Incidence and characterization of *Staphylococcus aureus* in fishery products marketed in Galicia (Northwest Spain)

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Abstract

A total of 298 fishery products purchased from retail outlets in Galicia (NW Spain) between January 2008 and May 2009 were analyzed for the presence of *Staphylococcus aureus*. *S. aureus* was detected in a significant proportion of products (~25%). Incidence was highest in fresh (43%) and frozen products (30%), but it was high in all other categories: salted fish (27%), smoked fish (26%), ready-to-cook products (25%), non-frozen surimis (20%), fish roes (17%) and other ready-to-eat products (10%). A significant proportion of smoked fish, surimis, fish roes and other ready-to-eat products did not comply with legal limits in force.

RAPD-PCR of 125 *S. aureus* isolated from fishery products was carried out using three primers (AP-7, ERIC-2 and S). Isolates displayed 33 fingerprint patterns. Each pattern was attributed to a single bacterial clone. Cluster analysis based on similarity values between RAPD fingerprints did not find relationship between any RAPD pattern and any product category.

Isolates were also tested for *se* genes and susceptibility to a range of antibiotics (cephalothin, clindamycin, chloramphenicol, erythromycin, gentamicin, oxacillin, penicillin G, tetracycline, vancomycin, methicillin, ciprofloxacin and trimethoprim-sulfamethoxazole). Most isolates (88%) were found to be *sea* positive. Putative enterotoxigenic strains counts reached high risk levels in 17 products. No relationship was found between the presence of *se* genes and RAPD patterns. All isolates were resistant to penicillin, chloramphenicol and ciprofloxacin, and most to tetracycline (82.4%), but none was methicillin-resistant.

A revision of pre-requisite programs leading to improve hygienic practices in handling and processing operations from fishing or farming to retail is recommended to ensure fishery products safety.

Keywords: *Staphylococcus aureus; fishery products; retail level; enterotoxin genes; antibiotic resistance.*

1. Introduction

Although it is necessary to ensure food safety for the health of consumers and industry, *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and pathogenic vibrio species have been repeatedly detected in a diverse variety of fishery products (EFSA, 2010; Garrido et al., 2009; Herrera et al., 2006; Kumar et al., 2009; Novotny et al., 2004; Papadopoulou et al., 2007; Yang et al., 2008). Novel trends in food production such as minimal processing, mass production and globalization, among others, have additionally introduced new factors and conditions that can enhance the presence and subsequent growth of bacterial pathogens (Abee and Wouters, 1999; Cebrián et al., 2007; Rendueles et al., 2011).

S. aureus is one of the major bacterial agents causing foodborne diseases in humans worldwide (EFSA, 2010; Le-Loir et al., 2003). Staphylococcal food poisoning is usually self-limiting and resolves within 24 to 48 h after onset. Most cases are therefore not reported to healthcare services. As a result, the actual incidence of staphylococcal food poisoning is known to be much higher than reported (Lawrynowicz-Paciorek et al., 2007; Smyth et al., 2004). In addition, the notification of staphylococcal intoxications is not mandatory in a number of member states of the European Union. Staphylococcal food poisonings result from the ingestion of food containing staphylococcal enterotoxins (SEs) preformed by enterotoxigenic strains (Kérouanton et al., 2007; Le-Loir et al., 2003). SEs are resistant to proteolysis and heat-stable, so the presence of SEs involves a significant food safety risk (Omoe et al., 2005).

The widespread use of antibiotics has evolved the emergence of multi-drug resistant strains, and it makes eradication more difficult and incidence to increase. Multi-resistant *S. aureus* is rather common in hospital settings and farms (Livermore, 2000; Sakoulas and Moellering,

2008). Community-associated multi-resistant *S. aureus* is becoming an emerging problem too (Popovich et al., 2007; Ribeiro et al., 2007; Stankovic et al., 2007). Antibiotic-resistant strains of *S. aureus* have been detected in food animals (Lee, 2003) and food like meat (Normanno et al., 2007; Pesavento et al., 2007), milk and dairy products (Gündogân et al., 2006; Peles et al., 2007; Pereira et al., 2009) and also fishery products (Beleneva, 2011), and it may be very hazardous for human health.

The identification of bacterial clones with enhanced virulence or increased ability to spread is important. Nowadays, PCR-based techniques are commonly used for typing, as they are easy, fast and cost-effective. Among such techniques, random amplified polymorphic DNA (RAPD-PCR) has been considered a very useful tool for rapid differentiation of clones with no prior information of the gene sequence (Fueyo et al., 2001; Nema et al., 2007; Nikbakht et al., 2008; Shehata, 2008; Van-Belkum et al., 1995).

Nowadays, Spain is the largest fishery producer, particularly in Galicia (NW Spain), and the second largest consumer in the European Union (Eurostat, 2007). However, no results have been found on the incidence of bacterial pathogens in fishery products made or sold in Galicia, apart from one study on molluscan shellfish farmed in Galician waters (Martínez et al., 2009). The situation is not different for fishery products marketed in other parts of Spain, and only two studies on smoked fish (Garrido et al., 2006; Herrera et al., 2006) and another study on freshwater fish (González-Rodríguez et al., 2002) have been carried out in the last decade.

Therefore, the present study was aimed to determine the incidence of *S. aureus* in fishery products marketed in Galicia and subsequently identify most common clones by RAPD-PCR, as well as cases of increased risk according to the presence of enterotoxin genes and antibiotic-resistance of isolates.

2. Materials and Methods

2.1. Sampling

A total of 298 fishery products marketed at different retail outlets in Vigo (Galicia, Northwest Spain) were purchased and analyzed between January 2008 and April 2009. Fourteen samplings (approximately one each month) were carried out. Products were classified into eight different categories: fresh products, frozen products, salted fish, ready-to-cook products, smoked fish, fish roes, non-frozen surimis and other ready-to-eat products (seafood salads, pâtés and anchovies in oil). Between 24 and 43 products of each category were analyzed.

2.2. Isolation and identification of S. aureus

About 50 g of product mixed with 200 ml of peptone water was homogenized in a stomacher masticator (IUL instruments, Barcelona, Spain). Subsequently, homogenates were serially diluted in peptone water (1:50 and 1:500). Aliquots (0.5 ml) of each dilution spread onto Baird Parker agar supplemented with egg yolk tellurite emulsion (Biolife, Milan, Italy) (BP-EY). Plates were incubated at 37°C for 48 h.

Typical colonies of *S. aureus* as well as non-typical colonies (showing no white margin and smaller than 2 mm) were counted. Between 1 and 9 colonies from each product were selected and sub-cultured twice on BP-EY agar for isolation of single colonies (isolates).

Isolates cultured in Brain Heart Infusion broth (Biolife) (BHI) for 24 h at 37ºC were subjected to three different biochemical tests: coagulase, DNAse and mannitol fermentation.

Coagulase production was tested by adding 100 µl of bacterial culture into 300 µl of reconstituted rabbit plasma with EDTA (Bactident® Coagulase rabbit, Merck, Darmstadt, Germany) followed by incubation of tubes at 37ºC. Clotting of plasma was assessed at 1-h intervals during 6 h and after 24 h of incubation.

Isolates were streaked onto DNAse agar (Cultimed, Panreac Quimica, Barcelona, Spain) supplemented with D-mannitol and bromothymol blue and plates were incubated at 37ºC for 24 h. The surface of the plates was then flooded with 0.1 N HCl during 15-20 min for DNA precipitation. DNAse activity was observed by the presence of a transparent halo around the colonies on the agar. Mannitol fermentation was observed as a colour change of the pH indicator -from blue to yellow- due to acid production.

