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Diagnosis Of Motile Aeromonas Sobria From Cat Fishes With Septicemia By PCR

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Abstract : *Aeromonas septicemia is a life threatening bacterial infection in fishes, concurrently in human with weakened immune systems. Present research work aims to isolate and identify the virulent strains of motile Aeromonas species linked with the septicemia in 12 fresh water cat fishes (Clarius batrachus) in the locality of Sathyamangalam, Tamil Nadu. A total of 72 samples consisting of intestinal contents, spleen, kidney, gills, liver and skin were collected aseptically from the infected cat fishes. Upon detailed bacteriological investigation, 56(77.78%) isolates were tentatively identified as A. sobria. All these isolates were further screened for hemolysin gene (ahh1), A. hydrophila aerolysin gene (area), A. sobria hemolysin gene (asa1) and A. caviae hemolysin gene (cav1) by polymerase chain reaction. In PCR, 43(76.78%) of 56 isolates, were found positive for single asa1 gene, while, 3(5.35%) were also positive for both asa1 and ahh1 genes. None of the isolates were positive for aerA and cav1 genes. PCR result suggested the presence of virulence genes in 46(82.14%) isolates of A. sobria. The asa1 gene has proven to be the predominant virulence factor in A. sobria associated with Aeromonas septicemia in cat fish. PCR based identification of virulent strains of A. sobria appeared to be very useful, sensitive and less time consuming method than traditional microbiological identification system*.
Keywords : *Aeromonas sobria, Clarius batrachus, hemolytic, PCR, septicaemia*

I. INTRODUCTION

Aeromonas septicemia is a fatal infectious disease of cold-blooded animals like fish, reptiles, amphibians [1] and in human [2], often caused by the motile *Aeromonas*, particularly *A. hydrphila*, *A. sobria* and *A. caviae*. These organisms have also been implicated as primary pathogens in cases of acute diarrheal disease in immunocompetent humans of all age groups around the world [3]. Motile *Aeromonas* are aerobic, Gram negative, rod-shaped, bacteria, commonly isolated from a variety of aquatic environments, including freshwater, estuarine, brackish, and salt waters. These organisms have also been isolated from a variety of foods, including red meats (beef, pork, lamb), poultry, produce, fish, and shellfish. These bacteria are observed to spread through water into the neighbouring states and cause significant loss to fish farmers. The septicemia in fresh water fishes predominantly in cat fishes is mostly found in farms where trash fish is used as fish feed or in multiple cultures system where motile *Aeromonas* is involved [4].

The occurrence of septicemia by these bacteria is mainly contributed by the release of two important virulence factors namely extracellular hemolysin and aerolysin [5, 6]. Although, these virulence factors are produced by different species of motile Aeromonas, single isolates often carry the genes encoding multiple virulence factors and therefore, it is now very essential to generate a protocol for screening such virulent strains from the clinical cases of septicemia. In recent years, polymerase chain reaction has been used to detect the toxin genes and to identify causative microorganisms from the clinical cases of abdominal dropsy from fresh water fishes [7] and motile *Aeromonas* septicaemia from cat fishes [8] from India and also from other countries. Present study is therefore focused to isolate and identify the virulent strains of *Aeromonas* from the cases of septicemia from fresh water cat fishes.

II. MATERIAL AND METHOD

2.1 Sample details

A total of 12 fresh water cat fish (*Clarias batrachus*) of body weighed ranging from 320g to 355g showing the symptoms of *Aeromonas* septicemia were captured during the months of September to December 2012 by casting net from Kodivery and Bhavani Sagar Dam of Sathyamangalam, Tamil Nadu. Infected fishes were thoroughly examined and any macroscopic and gross lesions observed were recorded. Almost in every case, post-mortem was performed within one to two hours duration after death. Samples were collected aseptically from intestinal contents, spleen, kidney, gills, liver and skin for microbial investigation.

