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UNDER THE MICROSCOPE

A decade with nucleic acid-based microbiological methods in safety control of foods

T. Kuchta[1], R. Knutsson[2], A. Fiore[3], E. Kudirkiene[4], A. Höhl[5], D. Horvatek Tomic[6], V. Gotcheva[7], B. Pöpping[8], S. Scaramagli[9], A. To Kim[10], M. Wagner[11] and D. De Medici[3]

1 Department of Microbiology and Molecular Biology, Food Research Institute, Bratislava 26, Slovakia
2 Security Department, SVA National Veterinary Institute, Uppsala, Sweden
3 Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Rome, Italy
4 Department of Food Safety and Quality, Lithuanian University of Health Sciences, Kaunas, Lithuania
5 Institute of Food Science, Department of Food Science and Technology, University of Natural Resources and Life Sciences, Vienna, Austria
6 Department of Poultry Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia
7 Department of Biotechnology, University of Food Technologies, Plovdiv, Bulgaria
8 Eurofins CTC GmbH, Hamburg, Germany
9 Laboratorio COOP Italia, Casalecchio di Reno, Bologna, Italy
10 School of Biotechnology and Food Technology, Hanoi University of Science and Technology, Hanoi, Vietnam
11 Department for Farm Animals and Veterinary Public Health, Institute for Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine, Vienna, Austria

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Correspondence
Tomáš Kuchta, Department of Microbiology and Molecular Biology, Food Research Institute, Priemyselná 4, PO Box 25, SK-82475 Bratislava 26, Slovakia.
E-mail: kuchta@vup.sk

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Abstract

In the last decade, nucleic acid-based methods gradually started to replace or complement the culture-based methods and immunochemical assays in routine laboratories involved in food control. In particular, real-time polymerase chain reaction (PCR) was technically developed to the stage of good speed, sensitivity and reproducibility, at minimized risk of carry-over contamination. Basic advantages provided by nucleic acid-based methods are higher speed and added information, such as subspecies identification, information on the presence of genes important for virulence or antibiotic resistance. Nucleic acid-based methods are attractive also to detect important foodborne pathogens for which no classical counterparts are available, namely foodborne pathogenic viruses. This review briefly summarizes currently available or developing molecular technologies that may be candidates for involvement in microbiological molecular methods in the next decade. Potential of nonamplification as well as amplification methods is discussed, including fluorescent *in situ* hybridization, alternative PCR chemistries, alternative amplification technologies, digital PCR and nanotechnologies.

Diseases caused by foodborne pathogens constitute a major burden to consumers, food business operators and national governments. Prevention and control of these diseases are international public health goals. Microbiological analysis is an integral part of technological food safety quality control and monitoring systems and of the management of microbial safety of the food chain. It is also an important tool for evaluation of critical control points and for evaluation of procedures of good hygienic practices (GHP) and standard operating procedures (SOP; Jacxsens *et al.* 2009; Newell *et al.* 2010).

Traditional methods of microbiological analysis of pathogenic bacteria in food, which constitute the basis of the current standard methods, involve isolation by agar culture. The selective agar medium may be inoculated directly, or after enrichment, which selectively increases the numbers of target organisms. If an improvement in the recovery of damaged organisms is required, pre-enrichment is used prior to selective enrichment. The entire procedure requires several days to accomplish, but is generally reliable and its analytical parameters are well defined. However, due to the time demand, these

methods are often ineffective because they are incompatible with the speed at which the food products are manufactured and with their (short) shelf life. To meet the requirement for faster analysis, several 'rapid' (faster than their classical counterpart) methods have been developed in recent decade, mostly on the basis of simplified enrichment coupled to real-time polymerase chain reaction (PCR; Gracias and McKillip 2004; Hoorfar 2011).

