Genotyping of *Listeria Monocytogenes* in Iraqi Women with Spontaneous Abortion Using Pulse Field Gel Electrophoresis

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Abstract

Background   *Listeria monocytogenes* (*L. monocytogenes*) is a Gram-positive, facultative intracellular bacterial pathogen that can cause a severe invasive disease (listeriosis) mainly in pregnant women. Based on genetic content, *L. monocytogenes* can be divided into 3 lineages I, II and III. Several molecular methods have been developed to assist in the characterization of *L. monocytogenes*, macrorestriction analysis by pulse-field gel electrophoresis (PFGE) is one of the most used methods for the genotyping of *L. monocytogenes*.

Objective   To determine the predominant genotype of *L. monocytogenes* isolated from clinical cases in a group of aborted Iraqi women.

Methods   A study was designed and included 15 clinical isolates of *L. monocytogenes* and one isolate from locally made cheese. The PFGE protocol was performed as described by Graves and Swaminathan. Briefly, bacterial suspensions adjusted to optical density of 1.3 at 610 nm were embedded in 1.2% Sea Kem Gold agarose plugs. The lysed, washed five times and a 2 mm thick piece was cut, equilibrated and digested with 200 µl of AscI enzyme master mix at 37 °C for 2 h. The macrorestriction fragments were separated by electrophoresis on a CHEF-DRIIBIO-RAD. Images were analyzed with the software Bio Numerics Gel Compar II version 6.6.11 (Applied Maths).

Results   All *L. monocytogenes* isolates displayed AscI digestion profiles after incubation with the enzyme, about 6-12 majorfragments of 30 to 675 kb were obtained, sixteen isolates have been used in this study, however, 11 different AscI profiles were encountered, the distribution of the isolates according to the restriction profiles showed that two profiles were the most predominant A, A2 with a percentage of similarity of 100%.

Conclusion   *L. monocytogenes* genotyping showed profiles that can be well used in tracing down infection source and outbreak of this bacterium. PFGE represents a great discrimination and reproducibility method for molecular sub-typing of *L. monocytogenes* and it is considered the gold standard.

Keywords   *Listeria monocytogenes*, genotyping, placenta, aborted women, pulse field gel electrophoresis

List of abbreviations: CHEF = Contour-clamp homogeneous electric field, PFGE = Pulse field gel electrophoresis, RFLP = Restriction Fragment Length Polymorphism, UPGMA = Unweighted-pair group method with arithmetic averages

Introduction

*L*isteria monocytogenes (*L. monocytogenes*) is a Gram-positive intercellular, rod shaped, nonspore-forming, motile, facultative anaerobic, bacterium. It causes a severe invasive disease [1].

Listeriosis during pregnancy may lead to intrauterine infection, which may result in severe complications like preterm labor, spontaneous abortion, and stillbirth and or infection of the neonate which may result in high morbidity and mortality rate [2]. Of several molecular methods currently available like

polymerase chain reaction (PCR), amplified fragment length polymorphism detection, DNA sequencing and others, macrorestriction analysis by pulse-field gel electrophoresis (PFGE) is one of the most used methods for the subtyping of *L. monocytogenes*. The using of restriction endonuclease AscI, as advised by Pulse Net USA, has shown excellent discrimination for *L. monocytogenes* and the technique is shown to be reproducible. PFGE is considered to be the international standard for subtyping [3].

PFGE is a form of RFLP (Restriction Fragment Length Polymorphism) typing in which, bacterial genome is digested with a rare cutting restriction enzymes that cut the bacterial genomic DNA infrequently and therefore generate a small number of DNA fragments between (10-20 bands); these fragments are of a large sizes, from 20 kb to 10,000 kb, and are separated using specific electrophoresis techniques [4] for *L. monocytogenes*, the enzymes are AscI or ApaI, these enzymes may generate between 6 to 12 and 14 to 17 fragments respectively separated through the PFGE. Combinations of the two profiles generated by the enzymes are used to characterize L. monocytogenes strains [5].