2.2 Isolation and identification of motile Aeromonas

Clinical samples were aseptically inoculated in sterile Brain heart infusion broth (Himedia, Mumbai) and incubated aerobically at 30°C for 24h. Bacterial cultures from BHI broth were inoculated in Aeromonas selective agar (Himedia, Mumbai) and Aeromonas dextrin agar (Himedia, Mumbai) supplemented with specific nutrient supplements and incubated at 30°C for 24h. Suspected bacterial colonies were purified based on the size, shape, colour and patterns of hemolysis and non hemolysis on 5% sheep blood agar and were subjected to Gram's and flagella staining. All the isolates were identified based on the indole production, esculin hydrolysis, Voges–Proskauer test, cytochrome oxidase, catalase test, acid production from L-arabinose, lactose, sucrose, salicin, m-inositol, D-manitol, triple sugar iron agar slant test for gas and acid production from glucose, sucrose and lactose and hydrogen sulphide production [9].

2.3 Oligonucleotide primers for PCR

Isolates of motile *Aeromonas* were screened with *A. hydrophila* extracellular hemolysin gene (*ahh*1), *A. hydrophila* aerolysin gene (*aer*A), *A. sobria* hemolysin gene (*asa*1) and *A. caviae* hemolysin gene (*cav*1) by PCR. The specific forward and reverse primer pairs for *ahh*1 genes of 130bp were 5′-gccgagcgcccagaaggtgagtt-3′ and 5′-gagcggctggatgcggttgt-3′ [3], *aer*A gene of 309bp were 5′-caagaacaagttcaagtggcca-3′ and 5′-acgaaggtgtggttccagt-3′ [3], *asa*1 gene of 249bp were and 5′-taaagggaaataatgacggcg-3′ and 5′-ggctgtaggtatcggttttcg-3′ [3] and *cav*1 gene of 381bp were 5′-gagccagtcctgggctcag-3′ and 5′-gcattcttcatggtgtcggc-3′ [10] were commercially synthesized (Eurofins Genomics India Pvt. Ltd., Bangalore). *A. sobria* (MTCC 3613) and *Escherichia coli* (MTCC 723) were used as a positive and negative controls respectively.

2.4 PCR condition

Freshly grown bacterial colonies from nutrient agar (Himedia, Mumbai) plates were suspended in 200µl of Milli-Q water in a micro centrifuge tube, gently vortexed and boiled for 15 min in a water bath, after centrifugation at 10000 g for 5 min, supernatant was used as a template DNA. The amplification was carried out in 25µl reaction volume containing 12.5 µl of 2× PCR master mix (Promega, USA) containing 4mM magnesium chloride, 0.4 mM of deoxynucleotide triphosphates (dNTPs), 0.5U of *Taq* DNA polymerase, 150mM trishydrochlroric acid, pH 8.5 (Promega, USA), 2µM of primers (ahh1-F and ahh1-R), 1.5µM of primers (aerA-F and aerA-R; asa1-F and asa1-R) and 1µM of primers (ahh1-F and ahh1-R) and 2.5µl of template DNA. The PCR reactions were performed in thermal Cycler (Eppendorf, USA). After initial denaturation at 94°C for 5 min, the amplification cycle (35×) for *ahh*1, *aer*A and *asa*1 genes had denaturation, annealing and extension at 94°C, 59°C and 72°C for 30s, 30s and 30s respectively, while amplification cycle (30×) for *cav*1 gene had denaturation, annealing and extension at 94°C, 65°C and 72°C for 2 min, 1 min and 1 min respectively. In each PCR, final extension was performed at 72°C for 10 min.

2.5 Agarose gel electrophoresis

The PCR amplicons (5µl) were electrophoresed in 1.5% agarose gel in TAE (tris-acetic acid-EDTA, pH 8) buffer, stained with ethidium bromide (0.4 µg/ml) and observed under gel doc system (Universal Hood, BIORAD, Italy).

