Nucleic acid based assays for rapid detection of emerging and reemerging foodborne pathogens in food industry

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Emerging and reemerging of many microbial pathogens increased efforts of governments and the food industry to support research institutes in developing molecular biology diagnostic methods. The most emerging bacterial strains isolated in foodborne outbreaks belongings to *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* or *Campylobacter*. Routine diagnostic tests for *L. monocytogenes* can takes 7 – 14 days, while DNA hybridization tests offer reliable results in 1 – 3 days, an optimal period to certificate the safety of products with short shelf life. The large variety of virulence factors described in the *E. coli* pathotypes restricts many molecular diagnostic methods, like DNA hybridization and multiplex PCR, but not the DNA microarray technology. Detection of *Salmonella* spp. in food products can be done by different PCR or qPCR methods, but the newest molecular diagnostic methods like PMA-LAMP (propidium monoazide with loop-mediated isothermal amplification) seem to be more sensitive and results are obtained in only 3 hours. Also, viable *Campylobacter* can be detected in chicken carcass rinse by Real-Time PCR and propidium monoazide treatment in less than 3 hours.

Keywords *L. monocytogenes*; *E. coli*; *Salmonella*; *Campylobacter*; PCR; DNA hybridization; microarray; PMA-LAMP

1. Introduction

Several nucleic acid based detection systems are developed for food-borne pathogens [1-7], most of them designed to supply rapid results with excellent detection limits and specificity [1]. Techniques important for detecting food-borne pathogens by using PCR are able to isolate bacterial DNA from different feed and food samples with previously growing on agar and in broth [2, 3]. This preliminary growing step will facilitate the accurately determination of bacterial cells when they are in low number, well as differentiation of live and dead bacterial cell. To eliminate this growth stage, methods for selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology [4], real-time PCR [5], loop-mediated isothermal amplification [6] or other DNA-based molecular detection tools have been developed. Also, the diagnostic systems based on PCR or other molecular tools for microorganisms' detection directly from food samples were developed; these are useful in detection of fastidious and uncultivable bacteria [10, 11].

Although the nucleic acid amplification and detection techniques were designed and implemented long time ago, their use as an alternative to classical bacteriological diagnosis was recommended only in the last 10-15 years. Rapid molecular diagnostic techniques are commonly used in screening, but increasing number of methods gain recognition in use as confirmatory methods. The development of sensitive, specific and rapid molecular methods for the revealing and recognition of food-borne bacteria, with simultaneous determination of their viability status, targeted both bacterial DNA and RNA. Initially, the researchers tried to find a correlation between the presence of longer intact DNA sequences and bacteria cells viability, but the precise correlation wasn't successful [12]. Masters *et al*. (1994) obtained strong PCR signals for long after *Escherichia coli* and *Listeria monocytogenes* cells became non-viable after starvation and desiccation, but not after acid or hydrogen peroxide treatment [13]. It's quite possible that *Salmonella* and *Campylobacter* will supply similar findings in similar conditions. The molecular methods designed for bacterial-RNA involved both mRNA and rRNA. Scientists supposed that because mRNA has a very short half-life, this could be used as marker of bacterial cell viability [12]. Detection of 16S rRNA was demonstrated as an excellent diagnostic method [14], but rRNA has longer half-life than mRNA and can resist at different bacterial stress treatments [12, 15].

The most used methods for DNA study are southern blotting, hybridization, isothermal amplification, PCR-based techniques and, in the last years, DNA-arrays. Usually, the diagnostic methods focused on detecting mRNA are northern blotting, reverse transcriptase PCR (RT-PCR) and NASBA (nucleic acid sequence-based amplification).

This chapter will provide information concerning nucleic acid based assays recommended in fast and sensitive detection of *L. monocytogenes*, *E. coli* patotypes (detection of virulence gene), *Salmonella* spp. and *Campylobacter* sp.

2. Nucleic acid based assays for rapid detection of *Listeria monocytogenes*

L. monocytogenes is one of the most virulent food-borne pathogens that can be isolated in various raw or processed food products, some of them with short shelf life (*e.g.* seafood). Several methods based on nucleic acid recognition are used today for screening or confirmation of *L. monocytogenes*, and to differentiate them from other *Listeria* species [16, 17]. Most of them are PCR-based protocols or use PCR products.

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2.1. PCR

Several research reports described various PCR-based protocols and proposed methods of genomic DNA extraction, *Listeria* genus detection, *L. monocytogenes* detection and serotyping. Also, many primers pairs were designed for target genes like *hly*A (Listeriolysin O), *Dth*18 and *iap*A (Betahemolysin). PCR is applied to either DNA isolated directly from samples or after one or two steps of *L. monocytogenes* pre-enrichment.

