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Gene expression in *Listeria monocytogenes* exposed to sublethal concentration of benzalkonium chloride

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ABSTRACT

In this study, tolerance at sublethal concentration of benzalkonium chloride and transcription levels of *mdr*L, *lad*R, *lde*, *sig*B and *bcr*ABC genes in *Listeria monocytogenes* strains were evaluated. Viable cells reduction occurred in 45% of strains and clinical isolates showed lower sensitivity than isolates from foods. An increased transcription of an efflux system encoding gene was found in 60% of strains, and simultaneous *mdr*L overexpression and *lad*R underexpression occurred in 30% of isolates. A significant association between reduced benzalkonium chloride activity and both *mdr*L and *sig*B overexpression was observed; *sig*B expression also correlated with both *mdr*L and *lad*R genes. The *bcr*ABC gene was only found in six strains, all isolated from foods and sensitive to benzalkonium chloride, and in four strains an underexpression was observed. Disinfection at sublethal concentration was less effective in clinical isolates, and *mdr*L and *sig*B expression was significantly affected by disinfection. Further insights are needed to understand the adaptation to benzalkonium chloride and to evaluate whether changes in gene expression could affect the *L. monocytogenes* virulence traits and persistence in the environment.

1. Introduction

Listeria monocytogenes is an opportunistic intracellular pathogen responsible of serious infections, usually by ingestion of contaminated foods [1]. The majority of human cases are caused by three (4b, 1/2a, and 1/2b) out of 13 known serovars [2]. Clinical signs can vary from self-limiting gastroenteritis among healthy people to invasive infections with high fatality rates in vulnerable population groups (neonates, pregnant women, elderly, AIDS patients and individuals under immunosuppressive treatment) [3,4]. *L. monocytogenes* can survive under adverse environmental conditions and during human gastrointestinal passage [5]. Infection of ventriculoperitoneal shunt, prosthetic joints and heart valves [6,7] further confirmed *L. monocytogenes* ability to form and survive within biofilms on indwelling medical devices. This food-borne pathogen is also a common contaminant of food processing plants where it may persist over extended periods of time [8], and

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the application of biocides such as quaternary ammonium compounds (QACs) represents a useful approach for the control of *L. monocytogenes* in the processing environment.

Hence, widespread distribution, natural resistance to stress conditions, and biofilm formation are of serious concerns for food industry [3,9] and healthcare settings, particularly if routine cleaning and disinfection procedures are not properly performed [10,11]. Moreover, an increased resistance to antimicrobials by Gram-positive and negative pathogens has been described [1,12], due to selective pressure for the extensive use of disinfecting agents and antibiotics.

Benzalkonium chloride (BAC), belonging to QACs, is widely used for surfaces sanitization or as antiseptic, and it is responsible for the dissociation of cell membrane lipid bilayers by altering cellular permeability and inducing leakage of cellular contents [13]. The bactericidal activity begins when a critical minimum concentration is surpassed below which such activity is too slow or absent; thus, the effective concentration of QAC compounds could not be lower than certain thresholds, even if time application is significantly extended. In industry, concentrations of about 1000 ppm are commonly used when applying QACs to machinery for disinfection [14].

Although high concentration of QACs ensures complete eradication of any pathogenic bacteria from the surface of industrial equipment, *L. monocytogenes* has been shown to survive and adapt when exposed to sublethal concentrations of these disinfectants [15]. Hence, BAC adaptation in *L. monocytogenes* strains is a significant public health emerging issue for the selection and persistence of resistant mutants [16]. The mechanisms involved in the intrinsic or acquired QACs resistance are not completely elucidated; however, previous studies provided evidences for both chromosomal determinants and plasmid-mediated BAC resistance.

A putative BAC resistance cassette, known as *bcr*ABC, was previously identified on a large plasmid (pLM80) of *L. monocytogenes* H7550 strain involved in the 1998–1999 United States listeriosis outbreak [17], as well as in other *Listeria* sequenced genomes. BAC-associated resistance cassette is composed by TetR family transcriptional regulator (*bcr*A) and two SMR (small multidrug resistance) genes (*bcr*B and *bcr*C), all essential for imparting BAC resistance. Dutta et al. [18] reported that transcription of *bcr*ABC in *L. monocytogenes* strains is induced by BAC sublethal concentration (10 μg/ml), and the analysis has shown that the majority of BAC-resistant strains had either the pLM80-type of organization of *bcr*ABC region or appeared to harbor *bcr*ABC on the chromosome, adjacent to novel sequences.

