

AN IN-HOUSE MULTIPLEX PCR METHOD TO DETECT OF PUTATIVE VIRULENCE FACTORS IN *Aeromonas* SPECIES

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Submitted: November 03, 2010; Returned to authors for corrections: March 04, 2011; Approved: May 30, 2011.

ABSTRACT

A pentaplex PCR was developed and optimised to detect the genes that encode the five most important putative virulence factors in *Aeromonas* isolates. It seems to be more efficient than previously reported techniques and promises to be a powerful tool for more accurate risk assessments and for monitoring pathogenic strains.

Key words: pentaplex-PCR, virulence potential, pathogenesis, *Aeromonas*.

Aeromonas spp. have been considered autochthonous inhabitants of aquatic environments. However, some species have a broad host spectrum, causing intestinal and extra-intestinal infections in humans, as well as furuncles, hemorrhagic septicaemia, and soft tissue infections in fish. In spite of the taxonomic expansion of the genus, which currently includes 25 species, only 3 species are of clinical importance in humans (3, 4, 5, 17, 20, 23, 25). Interest in the pathogenicity of these microorganisms has become relevant due to their increasing implications in human diseases. The pathogenic mechanism of *Aeromonas* spp. has not yet been elucidated. Some studies have indicated that their pathogenicity could entail a complex mechanism involving multiple virulence factors, in which these bacteria attach to and colonise the lumen of the intestine and give rise to several enterotoxins (15, 20, 23, 30). Moreover, no single virulence factor or combination of factors has been unequivocally correlated with virulence in the different species. Therefore, the continuous monitoring of several virulence factors in clinical, food, and

environmental *Aeromonas* isolates remains an important tool in the understanding of both pathogenesis and epidemiology of *Aeromonas* infections. The detection of genes coding for bacterial adhesins, biofilm formation, and secreted enzymes, such as lipases, haemolytic enterotoxins, proteases, and nucleases has been considered a crucial step to ascertain the virulence potential of isolates (2, 7, 10, 19, 26, 27, 28, 29).

In the last years, at least five different types of m-PCR have been described in the literature (11, 12, 22, 26, 28). Some authors have described their own approach to detect simultaneously two or more virulence genes in *Aeromonas* and have employed it in drinking water isolates (12), several kinds of food (11, 21, 22), and fish (26). In all of these publications, the isolates possessed a wide variety of virulence-related genes, suggesting the importance of examining as many isolates as possible to understand better the health risks posed by these bacteria (28). These studies described results reviewed the array of virulence factors within the *Aeromonas* species and supported the notion that different combinations, patterns, or

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genotypes of virulence determinants normally exist within the corresponding *Aeromonas* species. However, there is little consistency in the genes studied in all these reports; the most common were those encoding enterotoxins (*ast*, *alt*, *act*, *aer*), flagella (*fla*, *laf*), and lipases (*gcat*). There was even less consistency in the types of samples assayed, the number of species tested, or the reference strains investigated. Clinical samples were the less explored in the studies using m-PCR and the gene combinations were not particularly useful for detecting the most prevalent virulence genes of strains isolated from different geographical areas (2, 7, 10).

The above observations led us to develop a new *in-house* multiplex PCR, which we have termed pentaplex PCR (p-PCR), as a rapid and effective method for the simultaneous detection of five genes encoding virulence factors that have shown the strongest relationship to diarrhoea in a single reaction (2, 20). The utility of this kind of PCR as a tool in this context is well-documented because it allows for the simultaneous detection of numerous genes (13, 14). It can be used not only as a tool for the continuous characterisation of the virulence potential of strains and their monitoring in environmental, human, and animal-origin samples, but also to identify which genes are present in the isolated strains and to describe the abundance and variation of these genes in these strains. In this study, the p-PCR was developed and compared with previous single-PCR (s-PCR) assays (2).

