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Detection of Aerolysin and Hemolysin Genes in *Aeromonas* spp. Isolated from Environmental and Shellfish Sources by Polymerase Chain Reaction

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Abstract: Polymerase chain reaction (PCR) technique was used to assay for the detection of specific genes in the genomes of the *Aeromonas* spp. isolated from environmental and shellfish sources, particularly *aero* and *hlyA* genes, responsible for aerolysin and hemolysin toxins production in this genus. The results showed that: (i) the 1500 bp amplicon of the *hlyA* gene was detected in 20/38 of the *Aeromonas hydrophila*, 13/38 of the *A. caviae* and 6/9 of the *A. veronii* biovar *sobria* isolates; (ii) the 690 bp amplicon of the a*ero* gene was detected in 20/38 of *A. hydrophila*, 17/38 of *A. caviae* and 6/9 of *A. veronii* biovar *sobria* isolates; (iii) the nucleotide blast results of aerolysin gene sequences of the representative strains of *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* revealed a high homology of 94%, 95% and 95% with published sequences, respectively and ; (iv) the protein blast showed 97%, 94% and 96% homology when compared to the published sequences, respectively. The finding of *A. hydrophila* virulence genes in other members of the genus *Aeromonas*, may give a new perspective to the significance of these results. The method described here may be a useful detection tool to assist in further investigation of *aero* and *hlyA* genes in the genus *Aeromonas*, especially for food microbiologist.

Keywords: *Aeromonas*, PCR, hemolysin, aerolysin, sequencing

INTRODUCTION

Aeromonas spp. are Gram-negative, rod shaped, mainly motile, facultative anaerobic, oxidase positive and glucose fermenting bacteria (Nordmann and Poirel, 2002). They have recently been transferred from *Vibrionaceae* to their own family *Aeromonadaceae* (Ormen *et al.*, 2005). *Aeromonas* spp. has been involved in wound infections, sepsis, outbreaks of water- and food-borne gastroenteritis (Guadalupe *et al.*, 2005). Three species, *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* have been suggested as the causes of human gastroenteritis. A variety of potential virulence factors and toxins have been characterised (Biscardi *et al.*, 2002). Some strains are reported to be invasive to epithelial cells and one of the major virulence factors in gastroenteritis is aerolysin (Chu and Lu, 2005). The difficulty in recognizing potentially significant *Aeromonas* strains in foods poses a dilemma for the public health authorities (Kirov, 1993). The conventional micro-biological procedures for isolating and

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identifying *Aeromonas* spp. from food are both laborious and time consuming (ICMSF, 1996). Some novel approaches like polymerase chain reaction (PCR) (Kingombe *et al.*, 1999; Wang *et al.*, 2003), outer membrane protein based immunoassays (Sachan and Agarwal, 2002), DNA/RNA probes (Dorsch *et al.*, 1994) and flow cytometry (Diaper *et al.*, 1992) have been used for the detection and/or identification of the *Aeromonas* spp. from food and environmental or clinical samples.

Two haemolytic toxins, haemolysin and aerolysin have been described in *A. hydrophila*. In this study, a search was made for the presence of the *A. hydrophila* virulence genes *hlyA* and *aero*, described by Xia *et al.* (2003) and Hosin Ali (personal communication) in the genomes of *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* strains by using the polymerase chain reaction method.

MATERIAL AND METHOD

Bacterial Strains and DNA Extraction

The eighty five isolates of the *A. hydrophila* (n=38), *A. caviae* (n=38) and *A. veronii* biovar *sobria* (n=9) which were isolated from the fresh water and seafood from different locations in Malaysia using the method as described elsewhere (Son *et al.*, 2003) were used and the control strains of *A. hydrophila*, *A. caviae*. and *A. veronii* biovar *sobri* were grown in the LB broth with shaking at 120 rpm for 18-24 hours at 37°C. Using a 1.5 ml micro centrifuge tube, 1 ml of the cell culture from overnight grown culture in the LB broth was spun at 10 000 rpm for 5 min. The supernatant was discarded and 1 ml of sterile distilled water was added to re-suspend the cell pellet before it was re-centrifuged again at 10 000 rpm for 5 min. Next, the supernatant was discarded and 1 ml of sterile distilled water was added. The suspension was boiled for 10 min at 99°C to allow cell lyses and release of the DNA. After that, the tube was immediately placed in ice for 10 min. The cell lysates were centrifuged again and the clear supernatant was transferred to a new tube. The supernatant containing bacterial template DNA was used directly in the specific-PCR for the detection of aerolysin (a*ero*) and hemolysin (*hly*) genes.