Colonies found to be coagulase positive, DNAse positive and able to ferment mannitol (suspected *S. aureus*) were confirmed to be *S. aureus* by species-specific 23S rDNA PCR. Genomic DNA was extracted from 24 h cultures in BHI using an InstaGene™ Matrix kit (Bio-Rad Laboratories, S.A., Madrid, Spain) following manufacturer's instructions. DNA was quantified by assuming that an absorbance value at 260 nm of 0.100 corresponds to 5 µg/ml of DNA. Primers staur4 (5´-ACGGAGTTACAAAGGACGAC-3´) and staur6 (5´-AGCTCAGCCTTAACGAGTAC-3´) (Straub et al., 1999) were used for each strain. Expected size of amplified PCR products was 1250 bp. Each PCR mixture contained 100 ng DNA, 1x Taq Buffer Advanced, 2.5 U Taq DNA polymerase (5 Prime, Hamburg, Germany), 40 nmol of each dNTP (Bioline, London, United Kingdom), 0.25 nmol of forward and reverse primers (Thermo Fisher Scientific, Ulm, Germany) and sterile Milli-Q water up to a final volume of 50 µl. PCR was performed with a MyCycler™ Thermocycler (Bio-Rad). The conditions proposed by Vautor et al. (2008) were used to target the 23S rDNA gen. An initial step of 5 min at 94ºC was followed by 30 cycles of 30 s at 94ºC, 30 s at 58ºC and 75 s at 72ºC, and a final step at 72ºC for 5 min. PCR products were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide for 90 min at 75 V and 100 mAmp. Gels were photographed in a Gel Doc XR system (Bio-Rad) using the Quantity One® software (Bio-Rad). A DNA ladder of 50-2000 bp (Hyperladder II, Bioline) was included as a molecular size marker.

Stock cultures of *S. aureus* isolates were maintained in 50% glycerol (w/w) at -80 °C. When needed, stock cultures were thawed and subcultured twice in tryptic soy broth (Cultimed) for 24 h at 37 °C prior to being used.

2.1. RAPD

Genotypic characterization of isolates was performed by RAPD-PCR. DNA was extracted and quantified as previously described from two different cultures of each isolate to check reproducibility of banding profiles. Primers S (5´-TCACGATGCA-3´) (Martín et al., 2004), AP-7 (5´-GTGGATGCGA-3´) and ERIC-2 (5´-AAGTAAGTGACTGGGGTGAGCG-3´) (Van-Belkum et al., 1995) were individually used in separate reactions with each isolate. Each PCR mixture consisted of 200 ng DNA; 1x Taq Buffer Advanced and 2.5 U Taq DNA polymerase (5 Prime); 40 nmol of each dNTP (Bioline); 0.25 nmol primer (Thermo Fisher Scientific) and sterile Milli-Q water up to a final volume of 50 µl. PCR mixtures with primers AP-7 and ERIC-2 were further supplemented with 1mM $MgCl_2$ (5 Prime). RAPD-PCR was performed with a MyCycler™ Thermocycler (Bio-Rad). PCRs containing primer S consisted of an initial cycle at 95ºC for 5 min, followed by 35 cycles of 95ºC for 1 min, 37ºC for 1 min and 72ºC for 2 min, with a final extension of 5 min at 72ºC. Amplification conditions for AP-7 and ERIC-2 included a denaturation cycle at 94ºC for 4 min, 35 cycles of 94°C for 1 min, 25°C for 1 min and 72°C for 2 min, and a last extension at 72ºC for 7 min. PCR products were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide as aforementioned. A DNA ladder of 50-2000 bp was included in all gels.

A second-order polynomial relationship between molecular size and mobility was obtained for each gel ($r > 0.99$) and used to determine the molecular size of DNA bands. A RAPD pattern was described as different when at least one band difference was found. Reproducibility of patterns was checked twice using independent DNA samples. Variations in band intensity were not considered. Bands too faint to be reproduced were not considered. A

binary value (0 or 1) denoting absence or presence of each band was assigned to each pattern. Similarity analysis determining the Dice coefficients (Struelens et al., 1996) was performed by IBM SPSS 19.0. Cluster analysis by UPGMA (Sneath and Sokal, 1973) and dendrograms were performed with StatistiXL 1.8.

2.2. Detection of sea-see and seg-sei genes

A slight modification of the method described by Omoe et al. (2002) was followed to analyze the presence of staphylococcal enterotoxin (*se*) genes. Two multiplex PCR detecting *sea-see* and *seg-sei* genes were performed for each strain. DNA was extracted and quantified as previously described. Primer nucleotide sequences and expected sizes of amplicons are shown in Table 1. Each PCR mixture contained 100 ng DNA; 10x KCl reaction buffer and 40 nmol of each dNTP (Bioline); 40 pmol SEC-3/SEC-4 primers, 80 pmol SEB-1/SEB-4 primers and 20 pmol for other primers (Thermo Fisher Scientific); 2.5 U Taq DNA polymerase (5 Prime) and sterile Milli-Q water up to a final volume of 50 µl. *S. aureus* ATCC 12600 was used as a negative control in all PCRs, whereas *S. aureus* ATCC 13565 was used as a positive control for *sea* and *sed*, *S. aureus* ATCC 19095 for *sec*, *seg*, *seh* and *sei*, *S. aureus* ATCC 14458 for *seb*, and *S. aureus* ATCC 27664 for *see*. All these strains were obtained from the Spanish Type Culture Collection (CECT). All PCRs were performed with a MyCycler™ Thermocycler (Bio-Rad) as follows: an initial cycle of 95ºC for 2 min, 55ºC for 1 min and 68ºC for 2 min, followed by 28 cycles of 95ºC for 1 min, 55ºC for 1 min and 68ºC for 2 min, and a final cycle of 95ºC at 1 min, 55ºC for 1 min and 68ºC for 5 min. PCR products were subjected to electrophoresis on 2.5% agarose gel containing ethidium bromide. Run conditions and gel display were as aforementioned. A DNA ladder of 50-2000 bp was also included in all gels.

2.3. Antibiotic susceptibility test

Minimal inhibitory concentration (MIC) of twelve antibiotics was determined against *S. aureus* isolated. The EUCAST guidelines (2003) for broth microdilution and disk diffusion testing were followed.

Broth microdilution test was performed with nine antibiotics: cephalothin, oxacillin, penicillin G and vancomycin (Sigma-Aldrich Química, Madrid, Spain); clindamycin and erythromycin (Acofarma, Barcelona, Spain); and chloramphenicol, gentamicin and tetracycline (Fagron Iberica, Terrasa, Spain). Adjusted cultures of each isolate to $5 \cdot 10^5$ CFU/ml in Muller Hinton broth (Cultimed) supplemented with $CaCl_2 \cdot 2H_2O$ (25 mg/ml) and $MgCl_2 \cdot 6H_2O$ (12.5 mg/ml) were exposed to each antibiotic into a microtiter plate (Falcon®, Becton Dickinson Labware, USA) for 18-20 h (or 24h for oxacillin and vancomycin) at 35ºC. The OD_{655nm} was measured in an iMark Microplate Reader through Microplate Manager 6® software (Bio-Rad). MIC was defined as the minimum antibiotic concentration at which no growth was observed.

The susceptibility of isolates to methicillin (Oxoid, Basingstoke, United Kingdom), ciprofloxacin and trimethoprim-sulfamethoxazole (bioMerieux España, Madrid, Spain) was determined by a disk diffusion test. Adjusted cultures were evenly spread on Muller Hinton agar (Cultimed) and commercially prepared antibiotic disks were placed on the agar surface. The length of the inhibition halo was measured after 16-18 h (24 h for methicillin) at 35ºC.

The reference strains *S. aureus* ATCC 29213 and *S. aureus* ATCC 43300 purchased from the CECT was used in all these tests as negative control and positive control respectively. Antibiotic susceptibility was classified as sensitive, intermediate or resistant on the basis of the breakpoints reported in Table 2.

