Phylogenetic Diversity of *Aeromonas* from “Alheira,” a Traditional Portuguese Meat Product

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Abstract

“Alheira” is a traditional smoked meat sausage produced in the north of Portugal, representing an important economic resource for the region. This meat product has been subjected to research studies with the aim of detecting the presence of common foodborne pathogens, but, to our knowledge, isolation of emerging foodborne *Aeromonas* from alheira has never been previously described. Present work attempts to evaluate the *Aeromonas* species diversity of 84 isolates of *Aeromonas* spp. collected from 32 alheira samples. All presumptive *Aeromonas* isolates were subjected to genotyping by enterobacterial repetitive intergenic consensus–polymerase chain reaction analysis. The isolates presenting a different pattern were subjected to *gyrB* gene sequencing for species classification, and the species *A. hydrophila*, *A. salmonicida*, *A. caviae*, *A. media*, and *A. allosaccharophila* were identified. The *Aeromonas* species diversity found has not been previously described in any other meat product evaluated in previous studies. It is also important to highlight the presence of *A. hydrophila* and *A. caviae* because they were previously associated with illness in humans, including gastroenteritis.

Introduction

ALHEIRA IS A TRADITIONAL meat product produced in the North of Portugal, which is part of the diet in rural areas but also very popular among urban consumers. This traditional smoked meat sausage, which represents an important economic resource for the region, is produced from a mix of different types of meat (pork, chicken, turkey, duck, and beef) and wheat bread. The various meats are boiled in water with salt and spices, minced by hand, and mixed with sliced wheat bread previously moistened in the cooking broth. Garlic and olive oil are added to the mixture. The final homogeneous paste is used to fill the cattle or pig casings, and the horseshoe-shaped sausage obtained is submitted to a smoking process for a few days (Esteves *et al.*, 2008).

Several studies investigated the microbiological content of alheira. This product presented high microbial counts, and lactic acid bacteria represented the dominant microflora (Esteves *et al.*, 2008; Ferreira *et al.*, 2006). In addition, several foodborne pathogens such as *Staphylococcus aureus*, *Salmonella* spp., *Clostridium perfringens*, *Listeria monocytogenes*, *Bacillus cereus*, and *Yersinia enterocolitica* have been isolated from this traditional sausage (Esteves *et al.*, 2007a,b, 2008; Ferreira *et al.*, 2006).

Over the past 25 years, mesophilic *Aeromonas* have received increasing attention as an emergent agent of foodborne disease. In Spain, *Aeromonas* spp. occupied fourth place among

the list of causes of gastrointestinal disease reported each year over the period 1997–2006 (Pablos *et al.*, 2009). Some *Aeromonas* species are responsible for producing intestinal and extraintestinal infections worldwide, and gastroenteritis represents the most common clinical presentation associated with *Aeromonas*, followed by localized soft-tissue infections and bacteremia (Abbott *et al.*, 2003; Janda and Abbott, 2010; Schlenker and Surawicz, 2009; Vila *et al.*, 2003). Bacteremia mainly occurs in patients with underlying diseases such as hepatobiliary disorders, cancer, and diabetes (Figuera, 2005).

The genus *Aeromonas* belongs to the family *Aeromonadaceae* and currently comprises 26 species: *A. allosaccharophila*, *A. aquariorum*, *A. bestiarum*, *A. bivalvium*, *A. caviae*, *A. encheleia*, *A. eucrenophila*, *A. fluvialis*, *A. hydrophila*, *A. jandaei*, *A. media*, *A. molluscorum*, *A. piscicola*, *A. popoffii*, *A. rivuli*, *A. salmonicida*, *A. sanarellii*, *A. schubertii*, *A. simiae*, *A. sobria*, *A. taiwanensis*, *A. tecta*, *A. trota*, *A. veronii*, and also *Aeromonas* sp. HG11 and *Aeromonas* sp. HG13 (Enteric Group 501) (Alperi *et al.*, 2010a,b; Beaz-Hidalgo *et al.*, 2009; Demarta *et al.*, 2008; Figueras *et al.*, 2011; Harf-Monteil *et al.*, 2004; Martin-Carnahan and Joseph, 2005; Martínez-Murcia *et al.*, 2008; Miñana-Galbis *et al.*, 2004, 2007). Recently, the name *A. diversa* was proposed to designate *Aeromonas* sp. HG13 (Miñana-Galbis *et al.*, 2010). Martínez-Murcia *et al.* (2011) report that two additional potential new species “*A. cavernicola*” and “*A. lusitana*” have been recently recognized and are in the process of being described.

