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Detection of *Aeromonas* sp. from Chicken and Fish Samples by Polymerase Chain Reaction

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Abstract: A polymerase chain reaction was standardized for the identification of *Aeromonas* sp. using primers against 16S rRNA gene and aerolysin gene. The primers used were found to be highly specific for *Aeromonas* sp. and did not give positive result with other bacteria including other Gram positive and Gram-negative bacteria. The minimum detection level of PCR was found to be 10^2 and 10^4 cells mL^{-1} in case of 16S rRNA and aerolysin gene targeted assay, respectively. Suitability of the enrichment broth (Alkaline peptone water-cephalothin, APW-C) when tested to detect *Aeromonas* from the spiked samples gave good results on direct usage of the broth for template preparation without any subsequent treatment. The kinetics of the spiking study indicated that a minimum of 24 h enrichment was required for the detection of *Aeromonas* by cultural and PCR method. Among two PCR assays detection limits achieved by PCR targeting16S rRNA gene were better than aerolysin gene PCR assay. The results were comparable to cultural method. A total of 100 samples comprising of 50 each of chicken and fish samples were screened by cultural and PCR methods for the presence of *Aeromonas*. Two chicken samples and three fish samples turned out to be positive by both cultural method and PCR targeting 16S rRNA. From this study it was concluded that PCR assay targeting 16S rRNA gene can be used for the rapid detection of *Aeromonas* from chicken and fish samples after one step enrichment in APW-C.

Key words: *A. hydriphila*, fish, meat, sensitivity, specificity

Introduction

The genus *Aeromonas* is widely distributed in aquatic environment and increasingly reported as a primary pathogen of human and lower vertebrates. *Aeromonas* has been reported as an etiological agent in a variety of human infections including gastroenteritis and extra intestinal infections (Janda and Abbott, 1998). In India, Aeromonads are common contaminants in a wide spectrum of foods namely fishes and other seafoods, raw and cooked meat, poultry, vegetables, milk and milk products (Agarwal *et al*., 2000; Bachhil *et al*., 2002; Khurana and Kumar, 1997). These foods play an important role in the dissemination of the pathogenic *Aeromonas* to humans. The traditional classical microbial procedures for the detection of *Aeromonas* sp. are laborious and time consuming or do not allow quantitative assessment of there organisms (ICMSF, 1996). In this context the detection of the pathogen requires rapid and specific methods, which assist in the control of pathogenic organisms from various food sources to human population, which are mostly regarded as the main transport vector to human population. Although PCR is very effective with pure culture, its application to food samples is limited by the complex composition of food matrices that can inhibit the assay. In addition, PCR cannot differentiate the DNA from live cells or dead cells. To overcome these problems, enrichment of sample and a sample preparation step prior to the PCR analysis is necessary.

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Keeping in view the above points this study was envisaged to develop a rapid, sensitive and specific PCR based assay to detect *Aeromonas* from chicken and fish samples and also to assess the comparative efficacy of PCR assay vis-a-vis conventional isolation and identification method for detection of *Aeromonas* from artificially and naturally contaminated chicken and fish samples.

Materials and Methods

Bacterial Strains

The bacterial strains used in this study are listed in Table 1.

Oligonucleotide Primers

The primers for conserved regions 16S rRNA gene (Graf, 1999) and aerolysin gene (Santos *et al*.,1999) of *Aeromonas* used in this study were got synthesized from Bangalore Genei, Bangalore. The details of the primers used are as follows:

Primer set No.		Product size
	16 S rRNA (Graf, 1999)	
1. Forward primer	5'-TCATGGCTCAGATTGAACGCT-3'	
Reverse primer	5'-CGGGGCTTTCACATCTAACTTATC-3'	599 bp
	Aerolysin gene (Santos *et al.*, 1999)	
2. Forward primer	5'-GCAGAACCCATCTATCCAG-3'	
Reverse primer	5'-TTTCTCCGGTAACAGGATTG-3'	252 bp

Standardization of Polymerase Chain Reaction

Template DNA Preparation

Three different methods of cell lysis and release of DNA as described below were compared for their use in PCR assay.