PCR Protocol

A. hydrophila, *A. caviae* , and *A. veronii* biovar *sobria* strains isolated from food and the environment in this study were examined by the use of the specific PCR to determine the presence of aerolysin (*Aero)* gene and hemolysin (*hly*) gene. The primers used to detect the targeted genes were:

(i) *Aero* 1, 5'-ATGCTGCAGAAATGATGA ATAGAATAATTACCGC-3' and Aero 2,'5'-A T G C A A G C T T G C C C C A T A ATCTCCCAGCGAT-3' for the aerolysin gene (Hosin Ali, personal communication) and

(ii) Hly 1, 5'-CTATGAAAAAACTAAA AATAACTG-3' and Hly 2, 5'-CAGTATAAG TGGGGAAATGGAAAG-3' for the hemolysin gene (Xia'*et al.*, 2003). The PCR assay was carried out in 0.5 ml micro centrifuge tubes, with 20.0 µl of reaction mixture consisting of 11.9 µl sterile distilled water, 2.0 µl 10x PCR buffer, 1.6 µl 25 mM $MgCl_2$, 0.8 µl 2.5 mM deoxyribonucleotide phosphate (dNTP), 10 pmol of each of the primers (1.0 µl each) and 0.5 U of *Taq* DNA polymerase (0.5 µl) and template DNA 1.2 µl. The *A. hydrophila*, *A. caviae*, and *A. veronii* biovar *sobria* strains obtained from the Institute for Medical Research, Kuala Lumpur were used as the positive controls for the detection of *Aero* and *hyl* genes. The solution mixtures were placed in the PTC 100 thermocycler (M.J. Research, Watertown, M.A, USA) and subjected to 35 cycles.

Agarose Gel Electrophoresis

For the detection and confirmation of the PCR products by the gel electrophoresis, 10 ml of the amplification product mixture was subjected to electrophoresis through 1.5% agarose gel. The amplified DNA fragments of specific sizes were visualized by UV fluorescence after being stained with ethidium bromide and recorded using the gel documentation system (Syngene).

Sequencing of the* Aero *and* hlyA *Genes

After gel electrophoresis, the amplicons from representative strains of the *Aeromonas* spp. were purified using the QIAquick™ gel extraction kit (Qiagen®, Germany) and was subjected to the DNA sequencing analysis. The primer for the aerolysin gene that was designed by our colleague (Hosin Ali, personal communication) was used to analyse the specificity and confirm the PCR product on an ABI PRISM 377 DNA automated sequencer (Applied Biosystem, USA) at First Base Laboratories Sdn. Bhd. (Malaysia). The BLAST program (Basic Local Alignment Search Tool) accessed through the Internet *(http://www.ncbi.nlm.nih.gov/)* was used to analyse the sequence. The alignment of the sequences with several closely related genes was done using the CLUSTAW program from the BioEdit version 6.0.7 released on 19th May 2004.

RESULTS

The specific-PCR was performed using the primers H1, H2 and Aero1 and Aero 2 to determine whether a 1500 bp or 690 bp of the *hlyA* gene or a*ero* gene fragment, respectively, could be detected among the 85 *Aeromonas* isolates. The PCR analysis for the detection of aerolysin (*Aer*) and hemolysin *(hly)* showed that 45.9% of the isolates carried hemolysin (*hly*) gene and 50.5% of the isolates contained aerolysin (*Aer*) gene. Out of the 85 strains, 20/38 of *A. hydrophila* (Figure 1), 13/38 of *A. caviae* (data not shown) and 6/9 of *A. veronii* biovar *sobria* (Figure 2) isolates were PCR positive for the *hly*A gene. On the other hand, 20/38 of *A. hydrophila* (Figure 3) 17/38 of *A. caviae* (Figure 4) and 6/9 of *A. veronii* biovar *sobria* (data not shown) isolates were PCR positive for the *aero*A gene. The purified *aero*A gene PCR products using the QIAquick® gel extraction kit (Qiagen®, Germany) were subjected to direct DNA sequencing. The sequencing result of the *Aero* gene was compared to the Genbank database of the National Center for Biotechnology Information (NCBI) by using the BLASTN program *(http://www.ncbi.nlm.nih.gov/)*. The nucleotide blast results of the aerolysin gene sequences of representative *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* revealed high homology of 94%, 95% and 95% with published sequences (data not shown), respectively. The representative protein blast *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* showed homology of 97%, 94% and 96% (data not shown), respectively, when compared to the published sequences in the Genbank database of the National Centre for Biotechnology Information (NCBI).