At the beginning of the second millennium, molecular methods were considered not suitable for routine testing of food products for contamination with pathogens, because these techniques looked good and worked well only if used in research laboratories with skilful technicians (Rijpens and Herman 2002). However, in the last decade, nucleic acid-based methods gradually started to replace or complement the culture-based methods and immunochemical assays in routine laboratories involved in food control. Besides other development, the reduction of the cost for the real-time polymerase chain reaction (PCR) equipment catalyzed wider spread of molecular methods in routine laboratories, together with availability of validated kits for the detection of the most important foodborne pathogens and commercialization of different user-friendly platforms that require no significant user skills or training. Real-time PCR is more rapid, sensitive and reproducible, and is less endangered by carry-over contamination as specific amplicons are detected in a closed tube. Another advantage of the new technology is the reduction of the need of different rooms for post-PCR processing and avoiding the use of mutagenic ethidium bromide (Jasson *et al.* 2010; Rodríguez-Lázaro *et al.* 2013).

Methods based on simplified enrichment coupled to real-time PCR represent an important step ahead in the field, because they are clearly faster by one or several days than their classical counterparts, their analytical parameters are well defined, they are technically developed to the level that makes them suitable for routine use, they can be used along with the standard methods if necessary, and the legal status of the employed technology makes them open for public use. Besides being faster, they can often be automated and, in prospect, may become cheaper than the classical methods. However, one can see that despite the given benefits, the novel methods are not being widely implemented. One of the reasons is the delay in full validation and standardization. On the other hand, one has to admit that the technology of the methods is not always ideal and that PCR-based methods have various drawbacks: they require preprocessing step (sample preparation, nucleic acid extraction), amplification control must be developed, they cannot discriminate DNA from live cells, multiplexing is limited, and they are unable to quantify the target in the required range. So, employment of other new technologies may lead to better methods and a higher level of implementation to the routine microbiological control. New technologies and methods also have to reflect the emerging needs for added information, such as subspecies identification, presence of genes important for virulence or antibiotic resistance, but also to detect important foodborne pathogens for which no classical counterparts are available, namely foodborne pathogenic viruses.

Regarding the range of molecular methods that have become available for the detection of foodborne pathogens, however, a question may be asked whether they reflect practical needs, whether they provide any scientific, technical or economical advantage or whether they are just commercially motivated alternatives to the patented mainstream detection methods. The answer to this question is connected to identification of the bottlenecks of implementation of nucleic acid-based microbiological methods in safety control of foods.

Nonamplification methods

The most often used principle of nonamplification methods is DNA–DNA hybridization. DNA from the sample is hybridized to oligonucleotide probes that may be bound to various solid supports – wells of a microplate, glass microscopy slide (as spots in DNA-microarray technology) or coded plastic microbeads in xMAP technology. The methods are characterized by good specificity, high level of automation, good throughput, but comparatively low detection sensitivity – apparently insufficient for direct use for the detection of pathogens in food (Dunbar 2006; Dwivedi and Jaykus 2011).

Fluorescent *in situ* hybridization

Fluorescent *in situ* hybridization (FISH) is a method based on whole cell visualization of the target pathogen using fluorescence microscopy. The method uses fluorescently labelled probes, typically targeting 16S ribosomal RNA (16S rRNA; Amann *et al.* 1995). Recently, peptide nucleic acid (PNA) probes are preferentially used in FISH, as these more easily traverse cell membranes. FISH-based methods for the detection of pathogens in food have been developed, but did not provide better detection sensitivity or specificity than PCR (Bottari *et al.* 2006; Almeida *et al.* 2010).