A total of 23 reference and type *Aeromonas* strains were analysed, and 54 clinical isolates from patients with diarrhoea were surveyed. All isolates were previously identified to the species level by the *16S rRNA* restriction fragment length polymorphism (RFLP) method (2,6,16). The identified isolates (2) were grouped as follows: *A. caviae* (n = 25), *A. hydrophila* (n = 18), and *A. veronii* (n = 11). *Pseudomonas aeruginosa* ATCC 25668 and *Vibrio parahaemolyticus* CECT 611 and CECT 612 were used as controls for PCR amplifications. All strains were grown on tryptose soy agar (Oxoid, Cambridge, England) at 37°C for 18 h.

PCR amplifications of *gcat*, *ast*, *alt*, *aerolysin/haemolysin*, and *lafA* virulence genes were performed as previously described (supplementary Table) by Aguilera-Arreola *et al.* (1) and Soler *et al.* (31). The generic term “aerolysin/haemolysin genes” is applied here because the primers reported by Soler *et al.* (31) amplify a number of genes encoding toxins known as aerolysin, aerolysin/haemolysin, haemolysin/aerolysin, haemolysin, and cytolytic enterotoxin. The primers *lafA-F* and *lafA-R* amplify similarly sized fragments of the consensus region of the genes *lafA*, *lafA1*, and *lafA2* as they share > 80% similarity according to the CLUSTAL W analysis. Degenerated primers design was used to amplify these genes in different species. We used the basic local alignment search tool (BLAST) to show that the primers were unique and specific to their respective genes from different *Aeromonas* species, and did not match those of any other genus or species. PCR amplifications were performed in a final volume of 50 µl, containing 3 µg of DNA template, 0.3 mM deoxynucleotide triphosphate (dNTP) mix, 1X PCR buffer, 1.8 mM MgCl₂, 0.3 µM primers, and 2.5 units of *Taq* polymerase (Gibco-BRL, Gaithersburg, MD). All reactions were carried out in a Perkin-Elmer DNA Thermal Cycler PCR System using programs previously described (1, 31). A simple (one gene) PCR was first performed for each of the reference and type strains of *Aeromonas* and subsequently for the non-*Aeromonas* reference strains. Gene fragments from the lateral flagella (*lafA*, 736-743 bp), aerolysin-hemolysis (*aer/hem*, 431 bp), heat labile enterotoxin (*alt*, 320 bp), heat-stable enterotoxin (*ast*, 504 bp), and glycerophospholipid acyltransferase (*gcat*, 237 bp) genes were amplified from genomic DNA. Two replicates were performed for each reaction. To verify the presence or absence of amplification products, 10 µl from each PCR was electrophoresed on a 1% agarose gel. The remaining 40 µl of the amplification reaction was kept frozen for other potential uses. Two random samples of the PCR amplification products for each of the target genes were authenticated by DNA sequencing with an ABI-PRISM™ 310 (Applied Biosystems,

Foster City, CA) following the standard methodology outlined by the manufacturer.

The standard multiplex PCR was performed in a buffer containing 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 1.8 mM MgCl₂, 300 μM of each dNTP, 2.5 U *Taq* polymerase, and 100 pg of genomic DNA in a 5-μl volume. The primers for the *lafA*, *aer/hem*, and *alt* genes were used at a concentration of 0.15 μM, those for the *ast* gene were used at 0.10 μM, and those for the *gcat* gene were used at 25 μM. The cycling conditions were designed in two phases; in the first phase of 20 cycles, the *gcat*, *aer/hem*, and *lafA* genes were amplified; in the second phase of 15 cycles, the *alt* and *ast* genes were amplified. The cycling conditions in the first phase consisted of an initial single cycle at 95°C for 5 min, followed by 20 cycles of melting at 94°C for 1 min, annealing at 56°C for 1 min, and

elongation at 72°C for 1 min. The second phase consisted of 15 cycles of melting at 94°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 1 min. A final and unique cycle at 72°C for 10 min was included. The p-PCR was first performed in each of the reference and type strains and subsequently in the non-*Aeromonas* reference strains. Then, the p-PCR was performed in the 54 clinical strains for specificity and sensitivity calculations (Table 1). To verify the presence or absence of amplification products, 10 μl from each PCR was electrophoresed on a 1% agarose gel. The products of simultaneous p-PCR reactions of each gene were confirmed via DNA sequencing on an ABI-PRISM 310 (Applied Biosystems, Foster City, CA) following the standard methodology outlined by the manufacturer. As in the single PCR assay, each reaction was performed in duplicate.

