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PCR-Based Detection of *Aeromonas* from Milk Samples

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Abstract: A set of primers targeting 16S rRNA gene and aerolysin gene was employed to standardize PCR assay for detection of *Aeromonas* from milk samples. The primers used were found to be highly specific for *Aeromonas* spp and did not give positive result with other Gram positive and Gram-negative bacteria. The minimum detection level of PCR was found to be 10^2 cells mL^{-1} and 10^4cells 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 hrs 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. Examination of 50 milk samples(Pooled samples) revealed two samples to be positive by cultural method and PCR targeting 16S r RNA.Thus the standardized single –step enrichment PCR protocol holds promise as a reliable and rapid method for the detection of *Aeromonas* from milk samples.

Key words: *A. hydriphila*, milk, sensitivity, specificity

INTRODUCTION

Mesophilic aeromonads, the gram-negative bacteria of ubiquitous nature, are considered emerging food borne pathogen[1]. The genus *Aeromonas* has been reported as an etiological agent in a variety of human infections including gastroenteritis and extra intestinal infections[2]. In India, Aeromonads are common contaminants in a wide spectrum of foods namely fishes, raw and cooked meat, poultry, vegetables, milk and milk products[3,4]. These foods play an important role in the dissemination of the potentially pathogenic *Aeromonas* to humans. Conventionally, *Aeromonas* sp. is isolated by time and labour intensive cultural methods. In this context the detection of the pathogen requires rapid and specific methods, which assist in the control of potentially pathogenic organisms from various food sources 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.

Keeping in view the above points this study was envisaged to develop a rapid, sensitive and specific PCR based assay to detect *Aeromonas* from milk 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 milk 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 16 S rRNA gene[5] and aerolysin gene[6] of *Aeromonas* used in this study were got synthesized from Bangalore Genei, Bangalore.

S.No.	Name of the organism, serotype and code	Source
1.	*Aeromonas* hydrophila (MTCC 646)	IMTECH, Chandigarh
2.	*Aeromonas* caviae	VPH Division, IVRI
3.	*Aeromonas* sobria	do
4.	*Aeromonas* jandeai	VPH Division, IVRI
5.	*Escherichia coli* (MTCC 443)	-do
6.	*Salmonella dublin*	-do
7.	*Salmonella arizonae*	-do
8.	*Vibrio cholerae* IMTECH, Chandigarh	
9.	*Klebsiella* sp.	-do
10.	*Bacillus cereus* (MTCC 1272)	-do
11.	*Streptococcus faecalis* (MTCC 439)	-do
12.	*Rhodococcus equi* (MTCC 1135)	PHLS,London
13.	*Staphylococcus aureus*	-do
14.	*L. monocytogenes*1/2b (NCTC 10867)	-do
15.	*L. monocytogenes* 4b (NCTC 11994)	-do
16.	*L. innocua* (NCTC 11288)	-do
17.	L. welshimeri (NCTC 11857)	-do

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The details of the primers used are as follows:

Primer set No.	16 S rRNA[5]	Product size
1. Forward primer	5'-TCATGGCTCAGATTGAACGCT-3'	599bp
Reverse primer	5'-CGGGGCTTTCACATCTAACTTATC-3'	
	Aerolysin gene[6]	
2. Forward primer	5'-GCAGAACCCATCTATCCAG-3'	252bp
Reverse primer	5'-TTTCTCCGGTAACAGGATTG-3'	

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 5µL 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 10x Taq DNA polymerase buffer, 1.5mM $MgCl_2$ (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.

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 540nm 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.

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

Treatment	16SrRNA gene targeted PCR	Aerolysin gene targeted PCR
PCRWithout any treatment	+	+
Pellet washed once with PBS	+	+
Pellet washed twice with PBS	+	+

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 and 1 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 in the milk to assess the efficacy of the standardized PCR for the detection of *Aeromonas* from milk after one-step enrichment of samples.

Adjustment of concentration to $1x10^8$cells mL^{-1} and further serial ten fold dilutions of standard *Aeromonas* culture was done as described above .The milk samples (pooled samples) collected randomly from different private dairy farms in and around Bareilly were screened for the presence of *Aeromonas*. Samples, which were - negative for *Aeromonas* were used for experimental inoculation studies. Milk samples (*Aeromonas* free) were taken in eight different tubes with one tube used as negative control. In the remaining tubes different dilutions ($1x10^7$ to 10) of culture were inoculated. After thoroughly mixing the samples 1 mL each was transferred separately to 9 mL of APW-C for enrichment and incubated at 37°C About one mL of the sample was collected at12, 18, 24 hrs of incubation. They were simultaneously subjected to PCR assay and streaked onto ADA plates for cultural confirmation.