Sodium Dodecyl Sulphate (SDS) Treatment

About 1 mL of the overnight incubated culture of *Aeromonas* was centrifuged and the pellet was suspended in about 20 µL of 0.05% SDS and heated in a boiling water bath for 5 min and snap chilled. From this about 5 µL was used as template in PCR.

Sonication

About 1 mL of the overnight incubated culture of *Aeromonas* was centrifuged at 5000 g for 20 min and the pellet was resuspended in distilled water, subjected to sonication (MSE Sanyo Sonicator) 10 µ amplitude, 60 sec/cycle, 5 cycles and was used in PCR.

Boiling and Chilling

In this method about 0.5-1.0 mL of the overnight-incubated culture of *Aeromonas* was subjected to vigorous heating in a boiling water bath for 10 min and then snap chilled. From this about 5 µL was used as template in PCR.

PCR Protocol

The PCR was set up in 50 µL reaction volume. The reaction mixture was optimized as follows 5.0 µL of 10× Taq DNA polymerase buffer, 1.5 mM $MgCl_2$ (3 µL), 0.22 mM of dNTP mix (6 µL), 10 pM of forward and reverse primer (2 µL each), 1U of Taq DNA polymerase, 5 µL of cell lysate. The cycling conditions included an initial denaturation at 94°C for 5 min followed by 30 cycles each of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min elongation at 72°C. It was followed by a final extension for 5 min at 72°C. The samples were analysed in 1.5% agarose gel electrophoresis with ethidium bromide.

Table 1: List of bacterial strains

Name of the organism, serotype and code	Source
Aeromonas hydrophila (MTCC 646)	IMTECH, Chandigarh
Aeromonas caviae	VPH Division, IVRI
Aeromonas sobria	do
Aeromonas jandeai	VPH Division, IVRI
Escherichia coli (MTCC 443)	-do
Salmonella dublin	-do
Salmonella arizonae	-do
Vibrio cholerae	IMTECH, Chandigarh
Klebsiella sp.	-do
Bacillus cereus (MTCC 1272)	-do
Streptococcus faecalis (MTCC 439)	-do
Rhodococcus equi (MTCC 1135)	-do-
Staphylococcus aureus	-do
*L. monocytogenes*1/2b (NCTC 10867)	PHLS London
L. monocytogenes 4b (NCTC 11994)	-do
L. innocua (NCTC 11288)	-do
L. welshimeri (NCTC 11857)	-do

Specificity of PCR Assay

The specificity of primer set No. 1 (16S rRNA) and set No. 2 (aerolysin gene) were tested with different Gram positive and Gram-negative bacteria as listed in Table 1

Sensitivity of the PCR

Trials were conducted to evaluate the lowest quantum of bacterial cells that would produce a visible signal by the PCR assay. The standard strain of *Aeromonas* (MTCC 646) was inoculated in BHI broth and grown overnight at 37°C. The cells were pelleted by centrifugation at 6000 rpm for 10 min and the cell concentration was adjusted to an approximate concentration 10^8 cells mL^{-1} based on spectrophotometer absorbance reading of 0.6 at 540 nm and then ten fold serial dilutions were made and subjected to PCR assay according to standardized protocol. The total viable count (log cfu mL^{-1}) of the bacterial suspension was confirmed by spread plate method.

Evaluation of Enrichment Broth

Suitability of the enrichment broth (APW-C) was assessed to test whether any ingredient of medium will hinder the PCR. The organism was grown overnight in the broth and1 ml aliquots was taken in triplicate from the broth and were subjected to three different treatments as described in Table 2 before it was used in PCR.

Experimental Inoculation/spiking Studies

The experimental inoculation studies were carried out to assess the efficacy of the standardized PCR method for the detection of *Aeromonas* from the chicken meat samples after one-step enrichment of samples. The meat samples (chicken) were collected randomly from different parts of the carcass from the local markets (Retail outlets) in and around Barielly. Adjustment of concentration to 1×10^8cells mL^{-1} and further serial ten fold dilutions of standard *Aeromonas* culture was done as described above. The spiking of meat samples was carried out as shown in flow diagram (Fig. 1). The experimental inoculation of chicken meat and subsequent identification of *Aeromonas* was repeated five times to ascertain the repeatability of the cultural and PCR results Table 3.