Table 1. Combinations of enterotoxins and adherence related genes harboured in 54 clinical *Aeromonas* isolates.

Possible combinations	Number positive strains for analysed combination			Total positive strains for p-PCR/s-PCR
	p-PCR*/s-PCR			
	<i>A. hydrophila</i> (n= 18)	<i>A. caviae</i> (n= 25)	<i>A. veronii</i> (n=11)	
<i>None</i>	0/0	3/3	0/0	3/3
<i>aer/hem</i>	0/0	13/13	7/7	20/20
<i>aer/hem</i> + <i>alt</i>	0/0	1/1	3/3	4/4
<i>aer/hem</i> + <i>ast</i>	0/0	1/1	0/0	1/1
<i>aer/hem</i> + <i>lafA</i>	0/0	5/5	1/1	6/6
<i>alt</i> + <i>ast</i>	1/0	0/0	0/0	1/0
<i>alt</i> + <i>lafA</i>	0/0	1/1	0/0	1/1
<i>aer/hem</i> + <i>alt</i> + <i>lafA</i>	0/0	1/1	0/0	1/1
<i>alt</i> + <i>ast</i> + <i>lafA</i>	6/6	0/0	0/0	6/6
<i>aer/hem</i> + <i>alt</i> + <i>ast</i>	0/1	0/0	0/0	0/1
<i>aer/hem</i> + <i>alt</i> + <i>ast</i> + <i>lafA</i>	11/11	0/0	0/0	11/11
Total	18	25	11	54/54
% Sensitivity	100	100	100	100%
% Specificity	50	100	100	83.3%

Genes encoding: aerolysin/hemolysin (*aer/hem*), thermo labile cytotoxic enterotoxin (*alt*), thermo stable cytotoxic enterotoxin (*ast*), and lateral flagella (*lafA*).
*result obtained from two replicates.

The sensitivity of the p-PCR assay was evaluated by comparing the results of the multiplex PCR with those obtained by single PCRs. The sensitivity and specificity, as well as the positive and negative predictive values, were calculated by 2 × 2 contingency tables in a Microsoft Excel® spreadsheet.

All 23 reference and type strains used for the simple PCR produced at least some of the expected amplicons. These

strains harbour several combinations of virulence factor genes. The strains with the greatest number of virulence genes were *A. hydrophila*, *A. bestiarum*, *A. salmonicida* subsp. *mausocida*, and *A. salmonicida* subsp. *achromogenes*, whereas the strains with the fewest were *A. media*, *A. schubertii*, and the *A. HG11* group.

In general, there was a wide variety in the combinations of

virulence factors in the *Aeromonas* clinical isolates. The general abundance of the genes has been previously demonstrated (2). Table 1 shows the different combinations of enterotoxin and adherence-related genes detected by s-PCR in the previously mentioned study and the replicates did likewise in the present study.

The accuracy of the p-PCR assay was evaluated by comparison with the results from the s-PCR, revealing a specificity of 96% and a sensitivity of 91.84%. Therefore, use of the primer combination for the clinical isolates was justified by the reliability of the p-PCR design method (Figure 1). The primer combination's specificity for *Aeromonas* was

demonstrated by the negative PCR results obtained with all of the non-*Aeromonas* reference strains. The sensitivity and specificity were higher in the reference and type strains and in the clinical ones (Table 1). Two replicates for each reaction (strain) was performed and results were consistent in both. The major incongruities between p-PCR and s-PCR were found in the genes *aer/hem* since in five reference strains there was no correlation, in contrast to the clinical strains where only one inconsistency between both methods was detected. In the *lafA* genes, a single inconsistency was observed in *A. salmonicida* subsp. *achromogenes* reference strain; whereas no inconsistencies were detected in the other three genes tested.

