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Additional Information

Prevalence and antimicrobial resistance of *L. monocytogenes* and *Salmonella* strains isolated in ready-to-eat foods in Eastern Spain

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Abstract

Antimicrobial resistance is a major global public health concern and a food safety issue considered in the framework of Horizon 2020. Bearing this in mind, the current study determined the prevalence of *L. monocytogenes* and *Salmonella* strains isolated in ready-to-eat food sampled in industry and retail between 2006 and 2012 by the Official Food Control Services of the Valencian administration (Spain). The presence of *L. monocytogenes* was analysed in a total of 2864 samples including pasteurized cheese (624); cooked ham (487); dried pork sausages (192); ice cream (758) and smoked salmon (803). The presence of *Salmonella* was analysed in a total of 1264 samples: pasteurized cheese (289); cooked ham (316); dried pork sausages (78); ice cream (376) and smoked salmon (205). The results showed that *L. monocytogenes* was present in 3.8% of the samples, being most common in smoked salmon. *Salmonella* was not found in any of the products studied with the exception of 7 out of 78 samples of dried pork sausage. Both *L. monocytogenes* and *Salmonella* showed resistance to 4 antimicrobials (ampicillin, cephalothin, tetracycline and trimethoprim-sulfamethoxazole). Moreover, the former was resistant to amikacin, ciprofloxacin, erythromycin and vancomycin while the latter showed resistance to amoxicillin-clavulanate and chloramphenicol. Furthermore, multi-resistance was found for both microorganisms.

1. Introduction

Listeria monocytogenes and *Salmonella* are pathogenic bacteria that can contaminate food products during or after processing. Neither of them are heat resistant and are therefore inactivated by industrial cooking. However, their presence in ready-to-eat food, which does not undergo any post-sale treatment to ensure its safety before consumption, implies a health threat for the consumer (Cabedo et al., 2008).

Listeriosis, caused by *Listeria monocytogenes*, is a disease that results in nearly 1600 illnesses each year in the United States with more than 1400 hospitalizations and 250 deaths (Scallan et al., 2011). In the European Union the number of confirmed cases of listeriosis in 2012 was 1642. The overall notification rate of listeriosis was 0.41 cases per 100,000 population and the case-fatality rate was 17.8% (EFSA & ECDC, 2014). Listeriosis may cause either a serious invasive illness affecting children, pregnant women, the immunocompromised and the elderly, with symptoms like meningitis, encephalitis, septicaemia, or, in healthy adults, a non-invasive febrile gastroenteritis or/and influenza-like symptoms (Pesavento et al., 2010). To date, 13 serovars of *L. monocytogenes* have been identified: “4b” is responsible for the majority of human listeriosis cases, and serovar “1/2a” is the most prevalent serovar in food (Jamali & Thong, 2014).

Listeria monocytogenes is a foodborne pathogen widely distributed throughout the natural environment and is consequently found in many types of food products. The EFSA results, published in 2013 indicated that in the European Union the highest frequency of positive samples in ready-to-eat food was found in fish (10.4%), followed by meat (2.1%) and cheese (0.5%) (EFSA, 2013). Higher values had been published by other authors since 2000, for instance, 21% to 34.1% was found for smoked fish at retail, (Uyttendaele et al., 2009; Di Pinto et al., 2010; Doménech et al., 2012); 2.7% to 8.5% in deli meat products (Garrido et al., 2009) and 12.5% to 16.9% in cooked ham and cured dried pork sausage respectively (Cabedo et al., 2008). In relation to cheese, the EFSA results for *L. monocytogenes* are in accordance with other published surveys (0 to 2%) (Vitas et al., 2004; Manfreda et al., 2005; Doménech et al., 2013). These variations in the percentage of prevalence may be due to the intrinsic properties of the products, processing conditions and the conditions in the food chain.

Salmonella is also a well-known pathogen which has been implicated in a large number of

outbreaks of foodborne disease. In fact, 91,034 human cases were confirmed in Europe in 2012 (EFSA & ECDC, 2014), and 1 million people in the United States contract *Salmonella* each year, with an average of 19,000 hospitalizations and 380 deaths (Scallan et al., 2011). Most human *Salmonella* infections result in a self-limiting gastrointestinal illness characterized by diarrhea, fever and abdominal cramps which do not warrant antimicrobial therapy (Mølbak, 2005).