Detection of *Aeromonas* from natural samples: A total of 50 milk samples (pooled samples) were collected randomly from different private dairy farms in and around Bareilly. The samples were subjected for isolation/ detection of

Aeromonas by cultural method as well as PCR method as described above, after one-step enrichment.

RESULTS AND DISCUSSION

Comparison of different protocols for DNA extraction revealed that sonication and heat lysis method gave a clear-cut amplicon, whereas template prepared by SDS lysis buffer also gave positive amplicon but it was of lesser in intensity than other methods. Among heat lysis 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[7,8]. 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 bp and 252 bp fragments without any spurious product for both the primers targeted against 16S rRNA and aerolysin genes (Fig. 1). The standardized PCR assay was highly specific and yielded a specific PCR products of desired lengths i.e. 599 bp and 252 bp with all the *Aeromonas* sp. tested in study, whereas none of the non-*Aeromonas* gave any amplification product. These findings are in confirmation with the results obtained by Gonazalez-Rodriguez et al.[7] The sensitivity of the PCR assay was evaluated by subjecting serial ten fold dilutions of pure culture of *Aeromonas* ranging from 10^9

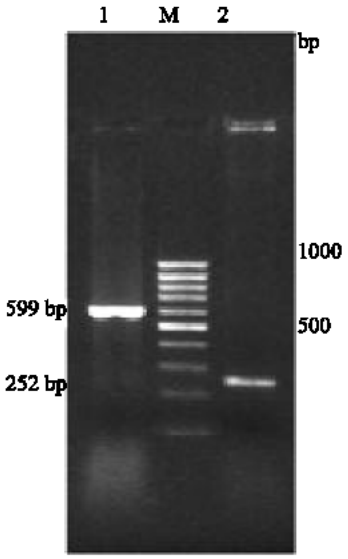

Fig. 1: Standardization of PCR for detection of *Aeromonas*, Lane M: 100 bp ladder, Lane 1: Product obtained with primer for 16 S rRNA gene, Lane 2: Product obtained with primer for Aerolysin gene

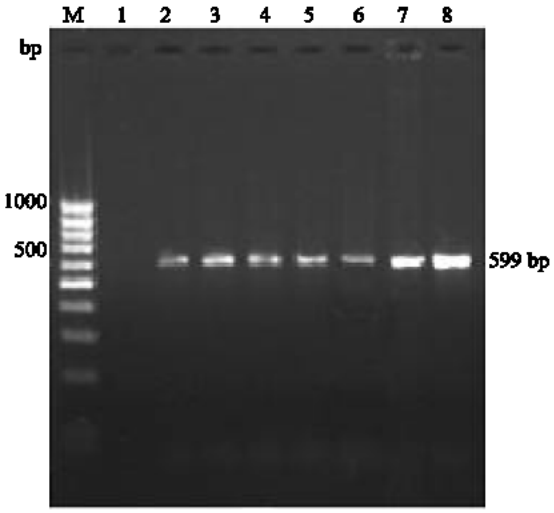

Fig. 2: Minimum detection levels of *Aeromonas* cells by using primer for 16S, rRNA gene, Lane M: 100 bp DNA ladder plus, Lane 1 : 10 cells mL^{-1}, Lane 2: 10^2 cells mL^{-1}, Lane 3: 10^3 cells mL^{-1}, Lane 4: 10^4 cells mL^{-1}, Lane 5: 10^5 cells mL^{-1}, Lane 6: 10^6 cells mL^{-1}, Lane 7: 10^7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

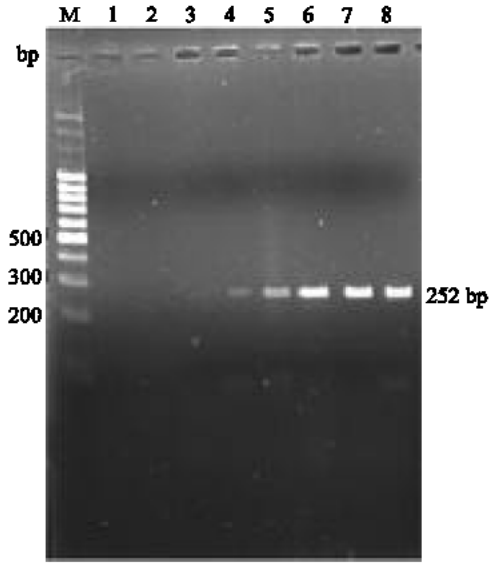

Fig. 3: Minimum detection levels of *Aeromonas* cells by using primer for Aerolysin gene, Lane M : 100 bp DNA ladder plus, Lane 1: 10 cells mL^{-1}, Lane 2: 10^2 cells mL^{-1}, Lane 3: 10^3 cells mL^{-1}, Lane 4: 10^4 cells mL^{-1}, Lane 5: 10^5 cells mL^{-1}, Lane 6: 10^6 cells mL^{-1}, Lane 7: 10^7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

cells mL^{-1} to 10 cells mL^{-1}. The minimum detection level was found to be 10^2 cells mL^{-1} for 16S rRNA gene and

