Original article

DETERMINATION OF BETA-HAEMOLYTIC ACTIVITY AND MINIMUM INHIBITORY CONCENTRATIONS OF ANTIMICROBIAL DRUGS AGAINST AEROMONAS HYDROPHILA STRAINS ISOLATED FROM FISH PRODUCTS

D. STRATEV¹, I. VASHIN¹ & H. DASKALOV²

¹Department of Food Hygiene and Control, Veterinary Legislation and Management, Faculty of Veterinary Medicine, Stara Zagora, Bulgaria; ²NDRVMI, Bulgarian Food Safety Agency, Sofia, Bulgaria

Summary


The purpose of the study was to investigate the prevalence of β-haemolytic Aeromonas hydrophila strains in 83 samples from cooled horse mackerel, rainbow trout, Black sea roach, silver carp, vacuum-packed rainbow trout fillets and 20 samples frozen rainbow trout and to establish the minimum inhibitory concentrations (MIC) of several antimicrobial drugs against these strains. Forty-five Aeromonas spp. strains were isolated, 36 (80%) of them with β-haemolytic activity. Identification of biochemical properties and determination of MICs were performed with 20 β-haemolytic A. hydrophila strains. The MICs of cefoxitin (≤0.5–32 µg/mL), azithromycin (≤0.5–16 µg/mL), chloramphenicol (≤2–16 µg/mL), tetracycline (≤4–16 µg/mL), ceftriaxone (≤0.25–32 µg/mL), amoxicillin/clavulanic acid (≤8/4–32/16 µg/mL), ciprofloxacin (≤0.015–1 µg/mL), gentamicin (≤1–4 µg/mL), nalidixic acid (≤0.5–8 µg/mL), ceftiofur (≤1–8 µg/mL), sulfisoxazole (≤64–256 µg/mL), trimethoprim/sulfamethoxazole (≤0.12/2.38–1/19 µg/mL), kanamycin (≤8–16 µg/mL) and streptomycin (≤32 µg/mL) were determined using Sensititre® CMV2AGNF microtitre plates (Trek Diagnostic Systems, USA). Tested strains were resistant to ampicillin concentrations in the plate: >32 µg/mL.

Key words: Aeromonas hydrophila, β-haemolysis, fish products, minimum inhibitory concentrations

INTRODUCTION

Microorganisms of genus Aeromonas are a part of the Aeromonadaceae family (Janda & Abbott, 1998). They have been isolated from various foods as fish, milk and dairy products, meat and meat products, vegetables (Palu et al., 2006; Kashtedikar & Chhabra, 2010). Some strains are enteropathogenic and possess virulence factors as enterotoxins, cytotoxins, haemolysins and invasins. A relationship
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between haemolysin production and enterotoxicity is reported, as 97% of enterotoxigenic strains are haemolysin producers (Burke et al., 1982). Aeromonas spp. could grow and release enterotoxin and haemolysin at fridge temperature conditions (Sharma & Kumar, 2011). A number of researchers have confirmed the presence of β-haemolytic aeromonads in fish products (Tsai & Chen, 1996; Wang & Silva, 1999; Radu et al., 2003; Thayumanavan et al., 2003; Yucel et al., 2005; Erdem et al., 2010). A special attention is paid on A. hydrophila as a causative agent of foodborne gastroenteritis, and fish products as the commonest vector of this microbial pathogen (Thayumanavan et al., 2003; Hatha et al., 2005; Paniagua et al., 2006). The wide spread of these bacteria is considered a public health threat due to the fact that Aeromonas infections are provoked by consumption of contaminated water and food (Kirov, 2001; Ottaviani et al., 2006). Gastroenteritis are the commonest infections provoked by Aeromonas spp., which may appear as diarrhoea, cholera-like disease or colitis (Janda & Abbott, 1998). According to Parker & Shaw (2010) 85% of human Aeromonas-related gastroenteritis are caused by A. hydrophila, A. caviae and A. veronii bv sobria.

