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Incidence and identification of mesophilic *Aeromonas* spp. from retail foods

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K. NEYTS, G. HUYS, M. UYTTENDAELE, J. SWINGS AND J. DEBEVERE. 2000. Sixty-eight food samples were examined for the presence of mesophilic *Aeromonas* species both qualitatively and quantitatively. Aeromonads were isolated from 26% of the vegetable samples, 70% of the meat and poultry samples and 72% of the fish and shrimps. Numbers of motile aeromonads present in the food samples varied from $< 10^2$ cfu g^{-1} to $> 10^5$ cfu g^{-1}. GLC analysis of FAMEs was used to identify a selection of presumptive *Aeromonas* colonies to fenospecies or genomic species level. *Aeromonas* strains belonging to the *Aer. caviae* complex, which also includes the potentially pathogenic genospecies HG4, were mostly isolated from vegetables but were also found in meat, poultry and fish. In addition, three strains of the virulent taxon *Aer. veronii* biovar sobria HG8 were isolated from poultry and minced meat. All members of the *Aer. hydrophila* complex, predominant in the fish, meat and poultry samples, were classified in the non-virulent taxon HG3. Although the significance of *Aeromonas* in foods remains undefined, the isolation of *Aeromonas* HG4 and HG8 strains from a variety of retail foods may indicate that these products can act as possible vehicles for the dessimination of food-borne *Aeromonas* gastroenteritis.

INTRODUCTION

Aeromonas species are widely distributed in the aquatic environment, including raw and processed drinking water (Holmes *et al.* 1996), and have been frequently isolated from various food products such as fish and shellfish, raw meat, vegetables and raw milk (Palumbo 1996). Motile aeromonads are considered as emerging food-borne pathogens because it was shown that some *Aeromonas* food isolates can produce different virulence factors, not only at optimal growth temperature, but also at refrigeration temperatures (Merino *et al.* 1995). This may be of importance for refrigerated food products that usually have an extended shelf-life at this temperature. Although the exact role of these virulence factors in the pathogenesis of motile *Aeromonas* species is still not fully elucidated, representatives of these taxa have been incriminated in cases of human gastroenteritis, particularly in children younger than 2 years, the elderly and immunocompromised patients (Joseph 1996). Further, *Aeromonas* species are frequently associated with travellers' diarrhoea (Hänninen *et al.* 1995; Yamada *et al.* 1997). Consequently, aeromonads should be carefully monitored in foodstuffs as a possible source of food-borne infections.

Phenotypic characteristics of *Aeromonas* spp. have been used to differentiate between environmental strains and those strains causing gastroenteritis. Historically, motile aeromonads were phenotypically classified into the species *Aer. hydrophila*, *Aer. sobria* and *Aer. caviae*, according to the criteria of Popoff (1984). With the introduction of DNA–DNA hybridization techniques (Popoff *et al.* 1981), the taxonomy of the genus *Aeromonas* has become much more refined and consequently, the use of biochemical characteristics alone for the reliable identification of unknown *Aeromonas* isolates is no longer adequate. Currently, the genus *Aeromonas* comprises at least 14 genomic species or DNA hybridization groups (HG), of which some have not yet been named (Huys *et al.* 1996). Interestingly, most pathogenic *Aeromonas* strains group predominantly in three of these genomic species, *Aer. hydrophila* HG1, *Aer. caviae* HG 4 and *Aer. veronii* biovar sobria HG 8 (Altwegg *et al.* 1990). As most surveys used mainly phenotypic techniques for the identification of *Aeromonas* food isolates, few reliable data are available on the prevalence of these *Aeromonas* taxa in foods.

The objectives of this study were: (i) to determine the prevalence of *Aeromonas* spp. in a wide variety of retail foods, both qualitatively (absence/presence per 25 g) and

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quantitatively (cfu g^{-1}) and (ii) to assess the taxonomic diversity of the isolated strains to genomic species (HG) level using gas–liquid chromatographic analysis of cellular fatty acids.