The discovery of antibiotics in the mid-twentieth century revolutionised the management and treatment of bacterial infections. However, these gains are in serious jeopardy due to the increase in antibiotic resistance and the potential global spread of genes resistant to pathogenic bacteria (Sharma et al., 2014). In some European countries, resistance rates to a single antibiotic exceed 40-50%. In fact, Antimicrobial Multiple Resistance (AMR) is estimated to cause some 25,000 deaths each year (UE 2011). Increasing global trade and travel favours the spread of antimicrobial resistance between countries and continents. Therefore, antimicrobial resistance is a global public health concern. Actions to address threats posed by antimicrobial resistance include programs to monitor antimicrobial resistant microbes throughout the food chain.

According to the European Commission, a co-ordinated research effort is required to amass more information related to resistance rates. With this aim in mind, one of the main goals of Horizon 2020 “Health, Demographic Change and Wellbeing”, is the reduction of antibiotic resistance in the food chain. Therefore, the objective of this work was to investigate the prevalence of *L. monocytogenes* and *Salmonella*, obtained by the official control carried out by the Valencian administration on ready-to-eat food products (pasteurized cheese, cooked ham, dried pork sausage and smoked fish) and to determine the susceptibility of these microorganisms to different antimicrobial agents.

2. Material and methods

Sample collection

A survey on *Listeria monocytogenes* and *Salmonella* was carried out by the Official Food Control Services of the Department of Health of the Valencian administration between 2006 and 2012 with the aim of estimating the prevalence and antimicrobial resistance of both microorganisms in ready-to-eat foods.

A total of 2864 samples were analysed for the presence of *L. monocytogenes* [pasteurized cheese (624); cooked ham (487); dried pork sausages (192); ice cream (758) and smoked salmon (803)] and 1264 for *Salmonella* [pasteurized cheese (289); cooked ham (316); dried pork sausages (78); ice cream (376) and smoked salmon (205)], table 1.

The number of samples, the place where these must be taken (industry or retail) and type of food products to be analyzed were determined by the Valencian health administration as part of the official control process. Two main aspects were taken into account, on one hand Regulation (EC) 882/2004 and on the other hand, information about consumption, the risk related to the product, data from the Rapid Alert System for Food and Feed, and data collected in previous years.

Approximately 250g of samples were stored in insulated containers at -18°C for ice cream and 4°C for the rest of the products and sent to the laboratory for analysis. The transport time was no more than one hour in any case. A record of the name of the company, the batch, expiry date, date of manufacture, storage conditions, etc., was generated in each case.

Sample examination

Samples were examined by official control laboratories, which are accredited following the standard ISO/IEC 17025:2005. This describes the general requirements for the competence of testing and calibration laboratories.

The detection of pathogens was performed following official methods: *Salmonella* spp. (ISO 6579:2002) and *Listeria monocytogenes* (ISO 11290-1:1996 and ISO 11290-2:1998).

Antimicrobial susceptibility test

Antimicrobial susceptibility testing of *Listeria* and *Salmonella* was performed by the disk diffusion method according to the CLSI guidelines (CLSI, 2012). Susceptibility to fourteen antibiotics, including those used to treat human listeriosis and salmonellosis, (Oxoid) was determined: amikacin (AK: 30µg), ampicillin (AMP: 10µg), amoxicillin-clavulanate (AMC: 20/10µg), sulfamethoxazole-trimethoprim (STX: 1.25/23.75µg), ceftriaxone (CRO: 30µg), ciprofloxacin (CIP: 5µg), chloramphenicol (C: 30µg), kanamycin (K: 30µg), gentamicin (CN: 10µg), nalidixic acid (NAL: 30µg), tetracycline (TE: 30µg), cephalothin (KF: 30µg), vancomycin (VA: 5µg) and erythromycin (E: 15µg). CRO and K were only tested on *Salmonella* strains and VA and E on *Listeria* strains. The antimicrobials used were grouped

into three categories based on their importance in human medicine (Veterinary Drugs Directorate, 2005). The category I antimicrobials with high importance in human medicine are AMC, CRO, CIP and VA; category II antimicrobials are AK, AMP, CN, E, K, KF, NAL and SXT (moderate importance); category III antimicrobials are C, and TE (low importance). *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains.

