

MULTIPLEX PCR DETECTION OF HAEMOLYSIN GENES IN β -HAEMOLYTIC *AEROMONAS HYDROPHILA* STRAINS ISOLATED FROM FISH AND FISH PRODUCTS

D. STRATEV^{1*}, E. GUROVA², I. VASHIN¹ and H. DASKALOV²

¹ Department of Food Hygiene and Control, Veterinary Legislation and Management, Faculty of Veterinary Medicine, Trakia University, BG-6000 Stara Zagora, Bulgaria

² National Diagnostic and Research Veterinary Medical Institute, National Centre of Food Safety, Bulgarian Food Safety Agency, BG-1000 Sofia, Bulgaria

Abstract

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The goal of this study was to establish the presence of genes coding aerolysin (*aerA*) and haemolysin (*ahh1*) in β -haemolytic *Aeromonas hydrophila* strains isolated from fish and fish products available for human consumption throughout food stores. Aerolysin and haemolysin are viewed as important virulence factors related to the enterotoxigenicity of *A. hydrophila*. A multiplex PCR was applied to 26 β -haemolytic *A. hydrophila* strains isolated from live carp, water, cooled horse mackerel, trout, silver carp cutlets, vacuum-packed trout filets and frozen trout. The study used a reference *A. hydrophila* strain (ATCC 7965) and an *A. hydrophila* strain isolated from a dead anaconda, as positive controls with strong β -haemolytic activity. All strains (100%) possessed the chromosome gene 16S rRNA (356 bp) specific for *A. hydrophila*. In all (100%) *A. hydrophila* strains the gene *ahh1* was present (130 bp). The gene *aerA* was confirmed (309 bp) in 26 *A. hydrophila* strains (93%). The presence of β -haemolytic *A. hydrophila* strains in fish and fish products available for human consumption throughout food stores represent a risk to the consumer's health.

Key words: *A. hydrophila*, PCR, aerolysin, haemolysin, β -haemolysis

Introduction

A. hydrophila is a normal inhabitant of water environments, viewed as an opportunistic pathogen causing gastroenterites, wound infections and septicaemia in humans, as well as haemorrhagic septicaemia in fish. The prevalence of *A. hydrophila* among fish and fish products available at food stores is well known. These food products are highly prone to contamination and become a means of transmitting pathogenic bacteria (Yogananth et al., 2009; Seethalakshmi et al., 2010). Studies indicate that fish is most often and most severely contaminated with microorganisms of the *Aeromonas* genus. The

prevalence of samples of fish positive for *Aeromonas* spp. varied from 37.3% (Thayumanavan et al., 2003) to 93% (Hanninen et al., 1997). The identification of the *Aeromonas* spp. isolated from fish and fish products indicated *A. hydrophila* as predominant species was (Kumar et al., 2000; Hatha et al., 2005; Herrera et al., 2006; Salah El-Dien et al., 2009; Erdem et al., 2010; Sharma and Kumar, 2011).

A. hydrophila forms virulence factors such as haemolysin, aerolysin, proteases, lipases, DNAses, which are important in the pathogenesis of human and fish diseases (Castro-Escarpulli et al., 2003; Cagatay and Sen, 2014). *A. hydrophila* can form virulence factors not only under

*E-mail: deyan.stratev@trakia-uni.bg

optimal growth temperatures, but also under refrigerator conditions (Neyts et al., 2000). *A. hydrophila* is one of the most common causes of gastroenteritis in humans, in relation to this genus of microorganisms (Vasaikar et al., 2002; Aslani and Alikhani, 2004; Guerra et al., 2007). Moreover, isolated *A. hydrophila* strains from patients with gastroenteritis are haemolytic (Wang et al., 2003; Orozova et al., 2007; Wejdan et al., 2014). Gastroenteritis occur after intake of the pathogen via contaminated food or water (Yogananth et al., 2009).