MATERIALS AND METHODS

Sampling of foods

A total of 68 food samples, 27 vegetable samples, 23 poultry, red meat and meat product samples, and 18 samples of fish and shrimps, were collected from seven randomly selected local retail shops and supermarkets in Flanders, Belgium. Foods were purchased in regular consumer packages and immediately transferred to the laboratory for analysis.

Quantitative analysis (enumeration of *Aeromonas* spp.)

Food samples (30 g) were transferred aseptically to sterile stomacher bags, diluted 10-fold in peptone saline solution [8·5 g NaCl l^{-1} (Vel), 1 g peptone l^{-1} (Oxoid), pH 7·0], homogenized, and a 10-fold serial dilution prepared. Two selective culture media were used for enumeration, namely ADA pH 8·0 (Ampicillin–Dextrin Agar) and modified BIBG pH 8·7 (modified Bile Salts–Irgasan–Brilliant Green Agar) (Neyts *et al.* 2000).

Both media were incubated for 24 h at 30 °C and presumptive *Aeromonas* colonies enumerated. *Aeromonas* strains typically produce yellow colonies on ADA medium and purple colonies on mBIBG medium. Three presumptive *Aeromonas* colonies were subcultured on Tryptic Soy Agar (Oxoid) for 24 h, 30 °C, for purification and subsequently identified to genus level by oxidase and catalase testing, Gram coloration and the BBL Crystal ID kit (Becton Dickinson Cockeysville, USA).

Qualitative analysis (presence/absence of *Aeromonas* spp.)

Food samples (25 g) were transferred aseptically to sterile stomacher bags, diluted 10-fold in Alkaline Peptone Water pH 8·7 (Oxoid), homogenized, and incubated for 24 h at 30 °C prior to inoculation onto ADA and mBIBG medium. Presumptive *Aeromonas* colonies were identified as described above. Food samples were defined as being positive for *Aeromonas* if confirmed *Aeromonas* colonies were isolated on either ADA or mBIBG media.

FAME identification of *Aeromonas* isolates

Gas–liquid chromatographic (GLC) analysis of cellular fatty acid methyl esters (FAMEs) was used to further determine the taxonomic diversity among 130 *Aeromonas* isolates from fish (73), vegetables (41), and meat and poultry(16). FAME profiles were determined as described by Huys *et al.* (1995). Essentially, overnight cultures were inoculated on Tryptic Soy Agar (15 g l^{-1}; Difco) according to the quadrant streak method (MIDI, 1999). Cells were harvested from the third quadrant after 24 h. Following saponification of the cell lipids, methylation of the fatty acids and extraction of FAMEs, the washed extracts were analysed by a GLC equipped with a flame ionization detector. The resulting peak patterns were automatically identified and compared with the predetermined library profiles of a representative database, AER48C, which contains the mean FAME profiles of all currently recognized DNA hybridization groups (HGs) or genomic species within the genus *Aeromonas*.

RESULTS

Prevalence of *Aeromonas* sp. in vegetables, poultry and meat, fish and shrimps

Mesophilic aeromonads were identified in 26% of the vegetable samples examined (Table 1). Only two samples (fennel and garden sorrel) were positive by direct plating (detection limit 10^2 cfu g^{-1}), indicating aeromonad counts of between $2{\cdot}9 \times 10^2$ cfu g^{-1} and $4{\cdot}9 \times 10^3$ cfu g^{-1}. A relatively high percentage of positive samples (70%) was found for meat and poultry ($n = 23$) (Table 1). Detection of mesophilic aeromonads by direct plating was only positive for five of these samples (bacon, chicken breast meat, hamburger, minced meat 2×). However, in one sample, aeromonad counts exceeded 10^5 cfu g^{-1}. Thirteen (72%) samples of fish and shrimps ($n = 18$) were contaminated with *Aeromonas* and in 10 of these samples, the aeromonad populations ranged from $1{\cdot}9 \times 10^2$ to $2{\cdot}4 \times 10^5$ cfu g^{-1} (Table 1).

