Potentially pathogenic *Listeria* spp. in refrigerated raw milk
Listeria spp. potencialmente patogênicas em leite cru refrigerado

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ABSTRACT

The aim of this paper was to determine the occurrence of potentially pathogenic and antimicrobial-resistant *Listeria* ssp. in refrigerated raw milk. Of the 140 presumptive isolates, 32.85% (46/140) were confirmed as belonging to the genus *Listeria*. Of these *Listeria* isolates, 6.89% (4/46) presented the three virulence genes investigated (*hlyA*, *inlA* and *actA*), indicating that these isolates have properties of a virulent strain. According to the antimicrobial resistance profile of the *Listeria* tested, vancomycin, gentamicin and tetracycline are the most effective drugs against these bacteria. Ampicillin was the antimicrobial with the highest resistance index, presented by 60.87% of the isolates. In conclusion, the milk produced and stored refrigerated in the Agreste region of Pernambuco constitutes a risk to public health and to the dairy production chain of the State due to the presence of potentially pathogenic and multidrug-resistant *Listeria* spp.

Keywords: public health, zoonosis, microbiology, dairy, *Listeria monocytogenes*

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1. INTRODUCTION

Listeria spp. are Gram-positive bacilli, which are non-sporulating, widely distributed and well adapted to the environment, food and animals (Shamloo et al., 2018). They are relatively resistant to adverse environmental conditions, such as high salt concentrations or acidity as well as low humidity and oxygen concentrations. And as psychrotrophic bacteria, they are able to survive and develop at refrigeration temperatures (Jung, 2009; Buchanan et al., 2017).

Although the genus *Listeria* contains seven species, *L. monocytogenes* is considered the most important because it is the main causative agent of listeriosis, recognized as one of the most serious, emerging, zoonotic bacterial diseases capable of infecting humans and animals. The disease is generally expressed as a mild febrile gastroenteritis or with symptoms similar to influenza but may develop into an invasive systemic form resulting in fetal miscarriage, septicemia, meningitis and encephalitis, with a high mortality rate among the susceptible population, which includes the elderly, pregnant women and immunocompromised individuals (Elshinawy et al., 2017; Shamloo et al., 2018). It is estimated that 99% of listeriosis cases are food-borne. Foods favorable to the growth of the bacteria and those consumed in large quantities are the ones with the greatest risk of causing the disease. Among these are milk, pasteurized or not, unripened soft cheeses and dairy products with high fat content (Buchanan et al., 2017; Jung, 2009).

The ability of a microorganism to cause disease depends on its ability to enter, multiply and propagate among host cells. The process of cellular infection by *L. monocytogenes* has been well characterized and essential genetic products have been identified. The main virulence factors of *Listeria* spp. are codified in a chromosomal segment known as the *Listeria* Pathogenicity Island 1 (LIPI-1), present in the species *L. monocytogenes* and *L. ivanovii* (Jung et al. 2009).

The listeriolysin protein O (LLO), determined by the *hly* gene, is the main factor of virulence of *L. monocytogenes* due to its hemolytic activity (hemolysin) and its mediation of the vacuole lysis (formation of pores), allowing its survival inside the cell (Borges et al., 2009; Vera et al., 2013). The *actA* protein is the main factor involved in intra- and intercellular movement, i.e., dissemination of the bacterium in the infection process (Borges et al., 2009; Vera et al., 2013). Both the *hly* and *actA* genes are located at LIPI-1, also called *prfA* virulence gene cluster (pVGC). Internalin A (*inlA*) is a surface protein that determines the binding and invasion of *L. monocytogenes* at the first binding site of infection, the host intestinal epithelial cell. The genes responsible for the production of internalins (*inl*) are outside of LIPI-1 (Jung, 2009; Vera et al., 2013). These virulence genes have been frequently used to identify *L. monocytogenes* by several authors (Aksoy et al., 2018; Pournajaf et al., 2016; Abdelllrazed et al., 2014; Peres et al., 2010).

Antimicrobial resistance, the ability of a microorganism to impede the action of an antimicrobial, is considered one of the challenges to contemporary health systems. It is estimated that 700,000 deaths are caused annually by antimicrobial resistance, which has as the most severe consequences ineffective treatments and persistent infection. In 2014, antimicrobial resistance was recognized as a threat to global sustainability and development efforts by the UN General Assembly (Estrela, 2018).

