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Isolation and PCR Amplification of *E. coli* from Freshwater Fish (*Cirrhinus cirrhosis*) and its PCR Amplification of SHV Gene

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A B S T R A C T

Aquaculture products can harbor pathogenic bacteria which are part of the natural microflora of the environment. Feeding infected fish with antibiotic- medicated food is a general practice but has led to antibiotic resistance development in bacterial pathogen, resulting in a higher dose requirement for effective control, a matter of increasing public concern. Resistance of pathogens to antibiotics occurs due to the presence of β-lactamase enzyme, thereby resulting in Multiple Drug Resistance (MDR). These enzymes are present in *Enterobacteriaceae such as E. coli*. This study was conducted aiming at the isolation of *E. coli* from the intestines, muscle, head and tail of apparently healthy freshwater fish *Cirrhinus cirrhosis* collected from the local dam in Coimbatore district, Tamilnadu. Differentiation and characterization of *E. coli* isolates was based on their growth characteristics on specific culture media. *E. coli* was observed to be present in higher amounts in all parts of the fish. *E. coli* was identified and PCR amplification was performed for the isolated *E.coli*. The Hi Comb assay was also performed.

Introduction

Fish is a vital source of food for people and contributes about 60% of the world's supply of protein. 60% of the developing countries derive 30% of their annual protein from fish. It is man's most important source of high quality protein, providing approximately 16% of the animal protein consumed by the world's population. In Africa, fish supplies 17% of protein and it is one of the cheapest sources of protein in Africa. The advantage of fish as food is as a result of its easy digestibility and high nutritional value. However fish are susceptible to a wide variety of bacterial pathogens, most of which are

capable of causing disease and are considered by some to be saprophytic in nature. According To the microbiological diversity of fresh fish muscle depends on the fishing grounds and environmental factors around it. The non-indigenous contaminate the fish or the habitat one way or the other and examples include *Escherichia coli, Clostridium botulinum, Shigella dynteriae, Staphylococcus aureus, Listeria monocytogens and Salmonella*. *E. coli* is a gram negative rod shaped bacterium. It is most commonly found in lower intestine of warm-blooded organisms. Most *E. coli* stains are harmless,

but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for products recalls due to food contamination. The indigenous bacterial pathogens are found naturally living in the fish's habitat for example *Vibrio* species and *Aeromonas* species. Thampuran *et al.,* (2005) observed the prevalence and characterization of typical and atypical Escherichia coli from fish sold at retail in Cochin, India. The bacteria from fish only become pathogens when fish are physiologically unbalanced, nutritionally deficient, or there are other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to prevail. Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include *Mycobacteium, Streptococcus spp., Vibrio spp., Aeromonas spp., Salmonella spp.* and others (Lipp and Ross, 1997). *Cirrhinus cirrhosis* was used for this study since it is abundant and it is one of the most consuming fishes.

PCR has become a very rapid and reliable tool for the molecular biology-based diagnosis of a variety of infectious disease. PCR has been applied for the detection of microorganisms from microbial cultures and tissues and directly from clinical samples. Conventional methods for the detection and identification of *Escherichia coli* O157:H7 include growth of non-sorbitol-fermenting *E. coli* colonies on sorbitol MacConkey agar culture (SMAC), followed by serological confirmation with O157- and H7-specific antisera. PCR have overcome problems associated with culture-based techniques, enabling the detection of microorganisms directly in clinical samples without the need for previous culturing. Many PCR methods have been developed for the identification of bacterial pathogens in aquacultures. In this study, PCR was performed in order to detect the presence of Multi Drug Resistance gene in the fish pathogen mainly *E. coli.*

*E. coli*is a common disease causing pathogen in freshwater fish especially under cultured conditions (Baya and White1997), and plays a big role in economic losses among fish industry. The study was carried out to isolate the *E. Coli* from freshwater fish (*Cirrhinus cirrhosis*) and to perform the PCR to identify the presence of Multi Drug Resistance gene (MDR) using the specific primer.

Hanson *et al.,* 2008 reported that, the *E. coli* was firstly discovered in the intestine of wild fish. We have isolated the *E. coli* from the muscle and the intestine of the fish. Soliman *et al.,* 2010 has used a standard procedure for the isolation of *E. coli*. In this isolation procedure, Nutrient medium is used for incubation of sample before it is inoculated into the solid medium (Buinn *et al.,* 1994). Here, we have used Mac Conkey agar (Oxoid *et al.,* 1997) as a selective medium for the isolation of *E. coli* which is a member family of *Enteriobactriaceae*.