III. RESULTS

Most of the bacterial colonies were found small and medium sized, mucoid and deep green or greenish yellow in colour in Aeromonas selective agar, whereas, medium sized, mucoid, dark yellow, with pigmentation or light silver in colour in Aeromonas dextrin agar after 24hr of incubation at 30°C. On 5% sheep blood agar, 45(80.35%) *Aeromonas* isolates were found β-hemolytic, whereas, 11(19.64%) isolates were found non hemolytic. All the isolated bacteria were motile, Gram negative, rod shaped, flagellated, positive for indole, catalase, oxidase, produced gas from glucose but did not hydrolyze esculin (Fig. 1). Other biochemical tests were found negative for all the isolate tested. All the biochemical tests were repeated two to three times to eliminate false results. Upon detailed bacteriological investigation, 56(77.78%) isolates were tentatively identified as *A. sobria*.

In PCR, 43(76.78%) of 56 isolates showed positive amplification for *asa*1 gene resulting 249bp fragment (Fig. 2). But 3(5.35%) of 56 isolates were also positive for both *asa*1 gene of 249bp and *ahh*1 genes of 130bp (Fig. 3). Other genes specific primers did not produce any desired amplification.

IV. DISCUSSION

Infected fishes had swimming abnormalities, pale gills, bloat and skin ulcerations prior to death and were thoroughly examined for gross lesions in the body which suggested the *Aeromonas* septicaemia [11, 8]. On blood agar plates, both beta hemolytic and non hemolytic colonies showing similar to the morphology of *Aeromonas sp.* were picked up and purified by repeated streaking and restreaking on fresh blood agar media

plates until the pure cultures were obtained [8]. Isolates were found to be motile and flagellated, Gram negative, rod shaped positive for indole, catalase, oxidase tests, produced gas from glucose but did not hydrolyze esculin. Upon detailed bacteriological investigation, 56(77.78%) isolates were tentatively identified as motile *A. sobria* which can be differentiate from other *Aeromonas sp.* as it can produce gas from glucose but does not hydrolyze esculin [12], whereas, other *Aeromonas sp.* produce gas from glucose and hydrolyze esculin. Similar identification protocols for identification of *Aeromonas sp.* were also followed by other researcher [9, 8]. Of the 56 isolates, 11(19.64%) isolates were found non hemolytic and remaining 45(80.35%) were found β-hemolytic on 5% sheep blood agar. The reproducibility of the hemolysis data for all isolates was demonstrated in triplicate on agar plate assays containing sheep blood, and in every cases, the production of hemolysin were found clear and improved after 72h of incubation at 4°C.

Out of 56 isolates, 43(76.78%) isolates were found positive for single *asa*1 gene of 249bp fragment (Figure 1), while, 3(5.35%) isolates were positive for both *asa*1 and *ahh*1 gene of 130bp fragment (Figure 2). None of the isolates were positive for *aer*A and *cav*1 genes. Similar detection of *asa*1 and *ahh*1 genes from the isolate of *Aeromonas* by PCR has been reported earlier [13, 3]. PCR result suggested the presence of virulence genes in 46(82.14%) isolates of *A. sobria*. The three isolates which were positive for both *asa*1 and *ahh*1 genes were also β-hemolytic in 5% sheep blood agar. However, among the 43 isolates positive for single *asa*1 gene, 40(93.02%) were β-hemolytic in 5% sheep blood agar, whereas, 2(4.65%) were non hemolytic in the blood agar. In view of the present result, Wang *et al.* [3] has been reported that the isolates which were phenotypically tested negative for hemolysin production, eventually found positive for that gene by PCR, indicating that other factors affect gene expression. Researchers have suggested that many enzymes such as lipase, PLC, protease, and RNase are putative virulence factors for *Aeromonas sp*. [14, 15] and these factors may interfere in hemolysin production, even hindered by a number of environmental factors or even growth conditions. It is possible that the non hemolytic isolates carried hemolysin genes either that could not be expressed or that had mutations affecting domains responsible for the hemolytic phenotype [3]. In the present study, it has also been observed that 2 of 45(4.44%) isolates of *A. sobria* did not amplify any of the hemolysin genes tested in the PCR but showed weak hemolytic phenotypic activity, suggesting that other virulence traits exist. Findings also suggested that 3 of 56(5.35%) isolates were carrying genes for two toxins and might be more virulent than the other isolates carrying single gene. In compare to our earlier study [8], the detection of single and multiple toxin genes (*ahh*1 and *aer*A) was possible only from the isolates of *A. hydrophila* in association with *Aeromonas* septicemia in cat fishes. In this regard, present report is very noteworthy in establishing the involvement of motile *A. sobria* from the clinical cases of *Aeromonas* septicemia in cat fishes. PCR results also suggested that *asa*1 gene is the predominant virulence factor in *A. sobria* might play a significant role in association with *Aeromonas* septicemia in cat fish. The *asa*1 gene specific PCR described in this study might proven to be a useful method for the identification of *A. sobria* when screening for the major species-specific isolates of *Aeromonas* species is carried out from the clinical cases of septicemia in fishes.