Border *et al*. (1990) proposed the following pair of primers in *Listeria* genus detection 5'-CTCCATAAAGGTGACCCT-3' and 5'-CAGCMGCCGCGGTAATWC-3' [18]. These primers were subsequently taken over by Lawrence and Gilmour (1994) and used in first PCR of an improved nested PCR protocol [19]. Lawrence and Gilmour (1994) used for the nested reactions 5'-ACGACCGCAADGTTGAAACT-3' and 5'-GACGTCATCCCCACCTTCCT-3' [19]. Furrer *et al*. (1991) used in detection of *L. monocytogenes* by PCR the following pair of primers 5'-CGGAGGTTCCGCAAAAGATG-3' and 5'-CCTCCAGAGTGATCGATGTT-3' that target gene coding the hemolysin [20]. Doumith *et al*. (2004) proposed pairs of primers to separate *L. monocytogenes* in four major serovars [21]. The first group included serovars 1/2a, 1/2c, 3a and 3c, the target gene was *lmo0737*, and the pairs of primers were 5'-AGGGCTTCAAGGACTTACCC-3' and 5'-ACGATTTCTGCTTGCCATTC-3'. The second group included serovars 1/2c and 3c, the target gene was *lmo*1118, and the pairs of primers were 5'-AGGGGTCTTAAATCCTGGAA-3' and 5'-CGGCTTGTTCGGCATACTTA-3'. The third group included serovars 1/2b, 3b, 4b, 4d, and 4e, the target gene was *ORF*2819, and the pairs of primers were 5'-AGCAAAATGCCAAAACTCGT-3' and 5'-CATCACTAAAGCCTCCCATTG-3'. The fourth group included serovars 4b, 4d, and 4e, the target gene was *ORF*2110, and the pairs of primers were 5'-AGTGGACAATTGATTGGTGAA-3' and 5'-CATCCATCCCTTACTTTGGAC-3' [21].

PCR parameters used in amplification have been different in several proposed protocols: from 94°C to 97°C for denaturation, from 54°C to 65°C for primer annealing, from 70°C to 72°C for extension, and 30-40 cycles. The amplified product are resolved on agarose gel (2-3%) and stained with a dye for DNA detection in gels (*e.g.* ethidium bromide, GelStar®, SYBR® Green). However, classical PCR protocols are becoming more rarely used in current diagnosis of *L. monocytogenes*. Automated electrophoresis systems (*e.g.* Experion™ Automated Electrophoresis System, Bio-Rad Laboratories, USA) and PCR methods that avoid pre-enrichment of *L. monocytogenes* (*e.g.* magnetic capture hybridization-PCR assays) have been proposed to reduce working time and to increase the confidence [22, 23]. Delibato *et al.* (2009) obtained very good results with a micro-fluidic chip-based automated electrophoresis system; identical with those obtained by standard horizontal method for the detection of *L. monocytogenes* (ISO 11290-1: 1996) but more accurate than classical gel electrophoresis on 24 hours culture enrichment [22]. Amagliani *et al.* (2006) proposed a sensitive and specific method for the detection of pathogens from milk avoiding bacterial enrichment culture and colony isolation by magnetic-capture hybridization of target pathogen directly on samples. In their study hybridization capture was carried out with 21-mer NH_2-labelled DNA probe linked to paramagnetic nanoparticles that were more sensitive than Dynabeads M-280 nanoparticles [23].

2.2. Real Time PCR

Real-time PCR permit to rapidly detect nucleic acids without too much manipulation, and the amplified DNA is detected as the reaction progresses in real time. The technique eliminate laborious gel electrophoresis step and reduce the risk of cross-contamination, but cannot discriminate viable and non-viable bacterial cells when the target is a genomic DNA sequence [24]. Like in classical PCR, the improved real-time PCR protocols that use reverse transcription PCR for mRNA [25] or new pre-PCR steps of sample processing [26-29] were designed to discriminate live and dead *L. monocytogenes* cells. Processing of samples for DNA extraction procedures can be done by BAX lysis method [26], by incubating 15 min at 100 °C [27], by FTA filter-based DNA isolation [28] or by lysis buffer, incubation 15min at 60 C, filtration of the lysate and 3-times washing followed by resuspension of the filter with 100 ml of dd-water [29].

Yea *et al.* (2012) developed a new procedure to specifically isolate RNA of *L. monocytogenes* by quantitative real-time RT-PCR assay without preenrichment that use the same gene target (*hly*) used in TaqMan® probe real-time PCR assays by Amagliani *et al.* (2010) [25, 30]. This protocol completes the list of most used RT-PCR commercial systems for rapid *Listeria* screening: BAX® System PCR assays for *L. monocytogenes* (DuPont, SUA); iQ-Check *L. monocytogenes* II kit (Bio-Rad Laboratories, France), LightCycler® foodproof *L. monocytogenes* Detection Kit (Roche Diagnostics, Germany), MicroSeQ® *L. monocytogenes* detection Kit (Applied Biosystems, SUA), and BAM Protocol: Simultaneous Confirmation of *Listeria* species and *L. monocytogenes* isolates by real-time PCR (FDA, SUA) [31-36].