Polymerase chain reaction – PCR

In vitro amplification of nucleic acids using the polymerase chain reaction (PCR) has become a powerful diagnostic

tool for the analysis of bacteria in food samples. Numerous PCR protocols targeting different genes were developed over last decades to detect and identify different pathogenic bacteria. However, the usual detection limit of conventional PCR, which uses gel electrophoresis for the detection of the amplicon, is 10^3–10^5 CFU ml^{-1}, and it makes the microbiological PCR-based methods dependent on enrichment (Rijpens and Herman 2002). For several bacterial pathogens, complete detection procedures including enrichment were developed and validated (Jasson *et al.* 2010; Rodríguez-Lázaro *et al.* 2013). PCR, coupled to reverse transcription, has been applied to the detection of viruses in different food matrices. However, different authors demonstrated that a single-round PCR need not be sensitive enough to detect enteric viruses in food because of the possible low numbers present (De Medici *et al.* 1998; Formiga-Cruz *et al.* 2005). A second round of amplification is necessary, in a format of either nested PCR, semi-nested PCR or booster PCR (De Medici *et al.* 2004).

Real-time PCR

A major technical improvement in diagnostic PCR is real-time PCR, a technique where amplification is monitored during its progress. No additional processing of the PCR product is necessary after the end of the reaction. The technique provides quantitative data, which are available sooner, and is better contained as it takes place inside a closed microtube without a need to open it. Currently, the most useful and robust chemistries include Sybr-Green, TaqMan probes, Molecular beacon probes and FRET probes. Using these chemistries, real-time PCR is sufficiently specific and sensitive as the detection step for most of the food microbiology applications. However, the usual detection limit of real-time PCR, which is 10^2–10^3 CFU ml^{-1}, makes the microbiological real-time PCR-based methods dependent on enrichment (Krascsenicsová *et al.* 2008; Hoorfar 2011).

Multiplex PCR

Multiplexed analytical methods combining enrichment and PCR are attractive for rapid routine detection of pathogenic micro-organisms. These methods are intended to detect several pathogens in one food sample in one assay, which may save chemicals, time and labour. It has been demonstrated that 2–6 DNA markers, specific for the selected genus/species/serovar, can be detected in one assay, if certain prerequisites are fulfilled (De Medici *et al.* 2009; Mayr *et al.* 2010; Fratamico *et al.* 2011). Individual targets are discriminated by the use of different fluorescent dyes to label the probe. Independent detection of individual dyes, without crosstalk, is technically quite challenging and is actually not achieved in most of the PCR cyclers. Only advanced optical configurations are capable of true independent detection of several dyes in a mixture, which is encountered in real-time PCR. Good results are obtained only if the detection is done by a spectrograph, supported by dedicated software for appropriate processing of the spectra, or individual channels are equipped with separate narrow-band excitation and emission filters. The detector should then be supported with a photomultiplier, because narrow-band filters transmit only a minute fraction of light. Certain problems at detecting fluorescence in a mixture of dyes may be caused also by the fact that some dyes (FAM) are very strong and others rather weak (Kuchta *et al.* 2007). Taking into account that one dye/channel has to be reserved for internal control, the number of targets that can be multiplexed on this platform is apparently insufficient for universal applications and probably cannot be increased for principal physicochemical and technological reasons.

As the number of dyes/channels available in real-time PCR cannot be practically increased, some useful platforms have been proposed to distinguish between multiple different amplified DNA fragments, to increase the potential for multiplexing. PCR coupled to (low-density) DNA microarrays, which is the technology widely used in molecular–biological research, seems too expensive and impractical, having an unnecessarily high capacity for different targets (Call *et al.* 2003; Litrup *et al.* 2010). As a cheaper and more versatile technology, DNA–DNA hybridization on fluorescently labelled microbeads, so-called xMAP technology, has been developed and introduced in clinical microbiology. Depending on the resolution of the instrument, 100–500 targets can be theoretically discriminated in one assay (Dunbar 2006). However, there is a vast disproportion between the potential of the optical system and its real applicability to analysing mixtures of targets. The problems reside mainly in cross-reactivity of oligonucleotides during PCR, production of side products, primer–dimers, exhausting of monomers and other processes, which lead to a significant decrease in the sensitivity of the assay for individual analyte or to inability to detect minor components in the presence of a dominant target. Certain technologies have been developed to fight this problem, for example template enrichment PCR, but even using this technique, not more than 8-plex reaction can be effectively performed. Probably, a multiplex PCR with a separate pair of primers targeting one DNA marker sequence per one pathogen is not a productive approach. It might be better to use one pair or a few pairs of primers to amplify markers (e.g. 16S rDNA) in all target pathogens. Unfortunately,