2.4. Detection of blaZ and mecA genes

A slight modification of the methods described by Baddour et al. (2007) and Olsen et al. (2006) was followed for detecting genes encoding penicillin (*blaZ*) and methicillin resistance

(*mecA*), respectively. DNA was extracted with DNeasy® kit (Qiagen, Hilden, Germany) according to the manufacturer. Extraction was tested by using λ *Hind*III DNA Ladder as a reference (564-23130 bp) (New England BioLabs™, Ipswich, USA). Primers blaZF487 (5´-TAAGAGATTTGCCTATGCTT-3´) and blaZR373 (5´-TTAAAGTCTTACCGAAAGCAG-3´) for *blaZ* gen, and mecA1-F (5´-TGGCTATCGTGTCACAATCG-3´) and mecA2-R (5´-CTGGAACTTGTTGAGCAGAG-3´) for *mecA* gen, were used. Expected sizes of amplified PCR products were 377 bp for *blaZ* gen and 309 bp for *mecA* gen. PCR mixtures were composed of 20 ng of DNA; 5 nmol of each dNTP (Invitrogen Corporation, Carlsbad, USA); 2.5 µl of Dynazym buffer 10x and 1.2 U of Dynazym Hot Start (Bio-Rad); 10 pmol of forward and reverse primer and sterile Milli-Q water up to a final volume of 25 µl. PCRs were performed with an 80 Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, USA). For *mecA* gen, PCR consisted of an initial denaturation at 94ºC for 5 min, followed by 30 cycles at 94ºC for 1 min, 54ºC for 1 min and 72ºC for 1 min, and a final cycle at 72ºC for 7 min. Conditions for *blaZ* gen detection consisted of denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 1 min, 54ºC for 1 min and 72ºC for 1 min, and a last extension at 72ºC for 10 min. *S. aureus* ATCC 29213 was used as negative control, whereas *S. aureus* ATCC 43300 was used as positive control. Amplicons were subjected to electrophoresis on 1.2% agarose gel containing ethidium bromide for 30 min at 100 V and 200 mAmp. Gels were visualized and saved in a Typhoon Scanner 8600 (Molecular Dynamics, GE Healthcare, Little Chalfont, UK). A DNA ladder of 154-2176 bp (DNA Molecular Weight Marker VI, Roche Applied Science, Indianapolis, USA) was included in all gels.

3. Results

3.1. Incidence in fishery products

Colonies were observed on BP-EY for 167 out of 298 fishery products. A total of 728 colonies were picked up, isolated and subjected to phenotypic (coagulase, DNAse and mannitol fermentation) confirmation tests. Out of them, 125 were positive by all tests and thus identified as *S. aureus*. Additionally, one representative isolate of each RAPD global pattern (see below) was analyzed by species-specific 23S rDNA PCR. All of them were confirmed as *S. aureus*. These isolates were obtained from 75 fishery products, which represented an incidence of 25.16%.

As shown in Figure 1, incidence was different in each product category, and it decreased in the following order: fresh products (43%), frozen products (30%), salted fish (27%), smoked fish (26%), ready-to-cook products (25%), non-frozen surimis (20%), fish roes (17%), and lastly other ready-to-eat products (10%).

A significant proportion of surimis (9%), fish roes (4%) and other ready-to-eat products (5%) as well as ready-to-cook products (13%) exceeded 10^2 CFU/g of food, which was the maximum number of *S. aureus* allowed by legislation in force when the study was conducted (O. 2/8/1991, RD 3484/2000 and Commission Regulation (EC) No 2073/2005). Additionally, counts were higher than 10^3 CFU/g in 9 out of 35 surimis, 5 out 41 other ready-to-eat products and 8 out of 40 ready-to-cook products. In the same way, *S. aureus* must have been absent in anchovies in oil, but it was detected in 2 out of 4 products.

The present results have also shown that a significant proportion (18.6%) of smoked products exceeded the limit set for smoked fish products in O. 2/8/1991, that is, $2 \cdot 10^1$ CFU/g of food. Later, Commission Recommendation 2001/337/EC proposed that counts higher than 10^2 CFU/g (M value) should not be permitted in smoked fish. The value set for M value was

exceeded in 16.3% of the smoked products tested in this study. Additionally, counts were higher than exceeded 10^3 CFU/g of food in 3 out of 43 smoked products (7%).

Although it is not subject to legal regulations, it is also worthy to mention that the number of *S. aureus* was higher than 10^2 CFU/g of food in 19.4% of fresh products, 14% of frozen products and 13.3% of salted fish, and 10^3 CFU/g of food in 7% of frozen products, 4.8% of fresh products and 6.7% of salted fish.

3.2. RAPD-PCR

Genotypic characterization of isolates was performed by RAPD-PCR with three different primers (S, AP-7 and ERIC-2). As shown in Figure 2, RAPD analysis with primer S yielded 13 visually different banding profiles, whereas 12 profiles were obtained with each of the two other primers. A total of 31 different bands with a size between 115 and 2292 bp were amplified by primer S, whereas primer AP-7 and ERIC-2 amplified 19 and 18 different bands ranging from 127 to 2023 bp and 125 to 1420 bp, respectively. A good reproducibility of patters was achieved when DNA from different cultures of each isolate was used as a template.

The combination of RAPD fingerprints obtained in separate reactions with different primers has been employed as a strategy to increase the discriminatory power of the analysis (Byun et al., 1997; Nema et al., 2007). The combination of the patterns obtained with the three primers generated a higher discriminatory power (D=0.926) than those of single primers (0.818 for primer S, 0.698 for ERIC-2 and 0.622 for AP-7) as well as of pairwise-combinations of primers (0.883 for S and ERIC-2, 0.878 for S and AP-7 and 0.850 for AP-7 and ERIC-2). As a result, 33 combined or global fingerprints were distinguished. A three-digit code number were assigned to combined patterns, each one corresponding to the number assigned to patterns obtained with primers S, AP-7 and ERIC-2, respectively. The most common

combined pattern was 4.4.1., which was characteristic of 23 isolates. Also, two other patterns (1.1.1 and 13.1.11) were shared by 12 and 16 isolates, respectively. Thus, 40.8% of isolates were included in one of these three patterns. In contrast, 18 patterns were specific to one isolate. Isolates showing identical combined RAPD-PCR fingerprints were considered to be a single genetic type, that is, a single bacterial clone or strain (Fueyo et al., 2001). Thus, strains St.1.10 and St.1.31 were the most prevalent in fishery products, being found in 15 and 16 products, respectively.

Cluster analyses based on similarity measurements from RAPD fingerprints were carried out with the aim of finding possible relationships between RAPD patterns and product categories. Cluster analysis of combined RAPD patterns classified isolates into 17 groups at a relative genetic similarity of 0.84 (Figure 3). Clusters 1 and 2 were the largest ones and contained 4 and 11 patterns, respectively, which included most isolates (31 and 57, respectively) from all product categories. In contrast, there were 10 single clusters which were formed by only one isolate. The other two single clusters (13.5.11 and 9.4.7) were composed of 3 and 4 isolates.

The validity assessment of cluster analyses rendered low values of hierarchical F-measures for banding patterns generated with primers S (0.314), ERIC-2 (0.311) and AP-7 (0.289). Similarly, low values were also obtained for F-measure, precision and recall in all cases. Hierarchical F-measure did not increase when banding patterns generated with each primer were combined (0.329). Values of F-measure, precision and recall did not increase in this case either. This assessment showed that no cluster was relatively pure and included most isolates of only one product category. No relationship was therefore found between any RAPD pattern and any product category.