2.3. Nucleic Acid Hybridization Probe

Routine diagnostic tests for *L. monocytogenes* can takes 7 – 14 days, while DNA hybridization tests offer reliable results in 1 – 3 days, an optimal period to certificate the safety of products with short shelf life. However, tests are laborious and required highly qualified personnel, and some DNA hybridization protocols are used only in screening studies and research activities. Chapter 2.9.7. of OIE Terrestrial Manual (2008) listed three commercial kits for DNA hybridization: GENE-TRAK *Listeria* assay (GENE-TRAKTM Systems, USA), GENE-TRAK test for *L.*

monocytogenes (GENE-TRAK™ Systems, USA) and Gen-Probe (AccuProbe®) *L. monocytogenes* Confirmatory Test (Gen-Probe, SUA) [31]. GENE-TRAK tests are used in screening and manufacturer recommends that all positive samples must be confirmed by standard cultural methods. However, Gene-Trak and GenProbe are preferred confirmation method for identification of atypical hemolytic *Listeria* isolates, which gives a positive test result for *L. monocytogenes* [37].

2.4. DNA fingerprinting methods

In the last years have been developed several procedures for molecular typing and genetic diversity study of *L. monocytogenes*, of which are retained: repetitive element sequence-based PCR (rep-PCR) with two variants (ERIC-PCR and REP-PCR), multilocus sequence typing (MLST), multi virulence locus sequence method (MVLST), multilocus variable number tandem repeat analysis (MLVA), amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA PCR (RAPD-PCR), arbitrarily primed PCR (AP-PCR), infrequent restriction site PCR (IRS-PCR) and chromosomal DNA restriction endonuclease analysis (*e.g.* SAU-PCR) [38-53].

Repetitive element sequence-based PCR (rep-PCR) generates DNA fingerprints that allow the bacterial strains differentiation [38] and epidemiological analysis in infectious diseases outbreaks [39]. This was alternative method to characterize *L. monocytogenes* strains isolated from foods [39, 40].

Ragon *et al.* (2008) used the following seven housekeeping genes to characterize *Listeria* strains by multilocus sequence typing (MLST): *acb*Z (ABC transporter), *bgl*A (beta-glucosidase), *cat* (catalase), *dap*E (Succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (lactate deshydrogenase), and *lhk*A (histidine kinase) [41]. Zhang *et al*. (2004) proposed for *L. monocytogenes* typing a multi virulence locus sequence method (MVLST). In this MVLST were used three virulence genes (*prf*A, *inl*B, and *inl*C) and three virulence-associated genes (*dal*, *lis*R, and *clp*P) of *L. monocytogenes* that were previously described in studies of Glaser *et al*. (2001) [42, 43].

In Denmark and Norway, in-house multilocus variable number tandem repeat analysis (MLVA) methods were developed. MLVA is used in Statens Serum Institut (Denmark) and Norwegian Institute of Public Health (NIPH) as genotyping tool for *L. monocytogenes* in order to investigate clusters and detect outbreaks [44].

Numerous restriction fragment length polymorphism (RFLP) methods have been utilized in bacterial genotyping. Keto-Timonen *et al*. (2003) performed an AFLP protocol that use two restriction enzymes (*Hind*III and *HpyCH*4IV) and primer combination *Hind*A and *Hpy*A [45], while Corcoran *et al*. (2006) described a single-enzyme AFLP protocol with one restriction enzyme (*Eco*RI) and one primer (*Eco*G) for comparison of *L. monocytogenes* strains isolated in different foods [46].

RAPD-PCR is one of the most used methods in characterization of *L. monocytogenes* strains for epidemiological purposes [47]. Cocolin *et al.* (2005) obtained the best results with the primer D8635 and amplification parameters proposed by Andrigetto *et al.* (2001) (primer D8635: 5'-GAGCGGCCAAAGGGAGCAGAC-3'; PCR parameters: denaturation - 94 °C for 1 min, annealing - 42 °C for 1 min, extension - 72 °C for 2 min, 35 cycles) [47, 48]. RAPD-PCR with primer D8635 differentiated the *L. monocytogenes* isolated in farm animals from *L. monocytogenes* isolated in food processing plant, and was able to group studied strains by their origin [47].

Arbitrarily primed (AP)-PCR produce a genetic profile of unknown DNA fragments with a single primer of an arbitrary sequence (*e.g.* primer M13 derived from the core sequence of the bacteriophage M13 genome: 5'-GTTGTAAAACGACGGCCAGT-3') [49, 50, 51]. Franciosa *et al.* (2001) use primer M13 for detecting DNA polymorphisms of *L. monocytogenes* strains and two profile cycles of temperature (denaturation - 94 °C for 5 min, annealing - 40 °C for 5 min, extension - 72 °C for 5 min, 2 cycles followed by denaturation - 94 °C for 1 min, annealing - 60 °C for 1 min, extension - 72 °C for 2 min, 40 cycles) [50]. Comparatively with other molecular typing methods AP-PCR seems to be faster and economical [51].

An infrequent-restriction-site (IRS)-PCR for *L. monocytogenes* isolates has been proposed by Franciosa *et al*. (2001) [50]. IRS-PCR consists of selective amplification of DNA restriction fragments, and for the selective amplification of the *Xba*I-*Pst*I restriction fragments Franciosa *et al*. (2001) used the primers PS1: 5'-GACTCGACTCGCATGCA-3' and PX-G: 5'-AGAGTCTGCCAGTACTAGAG-3', and the cycling conditions described in studies of Mazurek *et al*. (1996) (initial denaturation - 94 °C for 5 min, 30 cycles of denaturation - 94°C for 30 sec, annealing - 60°C for 30 sec, and extension - 72°C for 90 sec; final extension - 72°C for 5 min) [52].

