBR green is a non-sequence-specific intercalating fluorescent double-stranded DNA (dsDNA)-binding dye. It emits little fluorescence but the fluorescence signal is amplified when bound to the minor groove of the DNA double helix (Fukushima et al., 2003; Levin, 2005; Singh et al., 2009; Law et al., 2015). TaqMan probes are oligonucleotides which contain a fluorophore. Fluorophore acts as reporter dye at the 5′-end, and quenching dye at the 3′-end. It is very important that they are positioned closely together so that they pre-

vent the fluorescence of the emitted reporter (Hein et al., 2001; Levin, 2005). Molecular beacons are oligonucleotides as well as TaqMan probes, but here the reporter and quencher dye are attached to different ends of the probe. The signal is activated after the hybridization (in which spontaneous conformational change occurs, separating the two dyes), and this allows fluorescence of the probe to occur on its complementary nucleotide sequence in the amplicon (Leone et al., 1998; Chen et al., 2000; Liming and Bhagwat, 2004; Levin, 2005; Law et al., 2015). Furthermore, in their review, Law et al. (2015) present different commercial qPCR kits with valuable details regarding their advantages and disadvantages, with special detail on detection limits for various foodborne pathogens originated from different foods. Priyanka et al. (2016) points out that qPCR can generate results in only 30 minutes from the start of the thermal cycling if and when combined with a rapid cycling platform. Considering all advantages listed, it is reasonable to conclude that qPCR nowadays is considered the method of choice for the detection and quantification of microorganisms.

Digital PCR (dPCR)

In their recent technology review, QUAN et al. (2018) present digital PCR (dPCR) as a novel PCR method which can be used for the absolute quantification of target nucleic acids while mitigating the shortcomings of qPCR (Sykes et al., 1992; Kalinina et al., 1997). Although both methods are quantitative, the most important difference is found in the process of measuring the amount of target sequence. In contrast to qPCR, dPCR collects fluorescence signals via end-point measurement and uses the number of positive partitions over the total to back-calculate target concentration. Quantification of dPCR subdues the enumeration of a series of positive and negative outcomes to permuting the signals (continuous or analogue) into a series of binary or digital signals, following the theory that the random distribution of molecules in many partitions follows the Poisson statistical distribution (Dube et al., 2008; Whale et al., 2013). In their conclusion, the authors highlight several advantages of dPCR, such as elasticity to inhibitors and greater precision in quantifying relative abundance of target sequences, but also acknowledge that dPCR currently lacks the sample multiplexing of qPCR, and exhibits lower sensitivity compared to qPCR (Quan et al., 2018).

Novel approaches

Biosensors

Biosensors are a group of novel analytical methods/devices with great detection limits, speed of analysis, relatively low costs and high sensitivity to identify various foodborne risks such as allergens, toxins and microorganisms. They are most often consisted of two (or three) elements. Firstly, some form of a bioreceptor (probe, aptamer, enzyme, antibody) (1), then a transducer for converting the "bioreceptor-target" physico-chemical or biological interaction into a measurable signal (2) and finally an output data source (3). Demand for biosensor methods and technologies is on the rise in all agri-food sectors, as they are able to provide rapid screening used for food safety assurance (Luong et al., 1997; Ahmed et al., 2008; Priyanka, 2016; Malvano et al., 2020). Biosensors can be divided into several groups according to sensing elements or transducers, which play an important role in the detection of pathogens from food (Jannat et al., 2016).

Optical biosensors

Optical biosensor methods are very useful in detecting pathogen microorganism, food hygiene monitoring, and other tasks important in ensuring food safety. The measured output signal of optical-based biosensors is the emission of light, which then enables direct (label-free) detection of foodborne pathogens. When the "bioreceptor-target" complex is immobilized on the transducer surface, the sensors are able

to detect minute changes. Optical diffraction and electrochemiluminescence are standard technologies for optical biosensors (Kovacs, 1998; Jannat et al., 2016). Optical biosensor methods are classified into a large number of sub-categories, but two methods predominantly used for detection of foodborne pathogens are surface plasmon resonance (SPR) and fluorescence-based optical biosensors. The sensitivity and specificity of fiber optic sensors can be enhanced by combining other techniques such as immunomagnetic separation, bacteriophages and chemiluminescent in situ hybridization with these phenomena. Further developments in the technology of optics and microfluidics will enhance miniaturization and wider use of optical biosensors in food safety for real-time monitoring of various food risks during the whole production process (Velusamy et al., 2010; Narsaiah et al., 2012; Priyanka et al., 2016; Malvano et al., 2020).

Electrochemical biosensors

Electrochemical biosensors can be classified as amperometric, potentiometric, or conductometric, depending on the system of analysis. Due to their advantages such as low instrumentation costs, high sensitivity, ease of miniaturization and relatively simple instrumentation they are becoming more and more interesting for the agri-food industry. Unfortunately, the application of electrochemical impedance spectroscopy to a label-free technique is still very poor, but several electrochemical biosensors for the detection of pathogenic bacteria have been developed in the last couple of years (Velusamy et al., 2010; Malvano et al., 2020).

Bacterial typing

Pulsed-field gel electrophoresis (PFGE)

Bacterial typing is the technology used to differentiate different bacterial strains; it is an important tool for outbreak investigation, surveillance, and phylogenetic studies, and PFGE is considered the "gold standard" for bacteria typing (Neoh et al., 2019). Pulsed-field gel electrophoresis includes several steps – enzyme restriction of bacterial DNA, separation of bacterial DNA bands using a pulsed-field electrophoresis chamber which is then followed by clonal assignment of bacteria based on PFGE banding patterns. Similar to PCR, a vast number of PFGE protocols have been developed for typing various bacteria, which includes foodborne pathogens.

Due to its possibility to track and subtype zoonic foodborne bacteria it can be internationally validated and standardized, which makes PFGE the leading and probably most widely used method for phylogenetic studies, especially in food safety surveillance. Still, the method is lengthy and laborious when compared to some novel PCR methods and whole-genome sequencing (WGS), but it will probably remain an affordable and relevant technique for small laboratories in years to come (Goering, 2010; Carleton and Gerner-Smidt, 2016; Nyman et al., 2013; Ribot and Hise, 2016; Neoh et al., 2019).