DISCUSSION

Little is known about the virulence of the possible enterotoxins of *Aeromonas* spp. There is no doubt that at least three different species of *Aeromonas* have been involved in food poisoning (*A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria*) (Kirov, 1997) but there is no documentation of which enterotoxin(s) is responsible. The two potential candidates have been characterized after cloning (Chopra *et al.*, 1993), but these do not exclude other enterotoxins. Several papers have pointed towards a haemolysin as a good candidate for the main enterotoxin and it has been claimed that this might be a modified aerolysin. Granum *et al.* (1998), however, showed that aerolysin without the modification as described by Chopra *et al.* (1993) is cytotoxic to Caco-2 cells, and it is responsible for at least most of the cytotoxic activity. It is not unlikely that more than one enterotoxin may be involved in the food poisoning of humans, but addressing this question needs much more research, preferably on strains that have been involved in food poisoning. Although the involvement of *Aeromonas* spp. in food poisoning is still controversial, an increasing number of research reports point in the direction of direct involvement. The number of potential enterotoxins that has been suggested over the last years (Ormen *et al.*, 2005) may show the diversity of the different *Aeromonas* strains. They are pathogenic to a

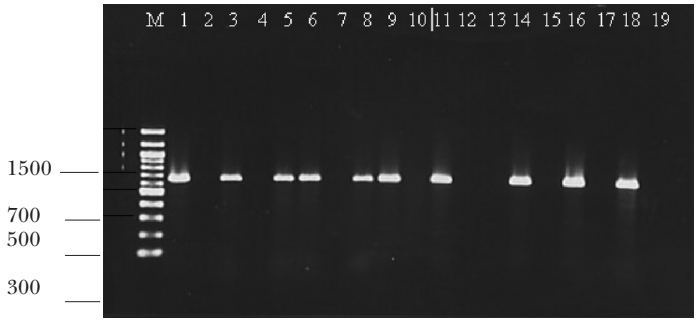

Figure 1: Representatives of the detection of hemolysin gene in *A. hydrophila* on 1.5% agarose gel. Lanes: 1, Control positive; 2, Control negative; : 3, UH1; 4, UH2; 5 , UH3 ; 6, UH4; 7, UH5; 8, UH6; 9, UH7; 10, UH8; 11,UH9; 12, UH10; 13, UH11; 14, MH12; 15, MH13; 16, MH14; 17, MH15; 18, MH16; 19, MH17

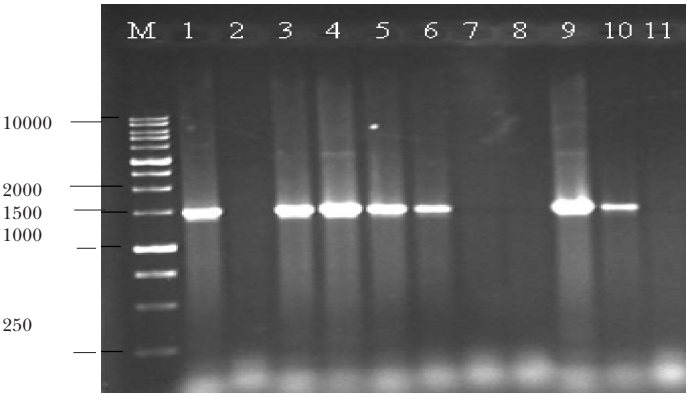

Figure 2: Representatives of the detection of hemolysin gene in *A. veronii* biovar *sobria* on 1.5% agarose gel. Lanes: 1, Control positive; 2, Control negative; 3, US1; 4, US2; 5, US3; 6, FS4; 7, SS5; 8, SS6; 9, SS7; 10, SS8; 11,SS9