In the qualitative analysis of foods, no significantly different results were obtained using the ADA or mBIBG medium (Table 1). Significantly more samples showed a high level of contamination using the ADA medium than the mBIBG medium for direct plating of food homogenates (Table 1). If typical *Aeromonas* was present on both media, enumeration was about 0·5–1·0 log unit higher on the ADA medium than on the mBIBG medium. However, the background flora was significantly reduced on the mBIBG medium (0·5–2·0 log units for the vegetable samples, results not shown). This indicates that mBIBG is a more selective medium. All presumed *Aeromonas* colonies from mBIBG medium were confirmed as *Aeromonas* sp., whereas

Table 1 Prevalence of *Aeromonas* spp. in different foods

Source	No. samples with *Aeromonas* spp.				
	Total	per 25 g		$>100\ g^{-1}$	
		ADA	mBIBG	ADA	mBIBG
Vegetables	7/27 (26)*[a]	6/27	6/27	2/27	1/27
Poultry and meat	16/23 (70)*[b]				
Poultry	5/6	4/5	4/5	1/5	0/5
Red meat	9/14	8/13	8/14	1/13	2/14
Meat products	2/3	2/3	1/3	1/3	0/3
Fish and shrimps	13/18 (72)*[b]				
Fish	11/14	9/12	11/14	9/12	5/14
Shrimps	2/4	0/2	2/4	0/2	0/4
Total	36/68 (53)*	29/62 (47)*[k]	32/67 (48)*[k]	14/62 (23)*[x]	8/68 (12)*[y]

*Figures in parentheses indicate percent values.
[a,b–k–x,y]Different characters indicate significantly different results ($P < 0{\cdot}05$ Binary Logistic Regression, SPSS 9·0 for Windows).

six typical colonies picked from ADA were identified as *Pseudomonas* sp. This indicates that mBIBG better differentiates *Aeromonas*.

Cross-inoculation of strains picked from either media on the other medium revealed that both media are complementary; two of 64 *Aeromonas* isolates taken from mBIBG did not grow on ADA, and another three of the 64 isolates did not produce typical *Aeromonas* colonies on ADA. On the other hand, 13 of 99 isolates taken from ADA did not grow on mBIBG, and one of the 99 isolates did not produce typical colonies on mBIBG (results not shown).

Identification of *Aeromonas* isolates from vegetables, meat and poultry, and fish

From the present survey, 216 mesophilic *Aeromonas* isolates were obtained. FAME was used to further determine the taxonomic diversity among 130 of these isolates (73 isolates from fish, 41 from vegetables and 16 from meat and poultry). The predominant hybridization group (HG) of motile aeromonads isolated from fish samples was *Aer. hydrophila* HG3 (59%), followed by representatives of the *Aer. caviae* complex (HG4 and HG5A) (12%). A similar result was obtained for the meat and poultry samples, i.e., 37% of the isolates were identified as *Aer. hydrophila* HG3 and 12% as belonging to the *Aer. caviae* complex (HG4 and HG5A). Interestingly, three strains (19%) isolated from meat and poultry could be assigned to HG8 (*Aer. veronii* biovar sobria). In contrast, isolates obtained from vegetables were dominated by strains belonging to the *Aer. caviae* complex (71%) (HG4, HG5A and HG5B), followed by *Aer. hydrophila* HG3 (7%) and *Aer. bestiarum* HG2 (5%).

