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Occurrence and antimicrobial resistance patterns of *Listeria monocytogenes* isolated from vegetables

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ABSTRACT

Although the consumption of fresh and minimally processed vegetables is considered healthy, outbreaks related to the contamination of these products are frequently reported. Among the food-borne pathogens that contaminate vegetables is *Listeria monocytogenes*, a ubiquitous organism that exhibits the ability to survive and multiply at refrigerated temperatures. This study aimed to evaluate the occurrence of *L. monocytogenes* in vegetables as well as the antimicrobial resistance of isolates. The results showed that 3.03% of samples were contaminated with *L. monocytogenes*, comprising 2.22% of raw vegetables and 5.56% of ready-to-eat vegetables. Multiplex PCR confirmed the virulence potential of the isolates. Antimicrobial resistance profiling showed that 50% of the isolates were susceptible to the antibiotics used. The resistance of one isolate to penicillin G, a commonly employed therapeutic agent, and the presence of serotype 4b, a serotype commonly associated with food-borne outbreaks, could be potential health hazards for consumers.

Introduction

Food safety is an increasingly relevant issue in the daily lives of consumers. The search for a healthier diet and, at the same time, faster preparation has favored the consumption of fresh and ready-to-eat vegetables. Because they are not subjected to treatments that considerably reduce microbiological hazards, these essential foods are potential vehicles for the transmission of pathogenic microorganisms.[1]

To inhibit microbial multiplication and ensure adequate conservation, certain vegetables are stored and transported at cool temperatures. However, these conditions facilitate the growth of some microbial pathogens, such as *Listeria monocytogenes*, a psychotropic microorganism that is highly relevant to public health.[1,2]

L. monocytogenes is a ubiquitous bacterium that can be found in the irrigation water, soil and fertilizer used on farms and in decaying plant matter, making the presence of this bacterium in vegetables a continual risk.

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The disease caused by *L. monocytogenes*, known as listeriosis, is particularly troublesome for vulnerable populations. This group of people, including pregnant women and their fetuses, the very young and the elderly, is particularly susceptible to invasive listeriosis, with mortality rates ranging between 20 and 40%.[3]

Outbreaks and sporadic cases of listeriosis have been associated with the contamination of various food items, including milk; soft cheese; meat and meat products; vegetables; seafood products; ready-to-eat foods[4]; and, recently, cantaloupes.[5]

It is well established that food product contamination is associated with food-processing environments harboring *L. monocytogenes* and subsequent post-processing transfer to finished products.[6,7]

In Brazil, food-borne listeriosis outbreaks have not been documented, but in recent studies, the presence of *L. monocytogenes* has been described in several products, including ready-to-eat vegetables, such as watercress and escarole[8]; leafy salads[9]; chopped kale and a mixture of spring onion/parsley[10]; salad mix, lettuce, collard greens, a mix for yakisoba, watercress, escarole, cabbage, spinach, and a mix for sukiyaki.[11]

L. monocytogenes is naturally susceptible to a range of antibiotics that act on Gram-positive bacteria.[12] Human strains of *L. monocytogenes* are sensitive to a group of antibiotics that includes penicillin, ampicillin, amoxicillin, gentamicin, erythromycin, tetracycline, rifampicin, co-trimoxazole, vancomycin and imipenem.[13,14] However, most strains of *L. monocytogenes* show natural resistance to current fluoroquinolones and cephalosporins, and especially those of the third and fourth generations, such as cefotaxime and cefepime, and to fosfomycin, oxacillin and lincosamides.[13]

Clinicians usually treat listeriosis with aminopenicillins in combination with an aminoglycoside, such as gentamicin. Additionally, in cases in which reduced sensitivity or resistance to beta-lactams is encountered, a number of agents that are active against Gram-positive bacteria may be used.[14]

Studies performed by the Clinical and Laboratory Standards Institute (CLSI)[15] using human strains and, to a lesser extent, foodstuff strains have not revealed an increase in resistance to antibiotics among the circulating strains of *L. monocytogenes*.[16,17] However, since the isolation of the first multi-resistant strain of *L. monocytogenes* in 1988, interspecies variation in antimicrobial susceptibilities has been reported among *Listeria* species.[12,18]

The purpose of this study was to verify the occurrence of *L. monocytogenes* in raw, frozen and ready-to-eat vegetables commercialized in different locales in Salvador, BA, Brazil, and to characterize *L. monocytogenes* strain using the multiplex PCR method. Furthermore, the resistance/susceptibility of the *L. monocytogenes* strains to eight antibiotics used in human and veterinary medicine was investigated.