3.3. Presence of enterotoxin genes

Over 91% of *S. aureus* isolates (n=114) carried enterotoxin genes, from which 112 were *sea* positive. However, only four isolates carried several enterotoxin genes. In two isolates, the *seg* and *sei* genes were detected, whereas two others carried the *sea*, *sec* and *seh* genes. Each of these isolates shared a different combined RAPD pattern, so they were different bacterial strains. Nevertheless, no further relationship was found between RAPD patterns of *se*-carrying isolates.

All isolates sharing an identical global RAPD pattern carried the same *se* gen pattern, and this supports the thesis that they are bacterial clones. A total of 26 out of the 33 strains identified by RAPD-PCR analysis were *se* gene carriers. *S. aureus* St.1.10 and St.1.31, which were most prevalent, were *se* positive. No *se*-carrying strain was characteristic of a single product category.

Non-*se*-carrying strains showed distinct RAPD fingerprints, which were not shared by any *se*-carrying strain. However, no further relationship was found between the presence of *se* genes and any RAPD pattern. Cluster analysis did not discriminate any cluster comprising either all non-*se*-carrying strains or all multi-*se*-carrying strains only.

A significant proportion of the fishery products tested (23.5%) were contaminated with *se*-positive *S. aureus*. Furthermore, counts of *se*-carrying *S. aureus* exceeded 10^2 and 10^3 CFU/g of food in 34 and 18 products, respectively, and were even higher than 10^5 CFU/g of food in two dried-salted tuna loin products (i.e. mojama) and one surimi product.

The incidence of *se*-positive *S. aureus* was different in each product category (Figure 4). Isolates carrying *se* genes were found in all salted fish (n=8), surimis (n=7) and fish roes (n=4) which were contaminated with *S. aureus.* The presence of *se*-positive isolates was also very high in fresh products (94%), smoked fish (91%), ready-to-cook products (90%), frozen products (85%) and other ready-to-eat products (75%) in which *S. aureus* was detected.

The presence of *se*-positive and *se*-negative isolates was not detected in a same fishery product, except in one frozen hake nuggets product and one fresh perch fillet, in which both types were found. Moreover, one cod pâté with pepper carried *sea* positive and *sea*, *sec* and *seh* positive isolates.

3.4. Antibiotic sensitivity

No differences were found among isolates with regard to sensitivity profiles, except for tetracycline. All strains were thus found to be resistant to penicillin G, chloramphenicol and ciprofloxacin. However, differences were found with regard to MIC values. Thus, strains St.1.11 and St.1.18 were the most resistant to penicillin G, St.1.01 and St.1.31 were the most resistant to chloramphenicol and St.1.26 and St.1.33 showed the highest MIC value (1.58 µg/ml) for ciprofloxacin. On the contrary, none of the strains was found to be resistant to beta-lactam antibiotics such as oxacillin and methicillin, but they all had intermediate resistance to methicillin (MIC from 9.5 to 14.5 µg/ml). Strains St.1.11 and St.1.13 showed the highest levels of resistance to methicillin (MIC=14.5 µg/ml). No strain was resistant to vancomycin, cephalothin, clindamycin, erythromycin, gentamicin or trimethoprim-sulfamethoxazole.

Tetracycline was the only antibiotic on which both resistant- and sensitive-strains were found. Thus, 17 out of the 33 strains (51.5%) identified by RAPD-PCR analysis were tetracycline-resistant, being *S. aureus* St.1.20 and St.1.31 the most resistant. However, over 82% of isolates were tetracycline-resistant. Tetracycline-resistant isolates were detected in 65 out of 75 fishery products contaminated with *S. aureus* (86.7%). St.1.10 and St.1.31, which were most prevalent, were also tetracycline-resistant. No differences in tetracycline susceptibility of isolates sharing identical global RAPD patterns with all three primers were found. However, no relationship was found between any RAPD pattern and susceptibility to tetracycline.

The incidence of tetracycline-resistant *S. aureus* was different in each product category (Figure 5). Tetracycline-resistant isolates were detected in all salted fish and fish roes which were found to be contaminated with *S. aureus*. Tetracycline-resistant isolates were also found in most contaminated fishery products of other categories, with an incidence decreasing in the following order: surimis (93.7%), ready-to-cook products (87.5%), smoked fish (82.4%), other ready-to-eat products (80.0%), fresh products (76.0%) and frozen products (64.3%).

Additionally, one representative isolate of each *se*-carrying strain was tested for the presence of *blaZ and mecA* genes, which are major determinants of the resistance of staphylococci to penicillin (Olsen et al., 2006; Vesterholm-Nielsen et al., 1999) and methicillin and all other beta-lactam antibiotics (Baddour et al., 2007; Strommenger et al., 2003), respectively. No PCR product was detected for *mecA* in any of the 26 isolates tested, whereas *blaZ* was detected in all of them (data not shown). These results are in agreement with those of antibiotic sensitivity testing.

Discussion

A high incidence of *S. aureus* was found in fishery products marketed in Galicia (25.16%) in the present study. Although data on the incidence of *S. aureus* in fishery products was scarce, a high incidence had also been reported over the last ten years in some studies (Abrahim et al., 2010; Herrera et al., 2006; Oh et al., 2007; Papadopoulou et al., 2007; Simon and Sanjeev, 2007), and only one work on fishery products collected at retail outlets in Italy found a low incidence, i.e. < 3% (Normanno et al., 2005). Only a few studies on microbial safety of fish products marketed in Spain have been carried out in the last decade (Garrido et al., 2009; González-Rodríguez et al., 2002; Herrera et al., 2006; Martínez et al., 2009). Considering the importance of fishery products at national level, the lack of information available on microbial safety in fishery products made or sold in Spain, and particularly in Galicia, was found surprising. Only Martínez et al. (2009) tested for the presence of bacterial pathogens in fishery products made in Galicia, specifically in farmed molluscan shellfish.

A wide range of product categories has been examined in the present work, comprising between 24 and 43 products of each one. In contrast, previous studies on the incidence of *S. aureus* in fish products have focused in only one or two product categories. Thus, Da-Silva et al. (2010), Herrera et al. (2006), Oh et al. (2007) and Papadopoulou et al. (2007) only tested fresh fishery products obtained from retail stores in Brazil, Spain, Korea and Greece, respectively, Simon and Sanjeev (2007) focused on frozen products and dried fish products sold in India, and Basti et al. (2006) examined smoked and salted Iranian fish products. Similarly, González-Rodríguez et al. (2002) only surveyed vacuum-packed cold-smoked freshwater fish.

It was also found surprising that most previous studies dealt with low risk fishery products, such as fresh fish, frozen products and salted or dried fish products, and hardly a work on the incidence of *S. aureus* in fishery products having to comply with legal regulations in Spain or

other European Union member states was found to be published in the last ten years. As an exception, a study on vacuum-packed cold-smoked freshwater fish by González-Rodríguez et al. (2002) reported that 3 packages out of 54 were contaminated with *S. aureus*. In addition, Normano et al. (2003) reported an incidence of 10% in a particular raw-eaten Italian fishery product, i.e. strips of cuttlefish, and Alarcón et al. (2006) detected the presence of *S. aureus* in 1 out of 10 ready-to-eat products within a study aimed to establish a RTQ-PCR procedure suitable for detection and quantification of *S. aureus* in food.

Incidence was found to be high in all categories (10-43%), but notable differences were found among them. Interestingly, the incidence was higher in those categories not covered by legislation, that is, fresh products, frozen products and salted fish, than in those having to comply with legal regulations in force at the time the study was conducted, i.e. ready-to-eat products and ready meals. This result seems to underline the effectiveness of regulations on the efforts of the industry to ensure food hygiene.

