



DETECTION OF *LISTERIA* PATHOGENS BY GRADIENT/ CONSTANT DENATURING GEL ELECTROPHORESIS

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Summary

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Listeriosis is a serious, but preventable disease, and the virulence factor of this disease is produced only by the two pathogenic species *L. monocytogenes* and *L. ivanovii* in humans and/or animals. In this study, we used both denaturing gradient gel electrophoresis (DGGE) and constant denaturing gel electrophoresis (CDGE) as molecular methods combined with Polymerase Chain Reaction (PCR) for the detection and identification of *Listeria* pathogens on 543 samples of raw milk collected from all Syrian provinces. The two methods are based on the PCR amplification of a fragment of the *InlC* gene (virulence gene) from the studied *Listeria* species and on the analysis of the PCR products obtained by DGGE/CDGE. Based on the differences present in the sequences amplified, it was possible to obtain species-specific DGGE/CDGE migration that allowed fast and easy identification of the virulence and pathogenicity of *L. monocytogenes* and *L. ivanovii* in humans and/or animals, in order to reduce the incidence of *Listeria* bacteria in the environment and foods and to prevent the occurrence of listeriosis in animal and human hosts.

Key words: constant denaturing gel electrophoresis (CDGE), denaturing gradient gel electrophoresis (DGGE), listeriosis, polymerase chain reaction (PCR), raw milk, virulence

INTRODUCTION

Listeriosis is a dangerous disease. *Listeria* spp. are ubiquitous Gram-positive bacteria present in a diversity of environments, including soil, vegetation, and water, where they survive as saprophytes (Al-Mariri *et al.*, 2013). Extensive research efforts in the preceding decades led to determination of two *Listeria* pathogens. *L. monocytogenes* was first described in the 1920s, but its impact as a food-borne

pathogen was not fully appreciated until the 1980s (McLauchlin, 1997), although it is apparent that *L. monocytogenes* causes an extremely serious, invasive, and often life-threatening food-borne disease (listeriosis). In very rare instances, this disease is due to *Listeria ivanovii* (previously known as *L. monocytogenes* serotype 5) (Guillet *et al.*, 2010). Although some early clinical manifestations of human listerio-

sis are mild, nonspecific, and influenza-like (e.g., chills, fatigue, headache, muscular and joint pain, as well as gastroenteritis), serious systemic illness occurs, including septicaemia, meningitis, encephalitis, abortions, and, in some cases, death (Liu *et al.*, 2012).

The average mortality rate from human listeriosis around the world is 30%, which is higher than that for most of other common food-borne pathogens such as *Salmonella enteritidis* (with a mortality of 0.38%), *Campylobacter* species (0.02–0.1%), and *Vibrio* species (0.005–0.01%), (Mead *et al.*, 1999).

The early generation laboratory diagnostic tests for *L. monocytogenes* are largely phenotype-based and usually assess *L. monocytogenes* gene products by biological, biochemical, and serological means. As the phenotypic properties of *Listeria* may vary with the constantly changing external conditions, growth phase, and spontaneous genetic alterations, use of the phenotype-based diagnostic procedures may lead to equivocal results at times. In addition, since many phenotypic tests are dependent on lengthy *in vitro* culture procedures, they are notoriously time consuming, thus delaying result availability. To circumvent the drawbacks of the phenotype-based procedures, new generation genotype-based methods targeting the nucleic acids (DNA or RNA) of *Listeria* have been developed over the preceding decade. As nucleic acids (particularly DNA) are intrinsically more stable than proteins and less prone to influences by external factors, the genotypic (or molecular) diagnostic procedures are much more precise and less variable than the phenotype-based methods. Furthermore, as many of the genotypic techniques involve template and/or signal amplification, they are also much faster and

more sensitive. Application of these state-of-the-art genotypic identification procedures such as denaturing gradient gel electrophoresis (DGGE) and constant denaturing gel electrophoresis (CDGE) attaching with PCR technology have the potential to overcome the problems associated with the phenotype-based tests, providing unprecedented levels of sensitivity, specificity, and speed for laboratory detection and identification of *Listeria* without the need to culture the suspect microorganism(s) (Cocolin *et al.*, 2002).

Denaturing gradient gel electrophoresis (DGGE) is one of several methods that can be used to screen DNA fragments for small sequence changes or point mutations. The separation principle of DGGE is based on the melting behaviour of DNA molecules, whereas CDGE is a modification of denaturing gradient gel electrophoresis.