Materials and methods

Sample collection

Raw, frozen and ready-to-eat vegetables were purchased at a local supermarket and at fast food outlets in Salvador, BA, in northeastern Brazil, from October 2013 through January 2014. Overall, 132 samples were collected, comprising 45 raw vegetables, 33 frozen vegetables and 54 ready-to-eat vegetables (salads). The raw vegetables included lettuce, broccoli, white cabbage, purple cabbage and arugula (nine samples of each). The frozen vegetables consisted of mixed vegetables (peas, carrots, green beans and maize), peas and broccoli (11 samples of each). The ready-to-eat vegetables included salads containing carrot, purple cabbage and lettuce; salads containing lettuce, beet and purple cabbage; and salads containing purple cabbage, white cabbage, lettuce and beets (18 samples each). Representative sampling was ensured by taking samples from the most consumed brands that contained at least one of the most consumed vegetables in Brazil.[19]

Detection of L. monocytogenes

For the isolation of *L. monocytogenes*, approximately 25 g of each sample was homogenized with 225 mL of half-concentrated Fraser broth (FB; Merck, Darmstadt, Germany) in a stomacher (240 bpm; ITR model 1204, series 126; São Paulo, SP, Brazil) for 2 min in a class II biosafety cabinet (Labconco Purifier Class IIb, Total Exhaust, model 36210-04, certified ISO 9002; Labconco Corporation, Kansas City, MO, USA). This homogenate was then incubated at 30 °C for 24 h. An aliquot of 1 mL was transferred to tubes containing FB supplemented with Fraser selective supplement and incubated at 37 °C for 48 h. The cultures were streaked onto plates containing the *Listeria* agar Ottaviani & Agosti (ALOA™, Laborclin, Pinhais, PR, Brazil) and incubated at 37 °C for 24 h.[20] Afterward, 3–5 suspect colonies (blue, diameter less than 3 mm and with a regular white halo) were selected for confirmation. The confirmation of *L. monocytogenes* colonies in isolation media was based on several methods, including Gram staining and measurement of hemolytic activity on sheep blood agar (Columbia agar supplemented with 5% defibrinated horse blood; HiMedia, São Paulo, SP, Brazil), the carbohydrate utilization pattern (0.5% mannitol, 0.5% rhamnose and 0.5% xylose), the catalase reaction and tumbling motility.[21] One *L. monocytogenes* Scott A (serotype 4b; ATCC 15313) positive control and one uninoculated-medium negative control were used for each set of concurrently analyzed samples.

Antigenic characterization was performed in the Laboratory of Bacterial Zoonoses (LABZOO/IOC/FIOCRUZ) using somatic and flagellar serotyping antisera produced in the same laboratory, as described by Seeliger and Höhne.[22] All strains are deposited in the Collection of *Listeria* (CLIST) from LABZOO and maintained in BHI broth with 20% glycerol at −20 °C.

Multiplex PCR confirmation of isolates

Multiplex PCR was performed to confirm genus, species, lineage and serotypes, and the conditions and the set of primers are summarized in Table 1. The DNA was extracted using the DNeasy Blood & Tissue kit® (Qiagen, Hilden, Germany) following the manufacturer's instructions. The PCR assays in a final volume of 50 μL were done using the following: 1× reaction buffer, $MgCl_2$ 1.5 mM, 0.4 mM of each dNTP, 10 pmol/μL of each primer and 0.5 U/μL of HotStar®Taq polymerase (Qiagen,

Table 1 – Primers, amplicon size and amplification conditions for the PCR assays.