Cocolin *et al*. (2005) obtained appreciable results when performed cluster analysis of the profiles obtained by SAU-PCR method for *L. monocytogenes* strains isolated from domestic animals and food processing plants [47]. SAU-PCR is based on the digestion of the genomic DNA with restriction endonuclease Sau3A and amplification with a primer designed on the restriction site of the enzyme (*e.g.* primer SAG-1: 5'-CCGCCGCGATCAG-3'; PCR parameters: denaturation - 94 °C for15 sec, annealing - 46 °C for 1 min, extension - 65 °C for 2 min, 35 cycles) [53].

PCR-based typing methods that generate DNA fingerprints have proven to be among the most effective for assessing genetic heterogeneity of bacterial isolates [54].

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2.5. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is used in genetic subtyping of *L. monocytogenes* [51] and along with RAPD-PCR is frequently used in epidemiological studies [47]. Extensive studies concerning *L. monocytogenes* pulsotypes detection in foods were performed by Autio *et al.* (2002) [55]. PulseNet selected PFGE as the primary genotyping method [55]. PulseNet protocol recommends for casting plugs a concentration of *L. monocytogenes* cell suspensions of OD610=0.8-1.0 and a volume of 400 µl cell suspension in 400 µl melted 1% SeaKem Gold agarose with 20 µl lysozyme and 20µl proteinase K [56]. Restriction enzymes used for *L. monocytogenes* are *Asc*I and *Apa*I. For regular typing surveillance can be use only one restriction enzyme, but in the *L. monocytogenes* outbreaks are recommended both restriction enzymes [57-58]. Macrorestriction fragments will be separated in 1% SeaKem Gold (SKG) agarose in freshly prepared 0.5X TBE cooled to 14°C in a PFGE-system (e.g. CHEF Mapper). CDC protocol use *Apa*I or *Asc*I enzymes, and the following running time in CHEF Mapper electrophoresis system: auto algorithm, 49 kb - low MW, 450 kb - high MW; select default values except where noted by pressing "enter"; initial switch time = 4.0 s; final switch time = 40.0 s; change run time to 18 - 19 h [56].

2.6. PCR-ribotyping

Characterization of *L. monocytogenes* strains with different molecular techniques, such as mixed-genome DNA microarray, pulsed-field gel electrophoresis, ribotyping and multilocus sequence typing, revealed different resolutions. Unfortunately, ribotyping proved to has the lowest subtyping resolution, when the results were compared with those obtained by microarray and PFGE [59]. However, Franciosa *et al.* (2001) obtained for RAPD less robust results than PCR- ribotyping when *L. monocytogenes* isolates were subtyped [50].

2.7. Microarray analysis

Several DNA microarrays for detection and comparative analysis of *L. monocytogenes* genome have been developed [59-61]. Borucki *et al*. (2004) obtained high resolution values by microarray (similar to that of PFGE and better than those of MLST), and they were able to group in phylogenic divisions the studied serotypes of *L. monocytogenes* by using 22 probes that simultaneously distinguish divisions, serotypes, and subtypes [59].

3. Nucleic acid based assays for rapid detection of *Escherichia coli* patotypes

3.1. PCR for VT genes and other virulence markers

Detection of the VTEC relevant virulence factors with PCR assays designed to detect target diagnostic genes [*e.g. vtx*1 (*stx*1), *vtx*2 (*stx*2), *eae*, *saa*] has been described in several scientific papers and manuals of diagnostic [62-65].
Even if DNA-based toxin gene detection is not recommended in diagnostic of human infection [66], the short time (from 3 hours to 36 hours) required to obtain results recommend the methods at least in the identification and characterization of suspect colonies for epidemiological studies, in detection of the VT genes and other virulence markers by non-culture techniques or in the screening faeces for *E. coli* O157:H7 by multiplex PCR for VT1, VT2 and *eae* [62].

In epidemiological studies, subtyping of *E. coli* O157 can be done by random amplification of polymorphic DNA (RPDA); repetitive DNA element PCR and amplified fragment length polymorphism analysis.
RPDA has been extensively used in *E. coli* O157 typing with primer M13: 5'-GAGGGTGGCGGTTCT-3' [67-69], which provided very good results in intraserotype genetic diversity studies [68, 69]. Krüger *et al.* (2006) were able to find three different RAPD profiles among six strains belonging to serotype O157:H7 when preparation of templates was conducted following recommendations of Padola *et al.* (2002) and amplification was done as follows: initial denaturation - 94°C for 5 min, denaturation - 94°C for 1 min, annealing - 50°C for 90 sec, extension - 72°C for 90 sec, 40 cycles; the thermal cycler was programmed with maximum heating/cooling rates between steps [69].