Haemolysins belong to a large group of pore-forming bacterial cytolysins, which can cause cytoplasmic content leakage by breaking the cellular membrane, and ultimately, cell death (Heng et al., 2005). Two haemolytic toxins (haemolysin and aerolysin) have been described in *A. hydrophila* (Pandey et al., 2010). Aerolysin and haemolysin are viewed as important virulence factors of *A. hydrophila*. Aerolysin is an extracellular, soluble, hydrophilic protein exhibiting haemolytic and cytolytic properties. It binds to specific glycoprotein receptors on the surfaces of eukaryote cells and forms pores (Uma et al., 2010; Samal et al., 2014). Singh and Sanyal (1992) studied the haemolytic activity and its correlation with the enteropathogenicity of *A. hydrophila*. Their study revealed that β -haemolytic strains caused a stronger fluid accumulation in rabbit ileal loops. Wong et al. (1998) emphasised the presence of two β -haemolytic toxins in *A. hydrophila* and proved their role in the stronger virulence of strains possessing genes responsible for their production. Ottaviani et al. (2011) reported that the *aerA* gene and the production of haemolysin are evident in most toxigenic strains, regardless of their origins. *Aeromonas* strains, isolated from food and water, often contain genes coding for toxins similar to the ones in the clinical strains, and exhibited virulence properties at the temperature of the human body. For this reason, we aimed to examine the presence of genes encoding aerolysin and haemolysin only in β -haemolytic *A. hydrophila* strains isolated from fish and fish products available at food stores for human consumption.

Table 1
Primers, target genes and specificity of primers included in the PCR

| Primers | Sequence (5'-3') | Gene | Localisation into the gene | Product size (bp) |
|----------|-------------------------|----------|----------------------------|-------------------|
| AHH1F | GCCGAGCGCCCAGAAGGTGAGTT | ahh1 | 961-983 | 130 |
| AHH1R | GAGCGGCTGGATGCGGTTGT | | 1090-1071 | |
| AH-aerAF | CAAGAACAAGTTCAAGTGGCCA | aerA | 1323-1344 | 309 |
| AH-aerAR | ACGAAGGTGTGGTTCCAGT | | 1631-1613 | |
| A16SF | GGGAGTGCCTTCGGGAATCAGA | 16S rRNA | 1020-1041 | 356 |
| A16SR | TCACCGCAACATTCTGATTTG | | 1375-1355 | |

Materials and Methods

Experimental strains

The investigation was carried out in November 2014 with 26 β -haemolytic isolates with strong β -haemolytic activity identified biochemically by means of API 20NE (bio-Mérieux, France) as *A. hydrophila* isolated from live carp (n = 132), water (n = 6), cooled horse mackerel (n = 20), rainbow trout (n = 11), silver carp cutlets (n = 20), vacuum-packed trout fillets (n = 20) and frozen trout (n = 20), described in previous studies of ours (Stratev et al., 2015a; Stratev et al., 2015b). A reference *A. hydrophila* (ATCC, 7965) strain purchased from the National Bank for Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria) and one *A. hydrophila* strain isolated from a dead anaconda provided by the National Reference Lab on Fish, Marine Molluscs and Crustacean Diseases (Sofia, Bulgaria) were used as positive controls with strong β -haemolysis. All studied isolates were collected from 2010 to 2014 in different fish matrices, object of scientific experiments.

Nutrient media, chemicals and reagents

Nutrient media and chemicals were purchased from Merck (Germany) and the PCR – MyTaq HS Red Mix kit – from Bioline (UK). The primers and reaction conditions were according to the method described by Wang et al. (2003). Primers were obtained by Metabion International (Germany) (Table 1).

DNA extraction from experimental strains

Bacteria DNA preparation kit - Spin column based genomic DNA purification (Jena Bioscience, Germany) was utilised for DNA extraction from experimental strains according to manufacturer's instructions.

PCR conditions

The multiplex polymerase chain reaction included 3 pairs of primers. The PCR reaction mixture (25 μ l volume) contained: 2X MyTaq HS Red Mix (Bioline, UK) – 12.5 μ l, and

1.0 µl of each *ahh1* and *aerA* primers, 0.2 µl of 16S rRNA primers and 1 µl extracted DNA.

PCR was conducted in a Techne TC 412 (Techne) thermocycler under the following conditions: initial denaturation at 95°C za 5 min., 50 cycles at 95°C for 30 sec., 59°C za 30 sec., 72°C za 30 sec., followed by final elongation at 72°C for 7 min.

Amplified DNA fragments were visualized after horizontal electrophoresis on 2% agarose gel (TopVision Agarose, Thermo Scientific) in 1X TAE buffer (0.04 M Tris, 0.02 M Acetic acid, 0.002 M Na₂ EDTA) at 100 V for 45 min with 8 µl PCR product.

The gel was stained with ethidium bromide (1 µg/ml) for 30 min and observed under UV illumination (> 2500 µW/cm²).

Gene Ruler 100 bp DNA Ladder (Thermo Scientific) was used for molecular weight determinations.

