DETECTION OF VIRULENCE-ASSOCIATED GENES IN *Listeria monocytogenes* ISOLATES FROM PORTUGUESE READY-TO-EAT MEAT-BASED FOOD INDUSTRY

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Abstract – The purpose of this work was to characterize *L. monocytogenes* isolates from surfaces swabs and from ready-to-eat (RTE) foods collected in RTE meat-based food industries, in order to identify serogroups and virulence factors and assess its potential for developing consumer's disease. A total of 62 *L. monocytogenes* isolates were evaluated regarding their serogroup by a multiplex PCR assay in order to cluster isolates into five serogroups: IIa, IIb, IIc, IVa and IVb, related with the most common serovars isolated from food and patients; secondly, a multiplex PCR for *inlA*, *inlB*, *inlC* and *inlJ* primers was used for virulence screening; a multiplex PCR assay was also performed for detection of four virulence associated-genes namely, *plcA*, *hlyA*, *actA* and *iap*. Results showed that the main serogroup in food items was IVa, while in surfaces the most common serogroups were IIb and IVa. Regarding virulence profile, a minimum of five virulence factor genes were present in 40% of the isolates, while in the other 60% of the isolates all of the assessed virulence factor genes were present. The above-mentioned results suggest that the studied *L. monocytogenes* isolates may have virulence potential to cause human food-borne listeriosis.

Key Words – *Listeria monocytogenes*, Ready-to-eat meat-based food products, Serogroups, Virulence factors

- INTRODUCTION

In the last decade, European member states have reported thousands of human listeriosis confirmed cases per year, with high fatality rates among these cases [1]. This trend has also been reported by the Centers for Disease Control and Prevention in United States of America [2].

RTE meat-based food products are one of the most consumed food products around the world [3]. Their long shelf-lives in refrigerated storage, as well as the fact that they do not need to undergo any heat-treatment prior to consumption, makes them an important source of food-borne disease, especially by *L. monocytogenes* [1].

There are several ways by which *L. monocytogenes* can remain in the finished RTE product, namely by its recontamination following a listericidal treatment [4] as a result of the contact with contaminated processing equipments or surfaces [5; 6; 7]. Therefore, tracing isolates within the food plant environment is of primary importance [10] to delineate strategies for contamination prevention.
Because of the importance of *L. monocytogenes* epidemiology to human health [8] and the notable diversity in the pathogenicity among its strains [9], subtyping and virulence characterization are of upmost importance. The four main *L. monocytogenes* serotypes identified in food and human patients are 1/2a, 1/2b, 1/2c and 4b, being the last one related to more disease cases. The IIa molecular serogroup includes 1/2a and 3a serotypes isolates, the IIb includes 1/2b and 3b, the IIc serogroup includes 1/2c and 3c, the 4b isolates are yielded in the IVb serogroup and the IVa includes 4c isolates [10]. Each serogroup has a different virulence potential due to factors that play an important role in this bacterium survival, environmental

persistence and pathogenesis.

Internalin A (InlA) and internalin B (InlB) are species-specific surface protein with essential roles in host cells entry; Internalin C (InlC) contributes to the post-intestinal stages of *L. monocytogenes* infection and internalin J (InlJ) is directly involved in the pathogen passage through the intestinal barrier and subsequent stages of infection [9].

The *plcA* gene codifies phosphatidyl-inositol-specific phospholipase C and *actA* gene mediates actin-based motility and cell-to-cell spread; *hly* gene is responsible by Listeriolysin O production, which has an important role in intracellular parasitism. *L. monocytogenes* pathogenicity is also related with the presence of the invasion-associated protein p60, which is encoded by the *iap* gene [15, 19].

The aim of this study was to genetically characterize *L. monocytogenes* isolates from RTE foods and from food-contact surfaces swabs collected in different Portuguese RTE meat-based food industries by the identification of serogroup and virulence factors in order to assess its virulence potential for developing consumer's disease.

- MATERIALS AND METHODS

Industrial units characterization:

Ten industrial units producing RTE meat-based products in Portugal were assessed during 2012.
Environment and food sample collection: In each industrial site, two RTE products of meat origin in their final package were collected. Three food contact equipment surfaces were sampled (500cm^2) with a sponge (MWE medical wire, MW729A) according to ISO 18593:2004, while in use and after routine cleaning and disinfection procedure. The samples were transported in an isothermic box (below 5ºC) to the laboratory in less than 2 hours.
Microbiological methods: Food samples were prepared according to ISO 6887-2:2003 and equipment surface samples according to ISO/DIS 18593:2004. Detection of *Listeria monocytogenes* was performed according to ISO11290-1:1995. *L. monocytogenes* identification was confirmed by PCR, according to Simon *et al*. (1996). *L. monocytogenes* isolates (n=62) serogrouping was done by multiplex PCR assay, according to Kérouanton *et al*. (2010). For inlA, inlB, inlC and inlJ gene detection a multiplex PCR assay was done according to Liu *et al.* (2007), and for virulence associated-genes detection (*plcA, hlyA, actA* and *iap)* the protocol proposed by Rawool *et al.* (2007) was used.