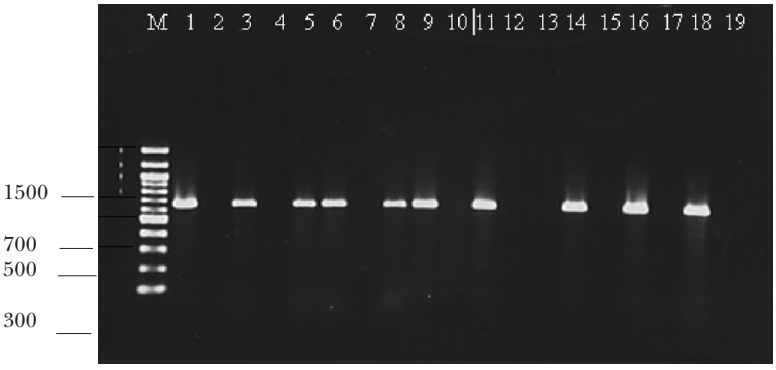

Figure 3: Representatives of the detection of aerolysin gene in *A. hydrophila* on 1.5% agarose gel. Lanes: 1, Control positive; 2, Control negative; : 3, UH1; 4, UH2; 5 , UH3 ; 6, UH4; 7, UH5; 8, UH6; 9, UH7; 10, UH8; 11,UH9; 12, UH10; 13, UH11; 14, MH12; 15, MH13; 16, MH14; 17, MH15; 18, MH16; 19, MH17

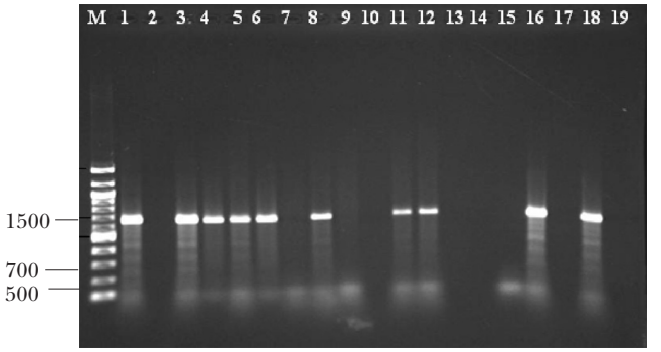

Figure 4: Representatives of the detection of aerolysin gene in *A. caviae* on 1.5% agarose gel. Lanes: 1, Control positive; 2, Control negative; 3, MC20; 4, FC21; 5, FC22; 6, FC23; 7, FC24; 8, FC25; 9, FC26; 10, FC27; 11,SC28; 12, SC29; 13, SC30; 15, SC31; 16, SC32; 17, SC33; 18, SC34; 19, SC35

variety of different animal species living under very different conditions (from fish to man). It is also not unlikely that the different virulence factors are important under the different conditions (Ormen *et al.*, 2005).

The two haemolytic toxins, haemolysin and aerolysin (Howard *et al.*, 1987) have been described in *A. hydrophila*. When the PCR was performed to detect aerolysin gene (*aer*A), we found that *aer*A were mainly associated with *A. veronii* biovar *sobria* (66.6%), while 52.6% and 44.7% of *A. hydrophila* and *A. caviae* harbored *aerA*. It was interesting to note that primers designed from the *aer*A gene sequence of the *A. hydrophila* were found to give the expected size of the amplicon with *A. caviae* and *A. veronii* biovar *sobria* isolates. *In vitro* toxicity may suggest that there is also a probable pathogenicity *in vivo*, although information about the lowest dose to cause infection is still unclear. This hypothesis could be correlated with many studies which associated *Aeromonas* with various diseases: gastroenteritis (Chopra *et al.*, 1999), endocarditis (Brouqui and Raoult, 2001), wound infection (Ouderkirk *et al.*, 2004), acute suppurative cholangitis (Chan *et al.*, 2000), cellulites (Grobusch, 2001), abscess (Halley and Mattano, 1999), peritonitis (Cordoba Lopez *et al.*, 1999), septic arthritis (Janda and Abbott, 1998), pneumonitis (Goncalves *et al.*, 1997), appendicitis (Dionisio *et al.*, 1997), osteomyelitis (Karam *et al.*, 1983), corneal ulcer (Carta *et al.*, 1994), meningitis (Lin and Cheng, 1998), septicemia (Chang *et al.*, 1997), myonecrosis (Moses *et al.*, 1995), and bacterial empyema (Wang *et al.*,2003).