Table 2

Incidence of β-haemolytic *A. hydrophila* strains in fish and fish products

| Strains origin | Number of samples | <i>Aeromonas</i> strains | β-haemolytic <i>A. hydrophila</i> strains |
|-----------------------------|-------------------|--------------------------|---|
| Live carp | 132 | 127 | 13 |
| Water | 6 | 6 | 1 |
| Cooled horse mackerel | 20 | 13 | 3 |
| Cooled trout | 11 | 11 | 3 |
| Cooled silver carp cutlets | 20 | 17 | 7 |
| Vacuum-packed trout fillets | 20 | 20 | 7 |
| Frozen trout | 20 | 2 | 2 |
| Total | 229 | 196 | 36 |

Table 3

Presence of haemolysin encoding genes in β-haemolytic *A. hydrophila* strains

| Strains origin | Number of strains | β-haemolytic activity | <i>ahh1</i> (%) | <i>aerA</i> (%) | 16S rRNA (%) |
|------------------------------------|-------------------|-----------------------|-----------------|-----------------|--------------|
| <i>A. hydrophila</i> (ATCC 7965) | 1 | ++++ | 1 (100) | 1 (100) | 1 (100) |
| <i>A. hydrophila</i> from anaconda | 1 | ++++ | 1 (100) | 1 (100) | 1 (100) |
| Live carp | 4 | ++++ | 4 (100) | 3 (75) | 4 (100) |
| Water | 1 | ++++ | 1 (100) | 1 (100) | 1 (100) |
| Frozen trout | 2 | ++++ | 2 (100) | 2 (100) | 2 (100) |
| Cooled trout | 3 | ++++ | 3 (100) | 3 (100) | 3 (100) |
| Cooled silver carp cutlets | 7 | ++++ | 7 (100) | 7 (100) | 7 (100) |
| Cooled horse mackerel | 3 | ++++ | 3 (100) | 3 (100) | 3 (100) |
| Vacuum-packed trout fillets | 6 | ++++ | 6 (100) | 5 (83) | 6 (100) |
| Total | 28 | | 28 (100) | 26 (93) | 28 (100) |

Results

The incidence of β-haemolytic *A. hydrophila* strains in fish and fish products is shown in Table 2. After conducting of the complex PCR, it was established that the tested 28 β-haemolytic strains possessed the chromosome gene 16S rRNA specific for *A. hydrophila*. All *A. hydrophila* strains (100%) had the *ahh1* gene. The *aerA* gene was detected in 26 (93%) of *A. hydrophila* strains. In one (25%) strain from carp blood and 1 (17%) strain of cooled vacuum-packed trout fillet, the *aerA* gene was not present (Table 3). PCR amplification products for the 16S rRNA gene (356 bp), the *ahh1* gene (130 bp) and the *aerA* gene (309 bp) are presented on Figures 1, 2 and 3.

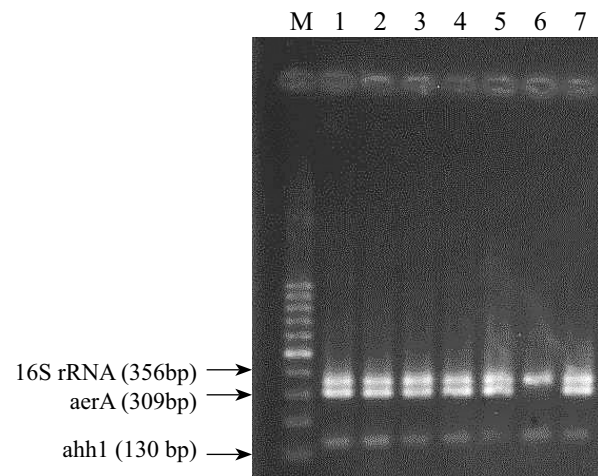


Fig. 1. Multiplex PCR of β-haemolytic *A. hydrophila* strains isolated from live carp and water
M – molecular weight marker DNA Ladder 100 bp,
1 – *A. hydrophila* (ATCC 7965) – positive control, 2 – *A. hydrophila* from anaconda – positive control, 3 – water,
4 – kidney, 5 – skin, 6 – blood, 7 – skin