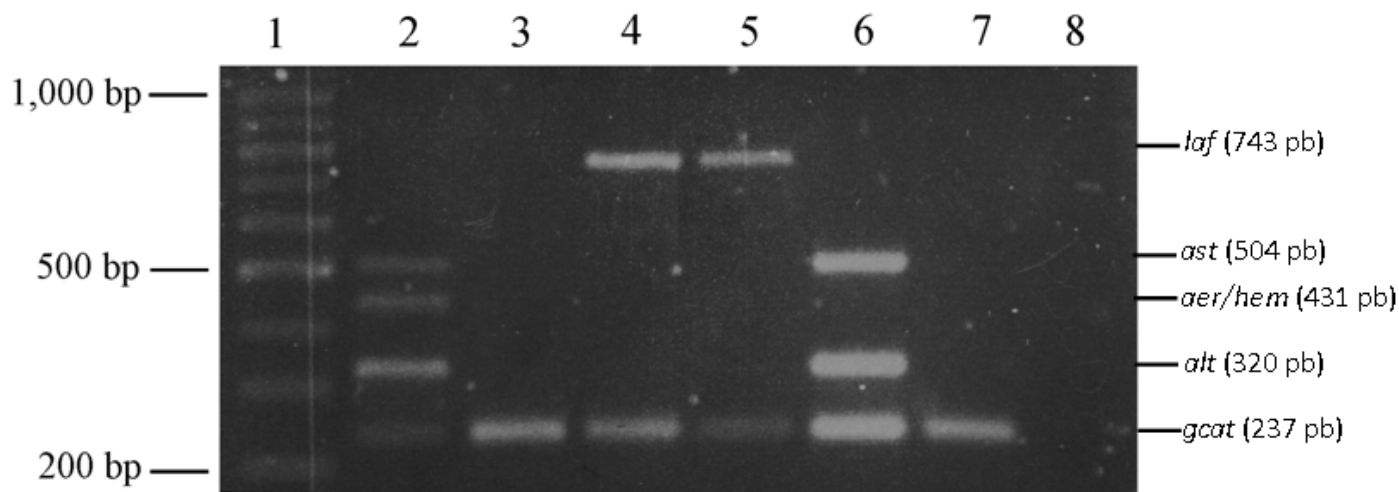


Figure 1. Pentaplex-PCR amplifications in *Aeromonas* type and clinical isolates. Lanes: 1, 100 pb DNA Molecular size marker (Amresco®); 2, *A. hydrophila* CECT 839; 3 and 7, *A. caviae* 44H; 4 and 5, *A. caviae* CECT 838; 6, *A. hydrophila* 180H; 8, negative control.

Because it has been impossible, so far, to establish a hierarchical classification of virulence factors according to their role in *Aeromonas* pathogenesis, several studies, via PCR or hybridisation assays, have been performed to find one single virulence factor (s-PCR) or defined combinations of virulence factors (m-PCR) in the different *Aeromonas* species. The early work performed by Sen and Rodgers (28) reported the

distribution of six virulence factors in *Aeromonas* from drinking water utilities, achieving this result through three sets of duplex PCR (d-PCR) addressed to search for two enterotoxin genes (*act* and *alt*), one gene of constitutive adherence (*fla*), and two genes considered accessory virulence genes (lipase and elastase genes), whereas the p-PCR, described herein, detects also five genes, it does so in a single

reaction and the detected genes are the ones related with diarrhoea, *alt*, *ast*, and *aer/hem* enterotoxin genes; *laf* genes related with adherence and biofilm mechanisms and *gcat*, an unusual lipase gene, which participates in pathogenicity and is useful as a tool for genus identification (9). Other authors have proposed a simpler design, standardising a d-PCR to detect specifically pathogenic *A. hydrophila* strains in fish; their d-PCR is capable of amplifying the *16S rRNA* gene and the aerolysin gene. This method has been useful for the direct detection of pathogenic strains of *A. hydrophila* (12) while the current p-PCR proposed herein is addressed to most species of the *Aeromonas* genus.