Detection of Aeromonas from Natural Samples

A total of 100 samples comprising of 50 meat (chicken) and 50 fish samples (gills) were purchased from the local market (Retail outlets) of Barielly. The samples were subjected for isolation/ detection of *Aeromonas* by cultural method as well as PCR method as described above, after one-step enrichment.

Table 2: Suitability of enrichment broth for detection of *Aeromonas* from foods

Treatments	16S rRNAgene targeted PCR	Aerolysin gene targeted PCR
Without any treatment	+	+
Pellet washed once with PBS	+	+
Pellet washed twice with PBS	+	+

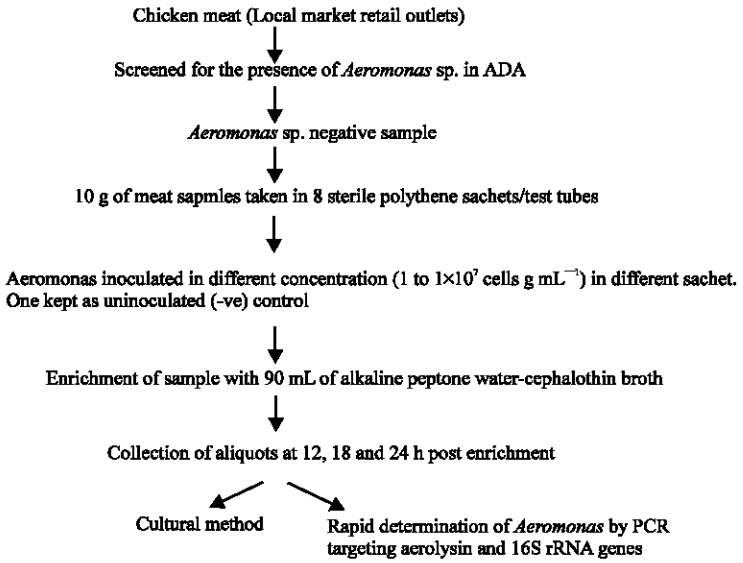

Fig. 1: Flow diagram for experimental inoculation studies

Results and Discussion

Comparison of different protocols for DNA extraction revealed that sonication and heat lysis method gave a clear-cut amplicon of 599 and 252 bp length product on PCR assay targeted for 16S rRNA and aerolysin genes, respectively. Template prepared by lysis buffer also gave positive amplicon but it was lesser in intensity than other methods. Among snap chilling after boiling and sonication method, the former method was used in all subsequent experiments of study as it was found to be very simple, rapid and gave good results. Some previous workers have also successfully used this method for the release of DNA (Gonazalez-Rodriguez *et al*., 2002; Surendran *et al*., 2003). The PCR protocol was standardized by optimizing annealing temperature, primer, $MgCl_2$ concentrations, template volume and cycling conditions. Electrophoretic analysis of the PCR product revealed the specific amplification of 599 and 252 bp fragments without any spurious product for both the primers targeted against 16S rRNA and aerolysin genes (Fig. 2). The specificity of the standardized PCR assay was tested by subjecting to different Gram positive and Gram-negative organisms (Table 1) to PCR assay. Only *Aeromonas* cultures yielded a specific PCR product of desired length i.e., 599 and 252 bp. These findings are in confirmation with the results obtained by Gonazalez-Rodriguez *et al*. (2002) who have reported no cross-reactions with other bacteria using the primer against aerolysin gene. These findings are in confirmation with the results obtained by Gonazalez-Rodriguez *et al*. (2002). The sensitivity of the PCR assay was evaluated by subjecting serial ten fold dilutions of pure culture of *Aeromonas* ranging from 10^9 to 10 cells mL^{-1}. The minimum detection level was found to be 10^2 cells mL^{-1} for 16S rRNA gene and 10^4 cells mL^{-1} for aerolysin gene (Fig. 3 and 4). Similar sensitivity of PCR has been reported in literature (Khan and Cerniglia, 1997; Gonazalez-Rodriguez *et al*., 2002). Better detection limits with PCR for 16S rRNA gene than aerolysin gene PCR

