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Research Note

Prevalence and Molecular Characterization of *Aeromonas* spp. in Ready-to-Eat Foods in Italy

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ABSTRACT

A survey was carried out in Italy to ascertain the prevalence of *Aeromonas* spp. in ready-to-eat foods (vegetables, cheeses, meat products, and ice creams) and the level of molecular heterogeneity of the isolates found by macrorestriction analysis of genomic DNA with pulsed-field gel electrophoresis (PFGE). In total, 46 (14.4%) of the 320 food samples examined were found positive for *Aeromonas* spp. The highest percentages of isolation were discovered in vegetables, particularly lettuce (45.0%), endive (40.0%), and rucola (20.0%). Ricotta was the only cheese type analyzed that showed a high frequency of isolation (45.0%). Among meat products, salami and raw ham (25.0% of samples positive) and, to a lesser extent, baloney (5.0%) were found positive for *Aeromonas* spp. *Aeromonas hydrophila* was the most common isolate from foods of animal origin, whereas *Aeromonas caviae* was the dominant species in vegetables. No motile aeromonads were found in ice cream samples. *Aeromonas* isolates showed a high level of genetic heterogeneity, because 24 PFGE patterns were identified among 27 *A. hydrophila* strains and 20 PFGE patterns were found in 23 *A. caviae* isolates. In conclusion, consumers of ready-to-eat foods in Italy are regularly exposed to many genetically distinct strains of *A. hydrophila* and *A. caviae* without evident signs of malaise, and therefore, few of these strains, if any, are likely to be pathogenic.

There is an increasing amount of evidence that at least some strains of *Aeromonas* spp. are involved in the etiology of gastrointestinal diseases in human beings and that they cause serious infections in immunocompromised hosts *(17–19, 38)*. Although the organisms are considered natural inhabitants in the aquatic environment, *Aeromonas* spp. can be isolated from a variety of foods, including meat, poultry, milk and milk products, fish and shellfish, and vegetables *(1, 2, 5, 7–11, 13–16, 20–23, 25, 28–32, 34, 39)*.

Until questions concerning *Aeromonas* pathogenicity are definitively answered, the presence of large numbers of aeromonads in food and water should be regarded as a potential health threat, particularly for immunocompromised individuals. Obviously, ready-to-eat foods are of particular concern, because these foods are consumed without a further heat treatment capable of reducing or completely abating the *Aeromonas* microbial load.

In this scientific note we report the results of an investigation aimed to determine the prevalence of *Aeromonas* spp. in ready-to-eat foods commonly consumed in Italy and the level of molecular heterogeneity of the isolates found by macrorestriction analysis of genomic DNA with pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Samples. A total of 320 samples of ready-to-eat foods were purchased from local retail outlets in Naples (Italy). The samples examined included vegetables (100), cheeses (100), meat products (100), and ice creams (20). Food samples were transferred to the laboratory in ice chests at 4 to 7°C and analyzed on the same day.

Microbiological analysis. Samples (10 g) were weighed aseptically in stomacher bags containing 90 ml of alkaline peptone water (pH 8.7 ± 0.1; Oxoid Italiana, Milan, Italy) and homogenized for 2 min using a Stomacher 400 (PBI, Milan, Italy). After decimal dilution, 0.1 ml from each dilution tube was spread onto Ryan medium (Oxoid) and starch ampicillin agar (Biolife, Milan, Italy). Plates were incubated aerobically for 48 h at 30°C. The remaining alkaline peptone water was incubated for 24 h at 30°C, and 0.1 ml was subsequently plated on the surface of the same media and incubated as described.

Colonies typical for *Aeromonas* spp. were counted (in the quantitative assay), purified, Gram-stained, and confirmed on the basis of the following tests: oxidase test, oxidation/fermentation test, resistance to vibriostatic agent O/129 and API 20E. Additional tests (hemolytic activity, gas production from glucose, H_2S production from cysteine, aesculin hydrolysis, suicide phenomenon) were performed for the identification at the species level according to the criteria of Popoff *(33)*, Namdari and Bottone *(27)*, and Carnahan et al. *(6)*.

Molecular typing of isolates. A 5-ml culture grown overnight in tryptone soy broth (Oxoid) at 30°C was pelleted, washed in 500 μl of EET buffer (100 mM EDTA, 10 mM EGTA, 10 mM Tris pH 8), and resuspended in 200 μl of the same buffer. Concentrations were adjusted to an optical density at 620 nm of 10.0. This cell suspension was then diluted 1:1 with 150 μl of 1.5% low-gelling-temperature agarose (Sea Plaque, FMC Bioproducts, Rockland, Maine) in EET buffer; disks of 20 μl were allowed to solidify for 5 min at −20°C. The cells were lysed by incubation of the disks at 50°C