Although incidence was lower in fishery products subject to legal regulations, the presence of *S. aureus* was also detected in a high proportion of ready-to-cook products, smoked fish, non-frozen surimis, fish roes and other ready-to-eat products. Fishery products of these categories do not need to be cooked prior to being consumed. Therefore, the risk for the consumer can become significant if *S. aureus* is above regulatory limits, and food exceeding legal limits cannot be placed on the market or must be recalled. However, this study has revealed that a significant proportion of fishery products marketed in Galicia (11.3%) did not comply with regulatory limits in force.

A previous work on cold-smoked fish obtained at retail level in a nearby location showed that 3 out of 54 packages (5.5%) were contaminated with *S. aureus* at levels lower than 4 log CFU/g (González-Rodríguez et al., 2002), but authors did not report if levels were higher than regulatory limits (O. 2/8/1991) or laid down in Commission Recommendation 2001/337/EC).

In the present study, a higher proportion of smoked fish (18.6%) was found not to comply with legal regulations.

Incidence was highest in fresh products, followed by frozen products. Incidence values reported for fresh products in previous studies were also high, ranging between 10 and 30%, with the highest value for those marketed in Northwest Spain (Herrera et al., 2006). These results were slightly lower to those presented in this work. Incidence was also reported to be slightly lower in frozen products sold in India, i.e. 17% (Simon and Sanjeev, 2007). In the present work, samples were homogenized in a lower volume of diluent and the volume of bacterial suspension spread on agar plates was higher than or equal to those used in all aforementioned studies. These slight differences in the methodology resulted in a higher limit of detection, and it could account for at least part of the differences.

Conditions such as low-temperature storage, particularly in frozen fish, a low water activity typical of frozen and salted products or the activity of specific spoilage microorganisms of fresh fish prevent *S. aureus* to grow and, as a result, enterotoxin production. Also, fresh, frozen and salted products are commonly cooked prior to being consumed and it should destroy all or most *S. aureus*. The risk for the consumer is thus lower and no regulatory limits have been laid down for these fishery products neither in Spanish nor European legislation. However, an improper storage (temperature abuse) or processing (e.g. long desalting), can enable SEs to be formed. For instance, desalting at 20°C was found to result in unsafe levels of *S. aureus* ($\geq 10^6$ CFU/g) in cod, and possible toxin formation (Pedro et al., 2004). The risk can increase if the number of microorganisms is low (such as in thawed products), shelf-life is long (such as in salted products) or the product is consumed raw, undercooked or, in general, lightly processed. Additionally, staphylococcal enterotoxins (SEs) are highly heat-resistant and therefore, in many cases, thermal processes cannot be used as a measure to prevent staphylococcal food poisoning (Balaban and Rasooly, 2000; Cremonesi et al., 2005).

Most of the *S. aureus* isolates found in this work carried *se* genes so their incidence in fishery products marketed in Galicia was high (23.5%). A lower proportion of *S. aureus se* positive had been previously found among isolates from fishery products in other geographical regions (Normanno et al., 2005; Oh et al., 2007; Simon and Sanjeev, 2007).

At present, nine different serological types of SEs (SEA-SEE and SEG-SEJ) have been proven to have emetic activity (Ortega et al., 2010). Except for two, all *se*-carrying isolates found in this study carried the *sea* gene, and therefore could produce SEA, but none was found to be *seb*-*see* positive. Classical staphylococcal enterotoxins (SEA-SEE) have been reported to cause 95% of staphylococcal food poisoning. Among them, SEA is the most common in staphylococcus-related food poisoning (Pinchuk et al., 2010), probably due to a very high resistance to proteolytic enzymes (Le-Loir et al., 2003). Several studies have reported that a high proportion of isolates from outbreaks of staphylococcal food poisoning occurring in South Korea, France, Japan and United Kingdom could produce SEA, either alone or with another toxin (Cha et al., 2006; Kérouanton et al., 2007; Shimizu et al., 2000; Wieneke et al., 1993). In contrast, *sea* had been found not to be the most predominant *se* gene in fishery products in some previous studies (Normanno et al., 2005; Simon and Sanjeev, 2007).

Only four of the isolates were multi-*se*-carriers, two harboured the *seg* and *sei* genes and two others, the *sea*, *sec* and *seh* genes. In contrast, Cha et al. (2006) found that most isolates (ca. 85%) from staphylococcal food poisoning incidents in South Korea were multi-*se*-carriers and detected *seg*-*sei* genes in a significant proportion of isolates, either alone (ca. 4%) or along with other *se* genes (17.5%). Nonetheless, SEG and SEI have been considered to play minor role in food poisoning (Chen et al., 2004).

A dose lower than 1 µg of SE has been reported to make symptoms of staphylococcal food poisoning to appear within 1-6 h after consumption of contaminated food in an adult healthy

individual (Pinchuk et al., 2010; FDA, 2009). This toxin level can be reached when cell number exceeds 10^5 CFU/g of food (Bathia and Zahoor, 2007). As a preventive measure, legal limits of 10^2-10^3 CFU/g had been set for *S. aureus* in different fishery products. However, in this study, counts of *se*-carrying *S. aureus* exceeded such limits in a significant number of products and in some of them even by one or two orders of magnitude.

S. aureus has been reported as the third major causative agent of foodborne illness by fish and fishery products in the European Union (EFSA, 2009). Moreover, the actual incidence of staphylococcal food poisoning is known to be much higher than reported (Lawrynowicz-Paciorek et al., 2007; Smyth et al., 2004). Thus, the notification of staphylococcal intoxications is not mandatory in many member states of the European Union and most cases are not reported to healthcare services as they resolve within 24 to 48 h after onset -hospitalization rate was 19.5% for verified outbreaks caused by *S. aureus* in 2008 (EFSA, 2010)-.However, microbiological criteria laid down in national regulations (O. 2/8/1991 and RD 3484/2000) have been recently repealed (RD 135/2010) following Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuff. Coagulase positive staphylococci (mainly *S. aureus*) are thus no longer a microbiological criterion for ready-to-cook products and most ready-to-eat products. At present, *S. aureus* is set as a process hygiene criterion only for shelled and shucked products of cooked crustaceans and molluscan shellfish, with a value for M of 10^3 CFU/g. In the present study, 3 out of 12 products (25%) exceeded this value.

The emergence of multi-drug resistant pathogens is recognized as an environmental hazard to the food supply and human health, as it makes eradication more difficult and incidence to increase (Livermore, 2000; Popovich et al., 2007; Ribeiro et al., 2007). *S. aureus* has developed multidrug resistance worldwide, but wide variations in incidence exist regionally (Gündogân et al., 2006; Normanno et al. 2007; Peles et al., 2007; Pesavento et al., 2007). All

isolates found in fishery products marketed in Galicia were resistant to penicillin, chloramphenicol and ciprofloxacin and most of them were resistant to tetracycline too. Beleneva (2011) also found a high incidence of ciprofloxacin-resistant *S. aureus* (84.7%) in fishery products from the Sea of Japan and South China Sea, but the percentage of penicillin- and tetracycline-resistant strains was lower (47.2% and 27.5%, respectively). Variations in antibiotic resistance are also the result of other different factors. For instance, Pereira et al. (2009) isolated a high number of penicillin-resistant *S. aureus* (73%) from meat and dairy products in a nearby geographical area (North of Portugal), but the number of chloramphenicol-, ciprofloxacin- and tetracycline-resistant isolates was extremely low (0-2%). Tetracycline was the only antibiotic on which both resistant- and sensitive-strains were found. Tetracycline-resistance seemed to enhance the presence of *S. aureus* in fishery products obtained at retail level in Galicia.

