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Characterization of protein profiles of *Aeromonas hydrophila* and *Aeromonas caviae* isolates from fish

Charakterisierung der Proteinprofile von Aeromonas hydrophila und Aeromonas caviae Isolaten aus Fischen

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Summary

In this study, sixteen *Aeromonas* (*A.*) *hydrophila* and *A. caviae* isolates obtained from fish in Ankara, Turkey were described and compared with two American type strains (*A. hydrophila* ATCC 7966 and *A. caviae* ATCC 15468) and characterised by various methods including biochemical, physiological tests and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Whole cell protein (WCP) profiles of these isolates were analyzed using SDS-PAGE. We concluded that the study showed that there was a similar clonal group origin of the *A. hydrophila* isolates and the reference strain (ATCC 7966). Likewise, there was also a similarity between the *A. caviae* isolates and the reference strain (ATCC 15468). The results show that SDS-PAGE may be an efficient method to differentiate *Aeromonas* isolates.

Keywords: *Aeromonas hydrophila*, *Aeromonas caviae*, SDS-PAGE, fish

Zusammenfassung

In dieser Studie werden sechzehn *Aeromonas* (*A.*) *hydrophila* und *A. caviae* Isolate beschrieben. Die Isolate stammten von Fischen aus Ankara (Türkei). Sie wurden mit zwei Referenzstämmen (*A. hydrophila* ATCC 7966 und *A. caviae* ATCC 15468) verglichen und mittels verschiedener biochemischer und physiologischer Methoden sowie Natrium-Dodecylsulfat-Polyacrylamid-Gel-Elektrophorese (SDS-PAGE) charakterisiert. Die Studie ließ die Schlussfolgerung zu, dass ein gemeinsamer klonaler Ursprung der *A. hydrophila* Isolate und des Referenzstamms (ATCC 7966) anzunehmen ist. Ebenso lag eine Ähnlichkeit zwischen den *A. caviae* Isolaten und dem Referenzstamm (ATCC 15468) vor. Die Ergebnisse zeigten zudem, dass SDS-PAGE eine brauchbare Methode zur Differenzierung von *Aeromonas* Isolaten darstellt.

Schlüsselwörter: *Aeromonas hydrophila, Aeromonas caviae,* SDS-PAGE, Fische

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Introduction

Aeromonas (*A.*) species are common inhabitants of aquatic ecosystems, although they have also been described in connection with fish and human diseases (Janda and Abbott, 1995; Austin and Adams, 1996). Two phenotypically distinct groups are well known within the genus *Aeromonas*: (i) psychrophilic and non-motile *A. salmonicida*, which are relatively homologous phenotypically and (ii) mesophilic and motile aeromonads, which are heterologous. Within the latter group three phenospecies have been recognized: *A. hydrophila*, *A. caviae* and *A. sobria* (Popoff, 1984). The most common, *A. hydrophila*, is an important pathogen causing disease in humans and animals, including fish. In recent years, the classification of the genus *Aeromonas* has undergone major changes. Extended DNA-DNA hybridization studies or other molecular techniques and improved phenotypic methods have resulted in the recognition of 14 well-defined genomic species within the genus (Janda, 1991; Joseph and Carnahan, 1994; Ali et al., 1996). These species are as follows: *A. hydrophila*, *A. bestiarum*, *A. salmonicida* (comprising non-motile and psychrophilic as well as motile and mesophilic strains), *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii*, (with two biotypes: *sobria & veronii*), *A. jandaei*, *A. trota*, *A. schubertii*, *A. encheletia*, *A. allosaccharophila* and *A. popoffii*. A wide range of *Aeromonas* species have been found in association with diseased or healthy fish (Huys et al., 1996), but only some of them have been documented as fish pathogens (Torres et al., 1993; Esteve et al., 1995). Since the changes in *Aeromonas* classification, the importance of recently described species in fish pathology is mostly unknown. The taxonomy of the genus *Aeromonas* is still confusing and controversial (Carnahan and Altwegg, 1995). SDS-PAGE of whole cell protein (WCPs protein) have proved to be useful for typing several bacterial species, including aeromonads (Stephenson et al., 1987; Millership and Want, 1989). 16S rDNA restriction fragment length polymorphism (RFLP) methods have been developed for the identification of all known *Aeromonas* species. More sensible approaches might be needed to identify strains belonging to the species *A. hydrophila*, *A. sobria* or *A. caviae*, for example the use of RFLP for further characterisation (Popoff, 1984; Borrel et al., 1997; Figueras et al., 2000).