Genetic comparisons among isolates carry out by using the differences in the restriction profiles. Computer-based analysis is very simple and enables rapid comparison on strains. Currently, PFGE represent the "gold standard" of the molecular typing techniques for foodborne pathogenic bacteria such as *Salmonella, E. coli, Yersinia, Vibrio* and *Listeria* [6].

The aim of this study and the benefit of it is to assess the extent of *L. monocytogenes* in causation of human spontaneous abortion in a sample of Iraqi women, and to determine the genetic subtypes of listeria monocytogenes associated with abortion.

Methods

A cross-sectional study was designed that included 250 placental tissues obtained from aborted women (which their acceptance was taken before sample collection) attended Al-Imamein AL-Kadhimein Teaching Hospital in Baghdad during the period from June 2014 to November 2015. Out of total number of the placentas samples, only fifteen isolates of *L. monocytogenes* were identified. The isolation and identification of *L. monocytogenes* were performed according to Collee et al. 1996 and McFaddin, 2000 [7,8].

PFGE of *L. monocytogenes*
The laboratory Protocol for molecular subtyping of *L. monocytogenes* by PFGE includes the following:
The Culture growth: An isolated colony from a pure culture of *L. monocytogenes* was streaked onto nutrient agar (NA) plates and the plates were incubated at 37 °C for 14-18 h.
Pulse field gel electrophoresis: The PFGE protocol was performed as described by Graves and Swaminathan (2001) [6]. Briefly, bacterial suspensions adjusted to optical density of 1.3 at 610 nm were embedded in 1.2% SeaKem Gold agarose plugs. The lysed, washed five times and a 2 mm thick piece was cut, equilibrated and digested with 200 µl of AscI enzyme master mix at 37 °C for 2 h. The macrorestriction fragments were separated by electrophoresis on a CHEF-DR II BIO-RAD (USA) in a 1% Pulse Field Certified Agarose gels (Ultrapure DNA grade agarose) at Initial switch time: 4.0 s; Final switch time: 40.0 s; Voltage: 6 V; Included Angle: 120° Run time: 18-19 hours.
Staining and Documentation of an Agarose gel: After electrophoresis run was over, gel was removed and stained with ethidium bromide for 20-30 min in covered container. The use of restriction endonucleases AscI, is advised by Pulse Net USA, and has shown excellent discrimination for *L. monocytogenes*.
Images were analyzed using Gel Compare II evaluation software version 6.6.11 from Applied Maths.
The similarity between fingerprints was determined using number of different bands coefficient with a 1% tolerance between band positions. The cluster analysis and generation of Dendrogram was performed using

Unweighted-pair group method with arithmetic averages (UPGMA) with a similarity score value of 100% as the cutoff.

Results

All isolates of Listeria monocytogenes were digested with AscI restriction endonuclease...GG▼CGCGCC... .

...CCGCGC▲GG...

Only one was isolated from local made cheese, which presented in lane number 1 (Figure 1). Isolates of *L. monocytogenes* were typed using PFGE. Analysis of the AscI restriction profiles by automated cluster analysis was done using number of different bands as a similarity coefficient and tolerance: 1 %.

All the isolates displayed AscI digestion profiles after incubation with the enzyme (Figure 1), about 6-12 major fragments of 30 to 675 kb were obtained following the digestion using AscI.

Sixteen isolates have been used in this study, fifteen represent the clinical isolates and the