The diameters of growth inhibition zones were measured and interpreted according to the breakpoints recommended by the CLSI for the various types of antibiotics, and the strains were classified as sensitive, intermediate (reduced susceptibility) or resistant. The resistant strains were confirmed by determining the minimum inhibitory concentrations (MIC) by graded-concentration antibiotic strips (E-test AB Biodisk), the interpretation of results was made according to CLSI guidelines.

Statistical analysis.

Descriptive analyses of the data were undertaken using Statgraphics Centurion XVI.II (Statpoint Technologies, Inc. Warrenton, Virginia). Relative proportions were compared using the Chi-squared test (X^2) and Fisher's exact test. Also, comparisons of means were made. A probability value of less than 5% was deemed to be significant.

3. Results

This study determined the prevalence of *L. monocytogenes* and *Salmonella* isolated from ready to eat products (Table 1). *L. monocytogenes* was found in 109 out of 2864 analyzed samples, which represents 3.8%. Prevalence in smoked salmon had the highest percentage: 69 out of 803 samples (8.6%), followed by dried pork sausages, 12 out of 192 (6.3%). A lower percentage was obtained for *Salmonella* (0.5%), as a result of 7 out 1264 samples found in one only product, dried pork sausages. An important result was that all pasteurized cheese, cooked ham, ice cream and smoked salmon samples were negative for *Salmonella*. There was a non-significant difference between the prevalence and the microorganism, *L. monocytogenes* and *Salmonella* (p-value 0.3145) and the prevalence according the type of product (p-value 0.2258).

Overall 89% (98/109) of the *L. monocytogenes* isolates were susceptible to all tested antimicrobials, which implies that 10% of strains were resistant. Resistance to amoxicillin-clavulanate, chloramphenicol and gentamicin

was not found in any *L. monocytogenes* strain. Moreover, no resistance was found in *L. monocytogenes* isolated in cooked ham.

Fig. 1 shows the susceptibility profiles of the four ready-to-eat products to the eleven antibiotics tested in this study. Pasteurized cheese had the highest percentage of resistance, 35.7% (5/14): one sample of hard cheese, three of fresh cheese and one semi-hard cheese. Moreover, this product was resistant to 6 out of 11 antimicrobials tested. A total of 3 out of 14 isolates (21%) were resistant to amikacin, cephalothin, tetracycline and vancomycin; 14.3% (2/14) to erythromycin and 7.1% (1/14) to ampicillin. Intermediate susceptibility was displayed in vancomycin 35.7% (5/14) followed by amoxicillin-clavulanate, ampicillin and cephalothin 7.1% (1/14). Finally, all *L. monocytogenes* strains were sensitive to chloramphenicol, ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole.

A total of 10 out of 12 (83.3%) *L. monocytogenes* strains isolated in dried pork sausages were sensitive or showed an intermediate susceptibility. Resistance to amikacin and trimethoprim-sulfamethoxazole was concurrently present in 16.7% of samples (2/12) followed by tetracycline 8% (1/12).

L. monocytogenes in ice-cream showed sensitivity to 10 out of 11 antibiotics tested. Only 2 out of 6 of *L. monocytogenes* isolates were resistant to ciprofloxacin (28.6%). Also two of them had intermediate susceptibility to cephalothin (28.6%).

Finally, smoked salmon had the lowest percentage of resistance, 1.4% (2/69) of the *L. monocytogenes* isolates. One of them was resistant to ampicillin and the other one to cephalothin. Intermediate susceptibility to vancomycin was found in 5.9% (4/69) of the isolates.

Differences between the resistance and type of antibiotic were not significant (p-value 0.6953). However, differences between resistance and type of microorganism (p-value 0.0000) and type of product (p-value 0.0137) were significant in both cases.

All of the *Salmonella* isolates were found in samples of dried pork sausages, and all of them were sensitive to amikacin, ceftriaxone, ciprofloxacin, gentamicin and kanamycin. Fig 2. shows the resistance rates to the different antibiotics tested. The *Salmonella* strains were most resistant to Tetracycline 85.7% (6/7). Resistance to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole was found in 71.4% (5/7) of the isolates. The next was cephalothin, ineffective in four out seven cases (57.1%). Finally, amoxicillin-clavulanate was ineffective in two out of seven isolates (28.6%).