Whole-Genome Sequencing (WGS)

In an effort to bring outbreaks under control as soon as possible and find out the causes, public health institutes have at their disposal new techniques for proving the causes of foodborne infections and intoxications. One of them is Whole-genome sequencing (WGS). Next-generation sequencing (NGS) technologies make WGS a powerful tool for determining the relatedness of bacterial isolates in foodborne illness detection and outbreak investigations. WGS has been applied to national outbreaks but has rarely been used in smaller local outbreaks (Oakeson et al., 2018). Some authors consider that although whole-genome sequencing has been found to improve discrimination of outbreak clusters, is not certain whether it can be applied in real time in public health laboratories (Den Bakker et al., 2014). The major benefit of WGS is to analyze data without the need of further tradition-

al confirmatory tests, including serotyping or PCR sequencing (McDermott et al. 2016). WGS methods are a new approach to pathogen monitoring. WGS can be an important part of a system of pathogen control networks in food products internationally. The assumption is to establish an international database that would connect systems for detecting pathogen strains in the form of a comprehensive network of sequencers. When it comes to the benefits of WGS in public health, the next step is direct application in food for the most important causes of epidemics (Allard et al., 2016), while the connection of the existing WGS network with other international networks should be considered in order to strengthen the role of health care institutions. Despite some remaining limitations, comprehensive information provided by WGS will greatly enhance the monitoring of antimicrobial-resistant strain types and genes circulating in humans, foods, animals, and environments. In addition, genomic data will bolster the efforts to understand the sources of infection, identify and characterize outbreaks, and better understand the consequences of antibiotic use, according to McDermott et al. (2016).

Omics

Food-transmitted bacterial pathogens are susceptible to stress, sublethal injuries and often, depending on the change in their virulence, react differently in the environment and especially when ingested into the human body. This will depend on the type of food and performance criteria during its production. Therefore, the state of the pathogen under different conditions is important for understanding the bacterial response to stress in food. The physiological state of pathogens in such conditions can be indicated by modulation of gene and protein expression, and the application of omics disciplines such as genomics, transcription, proteomics and metabolomics in this context offers significant potential to improve the understanding of stress responses and virulence of pathogenic bacteria (Wesche et al., 2009; Yoshida et al., 2001; Greppi and Rantsiou, 2016). Transcriptome refers to the total RNA in a cell or organism. In a bacterial transcriptome, there are classical mRNAs, a large number of cis-antisense RNAs, non-coding RNAs, over-lapping transcripts and RNA elements that regulate transcription such as riboswitches (Stazic and Voß, 2015). The role of non-coding RNAs in the regulation of gene expression, especially genes associated with adaptation and virulence, appears to be very important (Mellin and Cossart, 2012; Cossart et al, 2014). The importance of transcriptomics lies in the potential to link specific changes in gene expression to the phenotype of interest. Transcription of DNA into RNA is the first step in controlling expression that will ultimately affect the phenotypic characteristics (Yoshida et al., 2001). Metabolomics focuses on the analysis of cell metabolites (Villas-Bôas et al., 2005). Metabolomics can be applied to study stress responses and growth patterns of pathogens in food. Furthermore, metabolomics has been proposed as an analytical approach to detect pathogen (Greppi and Rantsiou, 2016).

Proteomics allows the characterization of the functional significance of proteins. The barriers it encounters are related to protein abundance and proteome complexity. Proteins with low abundance are difficult to detect; proteins rarely act alone but rather in interaction with other proteins, which is crucial for cellular activity (Betzen et al., 2015). However, the interactions that could occur between food proteins and protein of microorganisms should be equally considered (Greppi and Rantsiou, 2016).

Proteomics and metabolomics are considered to be useful tools in the analysis of raw materials and changes in molecular profiles during food production (Herrero et al., 2012), so analytical procedures can be used in detecting chemical as well as biological contamination (Aung and Chang, 2014). The methods are applicable in food microbiology and the detec-

tion of food-borne microorganisms and their toxins, even at low analyte levels (Martinović et al., 2015).

Discussion

Major interest in the development of rapid microbiological methods emerged in the mid-1960s.Initiating in human microbiology, it was embraced in the 1970s, continued into the 1980s and 1990s, and remains strong in the present day. On the other hand, interest in the development of rapid microbiological methods in food microbiology has been historically somewhat slower, but today it is just as important as in human microbiology (Fung, 2002; Straub et al., 2005; Hein et al., 2006; Patel et al., 2006). The time period from 1965 to 1975 is the period of "kit" development, primarily for microbiological diagnostics. From 1975 to 1985 came the years of immunological tests development, and the decade from 1985 to 1995 was the period of revolutionary development of molecular microbiological methods (genetic testing, molecular testing and PCR). It is culture-based diagnostics that has been developed intensively in terms of the application of immunological (ELISA) and molecular methods (PCR), with the aim of establishing rapid, sensitive, specific and cost-effective new methods. This goal is still common, whether we are referring to cultivation methods or futuristic biosensor methods (Priyanka et al., 2016).

The very definition of the "fast" method is not precisely explained, but it implies any microbiological method that gives faster results than classical, culture-based procedures. All these methods have their advantages and disadvantages. Thus, after the use of immunomagnetic separation, ELISA, nucleic acid methods and polymerase chain reaction, membrane filtration, ATP bioluminescence techniques, a major part of methodology research has focused on the use of different combinations of microbiological methods.

The development of methods has progressed rapidly in this century, and continues today with the implementation of computer micro-chip technology and biosensors. Research on the evidence of pathogenic microorganisms at the molecular level is deepening in terms of finding faster and more reliable methods that will quantify the number of bacteria and identify specific bacterial species (*Listeria* spp., *Campylobacter* spp., *Salmonella* spp., *E. coli*, *S. aureus*) in contaminated food (Fung, 2002; Umesha and Manukumar, 2018). Rapid identification of foodborne pathogens is still a challenge both in the basic science and in the whole agri-food sector. Therefore, rapid evidence regarding the presence and number of pathogens in food is still a challenge and of paramount importance. Traditional plate counting methods are time consuming procedures, requiring several working days to be completed. Their advantage is that only the cells which can be readily cultivated in vitro recover. Along with the dead or damaged bacteria, the presence of viable but non-culturable (VBNC) cells, that can occur as an adaptation to environmental stress, may lead to an underestimation of the actual numbers of foodborne pathogens in the investigated samples (Thomas et al., 2002).