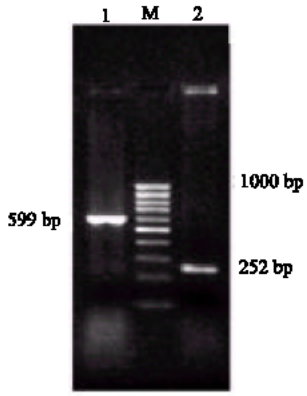

Fig. 2: Standardization of PCR for detection of *Aeromonas*. Lane M: 100 bp ladder, Lane 1: Product obtained with primer for 16S rRNA gene, Lane 2: Product obtained with primer for Aerolysin gene

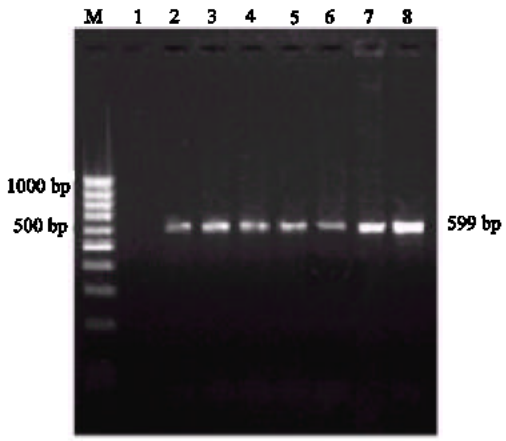

Fig. 3: Minimum detection levels of *Aeromonas* cells by using primer for 16S rRNA gene. Lane M: 100 bp DNA ladder plus, Lane 1: 10 cells mL^{-1}, Lane 2: 10^2 cells mL^{-1}, Lane 3: 10^3 cells mL^{-1}, Lane 4: 10^4 cells mL^{-1}, Lane 5: 10^5 cells mL^{-1}, Lane 6: 10^6 cells mL^{-1}, Lane 7: 10^7 cells mL^{-1}, Lane 8: 10^8 cells mL^{-1}

may be attributed to the multiple copies of ribosomal RNA that are present in a single bacteria (Rahman, 2002, Gonzalez-villasenor and Manak, 1998). It has been previously reported that certain media components inhibit PCR reaction (Rossen *et al*., 1992). Hence suitability of enrichment medium viz., APW-C and three different treatments for extraction and concentration of target organisms in small volume were tested. The results revealed that all the three treatments yielded PCR products of equal intensity indicating no PCR inhibitory substance was present in the medium. Our results are in agreement with previous findings of (Sachan and Agarwal, 2000) for the isolation of this organism.

Spiking studies in meat (chicken) were done to determine the ideal enrichment protocol, which could detect the least concentration of inoculum at the earliest by PCR assay and also compare the results with that of cultural method. As reported by several workers (Wang *et al*., 1997; Boer and Beumer, 1999; Olsen, 2000), we opted for an enrichment step prior to PCR to avoid the possibility

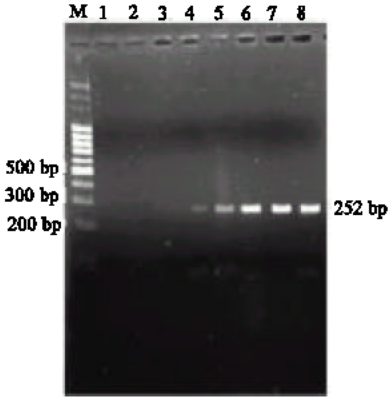

Fig. 4 : Minimum detection levels of *Aeromonas* cells by using primer fsor. Aerolysin gene, Lane M: 100 bp DNA ladder plus, Lane 1: 10 cells mL^{-1}, Lane 2: 10^2 cells mL^{-1}, Lane 3: 10^3 cells mL^{-1}, Lane 4: 10^4 cells mL^{-1}, Lane 5: 10^5 cells mL^{-1}, Lane 6: 10^6 cells mL^{-1}, Lane 7: 10^7 cells mL^{-1}, Lane 8: 10^8 cells mL^{-1}