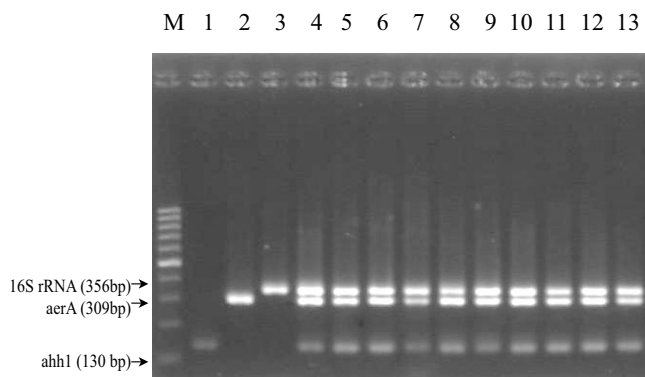


Fig. 2. Multiplex PCR of *β*-haemolytic *A. hydrophila* strains isolated from fish products

M – molecular weight marker DNA Ladder 100 bp, **1** – **ahh1** gene control, **2** – **aerA** gene control, **3** – 16S rRNA gene control, **4** – *A. hydrophila* (ATCC 7965) – positive control, **5** – frozen trout, **6** – frozen trout, **7** – cooled trout, **8** – cooled trout, **9** – cooled trout, **10** – cooled silver carp cutlet, **11** – cooled silver carp cutlet, **12** – cooled silver carp cutlet, **13** – cooled silver carp cutlet

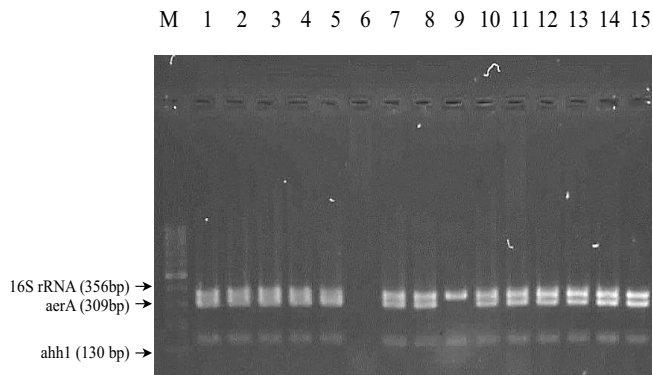


Fig. 3. Multiplex PCR of *β*-haemolytic *A. hydrophila* strains isolated from fish products

M – molecular weight marker DNA Ladder 100 bp, **1** – cooled silver carp cutlet, **2** – cooled silver carp cutlet, **3** – cooled vaccum-packed trout filet, **4** – cooled vaccum-packed trout filet, **5** – cooled vaccum-packed trout filet, **6** – negative control, **7** – cooled vaccum-packed trout filet, **8** – cooled vaccum-packed trout filet, **9** – cooled vaccum-packed trout filet, **10** – cooled horse mackerel, **11** – cooled horse mackerel, **12** – cooled horse mackerel, **13** – cooled silver carp cutlet, **14** – *A. hydrophila* from anaconda – positive control, **15** – *A. hydrophila* (ATCC 7965) – positive control

Discussion

PCR has been one of the most preferred methods for establishing pathogens used in food microbiology over the recent years, due to its speed, high sensitivity and specificity. Furthermore, when using specific virulence gene primers, pathogenic strains can be differentiated from non-pathogenic ones (Osman et al., 2012). In this study, we used specific primers to confirm *A. hydrophila* and primers for the genes of aerolysin and haemolysin, which are the primary virulence factors of *A. hydrophila* (Gonzalez-Serrano et al., 2002; Castro-Escarpulli et al., 2003). It is widely accepted that the pathogenesis of *Aeromonas* infections is multifactored and virulence factors cannot be categorised per the importance of their role in the infectious process. One virulence factor or a combination of several of them can be equally related to virulence in different *Aeromonas* species. It is for this reason that more research is needed into several virulence factors in *Aeromonas* species, for better understanding of the pathogenesis and epidemiology of *Aeromonas* infections (Yadav et al., 2014). In spite of this, throughout the last few years scientific literature has focused its attention on haemolysins as one of the primary virulence factors of *Aeromonas* spp. The haemolytic activity established by a number of researchers is viewed as proof of their pathogenic potential, with the most intensively studied haemolysin being aerolysin.

According to Singh et al. (2008) aerolysin is a haemolytic toxin, which is encoded by the *aerA* gene and plays a key role in the pathogenesis of the infection caused by *A. hydrophila* in fish. Aerolysin is a major virulence marker of *A. hydrophila* and pathogenic *A. hydrophila* strains produce aerolysin, which causes lysing of the erythrocytes and haemorrhages in the skin and internal organs of fish. Aerolysin has been found in all *A. hydrophila* strains isolated from fish with haemorrhagic septicaemia, and in 60% of the *A. hydrophila* strains isolated from healthy fish. This supports the thesis that a positive correlation exists between the presence of aerolysin and the pathogenicity of *A. hydrophila*.

