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Prevalence of *Listeria monocytogenes* in Raw Cow's Milk in the Region of Batna, Algeria

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Abstract: The aim of this study is the detection of the presence of *L. monocytogenes* in raw milk in the region of Batna using the real-time polymerase chain reaction (real-time PCR) technique, in order to assess the riskrun by the consumer of raw milk in the study area. 65 milk samples were investigated for the detection of *L. monocytogenes*. The detection of the *hlyA* gene in all samples was done by real-time PCR assay by comparative cycle threshold method. The results obtained were negative for all the samples analyzed, therefore the prevalence of *L. monocytogenes* in the 65 samples collected in this study is 0%. The absence of *L. monocytogenes* in the samples could be linked to the good health of the selected cattle. In fact, *L. monocytogenes* is considered to be very widespread in industrialized countries and rare in North Africa and Algeria.

Keywords: *Listeria monocytogenes*, Real-Time PCR, Raw Milk, Public Health

Introduction

L. monocytogenes is a foodborne pathogen responsible for listeriosis in humans due to its presence in raw and ready-to-eat foods which are stored at refrigeration temperature (Shi *et al.*, 2015). *L. monocytogenes* is the only species of the genus *Listeria* to be considered as pathogen in humans (Snapir *et al.*, 2006).It has been implicated in the original infection of environmental samples such as soil, water, manure and vegetation (Strawn *et al.*, 2013).

The main routes of contamination for animals (especially livestock) are silage (Welchman *et al.*, 1997) and the animals themselves. This animal carriage of *L. monocytogenes* in the agricultural environment can lead to contamination of animal products such as raw milk (Hunt*et al.*, 2012) or meat (Dmowska *et al.*, 2013). Contamination of agricultural raw materials is a pathway for the pathogen to enter the food industry.

Contaminated food with *Listeria monocytogenes* is the predominant route of transmission of listeriosis to humans, a severe illness with a high mortality rate (Magalhães *et al.*, 2017). Milk constitutes one of the main vectors of *Listeria* transmission; more particularly raw milk from cows due to its wide consumption. Contaminated raw milk used in cheese-making, particularly those with soft cheeses, flowery rinds or washed rinds, promotes the multiplication of *Listeria* during ripening. *L. monocytogenes* can be isolated from soft or semi-soft, blue-veined, pressed or fresh cheese (Millet *et al.*, 2006).

Listeria is killed, like all other bacteria by pasteurization. However, contamination can then occur at any time, by the environment or by contact with another contaminated food: You should know that *Listeria* multiplies between 4 and 45°C and is able to survive freezing temperatures (Kaismoune, 2009).

Several methods for detecting L. monocytogenes have been highlighted, but conventional culture remains a reference method according to international standards. However, the culture takes a long time between 3 and 5 days, for this, easy and fast alternative methods have been improved in order to better meet the needs by reducing the detection time. Among these methods, the PCR technique is widely used.

The vast majority of PCR tests used to identify *L. monocytogenes* use the primers of the *hly A* gene sequence which codes for Listeroloysin O (LLO). *Hly A* is part of the virulence genes of *Listeria* (Liu*et al.*, 2004). LLO was the first virulence factor identified in *L. monocytogenes*

(Gaillard *et al*., 1986). The application of real-time PCR has also been developed as a specific quantitative detection method for *L. monocytogenes* (Hough *et al*., 2002)and has shown good potential for routine analysis. For real-time PCR, the specificity and the sensitivity are better, the duration of the analysis is shorter and it is the desired germ or the group of bacteria to be characterized which controls the system used (Chentouf, 2015)

In Algeria, there are few studies on the frequency of isolation of *L. monocytogenes* from raw milk intended for direct consumption or for the dairy industry. The aim of this study is to determine the prevalence of *L. monocytogenes* in raw milk collected in the region of Batna. The method used in this study is the technique of real-time PCR.

Materials and Methods

Samples of Milk

A total of 65 milk samples were taken from cows (ages 3-8 years) belonging to 3 dairy farms in the Wilaya of Batna (North-Eastern Algeria). The milk samples were collected aseptically from teats of the udder which had previously been cleaned with water and soap and then, the surface was sterilized with 70% ethanol. For each animal, the first jet of milk was removed and then 15 mL of milk were collected from 4 quarters in sterile Falcon tubes previously identified. The samples were immediately transported to the laboratory at +4°C for DNA extraction.

This study was conducted at the Laboratory of Microbiology and Molecular Biology of Constantine Biotechnology Research Center, Algeria.

Pre-Treatment of Milk

Initially, the milk had undergone a pre-treatment as follows: A test sample of 800 µL of the milk was centrifuged for 5 min at 10 000 g, then the supernatant was removed and the pellet was resuspended in 300 µL of Phosphate-Buffered Saline (PBS).

Extraction of DNA

Genomic DNA was extracted from raw milk using a BioExtract® Column purification kit according to the manufacturer's protocol (Cat N° BEC050).