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This genus consists of facultative anaerobic Gram-negative non-spore-forming motile bacilli or coccobacilli, predominantly motile by a single polar flagellum, which are oxidase and catalase positive, reduce nitrate to nitrite, and are resistant to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridina) (Martin-Carnahan and Joseph, 2005). *Aeromonas* are ubiquitous in nature, being considered autochthonous inhabitants in aquatic environments, and have also been isolated from a wide range of foods, including meat, poultry, milk and milk products, fish and shellfish, and vegetables (Fiorentini *et al.*, 1998; Hazen *et al.*, 1978; Neyts *et al.*, 2000; Pablos *et al.*, 2009). Furthermore, the isolation of *Aeromonas* spp. from meat products, including sausages, has also been reported (Hudson and De Lacy, 1991; Singh, 1997; Villari *et al.*, 2000). Some *Aeromonas* spp. are facultative psychrophiles and maintain the ability to express virulence factors at refrigeration temperatures (Kirov *et al.*, 1993; Mano *et al.*, 2000). This fact, along with the ubiquitous nature of *Aeromonas* spp. and its presence in the food chain, suggested that food is a vehicle for illness caused by *Aeromonas* spp. (Isonhood and Drake, 2002).

Housekeeping gene sequencing analysis is considered as a high-resolution approach to ascertain phylogenetic relationships in bacteria (Stackebrandt *et al.*, 2002). During the past few years, the sequencing of *gyrB* gene (encoding the B-subunit of DNA gyrase, a type II DNA topoisomerase) has proved to be a useful marker for *Aeromonas* species identification and strain differentiation, even when using only a partial *gyrB* gene sequence (Fontes *et al.*, 2010, 2011; Martinez-Murcia *et al.*, 2008; Saavedra *et al.*, 2006, 2007; Soler *et al.*, 2004; Yañez *et al.*, 2003).

The present study was undertaken with two aims: to isolate and to describe the phylogenetic diversity of *Aeromonas* species in traditional Portuguese sausage alheira by using the partial *gyrB* gene sequencing.

Methods

Sample collection and isolation of *Aeromonas* species

A total of 32 samples of alheira were purchased from local retail establishments, in January and February 2007. They were immediately transported to the laboratory and analyzed within 2 h.

Twenty-five grams of each alheira sample were weighed aseptically, homogenized for at least 2 min in stomacher bags containing 225 mL of alkaline peptone water, and incubated for 24 h at 30°C. The homogenate was streaked on glutamate starch phenol-red agar (GSP; Merck, Whitehouse Station, NJ) plates. After incubation at 30°C for 24 h, yellow colonies were isolated and subcultured into tryptone soya agar (TSA; Oxoid, Hampshire, UK). Oxidase-positive colonies were taken as presumptive *Aeromonas* isolates. Strains were maintained on TSA (Oxoid) plates at 4°C and were stored in 10% glycerol at -80°C. Culture collection strains used in this study were purchased from the Spanish Collection of Type Cultures (CECT).

DNA extraction

A single colony from fresh cultures was resuspended in 100 µL of TE buffer (Tris-HCl 10 mM, pH 8; and EDTA 1 mM, pH 8), and 200 µL of Chelex 100 (BioRad) was added. The tube was vortexed at high speed for 1 min, incubated at 96°C for 10 min, kept at -20°C for 10 min, and this process was re-

peated three consecutive times. The tube was again vortexed and centrifuged for 5 min at 12,000×g. The supernatant was transferred to a fresh tube and stored at -20°C.

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

All isolates from the same sample were subjected to ERIC-PCR in order to recognize indistinguishable isolates and to discriminate dissimilar isolates, which have been chosen for sequencing the *gyrB* gene. The oligonucleotides selected for ERIC-PCR were ERIC 1R (5'-ATGTAAGCTCCTGGGGATT CAC-3') and ERIC 2 (5'-AAGTAAGTGAAGTGGGGTGA GCG-3') (Versalovic *et al.*, 1991). PCR amplification was performed using a PTC100™ Peltier Thermal Cycler (MJ Research, St. Bruno, Quebec, Canada). Each reaction was performed in a final volume of 25 µL, containing 2.5 µL of 10× PCR buffer, 50 pmol of each primer, 400 µM of deoxynucleotide triphosphates (dNTPs), 1 µL (1U) of Taq DNA polymerase, and 2.5 µL (50 ng) of template DNA. The reaction mixtures were subjected to an initial denaturation for 4 min at 94°C followed by 35 cycles, with a thermal cycling of 94°C for 15 s, 55°C for 30 s, 72°C for 45 s, and a single final extension for 5 min at 72°C. Following amplification, 8 µL of the PCR products were electrophoresed on 1.2% agarose gels in TAE buffer. Electrophoresis was performed for 80 min at 6 V/cm. A 1-kb DNA ladder was used as molecular size marker. Agarose gels were stained on ethidium bromide (0.5 µg/mL), visualized with an ultraviolet lamp, and photographed in the GelDocMega (Wolf Laboratories Limited, Pocklington, UK).