DISCUSSION

In recent years, the number of reports on the prevalence of *Aeromonas* in food products from various geographical regions has increased significantly. For instance, Ibrahim and MacRae (1991) reported that *Aeromonas* was present in 60, 58, 74 and 26% of investigated beef, lamb, pork and milk samples, respectively, whereas Krovacek *et al.* (1992) found aeromonads in 42% of the food samples originating from a random selection of retail outlets in Sweden. *Aeromonas* were also found in fish and fresh salads (Walker and Brooks 1993), freshly dressed lamb carcasses (Sierra *et al.* 1995), oysters (Tsai and Chen 1996), cheese and raw cow's milk (Melas *et al.* 1999). In the present study, mesophilic aeromonads were isolated from 26% of the vegetable samples, 70% of the meat and poultry samples and from 72% of the fish and shrimps. Because of the obvious differences in sampling period, geographical location, the origin of the samples and methodology for analysis, it is difficult to compare the level of *Aeromonas* incidence published by different authors. However, the present data clearly confirm the widespread distribution of motile aeromonads in retail foods, and also reveal a large variation in the level of contamination. Numbers of motile aeromonads present in the food samples varied from $<10^2$ cfu g^{-1} to $>10^5$ cfu g^{-1}. The latter might be a reflection of product-specific properties that can significantly influence the survival rate and growth characteristics of *Aeromonas*, i.e., initial contamination levels, type of processing, method of packaging and preservation (Palumbo 1996).

The biochemical classification of *Aeromonas* isolates into one of the three historically-defined fenospecies, i.e., *Aer. hydrophila*, *Aer. caviae* and *Aer. sobria*, is applied in the

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majority of surveys of motile aeromonads in foods (Hudson *et al.* 1992; Krovacek *et al.* 1992; Granum *et al.* 1998). On the other hand, very few studies have reported identification at the genomic species level. Because of the significant lack of data for the different taxa, rapid miniaturized or automated systems using a panel of biochemical tests are inappropriate for identification of motile aeromonads to genomic species level. As the majority of the virulent *Aeromonas* strains belong to *Aer. hydrophila* HG1, *Aer. caviae* HG4, or *Aer. veronii* biovar sobria HG8 (Altwegg *et al.* 1990), a reliable identification of food isolates is necessary to establish the risk associated with their prevalence in foods. In the current study, GLC analysis of FAMEs was used to identify a selection of presumptive *Aeromonas* colonies to fenospecies or genomic species level. *Aeromonas* strains belonging to the *Aer. caviae* complex, which also includes the potentially pathogenic genospecies HG4, were mostly isolated from vegetables but were also found in meat, poultry and fish. In addition, three strains of HG8 were isolated from poultry and minced meat. No representatives of HG1 were found among the selected colonies as all members of the *Aer. hydrophila* complex were classified in the non-virulent taxon HG3. The isolation of *Aeromonas* HG4 and HG8 strains from a variety of retail foods may indicate that these products can act as possible vehicles for the dessimination of food-borne *Aeromonas* gastroenteritis. However, it is clear that additional data on the production of virulence factors, such as haemolysins and cytolytic enterotoxins, are required to support this hypothesis. For example, Callister and Agger (1987) concluded that all *Aer. hydrophila* isolates from retail grocery store products were highly cytotoxic at 35 °C in comparison with the non-cytotoxic *Aer. caviae* strains. Interestingly, Granum *et al.* (1998) reported that the majority of the *Aer. caviae* strains isolated by Norwegian food control laboratories could produce cytotoxins at 30 °C but not at 37 °C. Although these results indicate that strains identified as *Aer. hydrophila* should be monitored in the epidemiology of *Aeromonas*-associated human gastroenteritis, it is unfortunate that the identification methods used in both studies did not allow discrimination among HG1, HG2 or HG3 of the *Aer. hydrophila* complex.

Despite the fact that potentially pathogenic aeromonads are present in food and food products, infection and the onset of gastroenteritis will only occur if the level of contamination exceeds the infective dose. However, there are currently insufficient clinical data to determine the infective dose. Moreover, it has been shown that the level of contamination at the moment of consumption depends upon the initial contamination and the opportunities for growth and/or survival during processing, preservation and preparation of the food (Palumbo 1996). Further research is also needed to establish contamination routes. So far, the significance of *Aeromonas* in foods remains undefined, although there is a growing concern about the consumption of *Aeromonas*-contaminated food by young children, the elderly and the immunocomprimised.

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