The most complex m-PCR was probably that described by Nam and Joh (26). These authors reported the rapid detection of virulence factors of *Aeromonas* isolated from a trout farm by a hexaplex PCR (h-PCR) designed to detect virulence accessory genes (nuclease, serine protease, and lipase) and aerolysin, *gcat*, and lateral flagella genes, the set of genes proposed by these authors is better suited to detect important virulence factors in sick fish (8,10). However, *ast*, *alt*, *act*, and *aer/hem* enterotoxin genes that show the strongest relationship with diseases in humans were not included. These authors concluded that their h-PCR could be used to rapidly screen and detect virulence factors from major *Aeromonas* species isolated from trout farms; whereas the p-PCR described by us could represent a more concrete method for both clinical and animal-origin isolates.

In contrast, Chang *et al.* (11) designed a triplex PCR (t-PCR) to amplify only the cytotoxic (*act*), heat-labile (*alt*), and heat-stable cytotoxic (*ast*) enterotoxin genes of aeromonads in 133 strains isolated from food and retail samples of poultry and fish. This method (t-PCR) has been useful for the detection and genotyping of enterotoxin genes of aeromonads. The analysed isolates were divided into six genotypes or patterns. *A. caviae* and *A. hydrophila* were the most frequently isolated species, and a significantly higher proportion of the former strains harboured all three of these enterotoxin genes. Recently, a

second t-PCR method for the detection of the same three enterotoxin genes (*act*, *alt*, and *ast*) was described by Kingombe *et al.* (22). Although the last two studies had similar designs, the latter characterised a wider range of reference strains, as well as foodborne isolates, finding seven gene pattern combinations; the *act* and *alt* genes were the most dominant in the analysed isolates. In this context, the proposed p-PCR includes all these enterotoxins and, in addition, allows for the amplification of two more suitable characteristics of virulence, important not only for food samples but also for clinical samples.

In previous studies, we demonstrated that the *lafA*, *ast*, *alt*, and *aer/hem* genes are the most frequent ones of clinical relevance in *Aeromonas* in Mexico and Spain (2). There, the use of independent PCR amplifications was a good approach. However, because of the large number of strains to be analysed in that study, a dot-blot hybridization assay was performed, and few discrepancies between the PCR and dot-blot detection were found. Due to the advantages that have been reported in the use of m-PCR, we decided to test the same eight primers used in our previous study to amplify these four target genes together in an m-PCR. The *gcat* gene was integrated into the p-PCR because it is marker gene for the *Aeromonas* genus as well as a significant virulence gene (8). The results were satisfactory; the pentaplex reaction showed good specificity and sensitivity in both type strains and clinical isolates. Thus, the p-PCR system developed in this study reduces the analyses time. It promises to be a powerful tool for more accurate risk assessments and for the monitoring of pathogenic strains of the most frequently isolated *Aeromonas* species, *A. hydrophila*, *A. caviae*, and *A. veronii* and other species. Of course, the reduced performance could be a problem of the number of samples analysed or the intraspecific variability previously shown in the *Aeromonas* genus (1,24); however, both hypotheses should be confirmed with a larger set of strains than tested currently.

We developed and evaluated a pentaplex PCR assay for the rapid detection of putative virulence factors in *Aeromonas*.

Although sophisticated surveys of pathogenicity mechanisms have already been conducted, the detection of enterotoxin genes by PCR remains a good approach to estimate virulence potential in *Aeromonas*. The p-PCR technique will be valuable as a simple tool to screen for the presence of virulent strains because the specific combination of genes employed facilitates the identification of the *Aeromonas* genus and characterize sick fish, particularly fish intended for human consumption, as well as waterborne bacteria and extra-intestinal clinical strains that are commonly analysed at clinical, research, or reference laboratories.

ACKNOWLEDGEMENTS

M.G.A.A. and G.C.E. received COFAA, EDI and SNI support. This work was financially supported by grants 20090729 and 20100252 from SIP-IPN, Mexico City. We would like to thank to Dr. Maria Jose Figueras from the Universitat Rovira i Virgili and Dra. Miroslava Sánchez from the Laboratorio de Salud Pública for kindly providing isolates.

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