According to Das et al. (2013) an *Aeromonas* spp. strain could carry genes encoding several virulence factors. The authors found the *ahh1* gene in 41% of the *A. sobria* strains and 55% of the *A. hydrophila* strains isolated from foods. The *aerA* and *ahh1* genes were found together in 68% of the *A. hydrophila* strains. Furthermore, Baloda et al. (1995) found the *aerA* gene only in the haemolytic *A. hydrophila* strains, which also exhibited cytotoxic properties. The haemolytic *A. sobria* strains and

the non-haemolytic *A. hydrophila* were not carriers of this gene. Yousef et al. (2007) found the *aerA* gene in 53% of the *A. hydrophila* strains isolated from fish. Herrera et al. (2006) found the aerolysin gene *aerA* and the haemolysin gene *hlyA* in 89% of the *A. hydrophila* strains isolated from sea fish. Similar results were obtained by Gonzalez-Rodriguez et al. (2002), who established the *aerA* gene in all, while *hlyA* - in 92% of the *A. hydrophila* strains isolated from fresh trout fillets and sliced salmon, as well as vacuum-packed cold-smoked trout and salmon fillets. The study by Castro-Escarpulli et al. (2003) indicated that all β -haemolytic strains of *A. hydrophila* isolated from frozen fish were carriers of the aerolysin gene. Contrary to these results, Pinto et al. (2012) did not establish the genes for haemolysin and aerolysin (*hlyA* and *aerA*) in *Aeromonas* strains isolated from sushi, hydrobiont salads, surimi and shrimps. All β -haemolytic *A. hydrophila* strains tested in our study carried the *ahh1* gene, while 93% of them had the *aerA* gene as well. One strain from carp blood and cooled vacuum-packed trout fillet did not carry the *aerA* gene.

Daskalov (2006) has voiced an opinion that the consumption of *A. hydrophila*-contaminated fish and fish products has a major significance for the occurrence of gastroenteritis. Most cases of illness are related to products from aquacultures or refrigerated foods ready for direct consumption. Furthermore, Wang et al. (2003) found out that the *Aeromonas* strains, which carry both haemolysin-coding *ahh1* and *aerA* genes form higher cytotoxic titres compared to other strains. Li et al. (2011) identified aerolysin as an important virulence factor of *A. hydrophila*. The pathogenicity of *A. hydrophila* is multifactorial and probably depends on the synergistic effect of various virulence genes. In this relation, Gonzalez-Serrano et al. (2002) found out that all *A. hydrophila* strains isolated from fish and patients with diarrhoea carried the *aerA* (100%) and *hlyA* (83%) genes. Wejdan et al. (2014) outlined the importance of haemolysins in the pathogenesis of *Aeromonas* infections in humans. The authors isolated six *A. hydrophila* strains from the faeces of patients with diarrhoea. All of them were β -haemolytic and carried the *ahh1* gene, while 50% of them also had the aerolysin-coding *aerA* gene.

Considering the rising number of cases with *Aeromonas* infections in immunocompetent and immunocompromised individuals, as well as the wide spread of *Aeromonas* spp., an intense and prolonged monitoring of potentially pathogenic *Aeromonas* spp. is strongly recommended for determining the risk to public health posed by the consumption of hydrobionts (Pinto et al., 2012). Ac-

ording to Das et al. (2013), the PCR identification of the virulent *A. hydrophila* strains is a very useful, sensitive and quick method, compared to the traditional microbiological identification systems. Our results confirmed that one *A. hydrophila* strain carried genes encoding several virulence factors. According to Das et al. (2013), these strains could be dangerous and are more likely to cause food poisonings in humans. The safety of fish products is affected by numerous factors, such as fish origins, products properties, processing method, and preparation before consumption. The risk from fresh fish is low after proper heat processing, yet it increases if the fish is consumed raw, insufficiently thermally processed or lightly treated. Fish contaminated with *A. hydrophila* could be hazardous, especially for sensitive populations, such as children, elderly persons and immunocompromised people (Herrera et al., 2006).

Conclusions

The present study confirmed the presence of β -haemolytic *A. hydrophila* strains in fish and fish products available at food stores for human consumption. Moreover, all these strains contain *ahh1* gene, while *aerA* gene is available in a majority of them. The presence of β -haemolytic *A. hydrophila* strains that contain haemolysin and aerolysin genes in fish and fish products poses a potential risk for consumers' health.

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