Listeria monocytogenes: A Micro-organism of Concern?

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Introduction

Listeria monocytogenes is a widely distributed bacterial pathogen, that has been isolated from a wide variety of foods, including meat, poultry, dairy, marine and vegetable products. The ubiquity of the organism and its ability to grow at refrigeration temperatures present distinct problems to the food industry. In this contribution, factors influencing the isolation, confirmation and identification of *L. monocytogenes*, and the behaviour of this pathogen in food and environment are discussed.

Detection of *Listeria monocytogenes*

There are three basic protocols for the detection of pathogens:

- direct plating on selective media
- direct selective enrichment and
- pre-enrichment.

Depending on the number of cells expected in a sample and/or the standards described (by law or otherwise), one or more of these procedures may be used for their detection. The success of all three basic protocols depends on:
the number and the state of the micro-organisms in the sample the selectivity of the media (a balance between inhibition of competitors and inhibition of the target organism) conditions of incubation (time, temperature, presence of oxygen) and the electivity of the isolation medium (the ease of distinction between the target organism and competitive microflora).

Of the six accepted species of *Listeria*, only *L. monocytogenes* is a human pathogen. Generally, microorganisms of the same genus are not equally affected by inhibitors used in selective enrichment media. Therefore, in procedures where all species of a genus are considered to be pathogen, e.g. *Salmonella*, only epidemiologically false information may be obtained. However, in case of detecting *L. monocytogenes*, other (faster growing) listerias can mask the presence of this pathogen (MacDonald and Sutherland 1994). The use of lithium chloride and other inhibitors in the enrichment procedure did not overcome this effect (Cox et al. 1990). Further studiès revealed that the use of acriflavine in enrichment media had both direct and indirect effects on the isolation of *L. monocytogenes*. Increasing acriflavine concentrations affected both lag time and generation time of *L. monocytogenes*, while hardly any effect was observed on *L. innocua*. Acriflavine binds to proteins in the samples, and with decreasing pH more acriflavine was bound. Growth promoting effects for *L. monocytogenes* are limited, because growth of this pathogen is restricted at low pH (Beumer et al. 1996a). It seems that the competitive microflora benefits more, since it has been reported that the predominant microflora in enrichment media for *Listeria* consists of lactobacilli and enterococci (Duffy et al. 1994).

All commercially available isolation media for *Listeria* suffer from the same disadvantage: *L. monocytogenes* cannot be differentiated from non-pathogenic listerias. Even the selection of five colonies from such media does not guarantee the detection of this pathogen, even though a few *L. monocytogenes* colonies are present on the plate. Enhanced hemolysis agar (EHA), a medium on which *L. monocytogenes* can be distinguished from other *Listeria* spp. on the basis of hemolysis, previously described by Cox et al. (1991a 1991b) was improved by Beumer et al. (1997). The use of EHA as direct plating- or isolation medium is to prefer to the use of media applying an upper layer with blood agar, because this doubles the incubation period.

The traditional methods for the detection of *Listeria* are both time consuming and labour intensive. Therefore, food producers and distributors as well as public health authorities have a great interest in rapid methods. It was shown that there are some *Listeria* and *L. monocytogenes* test kits on the market, which may be superior to the standard methods (Beumer et al. 1996b). The results were strongly dependent on the concentration of *Listeria* cells in the sample, the detection limits of the tests and the enrichment protocol used. Most rapid tests detected all samples with high numbers of listerias (>10 cfu g^{-1}). Because the outcome of collaborative and comparative studies is often used for promotion purposes, results obtained with samples with high numbers of *Listeria* should be excluded. Moreover, the use of identical enrichment protocols is recommended. In those cases where this is not feasible, cross-checking of the enrichment media is necessary (Noah et al. 1991). There are rapid tests for the detection of *Listeria* spp. as well as for the detection of *L. monocytogenes*. It is difficult, if not impossible to prefer one to another. Only methods detecting the human pathogen *L. monocytogenes* may be preferably. However, knowing the masking of *L. monocytogenes* in enrichment media by other listerias, it would be wise to opt for the detection of the genus *Listeria*.