Methicillin-resistant *S. aureus* (MRSA) are being increasingly found outside clinical settings (Popovich et al., 2007; Ribeiro et al., 2007; Stankovic et al., 2007). MRSA have thus been found in food animals (Lee, 2003) and different foods (Gündogân et al., 2006; Peles et al., 2007; Pereira et al., 2009; Pesavento et al., 2007) and also in fishery products recently (Beleneva, 2011). Although there is currently no evidence that eating food contaminated with MRSA may lead to an increased risk of humans becoming healthy carriers or infected with this bacterium (EFSA, 2010), it is important to take some preventive control measures. In the present study, however, no MRSA was isolated from fishery products and no isolate carried the *mecA* gene, though intermediate resistance to methicillin was detected in all isolates.

The identification of bacterial clones with enhanced virulence or increased ability to spread is important. RAPD is a fast and cost-effective PCR method for typing and differentiation of bacterial strains with no prior information of the gene sequence. However, a lack of standardization and a low reproducibility have been claimed as major drawbacks of RAPD

(Deplano et al., 2000; Van-Belkum et al., 1995). Nevertheless, RAPD-PCR binding patterns were found to be reproducible in this study when DNA from different cultures of a same isolate was used as a template. A good reproducibility has been also observed in epidemiological studies using RAPD for typing *S. aureus* isolates (Aras et al., 2012; Fueyo et al., 2001; Nikbakht et al., 2008; Shehata, 2008). Accordingly, RAPD could be used for initial screening of isolates in public health epidemiological studies (outbreak and endemic strains), prior to other complementary typing methods such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), which must be used for global epidemiology and population genetic studies of *S. aureus* (Al-Thawadi et al., 2003; Byun et al., 1997; Deplano et al., 2006).

The use of RAPD to discriminate strains has been also questioned. Morandi et al. (2010) has recently reported that multilocus variable number tandem repeat analysis (MLVA) is more powerful than RAPD-PCR for typing of *S. aureus* (discriminatory power of 0.99 and 0.94, respectively). Nonetheless, the discriminatory power of RAPD was determined by using only one primer (AP-4). In the present study, the combination of RAPD fingerprints obtained in separate reactions with three different primers allowed the discriminatory power of the analysis to be increased (increases of 11.7%, 24.6% and 32.8% for primers S, ERIC-2 and AP-7, respectively). Other authors have used even a much higher number of primers for strain differentiation (Byun et al., 1997; Nema et al., 2007). This allows strains to be differentiated when RAPD patterns are rather similar, i.e. low-yield patterns. However, the number of polymorphic bands generated by primers S, AP-7 and ERIC-2 was considered to be high enough to distinguish *S. aureus* strains among isolates found in this study. Nonetheless, it is not unlikely that the use of a higher number of primers had increased the number of different strains, but this had been time-consuming.

Isolates sharing a same global RAPD fingerprint also showed identical enterotoxin gene and antibiotic susceptibility patterns, and this supports the thesis that RAPD analysis allowed bacterial clones to be distinguished. Nema et al. (2007) also found that *S. aureus* isolates with a same RAPD fingerprint had identical *se* gen patterns and suggested a clonal origin of isolates.

Cluster analyses based on similarity measurements between RAPD patterns found no relationship between any RAPD pattern and any product category and, though *se*-negative and *se*-positive strains did not shared RAPD fingerprints, no further relationship was found between the presence of *se* genes and any RAPD pattern either.

The use of RAPD-PCR fingerprinting with several primers to assess the genetic relationship between *S. aureus* isolated from fishery products marketed in Galicia has therefore exclude a clonal origin.

4. Conclusions

A significant proportion (~25%) of fishery products surveyed from retail sector in Galicia in 2008 and 2009 was found to be contaminated with *S. aureus*, mostly with *se*-carrying strains. About 12% of products did not comply with regulatory limits, and a higher proportion of products not subject to regulations were contaminated too. These results suggest some effect of regulations on the efforts of the industry to ensure food hygiene.

However, a number of microbiological criteria laid down in national regulations have been recently repealed and coagulase positive staphylococci (mainly *S. aureus*) are thus no longer a microbiological criterion for ready-to-cook products and most ready-to-eat products. However, *S. aureus* has been reported as the third major causative agent of foodborne illness by fish and fish products in the European Union (EFSA, 2009). In addition, the actual incidence of staphylococcal food poisoning is known to be much higher than reported -the notification of is not mandatory in many member states of the European Union and most cases are not reported to healthcare services as they resolve within 24 to 48 h after onset-.

A revision of pre-requisite programs and an improvement of hygienic practices in handling and processing operations from fishing or farming to retail outlet is therefore recommended in order to ensure the safety of fishery products marketed in Galicia. Nonetheless, at present, *S. aureus* is still under surveillance by a significant part of the industrial sector.

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8 and salted seafood products in two coastal areas of eastern China. International Journal of
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Table 1. Nucleotide sequences of primer pairs and predicted sizes of resulting PCR products.

se gene	Primer	Nucleotide sequences (5´→3´)	Amplicons Size (bp)	Reference
sea	SEA-3	CCTTTGGAAACGGTTAAAACG	127	Becker et al. (1998)
	SEA-4	TCTGAACCTTCCCATCAAAAAC		
seb	SEB-1	TCGCATCAAACTGACAAACG	477	
	SEB-4	GCAGGTACTCTATAAGTGCCTGC		
sec	SEC-3	CTCAAGAACTAGACATAAAAGCTAGG	271	
	SEC-4	TCAAAATCGGATTAACATTATCC		
sed	SED-3	CTAGTTTGGTAATATCTCCTTTAAACG	319	
	SED-4	TTAATGCTATATCTTATAGGGTAAACATC		
see	SEE-3	CAGTACCTATAGATAAAGTTAAAACAAGC	178	
	SEE-2	TAACTTACCGTGGACCCTTC		
seg	SEG-1	AAGTAGACATTTTTGGCGTTCC	287	Omoe et al. (2002)
	SEG-2	AGAACCATCAAACTCGTATAGC		
seh	SEH-1	GTCTATATGGAGGTACAACACT	213	
	SEH-2	GACCTTTACTTATTTCGCTGTC		
sei	SEI-1	GGTGATATTGGTGTAGGTAAC	454	
	SEI-2	ATCCATATTCTTTGCCTTTACCAG		

Table 2. Antibiotic breakpoints used for interpretation of susceptibility tests (S: sensitive; R: resistant).

Antibiotic		[a]CEP	[a]MET	[a]OXA	[b]CIP	[b]CLI	[b]CLO	[b]ERY	[b]GEN	[b]PEN	[b]TET	[b]TRI-SUL	[b]VAN
Breakpoints (µg/ml)	**S ≤**	8	8	2	1	0.25	8	1	1	0.125	1	2	2
	R >	32	16	4	1	0.50	8	2	1	0.125	2	4	2

CEP: cephalothin; CLI: clindamycin; CLO: chloramphenicol; ERY: erythromycin; GEN: gentamicin; OXA: oxacillin; PEN: penicillin G; TET: tetracycline; VAN: vancomycin; MET: methicillin; CIP: ciprofloxacin; TRI-SUL: trimethoprim-sulfamethoxazole.

[a] Breakpoints from the CLSI (2011).

[b] Breakpoints from the EUCAST (2011).