Various systems for biochemical identification of members of genus Aeromonas are commercially available. These are API 20E, API ZYM, API 20NE, API 50 CH, API Rapid ID 32 (bioMérieux, France), Biolog MicroPlates GN2, GP2, AN (Biolog, Inc., USA), Enterotubes, BBL Crystal E/NF (Becton-Dickinson & Company, USA), Minitek (Becton-Dickinson & Company, USA), Bionor Aqua (Bionor, Skien, Norway) (Popovic et al., 2007). In the view of Wilcox et al. (1992) API 20NE could not distinguish A. hydrophila from A. caviae, and therefore, a test for β-haemolysis, which is positive for A. hydrophila and negative for A. caviae, is recommended. Aeromonas spp. are sensitive to cephalosporins, aminoglycosides, chloramphenicol, tetracycline, trimethoprim-sulphamethoxazole, aztreonam and fluoroquinolones (Ko et al., 2003) and resistant to penicillins (Awan et al., 2009). In these bacteria, antimicrobial resistance is chromosomally mediated but occasionally, β-lactamases produced by aeromonads could be encoded by plasmids or integrons (Aravena-Roman et al., 2012). The continuous increase of resistance to antimicrobial drugs is a serious concern which requires its periodic monitoring with respect to A. hydrophila in the different geographical regions in order to select a proper drug for therapy (Kaskhedikar & Chhabra, 2010).

At the background of the increasing role of microorganisms from the genus Aeromonas as agents of foodborne infections in men, we aimed at establishing the prevalence of β-haemolytic A. hydrophila strains in cooled and frozen fish products and to determine the minimum inhibitory concentrations of some antimicrobial drugs against these isolates.

MATERIALS AND METHODS

Sample collection

The study included 103 samples cooled and frozen fish products. Cooled fish products consisted of horse mackerel (20 samples), rainbow trout (11 samples), Black sea roach (12 samples), silver carp (20 samples), vacuum-packed rainbow trout fillets (20 samples). Frozen fish products included 20 rainbow trout samples. Samples were collected from retail
stores for fish and fish products and transported to the lab in a cooler bag.

*Aeromonas* spp. isolation

The isolation procedure was performed as per Evangelista-Barreto *et al.* (2006). Ten g muscle tissue with skin were collected aseptically from cooled fish and weighed in a Stomacher® 400 Circulator (Seward, England) bag. Ninety mL sample diluent (MRD, Merck) were added, and samples were homogenised at 256 rpm for 1 min. Tenfold dilutions were performed in tubes with 9 mL sample diluent (MRD, Merck). From each dilution, 0.1 mL were inoculated on two GSP agar plates (Merck, Germany). Plates were incubated at 28 °C over 24 h.

For isolation of *Aeromonas* spp. from frozen fish, tryptic soy broth (Merck, Germany) was employed for enrichment of samples. From the initial dilution, 10 mL homogenate were inoculated in 10 mL TSB at 28 °C for 24 h. After the incubation, inoculation on GSP agar (Merck, Germany) were performed and plates were incubated for another 24 h at 28 °C. Typical colonies were yellow, 2–3 mm in diameter, surrounded by a yellow zone. The reference *A. hydrophila* ATCC 7965 strain was used as positive control in the different tests with fish isolates.

**Haemolytic activity determination**

The haemolytic activity tests were performed on blood agar containing 5% sheep red blood cells. Typical colonies of each sample were grown for 4–5 h in brain heart infusion broth (Merck, Germany). The presence of haemolysis in agar plates was detected after 24-hour incubation at 37 °C according to Singh & Sanyal (1992).

**Identification of β-haemolytic *Aeromonas* spp.**

Isolates exhibiting β-haemolytic activity were identified by API 20 NE (bioMérieux, France).

**Determination of minimum inhibitory concentrations of antimicrobial drugs**

The minimum inhibitory concentrations of antimicrobial drugs against β-haemolytic *A. hydrophila* isolates were determined with Sensititre® CMV2AGNF microtiter plates (Trek Diagnostic Systems, USA) according to manufacturer’s instructions.

**RESULTS**

Table 1 presents the results from the haemolytic activity of *Aeromonas* spp. isolates. A total of 45 *Aeromonas* spp.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Number of isolates</th>
<th>Haemolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α</td>
</tr>
<tr>
<td>Cooled horse mackerel</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Cooled rainbow trout</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Cooled Black sea roach</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Cooled silver carp</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Cooled rainbow trout fillets</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Frozen rainbow trout</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>45</strong></td>
<td><strong>3</strong></td>
</tr>
</tbody>
</table>
Determination of beta-haemolytic activity and minimum inhibitory concentrations of antimicrobial drug MICs against beta-haemolytic Aeromonas hydrophila isolates. 