Gene target	Sequence (5′–3′)	Determinant	Amplicon	Amplification conditions	Reference
23S rRNA	F = GGGGAACCCACTATCTTTAGTC R = GGGCCTTTCCAGACCGCTTCA	Genus *Listeria*	239 bp	95 °C (1 min), 62 °C (1 min), 72 °C (1 min)	Hudson et al.[26]
D1	F = CGATATTTTATCTACTTTGTCA R = TTGCTCCAAAGCAGGGCAT	Division I or III	214 bp	95 °C (30 s), 56 °C (30 s), 72 °C (1 min)	Borucki and Call[27]
D2	F = GCGGAGAAAGCTATCGCA R = TTGTTCAAACATAGGGCTA	Division II	140 bp	95 °C (30 s), 56 °C (30 s), 72 °C (1 min)	Borucki and Call[27]
ORF2110	F = AGTGGACAATTGATTGGTGAA R = CATCCATCCCTTACTTTGGAC	Serotype 4b	597 bp	95 °C (1 min), 60 °C (1 min), 72 °C (1 min)	Doumith et al.[28]
lmo0737	F = AGGGCTTCAAGGACTTACCC R = ACGATTTCTGCTTGCCATTC	*L. monocytogenes* serovars 1/2a, 1/2c, 3a and 3c	691 bp	95 °C (1 min), 60 °C (1 min), 72 °C(1 min)	Doumith et al.[28]
lmo1118	F = AGGGGTCTTAAATCCTGGAA R = CGGCTTGTTCGGCATACTTA	*L. monocytogenes* serovars 1/2c and 3c	906 bp	95 °C (1 min), 60 °C (1 min), 72 °C(1 min)	Doumith et al.[28]
ORF2819	F = AGCAAAATGCCAAAACTCGT R = CATCACTAAAGCCTCCCATTG	*L. monocytogenes* serovars 1/2b, 3b, 4b, 4d, and 4e	471 bp	95 °C (1 min), 60 °C (1 min), 72 °C(1 min)	Doumith et al.[28]
Hly	F = GCCTGCAAGTCCTAAGACGCCAATC R = CTTGCAACTGCTCTTTAGTAACAGC	Listeriolysin O	706 bp	95 °C(1 min), 62 °C (1 min), 72 °C(1 min)	Hudson et al.[26]

Hilden, Germany). Standard strains of *L. monocytogenes* ATCC 19115 (serotype 4b), ATCC 19111 (serotype 1/2a), ATCC 19112 (serotype 1/2c) and CDC F4976 (serotype 1/2b), were used as positive controls, and *Listeria innocua* ATCC 12612 was used as a negative control.

The amplified fragments were subjected to electrophoresis on a 2% agarose gel (in TBE buffer), stained with ethidium bromide solution (10 mg/mL) (Sigma, São Paulo, Brazil) and visualized with a UV transilluminator coupled with a digital gel imaging system (Kodak EDAS 290).

Antimicrobial resistance of the isolates

Antimicrobial resistance was assessed by a disk diffusion assay according to CLSI guidelines[15] using the breakpoints of *Staphylococcus* species resistance because no resistance criteria exist for *Listeria* susceptibility testing in the CLSI guidelines.[23–25]

Cells were grown at 35 °C for 24 h in tryptic soy broth (TSB; HiMedia, São Paulo, SP, Brazil), suspended in a saline solution and diluted to 0.5 points on the McFarland scale (ca. 10^8 cfu). The suspension of cells was inoculated in Mueller-Hinton (MH) agar (HiMedia, São Paulo, SP, Brazil) using a swab. Bacterial growth was recorded after 24 h of incubation at 35 °C. One *Staphylococcus aureus* (ATCC 33591) positive control was used for each set of analyzed samples. The isolates were tested against a panel of eight antimicrobial agents: penicillin G (PEN) (10 IU), erythromycin (ERY) (15 μg), tetracycline (TET) (30 μg), oxacillin (OXA) (1 μg), cefoxitin (CEF) (30 μg), vancomycin (VCM) (30 μg), streptomycin (STR) (10 μg) and ciprofloxacin (CIP) (5 μg). The antibiotic discs were purchased from Laborclin (Pinhais, PR, Brazil). The zones of inhibition were measured (mm) at 24 h.

Results and discussion

Prevalence of L. monocytogenes in vegetables

In this work, we detected and identified *L. monocytogenes* in raw and ready-to-eat vegetables purchased at supermarkets located in the city of Salvador, Brazil, to show the existing risk to consumers.