Eleven PFGE types and two subtypes were distinguished with the restriction endonuclease AscI through visual inspection, using Unweighted-pair group method with arithmetic averages (UPGMA) with a similarity score value of 100% as the cutoff.
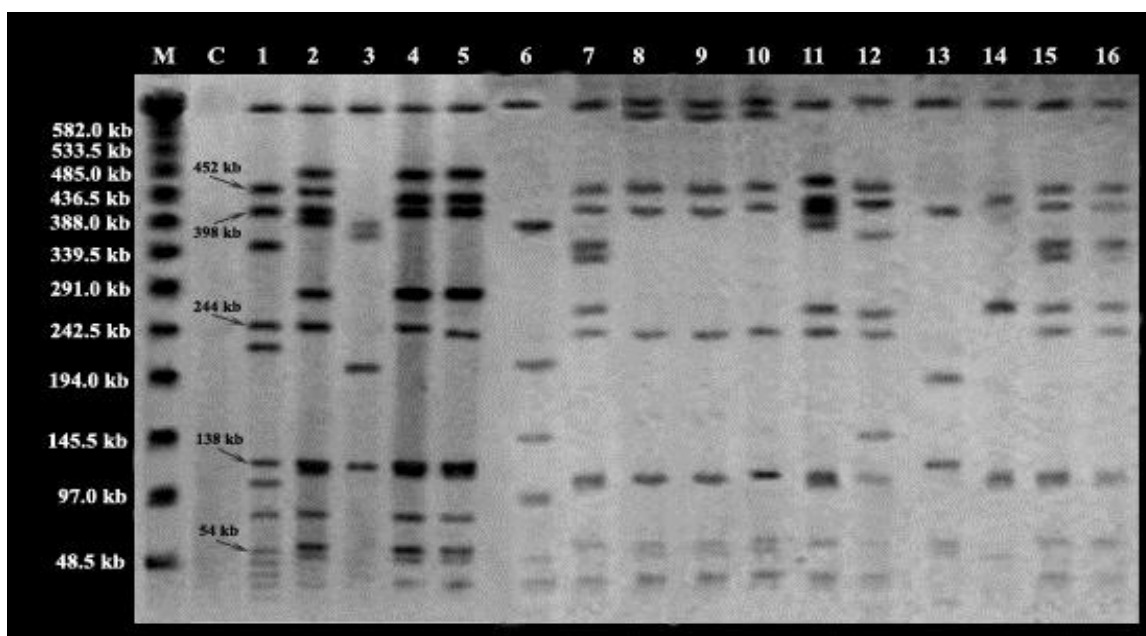
Figure 1. Genomic DNA restriction patterns of *Listeria monocytogenes* isolates, performed by PFGE after digestion with AscI