Detection of the VT genes and other virulence markers by PCR encounter a number of issues. First of all, the presence of VT genes or other virulence marker doesn't prove gene expression and toxin production by *E. coli* strains. Secondly, PCR sensitivity can be highly affected by faeces inhibitors and additional steps of bacteria enrichment will increase the working time, without giving higher values than immunomagnetic separation or Vero cell cytotoxicity assay [62]. Nielsen and Andersen (2003) proposed a duplex 5' nuclease PCR assay (real-time PCR) in detection and characterization of VTEC strains, which were isolated from possible sources of human infections (bovine fecal samples). Also, the comparison of the virulence profiles of bovine and human isolates has been done. Determination of the virulence profiles of VTEC and EPEC isolates involved 22 real-time PCR assays, designed for the detection of verocytotoxin genes (*vtx*1*, vtx*2), pO157-associated genes (*ehx*A*, kat*P*, esp*P, and *etp*D), adhesion gene (*saa*), seven subtypes of intimin genes (*eae*, *eae-α*, *eae-β*, *eae-γ*, *eae-δ*, *eae-ε*, *eae-ζ*), four subtypes of *tir*, and three subtypes of *esp*D [63]. In their paper, Nielsen and Andersen (2003) provide a large collection of probes and primers for automated 5' nuclease PCR assays [63].

Keer and Birch (2003) reviewed several molecular methods for the assessment of bacterial viability, emphasizing necessity of using such methods for food security and concluding that an effective monitoring system for the identification of viable cells should use the simultaneous analysis of multiple targets [12]. However, some protocols are laborious and may discourage the laboratories to implement, but multiplex or PMA/real-time PCR can be considered [62, 70].

Simple and fast multiplex PCR used to confirm the presence of virulence determinants VT1, VT2 and *eae* of *E. coli* in the screening faeces for O157:H7 is described in Chapter 2.9.11. of OIE Terrestrial Manual (2008) [62]. The pair of primers recommended for *vtx*1 gene is F: 5'-CGCTCTGCAATAGGTACTCC-3', and R: 5'-CGCTGTTGTACCTGGAAAGG-3'; for *vtx*2 gene is F: 5'-TCCATGACAACGGACAGCAG-3' and R: 5'-GCTTCTGCTGTGACAGTGAC-3'; and for *eae*A is F: 5'-GCTTAGTGCTGGTTTAGGATTG-3' and R: 5'-CCAGTGAACTACCGTCAAAG-3'. PCR parameters are following: initial denaturation - 94°C for 2 min; denaturation - 94°C for 1 min, annealing - 62°C for 90 sec, extension - 72°C for 2 min, 25 cycles; final extension - 72°C for 5 min [62].

Improved molecular methods for the assessment of bacterial viability by real-time-PCR assay with prior propidium monoazide (PMA) treatment of samples are already done. Recently Li and Chen (2012) proposed a sensitive and specific PMA/real-time PCR for *E. coli* O157:H7 that use the following primers and probe: Z3276-Forward: 5'-GCACTAAAAGCTTGGAGCAGTTC-3'; Z3276-Reverse 5'-AACAATGGGTCAGCGGTAAGGCTA-3' and Z3276-probe: FAM-CGTTGGCGAGGACC-MGBNFQ; the PCR conditions were: activation of TaqMan - 95°C for 10 min; denaturation - 95°C for 10 sec, annealing/extension - 60°C for 1 min, 40 cycles [70].

3.2. DNA fingerprinting methods

Amplified fragment length polymorphism analysis (AFLP) and restriction fragment length polymorphism analysis (RFLP) were used in *E. coli* genotyping as alternative methods to phenotyping methods in order to reduce the time required to detect a bacterial strain isolated from a farm, food product or human patient. To support the diagnostic laboratories were proposed standardized protocols but they did not spread widely. Detailed AFLP microbial fingerprinting protocol is supplied by Applied Biosystems [71]. The AFLP technique uses only two primers (*Eco*RI-0/*Mse*I-C or *Eco*RI-A/*Mse*I-C or *Eco*RI-G/*Mse*I-A or *Eco*RI-T/*Mse*I-C) and gives reproducible results. One on the most used restriction fragment length polymorphism analysis (RFLP) protocol has been described by Tyler *et al.* (1991). Using a polymerase chain reaction-restriction fragment length polymorphism procedure, Tyler *et al*. (1991) determined genotypes of VT2-producing strains of *E. coli* with restriction endonucleases *Hae*III, *Rsa*I and *Nci*I [72]. In other study, Indira *et al.* (1997) digested DNA with *Pvu*II and *Eco*RI in characterization of *E. coli* O157:H7 and other Shiga toxin-producing *E. coli* serotypes isolated from sheep [73].