The extracted DNA is then quantified using Nanodrop® ND 8000.

The DNA was then stored at 4°C until use.

Real-Time PCR Amplification

Real-time PCR was used to detect the presence of *L. monocytogenes* DNA in milk samples as described in the kit BactoReal® kit *Listeria monocytogenes* (REF: DVEB00813). This test has been developed and validated for ABI PRISM® 7500 (Fast) instrument (Applied Biosystems). This test allows rapid and sensitive detection of DNA of *L. monocytogenes* from purified milk samples.

BactoReal® Kit *Listeria monocytogenes* detects the gene *hlyA* of *L. monocytogenes.*

A Positive Internal Control of the system for the detection at Cy5 (667 nm) makes it possible to demonstrate the inhibitions of the PCR, resulting in false-negative results when interpreting the results due to the inhibition of PCR in real time.

The Reaction Mixture

The real-time PCR assay was performed by an ABI PRISM® 7500 instrument thermal cycler (Applied Biosystems) using 96-well optical barcode plates (Ref: 4306737) and Adhesive Optical Films Starter Kit (Ref: 4311971) following the manufacturer's instructions (Ingenetix, Austria). The real-time PCR amplification was carried out in a reaction mixture of 20 µL composed of 5 µL of sample (or genomic DNA) containing matrix DNA and 15 µL of Master Mix consisting of 3.0 µL of water, 10 µL of DNA mix reaction (1 µL of *Listeria monocytogenes* Mix Assay) for the detection of *L. monocytogenes* and 1 µL of CR mix assay (Primers and Probes (Cy5) for IPC detection).

At least one negative control (water), one positive control (*L. monocytogenes*) and one negative extraction included by PCR were ensured.

According to Ingenetix recommendations, all PCR analyzes are done in duplicate to optimize the probability of detecting pathogens and facilitate the interpretations of results.

Programming of the Temperature Profile

The reaction was carried out in a DNA thermocycler (Applied Biosystems) at a preliminary denaturing temperature of the DNA in dry at 95°C, followed by 45 cycles consisting of 5 s at 95°C for denaturation of DNA and 1 min at 60°C for polymerase-mediated primer extension.

Results

The results obtained were negative for all the samples analyzed, therefore the prevalence of *L. monocytogenes* in the 65 samples collected in this study is 0%.

The results are analyzed in real time using 7500 software V2.0.6 and represented in the form of a curve (Fig.1).

The Fig. 1 shows a multiplication of the FAM signal only for the positive controls (diluted and undiluted) which means that the DNA of *L. monocytogenes* of the positive controls has been amplified. However, the samples show no multiplication of the FAM signal, signifying that no *L. monocytogenes* DNA was detected in the sample. The samples are interpreted as negative.

On the other hand, a multiplication of the Cy5 signal is observed for all the samples, which means that the positive signal of the internal positive control excludes the possibility of inhibition of the PCR.

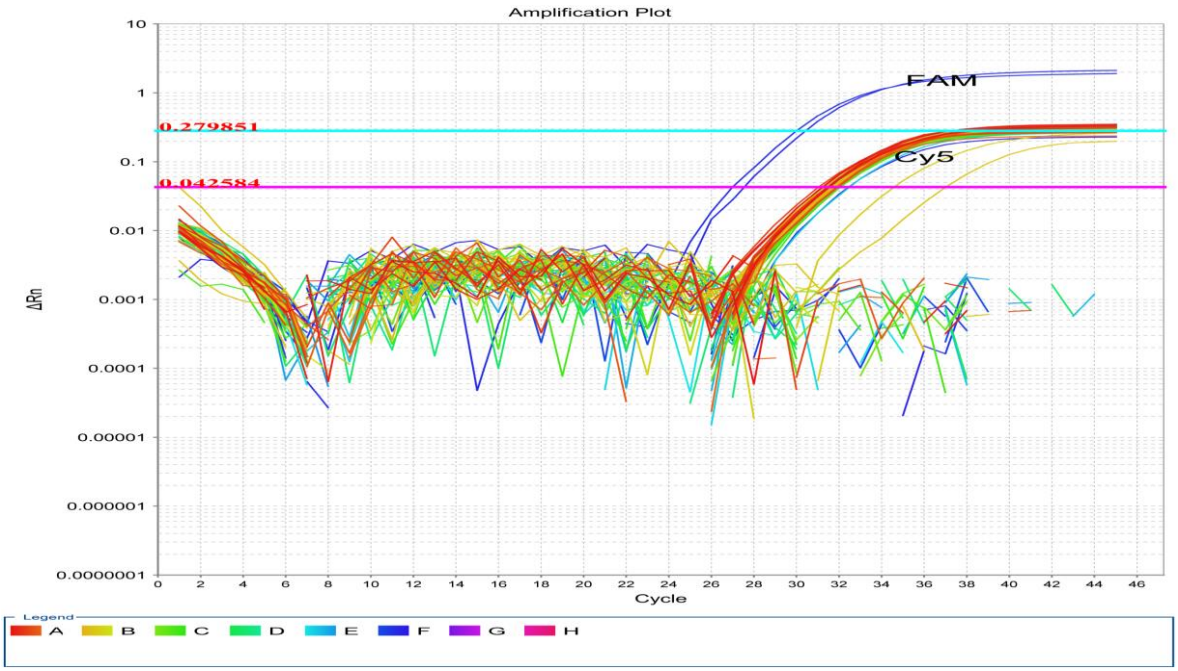

Fig. 1: Logarithmic curves for amplification of samples, positive and negative controls (FAM signal and Cy5)