Many studies have confirmed that numerous contemporary and novel approach methods can detect and identify foodborne pathogens when present in low numbers. Hudson et al. (2001) observed the growth of *L. monocytogenes* in a semi-durable meat product by a combination of IMS-PCR methods, detected 1 cfu/25 g of sample and concluded that the use of the IMS method increased the sensitivity of the PCR method, having obtained a result within 24 hours for the meat industry. Also, using a combination of the same procedures, *Salmonella* spp. was detected in minced meat in 24 hours. Samples with different numbers of bacteria and accompanying microflora were examined, and it was concluded that the IMS method makes it far more difficult to isolate a small number of target bacteria (1-3 cfu/g) in

the presence of a large number of accompanying microflora (104-105 cfu/g) and a large percentage of fat in the sample (meat), and thus reduces the sensitivity of the isolation process. In foods that do not contain a lot of fat or a large total number of bacteria, compared to the classical microbiological method, the IMS-PCR method gives fast and satisfactory results in the detection of *Salmonella* spp. (Jenikova et al., 2000; Hsih and Tsen, 2001). *E. coli* O157: H7 can be detected with immunoassay methods (ELISA) with a detection limit of 68 CFU / mL in phosphate buffer (PBS) to 6.8 x 10^3 CFU/mL in food samples – milk, vegetables and ground beef (Shen et al., 2014). Lateral Flow Immunoassay detects *Salmonella typhi* in food (meat, chicken, milk) with a limit of 10^4 - 10^5 CFU/mL (Kumar et al., 2008), while Shukla et al. (2014) established a detection limit of 30 cells / 25 g. Different nucleic acid based methods for pathogen detection like Multiplex PCR detect *Salmonella* spp. with a limit of 10^3 CFU/mL (Silva et al., 2011), as well as for *E. coli* O157: H7 and *L. monocytogenes* (Guan et al., 2013). Real time PCR can detect *L. monocytogenes* with a limit of <18 CFU/10 g (Suo et al., 2010) and 2 x 10^2 CFU/mL (Kawasaki et al., 2010), *Salmonella* spp. 5 CFU/25 g (Ruiz-Rueda et al, 2011) and *S. aureus* 9.6 CFU/g (MA et al., 2014). Pulsed-field gel electrophoresis (PFGE) has obtained its status as the gold standard for status as the gold standard for outbreak tracking and molecular subtyping of zoonotic foodborne bacteria such as *Salmonella enterica*, *Campylobacter* species, *E. coli, Shigella, Vibrio cholerae*, and *L. monocytogenes* (Swaminathan et al., 2001; Boxrud et al., 2010; Carleton and Gerner-Smidt, 2016).

In recent years scientists have changed their approach, turning towards increasingly sensitive methods capable of detecting pathogens at the level of the products of their metabolism, developing novel approach, advanced, more sensitive methods of detecting pathogens in which MS-based techniques play a crucial role. Foodomics is increasingly mentioned in the literature as a new discipline for research of food and food-related risks using omics technologies that require the MS technique. Applications of Foodomics include genomic, transcriptomic, proteomic, and/or metabolomic studies of foods which may be associated with food safety, detection of food contaminants, etc. Detection of pathogens and their toxins in food contaminated with microorganisms is important in food safety assessment, and GC-MS is used in this regard for the profiling of food product metabolites to determine volatile compounds associated with well-defined microbial activity. Using proteomics tools, MS is used to directly identify food-contaminating microorganisms (Herrero et al., 2012).

Despite all scientific and technological efforts to prevent food contamination, reports related to foodborne infections and intoxications tell us that pathogenic bacteria continue to cause diseases (EFSA, 2019). One of the reasons for this is the globalization in the agri-food chain and the trends of consuming food that is minimally processed, ready-to-eat and has a prolonged shelf life. This globalization presents a new challenge in food production technology, but at the same time it will furthermore drive the switch to standardization of fast and reliable, modern analytical methods for the detection and quantification of pathogens in food. On the other hand, the development of these same methods is important for reaching the fastest possible proof of the causes of epidemic outbreaks and monitoring the movement of diseases related to a particular type of food. Analysis of pathogens by new methods such as PFGE allows the detection of disease clusters and helps to confirm pathogens. By standardizing the method, results of tests from different laboratories can be compared, while additional technologies such as WGS both reduce costs, time of proof of the causative agent and are useful to public health laboratories in determining virulence and characterization of bacteria (Oakeson et al., 2018). On the basis of the above stated, it is necessary to realize that in order to satisfy both regulatory references

and requirements by industries and consumers, the scientific community will soon lead to a new era in the advancement in analytical methods and development of novel devices for a sensitive detection and quantification of biological risks (Malvano et al., 2020).

Conclusions

Due to their multipurpose possibilities (species-wise), high specificity, rapidity and sensitivity, all of the presented methods can be used for detection and quantification of major foodborne pathogen species (*Salmonella*, *Campylobacter*, *Escherichia* coli – STEC, *Yersinia* and *Listeria*), keeping in mind the specificities of the food sample. Novel approach methods, from biosensors, to bacterial typing and omics are becoming increasingly applicable for rapid *in vivo* surveillance in the whole agri-food production chain due to their ability to produce relevant results in a fraction of time needed for culture-based "gold standard" methods.

Thus, in the near future, the global food safety system will need to conduct the standardization and validation of the novel approach methods which will then become the new gold standard for the 21st century. These standards will represent a significant leap forward, but at the same time a great challenge to the overall organisation of public health and food safety. Nevertheless, once established, the new system will significantly affect the rapidity of the epidemiological response and assessment regarding microbiological foodborne risks and upgrade the control of the entire agri-food chain.