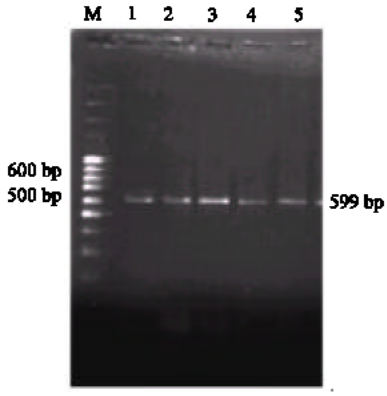

Fig. 5: PCR result for screening of natural samples against 16S rRNA gene. Lane M: 100 bp DNA ladder plus, Lane 1: Meat isolate, Lane 2: Meat isolate, Lane 3: Fish isolate, Lane 4: Fish isolate

Table 3: Results of detection of *Aeromonas* from spiked meat samples

	Enrichment time								
	12 h			18 h			24 h		
	PCR method			PCR method			PCR method		
Aeromonas conc. g^{-1} of meat	16 S rRNA	Aerolysin cultural	Cultural method	16S rRNA	Aerolysin cultural	Cultural method	16S rRNA	Aerolysin cultural	Cultural method
1 cell g^{-1}	1/5	0/5	1/5	1/5	0/5	2/5	2/5	0/5	2/5
10 cells g^{-1}	2/5	0/5	2/5	2/5	0/5	2/5	3/5	1/5	3/5
10^2 cells g^{-1}	2/5	1/5	1/5	2/5	1/5	2/5	3/5	2/5	3/5
10^3 cells g^{-1}	4/5	2/5	4/5	4/5	2/5	4/5	5/5	3/5	4/5
10^4 cells g^{-1}	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
10^5 cells g^{-1}	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
10^6 cells g^{-1}	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
10^7 cells g^{-1}	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Negative control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Numerator-No. of positive. Denominator-No. of trials

of false negative result. In order to further increase the validity and reproducibility of the experimental inoculation studies, a total of five trials were conducted as shown in Table 3. The results of detection of *Aeromonas* from spiked samples by cultural method revealed that 10^3 cells g^{-1} (4 out of 5) were positive from the post enrichment period of 12 and 18 h, whereas only some samples showed positive results from the samples spiked with the lower concentration (<10^2cells g^{-1}) at both 12 and 18h enrichment period. On further incubation at 24 h, the detection limit of the assay was increased and it was able to detect samples (3 out of 5) with initial concentration of upto 10 cell g^{-1} of meat, whereas only 2 out of 5 samples spiked with 1 cell g^{-1} of *Aeromonas* showed positive results on cultural examination. The results of detection of *Aeromonas* from the spiked samples by PCR indicated the superiority of PCR assay using primer for 16S rRNA over PCR assay targeting aerolysin gene. A good correlation was observed between results, PCR assay with 16S rRNA and cultural method.

From the kinetics study in milk samples it was observed that at 12 and 18 h enrichment the detection limits were 10^3 and 10^4 cells mL^{-1} for 16S rRNA and aerolysin gene, respectively. Enrichment for up to 24 h increased the detection limits of the 16S rRNA PCR assay, where it was able to detect 3 samples out of 5 which were spiked with as less as 10 cells mL^{-1}, whereas the detection limits remained same incase of aerolysin gene targeted PCR. Inconsistent results from the samples spiked with lower concentrations of *Aeromonas* sp. by both PCR and cultural method, may be attributed to the over growth of other competing micro flora (Lee *et al*., 1990). Similar detection limits from spiked food samples had been previously reported (Khan and Cerniglia, 1997; Gonzalez-Rodriguez *et al*., 2002).

Based on the results obtained from experimental inoculation/spiked studies, natural samples were screened for the *Aeromonas* sp. 50 chicken and 50 fish samples were screened for the presence of *Aeromonas*. Two samples from 50 meat and three samples from 50 fish samples turned out to be positive by cultural and PCR method targeting 16S rRNA (Fig. 5). This study of detection of *Aeromonas* from natural samples by cultural and PCR method has substantiated the results obtained from the spiking studies. A good correlation was observed between the cultural method and 16S rRNA targeted PCR assay. Thus from the study it is concluded that polymerase chain reaction targeting 16S rRNA can be used for rapid detection of *Aeromonas* from meat (chicken) and fish samples after single step enrichment in APW-C for 24 h.

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