in this case, individual amplification products may be very similar and cannot be discriminated in a rapid way, with sufficient specificity and sensitivity, by currently available technologies. It can be proposed that different marker sequences, common to a range of pathogens, with a favourable ratio of conservative and variable regions, have to be identified. These could be amplified by PCR with one or a few pairs of primers and subsequently selectively detected, possibly by a novel or adapted technology.

Light-upon-extension (LUX) fluorogenic primer-based alternative chemistry

Light-upon-extension (LUX) primer technology is based on PCR with the use of one fluorescently labelled, self-quenched primer and one normal primer. Typically 20–30 bases long, the LUX oligonucleotide primers are designed with the fluorophore close to the 3′-end in a hairpin structure. Upon annealing and extension of the labelled primer during PCR, it loses quenching and emits fluorescence whose intensity is proportional to the amount of the amplified polynucleotide. The primers may be labelled with different fluorophores, which facilitates multiplexing. The technology does not require probes, which may be an advantage with certain target DNA sequences. Melting curve analysis can be performed after PCR. As no change in marker sequences is necessary, this technology is available for a wide range of pathogens and can be effectively used in a multiplex format. Analytical parameters of LUX assays are comparable to those of PCR (Gašparič *et al.* 2008).

Plexor alternative chemistry

Plexor is an alternative real-time PCR chemistry, which is based on quenching during amplification. The technology employs the reaction between two modified molecules: (i) the primer labelled with a fluorescent dye at 5′-end, which contains a methylisocytosine (iso-C) modification, and (ii) the nucleotide carrying a quencher (dabcyl) and iso-dGTP modification, which is a component of the amplification mixture. Plexor technology visually differs from the other real-time PCR chemistries because the fluorescence signal decreases in proportion to the increase in the amount of PCR products during the course of amplification. This technology does not require probes, which may be an advantage with certain target DNA sequences. As no change in marker sequences is necessary, the technology is available for a wide range of pathogens and can be effectively used in a multiplex format. Analytical parameters of Plexor assays are comparable to those of PCR (Gašparič *et al.* 2008).

New and alternative amplification technologies

Amplification at a constant temperature represents an attractive and effective alternative method. Thus, a new generation of isothermal amplification techniques is gaining a wide popularity as a diagnostic tool due to simple operation, rapid reaction, easy detection and suitability for miniaturization. Different isothermal amplification methods, such as transcription-mediated amplification (TMA) or self-sustained sequence replication (3SR), nucleic acid sequence-based amplification (NASBA), signal-mediated amplification of RNA technology (SMART), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification of DNA (LAMP), isothermal multiple displacement amplification (IMDA), helicase-dependent amplification (HDA) and single primer isothermal amplification (SPIA), have been proposed for molecular detection of pathogens. All these non-PCR-based methods have the advantage of not requiring the use of a thermocycler, which represents a positive feature. For certain applications, in particular for virus detection, NASBA has better technical and analytical parameters than PCR-based methods.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a gene amplification technique that is simple and rapid; the amplification can be completed in <1 h at a constant temperature of 60–65°C. Gene amplification products can be detected by agarose gel electrophoresis as well as by real-time monitoring in an inexpensive turbidimeter. LAMP uses four primers that recognize six separate regions within a target DNA, and the amplification reaction occurs only when all these six regions are correctly recognized by the primers. This puts demands on the design of primers but, on the other hand, leads to a higher specificity than classical PCR. Sensitivity of LAMP is comparable with PCR (Song *et al.* 2005). LAMP assays have been successfully applied to detect different pathogenic micro-organisms, such as *E. coli* (Wang *et al.* 2012), *Salmonella* (Shao *et al.* 2011) and *Vibrio parahaemolyticus* (Yamazaki *et al.* 2011).