Cluster analysis of the AscI restriction digest profiles showed that PFGE subtype A and subtype A2 were predominant with a percentage of similarity of 100% for both followed by type A3 with a percentage of similarity of 99% then A5 and B with a percentage of similarity of 98%, D and A of 97.5%, A4 and A5 of 97%, A1 of 94.5%, F and E of 93.5%, I was of 93%, and G was presented with a percentage of similarity of 90%. Cluster analysis of L. monocytogenes isolates and the PFGE types assigned by visual inspection and by automated cluster analysis are shown in (Figure 2).
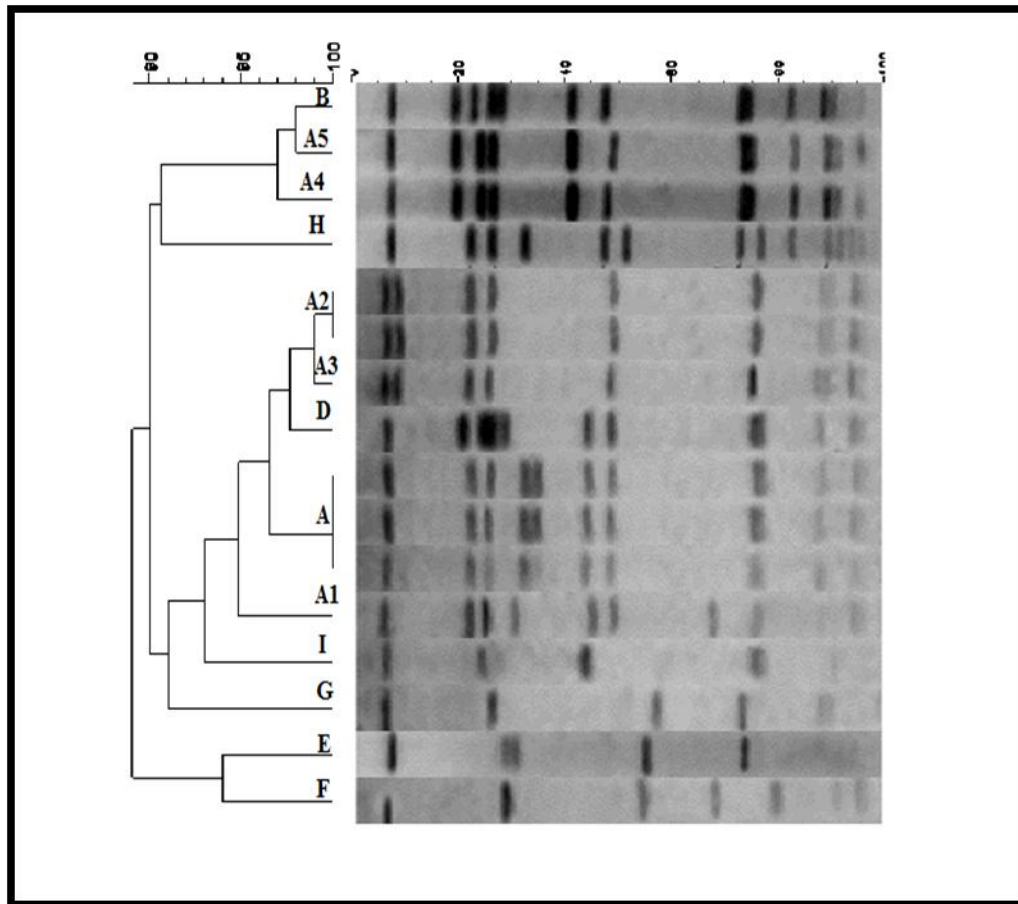
Figure 2. Dendrogram representing PFGE types assigned by Automated Cluster Analysis (ACA) of the AscI restriction digest profiles of *Listeria monocytogenes* isolates

Discussion

The large number of different PFGE types (sixteen isolates yield eleven PFGE types) may indicate that a variety of genetically distinct strains cause listeriosis in Iraq, however, this high number of PFGE types is in line with several published data for clinical cases [9,10], this variation in the number of PFGE types obtained in different studies may be due to that the clinical strains used in these studies not corresponded to sporadic cases; the choice of the rare cut endonucleases; and the analysis protocol used in the study. However, the current work was done using the Pulse Net method described by Graves and Swaminathan [6].

The cluster analysis results showed that there was two predominant subtypes A and A2, which include three isolates and two

respectively, those isolates were genetically indistinguishable from each other and also genetically related to other subtypes and or PFGE types with a percentage of similarity range from 99% to 89%, however those with high similarity may differ in a single band only while the lower similarity percentage was associated with isolates differ in more than one band.

Although PFGE profiles give a considerable degree of strain diversity in *L. monocytogenes* populations, the number of bands differences required for discrimination between strains is controversial [11]. Strains differing by more than one band are clearly distinct but some strains from geographically distinct sources may exhibit few or no differences in DNA profile and by the Turnover criteria could be considered epidemiologically related [12]. According to

Autio et al. (2002) [13], the isolation of indistinguishable PFGE types may mislead the investigation towards establishing the vehicle or the source of infection. However, many food products may carry genetically identical strains without any cross-contamination or common infection sources. Therefore, a suspected epidemic infection may consist of two or more independent sporadic cases.

In conclusion, human listeriosis in Iraq involves a great variety of genetically different isolates. However, in this study, indistinguishable isolates were isolated from more than one patient, suggesting the existence of more than one episodes of listeriosis. In all of the listeriosis episodes, the food vehicle was not determined, because of a lack of epidemiological evidence.

Authors Contribution:
Qassim collected the cases, performed the bacterial tests and analyzed the results. Dr. AL Attraqchi helped in the study design and supervising the work. Dr. Khatab participated collection of cases and performed the histopathological examination.

Conflict of interest
The authors declare no conflict of interest.

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