Of the 132 samples analyzed, *L. monocytogenes* was isolated from four (3.03%) samples, of which one (2.22%) came from raw vegetables and three (5.56%) came from ready-to-eat vegetables (salads) (Table 2).

Lettuce was the only raw vegetable contaminated by *L. monocytogenes*, whereas two types of mixed vegetable salads presented the microorganism. No sample of frozen vegetables was contaminated by *L. monocytogenes*.

The four obtained isolates recovered from the raw and ready-to-eat vegetable samples were identified as *L. monocytogenes* serotype 4b by both the conventional antigenic characterization and molecular serotyping methodologies. Multiplex PCR targeting the listeriolysin O genes (*hly*) of the *L. monocytogenes* recovered from the vegetable samples confirmed the virulence potential of the isolates.

The presence of *L. monocytogenes* in many types of raw and ready-to-eat vegetables intended for human consumption has been clearly demonstrated in many countries. In Brazil, the incidence of *L. monocytogenes* in vegetables has been reported in many studies by a number of researchers. The results found in the present work demonstrated higher levels of detection of

Table 2 – Occurrence of *L. monocytogenes* in raw, frozen and ready-to-eat vegetables.

Vegetable group	Samples	Positive samples		Origin
	n	*n*	%	
Raw	45	1	2.22	Lettuce (1 sample)
Frozen	33	ND	0.00	
Ready-to-eat (salads)	54	3	5.56	Lettuce, beets and purple cabbage (1 sample) Lettuce, beets, purple cabbage and white cabbage (2 samples)
Total	132	4	3.03%	

ND, not detected.

L. monocytogenes in salads than the levels previously reported by Froder et al. (0.55%),[9] Oliveira et al. (3.7%),[10] and Sant'ana et al. (3.1%).[11]

In countries other than Brazil, various results have also been reported for the occurrence of *L. monocytogenes* in vegetables. In Santiago, Chile, the bacterium was isolated from 10.2% of salad samples[29]; in Malaysia, from 22.5% of salad samples[30]; in Spain, from 4.18% of vegetable samples[31]; in Marmara, Turkey, from 13.6% of fresh vegetable samples[32] and in Germany, from only four samples of 1001 investigated vegetable samples.[33]

Work performed by Moreno et al.[31] in Valencia, Spain, showed that nine lettuce samples were contaminated by *L. monocytogenes*, with counts between 2.85 and 3.55 $\log_{10}$ viable cells/g of food. Additionally, only one positive sample, found in a dish called a 'Four Seasons Salad,' yielded a high number of viable cells, with a value of 4.35 log10 cells/g of food.

The presence of *L. monocytogenes* in vegetables, verified in the present study and in other studies reported by various authors, is cause for concern, as listeriosis cases are increasing at the global level, in many cases due to the cross-contamination of processed foods.[31] Therefore, Good Agricultural Practices and Good Manufacturing Practices must be addressed to guarantee the safety of food for consumers.

Antimicrobial resistance

In the current study, all *L. monocytogenes* isolates were susceptible to erythromycin (ERY), oxacillin (OXA), cefoxitin (CEF), vancomycin (VCM), streptomycin (STR) and ciprofloxacin (CIP) (Table 3).

Two *L. monocytogenes* isolates from ready-to-eat vegetables exhibited resistance to penicillin G (PEN) and tetracycline (TET).

In contrast, Kovacevic et al.[34] found that *L. monocytogenes* recovered from ready-to-eat fish possessed reduced susceptibility to ciprofloxacin and two of the three clonal 1/2b *L. monocytogenes* isolates were resistant to streptomycin. However, like the results of present study, the authors observed no resistance to erythromycin.

Studies performed by Davis and Jackson[25] with strains of *L. monocytogenes* recovered from diverse origins (i.e. clinical, animal, food, and environmental) showed similar results to those in the present work with regard to CIP and TET resistance. Also, Troxler et al.[35] in Germany tested 87 strains of *Listeria* spp. (19 *L. monocytogenes* from human and sheep and avian origin), predominantly isolated in the USA and different European countries, and grouped *L. monocytogenes* and *L. welshimeri* as naturally sensitive to CIP.

However, according to Kovacevic et al.[34], the extent of comparison between studies is hampered by differences in the methods used to verify resistance.

