Food Microbiology 46 (2015) 154–160

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

# Effects of osmotic pressure, acid, or cold stresses on antibiotic susceptibility of *Listeria monocytogenes*

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ARTICLE INFO

*Article history:*
Received 6 March 2014
Received in revised form
12 July 2014
Accepted 18 July 2014
Available online 7 August 2014

*Keywords:*
*Listeria monocytogenes*
Antibiotic susceptibility
Osmotic pressure
Acid stress
Cold stress
Stress adaptation

ABSTRACT

Prevalence of antibiotic resistance of *Listeria monocytogenes* isolated from a variety of foods has increased in many countries. *L. monocytogenes* has many physiological adaptations that enable survival under a wide range of environmental stresses. The objective of this study was to evaluate effects of osmotic (2, 4, 6, 12% NaC), pH (6, 5.5, 5.0) and cold (4 °C) stresses on susceptibility of three isolates of *L. monocytogenes* towards different antibiotics. The minimal inhibitory concentrations (MICs) of tested antibiotics against unstressed (control), stressed or post-stressed *L. monocytogenes* isolates (an ATCC strain and a meat and dairy isolate) were determined using the broth microdilution method. Unstressed cells of *L. monocytogenes* were sensitive to all tested antibiotics. In general, when *L. monocytogenes* cells were exposed to salt, cold and pH stresses, their antibiotic resistance increased as salt concentration increased to 6 or 12%, as pH was reduced to pH 5 or as temperature was decreased to 10 °C. Results showed that both meat and dairy isolates were more resistant than the ATCC reference strain. Use of sub-lethal stresses in food preservation systems may stimulate antibiotic resistance responses in *L. monocytogenes* strains.



## 1. Introduction

*Listeria monocytogenes* is a Gram-positive organism and one of 5 five species including *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria ivanovii*, and *Listeria grayi* which comprise this genus (Farber and Peterkin et al., 1991). Recently, two newly identified species (*Listeria marthii* and *Listeria rocourtiae*) were reported (Graves et al., 2010; Leclercq et al., 2009). *L. monocytogenes* is widely distributed and has been isolated from a variety of ready-to-eat and raw dairy, meat and meat products, sea foods and fresh produce (Bell and Kyriakides, 2005).

*L. monocytogenes* is a major concern for food producers, health regulatory officials, and consumers since it is considered one of the more virulent foodborne pathogens (McLauchlin et al. 2004). Although the incidence of listeriosis is rare compared to illness caused by other foodborne pathogens such as *Escherichia coli* O157:H7, *Campylobacter jejuni* or *Salmonella* spp., *L. monocytogenes* has been extensively studied during the past several decades because of its high (20%) case-fatality and high hospitalization (90%) rates (Lianou and Sofos, 2007). Furthermore, *L. monocytogenes* is a versatile organism that has the ability to survive for long periods under adverse conditions including cold storage, high NaCl concentrations and acidic pH (Rhoades et al., 2009).

Antibiotic treatment of infectious listeriosis is indicated and has proven to be successful. These treatments usually rely upon the use of β-lactam antibiotics such ampicillin or penicillin, alone or in combination with an aminoglycoside (gentamicin). However, with patients allergic to β-lactams, trimethoprim and a sulfonamide have been used as an alternative with success (Conter et al., 2009).

Even though it has been experimentally shown that *L. monocytogenes* strains are generally susceptible to a wide range of antibiotics, occasionally antibiotic resistant *L. monocytogenes* strains have been observed (Depardieu et al. 2007). The first multi-drug resistant strain of *L. monocytogenes* was reported in 1988 by Poyart-Salmeron et al. (1990). Since then, *L. monocytogenes* strains have been isolated from food, environmental and human clinical samples which have shown resistance to one or more antibiotics (Morvan et al., 2010).

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http://dx.doi.org/10.1016/j.fm.2014.07.015
0740-0020/

During food processing bacteria may encounter a variety of conditions that may cause chemical (acids, ethanol, alkaline, chlorine and salts) or physical (heat, radiation and pressure) stresses (Yousef and Courtney, 2003). When bacteria are exposed to mild forms of these stresses, opportunity is provided for them to improve their ability to adapt and become resistant to subsequent more extreme exposures through physiological adjustment, enabling reproduction (Depardieu et al. 2007; Hill et al. 2002). Furthermore, the adaptive responses to these stresses may enhance resistance to others such as exposure to antibiotics and lead to what is termed "cross-protection" (Doyle et al. 2006; McMahon et al. 2007).

*L. monocytogenes* may develop an adaptive response during exposure to sublethal food processing conditions (Hill et al. 2002). Lou and Yousef (1997) confirmed that *L. monocytogenes* adapted to pH 4.5–5.0 had increased resistance to lethal concentrations of $H_2O_2$, ethanol, and acid. In addition, *L. monocytogenes* is able to transfer antibiotic resistance genes to other strains of *Listeria* spp. as well as other pathogens, like *Staphylococcus aureus*, *Enterococcus* spp., and non-pathogenic bacteria (Courvalin, 1994; Walsh et al. 2001). Therefore, understanding the effects of stress during food processing on the antibiotic susceptibility of *L. monocytogenes* is important in developing strategies to facilitate effort to achieve clinical control over pathogens that originate from food. The objectives of the current study were to investigate the effect of osmotic, cold and acid stresses on the susceptibility of *L. monocytogenes* toward different antibiotics.