After a mutation has been identified by previous DGGE gels, a CDGE gel can be used to rapidly screen samples for the presence of a mutation. Then, DGGE, which is based on the discontinuous phenomenon of strand dissociation, allows the resolution of DNA fragments differing by as little as a single nucleotide substitution. In mutation analysis using parallel DGGE, field strength, temperature, and run time must be strictly controlled to achieve reproducible results.

DGGE is complicated by the difficulties of choosing the exact running time and gel conditions to achieve the optimal separation. By running a DGGE gel too long, an achieved separation decreases, and may even be lost. Mutations present in a homo- or hemizygote state, where heteroduplexes are not formed, will be missed. Several of these problems are eliminated by CDGE in which a single denaturing condition is used to melt a

fragment. In CDGE, wild-type and mutant fragments will melt partially into a certain configuration immediately after entering the gel. This configuration will be kept throughout the run, and the fragments will migrate with a constant rate. Therefore, the longer the gel is run, the wider the separation between the mutant and wild-type samples.

DGGE/CDGE are useful techniques to monitor microbial populations, without culture dependant methods (Adil, 2015). PCR-DGGE is sorted as part of the new discipline of microbial population as *Pyrenophora* and *Mycoplasma* species (Mcauliffe *et al.*, 2005; Mavragani *et al.*, 2011).

The combined DGGE/CDGE system was chosen as it might have the advantages of both systems; heteroduplex molecules may become visible in the DGGE component, while the CDGE component should prevent complete strand dissociation.

In our study, specific primers for listerial pathogens were targeted typically to a sequence of a gene coding for a virulence factor (Internalin "*InlC*") unique to *L. monocytogenes*, *L. ivanovii* (Engelbrecht *et al.*, 1998; Vazquez-B *et al.*, 2001) in order to highlight the ability of PCR-DGGE/CDGE for detection of *Listeria* in milk (Quigley *et al.*, 2013). Then the PCR products have been analysed by density gradient gel electrophoresis (DGGE) (perpendicular or parallel denaturing gel) which was able to separate DNA fragments of the same size when the fragments display differences in the sequence of the base pairs that yield different G+C content, while these fragments have been resolved as discrete bands on a gel, so the denaturant concentration that gives optimal resolution from a parallel or perpendicular DGGE gel is held constant. This means that the optimum concentra-

tion of denaturant to use for a CDGE is determined from the visible maximum split on the gel which distinguish between two *Listeria* pathogens.

In this study, we aimed to show the capability of DGGE/CDGE with respect to their use as a fast screening test to investigate the presence of *Listeria* pathogens in the studied samples.

MATERIALS AND METHODS

Bacterial strains

Listeria spp. strains in this study were isolated from 543 raw milk samples (370 bovines, 173 sheep) which were collected from different provinces of Syria (collected from unorganised sectors) from September 2010 to December 2013. Then, these strains were cultured on brain heart infusion (BHI; Oxoid) broth and incubated at 30 or 37°C overnight before being subjected to analysis by molecular methods.

Traditional isolation of Listeria pathogens

Previous isolations were identified by using traditional methods. In particular, Gram staining, catalase test, oxidase activity, H₂S production, mobility test, β-haemolysis, camp test, and production of acids from rhamnose and xylose were used (Iordache & Tofan, 2008; Al-Mariri *et al.*, 2013).

DNA extraction

Briefly, for the extraction of *Listeria* DNA, 1 mL of pure *Listeria* culture was centrifuged at 13.000×g for 5 min at room temperature. The DNA was then extracted using a genomic DNA purification kit (Fermentas, GmbH, Germany), and the DNA concentration was determined using

a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific mbH, Germany) at 260 nm.

PCR amplification of target regions

The PCR primers used for the amplification of DNA fragments spanning the *InlC* gene (member of Internalin multigene family) for *L. monocytogenes* and *L. ivanovii* were targeted at highly conserved regions of this gene. Primers for the detection of the *Listeria* spp. isolates are given in Table 1.

Five hundred ng of extracted genomic DNA were used for PCR amplification. The PCR reaction mixture (25 µL) contained 5 µL PCR buffer HotStar HiFidelity (Qiagen, Germany), 200 µM of each dNTP (Promega, USA), 2.5 mM MgCl₂, 25 pmol of each primer and 1.5 U HotStar HiFidelity DNA Polymerase (Qiagen, Germany) according to standard protocols (Innis *et al.*, 1990; Luo *et al.*, 2004).