In 2015, member states endorsed the "Global Plan of Action to Combat Antimicrobial Resistance", organized in partnership with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE), whose main objectives are to increase awareness of antimicrobial resistance, strengthen surveillance and research and optimize the use of antimicrobial agents in human and animal health. The understanding that resistant bacteria circulate among humans, animals and the environment justifies the need for the One Health approach

in addressing the problem, ensuring that the issue is addressed from the combined perspectives of human, animal and environmental health and thus has greater effectiveness of action (WHO, 2015).

Since the emergence of its first multi-resistant strain in 1988, *Listeria monocytogenes* has been monitored for antimicrobial resistance. Recently, antibiotic resistance among environmental strains or food isolates has increased, especially for those drugs commonly used in the treatment of listeriosis. Thus, monitoring changes in the resistance profile of *L. monocytogenes* is necessary due to the slow and gradual emergence of resistant strains (Olaimat et al., 2018; O'Connor et al., 2010). The aim of this article was to determine the occurrence of *Listeria* ssp. with pathogenic potential and antimicrobial resistance in refrigerated raw milk.

2. MATERIAL AND METHODS

2.1 Sample preparation

Refrigerated raw milk samples were collected from refrigeration tanks of 20 milk producing properties located in the Agreste region of Pernambuco. After packaging in isothermal boxes containing recyclable ice, they were sent for analysis at the Garanhuns Laboratory Center (CENLAG) of the Garanhuns Academic Unit (UAG) of the Federal Rural University of Pernambuco (UFRPE). The samples were kept in a refrigerator (7ºC) until completing 48 hrs under refrigeration, followed by the search for *Listeria* spp.

2.2 Detection and isolation of *Listeria* spp.

The detection of bacteria of the genus *Listeria* was based on the method described by Hitchins et al. (2016), with adaptations in the selective enrichment phase, performed by adding 25 ml of the sample to 225 ml of Half Fraser broth, with incubation at 30ºC for 48 hrs. For isolation, layers of enriched broth after 24 and 48 hrs of incubation were sown in Oxford agar, and the plates were incubated at 37ºC for 48 hrs. Colonies with characteristic *Listeria* development (black with black halo) were purified and isolated in Trypticase Soy Agar with yeast extract (TSA-YE). Gram staining was performed to evaluate characteristic morphology and purity. Presumptive isolates of *Listeria* spp. were stored in Tryptone Soy Broth (TSB) containing 25% glycerin at -80ºC for later molecular identification.

2.3 Molecular analyses

For molecular analyses, DNA was extracted as described by Abdellrazeq et al. (2014), with adaptations. Presumptive colonies of *Listeria* spp. were inoculated in TSB-YE broth and incubated at 37ºC for 24-48 hrs. The enriched broth was distributed in 1.5-ml microtubes, which were centrifuged at 10000 rpm for 2 min until forming pellets, which were re-suspended in ultrapure water with the aid of a vortex in a final volume of 50 µl. The microtubes were submitted to a water bath at 100ºC for 10 min and then cooled to -20ºC for 5 min. After thermal shock, the microtubes were centrifuged at 15,000 rpm for 5 min. The supernatant was collected and used in the PCR assays.

Confirmation of the genus was performed by amplification of a fragment of the 23S rRNA gene, *S2*, as described by Paillard et al. (2003). For DNA amplification 10 µl of Green Master Mix 2x (Promega), 0.5 µM of each primer, 1 µl of extracted DNA and ultrapure water were used to complete a final volume of 20 µl. Amplification conditions were the following: denaturation at 95ºC for 5 min, followed by 35 cycles at 94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min and a final cycle of 72ºC for 7 min (Paillard et al., 2003). The PCR product was visualized by electrophoresis in 1.5% agarose gel stained with Sybr Green (Bio Rad).

The isolates confirmed as being of the genus *Listeria* were tested for the presence of virulence genes (*hlyA, actA, inlA*) according to the conditions specified by Ciccio et al. (2012). For amplification reactions, 12.5 µl of Green Master Mix 2x (Promega), 0.9 µM of each primer, 2 µl of DNA and ultra-pure water were used to complete a final volume of 25 µl. In the thermal cycler, the parameters used were the following: 94ºC for 3 min for initial denaturation followed by 35 cycles of denaturation at 94ºC for 1.2 min, annealing at 55ºC for 1.3 min and extension at 72ºC for 2 min, with final extension at 72ºC for 10 min. The amplification products were stained with Sybr Green, submitted to 1.5% agarose gel electrophoresis and the results were visualized under UV light.

The positive control *Listeria monocytogenes* ATCC 15313 (Fiocruz) was used in all molecular analyses, genus confirmation and virulence gene research. All primer sequences used in PCR reactions as well as the size of the fragments formed are listed in Table 1.

