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Communication

[*Comunicação*]

Occurrence of *Listeria monocytogenes* in silages assessed by fluorescent *in situ* hybridization

[*Ocorrência de* Listeria monocytogenes *em silagens avaliadas por meio da hibridação* in situ *fluorescente*]

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Most domestic and wild animals are assymptomatic carriers of *Listeria monocytogenes* and play an important role in the dissemination of this microorganism (Sanaa et al., 1993; Bind and Delaval 1994). Animal listeriosis has a worldwide distribution, and the main source of contamination is through food (Bind and Delaval, 1994). Animals can be infected trough the consumption of several types of contaminated feed, but silages are the most frequently associated with ruminant listeriosis (Vázquez-Boland et al., 2001; Pople, 2003). The association between silage consumption and ruminant listeriosis was established for the first time in 1922 in Island (Gray, 1960). Since then, this relation has been registered in bovine and ovine (Vázquez-Boland et al., 1992; Wiedmann et al., 1999). Many cases result from the consumption of bad quality silage, subjected to inadequate fermentation, with pH values higher than 4.0, which allows the multiplication of *L. monocytogenes* (Donald et al., 1995; Ryser et al*.,* 1997).

The adoption of certain measures could prevent the presence of *Listeria* in silages: the maintenance of an anaerobic environment; the guaranty that fermentation lasts for two weeks; the use of grass with low humidity levels; the maintenance of a clean environment surrounding the silo; the elimination of silage which contacted with oxygen; and the development of rapid and reliable techniques for the detection of *L. monocytogenes* in these samples. The detection of *L. monocytogenes* in silages is therefore an extremely relevant procedure for the control of the sanitary safety of these products. The objectives of this study were the evaluation of the occurrence of *L. monocytogenes* in silage samples in silos produced in Portugal and the development of a fluorescent *in situ* hybridization (FISH) protocol for its rapid detection in these samples.

Seventy-four silage samples were collected from 37 big bale silos produced in locations from the north of Portugal: Vila Nova de Famalicão (n=14), Braga (n=10), Vila do Conde (n=26), Cambas (n=2), Póvoa do Varzim (n=22). The samples were analyzed according to a microbiological protocol previously optimized for the detection of *L. monocytogenes*, based on the NF EN ISO 11290-1.2 standard (M.M. Guerra et al, dados não publicados).

For the FISH protocol, a specific probe for the 16S rRNA of *L. monocytogenes*, RL-2 (5'-ATAGTTTTATGGGATTAGC-3'; *E. coli* 168→176; Wang et al., 1991; Oliveira et al*.*, 2003) was applied. The FISH protocol was performed as described elsewhere (Oliveira et al., 2003).

The statistical analysis of the liability of the results was performed according to the ISO/FDIS 16140:2000 (E) standard – "Microbiology of food and animal feeding stuffs – protocol for the validation of alternative method".

Using the microbiological method, it was observed that 11 (15.0%) silage samples were contaminated with *L. monocytogenes* (Table

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1).The occurrence is within the range observed by other studies in several countries (Laithier et al., 2000). Grønstøl (1979) described a much higher frequency by isolating *L. monocytogenes* in 28% of the samples studied. In Scotland, Fenlon (1986) detected *L. monocytogenes* in 2.5% of the samples analyzed in 1983 and in 5.9% of the samples analyzed in 1984. In the United States, Perry and Donnelly (1990) isolated *L. monocytogenes* in 2.9% of the silages studied.

The analysis of the silage samples by the FISH protocol revealed the presence of *L. monocytogenes* in 22 (29.7%) samples (Table 1). This observation may indicate that the detection in agar plates results in a sub-estimation of the bacteria number. The difference between the results obtained by direct microscopic observation and traditional method may be related to the presence of non-viable cells, the presence of bacteria aggregates, or the selective characteristics of the agar media used, that may difficult the microorganisms multiplication (Amann et al., 1995; Auty et al., 2001).

The relative accuracy, specificity and sensitivity of the methods were determined according to the ISO/FDIS 16140:2000 (E) standard (Table 1). The FISH method presented high accuracy (77.0%), specificity (92.5%) and sensitivity (72.7%). Other authors had already stated that the sensitivity of the FISH technique is higher than the one of the conventional bacteriologic methods (Auty et al., 2001).

Table 1. Occurrence of *L. monocytogenes* detection in silage samples as determined by the bacteriological and the fluorescent *in situ* hybridization methods and comparison between the results according to the ISO/FDIS 16140:2000(E) standard

Parameter	Formula	Result
Positive agreement (PA)	Bac + and FISH +	8
Negative deviation (ND)	Bac + and FISH -	3
Positive deviation (PD)	Bac - and FISH +	14
Negative agreement (NA)	Bac - and FISH -	49
N.º samples (N)	NA+PA+PD+ND	74
N.º negative results (N-)	NA+PD	53
N.º positive results (N+)	PA+ND	11
Relative accuracy (AC)	[(PA+NA)/N]x100%	77.0%
Relative specificity (SP)	(NA/N-)x100%	92.5%
Relative sensitivity (SE)	(PA/N+)x100%	72.7%

Legend: Bac+: positive result by the bacteriologic method; Bac-: negative result by the bacteriologic method; FISH+: positive result by the fluorescent *in situ* hybridization method; FISH-: negative result by the fluorescent *in situ* hybridization method.

The results from this study confirm the potential risk that silages represent in listeriosis transmission and suggest that the conventional bacteriologic method presents limitations for the detection of stressed microorganisms subjected to acidification, heating and dehydratation stresses during the production steps. The developed FISH methodology applied to fresh samples revealed to be adequate for the presumptive microbiological analysis of silage, being rapid, easy to perform, sensible, reproducible, and less expensive.

Keywords: silage, listeriosis, fluorescent *in situ* hybridization, *Listeria monocytogenes*, Portugal

RESUMO

Avaliou-se a qualidade microbiológica da silagem produzida em Portugal e otimizaram-se alguns aspectos relacionados com a eficácia de detecção de Listeria monocytogenes *mediante protocolo baseado na técnica de hibridação* in situ *fluorescente (FISH) para detecção directa desse microrganismo em amostras de silagens. O protocolo foi aplicado a 74 amostras, em simultâneo com o método bacteriológico convencional. Este último permitiu a detecção de* L. monocytogenes *em 11 silos (15%). Por meio do protocolo FISH, observou-se que 22 silos (29,7%) se encontravam contaminados. O método*

FISH apresentou precisão (77,0%), especificidade (92,5%) e sensibilidade (72,7%) elevadas, sendo adequado para a análise microbiológica presuntiva de silagens, uma vez que é um método rápido, fácl de realizar, sensível, reprodutível e pouco dispendioso.

Palavras-chave: silagem, listeriose, hibridação fluorescente in situ, Listeria monocytogenes

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