Table 2 shows the multiple antibiotic resistance pattern obtained for *L. monocytogenes* and *Salmonella* and the involved ready-to-eat products. A total of 11 out of 109 samples (10%) of all *L. monocytogenes* isolates showed resistance to at least one antimicrobial agent and 5 out of 109 (4.6%) showed multidrug resistance. In relation to *Salmonella*, this percentage was quite high, 28.6%, as a result of 2 isolates out of 7 which were multi-resistant.

4. Discussion

The results obtained showed that *L. monocytogenes* was detected in all the types of products analysed, with the highest percentage in smoked salmon (8.6%) followed by dried pork sausages (6.3%). The smoked salmon results are slightly lower than those found by Dauphin et al. (2001) who found 9.53% of *L. monocytogenes* in smoked salmon (n=141) and by Garrido et al., (2009) who reported 10.8% (n=11). The results found in the present study for dried pork sausages concur with Vitas et al., (2004).

In relation to cheese, a wide range of levels of *L. monocytogenes* have been reported by other authors: 6.3% in 195 samples of soft cheese and 4.4% in 45 samples of hard cheese (Rudolf and Scherer, 2001), 2.1% in 1656 samples of Gorgonzola cheese (Manfreda, et al., 2005); and 20% in 10 samples of fresh soft cheese (Pesavento et al., 2010). This variation in the percentage of prevalence may be due to the type of cheese, intrinsically properties, use of thermal treatment, personal hygiene, cleaning procedures, ventilation and processing conditions.

In relation to cooked meat such as ham, values reported by Uyttendaele et al. (2009) were in accordance with the present study (1.1%). However, around 12% was found by other authors (Van Coillie et al., 2004; Gilbert et al., 2009). Improper handling or ineffective maintenance of cutting equipment are often the main causes of increased contamination in these products, which can justify the differences between authors.

Finally, in the present study the lowest percentage of prevalence of *L. monocytogenes* was found in ice cream, with values similar to other authors, 0.4%-1% (Miettinen et al., 1999; Warke et al., 2000).

Considering *Salmonella*, the results of this study suggest a generally low prevalence of this microorganism in all analysed products, with the exception of dried pork sausages. Previous studies of ice cream and cheese reported levels

of less than 0.1% or no isolation (Ortolani et al., 2010; EFSA & ECDC 2014). *Salmonella* in meat preparations, intended to be eaten without any additional treatment, were reported by Cabedo et al., 2008 with 2% in cooked ham and 11.1% in cured dried pork sausage.

The first resistant strain of *L. monocytogenes* was isolated in 1988. Since then, resistant strains have been found in foodstuffs, food contact surfaces in the industry as well as in human samples (Gomez et al., 2014). In the present study, *L. monocytogenes* isolates in cooked ham, ice cream and smoked salmon were sensitive to amikacin. However, they were resistant in pasteurized cheese (21.4%) and dried pork sausages (16.7%). These findings were higher than those found by Arslam and Özdemir (2008) who reported only 4.3% resistance, although intermediate resistance reached 34%. The same authors did not find resistance to amoxicillin-clavulanate, coinciding with this study. This result is positive as amoxicillin-clavulanate is one of the preferred antibiotics used to treat listeriosis (Gomez et al., 2014). The present study showed that in pasteurized cheese this microorganism had 7.1% resistance to ampicillin. However, in relation to this antibiotic, a huge variety of results have been found by other authors: Pesavento et al., (2010) obtained 20% in raw meat and retail food. Higher percentages were obtained in dairy products by Rahini et al., 2010 (26.3%) and Harakeh et al., 2009 (60%) and in Malaysian ready-to-eat food (100%). (Marian et al., (2012).