Legends

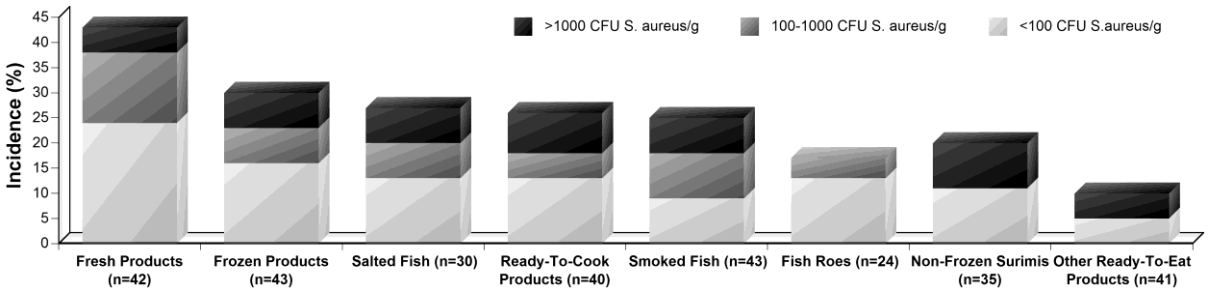
Figure 1. Incidence (%) of *S. aureus* in fishery products marketed at retail level in Galicia. The number of products surveyed in each category is shown (*n*).

Figure 2A-C. Agarose gels showing RAPD-PCR fingerprints obtained for *S. aureus* isolates using primers S (A), AP-7 (B) and ERIC-2 (C). Lane 1: DNA Molecular Weight Marker (HyperLadder II, 50-2000 bp; Bioline). Pattern number is shown on the top of each fingerprint.

Figure 3. Dendrogram from cluster analysis based on the global combination of RAPD-PCR patterns obtained with all three primers. Combined patterns were assigned a three-digit code number, with digits corresponding to numbers assigned to patterns obtained with primers S, AP-7 and ERIC-2, respectively.

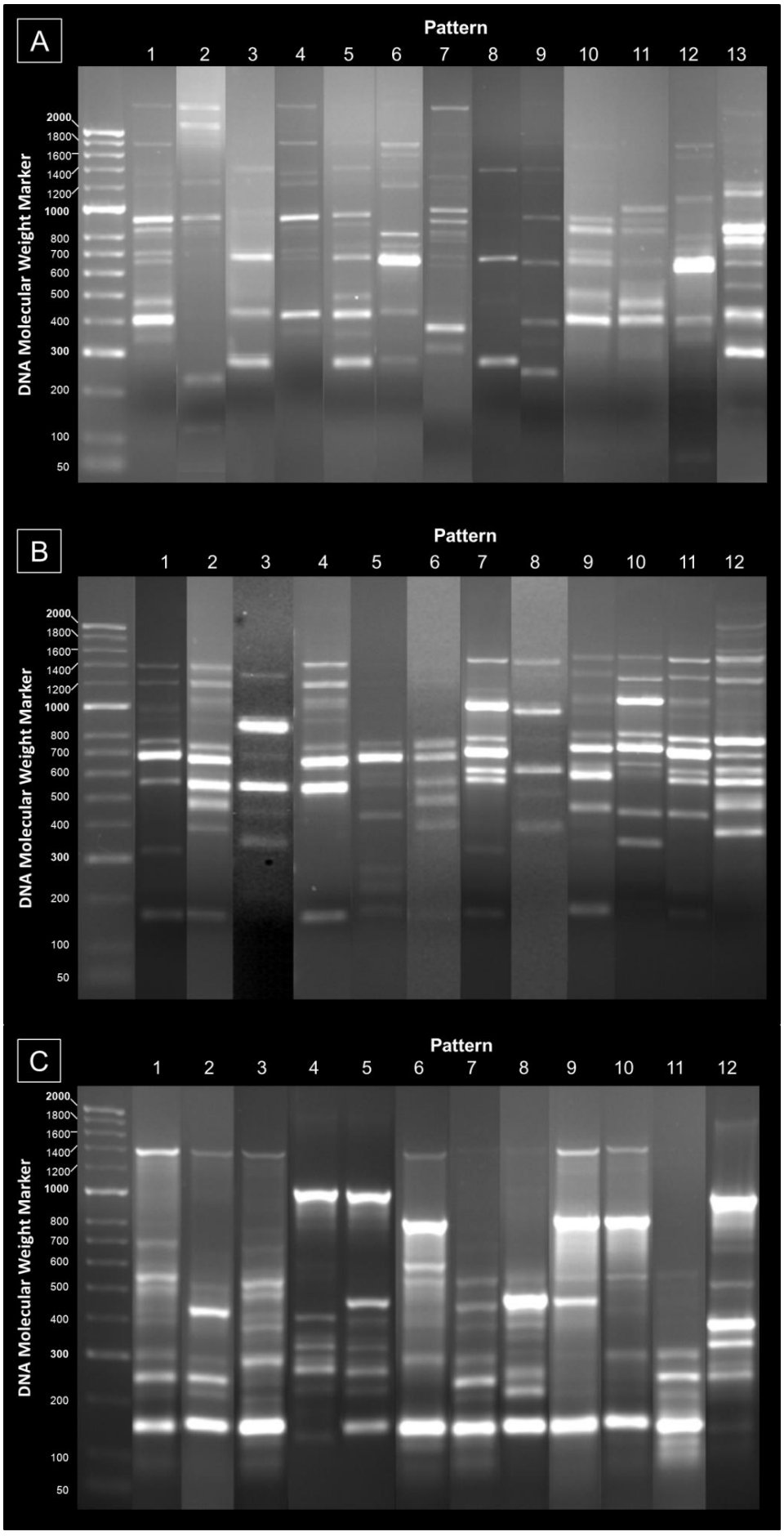
Figure 4. The presence of *se* genes (%) in *S. aureus* isolated from fishery products marketed at retail level in Galicia. Presence of *se* genes was defined as the number of *se*-carrying *S. aureus*-containing products respect to the number of *S. aureus*-containing products. The number of fishery products carrying *se* positive *S. aureus* in each category is shown (*n*).

Figure 5. Tetracycline resistance (%) of *S. aureus* isolated from fishery products marketed at retail level in Galicia. Resistance was defined as the number of tetracycline-resistant isolates with respect to the number of isolates. The number of isolates from each category is shown (*n*).