PCR amplification reactions were performed in a GeneAmp® PCR system 9700 (Applied Biosystems, USA), (30–40 pb) GC clamps were added to one of pair primers (5'- GCG GGC GGC GCG GGC

CGC GGG CAG GGC GGC GGG GGC GGG GG C GGC -3') to improve its sensitivity in the detection of DNA sequence by DGGE analysis as previously described. To minimise non-specific annealing of the primers, Taq polymerase was added after the first denaturing step, at a temperature of 80°C, a technique referred to as “Hot Start”. Another technique (Muyzer *et al.*, 1995) referred to as “Touchdown”, was also used to reduce the formation of spurious by-products (Wawer & Muyzer, 1995). This involved setting the annealing temperature (Tan) 10 °C higher than the calculated primer melting temperature for the first cycle, then decreasing it by 0.5 °C every cycle for 20 cycles, and finally annealing at the T_m during 9 subsequent cycles as shown in Table 2.

The data in the table explain that when the reaction of PCR was carried out with primer pair mon/ivaF (with GC clamp) and mon/ivaR, the annealing temperature (Touchdown) was set at 66 °C for the first cycle and decreased by 0.5 °C each cycle thereafter for 20 cycles, with the final 9 cycles performed at T_m of 56 °C as shown.

Table 1. Primers used for PCR and amplification of *InlC* region of *Listeria* spp. isolates

Primer sequence (5'-3')	<i>Listeria</i> spp.
GCGGGCGGCGCGGGGCGCGGGCAGGGCGGCGGGGGCGGGC	<i>L. monocytogenes</i>
CTCTTTTGTGTTTCTAAATTTTCATTTTGTAACCAATTATTTT	<i>L. ivanovii</i>

Table 2. Hot Start and Touchdown PCR protocol

94°C	3 min	
80°C	1 min	
66°C	1 min	Repeated 20 times with the annealing temperature decreased by 0.5°C every cycle
75°C	45 sec	
56°C	1 min	Repeated 9 times at T _m
75°C	45 sec	
94°C	1 min	
61°C	45 sec	
75°C	10 min	

In the final step of the reaction, the mixture was held at 75 °C for 10 min to allow the extension of incomplete products (Nübel *et al.*, 1996; Aznar *et al.*, 2002).

The amplified products were analysed by agarose gel electrophoresis as follows. Ethidium bromide (Sigma, Germany) was added to 2% agarose solution at a concentration of 0.5 mg/mL before the gel was poured in the mold. The gel was run for 1 h at 50 V, and the PCR product was visualised under UV-light. Then, the PCR product was purified using a QiaQuick PCR purification kit (Qiagen, Milan, Italy) and sequenced by a commercial facility (MWG Biotech, Edelsberg, Germany). The amplified products were stored at -20 °C until they were used for DGGE.

DGGE analysis

The Dcode Universal Mutation Detection system (Bio-Rad, Hercules, USA) was employed to separate the sequence-specific of the PCR products.

Perpendicular gradient gel analysis

This analysis uses a range of denaturants to separate the two *Listeria* pathogens DNA fragments. Electrophoresis was performed in a 1 mm-thick (16×16 cm) polyacrylamide gel (10% w/v acrylamide-bisacrylamide 37.5:1) containing 20 to 70% urea/formamide denaturing gradient (100% denaturant is a mixture of 7M urea and 40% v/v formamide) (McAuliffe *et al.*, 2005), increasing in the direction of the electrophoretic run. The gel was cast using a Bio-Rad Model 385 Gradient Delivery System (Bio-Rad Laboratories, Hercules, CA).

We added 100 µL 2× gel loading dye (70% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanole, 2 mM EDTA) to the 100 µL equal mixer of the samples (amplified DNA of *L. monocytogenes* and

L. ivanovii), and electrophoresed them on the Dcode universal mutation detection system at 130 V for 2 h at 65 °C. After electrophoresis, the gels were stained in a 50 µg/mL ethidium bromide in 1× TAE buffer for 3 min, then destained the gel in 1× TAE buffer for about 20 min. The gels were imaged under ultraviolet transillumination.