- RESULTS AND DISCUSSION

From the 20 food products analyzed, 5 samples were positive (25%) for *L. monocytogenes* presence. These findings are higher than those found in similar studies [16, 17]. This may be due to the fact that the tested food samples are RTE meat-based food products, commonly associated with food-borne outbreaks, and also because they undergone some handling after the listericidal treatment in the food industry.

Considering the analysed food contact surfaces, 6 while in use surfaces revealed *L. monocytogenes* presence, whilst after routine hygienization only 2 of those surfaces showed *L. monocytogenes* presence. This may be due to the presence of difficult access parts of the equipment, as well as to the sanitizer in use (active molecule and/or inappropriate dilution).

Later, 62 *L. monocytogenes* isolates (30 from food products, 3 from clean surfaces and 29 from in-use surfaces) from different RTE industries were assessed by multiplex PCR assays and results may be seen in Table 1.

Table 1 *L. monocytogenes* isolates characterization by multiplex PCR assays

Multiplex PCR assay		*L. monocytogenes* isolates		
		Food (n=30)	In use surfaces (n=29)	Clean surfaces (n=3)
Serogroups	IIa	3	8	1
	IIb	5	11	-
	IIc	4	1	-
	IVa	16	8	-
	IVb	2	1	2
Internalins genes	*InlA*	21	21	2
	InlB	14	18	1
	InlC	21	23	3
	InlJ	27	29	3
Other virulence factors genes	*plcA*	30	27	2
	actA	30	29	3
	hlyA	30	29	3
	iap	30	29	3

Of the 62 isolates, twelve (19%) belonged to serogroup IIa; sixteen (26%) belonged to serogroup IIb; five (8%) belonged to serogroup IIc; twenty-two (39%) belonged to serogroup IVa and five (8%) belonged to serogroup IVb. Above all, serogroup IVb presence is particularly disturbing, since this is the most frequently associated serotype with human listeriosis[16]. IVa was the most frequent serogroup in food samples, while in clean surfaces the most common one was IVb and in in use surfaces IIb serogroup had the highest frequency. According to international epidemiological data, serogroups IVb, IIa and IIb are the more commonly identified in human listeriosis cases [19]. These observations also suggest that specific *L. monocytogenes* serotypes may easily develop in particular substrates and are in accordance with some previous studies which suggested that *L.monocytogenes* serotypes differ in their associations with specific food and nonhost environments [18]. Some serotypes will develop better when in food items, while others will subsist for months in abiotic surfaces [20].

Regarding virulence factors genes, thirty-seven isolates had all of the virulence factors genes tested, while the others isolates varied in the virulence genes profile, but at least four of the studied genes were always present, including *inlJ*, *actA*, *hly* and *iap* genes. These genes are associated with the bacterium capability of passing the intestinal barrier, cell-to-cell spread and motility, cell invasion and intracellular parasitism [9, 15, 19].

The above-mentioned results show that *L. monocytogenes* serogroups have distinct virulence potential, which is also in accordance with previous studies [18, 19].
An understanding of the variability of strains encountered in humans, foods and food production environment and the range of virulence characteristics expressed by different strains will contribute to improved assessment of the public health risk posed by *L. monocytogenes* [18].

- CONCLUSION

L. monocytogenes presence in RTE foods is of public health concern as these food items are consumed without previous heat treatment.

The frequency of *L. monocytogenes* found in food samples was 25%, which is higher than similar studies results.
In our study, all of the assessed serogroups were present, but serogroup IVa was the most frequent in food samples, while in food contact surfaces the most common were IVb and IIb. All of the virulence factor genes assessed were present. Sixty percent of the isolates were positive to all of the assessed virulence factor genes, confirming its high virulence potencial.