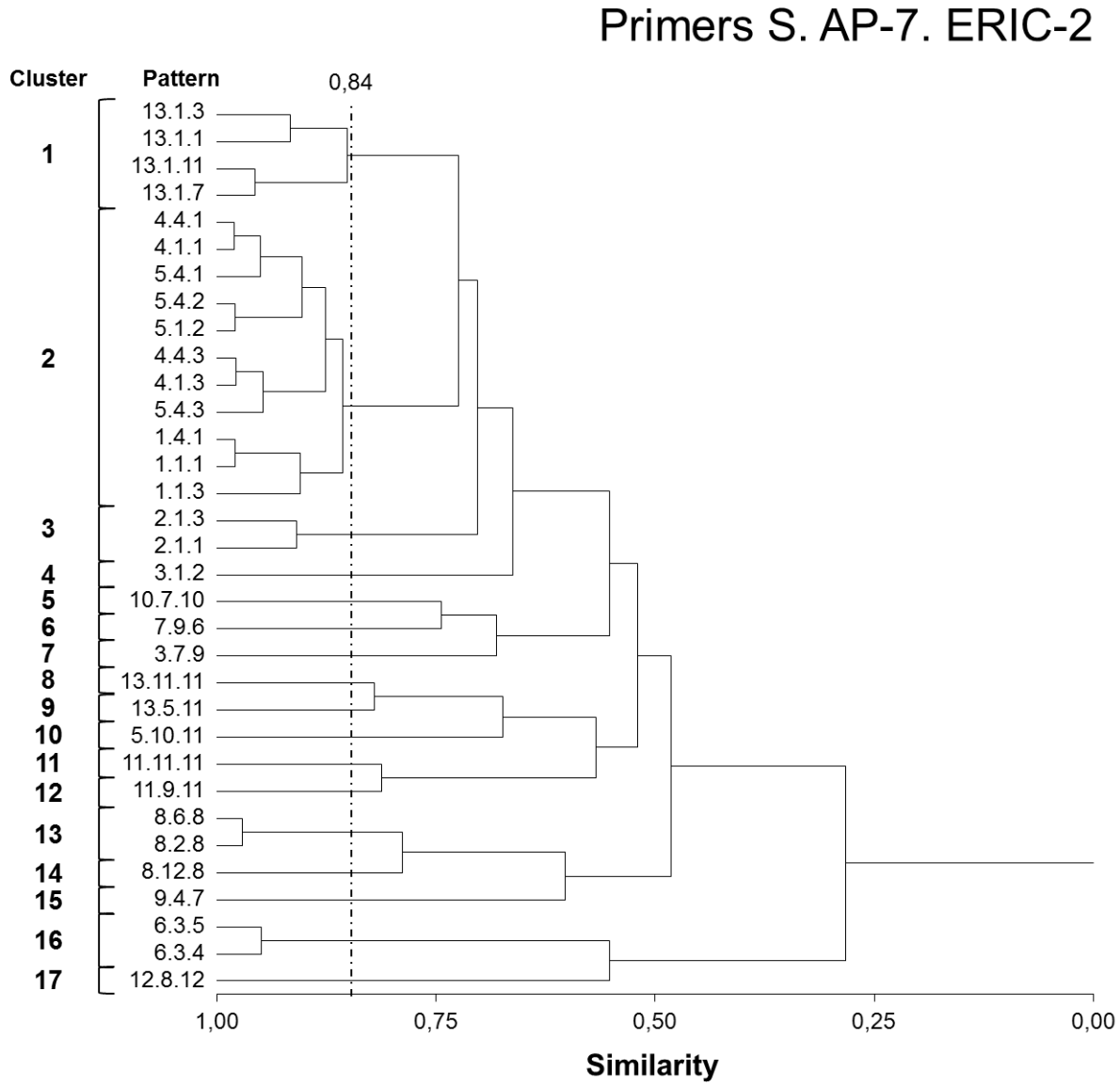

>1000 CFU S. aureus/g
100-1000 CFU S. aureus/g
<100 CFU S.aureus/g
Incidence (%)
45
40
35
30
25
20
15
10
5
0
Fresh Products (n=42)
Frozen Products (n=43)
Salted Fish (n=30)
Ready-To-Cook Products (n=40)
Smoked Fish (n=43)
Fish Roes (n=24)
Non-Frozen Surimis (n=35)
Other Ready-To-Eat Products (n=41)

1

2

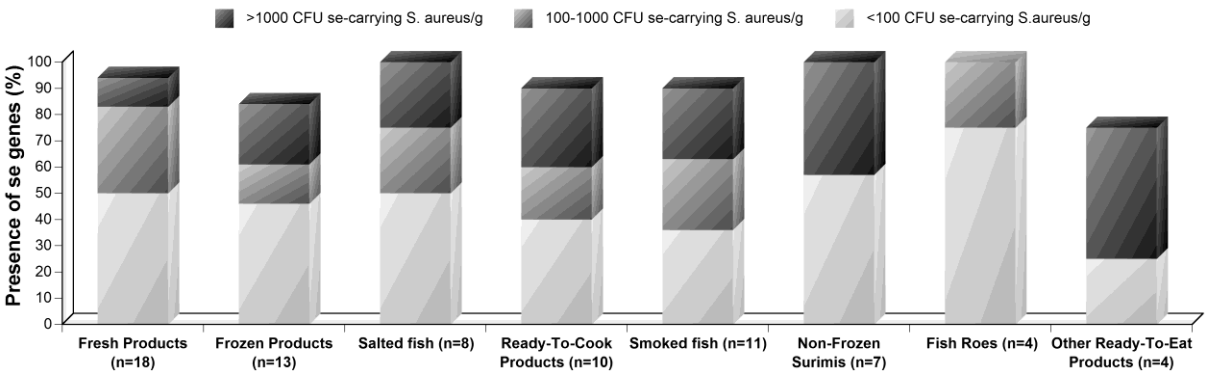

A
Pattern
1 2 3 4 5 6 7 8 9 10 11 12 13
DNA Molecular Weight Marker
2000 1800 1600 1400 1200 1000 800 700 600 500 400 300 200 100 50
B
Pattern
1 2 3 4 5 6 7 8 9 10 11 12
DNA Molecular Weight Marker
2000 1800 1600 1400 1200 1000 800 700 600 500 400 300 200 100 50
C
Pattern
1 2 3 4 5 6 7 8 9 10 11 12
DNA Molecular Weight Marker
2000 1800 1600 1400 1200 1000 800 700 600 500 400 300 200 100 50

1


Primers S. AP-7. ERIC-2
Cluster
Pattern
0,84
1
13.1.3
13.1.1
13.1.11
13.1.7
2
4.4.1
4.1.1
5.4.1
5.4.2
5.1.2
4.4.3
4.1.3
5.4.3
1.4.1
1.1.1
1.1.3
3
2.1.3
2.1.1
4
3.1.2
5
10.7.10
6
7.9.6
7
3.7.9
8
13.11.11
9
13.5.11
10
5.10.11
11
11.11.11
12
11.9.11
13
8.6.8
8.2.8
14
8.12.8
15
9.4.7
16
6.3.5
6.3.4
17
12.8.12
1,00
0,75
0,50
0,25
0,00
Similarity

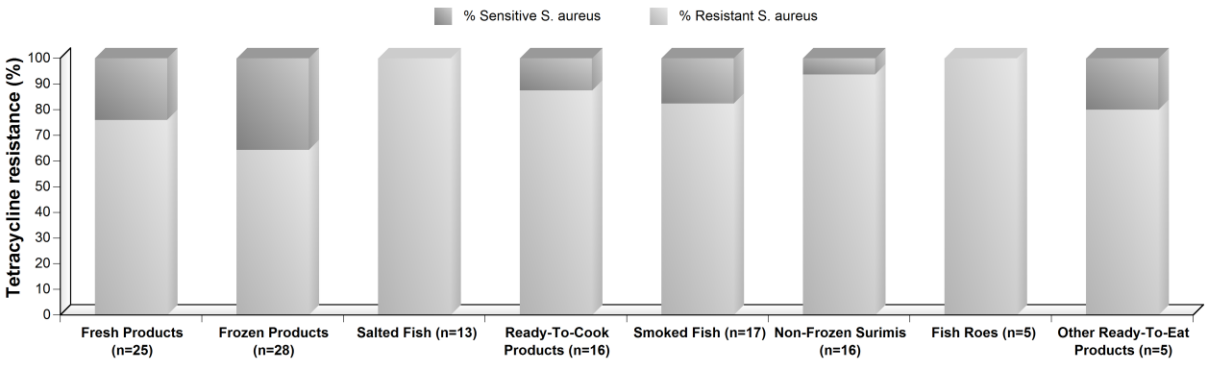
1

2


>1000 CFU se-carrying S. aureus/g
100-1000 CFU se-carrying S. aureus/g
<100 CFU se-carrying S.aureus/g
Presence of se genes (%)
100
90
80
70
60
50
40
30
20
10
0
Fresh Products (n=18)
Frozen Products (n=13)
Salted fish (n=8)
Ready-To-Cook Products (n=10)
Smoked fish (n=11)
Non-Frozen Surimis (n=7)
Fish Roes (n=4)
Other Ready-To-Eat Products (n=4)

1

2


% Sensitive S. aureus
% Resistant S. aureus
Tetracycline resistance (%)
100
90
80
70
60
50
40
30
20
10
0
Fresh Products (n=25)
Frozen Products (n=28)
Salted Fish (n=13)
Ready-To-Cook Products (n=16)
Smoked Fish (n=17)
Non-Frozen Surimis (n=16)
Fish Roes (n=5)
Other Ready-To-Eat Products (n=5)
Product categories

1