Parallel gradient gel analysis

For this analysis, the denaturant is parallel to the electrophoresis direction. A time course run is initially done for parallel gels to find the optimal run time for the *L. monocytogenes* and *L. ivanovii* DNA samples. Here, electrophoresis was performed in a 1 mm-thick (16×16 cm) polyacrylamide gel (10% w/v acrylamide-bisacrylamide 37.5:1) containing 40–65% urea/formamide denaturing gradient. Five µL of PCR products were mixed with 5 µL 2× gel loading dye, and electrophoresed on the Dcode universal system at 150 V for 2.5 h at 65 °C. After electrophoresis, the gels were stained in a 50 µg/mL ethidium bromide in 1× TAE buffer for 3 min, then destained in 1× TAE buffer for about 20 min, and imaged under ultraviolet transillumination (Abu Al-Soud *et al.*, 2003).

Constant gradient gel analysis

Constant denaturing gel electrophoresis is a modification of DGGE. In CDGE, the denaturant concentration that gives optimum resolution from a parallel or perpendicular DGGE gel is held constant. For this analysis, the optimal concentration of denaturant is determined from the maximum split between *L. monocytogenes* and *L. ivanovii* DNA, as seen in the perpendicular or parallel denaturing gel. Here, electrophoresis was performed also in a 1 mm-thick (16×16 cm) polyacrylamide

gel (10% w/v acrylamide-bisacrylamide 37.5:1) containing a constant urea/formamide denaturing 51% gradient, 180–300 ng of amplified DNA was mixed with an equal volume of 2× gel loading dye, and electrophoresed on the Dcode universal system at 130 V for 2.5 h at 65 °C. Then, after electrophoresis, the protocol was as mentioned in both previous analyses (Wu *et al.*, 1999; Farnleitner *et al.*, 2000).

Heteroduplexes preparation for DGGE analysis

It is important to optimise the PCR reaction to minimise unwanted products which may interfere with gel analysis. Heteroduplexes could be generated during PCR by amplifying DNA of *L. monocytogenes* and *L. ivanovii* samples in the same tube. The tube was heated at 95°C for 5 min, then placed at 65 °C for 1 h, and let slowly to cool to room temperature (Tchernitchko *et al.*, 1999).

RESULTS

Isolation of Listeria

Among the 543 samples, thirty-nine *Listeria* strains were identified: 29 as *L. monocytogenes* (43%), and 10 – as *L. ivanovii* (15%). All isolates were Gram positive bacteria, regular, short rods, 0.4–0.5 by 1–2 µm of size, with parallel sides, and blunt ends and usually occurred singly or in short chains in Oxford and Palcam agar cultures when incubated at 30 °C for 48 h, catalase-positive and oxidase-negative.

Results of amplification InlC gene as a target for specific Listeria pathogens

The set of primers was designed based on the partial alignments of the *InlC* gene from the *Listeria* pathogens (*L. monocytogenes*, *L. ivanovii*), the primers chosen

were able to prime the amplification of the two pathogens tested, due to their high or complete homology to the sequence of the *InlC* gene considered. More specifically, two primers (forward, reverse) were completely homologous to the reference sequence of the two *Listeria* species under consideration. So, these primers were able to amplify a region that contained only one significant difference (G: in *L. monocytogenes* – 5'CTCTTTT GTGTTTCTAA ATTTATTTTTAAGGAGTGGAGAATG TTGAAAAAAAAATAATTGGTTACAA AATG3'; A: in *L. ivanovii*: 5'CTCTTT TGTGTTTCTAAATTTATTTTTAAGG AGTGGAGAATATTGAAAAAAAAATA ATTGGTTACAAAATG3').

Primer pair (mono, iva)F/R yielded a single band on agarose gel corresponding to a 70 bp DNA fragment when the PCR reaction was carried out with Taq polymerase on DNA extracted from tested *L. monocytogenes*, *L. ivanovii* samples. That a single band was obtained with each primer pair indicated that only the target gene was amplified, with no non-specific primer attachment or heteroduplexes formed as seen in Fig. 1, which summarises the amplification results by PCR reaction of the target DNA of *InlC* gene of two *Listeria* strains using specific primers sets: lanes 1, 4 indicated standard *L. monocytogenes* gene and *L. ivanovii*, respectively (70 bp); lanes 2, 3 – tested *L. monocytogenes*, *L. ivanovii*, respectively (also 70 bp), whereas lane 5 – genus *Brucella* which in our lab was used as negative control.

All isolates were well-defined as *L. monocytogenes*, *L. ivanovii* by PCR reaction, in order to study them by DGGE analysis.