References

1. AHMED, M. U., M. M. HOSSAIN and E. E. TAMIYA (2008): Electrochemical Biosensors for Medical and Food Applications. Electroanal. 20, 616-626.
2. ALEXANDRE, D. L., A. M. A. MELO, R. F. FURTADO, M. F. BORGES, E. A. T. FIGUEIREDO, A. BISWAS, H. N. CHENG and C. R. ALVES (2018): A Rapid and Specific Biosensor for Salmonella Typhimurium Detection in Milk. Food Bioproc Tech. 11, 748-756. 10.1007/s11947-017-2051-2058.
3. ALLARD, M. W., E. STRAIN, D. MELKA, K. BUNNING, S. M. MUSSER, E. W. BROWN and R. TIMME (2016): Practical value of food pathogen traceability through building a whole-genome sequencing network and database. J. Clin. Microbiol. 54, 1975-1983.
4. AUNG, M. M. and Y. S. CHANG (2014): Traceability in a food supply chain: Safety and quality perspectives. Food Control 39, 172-184.
5. AYDIN, S. (2015): A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. Peptides. 72, 4-15 10.1016/j.peptides.2015.04.012
6. AYRAPETYAN, M. and J. D. OLIVER (2016): The Viable but Non-Culturable State and Its Relevance in Food Safety. Curr. Opin. Food Sci. 8, 127-133. 10.1016/j.cofs.2016.04.010.
7. BARTLETT, J. M. S. and D. STIRLING (2003): A short history of the polymerase chain reaction. Methods Mol. Biol. 226, 3-6. 10.1385/1-59259-384-4:3.
8. BETZEN, C., M. S. S. ALHAMDANI, S. LUEONG, C. SCHRODER, A. STANG and J. D. HOHEISEL (2015): Clinical proteomics: promises, challenges and limitations of affinity arrays. Proteomics Clin. Appl. 9, 342-347.
9. BOTTELDOORN, N., E. VAN COILLIE, V. PIESSENS, G. RASSCHAERT, L. DEBRUYNE, M. HEYNDRICKX, L. HERMAN and W. MESSENS (2008): Quantification of Campylobacter spp. in chicken carcass rinse by real-time PCR. J. Appl. Microbiol. 105, 1909-1918. 10.1111/j.1365-2672.2008.03943.x
10. BOXRUD, D., T. MONSON, T. STILES and J. BESSER (2010): The role, challenges, and support of pulsenet laboratories in detecting foodborne disease outbreaks. Public Health Rep. 125, (Suppl 2) 57-62.
11. BRIGMON, R. L., S. G. ZAM and H. R. WILSON (1995): Detection of Salmonella Enteritidis in Eggs and Chicken with Enzyme-Linked Immunosorbent Assay. Poult. Sci. 74, 1232-1236. 10.3382/ps.0741232.
12. CANGELOSI, G. A. and J. S. MESCHKE (2014): Dead or alive: molecular assessment of microbial viability. Appl. Environ. Microbiol. 80, 5884-5891. 10.1128/AEM.01763-14
13. CARLETON, H. A. and P. GERNER-SMIDT (2016): Whole-genome sequencing is taking over foodborne disease surveillance. Microbe 11, 311-317.
14. COSSART, P. and A. LEBRETON (2014): A trip in the ''New Microbiology'' with the bacterial pathogen Listeria monocytogenes. FEBS Lett. 588, 2437-2445.
15. CHEAH, Y. K., A. S. NOORZALEHA, L. H. LEE, S. RADU, S. SUKARDI and J. H. SIM (2008): Comparison of PCR fingerprinting techniques for the discrimination of *Salmonella enterica* subsp. *enterica* serovar Weltevreden isolated from indigenous vegetables in Malaysia. World J. Microbiol. Biotechnol. 24, 327-335. 10.1007/s11274-007-9474-8
16. CHEN, W., G. MARTINEZ and A. MULCHANDANI (2000): Molecular beacons: a real-time polymerase