Furthermore, Tn6188, an integrated chromosomally transposon, has been described to provide an increased tolerance of *L. monocytogenes* strains to BAC [19]. Tn6188 consists of three transposase genes (*tnp*ABC), genes encoding a putative transcriptional regulator and QacH, a small multidrug resistance protein family (SMR) transporter associated with export of BAC.

The induction of multidrug resistance efflux pump (MdrL) after BAC exposure has also been reported [20], and a putative protein, encoded by *orf*A gene, renamed as *lad*R [21], is considered as the *mdr*L transcriptional repressor [22]. The Listeria drug efflux transporter, encoded by *lde* chromosomal gene, belongs to the major facilitator superfamily of secondary transporters, and is involved in the excretion of toxic compounds from bacterial cells, including some antibiotics. Moreover, several studies confirmed the role of the alternative sigma factor B (σ^B), encoded by *sig*B gene, in the response to environmental stress conditions (*i.e.*, low pH, high bile and ethanol exposure, osmotic stress, *etc.*), and in BAC and peracetic acid resistance [23,24]. The induction of σ^B-dependent genes under environmental stress conditions may support the hypothesis that resistant isolates should be more pathogenic, with higher resistance to antimicrobials.

The aim of this study was to evaluate the antimicrobial activity of BAC, defined as the reduction of viable plate counts, at sublethal concentration, in *L. monocytogenes* isolated from foods and clinical cases in Italy, in order to evaluate the tolerance phenotype of the strains according to the isolation source. Changes in the relative expression of efflux systems encoding genes (*mdr*L and *lde*) and their regulators (*lad*R and *sig*B), as well as of *bcr*ABC cassette, in the same isolates upon exposure to BAC sublethal concentration, were also assessed in order to evaluate the extent of the disinfecting treatment on the transcription, and the interaction in the tolerance development.

2. Materials and methods

2.1. Bacterial strains and bio-molecular subtyping

Twenty *L. monocytogenes* strains from the Laboratory of Hygiene culture collection (University of Molise) and isolated by the Lombardia Regional Surveillance Network (North-Italy) were analyzed. The isolates were collected from both clinical cases ($n = 10$) and foods ($n = 10$) (Table 1). The serotypes were assigned by slide agglutination [25] and multiplex-PCR, developed by Doumith et al. [26], with some modifications [27]. The genomic division or lineage was identified, by PCR, according to Ward et al. [28] and some modifications [27].

Molecular subtyping of *L. monocytogenes* strains was performed by pulsed-field gel electrophoresis (PFGE), according to PulseNet USA protocol [29]. Briefly, the strains were grown overnight on Brain Heart Infusion (BHI; Biolife, Milan, Italy) agar, and cell suspension with optical density (OD_{620}) of 1.6–1.8 in Tris-EDTA (TE, pH 8.0) buffer was prepared. Cells were lysed with lysozyme at 37 °C for 10 min, and embedded in 1.2% Seakem® Gold agarose (Lonza, Milan, Italy). The cell plugs were washed and digested with both *Asc*I (10 U/μl) and *Apa*I (50 U/μl) at 37 °C for 4 h. Electrophoresis was carried out in 1% agarose gel (Seakem® Gold Agarose) with 0.5× Tris-borate-EDTA buffer in CHEF-DR II instrument (Bio-Rad, Milan, Italy) at 6 V/cm for 21 h at switch time of 4–40 s. Gel was stained with ethidium bromide solution, and visualized by UV transilluminator Fire-Reader (UVITEC Cambridge, Eppendorf, Milan, Italy). PFGE profiles were analyzed by using BioNumerics software (Applied Maths, Kortrijk, Belgium) version 6.0. Dendrograms were generated by the unweighted pair group method algorithm (UPGMA) [30] including all the