Perpendicular gradient gel analysis

In perpendicular denaturing gradient gel, a range of denaturants (20–70%) was used

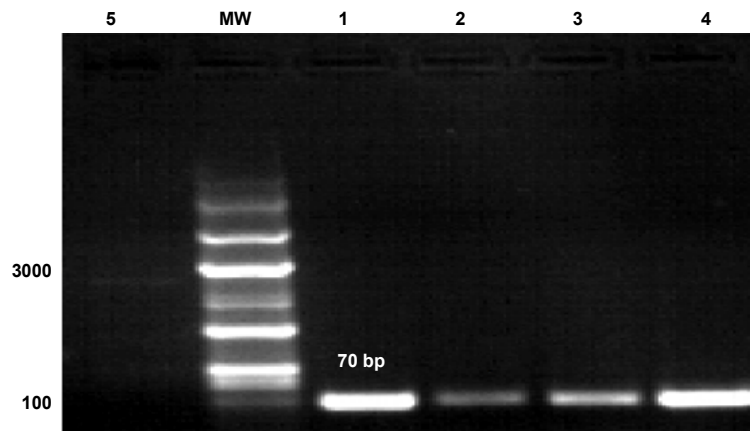


Fig. 1. Electrophoresis on 1.5% agarose gel showing the PCR products of *InlC* gene. MW: DNA ladder; lane 1: amplification of standard *L. monocytogenes*; lane 2: amplification of tested *L. monocytogenes*; lane 3: amplification of tested *L. ivanovii*; lane 4: amplification of standard *L. ivanovii*, lane 5: referred to genus *Brucella* which was classified in our lab as negative control.

to separate the *L. monocytogenes* and *L. ivanovii* strain DNA fragments of *InlC* gene. Both were electrophoresed on a perpendicular denaturing gradient gel, and the DNA pattern formed a "S" shape. A split (difference in migration) was formed between the *L. monocytogenes* and *L. ivanovii* strain DNA. This separation was caused by the *L. ivanovii* DNA melting sooner than the *L. monocytogenes* DNA of the *InlC* fragment as shown on Fig. 2. On this figure, the perpendicular gels had an increasing gradient of denaturants from left to right, perpendicular to the direction of electrophoresis. During electrophoresis, the migrating double-stranded fragment (arrow three) began to melt upon exposure of various domains of the fragment to an increasing denaturant concentration. Domains with a low G+C% (as *L. ivanovii* DNA, arrow one) melted at a low denaturant concentration, while domains with high G+C% (as *L. monocytogenes* DNA, arrow two) melted at higher denaturant concentrations further down the gel.

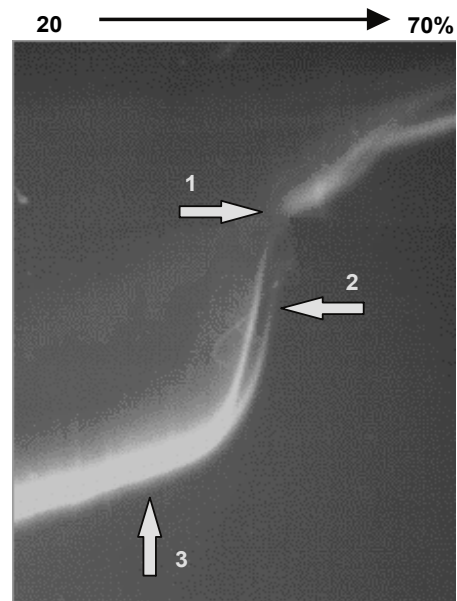


Fig. 2. Separation of *L. monocytogenes* and *L. ivanovii*-strain DNA electrophoresed on a 20–70% perpendicular denaturing gradient gel. The gel was 10% acrylamide:bis (37:5:1), run at 130 V in 1×TAE buffer, heated at 65 °C for 2 h (arrow 1: DNA *L. ivanovii*, arrow 2: DNA *L. monocytogenes*, arrow 3: DNA double stranded of both DNA of *L. monocytogenes* and *L. ivanovii*).