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REFERENCES

- European Food Safety Authority. European Centre for Disease Prevention and Control. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011. EFSA Journal 2013;11(4):3129.
- Hereu, A., Dalgaard, P., Garriga, M., Aymerich, T. & Bover-Cid, S. (2012). Modeling the high pressure inactivation kinetics of Listeria monocytogenes on RTE cooked meat products. Innovative Food Science and Emerging Technologies 16: 305-315.
- Pérez-Rodríguez, F., Castro, R., Posada-Izquierdo, G.D., Valero, A., Carrasco, E., García-Gimeno, R.M. & Zurera, G. (2010). Evaluation of hygiene practices and microbiological quality of cooked meat products during slicing and handling at retail, Meat Science 86: 479-485.
- Lambertz, S. T., Nilsson, C., Bradenmark, A., Sylvén, S., Johansson, A., Jansson, Lisa-Marie & Lindblad, M. (2012). Prevalence and level of *Listeria monocytogenes* in three categories of ready-to-eat foods in Sweden 2010. International Journal of Food Microbiology 160 (1): 24-31.
- Carpentier, B. & Chassaing, D. (2004). Interactions in biofilms between Listeria monocytogenes and resident microorganisms from food industry premises. International Journal of Food Microbiology, 97: 111 –122.
- Lin, C.-M, Takeuchi, K., Zhang, L., Dohm, C.B., Meyer, J.D., Hall, P.A. & Doyle, M.P. (2006). Cross-contamination between processing equipment and deli meats by Listeria monocytogenes. Journal of Food Protection 69 (1): 71-79.
- Harvey, J., Keenan, K. P. & Gilmour, A. (2007). Assessing biofilm formation by Listeria monocytogenes strains. Food Microbiology 24, 380–392.
- Borucki, M. K. & Call, D. R. (2003). Listeria monocytogenes serotype identification by PCR. Journal of clinical microbiology, 41 (12): 5537-5540.
- Liu D., Lawrence M. L., Austin F. W. & Ainsworth A. J. (2007). A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*. Journal of Microbiological Methods 71(2): 133-140.
- Kérouanton, A., Marault M., Petit L., Grout J., Dao T. T. & Brisabois A. (2010). Evaluation of a multiplex PCR assay as an alternative method for *Listeria monocytogenes* serotyping. Journal of Microbiological Methods 80(2): 134-137.
- ISO/DIS 18593:2004. Microbiology of food and animal feeding stuffs - Horizontal method for sampling techniques from surfaces using contact plates and swabs**.**
- ISO 6887-2:2003. Microbiology of food and animal feeding stuffs -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 2: Specific rules for the preparation of meat and meat products
- ISO 11290-1:1996: Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of Listeria monocytogenes - Part 1: Detection method.
- Simon, M. C., Gray, D. I. & Cook, N. (1996).DNA Extraction and PCR Methods for the Detection of *Listeria monocytogenes* in Cold-Smoked Salmon. Applied and environmental microbiology, 62 (3): 822–824.
- Rawool, D.B., Malik S.V.S., Barbuddhe S.B., Shakuntala I. & Aurora R. (2007). A Multiplex PCR for Detection of Virulence Associated Genes in *Listeria monocytogenes.* International Journal of Food Safety, 9: 56-62.
- Gamboa-Marín, A., Buitrago, S., Pérez-Pérez, K., Mercado, M., Poutou-Piñales, R. & Carrascal-

Camacho, A. (2012). Prevalence of *Listeria monocytogenes* in pork-meat and other processed products from the Colombian swine industry. Rev. MVZ Córdoba 17(1): 2827-2833.

- Bohaychuk, V.M., Gensler, G.E., King, R.K., Manninen, K.I., Sorensen, O., Wu, J.T., Stiles, M.E. & McMullen, L.M. (2006). Occurrence of pathogens in raw and ready-to-eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. J Food Prot. 69 (9):2176-82.
- Gray, M. J., Zadoks, R.N., Fortes, E.D., Dogan, B., Cai, S., Chen,Y., Scott, V. N., Gombas, D. E., Boor, K. J. & Wiedmann, M.(2004). *Listeria monocytogenes* Isolates from Foods and Humans Form Distinct but Overlapping Populations. Appl Environ Microbiol., 70(10): 5833–5841.
- Swaminathan, B. & Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. Microbes and Infection 9, 1236-1243
- Norton, D. M., M. A. McCamey, K. L. Gall, J. M. Scarlett, K. J. Boor & M. Wiedmann. (2001). Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. Appl. Environ. Microbiol. 67**:**198-205.
- Schuerch, D.W.; Wilson-Kubalek, E.M. & Tweten, R.K. (2005). Molecular basis of listeriolysin O pH dependence. In Procedings of the National Academy of Sciences of the United States of America 102(35), pp- 12537-12542, 30 August 2005.