The presence and frequency of the aerolysin (*aerA*) gene in the *Aeromonas* strains in this study was in broad agreement with an earlier PCR survey (Husslein *et al.*, 1991) that detected the *aerA* in all strains belonging to *A. hydrophila* and *A. veronii* biotype *sobria* species but in only half of *A. caviae* strains tested. In this study, the *aerA* gene was widespread in *A. veronii* biotype *sobria* and *A. hydrophila*, but less so in *A. caviae*. In contrast, the studies of Pollard *et al.* (1990) and Lior and Johnson (1990) showed that the *aerA* gene was only detected in hemolytic, cytotoxic and enterotoxic strains of *A. hydrophila* but not in *A. veronii* biotype *sobria* and *A. caviae*. Primer design divergences and limited numbers of strains, together with a lack of confirmatory Southern analyses on the PCR negative strains, may explain this anomaly.

When the genotypes of known virulent strains as defined in Wong *et al.* (1996) were compared, it was apparent that all the *A. hydrophila* isolates with the *hlyA* and *aerA* genotype were virulent in the suckling mouse model. These isolates also demonstrated ≤-hemolytic and cytotoxic activities. Due to the fact that the *aerA* and hemolysin genes were found in the vast majority of the diarrhoeal isolates from this species (Michelle *et al.*, 1999),

it is possible that these strains may possess other hemolytic/cytotoxic factors related to virulence that have not yet been described. The possibility of detecting the *Aeromonas* virulence gene followed by its characterization into the three main groups of the virulence markers (i.e. aerolysins, hemolysins, and enterotoxins) during a single PCR amplification from clinical, environmental or food isolates was promoted by Kingombe *et al.* (1999). In this study, the application of this method for the detection of *Aeromonas* virulence genes in food and environmental samples has proved to offer an interesting alternative for the rapid screening of potentially virulent aeromonads in food and the environment.

The nucleotide blast results of the aerolysin gene sequences representative of the *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* have shown that high sequence homologies exist between the aerolysin gene and GenBank database, thus confirming clearly the high level of DNA relatedness of the *Aeromonas* spp. virulence factors. In practice, the determination of the pathogenicity among *Aeromonas* food isolates is mostly based on the biological assays assessing the cytotoxicity or adherence to human or animal cell lines (Handfield *et al.*, 1996; Kirov *et al.*, 1984). Unfortunately, the majority of these systems are not always suitable for use on a routine basis. Although not linked to a biological assay, a positive reaction in our PCR-based detection method can be considered as an indication of the potential pathogenicity of the *Aeromonas* isolate under study. Furthermore, the PCR results obtained either by using the BLAST search of the GenBank database or by the alignment of the amplicon to the reference sequence (Chopra *et al.*, 1993; GenBank accession no. M8470) allowed the determination of the identity and sequence similarity of a given amplicon and to the complete collection of the *Aeromonas v*irulence markers in the GenBank.

In conclusion, these observations suggest that an evaluation of the *Aeromonas* virulence requires the assessment of virulence phenotypes and complete virulence gene set. Screening of specific cytotoxin and hemolysin genes appeared to be the most effective way of detecting and characterizing *Aeromonas* virulence factors. The high throughput and cost-effective specific-PCR system used in this study could provide a powerful supplement to the conventional methods for a more accurate risk assessment and monitoring of *Aeromonas* species in the food and environment.

REFERENCES

Biscardi, D., Castaldo, A., Gualillo, O. and de Fusco, R. 2002. The occurrence of cytotoxic *Aeromonas hydrophila* strains in Italian mineral and thermal waters. The Science of the Total Environment, 292: 255–263.

Brouqui, P. and Raoult, D. 2001. Endocarditis due to rare and fastidious bacteria. American Society for Microbiology Clinical Microbiology Reviews, 14: 177–207.

Carta, F., Pinna, A., Zanetti, S., Carta, A., Sotgiu, M. and Fadda, G. 1994 Corneal ulcer caused by *Aeromonas* species. American Journal of Ophthalmology, 118: 530- 531.

Chan, M.S., Maiden, M.C.J. and Spratt, B.G. 2001. Database-driven multi locus sequence typing (MLST) of bacterial pathogens. Bioinformatics, 17: 1077–1083.