V. CONCLUSION

Present research investigation suggested that a systematic evaluation of motile *Aeromonas* virulence requires the assessment of virulence phenotypes and complete virulence gene from the clinical isolates. The phenotypic methods may not detect the presence of toxins. The *asa*1 gene might be predominant virulence factor among the isolates of motile *A. sobria* associated with *Aeromonas* septicaemia in cat fish. PCR based identification of virulent strains of *A. sobria* appeared to be very useful, sensitive and less time consuming method than traditional microbiological identification system.

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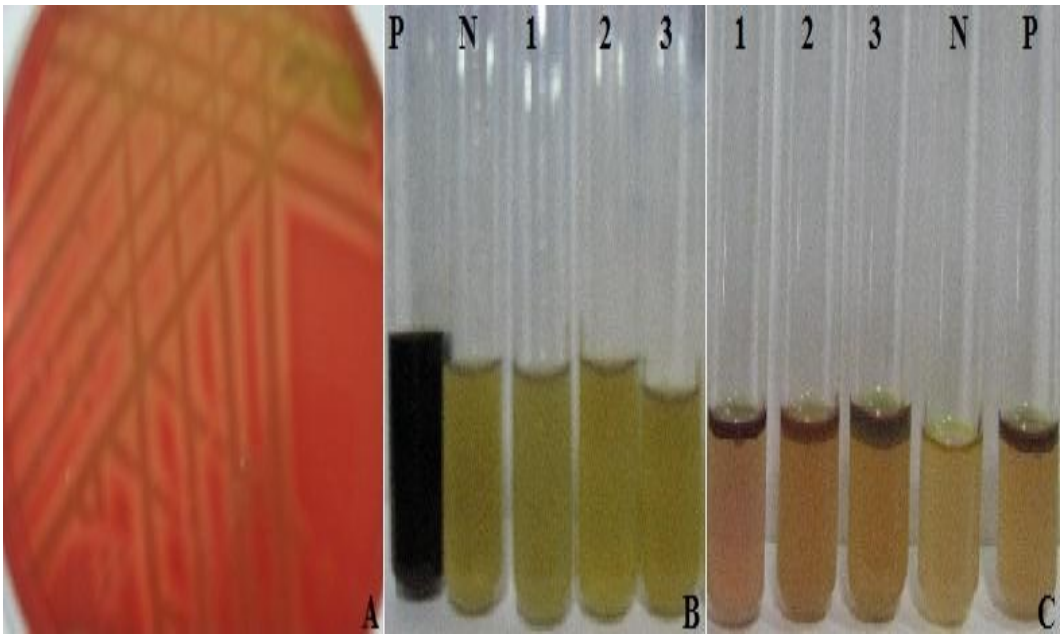

Figure 1: Morphological and biochemical tests of *A. sobria*

A: hemolysis of *A. sobria* on blood agar; B: esculin test (P: positive control [*A. hydrophila*, MTCC 646], N: negative control [*A. sobria* (MTCC 3613), tubes 1-3: *A. sobria* field isolates negative for esculin hydrolysis; C: indole test (P: positive control [*A. hydrophila*, MTCC 646], N: negative control, tubes 1-3: *A. sobria* field isolates positive for indole production