Table 1. Pairs of primers used in PCR reactions

Gene	Primer sequence (5'3')	Product size (bp)	Reference
S2	S2F: GCCTACAAGTAGTTAGAGCC S2R: ACTGGTACAGGAATCTCTAC	890	Paillard et al., 2003
hlyA	hlyAF: CCT AAG ACG CCA ATC GAA hlyAR: AAG CGC TTG CAA CTG CTC	702	Ciccio et al., 2012
ActA	ActAF: GACGAAAATCCCGAAGTGAA ActAR: CTAGCGAAGGTGCTGTTTCC	268 ou 385	Ciccio et al., 2012
inlA	inlAF: CCT AGC AGG TCT AAC CGC AC inlAR: TCG CTA ATT TGG TTA TGC CC	255	Ciccio et al., 2012

2.4 Antimicrobial susceptibility testing

All confirmed *Listeria* spp. isolates were evaluated for susceptibility to antimicrobials by the disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI). The drugs and concentrations were selected based on the antimicrobials used in human and veterinary therapy: ampicillin (10 µg), sulfamethoxazole/trimethoprim (25 µg), vancomycin (30 µg), erythromycin (15 µg), gentamicin (10 µg), rifampicin (5 µg) and tetracycline (30 µg).

Listeria isolates were reactivated in TSA-YE at 37ºC for 24 hrs and three to five colonies were transferred to 5 mL of Muller-Hinton broth until 0.5 turbidity was obtained on the McFarland scale. This suspension was inoculated into Muller-Hinton agar with a swab and the antibiotic discs dispensed over the agar. The plates were incubated at 37ºC for 18-24 hrs (NCCLS, 2003). The diameter of the growth inhibition zones was measured and interpreted using the limits established for *Staphylococcus* spp. in CLSI (2013), since there are no standards for *Listeria* spp. for all antibiotics tested.

2.5 Data analysis

Data regarding the occurrence of *Listeria* spp. and the frequency of virulence genes were analyzed using descriptive statistics.

The Multiple Antibiotic Resistance index (MAR) was calculated as the number of antimicrobials to which a given isolate was resistant over the total number tested, and the final value was multiplied by 100 to obtain the results in percentages. A MAR index above 0.2 (20%) indicates multiple resistance (Krumperman, 1983).

3. RESULTS AND DISCUSSION

Of the 140 presumptive isolates, 32.85% (46/140) were confirmed as belonging to the genus *Listeria*. The detection of *Listeria* spp. in this study is a relevant finding, since there are case reports of human listeriosis involving species other than *L. monocytogenes* (Rocourt et al., 1986; Cummins et al., 2004; Guillet et al., 2010). Furthermore, according to Ferronatto et al. (2012), the presence of other *Listeria* spp. in food is an indicator of inadequate hygiene and processing conditions and means an increased risk of contamination by pathogenic *Listeria* spp., since all live under the same conditions. Moura et al. (2018), when evaluating milk from expansion tanks in Alagoas, obtained 23.3% of the samples testing positive for bacteria to be of the genus *Listeria*, which is the closest result to the findings of this study.

On the other hand, Mattos et al. (2010), when assessing the quality of milk from the Agreste region of Pernambuco, did not find *L. monocytogenes* in their analyses. Rosa et al. (2018) obtained the same negative result in their analysis of fresh cheese. Elshinawy et al. (2017) found *Listeria* spp. in 10% of the dairy samples they analyzed. According to Rosa et al. (2018), a negative result does not guarantee the absence of the pathogen, since the low population of *L. monocytogenes* and the sublethal effects caused by processing are factors that hinder its recovery in the enrichment broth. The presence of coliforms can also inhibit the population of *L. monocytogenes*, hindering their proliferation and even their detection.

Dairy farms represent an important reservoir of *Listeria monocytogenes*, with inferior quality silage being the main source of environmental contamination. Healthy cattle often eliminate *L. monocytogenes* in feces, spreading the pathogen in the farm environment. Transmission of bacteria to refrigeration tanks occurs through direct fecal contamination or environmental contamination of the udder surface, indicating hygienic failures in milking management. The excretion of the pathogen directly into the milk from an infected udder is considered an unusual route of contamination of the refrigeration tanks due to the low incidence of mastitis from *Listeria* (Castro et al., 2018).

Despite the importance of ready-to-eat foods as vehicles for listeriosis, it is estimated that approximately half of the disease outbreaks are linked to contaminated dairy products. *L. monocytogenes* is a common contaminant of raw milk and its prevalence in bulk milk tanks is around 2 to 7%, thus the consumption of unpasteurized milk presents a risk of listeriosis, in addition to representing a danger of cross-contamination in the processing environment (Castro et al., 2018).