Chang, T.M., Liu, C.C. and Chang, M.C. 1997. Cloning and sequence analysis of the gene (*epr*A1) encoding an extracellular protease from *Aeromonas hydrophila*. Gene, 199: 225-229.

Chopra, A. K., Houston, C. W., Peterson, J. W. and Jin, G. F. 1993. Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. Canadian Journal of Microbiology, 39: 513–523.

Chopra, S., Brendel, V., Zhang, J.B., Axtell, J.D. and Peterson, T. 1999. Molecular characterization of a mutable pigmentation phenotype and isolation of the first active transposable element from Sorghum bicolor. Proceedings

of the National Academy of Sciences of the United States of America, 96: 15330–15335.

Chu, W.H and Lu, C.P. 2005. Multiplex PCR assay for the detection of pathogenic *Aeromonas hydrophila*. Journal of Fish Diseases, 28: 437–441.

Cordoba, L.A., Bueno, A., Alzugaray, F.R.J., Veiga, G.M.D. and Corcho, S.G. 1999. Spontaneous bacterial peritonitis and sepsis due to *Aeromonas hydrophila*. Gastroenterology and Hepatology, 22: 208-212.

Diaper, J.P., Tither, K. and Edwards, C. 1992. Rapid assessment of bacterial viability by flow cytometry. Journal of Applied Microbiology and Biotechnology, 38: 268-272.

Dionisio, L.P.C. and Borrego, J.J. 1995. Evaluation of media for the enumeration of faecal streptococci from natural water samples. Journal of Microbiological Methods, 23: 183–203.

Dorsch, M., Ashbolt, N.J., Cox, P.T. and Goddman, A.E. 1994. Rapid identifcation of *Aeromonas* species using 16S rDNA targeted oligonucleotide primers: a molecular approach based on screening of environmental isolates. Journal of Applied Bacteriology, 77: 722-726.

Goncalves, A.G., Campos, L.C., Gomes, T.A.T., Rodrigues, J., Sperandio, V., Whittam, T.S. and Trabulsi, L.R. 1997. Virulence properties and clonal structures of strains of *Escherichia coli* O119 serotypes. Infection and Immunity, 65: 2034-2040.

Granum, P.E., O'Sullivan, K., Tomas, J.M. and Ormen, O. 1998. Possible virulence factors of *Aeromonas* spp. from food and water. FEMS Immunology and Medical Microbiology, 21: 131-137.

Grobusch, M.P. 2001. Cellulitis and septicemia caused by *Aeromonas hydrophila* acquired at home. Journal of Infection, 29: 109-110.

Guadalupe, M.A., Cesar, H.R., Gerardo, Z., Marla, J.F. and Graciela, C.E. 2005. *Aeromonas hydrophila* clinical and environmental ecotypes as revealed by genetic diversity and virulence genes. FEMS Microbiology Letters, 242: 231–240.

Halley, M., and Mattano, L.A.J. 1999. *Aeromonas* abscess in an immunocompromised child. Journal of Pediatric Hematology and Oncology, 21: 551-554.

Handfield, M., Smard, P., Cuillard, M. and Letarte, R. 1996. *Aeromonas hydrophila* isolated from food and drinking water: hemagglutination, hemolysis, and cytotoxicity for a human intestinal cell line (HT-29). Applied and Environmental Microbiology, 62: 3459-3461.

Howard, S.P., Garland, W.J., Green, M.J. and Buckley, J.T. 1987. Nucleotide sequence of the gene of the hole-forming toxin aerolysin of *Aeromonas hydrophila*. Journal of Bacteriology, 169: 2869–2871.

Husslein, V., Chakraborty, T., Carnahan, A. and Joseph, S.W. 1992. Molecular studies on the aerolysin gene of *Aeromonas* species and discovery of a species-specific probe for *Aeromonas trota* species nova. Clinical Infectious Diseases, 14: 1061–1068.

ICMSF (International Commission on Microbiological Specifications on Foods). 1996. Microorganisms in foods 5. Microbiological specifications of food pathogens. London: Blackie A and P.

Janda, J.M. and Abbott, S.L. 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. Journal of Clinical and Infectious Diseases, 27: 332-344.