chain reaction assay for detecting *Salmonella*. Anal. Biochem. 280, 166-172. 10.1006/abio.2000.4518
17. CHEN, J., J. TANG, J. LIU, Z. CAI and X. BAI (2012): Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. J. Appl. Microbiol. 112, 823-830. 10.1111/j.1365-2672.2012.05240.x
18. DEBRETSION, A., T. HABTEMARIAM, S. WILSON, D. NGANWA and T. YEHUALAESHET (2007): Real-time PCR assay for rapid detection and quantification of Campylobacter jejuni on chicken rinses from poultry processing plant. Mol. Cell. Probes 21, 177-181. 10.1016/j.mcp.2006.10.006
19. DEN BAKKER, H. C., M. W. ALLARD, D. BOPP, et al. (2014): Rapid whole-genome sequencing for surveillance of Salmonella enterica Serovar Enteritidis. Emerg. Infect. Dis. 8, 1306-1314.
20. DUBE, S., J. QIN and R. RAMAKRISHNAN (2008): Mathematical analysis of copy number variation in a DNA sample using digital PCR on a nanofluidic device. PLoS ONE 3, e2876.
21. EUROPEAN FOOD SAFETY AUTHORITY - EFSA (2019): Plain Language Summary The European Union One Health 2018 Zoonoses Report. EFSA Journal 17, 1-2. 10.2903/j.efsa.2019.5926.
22. FENG, P. (1997): Impact of molecular biology on the detection of foodborne pathogens. Mol. Biotechnol. 7, 267-278
23. FUKUSHIMA, H., Y. TSUNOMORI and R. SEKI (2003): Duplex real-time SYBR green PCR assays for detection of 17 species of food or water borne pathogens in stools. J. Clin. Microbiol. 41, 5134-5146. 10.1128/JCM.41.11.5134-5146.2003
24. FUNG, D. Y. C. (2002): Rapid methods and automation in microbiology. Compr. Rev. Food Sci. Food Saf. 1, 3-22.
25. GOERING, R. V. (2010): Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. Infect. Genet. Evol. 10, 866-875.
26. GRACIAS, K. S. and J. L. MCKILLIP (2004): A review of conventional detection and enumeration methods for pathogenic bacteria in food. Can. J. Microbiol. 50, 883-890.
27. GREPPI, A. and K. RANTSIOU (2016): Methodological advancements in foodborne pathogen determination: From presence to behavior. Curr. Opin. Food Sci. 8, 80-88. 10.1016/j.cofs.2016.04.005
28. GUAN, Z. P., Y. JIANG, F. GAO, L. ZHANG, G. H. ZHOU and Z. J. GUAN (2013): Rapid and simultaneous analysis of five foodborne pathogenic bacteria using multiplex PCR. Eur. Food Res. Technol. 237, 627-637.
29. HARBOTTLE, H. (2018): "Advanced Methods for Detection of Foodborne Pathogens" in Advanced Techniques in Diagnostic Microbiology: Volume 2 Applications. Third Edition, eds. Y-W. Tang, C. W. Stratton (Springer eBook), 219-239. 10.1007/978-3-319-95111-9
30. HEIN, I., A. LEHNER, P. RIECK, K. KLEIN, E. BRANDL and M. WAGNER (2001): Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. Appl. Environ. Microbiol. 67, 3122-3126. 10.1128/AEM.67.7.3122-3126.2001
31. HEIN, I., G. FLEKNA, M. KRASSNIG and M. WAGNER (2006): Real-time PCR for the detection of Salmonella spp. in food: An alternative approach to a conventional PCR system suggested by the FOOD-PCR project. J. Food Microbiol. Methods 66, 538-547.
32. HERRERO, M., C. SIMÓ, V. GARCÍACAÑAS, E. IBÁÑEZ andA. CIFUENTES (2012): Foodomics: MS-based strategies in modern food science and nutrition. Mass Spectrom. Rev. 31, 49-69.
33. HSIH, H. Y. and H. Y. TSEN (2001): Combination of Immunomagnetic separation and polymerase chain reaction for the simultaneous detection of Listeria monocytogenes and Salmonella spp. in food samples. J. Food Prot. 64, 1744-1750.
34. HUDSON, J. A., R. J. LAKE, M. G. SAVILL, P. SCHOLES and R. E. MCCORMICK (2001): Rapid detection of Listeria monocytogenes in ham samples using immunomagnetic separation followed by polymerase chain reaction. J. Appl. Microbiol. 90, 614-621.
35. HUGGETT, J. F., S. COWEN and C. A. FOY (2015): Considerations for digital PCR as an accurate molecular diagnostic tool. Clin. Chem. 61, 79-88. 10.1373/clinchem.2014.221366
36. IRWIN, P. L., L. H. T. NGUYEN and C. Y. CHEN (2010): The relationship between purely stochastic sampling error and the number of technical replicates used to estimate concentration at an extreme dilution. Anal. Bioanal. Chem. 398, 895-903. 10.1007/s00216-010-3967-2
37. IVANOVA, M., R. SINGH, M. DHARMASENA, C. GONG, A. KRASTANOV and X. JIANG (2014): Rapid identification of Campylobacter jejuni from poultry carcasses and slaughtering environment samples by real-time PCR. Poultry Sci. 93, 1587-1597. 10.3382/ps.2013-03736
38. JANNAT, Y., R. A. MOHAMMED and C. BYOUNG-KWAN (2016): Biosensors and their applications in food safety: a review. J. Biosystems Eng. 41, 240-254.
39. JENIKOVA, G., J. PAZLAROVA and K. DEMNEROVA (2000): Detection of Salmonella in food samples by the combination of immunomagnetic separation and PCR assay. Int. Microbiol. 3, 225-229.
40. JOSEFSEN, M. H., C. LÖFSTRÖM, T. B. HANSEN, L. S. CHRISTENSEN, J. E. OLSEN and J. HOORFAR (2010): Rapid quantification of viable Campylobacter bacteria on chicken carcasses, using real-time PCR and propidium monoazide treatment, as a tool for quantitative risk assessment. Appl. Environ. Microbiol. 76, 5097-5104. 10.1128/AEM.00411-10
41. JOSIĆ, D., Ž. PERŠURIĆ, D. REŠETAR, T. MARTINOVIĆ, L. SAFTIĆ, L. KRALJEVIĆ and S. PAVELIĆ (2017): Use of Foodomics for Control of