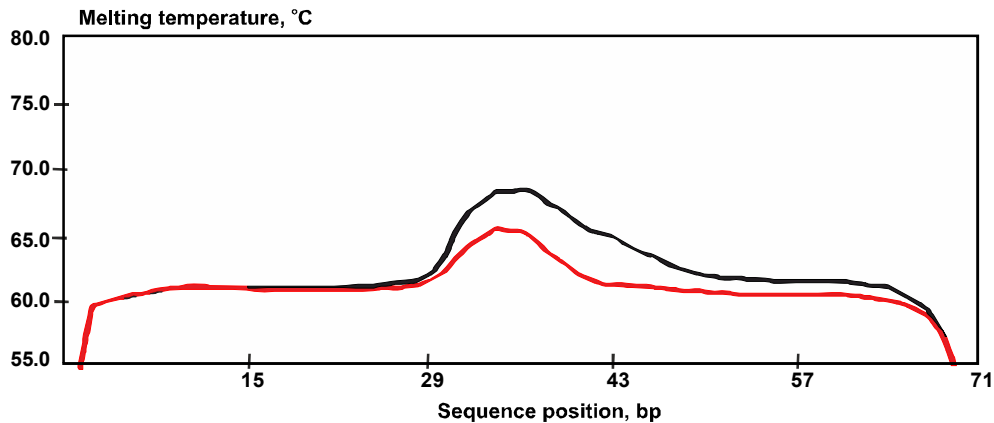


Fig. 3. The melting profile for the Internalin (*InlC*) sequence, it shows the split between the two sequences of *L. monocytogenes* (upper line) and *L. ivanovii* (lower line) DNA to determine the single base substitution (G to A), the melting profile for the Internalin sequence graphed using the 50% melted probability.

Therefore, the behaviour of a DNA fragment in a denaturing gradient gel could be predicted solely from the base-pair sequence. On the other hand, a computer program calculated the T_m for each base pair, and the result determined a calculated melting map (Fig. 3), which showed high- and low-temperature melting domains. Only single base substitution was detected (G in *L. monocytogenes*, A in *L. ivanovii*) in the low temperature melting domain of Internalin molecule as seen on Fig. 3, the split between the two sequences of *L. monocytogenes* and *L. ivanovii* DNA to determine the single base substitution (G to A), the melting profile for the Internalin sequence graphed using the 50% melted probability. The 50% melting probability is typically used for analysing melting profiles for denaturing gel electrophoresis. From the melting profile, it can be seen that there were two low-temperature melting regions and one high-temperature melting region in the middle of the sequence.

Parallel gradient gel analysis

In parallel DGGE, the denaturing gradient was parallel to the electric field, and the range of denaturant was narrowed to allow better separation of fragments. In this analysis, we used a range of denaturants (40–65%) to separate the *L. monocytogenes* and *L. ivanovii*-strain DNA fragments of internalin gene as shown on Fig. 4.

The parallel gels had an increasing gradient of denaturants from top to bottom, parallel to the direction of electrophoresis. Alignment of the base sequences of the *InlC* fragments targeted by the primer pair (mon, ivaF/R) had revealed one base that differed in the two fragments (G in *L. monocytogenes*, A in *L. ivanovii*), so this difference yielded a DNA fragment of *L. ivanovii* with lower G+C%, melting at a lower denaturant concentration to produce a DGGE band that migrated a shorter distance through the gel (lane 1) than the band produced from *L. monocytogenes* fragment (lane 2),

while lane 3 contained the two *Listeria* pathogens fragments.

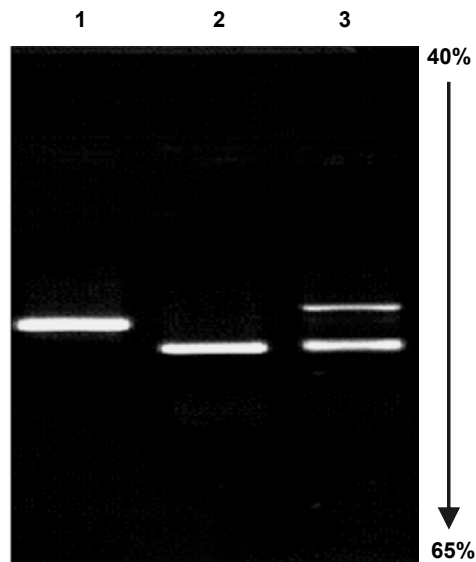


Fig. 4. Separation of *L. monocytogenes* and *L. ivanovii* strain DNA electrophoresed on a 40–65% parallel denaturing gradient gel. The gel was 10% acrylamide:bis (37:5:1), run at 150 V in 1×TAE buffer, heated at 65 °C for 2.5 h. Lane 1: DNA of *L.ivanovii*; lane 2: DNA of *L.monocytogenes*; lane 3: DNA of two *Listeria* pathogens.