Karam, G.H., Ackley, A. M. and Dismukes, W. E. 1983. Posttraumatic *Aeromonas hydrophila* osteomyelitis. Archives of Internal Medicine, 143: 2073-2074.

Kingombe, C.I.B., Huys, G., Tonolla, M., Albert, M.J., Swings, J. and Peduzzi, R. 1999. PCR detection, characterization and distribution of virulence genes in *Aeromonas* spp. Applied and Environmental Microbiology, 65: 5293–5302.

Kirov, S.M. 1993. Adhesion and piliation of *Aeromonas* spp. Medical and Microbiology Letters, 2: 274–280.

Kirov, S.M. 1997. *Aeromonas* and *Plesiomonas* species. In Doyle, M., Beuchat, L. and Montville, T. (Eds). Food Microbiology. Fundamentals and Frontiers, p. 265-287. Washington D.C.: ASM Press.

Kirov, S. M., Wellock, R. and Goldsmid, J. M. 1984. *Aeromonas* species as enteric pathogens. Australasian Microbiologist, 5: 210-214.

Lin, C.S. and Cheng, S.H. 1998. *Aeromonas hydrophila* sepsis presenting as meningitis and necrotizing fasciitis in a man with alcoholic liver cirrhosis. Journal of the Formosan Medical Association, 97: 498-502.

Lior, H. and Johnson, W.M. 1991. Application of the polymerase chain reaction (PCR) to detection of the aerolysin gene in whole cell cultures of β-hemolytic *Aeromonas hydrophila*. Experientia, 47: 421–424.

Michelle, C., Callegan, B.D., Jett, L.E. and Michael, S.G. 1999. Role of Hemolysin BL in the pathogenesis of extraintestinal *Bacillus cereus*–infection assessed in an endophthalmitis model. Infection and Immunity, 67: 3357–3366.

Moses , A.E., Leibergal, M., Rahav, G., Perouansky, M.R. and Shapiro, M. 1995. *Aeromonas hydrophila* myonecrosis accompanying mucormycosis five years after bone marrow transplantation. European Journal of Clinical Microbiology and Infectious Diseases, 14: 237-240.

Nordmann, P. and Poirel, L. 2002. Emerging carbapenemases in Gram-negative aerobes. Clinical Microbiology and Infection, 8: 321–331.

Ormen, O., Pereinar, G., Lassen, J. and Figueras, M.J. 2005. Lack of agreement between biochemical and genetic identification of *Aeromonas* spp**.** Acta Patolugica Microbiologica et Immunologica Scandinavica, 113: 203–210.

Ouderkirk, J.P., Bekhor, D., Glenn, S.T. and Raj, M. 2004. *Aeromonas* meningitis complicating medicinal leech therapy. Clinical Infectious Diseases, 38: 36-37.

Pollard, D.R., Johnson, W.M., Lior, H., Tyler, S.D. and Rozee, K.R. 1990. Detection of the aerolysin gene in *Aeromonas hydrophila* by the polymerase chain reaction. Journal of Clinical Microbiology, 28: 2477–2481.

Sachan, N. and Agarwal, R. 2002. A simple enzyme-linked immunosorbent assay for the detection of *Aeromonas* spp. Journal of Veterinarski Arhiv, 72: 327-334.

Son, R., Noorlis, A., Foo, H.L. and Reezal, A. 2003. Prevalence and resistance to antibiotics for *Aeromonas* species from retail fish in Malaysia. International Journal of Food Microbiology, 81: 261-266.

Wang, G., Clifford, G.C., Chenyi, L., Chad, P., Cindy, K. M., Tamara, M.A.C., Richard, C., Woodward, D.L. and Frank G.R. 2003. Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. Journal of Clinical Microbiology, 41: 1048–1054.

Wong, C.Y.F., Mayrhofer, G., Heuzenroeder, M. W., Atkinson, H. M., Quinn, D. M. and Flower, R.L.P. 1996. Measurement of virulence of aeromonads using a suckling mouse model of infection. FEMS Immunology and Medical Microbiology, 15: 233-241.

Xia, C., Ma, Z., Habibur, M.R. and Zhi-Guang, W. 2003. PCR cloning and identification of the haemolysin gene of *Aeromonas hydrophila* from freshwater fishes in China. Journal of Aquaculture, 229 : 45–53.