Food Processing and Assessing of Food Safety Adv. Food Nutr. Res. 187-229. 10.1016/bs.afnr.2016.12.001
42. KALININA, O., I. LEBEDEVA, J. BROWN and J. SILVER (1997): Nanoliter Scale PCR with TaqMan Detection. Nucleic Acids Res. 25, 1999-2004. 10.1093/nar/25.10.1999
43. KAWASAKI, S., P. M. FRATAMICO, N. HORIKOSHI, Y. OKADA, K. TAKESHITA, T. SAMESHIMA and S. KAWAMOTO (2010): Multiplex real-time polymerase chain reaction assay for simultaneous detection and quantification of Salmonella species, Listeria monocytogenes, and Escherichia coli O157: H7 in ground pork samples. Foodborne Pathog. Dis. 7, 549-554.
44. KHOO, C. H., Y. K. CHEAH, L. H. LEE, J. H. SIM, A. S. NOORZALEHA, M. S. SIDIK, S. RADU and S. SUKARDI (2009): Virulotyping of *Salmonella enterica* subsp. *enterica* isolated from indigenous vegetable s and poultry meat in Malaysia using multiplex-PCR. Antonie Van Leeuwenhoek 96, 441-457. 10.1007/s10482-009-9358-z
45. KÖSEOGLU, V. K., C. HEISS, P. AZADI, E. TOPCHIY, Z. T. GUVENER, T. E. LEHMANN, K. W. MILLER and M. GOMELSKY (2015): Listeria monocytogenes exopolysaccharide: origin, structure, biosynthetic machinery and c-di-GMP-dependent regulation. Mol. Microbiol. 96, 728-743.
46. KOVACS, G. (1998): Micromachined Transducers: Sourcebook. WCB/McGraw Hill, Inc.
47. KUMAR, S., K. BALAKRISHNA and H. V. BATRA (2008): Enrichment-ELISA for detection of Salmonella typhi from food and water samples. Biomed. Environ. Sci. 21, 137-143.
48. LAW, J. W., N. S. AB MUTALIB, K. G. CHAN and L. H. LEE (2015): Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. Front Microbiol. 5, 770.
49. LEONE, G., H. VAN SCHIJNDEL, B. VAN GEMEN, F. R. KRAMER and C. D. SCHOEN (1998): Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. Nucleic Acids Res. 26, 2150-2155. 10.1093/nar/26.9.2150
50. LEVIN, R. E. (2005): The application of real-time PCR to food and agricultural systems. A review. Food. Biotechnol. 18, 97-133. 10.1081/FBT-120030386
51. LIMING, S. H. and A. A. BHAGWAT (2004): Application of a molecular beacon-real-time PCR technology to detect *Salmonella* species contaminating fruits and vegetables. Int. J. Food Microbiol. 95, 177-187. 10.1016/j.ijfoodmicro.2004.02.013
52. LUONG, J. H., P. BOUVRETTE and K. B. MALE (1997): Developments and applications of biosensors in food analysis. Trends Biotechnol. 15, 369-377. doi:10.1016/
53. MA, K., Y. DENG, Y. BAI, D. XU, E. CHEN, H. WU and L. GAO (2014): Rapid and simultaneous detection of *Salmonella*, *Shigella*, and *Staphylococcus aureus* in fresh pork using a multiplex real-time PCR assay based on immunomagnetic separation. Food Control. 42, 87-93.
54. MALVANO, F., R. PILLOTON and D. ALBANESE (2020): Label-free impedimetric biosensors for the control of food safety–a review. J. Environ. Anal. Chem. 100, 468-491.
55. MANGAL, M., S. BANSAL, S. K. SHARMA and R. K. GUPTA (2016): Molecular Detection of Foodborne Pathogens: A Rapid and Accurate Answer to Food Safety. Crit. Rev. Food Sci. Nutr. 56, 1568-1584. 10.1080/10408398.2013.782483.
56. MARKOULATOS, P., N. SIAFAKAS and M. MONCANY (2002): Multiplex polymerase chain reaction: a practice alapproach. J. Clin..Lab..Anal. 16, 47-51. 10.1002/jcla.2058
57. MARTINOVIĆ, T., U. ANDJELKOVIĆ, M. ŠRAJER GAJDOŠIK, D. REŠETAR and DJ. JOSIĆ (2016): Foodborne pathogens and their toxins. J. Proteomics 147, 226-235.
58. MCDERMOTT, P. F., G. H. TYSON, C. KABERA, Y. CHEN, C. LI, J. P. FOLSTER, S. A. AYERS, C. LAM, H. P. TATE and S. ZHAO (2016): Whole-genome sequencing for detecting antimicrobial resistance in nontyphoidal Salmonella. Antimicrob. Agents Chemother. 60, 5515-5520.
59. MELERO, B., L. COCOLIN, K. RANTSIOU, I. JAIME and J. ROVIRA (2011): Comparison between conventional and qPCR methods for enumerating Campylobacter jejuni in a poultry processing plant. Food Microbiol. 28, 1353-1358. 10.1016/j.fm.2011.06.006
60. MELLIN, J. R. and P. COSSART (2012): The non-coding RNA world of the bacterial pathogen Listeria monocytogenes. RNA Biol. 9, 372-378.
61. MIYAMOTO, K. N., K. M. MONTEIRO, K. DA SILVA CAUMO, K. R. LORENZATTO, FERREIRA and A. BRANDELLI (2015): Comparative proteomic analysis of Listeria monocytogenes ATCC 7644 exposed to a sublethal concentration of nisin. J. Proteomics 119, 230-237.
62. NARSAIAH, K., N. J. SHYAM, B. RISHI, S. RAJIV and K. RAMESH (2012): Optical biosensors for food quality and safety assurance—a review. J. Food Sci. Technol. 49, 383-406 10.1007/s13197-011-0437-6
63. NEOH, H. M., X. E. TAN, H. F. SAPRI and T. L. TAN (2019): Pulsed-field gel electrophoresis (PFGE): A review of the "gold standard" for bacteria typing and current alternatives. Infect. Genet. Evol. 74, 103935. 10.1016/j.meegid.2019.103935
64. NYMAN, A. K., E. C. ÅGREN, K. BERGSTRÖM and H. WAHLSTRÖM (2013): Evaluation of the specificity of three enzyme linked immunosorbent assays for detection of antibodies against Salmonella in bovine bulk milk. Acta Vet. Scand. 55, 5.
65. OAKESON, K. F., J. M. WAGNER, A. ROHRWASSER, and R. ATKINSON-DUNN (2018): Whole-genome sequencing and bioinformatic analysis of isolates from foodborne illness outbreaks of Campylobacter jejuni and Salmonella enterica. J. Clin. Microbiol. 56, e00161-18. 10.1128/JCM.00161-18