In this work, the aim was to study the whole-cell proteins of *A. caviae* and *A. hydrophila* strains isolated from fish by SDS-PAGE and to determine the relationship between the protein profiles and the strains.

Material and Methods

Isolation and identification of *A. hydrophila* and *A. caviae* strains

Fish samples were obtained from the local market in Ankara. The following fish species were studied: 32 samples of freshwater fish (*Cyprinus carpio L.*) and 24 samples of seawater fish (*Engraulis encrasicholus*, *Sardina pilchardus*). Samples were immediately transferred to the laboratory in ice chests at +4 to +7 °C. They were sampled on the same day. Gills, intestinal and liver content of the fish samples (5 g) were aseptically swabbed using sterile cotton buds and inoculated into 45 ml of alkaline peptone water containing 30 µg/ml ampicillin (A-9393 Sigma Chemical Co., St. Louis, Mo., USA) (APW, pH 8.4) and homogenized for 2 min in sterile stomacher bags. After 18 h of incubation at 30 °C, 0.1 ml APW was streaked on glutamate starch phenol red agar (GSP agar, Merck, Darmstadt, Germany). After incubation at 30 °C for 24 h, yellow colonies surrounded by a yellow zone were picked and grown on fresh GSP agar for confirmation. The presumptive *Aeromonas* colonies were identified at genus and species level by testing their gram reaction, oxidase, catalase, motility, growth conditions in 0 % and 6 % NaCl, oxidation/fermentation (glucose), and resistance to vibriostatic agent O/129 (2,4-diamino 6,7-diisopropylpteridine; 150 µg/ml). All *Aeromonas* spp. were reidentified biochemically to species level using tests chosen from those described by Popoff (1984) and Carnahan et al. (1995), that is, esculin hydrolysis, production of H_2S from cysteine, production of gas from glucose, citrate utilization, salicin and arabinose fermentation, Voges-Proskauer reaction and lysine decarboxylase (Janda et al., 1995). The reference strains used in this work were *A. hydrophila* (ATCC 7966) and *A. caviae* (ATCC 15468). The isolates were stored in trypticase soy broth with 15 % (v/v) sterile glycerol at –70 °C until assayed.

SDS-PAGE

Electrophoresis was performed with a discontinuous buffer system in a Consort electrophoresis unit (Consort nv, Turnhout, Belgium). For SDS-PAGE analysis, *A. hydrophila* and *A. caviae* isolates were cultured on Nutrient Broth and incubated overnight at 30 °C for 24 h, and then washed in phosphate buffered saline (PBS; pH 7.2). The cells were sedimented at 3000 g for 15 min, resuspended in 15 % glycerol, 1 % sodium dodecyl sulfate (SDS) and 0.1 M Tris/HCl (pH 6.8), and denatured by treatment at 100 °C for 20 min. Nonsolubilized material was diluted

TABLE 1: *Prevalence of Aeromonas spp. in different body parts of fish samples examined*

Type of fish	Positive samples (%)	*A. hydrophila* (%)	*A. caviae* (%)	Unidentified *Aeromonas* spp. (%)
Freshwater fish				
Gill	7/10 (70.0)	2 (28.5)	1 (14.2)	4 (57.1)
Intestine, liver	13/16 (81.2)	4 (30.7)	1 (7.6)	8 (61.5)
Total	20/26 (76.9)	6 (30.0)	2 (10.0)	12 (60.0)
Seawater fish				
Gill	10/12 (83.3)	3 (30.0)	nd	7 (70.0)
Intestine, liver	15/18 (83.3)	4 (26.6)	1 (6.6)	10 (66.6)
Total	25/30 (83.3)	7 (28.0)	1 (4.0)	17 (68.0)
Total Freshwater + Seawater	45/56 (80.3)	13 (28.8)	3 (6.6)	29 (64.4)

nd: none detected.

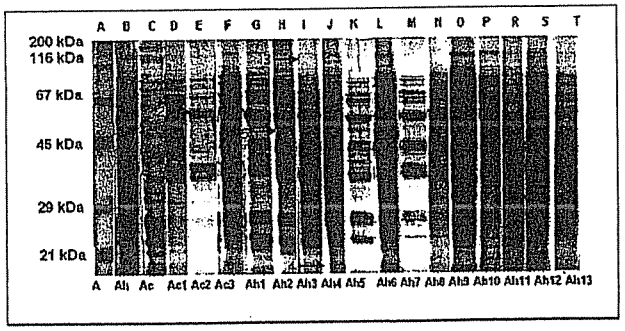

FIGURE 1: *SDS-PAGE of whole-cell proteins of A. hydrophila and A. caviae.*
Lane A: Molecular weight standard in kDa; Lane B: A. hydrophila ATCC 7966 (Ah); Lane C: A. caviae ATCC 15468 (Ac); Lanes D–F: A. caviae fish isolates (Ac1-Ac3); Lanes G–T: A. hydrophila fish isolates (Ah1-Ah13).