Constant gradient gel analysis

The *L. monocytogenes* and *L. ivanovii* amplified DNA run on a constant denaturing gel (CDGE). For this gel, a single denaturant concentration was used to melt the fragments of *Inlc* gene. The concentration of denaturant for CDGE was determined at the maximum split between the two studied *Listeria* species DNA, as seen in a perpendicular denaturing gradient gel.

The concentration of denaturant for CDGE gel was calculated first by placing a fluorescent ruler along the axis of the denaturant gradient when taking a photograph as seen in Fig. 5 which explained

that the distance along the gradient where the maximum split seen between two *Listeria* strains was 5 cm. This distance was divided by the length of the gel and multiplied by the denaturant range $(5-8) \times 50\% = 31\%$. Then, this number was added to the starting denaturant concentration to determine the optimum concentration to use for CDGE $(20\% + 31\% = 51\%)$. Therefore, the CDGE gel used a denaturant concentration of 51%.

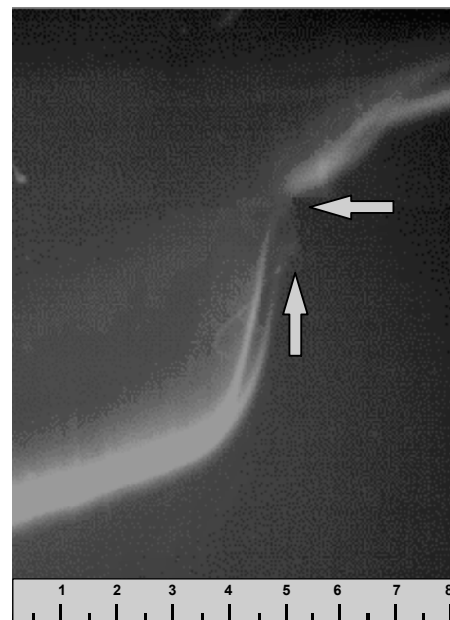


Fig. 5. Perpendicular DGGE gel used for determining the optimum denaturant concentration used in a CDGE gel.

Heteroduplexes analysis

Heteroduplex analysis (HA) was based on conformational differences in double-stranded DNA caused by the formation of heteroduplex molecules. Heteroduplex molecules had a mismatch in the double-strand, caused a distortion in its usual conformation and detected on parallel gel due to slower migration than the correspon-

ding homoduplex molecules. Heteroduplex molecule with as little as one mismatch could show a difference in mobility in a gel than homoduplex molecules as seen on Fig. 6 which showed that melting behaviour of the heteroduplexes was altered so that they melt at a lower denaturant concentration than the homoduplexes and could be visualised on a parallel denaturing gradient gel.

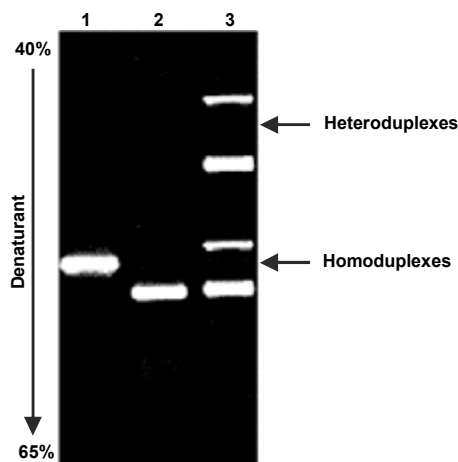


Fig. 6. *L. monocytogenes* and *L. ivanovii* DNA fragments that were denatured and re-annealed to generate four fragments run on a parallel denaturing gradient gel. Lane 1: *L. ivanovii* DNA, Lane 2: *L. monocytogenes* DNA, Lane 3: two heteroduplexes and two homoduplexes of *L. monocytogenes* and *L. ivanovii*.

DISCUSSION

Amplification of DNA by genus-specific primers that target DNA sequences and the subsequent differentiation of this DNA on DGGE gel is known as PCR-DGGE (Ercolini *et al.*, 2004; Dar *et al.*, 2005). It is a rapid and efficient separation technique of the same length DNA sequences (amplified by PCR), which may vary as little as a single base pair modification as

in our study on *Inlc* gene for the determination of the two *Listeria* pathogens.