66. OLIVER, J. D. (2005): The viable but nonculturable state in bacteria. J. Microbiol. 43, Spec No: 93-100.
67. OMICCIOLI, E., G. AMAGLIANI, G. BRANDI and M. MAGNANI (2009): A new platform for real-time PCR detection of Salmonella spp., Listeria monocytogenes and Escherichia coli O157 in milk. Food Microbiol. 26, 615-622. 10.1016/j.fm.2009.04.008
68. PAPIĆ, B., M. PATE, U. HENIGMAN, U. ZAJC, I. GRUNTAR, M. BIASIZZO, M. OCEPEK and D. KUŠAR (2017): New Approaches on Quantification of Campylobacter jejuni in Poultry Samples: The Use of Digital PCR and Real-time PCR against the ISO Standard Plate Count Method. Front. Microbiol. 8, 331. 10.3389/fmicb.2017.00331
69. PARK, S. H., M. AYDIN, A. KHATIWARA, M. C. DOLAN, D. F. GILMORE, J. L. BOULDIN, S. AHN and S. C. RICKE (2014): Current and emerging technologies for rapid detection and characterization of Salmonella in poultry and poultry products. Food Microbiol. 38, 250-262.
70. PATEL, J. R., A. A. BHAGWAT, G. C. SANGLAY and M. B. SOLOMON (2006): Rapid detection of Salmonella from hydrodynamic pressure-treated poultry using molecular beacon real-time PCR. Food Microbiol. 23, 39-46.
71. PRIYANKA, B., R. K. PATIL and S. DWARAKANATH (2016): A review on detection methods used for foodborne pathogens. Indian J. Med. Res. 144, 327-338. 10.4103/0971-5916.198677.
72. QUAN, P. L., M. SAUZADE and E. BROUZES (2018): dPCR: A Technology Review. Sensors. 18, 1271. 10.3390/s18041271
73. RAEISOSSADATI, M. J., M. D. NOOR, B. FAZLOLLAH, G. MEHRDAD, R. MOHAMMAD, A. KHALIL and M. T. SEYED (2016): Lateral flow based immunobiosensors for detection of food contaminants. Biosens. Bioelectron. 86, 235-246. 10.1016/j.bios.2016.06.061.
74. RENIER, S., I. CHAFSEY, C. CHAMBON, N. CACCIA, A. CHARBIT, M. HE´BRAUD and M. DESVAUX (2015): Contribution of the multiple Type I signal peptidases to the secretome of Listeria monocytogenes: Deciphering their specificity for secreted exoproteins by exoproteomic analysis. J. Proteomics 117, 95-105.
75. RIBEIRO, V. B., S. MUJAHID, R. H. ORSI, T. M. BERGHOLZ, M. WIEDMANN, K. J. BOOR and M. T. DESTRO (2014): Contributions of sB and PrfA to Listeria monocytogenes salt stress under food relevant conditions. Int. J. Food Microbiol. 177, 98-108.
76. RIBOT, E. M. and K. B. HISE (2016): Future challenges for tracking foodborne diseases: PulseNet, a 20-yearold US surveillance system for foodborne diseases, is expanding both globally and technologically. EMBO Rep. 17, 1499-505.
77. RUDI, K., H. K. HØIDAL, T. KATLA, B. K. JOHANSEN, J. NORDAL and K. S. JAKOBSEN (2004): Direct real-time PCR quantification of Campylobacter jejuni in chicken fecal and cecal samples by integrated cell concentration and DNA purification. Appl. Environ. Microbiol. 70, 790–797. 10.1128/AEM.70.2.790-797.2004
78. RUDI, K., B. MOEN, S. M. DRØMTORP and A. L. HOLCK (2005): Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl. Environ. Microbiol. 71, 1018-1024. 10.1128/AEM.71.2.1018-1024.2005
79. RUIZ-RUEDA, O., M. SOLER, L. CALVÓ and J. L. GARCÍA-GIL (2011): Multiplex real-time PCR for the simultaneous detection of Salmonella spp. and Listeria monocytogenes in food samples. Food Anal. Methods. 4, 131-138.
80. RYCHLI, K., T. GRUNERT, L. CIOLACU, A. ZAISER, E. RAZZAZI-FAZELI, S. SCHMITZ-ESSER, M. EHLING-SCHULZ and M. WAGNER (2016): Exoproteome analysis reveals higher abundance of proteins linked to alkaline stress in persistent Listeria monocytogenes strains. Int. J. Food Microbiol. 218, 17-26.
81. RYU, J., S. H. PARK, Y. S. YEOM, A. SHRIVASTAV, S. H. LEE, Y. R. KIM and H.-Y. KIM (2013): Simultaneous detection of Listeria species isolated from meat processed foods using multiplex PCR. Food Cont. 32, 659-664. 10.1016/j.foodcont.2013.01.048
82. SCHNIDER, A., G. OVERESCH, B. M. KORCZAK and P. KUHNERT (2010): Comparison of real-time PCR assays for detection, quantification, and differentiation of Campylobacter jejuni and Campylobacter coli in broiler neck skin samples. J. Food Protoc. 73, 1057-1063. 10.4315/0362-028X-73.6.1057
83. SHEA, S., K. A. KUBOTA, H. MAGUIRE, S. GLADBACH, A. WORON, R. ATKINSON-DUNN, M. R. COUTURIER, M. B. MILLER and P. H. GILLIGAN (2017): Clinical microbiology laboratories' adoption of culture independent diagnostic tests is a threat to foodborne-disease surveillance in the United States. J. Clin. Microbiol. 55, 10-19.
84. SHEN, Z., N. HOU, M. JIN, Z. QIU, J. WANG, B. ZHANG, X. WANG, J. WANG, D. ZHOU and J. LI (2014): A novel enzyme-linked immunosorbent assay for detection of Escherichia coli O157: H7 using immunomagnetic and beacon gold nanoparticles. Gut Pathogens 6, 14.
85. SHUKLA, S., H. LEEM, J. S. LEE and M. KIM (2014): Immunochromatographic strip assay for the rapid and sensitive detection of Salmonella Typhimurium in artificially contaminated tomato samples. Can. J. Microbiol. 60, 399-406.
86. SILVA, D. S. P., T. CANATO, M. MAGNANI, J. ALVES, E. Y. HIROOKA and T. C. R. M. DE OLIVEIRA (2011): Multiplex PCR for the simultaneous detection of Salmonella spp. and Salmonella Enteritidis in food. Int. J. Food Sci. Technol. 46, 1502-1507.
87. SINGH, J., V. K. BATISH and S. GROVER (2009): A molecular beacon-based duplex real-time

polymerase chain reaction assay for simultaneous detection of Escherichia coli O157:H7 and Listeria monocytogenes in milk and milk products. Foodborne Pathog. Dis. 6, 1195-1201. 10.1089/fpd.2009.0310

