Strain variability in *Listeria monocytogenes* with respect to pathogenicity after storage with no growth at refrigeration temperature

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Introduction

Two factors play the most important role in the epidemiology of foodborne listeriosis: host susceptibility and virulence of the organism, and both factors may vary considerably (Farber and Peterkin, 1991; Hof and Rocourt, 1992). The virulence/pathogenicity of *Listeria monocytogenes* can be affected by various substrate factors, i.e. in foods (Mekalanos, 1992; Datta, 1994), but these factors are no longer relevant when strains are transferred from foods to laboratory media. Therefore, in assessing the health risk posed by *L. monocytogenes* in foods, the relevant factor should be actual pathogenicity of the strains *in situ*, i.e. at the moment of ingestion by humans, rather than that of subsequent bacterial generations grown in laboratory media. It has been found that even though different *L. monocytogenes* strains are equally pathogenic when tested as fresh cultures, virulence of some strains may significantly decrease after their exposure to negative environmental factors, i.e. storage in nutrient-free substrate at refrigeration temperature (Buncic *et al.*, 1996; Buncic and Avery, 1996; Avery and Buncic, 1997), although the effects were strain-dependent. In the present study, we further investigated the variations in pathogenicity and subsequent growth characteristics at body temperature among *L. monocytogenes* strains after chilled, long-term storage.

Materials and Methods

Storage of *L. monocytogenes* strains - Cells from 24-h BHI broth cultures of 30 *L. monocytogenes* strains (Avery and Buncic, 1997) were pelleted by centrifugation (Labofuge 6000, Heraeus Christ, Germany) for 10 min at 4600 *g* and resuspended in 10 ml amounts of three different substrates: a) phosphate-buffered saline (PBS: 0.85% NaCl in 0.1 mol l^{-1} phosphate buffer) of pH 7.0; b) PBS of pH 5.5 and c) PBS of pH 5.5 plus potassium sorbate (Sigma, St. Louis, MO, USA), final concentration 0.3%. Bacterial suspensions were stored at 4°C in shaking water baths (Julabo SW 1, Seelbach, Germany) and samples for examination were taken immediately after preparation of the suspensions and after 4 weeks.

Pathogenicity testing - The pathogenicity of *L. monocytogenes* before and after 4°C storage was tested by inoculating 14-day-old chick embryos via the chorioallantoic membrane with a 0.1 ml volume of a 10^{-6} dilution of each bacterial suspension, prepared in 0.85% saline, as described previously (Buncic *et al.*, 1996).

Determination of *L. monocytogenes* growth kinetics at 37°C - Before and after 4°C storage, 0.1 ml volumes of appropriate dilutions of *L. monocytogenes* suspensions were transferred into 10 ml amounts of BHI broth and incubated at 37°C. The counts of *L. monocytogenes* in these BHI cultures were determined and growth kinetics (lag time and maximum specific growth rate) for each culture were calculated as described previously (Buncic *et al.*, 1996).

Results and Discussion

During our previous investigations performed over the past 3 years using 100 haemolytic strains of *L. monocytogenes*, we never observed mortality lower than 80% in chick embryos inoculated with freshly prepared cultures of this pathogen, using the technique described in this study. For the vast majority of fresh *L. monocytogenes* cultures, the mortality was 100%. Therefore, in this study, *L. monocytogenes* cultures with 80-100% mortality in chick embryos are considered fully pathogenic, while those with ≤70% mortality are considered to have attenuated virulence.

In this study, the 30 strains of *L. monocytogenes* behaved relatively uniformly before cold storage; all were fully pathogenic and all had a short lag phase when subcultured at body temperature (Table 1). However, large variations in pathogenicity between the different strains became visible after exposure to unfavourable storage conditions. After 4-weeks storage in PBS at pH 7.0, all 15 human strains and a minority of meat strains (6 of 15) maintained their original, high pathogenicity, while the majority of meat strains (9 of 15) had decreased virulence.

The attenuating effects of cold storage at a pH value typical for fresh meat (5.5), however, were much less remarkable. At pH 5.5 the pathogenicity of 14 human strains and most meat strains (12 of 15) was unaffected, and only 3 meat strains were attenuated. The most likely explanation of this phenomenon, that more strains maintained high virulence after cold storage at pH 5.5 than after storage at neutral pH, is activation of stress-mediated cellular mechanisms triggered by low pH (Mekalanos, 1992; Hill *et al.*, 1995; Buncic *et al.*, 1996).

Our previous investigation with only three strains of non-growing *L. monocytogenes* indicated that the attenuating effects of cold storage may be enhanced by the presence of sorbate (Buncic and Avery, 1996). In the present study, sorbate did not significantly affect pathogenicity of human *L. monocytogenes* strains stored at pH 5.5. However, at pH 5.5 significantly more meat strains showed decreased pathogenicity after cold storage in PBS with added sorbate (7 of 15) than in PBS without it (3 of 15). Nevertheless, these effects were weaker than that of cold storage in sorbate-free PBS of neutral pH.