Helicase-dependent amplification

Helicase-dependent amplification (HDA) is an isothermal amplification reaction inspired by the natural mechanism of the DNA replication fork. HDA technology mimics DNA replication *in vivo* using a DNA helicase to separate two complementary DNA strands (ds-DNA) into single-stranded templates for primers hybridization and

subsequent extension by a DNA polymerase. This isothermal amplification method uses the *Escherichia coli* UvrD helicase for unwinding the DNA double-strand and DNA polymerase I Klenow fragment for subsequent amplification (Vincent *et al.* 2004). A one-tube isothermal reverse transcription-thermophilic helicase-dependent amplification (RT-tHDA) system has also been developed. RT-tHDA has been used specifically for amplification of RNA targets. Inclusion of an extremely thermostable single-stranded DNA-binding protein (ET SSB) in the RT-tHDA reaction resulted in a rapid increase in the reaction speed (Goldmeyer *et al.* 2007). The HDA amplicons can be detected using gel electrophoresis, real-time fluorimetry, enzyme-linked immunosorbent assay (ELISA) or gold nanoparticle probes (Gill *et al.* 2008). Analytical parameters of this method are comparable to PCR (Andresen *et al.* 2009).

Isothermal amplification with continuous luminometry

Another DNA-targeted closed-tube system, isotermal amplification for the detection of pathogens in food, is being marketed by 3M. Based on the disclosed information, the system utilizes isothermal amplification at 60°C by *Bst* DNA polymerase, which is monitored luminometrically. For this purpose, primers labelled with inorganic pyrophosphate (PP_i) are used. Positive amplification leads to the release of PP_i, which is consumed to form ATP by ATP sulfurylase. The formed ATP is detected by luciferin–luciferase system. All reactions take place in one tube being placed in one robust instrument, which is a thermostated luminometer. For positive samples, a luminometric peak is typically observed in about 20 min; however, the recommended assay duration is 75 min, which probably reflects the undisclosed knowledge of the manufacturer on atypical amplification in 'problematic' samples (Anon. 2012a,b). The detection system is said to produce same results as real-time PCR in terms of sensitivity and detection limit after enrichment (Anon. 2012a, b). Kits for the detection of *Salmonella*, *Listeria*, *E. coli* O157 and external amplification control are currently available on commercial basis. Unfortunately, no details including marker identity and sequence information have been disclosed. The system may be facing certain problems with specificity, as reflected by the fact that only a genus-specific kit for *Listeria* is available, and the kit for *Salmonella* was found to be false-positive with some *Citrobacter* spp. (Kolackova and Karpiskova 2012). The use of a combination of biochemical technologies that leads to luminometric monitoring of amplification is interesting from the technical point of view, as it relies on less complex and more robust instrumentation, including better signal-to-noise characteristics. Unfortunately, shortage in information on the system does not allow to judge whether this apparently lower specificity is an intrinsic feature or it can be improved with different markers or primer sequences. This technology does not provide improvement in analytical parameters compared with real-time PCR.

Nucleic Acid Sequence-Based Amplification (NASBA)