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for 20 h in a solution of proteinase K (1 mg/ml) and sodium dodecyl sulfate (1%) in EET buffer. The agarose disks were washed five times in 14 ml of TE buffer (10 mM Tris, 1 mM EDTA pH 7.5) for 1 h with gentle agitation. After this the DNA was considered purified and was stored in TE buffer at 4°C. Restriction digestion was performed with *Xba*I (30 U/disk) according to the manufacturer's recommendations (New England Biolabs, Beverly, Mass.). The PFGE gels were prepared with 1% agarose (SeaKem LE, FMC Bioproducts) in 0.5× TBE (Tris-borate-EDTA) buffer. Disks containing the DNA and two molecular weight markers, i.e., lambda ladder and low-range markers (New England Biolabs), were deposited in the slots of the gel, and these were sealed with 0.75% low-gelling-temperature agarose in 0.5× TBE. Elecrophoresis was carried out in a Chef-DR II apparatus (BioRad, Hercules, Calif.) at 150 V at 10°C in the same buffer with pulse times of 20 s for 12 h and 5 to 15 s for 17 h.

RESULTS

Globally, 46 (14.4%) of the 320 food samples examined were found to be positive for *Aeromonas* spp. (Table 1). The biochemical identification of the isolates indicated that 27 samples (8.4%) were contaminated with *Aeromonas hydrophila,* 23 samples (7.2%) with *Aeromonas caviae,* and only 1 sample (0.3%) with *Aeromonas sobria.* On five occasions (1.6%) it was possible to isolate two different *Aeromonas* spp. from the same sample.

The highest percentages of isolation were found in vegetables, particularly lettuce (45.0%), endive (40.0%), and rucola (20.0%), with contamination levels ranging from 1 × 10^4 to 5.3 × 10^5 CFU/g. Ricotta was the only cheese type analyzed showing a high frequency of isolation (45.0%), with contamination ranges of 1 × 10^4 to 2.3 × 10^5 CFU/g. Among meat products, salami and raw ham (25.0% of samples positive) and, to a lesser extent, baloney (5.0%) were found positive for *Aeromonas* spp. with contamination levels lower than those noticed in vegetables and chesees. It is notable that, whereas *A. caviae* was the dominant species in vegetables, *A. hydrophila* was the most common isolate from cheeses and meat products. No motile aeromonads were found in ice cream samples (Table 1).

All *Aeromonas* isolates were typed with macrorestriction analysis of genomic DNA with *Xba*I and PFGE (Fig. 1). Assuming that a single base mutation in the genomic DNA could introduce maximally a three-fragment difference in their restriction pattern, strains showing more than three-fragment variations were assumed to represent major patterns (assignment of capital letters), while one- to three-fragment differences were considered subtypes (capital letters with numerical subcodes) *(36).* By these criteria, the 27 *A. hydrophila* strains belonged to 24 major PFGE patterns (A to X) (Fig. 1A). The only major PFGE patterns found in more than one isolate were pattern A, with two subtypes (A_1 and A_2) found in two samples of endive and lettuce, pattern F, exhibited by two strains of ricotta cheese and rucola, and pattern I, found in two samples of raw ham. Analogously, the 23 *A. caviae* isolates belonged to 20 PFGE patterns (A to T) (Fig. 1B). All these patterns were shown by single isolates, with the exception of pattern A, found in two different samples of endive, and pattern C, exhibited by three strains isolated from three separate samples of lettuce.

DISCUSSION

There are relatively few published cases in which *Aeromonas* species have been associated with foodborne gastroenteritis. Suspect foods presumably were either inadequately cooked before consumption or consumed after no or minimal cooking *(4, 18, 19).* In only one case were the isolates from food and feces typed (rRNA restriction pattern) in a manner to establish definitively the source of infection *(3).* The contamination by *Aeromonas* spp. of ready-to-eat foods is of particular concern, because these products receive no further cooking and therefore may represent a major source of infection for human beings.

The results of this study indicate that *Aeromonas* species are common in ready-to-eat foods consumed in Italy, confirming the findings of other surveys conducted in other countries, such as the United States *(5),* the United Kingdom *(8),* Japan *(28),* Denmark *(21),* New Zealand *(14, 15),* Switzerland *(9),* and Greece *(25).* In this study *A. hydrophila* and *A. caviae* were the predominant *Aeromonas* species isolated from foods of animal origin (meat and cheese) and vegetables, respectively. Other surveys reported similar species distribution *(8, 9, 14, 15, 25, 28),* although Callister and Agger found that *A. hydrophila* was the most common isolate in vegetables in the United States *(5).*

Digestion of DNA with rare cutting endonucleases and separation of the fragments by PFGE has been widely used in recent years for the typing of various bacterial species *(24, 36).* In this study on *Aeromonas* isolates from ready-to-eat foods, the typeability and the reproducibility of PFGE was optimal, because reproducible PFGE patterns were obtained for all strains tested. The discriminatory power of PFGE typing, defined as the ability to distinguish between unrelated isolates, was high. Finally, the stability of PFGE patterns was confirmed by typing two strains on first isolation and after several passages (up to 20) in culture (data not shown). However, in vitro conditions may be different from those encountered in the environment, where a variety of selective and nutritionally stressful situations may induce mutations to a greater extent. Therefore, the usefulness of PFGE as a tool in the epidemiological analysis on *Aeromonas* isolates—that does appear promising on the basis of the results of this study—should be definitively validated by further research showing the epidemiologically related isolates have indistinguishable or highly related PFGE patterns.