Although results of investigations conducted with nutrient-free substrate cannot be directly extrapolated to foods, this study shows that human *L. monocytogenes* strains are highly resistant to the effects of unfavourable storage conditions, while meat strains, as a group, are more sensitive to the attenuating effects of cold storage (Table 2). This phenomenon indicates that a "natural" population of *L. monocytogenes* in foods may not respond uniformly to chilled, long-term storage, with respect to pathogenicity. It is possible that, after chilled storage, a population may consist of a mixture of individual *L. monocytogenes* cells of various virulences. In that case, the crucial food safety question is what proportion of less-virulent cells occurs within the population after cold storage? The randomly isolated meat strains used in this study may more realistically reflect the composition of an initial, natural population of *L. monocytogenes* on meats than the human strains, which were isolated after causing human disease. If the meat strains are taken hypothetically as the "natural" population in meats, and if the strains suspended in PBS behave comparably to those whose growth is prevented on meat, then after chilled storage of such meat where *L. monocytogenes* cannot grow, approximately one-half of strains *in situ* would maintain their high pathogenicity, while the remainder would have decreased pathogenicity. Unfortunately, there are no methods available to directly differentiate and assess pathogenicity of *L. monocytogenes* cells *in situ* on meat at present, and isolating attenuated cells from foods by subculturing them probably changes their pathogenicity (Buncic *et al.*, 1996). Further research is, therefore, necessary to assess the real implications of strain variability in *L. monocytogenes* for the safety of chilled, long-term stored foods.

Table 1 - Variations in pathogenicity and lag phase at 37°C among different strains of non-growing *L. monocytogenes* stored at 4°C.

Mortality (%) of inoculated chick embryos	*L. monocytogenes* before cold storage		*L. monocytogenes* after cold-storage (4 weeks at 4°C)					
			In PBS of pH 7.0		In PBS of pH 5.5		In PBS of pH 5.5 plus potassium sorbate (0.3%)	
	Number of strains in pathogenicity range	Mean lag phase (h) at 37°C	Number of strains in pathogenicity range	Mean lag phase (h) at 37°C	Number of strains in pathogenicity range	Mean lag phase (h) at 37°C	Number of strains in pathogenicity range	Mean lag phase (h) at 37°C
Human strains (n=15)								
80-100	15	2.17 (1.83-2.75)	15	3.59 (2.19-4.55)	14	3.95 (2.89-5.13)	13	7.86 (5.47-12.07)
60-70	-	-	-	-	1	3.95	-	-
40-50	-	-	-	-	-	-	1	7.09
20-30	-	-	-	-	-	-	1	8.90
Meat strains (n=15)								
80-100	15	2.66 (1.81-2.96)	6	4.85 (4.11-6.65)	12	4.68 (4.21-5.30)	8	8.81 (7.56-10.19)
60-70	-	-	5	4.52 (3.83-5.06)	2	4.58 (3.74-5.43)	3	7.46 (6.96-8.79)
40-50	-	-	3	4.67 (3.78-5.58)	1	4.28	2	8.92 (7.34-10.50)
20-30	-	-	1	3.41	-	-	2	9.76 (9.24-10.31

Table 2 - Group characteristics of human and meat isolates of *L. monocytogenes* with respect to response to storage in nutrient-free substrate at refrigeration temperature.

Strains	*L. monocytogenes* before cold storage		*L. monocytogenes* after cold storage (4 weeks at 4°C)					
			In PBS of pH 7.0		In PBS of pH 5.5		In PBS of pH 5.5 plus potassium sorbate (0.3%)	
	Pathogenicity (% mortality of chick embryos)	Lag phase (h) when subcultured in BHI broth at 37°C	Pathogenicity (% mortality of chick embryos)	Lag phase (h) when subcultured in BHI broth at 37°C	Pathogenicity (% mortality of chick embryos)	Lag phase (h) when subcultured in BHI broth at 37°C	Pathogenicity (% mortality of chick embryos)	Lag phase (h) when subcultured in BHI broth at 37°C
Human strains[a] (n=15)	100.0±0.0	2.17±1.7	96.7±4.9^{NS}	3.59±0.57***	94.6±9.7^{NS}	4.06±0.75***	88.0±20.8*	7.68±1.94***
Meat strains[b] (n=15)	98.7±5.2	2.66±0.60	68.0±20.1***	4.61±0.71***	88.6±14.1*	4.73±0.56***	75.7±24.1***	8.54±1.30***
Total (n=30)	99.3±3.6	2.41±0.53	82.3±12.5**	4.10±0.64***	91.48±12.3*	4.38±0.73***	82.1±22.8**	8.09±1.69***

Significance of differences between means, as compared to strains before cold-storage: * P<0.05; ** P<0.01; ***P<0.001; NS No significant differences
[a] Strains (isolated from clinical specimens) that caused human listeriosis; [b] Strains (isolated from meats) that have not been involved in human listeriosis

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