Fish and fish products are of primary significance for men because of their nutritional and health values. They are very prone to contamination and could transmit microbial pathogens, which normally inhabit the aquatic environment (Seethalakshmi et al., 2010). A. hydrophila produces virulence factors as haemolysin, aerolysin, proteases, lipases, DNA-ases with important role in the pathogenesis of human and fish diseases (Castro-Escarpuli et al., 2003). Two haemolytic toxins (haemolysin and aerolysin) are described in A. hydrophila. Haemolytic proteins are isolated from microbial pathogens, and beta-haemolysins are among the essential virulence factors (Pandey et al., 2010). In our study, we isolated 45 Aeromonas spp. strains, 80% of which with beta-haemolytic activity. The tested 20 beta-haemolytic isolates were identified as A. hydrophila. A high percentage of haemolytic A. hydrophila strains in fish products was also reported by other researchers. Thayumanavan et al. (2003) found out that 78.4% of 255 A. hydrophila isolates produced haemolysin, whereas Radu et al. (2003) established haemolytic activity in more than 90% A. veronii bv sobria, A. hydrophila and A. caviae isolates. Tsai & Chen (1996) isolated 16 A. hydrophila strains from fish fillets, 14 of which produced haemolysin and cytotoxin. Yucel et al. (2005) confirmed haemolytic activity in more than 80% of A. veronii bv sobria and A. hydrophila isolates, and Erdem et al. (2010) – in 89% of A. hydrophila strains. Unlike us, Wang & Silva (1999) established haemolytic activity only in 5.8% of A. hydrophila strains isolated from channel catfish fillets.

Several authors demonstrated that all investigated A. hydrophila strains were resistant to ampicillin (Vila et al., 2002; Castro-Escarpuli et al., 2003; Radu et al., 2003; Hatha et al., 2005; Kaskhedikar & Chhabra, 2010). The results from the present study were comparable, and established MIC of ampicillin was over 32 µg/mL. This is attributed to the fact that aeromonads form three classes of beta-lactamases which determine their resistance to a broad spectrum to beta-lactams (Chen et al., 2012). In contrast, Vaseeharan et al. (2005) determined MIC for ampicillin in the range from 89.5 to 126.7 µg/mL. MIC of amoxicillin/clavulanic acid against A. hydrophila was 8/4–32/16 µg/mL, which is the same (8/4 µg/mL) as that reported by Aravena-Roman et al. (2012).

The European Committee on Antimicrobial Susceptibility Testing has set clinical breakpoints for gentamicin...
### Table 2. MICs of antimicrobial drugs for *A. hydrophila* \( (n=20) \)

<table>
<thead>
<tr>
<th>Antimicrobial drugs</th>
<th>Number of isolates with MIC (µg/mL) of:</th>
<th>Panel MIC range (µg/mL)</th>
<th>Number of isolates with MIC (µg/mL) beyond the panel range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.015 0.03 0.06 0.12 0.25 0.5 1 2 4 8 16 32 64 128 256</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1 1 0 0 0 0 9 3</td>
<td>0.5–32</td>
<td>6 (&gt;32)</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0 0 1 1 0 12 1 2</td>
<td>0.12–16</td>
<td>3 (&gt;16)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>16 3 0 1 0</td>
<td>2–32</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>13 1 5 0</td>
<td>4–32</td>
<td>1 (&gt;32)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>5 4 3 4 1 2 0 1 0</td>
<td>0.25–64</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>11 2 2 2 1 1 0 0</td>
<td>0.015–4</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0 0 2 15 3 0 0</td>
<td>0.25–16</td>
<td>0</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>12 0 0 1 3 0 0</td>
<td>0.5–32</td>
<td>4 (&gt;32)</td>
</tr>
<tr>
<td>Cefotaxim</td>
<td>0 0 0 2 1 1 14</td>
<td>0.12–8</td>
<td>2 (&gt;8)</td>
</tr>
<tr>
<td>Sulfisoxazole</td>
<td>0 0 0 1 2 6</td>
<td>16–256</td>
<td>11 (&gt;256)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>14 6 0 0</td>
<td>8–64</td>
<td>0</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0 0 0 0 0 0 0 1</td>
<td>1–32</td>
<td>20 (&gt;32)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>20 0</td>
<td>32–64</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 3. MICs of antimicrobial drug combinations for *A. hydrophila* \( (n=20) \)

<table>
<thead>
<tr>
<th>Antimicrobial drug combinations</th>
<th>Number of isolates with MIC (µg/mL) of:</th>
<th>Panel MIC range (µg/mL)</th>
<th>Number of isolates with MIC (µg/mL) beyond the panel range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/0.5 2/1 4/2 8/4 16/8 32/16 0.12/2.38 0.25/4.75 0.5/9.5 1/19 2/38 4/76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxicillin/Clavulanic acid</td>
<td>0 0 0 2 11 6</td>
<td>1/0.5–32/16</td>
<td>1 (&gt;32/16)</td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole</td>
<td>1 11 4 4 0 0</td>
<td>0.12/2.38–4/76</td>
<td>0</td>
</tr>
</tbody>
</table>
Determination of beta-haemolytic activity and minimum inhibitory concentrations of antimicrobial drugs