Confirmation and identification of *Listeria* species

For confirmation of typical colonies on isolation media, microscopy (Gram-stain) and traditional biochemical tests are used (Lovett 1988). These tests can also be replaced by rapid methods, such as latex tests and DNA-probes. For identification of *Listeria* spp., systems based on biochemical tests are available. The Microscreen *Listeria* latex test gives fast reliable results for the confirmation of suspected colonies on isolation media for *Listeria*. For the confirmation of *Listeria monocytogenes* the two DNA-probes (Accuprobe and Gene-Trak) perform equally well, and for further biochemical identification on species level both the API *Listeria* and the Micro-ID *Listeria* tests can be used. Preference may be given to the API test, since differentiation of *L. monocytogenes* from *L. innocua* is based on the absence of arylamidase, through which tests for hemolytic activity and/or CAMP reaction can be omitted (Beumer et al. 1997). For epidemiological studies further typing of the strains is necessary. Both serotyping (Seeliger and Höhne 1979) and phage-typing (Rocourt et al. 1985) are commonly used for this purpose. Unfortunately, serotyping

provides insufficient information (only three serotypes are responsible for the majority of listeriosis outbreaks) and, although phage typing allows further strain subdivision, not all isolates are typable with the existing set of phages. Moreover, only a small number of laboratories have the possibility to use this method. Multilocus enzyme electrophoresis (Harvey and Gilmour 1994) and DNA-based typing methods such as restriction fragment length polymorphism (RFLP) (Ridley 1995), randomly amplified polymorphic DNA (RAPD) (Farber and Addison 1994) and ribotyping (Wiedmann et al. 1996) are valuable alternatives for traditional typing protocols (Nørrung and Gerner-Smidt 1993, Boerlin et al. 1995).

Growth of *Listeria monocytogenes* on raw meat and sliced cooked meat products

Several studies have reported conflicting results concerning the survival and growth of *L. monocytogenes* on meat. Due to the use of different strains (Barbosa et al. 1995), inoculated on various types of meat, and storage temperatures varying from 0-30°C in different atmospheres, results cannot be compared directly (Buchanan and Klawitter 1991, Kaya and Schmidt 1991). Generally, it can be said that growth of *L. monocytogenes* on meat is strain dependent and mainly determined by initial pH of the meat and storage temperature. Abusive storage temperatures may result in the growth of this pathogen. For that reason, and because survival at low pH for a long period is likely (Farber and Peterkin 1991), raw meat that has been stored for a long time, should not be used for the production of ready-to-eat products such as steak tartare.

In cooked meat products *L. monocytogenes* should be absent. As raw meats generally contain low numbers of *Listeria* (less than 100 cfu g^{-1}), the heating of these products for 2 min at 70°C, or to core temperatures of 71°C, is likely to inactivate any *L. monocytogenes* present (Mackey et al. 1990). Still the incidence of *L. monocytogenes* on prepacked sliced cooked meat products has frequently been reported. From previous studies it is clear, that presence of this pathogen was most likely due to post process contamination rather than survival of the heating process (Wang and Muriana 1994). As these products are supposed to have a refrigerated shelflife of several weeks, it is important to know the growth potential of this pathogen on cooked meat products. In a study of Beumer et al. (1996d) it was shown that during the shelf life (4-6 weeks) of artificially contaminated sliced cooked meat products, the growth of *L. monocytogenes* in vacuum packaged products was similar to the growth in modified atmosphere (30% CO_2/70% N_2) packed products.

Despite improved transport of food products (fast, controlled conditions), there is a tendency towards extending storage life of raw or minimally processed foods through cooling and modified atmosphere packaging (MAP). The predominant microorganisms on the cooked meat products in that study were lactic acid bacteria. Even if lactic acid bacteria were present in concentrations one hundred times those of *L. monocytogenes*, the growth of this pathogen was only slightly inhibited. At the end of the shelflife, levels were still 10^7 cfu g^{-1}. To prevent outgrowth of *L. monocytogenes* to such high levels, it is necessary to prevent recontamination during slicing and packaging, and to shorten the rather long shelflife of these products. Addition of nisin in a concentration of 25 mg per kg to luncheon meat, led to an initial decrease in numbers of *L. monocytogenes*. After storage for one week at 7°C, the levels were about two log units lower compared to the controls, but at the end of the storage time (after 6 weeks) counts in both products exceeded 10^7 cfu g^{-1}. This phenomenon is due to the rapidly decreasing activity of nisin in food products (Chung et al. 1989, Fang and Lin 1994).