PCR amplification and sequencing of *gyrB*

PCR amplification was performed using a PTC100™ Peltier Thermal Cycler (MJ Research). Procedures and characteristics of oligonucleotide primers (*gyrB*3F 5'-TCCGGCG GTCTGCACGGCGT-3'; *gyrB*14R 5'-TTGTCCGGGTTGTAC TCGTC-3') for the PCR-based amplification and sequencing of the *gyrB* gene were performed as previously described (Soler *et al.*, 2004; Yañez *et al.*, 2003), except for using the BigDye Terminator V3.1 Cycle Sequencing Kit in the ABI 3100-Avant Genetic Analyzer (Applied Biosystems, Carlsbad, CA), according to the manufacturer's instructions.

Phylogenetic data analysis

The *gyrB* nucleotide sequences were aligned to previously published reference sequences (Saavedra *et al.*, 2006; Soler *et al.*, 2004; Yañez *et al.*, 2003) by using the Clustal X program, version 1.8 (Thompson *et al.*, 1997). Genetic distances were determined by Kimura's two-parameter model (Kimura, 1980), and an evolutionary tree was constructed by the Neighbour-Joining method (Saitou and Nei, 1987) with the Mega program (Kumar *et al.*, 2001).

Results

In this study, *Aeromonas* spp. were detected in 10 (31%) of the 32 samples analyzed, and a total of 84 presumptive *Aeromonas* isolates were obtained. The 84 isolates of *Aeromonas* spp. were subjected to ERIC-PCR analysis, and 20 isolates presented different and unique patterns. Nevertheless, isolate redundancy (i.e., pairs of strains with identical genetic pattern) was found in the remaining 64 isolates. Six of the 20

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TABLE 1. CHARACTERIZATION OF THE 20 ISOLATES WITH DIFFERENT ENTEROBACTERIAL REPETITIVE INTERGENIC CONSENSUS (ERIC) PATTERNS

Sample	Species	No. of isolates with the same ERIC pattern	Strains selected for <i>gyrB</i> gene sequencing	Partial <i>gyrB</i> gene sequence
Alheira 1	<i>Aeromonas caviae</i>	8	MDC2033	Unique
	<i>A. hydrophila</i>	3	MDC2024	Unique
Alheiras 2 and 4	<i>A. caviae</i>	2 + 13	MDC2177	Unique
Alheira 3	<i>A. hydrophila</i>	4	MDC2180	Unique
Alheira 5	<i>A. caviae</i>	1	MDC2198	Unique
	<i>A. media</i>	2	MDC2197	Unique
	<i>A. salmonicida</i>	1	MDC2199	Unique
Alheira 6	<i>A. caviae</i>	8	MDC2207	Unique
Alheiras 6 and 8	<i>A. media</i>	4 + 5	MDC2212	Unique
Alheira 7	<i>A. caviae</i>	2	MDC2216	Unique
	<i>A. caviae</i>	8	MDC2222	Unique
	<i>A. hydrophila</i>	1	MDC2214	Unique
Alheira 8	<i>A. media</i>	1	MDC2161	=MDC2212
	<i>A. salmonicida</i>	8	MDC2162	Unique
Alheira 9	<i>A. hydrophila</i>	2	MDC2363	Unique
	<i>A. hydrophila</i>	1	MDC2365	Unique
	<i>A. allosaccharophila</i>	3	MDC2362	Unique
Alheira 10	<i>A. media</i>	4	MDC2366	Unique
	<i>A. media</i>	1	MDC2367	Unique
	<i>A. media</i>	2	MDC2369	=MDC2367

different patterns included just one isolate each, three included two, and the number of isolates included in the other 11 different patterns varied between three and 15 (Table 1).