3.3. Pulsed field gel electrophoresis (PFGE)

The main genotyping methodology for the molecular subtyping of *E. coli* O157:H7 in PulseNet network is PFGE [74]. PulseNet protocol recommends for casting plugs in disposable plug molds a cell suspensions of OD610=0.8-1.0 and a volume of 200 µl cell suspension, 10 µl Proteinase K (20 mg/ml stock) and 200 µl of agarose [74]. Restriction enzymes used for *E. coli* O157 and non-O157 are *Xba*I (50U/ample) as the primary enzyme, *Bln*I/*Avr*II (30U/sample) as the secondary enzyme and *Spe*I (30U/sample) as the tertiary enzyme. Macrorestriction fragments will be separated in 1% SeaKem Gold (SKG) agarose in freshly prepared 0.5X TBE cooled to 14°C in a PFGE-system (*e.g.* CHEF Mapper). The electrophoresis running times for CHEF Mapper used at CDC for *E. coli* O157 strains restricted with *Xba*I or *Avr*II (*Bln*I) are: Auto Algorithm, 30 kb - low MW, 600 kb - high MW; select default values except where noted by pressing "enter"; change run time to 18 - 19 h; default values: initial switch time = 2.16 s; final switch time = 54.17 s. The electrophoresis running times for non-O157 Shiga toxin-producing *E. coli* (STEC) strains restricted with *Xba*I or *Avr*II (*Bln*I) (CDC-PulseNet protocol for CHEF Mapper) are: auto algorithm, 50 kb - low MW, 400 kb - high MW; select default values except where noted by pressing "enter"; change run time to 18 - 19 h; default values: initial switch time = 6.76 s; final switch time = 35.38 s [74].

3.4. Ribotyping

Some reference laboratories use ribotyping in epidemiological investigations of the human disease outbreaks with *E. coli* O157:H7 [62]. However, studies performed by different scientists' teams have done different recommendations on the usefulness of this method in practice [75, 76]. Tarkka *et al.* (1994) obtained in ribotyping different values from a serogroup to other while Martin *et al.* (1996) could differentiate serogroup O157 from O55 but could not differentiate phage types in serogroup O157 [75, 76].

3.5. Microarray analysis

Above has been described a simple and fast multiplex PCR designed to confirm presence of three virulence determinants [62]. An improved assay based also on DNA technology is DNA and oligonucleotide microchip

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(microarray) technology [77, 78]. Hybridization of PCR products to glass microchips proved to be rapid and reliable in characterization of *E. coli* strains [79, 80]. Bekal *et al.* (2003) proposed a rapid and sensitive DNA microarray analysis of the *E. coli* pathotypes with a wide range of applications in food security, drinking water testing, environmental research and most of all in medicine [80].

4. Nucleic acid based assays for rapid detection of *Salmonella* spp.

Identification and characterization of S*almonella* strains in samples collected throughout the food chain, with or without humane cases, suppose several diagnostic activities that consist in biochemical profiling, serotyping and phage typing [81-83]. Despite numerous molecular methods developed in the last twenty years, conventional methods of *Salmonella* typing are still commonly used [82-85]. Most molecular typing methods have been validated only on a subset of serovars. PFGE is accepted as the reference method in subserovar identification [74], and serovar identification need to be done by slide or tube agglutination tests, in order to determine the O factor(s) and the H antigen(s), and in special circumstances the Vi antigen (present in *S.* Typhi, *S.* Paratyphi C and *S.* Dublin) [86]. Recently it was developed DNA microarray for molecular epidemiology of *Salmonella* spp. that targets simultaneously several major genes [87]. These composite microarrays identify serovars and subserovars in 1-2 days.
Despite of these drawbacks, DNA-based methods are in continuous development, and detection of *Salmonella* spp. in food products can be done by different PCR, qPCR or PMA-LAMP (propidium monoazide with loop-mediated isothermal amplification) methods [1-3, 6, 7, 26-28, 57, 88-90].

4.1. PCR-based methods

Even if the PCR-based methods do not allow identification of all *Salmonella* serovars and subserovars, the last developed methods were designed to supply as sun as possible specific and precise results concerning food contamination and the most possible source on infection [6, 85, 91, 92]. Malorny *et al.* (2004) proposed a 5′ nuclease (TaqMan) real-time PCR for the specific detection of *Salmonella* in food, with the overall analysis time of 24 hours, but more reliable seem to be quantitative real-time PCR that use samples treated with ethidium monoazide or propidium monoazide [85, 93, 94]. Wang and Mustapha (2010) used in their EMA-real-time PCR the following pairs of primers and probes: *Salm*-1: 5'-GCGACTATCAGGTTACCGTGGA-3', *Salm*-2: 5'-AGTACGGCCTGCTTTTATCG-3' and *Salm*-probe: FAM-TAGCCAGCGAGGTGAAAACGACAAAGG-TAMRA for specific detection of *Salmonella* and IAC-for: 5'-GCAGCCACTGGTAACAGGAT-3', IAC-rev: 5'-GCAGAGCGCAGATACCAAAT-3' and IAC-probe: VIC-AGAGCGAGGTATGTAGGCGG-TAMRA for internal control. PCR amplification was done as follows: initial denaturation - 95°C for 2 min, denaturation - 95°C for 1 min, annealing - 60°C for 1 min, extension - 72°C for 1 min, 40 cycles [94].