Since spoilage may not be evident in MAP foods, consumers could judge such products as safe even in the presence of high numbers of pathogens. Processors should be aware of this and take measures to prevent recontamination of the products. Studies in factory ecology can be a valuable tool for this. As such steps will never fully guarantee the absence of a single pathogen, the shelflife of products supporting the growth of *L. monocytogenes* and/or other pathogens should be restricted to the period necessary for an initial contamination of *L. monocytogenes* to reach numbers of 10^2 cfu g^{-1} at the last day of consumption. In respect of this, challenge studies will be necessary with strains preferably isolated from similar naturally contaminated products, or from food processing environments (Notermans and In 't Veld 1994).

As the recontamination of food products with *L. monocytogenes* is usually limited, only few (micro) colonies will be formed during the storage. Therefore, it is recommended to examine large portions (e.g. whole packages) to increase the chance to detect *L. monocytogenes*.

Effect of osmoprotectants on the growth of *Listeria monocytogenes*

L. monocytogenes can grow in the presence of a wide range of salt concentrations in complex media and food products. To understand the ability of a microorganism to grow and survive in widely diverse environments, it is necessary to determine the nutritional requirements of that organism in a chemically defined minimal medium (MM). The MM is also important to study how these nutritional requirements are affected under various stress conditions. A MM that was based on a medium described by Premaratne et al. (1991), containing five essential amino acids, was used to determine the influence of incubation temperature, salt concentration and various osmoprotectants on the growth of *L. monocytogenes* (Beumer et al. 1994). In the absence and presence of NaCl (3 %) final cell numbers reached in minimal medium were 10^9 and 10^7 cfu ml^{-1}, respectively. Growth in the latter condition could not be detected by spectrophotometry measuring absorbance at 660 nm. Apparently this technique is not suitable for these experiments since the detection level is $>10^7$ cfu ml^{-1}.

Exogenously added proline, betaine and carnitine significantly stimulated growth under osmotic stress conditions in minimal medium both at 37 and 10°C. Betaine and carnitine are present in foods derived from plant and animal origin, respectively. These compounds can therefore contribute significantly to growth of *L. monocytogenes* in various foods at high osmolarities.

Listeria species in domestic environments

Although various food products have been implicated in outbreaks of listeriosis, the source and route of infection are usually unknown. The investigation of a case of listeriosis is often hampered by

both the limitations of traditional typing techniques and by the occurrence of strains unrelated to the outbreak strain in implicated food products and in the environment (Kerr et al. 1995). This can be contributed to the isolation methods used. However, it cannot be ruled out that unknown sources of *L. monocytogenes* may also be responsible for illness. Although presence of *Listeria* spp. in household environments has been demonstrated (Cox et al. 1989), these environments have not frequently been included in epidemiological studies. The occurrence of *Listeria* spp. was investigated in 213 households in The Netherlands (Beumer et al. 1996e). Samples were taken from six domestic environments such as dishcloths, washing-up brushes, tooth brushes, the bathroom (shower), the kitchen sink and the vegetable compartment of the refrigerator. Using a direct isolation method *Listeria* spp. were detected in 101 (47.4%) of the 213 houses investigated. In 45 (21.1%) *L. monocytogenes* was present. *Listeria* occurred at all sampling sites; dishcloths (37%) and surface samples around the drain in the bathroom (27.2%) were most frequently contaminated. Highest numbers (10^4-10^5 cfu/object) were found in dishcloths and washing-up brushes. From these data it is clear that *L. monocytogenes* may be present in high numbers in the domestic environment. Until now it is not clear if these numbers contribute directly or indirectly (via contamination of food by these objects) to listeriosis. Future epidemiological studies should also take the domestic environment into account. The use of appropriate typing methods may elucidate if these sources contribute to outbreaks of listeriosis. From the results of this study it seems that careful personal and domestic hygiene could be an important factor in the prevention of human listeriosis and other foodborne infections.

Conclusion

Fortunately, the numbers of listerias in most food products are low ($<10^2$ cfu g^{-1}). However, in ready-to-eat products with a long refrigerated shelf life numbers may increase to 10^6-10^8 cfu g^{-1}. As the pathogenicity of *L. monocytogenes* strains varies considerably (Hof and Rocourt 1992) and the susceptibility for this pathogen may vary from individual to individual, it remains obscure which dose is necessary to cause illness and which microbiological standards will guarantee safe food.