From a regional point of view, the presence of *Listeria* spp. in refrigeration tanks presents another aggravating factor. Part of the milk production is used in the manufacture of artisanal rennet cheese, a typical product of the Brazilian Northeast, which, according to state legislation (ADAGRO, 2018) can be manufactured from raw milk, even without ripening. This dairy derivative is often kept for prolonged periods under refrigeration and consumed without prior thermal heating, both risk factors for listeriosis. In Brazil, the absence of *Listeria monocytogenes* is determined in medium- to high-humidity, ripened, grated or powdered cheeses, and there are no parameters for other species of *Listeria* or other dairy products (BRASIL, 2001).

Listeriosis is an important public health disease due to its severe clinical condition and considerable mortality rate among immunocompromised individuals. The development of the condition is associated with the immune status of the susceptible patient and the ability of the bacterium to cause disease. In the current study, 6.89% (4/46) of the *Listeria* isolates detected had the three virulence genes which were investigated - *hlyA*, *inlA* and *actA*, indicating that these isolates have properties of a virulent strain. According to Abdellrazeq et al. (2014), the genetic information of

virulence genes may be divergent between strains of *L. monocytogenes* and other *Listeria* spp., which would explain the non-amplification of these genes in other *Listeria* isolates.

Several studies have used the *hlyA* gene to identify *L. monocytogenes* (Border et al, 1990; Aznar & Alarcón, 2003; Peres et al., 2010; Aksoy et al., 2018). For Pournajaf et al. (2016), the *inlA* gene is species-specific, present in all *L. monocytogenes* regardless of source and serotype, and absent in other *Listeria* spp. or in other bacteria. However, in previous studies by Abdelllrazed et al. (2014) it was found that the detection of only one gene associated with virulence is insufficient to identify *L. monocytogenes* isolates or their true pathogenic potential. The same authors were able to separate *Listeria monocytogenes* from other *Listeria* spp. by amplifying four virulence genes (*prfA*, *hlyA*, *actA* and *inlA*). Amplification of the sequences of the *iap*, *prfA*, *hly*, *inl* and *plcA* genes has already been reported as a method to identify the species *L. monocytogenes* (Pourjanaf et al., 2016). Jung (2009) determined that pVGC genes are reliable targets for the molecular differentiation of *L. monocytogenes* from other *Listeria* spp. Thus, the presence of the three virulence genes in the *Listeria* isolates of this study shows that they are virulent strains of *L. monocytogenes*.

Monitoring the antimicrobial resistance of *Listeria* spp. and *L. monocytogenes* is necessary to understand changes in commonly used resistance patterns, implement proactive control measures for the use of antimicrobial agents, and to prevent the propagation of resistance by strains (Abdellrazeq et al., 2014). With the antimicrobial resistance profile of the *Listeria* tested, it was demonstrated that vancomycin, gentamicin and tetracycline are the most effective drugs against these bacteria. The results of the antimicrobial susceptibility test can be seen in Figure 1. With a MAR index between 0.28 and 0.85, 36.95% of the isolates were characterized as multidrug-resistant. Ampicillin, erythromycin and sulfamethoxazole-trimethoprim were the drugs with the highest number of resistant isolates.