4.2. DNA fingerprinting methods

Among the most used molecular methods of *Salmonella* typing are multilocus sequence typing (MLST) and multiple-locus variable number tandem repeat analysis (MLVA) [89, 90]. Between these two methods, MLVA is often preferred because generates reproducible and clear results. Even more, in some aspects MLVA is more useful than PFGE, and its inclusion as a standard diagnostic method for some *Salmonella* serotypes would not be a surprise. A number of research teams have developed and manages database for *Salmonella* serotypes and other bacteria [*e.g.* MLST Databases at the ERI (University College Cork, Ireland): http://mlst.ucc.ie/mlst/dbs/Senterica; Institute Pasteur MLVA database (Paris, France): www.pasteur.fr/mlst; Institute Pasteur MLVA database (Paris, France): http://www.pasteur.fr/mlva]. In 2009 Larsson *et al*. proposed a new nomenclature for *S. typhimurium* based on 5-locus MLVA (STTR3, STTR5, STTR6, STTR9 and STTR10) to assigning standardized names for MLVA profiles [90].

4.3. Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a DNA amplification technique that uses four or six specially designed primers and a strand-displacing *Bst* DNA polymerase to produce a target-specific stem-loop DNA [6, 95]. Recently Chen *et al.* (2011) improved LAMP assay by previously treatment of samples with propidium monoazide [6]. PMA-LAMP simplicity, rapidity, sensitivity and quantitative capability can be exploited especially in the detection viable bacteria that do not require typing or in food security programs to better control of *Salmonella* spp [6]. Chen *et al.* (2011) used in PMA-LAMP the *Salmonella inv*A target gene and the following six primers: F3: 5'-CGGCCCGATTTTCTCTGG-3', B3: 5'-CGGCAATAGCGTCACCTT-3', FIP: 5'-GCGCGGCATCCGCATCAATA-TGCCCGGTAAACAGATGAGT-3', BIP: 5'-GCGAACGGCGAAGCGTACTG-TCGCACCGTCAAAGGAAC-3', Loop-F: 5'-GGCCTTCAAATCGGCATCAAT-3', Loop-B: 5'-GAAAGGGAAAGCCAGCTTTACG-3'; the reaction was carried out at 63°C for 40 min and terminated at 80°C for 5 min [6].

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4.4. Pulsed field gel electrophoresis (PFGE)

PFGE can be successfully used for epidemiological investigations in livestock production and foodborne outbreaks due to the ability of this method to distinguish between *Salmonella* isolates [96]. PulseNet developed a gold standard PFGE protocol to molecular *Salmonella* typing [74]. PulseNet protocol recommends for casting plugs in disposable plug molds, a cell suspensions of OD610=0.8-1.0 and a volume of 200 µl cell suspension, 10 µl Proteinase K (20 mg/ml stock) and 200 µl of agarose. Restriction enzymes used for *Salmonella* serotypes are *Xba*I (50U/ample) as the primary enzyme, *Bln*I/*Avr*II (30U/sample) as the secondary enzyme and *Spe*I (30U/sample) as the tertiary enzyme. Macrorestriction fragments will be separated in 1% SeaKem Gold (SKG) agarose in freshly prepared 0.5X TBE cooled to 14°C in a PFGE-system (*e.g.* CHEF Mapper). The electrophoresis running times for CHEF Mapper used at CDC for *Salmonella serotypes* restricted with *Xba*I or *Avr*II (*Bln*I) are: auto algorithm, 30 kb - low MW, 700 kb - high MW; select default values except where noted by pressing "enter"; change run time to 18 - 19 h; default values: initial switch time = 2.16 sec; final switch time = 63.8 sec [74]. However, analysis time for PFGE is 2-3 days, and the capacity of serovar identification is only partial [96].

4.5. Microarray analysis

Application of microarray analysis in *Salmonella* diagnostic for food industry is not yet in use, but the research studies are promising [87]. Huehn and Malorny (2009) designed a DNA microarray with 282 probes that targets *Salmonella enterica* subsp. *enterica* genes associated with pathogenicity, antibiotic resistance, fimbriae, prophages, H antigens, O antigens, plasmids, insertion sequence elements and metabolism [87].

5. Nucleic acid based assays for rapid detection of *Campylobacter* sp.

Campylobacter is foodborne pathogen that produces gastroenteritidis and sometimes bacteraemia, arthritis, Guillain–Barré syndrome and abortion in humans. The control of *Campylobacter* bacteria in the food chain is a major objective of the foodborne pathogens surveillance networks [97]. Simultaneously with classical methods of diagnostic has been developed molecular methods of *Campylobacter* detection in faecal and meat samples [97-101].

5.1. PCR-based methods

Routine *Campylobacter* screening in poultry slaughterhouses in Denmark use PCR-based methods [98], and real-time PCR commercial kits are already done [99, 100]. Josefsen *et al.* (2010) developed a rapid method, based on real-time PCR and propidium monoazide treatment, able to detect and quantify viable *Campylobacter* bacteria on chicken carcasses. The method has been considered for implementation in reference and research laboratories. The pair of primers targeted *C. jejuni* 16S rRNA. The primers and probes used were For: 5'-CTGCTTAACACAAGTTGAGTAGG-3', Rev: 5'-TTCCTTAGGTACCGTCAGAA-3', *Campylobacter* Probe: FAM-TGTCATCCTCCACGCGGCGTTGCTGC-TAMRA, IAC probe: VIC-TTCATGAGGACACCTGAGTTGA-TAMRA. The PCR profile proposed in 2010 by Josefsen *et al.* has been the following parameters: initial denaturation - 95°C for 3 min (10 min for colony PCR), denaturation - 95°C for 15 sec, annealing - 60°C for 60 sec, extension - 72°C for 30 sec, 40 cycles [101].