Discussion

The absence of *L. monocytogenes* in our raw milk samples is in agreement with the results obtained by Al Kassaa *et al.* (2016). Studies in Italy and Switzerland have also shown negative results (Jemmi and Stephan, 2006).

Our results are lower than those of other studies carried out in other countries such as Turkey and Korea, where the prevalence was 4% for the first city (Vardar-Ünlü *et al.*, 1998) and 2% for the second (Ha *et al.*, 2002). In Europe, the prevalence of listeriosis increased by 19.1% between 2008 and 2009 (EFSA, 2011). This increase had mainly occurred in people over 65 years of age (58%) (Goulet *et al.*, 2008). The prevalence of listeriosis is 6.5% in the United States (Van Kessel *et al.*, 2004), 13% in Brazil, 12% in Canada (Jemmi and Stephan, 2006).

In the United States, investigations have indicated that *L. monocytogenes* is present in approximately 4% of the raw milk samples examined, as well as in unpasteurized milks and cheeses made from unpasteurized milk products (Washington *et al.*, 2006).

The absence of *L. monocytogenes* in our raw milk samples and in those with similar results cited above may be due to unfavorable conditions for its colonization and its multiplication. Well-preserved silage may be one of the factors behind the absence of *L. monocytogenes* in cattle. Indeed, according to statistical analyzes by Vilar *et al.* (2007) the relationship between the poor quality of silage (due to the high pH) and the presence of *L. monocytogenes* in silage is confirmed. They note isolation rates of 29.5% versus 6.2% for a pH above or below 4.5, respectively. These observations are also confirmed by numerous authors who have shown an increase in the carriage of *Listeria spp.* in winter and spring (October to June, depending on the country), times when animals, according to breeding techniques, are most often fed with silage. The portage of *Listeria spp.* exists in most herds (cows), with a rate varying between 0.5 to 10% of animals, depending on the season (AFSSA, 2000).

Jaradat *et al*. (2002) claimed in their work that the majority of animal contaminations by *L. monocytogenes* occurs in cold geographic regions such as North American and North European countries. This phenomenon can be explained by the increased risk of contamination during the housing of animals (Nightingale *et al.*, 2005). The use of silage during the winter could also explain the increase in contamination cases during this period.

The absence of *L. monocytogenes* in our samples may be due to the sample collection period, taken during the summer in an aseptic manner thus avoiding the contamination of milk by environmental *Listeria*, to the presence of bacteriocins secreted by lactic acid bacteria belonging to the intestinal flora of cattle, or to the good health of selected dairy cows. Several research studies have confirmed the inhibitory effect of bacteriocins produced by *Enterococcus* strains against the growth and development of *Listeria Spp.* (Elotmani *et al.*, 2002).

Human listeriosis is present in industrialized countries, but it appears to be almost absent from developing countries. In addition to the differences existing in the means of diagnosis and health surveillance, this geographical distribution could be explained by better

hygiene and by the generalization of the cold chain in developed countries. Paradoxically, it seems that it is the good hygiene in the manufacturing processes and the development of the cold chain that are at the origin of an increase in the cases of listeriosis observed in the last forty years (FAO/WHO, 2002).

The increase in the incidence of human listeriosis in North American and North European countries could be explained by the aging of the population, the increase in the number of immunocompromised people (cancer patients, transplant patients, etc.), the evolution of eating habits favoring prepared meals, the significant consumption of risky foods (raw milk, cheeses, cold meats) and the increase in consumption deadlines (Desneux, 2015).

Conclusion

In conclusion, the prevention of listeriosis must be the subject of a community effort associating those responsible for veterinary medicine and public health. The systematic use of molecular techniques such as the real-time PCR technique would make it possible to detect all the animals that may be at the origin of human contamination and their elimination from consumption circuits.

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Authors' Contributions

Sabrina Rabehi: Drafted and prepared the study design, carried out the research and analyzed the data.

Asma Meghezzi and Sabrina Rabehi: Executed the extraction of DNA.

Khaoula Boushaba and Sabrina Rabehi: Executed the real-time PCR.

Taha Mossadak Hamdi: Revised the manuscript

Bakir Mamache: Revised the manuscript and approved the final version.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all authors read and approved the final manuscript.

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