Goni (2016) varied from less than 0.015–1 µg/mL and tramiphacin (S=≤2 mg/L; R=1 mg/L) and tobramycin (S=≤2 mg/L; R=4 mg/L) against A. hydrophila (EUCAST, 2014). All strains in our study were sensitive to gentamicin at concentrations of ≤4 µg/mL. Other researchers (Wang & Silva, 1999; Vila et al., 2002; Hatha et al., 2005; Ottaviani et al., 2006; Awan et al., 2009) have also confirmed that A. hydrophila was sensitive to gentamicin. Unlike us, Thyumanavan et al. (2003) showed a resistance to gentamicin (3.6%). The MIC of gentamicin according to Dibua & Okpokwasili (2006) is 50 µg/mL, of kanamycin – 45 µg/mL and of streptomycin –25 µg/mL vs. A. hydrophila. The MIC values obtained in this study for gentamicin (≤1–4 µg/mL) and kanamycin (≤8–16 µg/mL) were lower, and the results for streptomycin – comparable (≤ 32 µg/mL). A previous study of ours (Stratev et al., 2013) provided similar evidence as 6 out of 8 β-haemolytic A. hydrophila isolates were found to be sensitive to gentamicin at a concentration ≤ 4 µg/mL.

Quinolones are broad-spectrum antimicrobial drugs, widely used in human and veterinary medicine. They, as well as co-trimoxazole, are recommended as first-choice therapeutics for treatment of Aeromonas-induced infections. Less than 25% of aeromonads from the environment and less than 5% of clinical isolates are resistant to quinolones (Goni-Urriza et al., 2000). Our results support this statement with MICs of ciprofloxacin for all tested strains ≤0.015–1 µg/mL and trimethoprim/sulfamethoxazole ≤0.12/2.38–1/19 µg/mL, whereas the MIC of nalidixic acid varied from less than 0.5 up to 8 µg/mL. Goni-Urriza et al. (2000) established higher MIC values of ciprofloxacin (2 µg/mL) and nalidixic acid (16 µg/mL), while Overman (1980) reported the MIC of trimethoprim/sulfamethoxazole of ≤0.5–9.5 µg/mL against A. hydrophila.

The resistance to first- and second-generation cephalosporins is different, but more than 90% of Aeromonas spp. isolates are sensitive to the third generation of these antimicrobial drugs (Jones & Wilcox, 1995). Panigua et al. (2006) established MIC of ceftriaxone of 3.9 µg/mL, and Goni-Urriza et al. (2000) – 16 µg/mL of ceftoxitin. Our results were in agreement with these data, with MIC values for ceftoxitin of ≤0.5–16 µg/mL and for ceftriaxone of ≤0.25-32 µg/mL.

Dibua & Okpokwasili (2006) indicated that MIC of chloramphenicol vs. A. hydrophila was 15 µg/mL, and that of tetracycline – 80 µg/mL. Jones et al. (1988) reported that the MIC of azithromycin against A. hydrophila was 4 µg/mL. These values are comparable to the results of our study, where most of A. hydrophila strains had MICs of 4 µg/mL, MICs of chloramphenicol varied within 2–16 µg/mL, and MICs of tetracycline – from 4 to 16 µg/mL.

In conclusion, cooled and frozen fish products were often contaminated with A. hydrophila. Most strains belonging to this species possessed β-haemolytic activity, posing a risk from infection as A. hydrophila produces haemolysins during cold storage of fish products. The MICs of ceftoxitin, azithromycin, chloramphenicol, tetracycline, ceftriaxone, amoxicillin/clavulanic acid, ciprofloxacin, gentamicin, nalidixic acid, ceftiofur, sulfisoxazole, trimethoprim, kanamycin and streptomycin against β-haemolytic A. hydrophila isolates were determined. Tested strains were resistant to ampicillin concentrations in the plate: >32 µg/mL.
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**Correspondence:**

Dr. Deyan Stratev  
Department of Food Hygiene and Control,  
Veterinary Legislation and Management,  
Faculty of Veterinary Medicine,  
6000 Stara Zagora, Bulgaria  
e-mail: deyan.stratev@trakia-uni.bg