Another significant finding of this study is the high level of genetic heterogeneity of mesophilic *Aeromonas,* because 24 PFGE patterns were identified among 27 *A. hydrophila* strains and 20 PFGE patterns were found in 23 *A. caviae* isolates. These results are very similar to those obtained by Talon et al *(35),* who used PFGE to characterize a cluster of clinical and environmental *A. hydrophila* strains isolated in a French hospital and by Hänninen and Hirvelä-Kosky *(12)* in their analysis of 39 strains representative of the different hybridization groups found in mesophilic *Aeromonas.* These data are in sharp contrast with the global

TABLE 1. *Occurrence of* Aeromonas *spp. in ready-to-eat foods commonly consumed in Italy*

Food type	No. of samples	No. (%) of samples positive for *Aeromonas* spp.	Contamination ranges of samples positive (CFU/g)	No. of samples positive for:		
				A. hydrophila	*A. caviae*	*A. sobria*
Vegetables						
Lettuce	20	9 (45.0)	1×10^4–4.5×10^5	3	7	0
Endive	20	8 (40.0)	3.9×10^4–5.3×10^5	3	5	0
Rucola	20	6 (30.0)	1.3×10^5–2.3×10^5	4	2	0
Fennel	20	0	—	0	0	0
Chiroy	20	2 (10.0)	1.3×10^5–1.4×10^5	1	1	0
Total	100	25 (25.0)	1×10^4–5.3×10^5	11	15	0
Cheeses						
Ricotta	20	9 (45.0)	1×10^4–2.3×10^5	6	4	1
Mascarpone	20	0	—	0	0	0
Mozzarella	20	1 (5.0)	1×10^3	0	1	0
Fiordilatte	20	0	—	0	0	0
Treccia	20	0	—	0	0	0
Total	100	10 (10.0)	1×10^3–2.3×10^5	6	5	1
Meat products						
Salami	20	5 (25.0)	1.5×10^4–3.4×10^4	5	1	0
Baloney	20	1 (5.0)	9×10^3	1	1	0
Raw ham	20	5 (25.0)	2.8×10^4–3.2×10^4	4	1	0
Tinned meat	20	0	—	0	0	0
Wurstel	20	0	—	0	0	0
Total	100	11 (11.0)	9×10^3–3.4×10^4	10	3	0
Ice cream	20	0	—	0	0	0

genetic homogeneity of some psychrophilic *Aeromonas,* such as *Aeromonas salmonicida* ssp. *salmonicida,* shown through various genetic methods, including PFGE *(12, 26, 37).*

In conclusion, substantial clinical and epidemiological research now supports the epidemiological evidence that at least some strains of *Aeromonas* spp. can cause gastroenteritis in some individuals. The results of this survey show that consumers of ready-to-eat foods in Italy are regularly exposed to many genetically distinct strains of *A. hydrophila* and *A. caviae* without evident signs of malaise. Therefore, few of these strains, if any, are likely to be pathogenic. PFGE macrorestriction analysis of genomic DNA appears to be a promising tool in the epidemiological analysis of *Aeromonas* isolates. Because, at present, *Aeromonas* virulence mechanisms are not well understood, PFGE may be useful in linking human infections to contaminated foods, allowing the identification of strains that pose a threat to human health from the diversity of isolates present in foods.

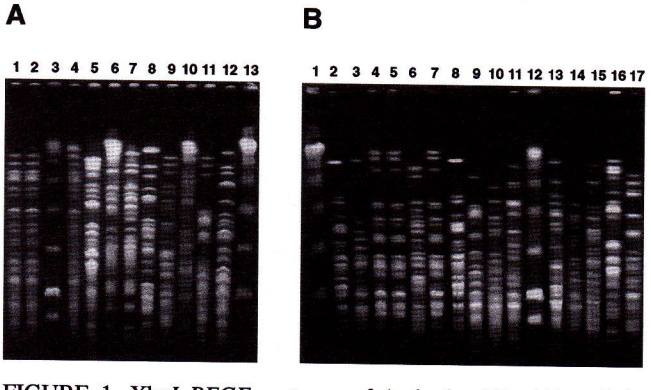

FIGURE 1. XbaI *PFGE patterns of* A. hydrophila *(A) and* A. caviae *(B) isolates from different ready-to-eat foods in Italy. (A) Lanes 1, 2, and 4 show subtypes of pattern A. Lanes 1 and 2 contain two strains with pattern* A_1 *isolated from the same sample of endive, and lane 4 contains one strain with pattern* A_2 *isolated from a different sample of lettuce. (B) Lanes 2 and 8 contain strains with pattern A and lanes 5, 6, and 7 show pattern C. Lanes 3 and 13 in A and lanes 1 and 12 in B contain molecular size markers (lambda ladder).*

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