Food safety can be defined as: 'All conditions and measures necessary in the whole food chain (from production to consumption), to ensure that food is a negligible risk to health'. It is currently considered that ready-to-eat-foods should be free from salmonellas, campylobacters, *Escherichia coli* O157 and other verotoxin producing strains of *E. coli* (VTEC). For the remaining pathogens (*Bacillus cereus*, *Clostridium perfringens*, *L. monocytogenes*) generally more liberal standards or guidelines are given.

The largest US listeriosis outbreak occurred in 1985 in Southern California (Linnan et al. 1988) and led to the formulation of the 'zero tolerance' for *L. monocytogenes* in ready-to-eat foods, adopted first by the Food and Drug Administration (FDA) and then by the US Department of Agriculture (USDA) (Klima and Montville 1995). More realistic criteria, including a sample plan, have been formulated by the International Commission on Microbiological Specifications for Food (Van Schothorst 1994). However, no sampling plan can ensure the absence of a pathogen in food (ICMSF 1986). The government, industry and consumer are the most important parties in ensuring food safety. The government should establish standards and develop codes of practice and ensure that these will be observed. Food producers and food preparators should produce safe food, which may be achieved by adequate hygiene standards, good manufacturing practices (GMP) and implementation of hazard analysis and critical control points (HACCP). Consumers, in particular those who buy food and prepare meals, should have a basic knowledge of safe food preparation.

Due to the great variety in protocols and media for the detection of *Listeria* and/or *L. monocytogenes* it is difficult to make the right choice. Fortunately, the proposed ISO method (Anonymous 1995) employs a medium that keeps its pH at neutral values during enrichment. However, one of the major drawbacks is the rather high (37°C) incubation temperature of the secondary enrichment broth. Many immunological tests are based on flagellar antibodies, and since *Listeria* spp. loose their motility at 37°C, it would be better to prescribe an incubation temperature of 30°C both for primary and secondary enrichment.

Presence of *L. monocytogenes* in heat-processed food products is due to recontamination rather than to survival of heat treatments. Therefore, not too much attention should be paid to the recovery of injured cells. Many studies indicating the presence of damaged cells, leading to lower recoveries on selective isolation media, were performed with unrealistically high initial numbers of *Listeria*. To control presence of *L. monocytogenes* in food knowledge of factory ecology is as important as knowledge about micro-organisms obtained in challenge experiments and both are indispensable in HACCP procedures. Many researchers are convinced that quantitative risk analysis also contributes to a lower incidence of listeriosis and other foodborne infections (Notermans and Jouve 1995, Todd and Harwig 1996). Certainly, hazard identification, followed by risk characterization (to estimate the severity of the hazard) are essential steps in risk management. However, obtaining reliable data for exposure assessment and dose response assessment is difficult, if not impossible. After foodborne outbreaks the numbers of pathogens in the incriminated products may have changed, due to outgrowth or die-off. Moreover, the detection methods used may hamper, due to high numbers of competitors or otherwise.

It has been suggested that the decreased incidence of listeriosis in the UK (McLauchlin 1992), Spain (Nolla-Salas et al. 1994) and in the USA (Tappero et al. 1995) can be contributed to the effectiveness of the preventive measures (zero tolerance, intensified clean-up programs and dietary recommendations). Therefore, international microbiological criteria (preferably standards, since guidelines are non-committal) should be established. As zero-tolerance for ready-to-eat foods is unrealistic, international agreement should be based on $<10^2$ *L. monocytogenes* cells per gram on the sell-by date. Persons at risk (immuno-compromized, pregnant) should be strongly advised not to eat food products likely to contain *L. monocytogenes*. Meanwhile, more attention should be given to challenge studies, factory ecology and sound cleaning and disinfection. From studies to evaluate dairy and food plant sanitizers it was concluded that many commonly used

sanitizers are effective at the recommended concentration. However, particularly in the presence of organic material not all disinfectants perform equally well against *Listeria* spp. (Best et al. 1990).

In conclusion, the problems caused by *L. monocytogenes* seem controllable, in contrast with these caused by *Salmonella* and *Campylobacter*.

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