The NASBA assay is a sensitive, transcription-based amplification system specifically designed for detecting RNA. The technology relies on the simultaneous activity of three different enzymes: a reverse transcriptase, RNase H and T7 RNA polymerase, which act in concert to amplify sequences from an original single-stranded RNA template. Oligonucleotide primers, complementary to sequences in the target RNA, are incorporated in the reaction. One primer also contains a recognition sequence for T7 RNA polymerase. The reaction contains both dNTPs and NTPs. The first primer binds to RNA, allowing the reverse transcriptase to form a complementary DNA strand. Then, RNase digests away RNA and the second primer binds to cDNA, allowing the reverse transcriptase to form a double-stranded cDNA copy of the original sequence. This double-stranded DNA then acts as a kind of mini 'gene', which is transcribed by T7 RNA polymerase to produce thousands of RNA transcripts that then cycle through the reaction. The reaction is performed at a single temperature (Compton 1991; Cook 2003). NASBA has several advantages over reverse transcriptase-PCR. It generates the same number of copies in a shorter time than RT-PCR because every cycle results in an exponential increase, whereas PCR progresses in a binary fashion (Chan and Fox 1999). Incubation times for NASBA are shorter, that is, 90–150 min *vs* 3–5 h for RT-PCR. NASBA showed good performance at assessment of viability of cells of foodborne bacteria (Uyttendaele *et al.* 1997; Simpkins *et al.* 2000).

Digital PCR

The ability to quantify foodborne pathogens with accuracy and precision is important for several applications, such as tracing pathogens in food processing environments or in tenacity studies monitoring survival of micro-organisms in food matrices. Although real-time PCR has found widespread use for one-step nucleic acid quantification, it has been found vulnerable to some bias caused by comparatively low precision of the standard curve that is required to quantify unknown samples. Digital PCR (dPCR) has been introduced to provide absolute quantification. The technique is based on endpoint PCR with a series of diluted template DNA solutions, which

are carried out as 15,000–20,000 parallel low-volume PCR reactions. The reactions take place either in a microdroplet format or in a microvolume chip, and the amplification product is detected fluorimetrically in endpoint mode in a microfluidic device and by a scanner, respectively. Calculation of the absolute quantities is based upon counting positive *vs* negative amplification results at an appropriate dilution level (Whale *et al.* 2013). Studies targeting low-copy-number genes, typically in the field of molecular oncology, demonstrated high sensitivity and precision of digital PCR (dPCR) compared with quantitative real-time PCR (Hindson *et al.* 2011). Applicability of this technology to food analysis deserves future research.

Controlling the analysis

Nucleic acid-based detection of pathogenic micro-organisms in food is a comparatively complex process involving several steps, all of them being possible sources for errors. Those include improper sample preparation (defective enrichment, cell lysis, nucleic acid extraction, removal of inhibitors) or failure of the detection (composition of reaction mixture, performance of the instruments). As a technical measure to monitor the performance of the analysis, various types of controls have been implemented. The most important approach is the positive exogenous internal control, which is, for example, used in PCR as an internal amplification control (IAC). IAC is added to each tube prior to analysis and is detected along with the target. Several formats of this type of control have been developed, based mostly on plasmids or phages. If carefully designed, the addition of a positive exogenous internal control should not affect the main analytical reaction (Anon. 2005; Fricker *et al.* 2007).

An ideal type of control would, however, be the one that controls the entire analytical procedure, including sample preparation. This might be, for example, a freeze-dried microbial culture that would be added to the food sample prior to enrichment, would pass all steps of the analysis (enrichment, lysis, nucleic acid extraction, molecular detection) and then would be selectively detected along with the target. The requirements suggest that it probably would be a micro-organism related to the target, able to grow in all enrichment media, but not faster than the target. Either well-defined wild or specifically designed engineered micro-organisms may suit this purpose (Rossmanith *et al.* 2011). However, further development of this type of entire-process controls for individual pathogens is necessary.

Nanotechnologies

Nanotechnologies, the combination of nano-objects and nanosystems, offer techniques to reach a single cell or molecule and, consequently, overcome several current technological problems of the detection of pathogens. Nanotechnologies offer also new tools for improving sample preparation and also for avoiding the necessity of target amplification and fluorescent labelling (Fournier-Wirth and Coste 2010). Nanoscale devices may represent a significant improvement of the detection of pathogens in the food chain (Valdés *et al.* 2009). The review of Lazcka *et al.* (2007) on methods for pathogen detection highlights the advantages of a nanoscale approach in biosensing, which include:

i the possibility of mass production and reduced unit costs;
ii working with sample volumes in the range of nanolitres, thus using less reagents and making the cost of the analysis not too high;
iii shorter analysis times;
iv multi-analyte analysis;
v safer and environmentally friendly devices