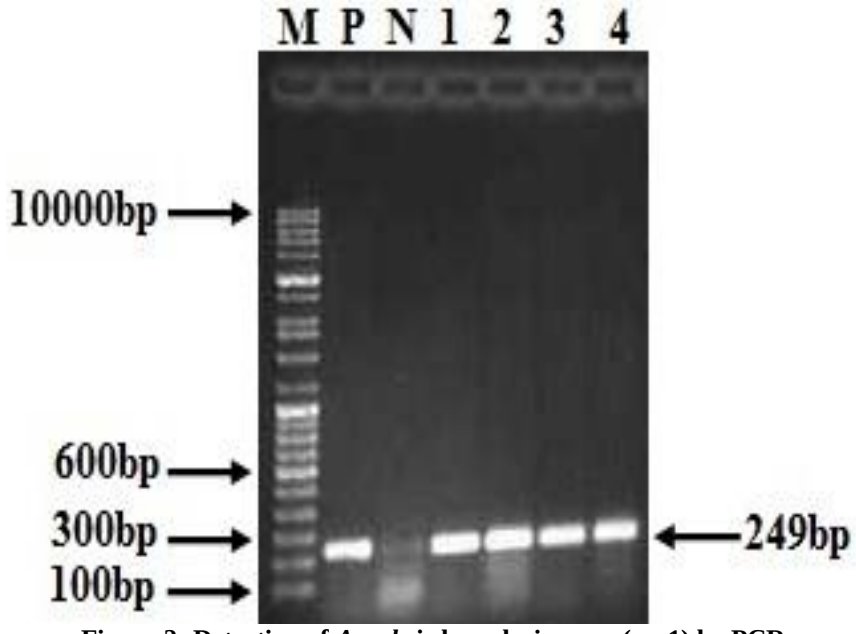

Figure 2: Detection of *A. sobria* hemolysin gene (*asa*1) by PCR
P: *A. sobria* (MTCC 3613) as Positive control; N: *E. coli* (MTCC 723) as Negative control; Lanes 1-4: Field isolates of *A. sobria*; M: High range DNA ladder (Genei, Bangalore)

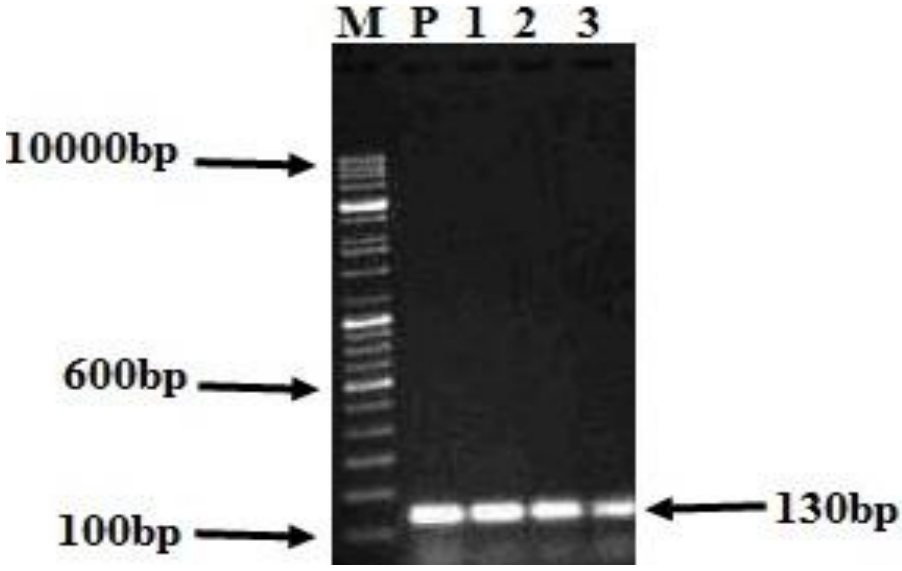

Figure 3: Detection of hemolysin gene (*ahh*1) of *A. sobria* by PCR
P: *A. sobria* (MTCC 3613) as Positive control; Lanes 1-3: Field isolates of *A. sobria*; M: High range DNA ladder (Genei, Bangalore)