1:1 with 20 % glycerol, 10 % 2-mercaptoethanol, 4 % SDS and 0.125 M Tris/HCl (pH 6.8) (Whittington et al., 1987). After incubation for a further 2 min at 100 °C, the samples were stored at –20 °C for electrophoresis. Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), with a stacking gel containing 4.5 % acrylamide and a resolving gel containing 12 % acrylamide. Then, 10 µl of the samples was loaded onto the gel. SDS-PAGE was performed at a constant voltage of 80 V for the stacking gel and 150 V for the resolving gel. The gels were fixed for 15 min in an aqueous solution containing 7 % glacial acetic acid and 30 % methanol and stained overnight in 0.1 % (w/v) Coomassie Blue R-250 solution. The gel was kept in destaining solution I (50 % methanol, 10 % acetic acid) for 1 h and then transferred to destaining solution II (7 % acetic acid, 5 % methanol). We used SDS-PAGE protein marker 6.5–200 kDa liquid mix (Serva, Heidelberg, Germany).

Results and Discussion

Table 1 presents the types, numbers and sources of the fish samples analyzed in this study.

Overall, 80.3 % (45/56) of the examined fish samples were positive for *Aeromonas* spp., 64.4 % (29/45) were positive for unidentified *Aeromonas* spp., 28.8 % (13/45) for *A. hydrophila* and 6.6 % (3/45) for *A. caviae*. Of these 45 samples, 13 *A. hydrophila* and 3 *A. caviae* isolates were selected for further analysis.

Figure 1 shows the whole-cell protein profiles of the *A. hydrophila* (Ah1-Ah13) and *A. caviae* isolates (Ac1-Ac3) obtained by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Most of the *A. hydrophila* and *A. caviae* isolates possessed the protein band marked by 1 (Fig. 1, lane C) at the top of each lane. All 3 *A. caviae* isolates and the *A. caviae* ATCC 15468 standard reference strain (Fig 1, line C) had the band marked by 2 (Fig. 1, lanes C, D, E and F). However, one (Ah2) isolate could be distinguished from the other isolates due to the presence of a band marked by 3 (Fig. 1, lane H). *A. hydrophila* ATCC 7966 (Fig. 1, lane B) and three isolates (Fig 1, lanes G, H and I) had crucial differences in protein bands, although they belong to the same species. The same protein band marked by 4 (Fig 1, lanes G-T) was detected in all of the *A. hydrophila* isolates (Ah1–Ah13). There were similarities between *A. hydrophila* isolates (Ah1, Ah2 and Ah3) and *A. caviae* isolates (Ac1,Ac2 and Ac3) by the presence of protein bands marked by 5 (Fig. 1, lanes G, H and I). According to Delamare et al. (2002), bands were considered identical only when their width, intensity and position were the same.

The taxonomy of the genus *Aeromonas* has been extended in recent years because of the description of new mesophilic species. These species can be accurately identified by molecular techniques but also biochemical systems for the discrimination of genomospecies have recently been developed (Borrel et al., 1998). Protein gel electrophoresis in microbial characterization has been established for 20 years (Vouterin et al., 1993). Several studies have been carried out on aeromonads by SDS-PAGE, and whole-cell protein profiles have been found useful for epidemiological studies (Stephanson et al., 1987; Millership and Want, 1989). In this study, we could show that all three of the *A. caviae* isolates were grouped by a protein band marked by 2 (Fig. 1, lanes D, E and F), but these bands did not exist in *A. hydrophila* isolates. A double band marked by 3 was common in all *A. hydrophila* isolates and may be used to distinguish *A. caviae* from *A. hydrophila* strains.

In our results, numerical analysis of one dimensional SDS-PAGE of the protein patterns of whole-cell of *A. hydrophila* and *A. caviae* isolates provided a useful approach towards clarifying the relationship within the *Aeromonas* species as both of the species had characteristically distinctive protein band patterns. These results agree with those of Delamare et al. (2002). We suggest that visual comparison of the principal bands provides a rapid means of identifying isolates from various sources, by combination of cellular protein patterns. We conclude that these isolates might have originated from the same contamination source, and that SDS-PAGE of whole-cell proteins could be a useful method for the characterization of *A. hydrophila* and *A. caviae* isolates as well as for epidemiological studies.

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