Conjugation of biomolecules with nanomaterials is the basis of nano-biorecognition. A variety of strategies, including antibody–antigen, adhesin–receptor, antibiotic and complementary DNA sequence recognition, have been studied for specific recognition of bacterial cells. The incorporation of bio-functionalized nanomaterials into current pathogen detection methods may lead to their improvement, facilitating nearly real-time pathogen detection (on a level of minutes), improved sensitivity (single bacterial cell) and simultaneous detection of multiple micro-organisms from either nutrient broth, liquid or solid food products, or biofilms (Yang *et al.* 2008).

The unique properties of nanomaterials make them promising also in the development of portable biosensors. Carbon strip or paste electrode transducers, supporting the DNA recognition layer, may be used with a highly sensitive chronopotentiometric transduction of the DNA analyte recognition event. Carbon nanotubes (CNTs) are effective as nanoscale sensors of food pathogens thanks to their unique mechanical, electrical and geometrical properties. CNTs can enhance electrocatalytic activity and reduce fouling on electrode surface, making them useful for electrochemical sensing. In addition, their large specific surface area can increase the biosensor response (He *et al.* 2006). A pathogen-detecting nanosensor was developed for the detection of *E. coli* O157:H7 on the basis of an electrochemical immunosensor with self-assembled peptide nanotubes. Attachment of antibodies to peptide nanotubes reduced the necessary steps for immobilization and, therefore, the duration of analysis (Chan Cho *et al.* 2008). A network of single-walled carbon nanotubes was used for the construction of a field effect transistor

biosensor for the detection of *Salmonella* Infantis, with the recognition process based on antigen–antibody interaction. The selective adsorption of *S.* Infantis was achieved with the detection of 100 CFU ml^{-1} in one hour (Villamizar *et al.* 2008). The company NanoRETE developed a platform for real-time detection of pathogens (anthrax, *E. coli, Salmonella, Mycobacterium tuberculosis*) by customized nanoparticle biosensors. The simple-to-use handheld device generated screening results in about an hour. An easy-to-use pathogen sensing device using anti-*E. coli* O157:H7 antibody-functionalized glass capillaries as solid substrates to perform a quantum dot-based sandwich assay was also implemented in a recently presented mobile phone-based *E. coli* detection platform for screening of liquid samples. The platform can also be applicable to other pathogens through the use of different antibodies (Zhu *et al.* 2012).

However, it is accepted that although the described approaches are promising, much research and development work is still needed before biosensors become a real and trustworthy alternative to current methods (Lazcka *et al.* 2007).

Conclusions

Currently available faster methods for the detection of bacterial pathogens in food, which are based on simplified enrichment and real-time PCR with probes, have analytical parameters compatible with the corresponding standard microbiological methods. Therefore, their implementation relies only on full validation and standardization and eventually on the format in which they will be available (open-formula as a cheaper version *vs* user-friendly kits). Further substantial improvement in speed will require new technologies for sample preparation, to process typically 25 g of food sample without enrichment by cultivation. New sample preparation technology will be necessary also for quantitative detection methods, which will have to be combined with a sensitive detection technology providing quantitative results selectively for live bacterial cells. Alternative PCR chemistries may be used for newly developed assays for emerging pathogens, as they may be advantageous at detection of specific marker sequences. Alternative amplification technologies may be useful for world regions where expensive thermal cyclers are not available or for miniaturized instruments. A range of complete methods for the detection of viruses in food have to be developed on the basis of techniques that have become recently available, and these need to be fully validated and standardized. Nanotechnologies require future development to transform their potential to an effective technical solution.

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Conflict of Interests

All authors have no conflict of interests to declare.

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