In this study, the development of a PCR-DGGE method to directly identify the pathogens of the genus *Listeria* (*L. monocytogenes* and *L. ivanovii*) in milk samples is described. This approach exploited the potential of PCR to amplify, with specific primers, variable regions within the *Inlc* gene as well as the discriminatory power of DGGE/CDGE to differentiate DNA molecules on the basis of differences in their sequence (Luo *et al.*, 2004). The *Inlc* gene has been demonstrated to be a reliable PCR target for the differentiation of *Listeria* spp., (Eruteya & Odunfa, 2014).

The fragment of studied *Inlc* gene was characterised from different *Listerial* pathogens and demonstrated the presence of conserved regions at the 5'/3' ends and a species-specific internal region. This gene was used to design primers that specifically amplify *Listeria* spp., (Moreno *et al.*, 2014) and that it could be used for specific identification using the DGGE/CDGE method. Two primers were identified for the region of the sequences that were conserved among different *Listeria* spp. The regions amplified contained a high degree of heterogeneity among the sequences considered, allowing their differentiation by DGGE/CDGE. The PCR products produced from studied samples were used to optimise the experimental conditions (denaturant gradients, temperature, voltage, and length of the electrophoretic run) of the DGGE (perpendicular-DGGE/parallel-DGGE and CDGE). Denaturants from 40 to 65% in the parallel-DGGE showed the best differentiation power, allowing the identification, on the basis of specific migration, of the two pathogens considered in the study which agreed with the study of Luca Cocolin *et*

al. (2002) about using DGGE in detecting the food-borne bacterial pathogen *L. monocytogenes* by the amplification the *iap* gene, while, the *iap* gene has been also demonstrated to be a reliable PCR target for differentiation of *Listeria* spp. (Rawool *et al.*, 2007). Simultaneously, Petersen *et al.* (2007) explained that the PCR combined with DGGE as capable to distinguish another species of bacteria as *Campylobacter*, *Helicobacter*, *Arcobacter* and related *Epsilobacteria* in saliva samples from humans and domestic pets.

Furthermore, this methodology can be utilised in diverse subject areas. For instance, DGGE allowed the identification of over 65 *Mycoplasma* species of human and veterinary origins in less than 24 hours (McAuliffe *et al.*, 2005). Mycoplasmas are fastidious organisms that require many weeks to culture and other serological tests to be identified. They cause various diseases associated with pneumonia, arthritis, conjunctivitis, infertility and abortion. Moreover, a total of 16 *Bifidobacterium* species were rapidly identified by PCR–DGGE method targeted on a 770-bp region of the *tuf* gene (Sheu *et al.*, 2013). These applications of PCR-DGGE could potentially allow considerable savings of time, life and treatment costs.

Also, Dang *et al.* (2002) assured the importance of DGGE which is one of the most consistently used mutation scanning methods by using of single base changes in the *p53* gene to detect minimal residual disease of breast cancer. Mark McCammon *et al.* (2005) demonstrated the power of DGGE in exposing of *pncA* mutations associated with pyrazinamide resistance in *Mycobacterium tuberculosis* isolates from the United states-Mexico Border Region. In addition, DGGE has been established to have a real potential in screening a

large number of patients for rapid and reliable identification of deleterious changes in the oncogenes *BRCA1* and *sBRCA2* (Van der Hout *et al.*, 2006). Hence PCR-DGGE allowed the detection of numerous mutations and revealed the existence of unclassified variants that were not reported before. Over and above, the recent studies by Liao *et al.* (2015) aimed to simultaneously construct PCR-DGGE-based predictive growth models of *Listeria monocytogenes* and *Vibrio parahaemolyticus* on cooked shrimps at 4 and 10°C, but it is inappropriate for inactivation models yet.

It is to be noted that PCR-DGGE became more important when combined with CDGE, which we tested in our study when the optimum concentration of denaturant on the electrophoresis gel was determined. This matter was explained by Wu *et al.*, (1999) whereas the combination between DGGE and CDGE had the advantages of both methods, because it prevented the dissociation of the total strand DNA, raised the capability of separation the heteroduplex molecules, and it was proved successful in the detection of DNA variants in several GC-rich fragments.

CONCLUSIONS

So, from all mentioned previous data, we concluded that using of DGGE/CDGE technique, diagnosis of emerging infections could become easier and faster, and identification of uncultivable pathogens can also now be facilitated, because, understanding complex microbial populations would certainly help us in rapid decision-making with regard to adequate treatment and other major interventions aiming at making the world a better and safer place to live.

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