The high levels of *L. monocytogenes* resistance to ampicillin could be explained by the fact that it is a first choice antibiotic, which is widely used in listeriosis treatment for humans (Conter et al., 2009). On the other hand, all strains were susceptible to chloramphenicol. The same result was found by Marian et al (2012) in RTE foods. However, values around 10% were found by Arslam and Özdemir (2008), and Rahini et al., (2010). Ciprofloxacin resistance was found in 2 out 7 samples of ice cream (28.6%). This result was quite similar to values reported by Rahini et al., (2010) (21.1%), however Arslam and Özdemir (2008) found no resistance. All *L. monocytogenes* were susceptible to gentamicin, which agrees with previous studies (Arslan & Özdemir, 2008; Conter et al., 2009 and Jamali, 2013). The high susceptibility of isolates to gentamicin may be due to the fact that this antimicrobial is not used anymore for therapeutic purposes in veterinary medicine nor as a growth promoter in conventional animal fattening (Harakeh et al., 2009). Resistance to erythromycin was only found in pasteurized cheese (14.3%). Similar

values were reported by Rahini et al., (2010) (15.8%) and Jamali et al., (2013) (11.1%). However, no resistance was found in cooked ham, dried pork sausages, ice cream and smoked salmon, which concurs with Conter et al., (2009) and Marian et al., (2012). In the present paper, resistance to cephalothin was found in pasteurized cheese (21.4%) and smoked salmon (1.4%). Similar values were reported by Pesavento et al., (2010) who found that 6 out of 40 samples were resistant (15%) to cephalothin in raw and ready-to-eat foods at retail.

L. monocytogenes isolated in dried pork sausages was resistant to trimethoprim-sulfamethoxazole; however, no resistance was found in the rest of the analysed products. These results have a great importance, since this antimicrobial agent is the second choice for treatment of listeriosis, especially in patients allergic to penicillins (Pesavento et al., 2010). The highest resistance to tetracycline was found in pasteurized cheese (21.4%). Results from other authors present a huge variation in resistance to this antimicrobial in dairy products: 15.8% (Rahini et al., 2010; 20% (Harakeh et al., 2009) and overall 70% (Jamali et al., 2013). These high differences could be due to widespread use of this antimicrobial, especially in farms. Finally, resistance to vancomycin was found in pasteurized cheese (21.4%) and intermediate resistance in dried pork sausages (8.3%) and smoked salmon (5.9%). These percentages are lower than those found by Harakeh et al., (2009) (26.6%), who consider that this percentage is worrying as vancomycin is considered the last therapeutic option to treat human *Listeria* infection. However, other authors found no resistance, or it was lower than 10% (Arslam and Özdemir, 2008; Conter et al., 2009; Pesavento et al., 2010; Rahini et al., 2010).

In the current study, resistance to amikacin, ceftriaxone, ciprofloxacin, gentamicin and kanamycin was not found in any *Salmonella* strain. Sensitivity to these antimicrobials has also been reported by others (CIPARS, 2011; NARMS, 2011). This is encouraging as ciprofloxacin is a drug used for treating salmonellosis in human patients (Aslam et al., 2012). On the contrary, the highest percentage of resistance was found for tetracyclines (85.7%). This is important as they are used parentally to treat a range of infections including bacteria, mycoplasma, and some protozoa. However, this percentage is not unusual. Resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines has been frequently observed in *Salmonella* isolates with a variety of origins,

varying between 23.5% and 83.1% (Van Boxstael 2012). These resistance levels are similar to those found in *Salmonella* strains from cases of salmonellosis in humans. In fact, resistance in human *Salmonella* isolates was high for ampicillin, tetracyclines and sulfonamides and was moderate for streptomycin and nalidixic acid. These are antimicrobials that are or have commonly been used for treatment in humans and animals. However, resistance to ciprofloxacin (widely used in human medicine), was relatively low (EFSA & ECDC, 2013).

Multi-resistant strains, which may represent potential threats to human health, were only found in pasteurized cheese and dried pork sausages in this study. Comparing the findings here with prior research, multi-resistance was also found in ready-to-eat food by Conter et al. (2009) who reported that 3.30% of *L. monocytogenes* strains isolated from milk and dairy products were multi-resistant to 5 antibiotics. Pesavento et al. (2010) isolated 27.5% *L. monocytogenes* multi-resistant strains from raw meat and retail food.

A high percentage of resistance and multi-resistance was obtained for the antimicrobials classified as being of “high” and “medium importance” in relation to human health, which can jeopardize the effectiveness of clinical treatments and increase the severity of diseases (Veterinary drugs directorate, 2005). This highlights the importance of continued surveillance and the usefulness of this information to take measures in the primary sector.

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