Table 3. Results of detection of *Aeromonas* from spiked milk samples

Sl. No.	*Aeromonas* conc. mL^{-1} of milk	Enrichment Time: 12 hrs, PCR Method: 16 S rRNA gene	12 hrs, PCR Method: Aerolysin gene	12 hrs: Cultural method	18 hrs, PCR Method: 16 S rRNA gene	18 hrs, PCR Method: Aerolysin gene	18 hrs: Cultural method	24 hrs, PCR Method: 16 S rRNA gene	24 hrs, PCR Method: Aerolysin gene	24 hrs: Cultural method
1.	1 Cell mL^{-1}	1/5	0/5	2/5	1/5	0/5	1/5	1/5	0/5	2/5
2.	10 Cells mL^{-1}	1/5	0/5	2/5	1/5	0/5	2/5	1/5	0/5	3/5
3.	10^2 Cells mL^{-1}	2/5	0/5	2/5	2/5	1/5	3/5	2/5	1/5	3/5
4.	10^3 Cells mL^{-1}	3/5	1/5	3/5	3/5	1/5	3/5	4/5	1/5	4/5
5.	10^4 Cells mL^{-1}	4/5	3/5	4/5	4/5	4/5	5/5	5/5	3/5	4/5
6.	10^5 Cells mL^{-1}	5/5	4/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5
7.	10^6 Cells mL^{-1}	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5
8.	10^7 Cells mL^{-1}	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
9.	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

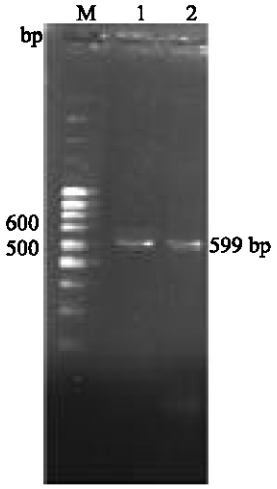

Fig. 4: PCR result for screening of natural samples against 16S rRNA gene, Lane M: 100 bp DNA ladder plus, Lane 1: Milk isolate, Lane 2: Milk isolate

10^4 cells mL^{-1} for aerolysin gene (Fig. 2 and 3). Similar sensitivity of PCR has been reported in literature[7,9]. Better detection limits with PCR for 16S rRNA gene than aerolysin gene PCR may be attributed to the multiple copies of ribosomal RNA that are present in a single bacteria[10,11]. It has been previously reported that certain media components inhibit PCR reaction[12]. 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[13] for the isolation of this organism.

Spiking studies in milk 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[14-16], we opted for an enrichment step prior to PCR to avoid the possibility 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 mL^{-1}(3 out of 5) were positive from the post enrichment period of 12 and 18 hrs, whereas only some samples showed positive results from the samples spiked with the lower concentration (<10^2cells mL^{-1}) at both 12 and 18 hrs 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 upto10 cell mL^{-1} of milk, whereas only 2 out of 5 samples spiked with 1 cell mL^{-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 hrs enrichment the detection limits were 10^3cells mL^{-1} and 10^4cells mL^{-1} for 16S rRNA and aerolysin gene, respectively. Enrichment for up to 24 hrs increased the detection limits of the 16S rRNA PCR assay, where it was able to detect 2 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* spp by both PCR and cultural method, may be attributed to the

over growth of other competing micro flora. Similar detection limits from spiked food samples had been previously reported[7,9].

Based on the results obtained from experimental inoculation/spiked studies, natural samples were screened for the *Aeromonas* sp. 50 milk samples were screened for the presence of *Aeromonas*. Two samples from 50 milk samples turned out to be positive by cultural and PCR method targeting 16S rRNA. (Fig. 4). 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.

CONCLUSIONS

Thus from the study it is concluded that polymerase chain reaction targeting 16S rRNA can be used for rapid detection of *Aeromonas* from milk samples after single step enrichment in APW-C for 24 hrs.

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