The isolation of DNA from the *E. coli* bacteria was performed as reported in (James Higgins *et al.,*2000). Phenol, Chloroform and Isoamyl alcohol method was used for the isolation of DNA. The presence of MDR gene was confirmed by performing PCR amplification and hi combs analysis. The specific primer (SHV gene) was used in PCR technique as reported in (F Di Cello et al, 1996) because this primer effectively identify the resistance gene which is present in the bacteria and amplifies those resistance genes.

Another method was performed for the detection of presence of MDR gene was Hi Comb analysis. In this method the antibiotic coated comb was placed on the *E. coli* streaked plate and the zone of inhibition was measured. The zone of inhibition indicates the presence of MDR gene (Albuquerque *et al.,* 2007).

Materials and Methods

Collection of sample

Freshwater fish (*Cirrhinus cirrhosis*) used in this study was collected from a local dam in Coimbatore district, Tamilnadu, India.

Isolation of *E. coli* from fish

About 2g of fleshy part of the head, intestine, muscle and tail region of the fish were dissected and inoculated into nutrient broth containing 5 ml in each test tube. The test tubes were incubated at 37°C for 24 hours. Loopfull of inoculated broth was streaked on Mac Conkey agar plate and incubated for 24 hours at 37°C. *E. coli* grown on the plate was isolated and sub-cultured in Luria bertoni broth for obtaining pure culture of *E. coli*.

Identification of *E. coli*

Indole test

Sterilized test tubes containing 4 ml of tryptophan broth was taken and 18 to 24 hrs old culture was aseptically inoculated. The tubes were incubated at 37°C for 24-28 hours. 0.5 ml of Kovac's reagent was added to the broth culture. Finally, the presence or absence of the ring was observed. Formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent is considered as Indole positive, which is an evidence for the presence of *E. coli* and no color change even after the addition of appropriate reagent indicates Indole negative, which is an evidence for the absence of *E. coli.*

Methyl Red (MR) test

The methyl red (MR) test detects the production of sufficient acid during the fermentation of glucose and the maintenance of conditions such that the pH of an old culture is sustained below a value of about 4.5, as shown by a change in the colour of the methyl red indicator which is added at the end of the period of incubation. MRVP broth (pH 6.9) was prepared and sterilized. The medium was inoculated with 18-24 hours old culture and incubated at 37°C for 24 hours. After the incubation period, 2 to 3 drops of Methyl red was added to the medium. An immediate change of the medium to a distinct red color indicates the presence of *E. coli.*

Voges-Proskauer (VP) test

The Voges-Proskauer (VP) test is used to determine if an organism produces acetyl methyl carbinol from glucose fermentation. If present, acetyl methyl carbinol is converted to diacetyl in the presence of ∝- naphthol, strong alkali (40% KOH), and atmospheric oxygen the broth then condense to form a pinkish red polymer. MRVP broth (pH 6.9) was prepared and sterilized. The medium was inoculated with 18-24 hours old culture and incubated at 37°C for 24 hours. After the incubation period, 6 drops of 5% alpha-naphthol was added and mixed well to aerate. Then 2 drops of 40% potassium hydroxide was added and mixed well to aerate. The tubes were observed for a pink-red color at the surface within 30 min.

Citrate test

Bacteria that can grow on citrate agar medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6. Simmon's citrate agar was prepared by adding sodium chloride (5.0gm), sodium citrate (dehydrate) (2.0gm), ammonium dihydrogen phosphate

(1.0gm), dipotassium phosphate (1.0gm), magnesium sulfate (heptahydrate) (0.2gm), bromothymol blue (0.08gm), agar (15gm). The medium was sterilized and cooled in slant position. The slant was streaked with the inoculums and incubated at 37°C for 4-7 days. Color change from green to blue along the slant was observed.

Triple Sugar Iron agar (TSI) test

This medium contains lactose, sucrose and glucose in the concentration of 10:10:1. Iron, phenol red and peptone are also the components of the TSI agar. The medium was prepared and sterilized. It was cooled in a slant position.