In general, antibiotic therapy is required for the treatment of listeriosis infections, with clinicians commonly using ampicillin in combination with gentamycin or using trimethoprim-sulfamethoxazole.[36] In the present study, resistance to penicillin G, a commonly employed therapeutic agent, was observed for one isolate of *L. monocytogenes*.

Table 3 – Resistance/susceptibility of *L. monocytogenes* isolated from vegetables.

Antimicrobial	Breakpoints (mm)			Isolates (number)		
	Resistant	Intermediate	Susceptible	Resistant	Intermediate	Susceptible
Cefoxitin	≤19	–	≥20	ND	–	4
Ciprofloxacin	≤15	16–20	≥21	ND	ND	4
Erythromycin	≤13	14–22	≥22	ND	ND	4
Streptomycin	≤19	–	≥23	ND	–	4
Oxacillin	≤17	–	≥18	ND	–	4
Penicillin G	≤28	–	≥29	1	–	3
Tetracycline	≤14	15–18	≥19	1	ND	3
Vancomycin	–	–	≥15	ND	–	4

ND, not detected.

According to Kovacevic et al.,[34] only three isolates of *L. monocytogenes* recovered from 4668 clinical samples in France were resistant to streptomycin (STR). Two isolates were resistant to lower concentrations (4 and 6 mg/mL), whereas one exhibited resistance at 256 mg/mL.[17] Regarding food and environmental strains, none of the 49 *L. monocytogenes* isolates from food and environmental samples tested in the U.S. in 2009 showed resistance to STR (1 mg/mL). In 2010, another study performed in U.S. with *L. monocytogenes* isolated from catfish filets and their respective processing environments reported reduced susceptibility to STR (10 mg) in 2% (2/80) of the isolates).[23] Has been reported that sublethal exposure to triclosan, a broad-spectrum biocide used into a variety of commercial products, promotes resistance to various aminoglycosides, including gentamycin, kanamycin, streptomycin and tobramycin.[34] In addition, resistance to low and high concentrations of the antibiotic has been suggested to be associated with possible ribosomal mutations and/or the production of 6-N-streptomycin adenylyltransferase, encoded by the *aad6* gene.[17,34] These finds is a cause for concern, considering that clinicians typically use aminopenicillins (e.g., ampicillin or amoxicillin) in combination with an aminoglycoside, such as gentamicin, for the treatment of invasive infections.[13,36]

Similar to the results presented in our study, Gómez et al.[13] found that one strain (0.5%) of *L. monocytogenes* recovered from a stainless steel surface in Spanish industry was resistant to tetracycline and eight strains (3.9%) recovered from meat products showed reduced susceptibility to penicillin G. According to the authors, the effectiveness of tetracycline diminished in the last decades owing to the widespread existence of resistance genes, probably because of the extensive and prolonged use of these antimicrobials in human medicine and as growth promoters in animals. In addition, regarding fluoroquinolones, intermediate susceptibility to ciprofloxacin was found in the mentioned work for one strain of the pathogenic species. The authors concluded that all *Listeria* strains were highly sensitive to the preferred antibiotics used to treat listeriosis, although special mention was made of the reduced susceptibility of eight strains of *L. monocytogenes* (3.9%) and of seven strains of *L. innocua* (5.4%) to penicillin G.

The isolates of *L. monocytogenes* investigated in the present study did not show multi-resistance.

Work performed by Korsak et al.[37] on 471 samples from different types of food and food-related sources in Poland demonstrated that one *L. monocytogenes* strain, isolated in 2005 from iced green beans, was resistant to tetracycline (MIC 16 μg/ml). Chen et al.,[23] who investigated ten samples of foods commercialized in China, reported similar results, with 2.7% of the isolates resistant to tetracycline and 8.1% resistant to penicillin. These results show that antimicrobial resistance in *L. monocytogenes* occurs at a low prevalence.

Conclusion

The presence of *L. monocytogenes* in fresh and ready-to-eat vegetables and the resistance of isolates to penicillin G, a commonly employed therapeutic agent, as verified in the present study, are cause for concern, as listeriosis cases are increasing at the global level. In addition, the presence of serotype 4b, more commonly associated with outbreaks, could be a potential health hazard for consumers.

Conflicts of interest

The content of this report solely reflects the opinions of the authors, and we report no conflicts of interest.

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