## 2. Materials and methods

### 2.1. Preparation of bacterial cultures

Three strains of *Listeria moncytogenes* were used in the current study; *L. moncytogenes* ATCC 7644 and two *L. moncytogenes* isolates from processed meat and dairy products, respectively, in Jordan. These isolates were obtained from the Department of Nutrition and Food Technology at Jordan University of Science and Technology and were kept frozen in glycerol. They were maintained on Typtic Soy Agar (TSA, Oxoid Ltd., Basingstoke, UK) slants at 4 °C and transferred bi-weekly to maintain viability. Working cultures were prepared by growth in 10 ml Tryptic Soy Broth (TSB, Oxoid) incubated at 37 °C for 20 h. For tests, 100 μl was transferred to 100 ml TSB and incubated at 37 °C for 20 h. The cultures were diluted in Mueller Hinton Broth (MHB, Oxoid) to give a final concentration of approximately 6 $\log_{10}$ CFU/ml in the reaction mixtures.

### 2.2. Antibiotics

The three *L moncytogenes* isolates were challenged with 9 antibiotics (Table 1), chosen according to their mode of action and use in clinical therapy. Those that inhibit cell wall synthesis included vancomycin 98%, ampicillin 84.5% (Bio Basic Inc., Markham, ON, Canada) and penicillin G sodium salt 98% (Biochemika Int., Hangzhou, China). Those that inhibit protein synthesis included tetracycline hydrochloride 90%, gentamicin sulfate 59%, streptomycin sulfate 65% (Bio Basic Inc.), and doxycycline HCL 99.5% (Tocelo Chemicals B.V., Hertogenbosch, Netherlands). Those that inhibit nucleic acid synthesis included enrofloxacin 99.39% (Tocelo Chemicals B.V.) and ciprofloxacin 98% (Biochemika Int.) (Yanoeyama and Katsumata, 2006; Al-Nabulsi et al. 2011).

### 2.3. Preparation of antibiotic stock solutions

Antibiotic stock solutions were prepared following the manufacturers' recommendations. Vancomycin, ampicillin, penicillin, tetracycline, gentamicin and streptomycin were dissolved directly in 10 ml distilled water. Doxycycline and ciprofloxacin were dissolved in sterile distilled water with a drop (≤0.05 ml) of 1 N HCL. Enrofloxacin was first dissolved in a few drops of 0.1 N NaOH and then diluted with sterile distilled water. All stock solutions were sterilized by using 0.20 μm disposable syringe filter units (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and serially diluted to provide the desired concentrations (0.01–1000 μg/ml) in the reaction mixtures.

**Table 1**
Minimal inhibition concentration (MIC) breakpoints used to determine antibiotic susceptibility.[a]

| Antibiotics | MIC[b] breakpoint (μg/ml) | | |
|---|---|---|---|
| | S[c] | I | R |
| Aminoglycosides antibiotics | | | |
| Streptomycin | ≤8 | >8, ≤16 | >16 |
| Gentamycin | ≤4 | >4, ≤8 | >8 |
| Tetracycline antibiotics | | | |
| Tetracycline | ≤4 | >4, ≤8 | >8 |
| Doxycycline | ≤4 | >4, ≤8 | >8 |
| Aminopenicillin antibiotics | | | |
| Ampicillin | ≤4 | >4, ≤16 | >16 |
| Penicillin | ≤0.25 | >0.25, ≤16 | >16 |
| Glycopeptide antibiotics | | | |
| Vancomycin | ≤4 | >4, ≤16 | >16 |
| Fluoroquinolone antibiotics | | | |
| Ciprofloxacin | ≤1 | >1, ≤2 | >2 |
| Enrofloxacin | ≤1 | >1, ≤2 | >2 |

[a] As proposed by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) in France (Acar et al., 1992).
[b] MIC: (Minimal Inhibitory Concentration) the lowest concentration of the antibiotics required to inhibit the visible growth of the microorganism.
[c] S: Susceptible, I: Intermediate, R: Resistant.

### 2.4. Preparation of stressed L. moncytogenes cultures

Preliminary tests showed that the mild stresses to be used in the current study would decrease the initial number of cells by approximately ≤1.0 $\log_{10}$ CFU/ml except for osmotic stress at 12% NaCl, where the initial number of cells was decreased by approximately 2 $\log_{10}$ CFU/ml. The number of survivors was determined by plating on TSA before and after exposure to stress.

#### 2.4.1. Osmotic stress

Four different levels of NaCl were used; 2%, 4%, 8%, and 12% (wt/vol) in TSB. The NaCl-supplemented suspensions were inoculated with *L. monocytogenes* and incubated at 37 °C for 24 h. *L. monocytogenes* cells were harvested by centrifugation (Herolab, UniCen M, Wiesloch, Germany) at 4000 g for 20 min and the pellet was resuspended and diluted to give a final concentration of approximately 6 $\log_{10}$ CFU/ml in the reaction mixtures for antibiotic challenge.

#### 2.4.2. Acid stress

Three different levels of pH were used in the current study; 6.0, 5.5 and 5.0. The acid-stressed cells were prepared as described by Francois et al. (2006) and Osaili et al. (2008). Briefly, 10 ml of an overnight culture of *L. monocytogenes* (pH 6.3) was harvested by centrifugation at 4000 g for 20 min and the pellet was resuspended with 10 ml 0.1M potassium phosphate buffer previously adjusted to pH 6.0, 5.5 or 5.0 with 85% lactic acid (Sigma, St. Louis, MO, USA) and then held at room temperature for 30 min. The culture was diluted to give a final concentration of approximately 6 $\log_{10}$ CFU/ml in the reaction mixtures for antibiotic challenge.