The 20 isolates with different patterns were taken for species identification by *gyrB* gene sequencing, and we have found 18 isolates with different *gyrB* sequences. The *gyrB* sequences obtained (427–486 nt in length) were aligned with those from reference strains of the *Aeromonas* species, and a phylogenetic tree was constructed (Figs. 1 and S1) (see supplementary Fig. S1 at www.liebertpub.com/fpd). The strains analyzed belong to five different species of *Aeromonas*: *A. hydrophila* (five strains), *A. salmonicida* (two strains), *A. caviae* (six strains), *A. media* (six strains), and *A. allosaccharophila* (one strain).

It was found that 60% of the positive samples presented more than one *Aeromonas* species, and in one of them we isolated and identified three different species: *A. salmonicida*, *A. caviae*, and *A. media*.

Discussion

Aeromonas are ubiquitous in nature, and their presence in food has been reported in an increasing number of studies, from different geographical regions. *Aeromonas* species have been detected in different food products, including meat products (Hudson and De Lacy, 1991; Neyts *et al.*, 2000; Singh, 1997; Villari *et al.*, 2000). In a study describing the distribution of mesophilic *Aeromonas* species in raw and ready-to-eat fish and meat products in Switzerland by Gobat and Jemmi (1993), sliced cooked ham, smoked cooked sausage, and mortadella showed a occurrence of 38.2%, 15.6%, and 12.9%, respectively, for *Aeromonas* spp. In Italy, 25% of salami and raw ham samples were positive for *Aeromonas* spp., with *A. hydrophila* and *A. caviae* being the only species detected in meat products (Villari *et al.*, 2000). Hudson and De Lacy (1991) reported the

presence of *A. hydrophila* and *A. caviae* in 18% and 4.5%, respectively, of the sausages analyzed. Singh (1997) isolated *Aeromonas* spp. from all sausage samples analyzed, which belong to the species *A. hydrophila*, *A. caviae*, and *A. sobria*. These *Aeromonas* species were also detected in pork sausages and ground pork by Okrend *et al.* (1987).

In the present study, 31% of the alheira samples analyzed, all from the same factory, were positive for *Aeromonas* spp., and five different *Aeromonas* species have been identified: *A. hydrophila*, *A. salmonicida*, *A. caviae*, *A. media*, and *A. allosaccharophila*.

The *gyrB* gene sequencing was very useful in this study for the identification of *Aeromonas* species, which confirms the usefulness of this methodology in discriminating *Aeromonas*, including the identification of strains of rarely isolated species such as *A. allosaccharophila* (Saavedra *et al.*, 2007).

Generally, alheira is characterized by high variability in its chemical, physical, and sensory attributes (Patarata *et al.*, 2008), and it is important to note that this traditional product is submitted to a smoking process at low temperature (generally <40°C) (Albano *et al.*, 2008). According to Ferreira *et al.* (2006), alheira exhibited pH and salt levels insufficient to assure microbiological safety, although this traditional meat product is not considered a ready-to-eat food and is usually eaten after being cooked (roasted or fried). Because of this, even when contaminated with pathogens, including *Aeromonas*, these would be inactivated. However, very often, alheira is consumed after being submitted to culinary treatments, whose time/temperature combination is insufficient to inactivate pathogens. On the other hand, there is always ample opportunity for post-cooking contamination (Ferreira *et al.*, 2006). In these cases, a contaminated alheira may represent a threat to consumers' health, especially to the young, the elderly, and the immunocompromised.

FIG. 1. Phylogenetic tree (NJ) showing all the strains of *Aeromonas hydrophila*, *A. salmonicida*, *A. caviae*, *A. media*, and *A. allosaccharophila* obtained (underlined) and other known *Aeromonas* species based on the *gyrB* gene sequences and the type strains of each corresponding species. Numbers shown next to each node indicate bootstrap values (percentage of 1000 replicates). Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) profiles of the strains obtained in this study are shown.

Conclusion

In this work, the phylogenetic diversity of *Aeromonas* isolated from alheira has been reported. Despite the increasing number of studies on the occurrence of *Aeromonas* in food, to our knowledge it is the first time that the presence and diversity of these bacteria are described in this traditional Portuguese meat product. We would like to emphasize the identification of clinically relevant species, i.e., *A. hydrophila* and *A. caviae*, among the species isolated. The results demonstrate the usefulness of *gyrB* sequencing in discriminating *Aeromonas* species.

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Disclosure Statement

No competing financial interests exist.

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Supplementary Data

FIG. S1. Clustal $\times$ (1.81) multiple sequence alignment of the *Aeromonas gyrB*-encoding gene sequences obtained.

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