88. ST CLAIR, V. J. and M. M. KLENK (1990): Performance of three methods for the rapid identification of Salmonella in naturally contaminated foods and feeds. J. Food. Prot. 53, 961-964. 10.4315/0362-028X-53.11.961.
89. STAZIC, D. and B. VOß (2015): The complexity of bacterial transcriptomes. J. Biotechnol. 232, 69-78. 10.1016/j.jbiotec.2015.09.041
90. SUO, B., Y. HE, G. PAOLI, A. GEHRING, S. I. TU and X. SHI (2010): Development of an oligonucleotide-based microarray to detect multiple foodborne pathogens. Mol. Cell. Probes. 24, 77-86.
91. STRAIN, M. C., S. M. LADA, T. LUONG, S. E. ROUGHT, S. GIANELLA, V. H. TERRY, C. A. SPINA, C. H. WOELK and D. D. RICHMAN (2013): Highly precise measurement of HIV DNA by droplet digital PCR. PLOS ONE 8:e55943. 10.1371/journal.pone.0055943
92. STRAUB, T. M., B. P. DOCKENDORFF, M. D. QUIÑONEZ-DÍAZ, C. O. VALDEZ, J. I. SHUTTHANANDAN, B. J. TARASEVICH, J. W. GRATE and C. J. BRUCKNER-LEA (2005): Automated methods for multiplexed pathogen detection. J. Microbiol. Methods 62, 303-316.
93. SYKES, P. J., S. H. NEOH, M. J. BRISCO, E. HUGHES, J. CONDON and A. A. MORLEY (1992): Quantitation of targets for PCR by use of limiting dilution. Biotechniques 13, 444-449.
94. SWAMINATHAN, B., T. J. BARRETT, S. B. HUNTER, R. V. TAUXE and CDC PulseNet Task Force (2001): PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg. Infect. Dis. 7, 382-389.
95. TAYLOR, A. J., V. LAPPI, W. J. WOLFGANG, P. LAPIERRE, M. J. PALUMBO, C. MEDUS and D. BOXRUD (2015): Characterization of foodborne outbreaks of Salmonella enterica serovar Enteritidis with whole-genome sequencing single nucleotide polymorphismbased analysis for surveillance and outbreak detection. J. Clin. Microbiol. 53, 3334-3340.
96. TAYLOR, S. C., J. CARBONNEAU, D. N. SHELTON and G. BOIVIN (2015): Optimization of droplet digital PCR from RNA and DNA extracts with direct comparison to RT-qPCR: clinical implications for quantification of oseltamivir-resistant subpopulations. J. Virol. Methods 224, 58-66. 10.1016/j.jviromet.2015.08.014
97. THOMAS, C., D. HILL and M. MABEY (2002): Culturability, injury and morphological dynamics of thermophilic Campylobacter spp. within a laboratory-based aquatic model system. J. Appl. Microbiol. 92, 433-442. 10.1046/j.1365-2672.2002.01550.x
98. UMESHA, S. and H. M. MANUKUMAR (2018): Advanced molecular diagnostic techniques for detection of food-borne pathogens: current applications and future challenges. Crit. Rev. Food Sci. Nutr. 58, 84-104. 10.1080/10408398.2015.1126701.
99. VELUSAMY, V., K. ARSHAK, O. KOROSTYNSKA, K. OLIWA and C. ADLEY (2010): An overview of foodborne pathogen detection: in the perspective of biosensors. Biotechnol. Adv. 28, 232-254.
100. VILLAS-BÔAS, S. G., S. RASMUSSEN and G. A. LANE (2005): Metabolomics or metabolite profiles? Trends Biotechnol. 23, 385-386.
101. WESCHE, A. M., J. B. GURTLER, B. P. MARKS and E. T. RYSER (2009): Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. J. Food Protect. 72, 1121-1138.
102. WHALE, A. S., S. COWEN, C. A. FOY and J. F. HUGGETT (2013): Methods for applying accurate digital PCR analysis on mLow copy DNA samples. PLoS ONE, 8, e58177
103. WU, X., W. WENBIN, L. LIQIANG, K. HUA and X. CHUANLAI (2015): Monoclonal antibody-based cross-reactive sandwich ELISA for the detection of Salmonella spp. in milk samples. Anal. Methods 7, 9047-9053. 10.1039/c5ay01923k.
104. YOSHIDA, K., K. KOBAYASHI, Y. MIWA, et al. (2001): Combined transcriptome and proteome analysis as a powerful approach to study genes under glucose repression in Bacillus subtilis. Nucleic Acids Res. 2001, 29, 683-692.
105. ZENG, D., C. ZI, J. YUAN, X. FENG and L. BAOGUANG (2016): Advances and challenges in viability detection of foodborne pathogens. Front. Microbiol. 7, 1-12. 10.3389/fmicb.2016.01833.
106. ZHAO, X., L. CHII-WANN, W. JUN and H. O. DEOG (2014): Advances in Rapid Detection Methods for Foodborne Pathogens. J. Microbiol. Biotechnol. 24, 297-312. 10.4014/jmb.1310.10013
107. ZHAO, S., G. H. TYSON, Y. CHEN, C. LI, S. MUKHERJEE, S. YOUNG, C. LAM, J. P. FOLSTER, J. M. WHICHARD and P. F. MCDERMOTT (2016): Whole-genome sequencing analysis accurately predicts antimicrobial resistance phenotypes in Campylobacter spp. Appl. Environ. Microbiol. 82, 459-466.
108. ZHOU, B., J. XIAO, S. LIU, J. YANG, Y. WANG, F. NIE, Q. ZHOU and Y. LI (2013): Simultaneous detection of six food-borne pathogens by multiplex PCR with GEXP analyzer. Food Cont. 32, 198-204. 10.1016/j.foodcont.2012.11.044

Suvremene metode i novi pristupi u identifikaciji i kvantifikaciji patogena koji se prenose hranom – pregledni rad

dr. sc. Tomislav MIKUŠ, docent, dr. sc. Željka CVRTILA, redovita profesorica; dr. sc. Lidija KOZAČINSKI, redovita profesorica, Veterinarski fakultet Sveučilišta u Zagebu, Zagreb, Hrvatska

Patogeni koji se prenose hranom stalni su problem koji privlači pozornost javnozdravstvenih institucija, mikrobiologa i proizvođača hrane diljem svijeta. Stoga je znanstvena zajednica godinama usmjerena na pronalaženje "novih" i "boljih" metoda, a sve u nastojanju da se patogeni koji se prenose hranom otkriju na brži i pouzdaniji način. Treba naglasiti da je razvoj molekularne genetike – posebice korištenje genetskih informacija bioloških makromolekula u rutinskom testiranju, doveo do revolucionarnog zaokreta u biološkim i znanstvenim istraživanjima. Gotovo svi postupci otkrivaju različite bakterijske genetske otiske ili biomarkere, zbog čega je izolacija bakterija nepotrebna. Međutim, ova novija istraživanja "brzih mikrobioloških metoda", koja zahtijevaju samo nekoliko sati (a ne nekoliko dana), nisu potisnula klasični način mikrobiološkog testiranja hrane, odnosno klasične mikrobiološke metode i „zlatni standard". Stoga se nove metode suočavaju s visokim zahtjevima kao što su: granice detekcije, brzina analize, relativno niski troškovi i visoka osjetljivost za identifikaciju različitih patogena koji se prenose hranom. Neke od njih imaju mogućnost praćenja patogena, što je važan dio uspostave mreže kontrole patogena u lancu poljoprivredno-prehrambene proizvodnje na međunarodnoj razini. U ovom opsežnom pregledu literature autori predstavljaju i pregled metoda identifikacije i kvantifikacije s naglaskom na najvažnije EU patogene koji se prenose hranom (*Salmonella*, *Campylobacter*, *Escherichia coli* – STEC, *Yersinia* i *Listeria*). Metode su podijeljene u dvije velike skupine: suvremene metode (imunotestovi i lančana reakcija polimerazom) i nove metode (biosenzori, bakterijska tipizacija i omika) te ocijenjene prema njihovoj funkcionalnosti, prednostima i nedostatcima.

Ključne riječi: *imunotestovi, PCR, biosenzori, PFGE, WGS, omika, sigurnost hrane*