5.2. DNA fingerprinting methods

Several DNA fingerprinting methods for *Campylobacter* spp. have been developed, but not all of them have been widely extended in diagnostic laboratories. Molecular epidemiology studies used AFLP, flagellin gene short variable region sequencing, the flagellin gene restriction fragment length polymorphism analysis or, more recently, 40-gene comparative genomic fingerprinting assays [102-105]. Only multi locus sequence typing (MLST) received a large and continuous database support which led to the implementation of a similar methodology in several laboratories [106, 107]. As at 5 April 2013, the *Campylobacter* PubMLST definitions database contains 157371 sequences, and 6509 MLST profiles [107]. MLST for *Campylobacter* uses internal fragments of the following genes: *asp*A, *gln*A, *glt*A, *gly*A, *pgm*, *tkt* and *unc*A [108].

5.3. Pulsed field gel electrophoresis (PFGE)

High genetic diversity and chromosomal rearrangements may limit molecular epidemiology analysis of *Campylobacter* strains by PFGE [105]. However, PulseNet developed for molecular subtyping of *C. jejuni* a PFGE tool [109]. The PulseNet protocol recommends for casting plugs in disposable plug molds, a cell suspensions of OD610=0.570 to 0.820 and a volume of 400 µl cell suspension in 400 µl melted 1% SeaKem Gold agarose with 20µl proteinase K. Restriction enzymes used for *C. jejuni* are *Sma*I as the primary enzyme and *Kpn*I as the secondary enzyme. Secondary enzyme is used where the PFGE patterns obtained with the primary enzyme cannot differentiate two or more isolates. Macrorestriction fragments will be separated in 1% SeaKem Gold (SKG) agarose in freshly prepared 0.5X TBE cooled

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to 14°C in a PFGE-system (*e.g.* CHEF Mapper) [109]. The electrophoresis running times for CHEF Mapper used at CDC for *C. jejuni* restricted with *Sma*I are: auto algorithm, 50 kb - low MW, 400 kb - high MW; select default values except where noted by pressing "enter"; change run time to 18 h; default values: initial switch time = 6.76 s; final switch time = 35.38 s. The electrophoresis running times for *C. jejuni* restricted with *Kpn*I are: two state; gradient – 6.0V; change run time to 18 hours; included angle – 120; initial switch time =5.2 s; final switch time =42.3 s; select default values except where noted by pressing "enter" [109].

5.4. Microarray analysis

The use of microarray in identification of *Campylobacter* spp has been proposes by several teams of researchers [110-112], but its widespread use in laboratories not yet been done. However, it is worth remembering the improved method proposed by Quinones *et al.*(2007) that it's had a sensitivity of 10.000 *C. jejuni* cells and an increased efficiently of *C. jejuni* detection directly both in package liquid from whole chicken carcasses and in enrichment broths [110].

6. Conclusion

The food industry requires fast and sensitive methods of analysis for the food production monitoring that can need a number of criteria for selection. In the last twenty years molecular typing methods experienced a rapid development and in combinations with phenotyping methods have been included in several new standards of pathogens diagnostic. The diagnostic protocols may include one or, much better, a combination of the following methods of bacterial genotyping groups:

1. PCR-based typing methods: randomly amplified polymorphic DNA PCR (RAPD-PCR);
2. DNA fingerprinting methods: multilocus sequence typing (MLST), multi-virulence-locus sequence method (MVLST), multilocus variable-number tandem repeat analysis (MLVA);
3. Restriction fragment length polymorphism versions: classical RFLP, conventional terminal restriction fragment length polymorphism (TRFLP or T-RFLP), two-dimensional electrophoresis separation T-RFLP (TDES-TRFLP), cleaved amplified polymorphic sequence (CAPS or PCR-RFLP), amplified fragment length polymorphism (AFLP or AFLP-PCR), three endonuclease AFLP (TE-AFLP), amplified ligation selected fragment length polymorphism (ALIS-FLP), complementary DNA amplified fragment length polymorphisms (cDNA-AFLP); robust ordered mRNA differential display (RoDD); infrequent-restriction-site PCR (IRS-PCR), melting curve analysis of SNPs (McSNP) or restriction fragment melting temperature PCR (PCR-RFMT), inverse PCR-based amplified restriction fragment length polymorphism (iFLP);
4. Nucleic acid sequences gel electrophoresis: pulsed-field gel electrophoresis (PFGE), temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE);
5. Ribotyping;
6. DNA microarray typing.

It is very possible that the above list is larger and the categories names better set out, but this does not influence the quality of diagnostic procedures. In addition, it is expected that in the near future new molecular methods will increasingly used in current diagnosis of food-borne pathogens. Further research will establish which it is the best combination of them for a fast and accurate diagnosis.

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