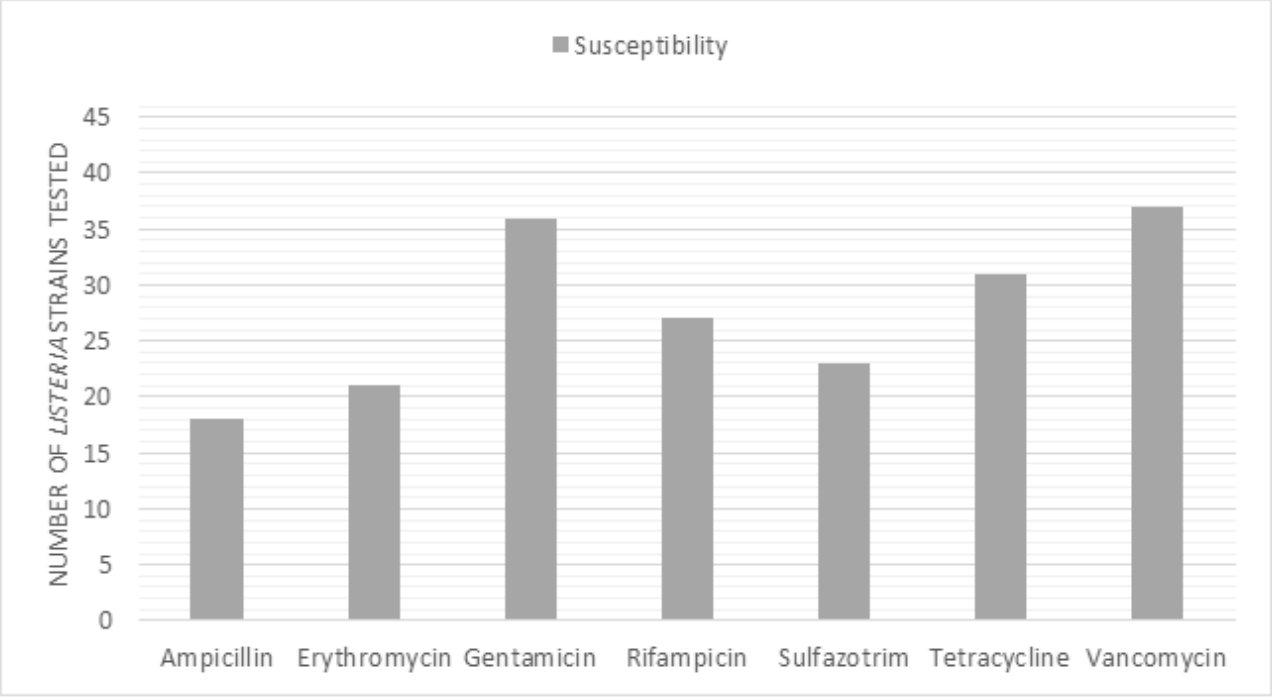

Figure 1. Susceptibility to antimicrobials of Listeria spp. isolated from refrigerated raw milk.

This result represents a public health concern since multidrug-resistant isolates can spread resistance genes to other *Listeria* spp. and other microorganisms. According to Ruiz-Bolivar et al. (2008), resistance to antimicrobials is due to an ecological imbalance caused by the indiscriminate use of these drugs on a large scale in the agricultural, aquaculture, veterinary and medical industries. In several studies on antimicrobial resistance in *Listeria* spp., it has been pointed out that the genetic material involved in short-term evolutionary adaptation can be successfully transferred between *Listeria* spp. and other evolutionarily related Gram-positive bacteria, such as *Enterococcus* spp.

In general, *L. monocytogenes* is susceptible to antimicrobials effective against Gram-positive bacteria, although resistance has been reported in isolates from several sources (Aksoy et al., 2018). Among the factors associated with the progression and spread of drug resistance by *L. monocytogenes*, the pre-exposure of these bacteria to low levels of antibiotics and other antimicrobials in the food production chain may lead to adaptations that allow *L. monocytogenes* to resist higher levels of antimicrobials. Other factors include the ability to form biofilms, co-selection of resistant strains by sublethal exposure to disinfectants and increased resistance by cross-protection against environmental stresses (Olaimat et al., 2018).

The drugs of first choice for the treatment of listeriosis consist of an association of ampicillin with gentamicin, and, in the second case, vancomycin and an aminoglycoside or sulfamethoxazole-trimethoprim associated with rifampicin (De Nes et al, 2010). Ampicillin was the antimicrobial with the highest resistance index, demonstrated by 60.87% of the isolates in this study, which may represent a limitation in the treatment options of a patient with the disease. Abdelllrazeq et al. (2014) also reported resistance to ampicillin and tetracycline in all isolates of *L. monocytogenes*.

Recent studies have shown a substantial increase in antimicrobial resistance in *L. monocytogenes*, but its prevalence varies geographically (Abdelrazeq et al. 2014). In milk isolates produced in southeastern Brazil, De Nes et al. (2010) found no resistant *Listeria monocytogenes* for the antibiotics tested, including tetracycline, ampicillin, gentamicin, vancomycin and erythromycin. In Turkey, Aksoy et al. (2018) found that 66.66% of *Listeria* isolated from raw milk and dairy products were sensitive to all antibiotics tested. These data disagree with the present study, where 89.11% of *Listeria* isolates showed resistance to at least one of the drugs tested. The difference in resistance rates can be influenced by the country involved and by regulations regarding the use of antibiotics, by production and processing practices, and by the types of samples (Abdellrazeq et al. 2014).

4. CONCLUSION

In view of the above, it is inferred that the milk produced and stored under refrigeration in the Agreste region of Pernambuco constitutes a risk to public health and to the dairy production chain of the State due to the presence of potentially pathogenic *Listeria monocytogenes* and *Listeria* spp. multi-resistant to antimicrobials.

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