TSI Agar was inoculated with the culture by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. The tubes were incubated at 35°C in ambient air for 18 to 24 hours. When lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in butt and in the slant. Some organisms generate gases, which producesbubbles/cracks on the medium.

Hi-comb assay

Zone of inhibition was calculated using hi-comb assay method. 24hrs old nutrient broth *E coli* culture (80μL) was swabbed in Muller Hinton agar plates. Ampicillin (30mcg) and Methicillin (10mcg) antibiotics comb were placed on the plates and incubated for 24 hours at 37°C.

DNA isolation

For *E. coli* isolation, pure culture was centrifuged at 5000rpm for 5 mins and the pellet was collected. Along with the pellet, 700μl of saline EDTA and 20μl of lysozyme

was added and mixed well and incubated it for 30 mins at 37°C.

After 30 mins, 150μl of 10% SDS was added and incubated it for 15 mins at 65°C in water bath and 180μl of phenol, 160μl of chloroform and 10μl of isoamyl alcohol were added and centrifuged.

The aqueous phase was collected and half the volume of sodium acetate and 5 volume of isopropanol were added and centrifuged for 10 mins at 12000rpm. The DNA pellet was collected and washed with 70% ethanol, air dried and re-suspended in 40μl of 1X TE buffer.

PCR amplification of SHV gene

The amplification of DNA was carried out in a reaction with the final volume of 20μl containing 1μl of total DNA, 1μl of each SHV primer (SHV-F 5'-GGT TAT GCC TTA TAT TCG-3' and SHV-R 5'-TTA GCG TTG CCA GTG CTC-3'), 6μl of PCR master mix, 4μl of PCR buffer and 6μl of distilled water.

A gradient PCR was followed in the experiment. The PCR reaction condition were as follows: 94ºC for 3 min, followed by 20 cycles of denaturation at 94ºC for 15 s, annealing at 53ºC, 55°C for 15 s at 30°C and extension at 72ºC for 2 min, before a final extension at 72ºC for 15 s. for 20 cycle. The PCR product was analyzed using 1.5% agarose gel.

Results and Discussion

Isolation of *E. coli* from fish

Among the 7 isolates one *E. coli* was isolated and identified. The isolated *E. coli* was stored on 1% nutrient agar slants and stored at $4^{°}$C for further studies.

Gram staining: Gram – ve rod

Table.1 Biochemical tests

Name of the tests	Result
Indole	+ve
MR	+ve
VP	-ve
Citrate	-ve
TSI	Acid slant and acid butt
H2S	-ve

After isolation and basic confirmation, the isolated bacteria were further streaked on MaC Conkey agar (0.5%bile salts) and presence of pink color colonies confirmed the *E. coli* presence.

Hanson *et al.,* (2008) reported that, the *E. coli* was firstly discovered in the intestine of wild fish. It was proved that *E. coli* was present in the intestine part of the fish. This study is aimed to isolate *E. coli* from various parts of the fish like head, tail, muscle and also the intestine. Fish was dissected into head, tail, muscle and intestine and incubated in nutrient broth for 24 hours in order to isolate the pathogen (*E. coli*). In the Present study we have isolated and identified from the incubated broth and they were sub-cultured in specific media for *E. coli* isolation. MaC Conkey agar was the specific medium used for the isolation of *E. coli* (Figs. 1-3).

Hi Comb assay test

Multi drug resistance to strains is defined as being resistant to four or more antimicrobial agents but sometimes as low as two antibiotics from different classes (Jyoti Tanwar *et al.,* 2014). The most common bacterial species in environmental specimens was *E. coli*, and it was more multidrug resistant than clinical specimens. Furthermore, the multidrug resistance was

evident for *β*-lactam drugs, aminoglycosides, monobactams, quinolones, sulfonamides and nitrofurantoin. Beta-lactam antibiotics are the largest and most commonly used group of antimicrobial agents worldwide. A previous study demonstrated that these microorganisms utilize antibiotics as nutrients (Israa Abdul Jabbar Ibrahim, 2015). The result of this study revealed the presence of multidrug resistant bacteria from fish. The below table represents the multi drug resistance pattern of the bacterial isolates to a number of antibiotics used in the study. Lomovskaya *et al.,* (2001) reported that zone of inhibition can be calculated by placing hi combs on the *E. coli* swabbed plate. In this study, no zone of inhibition was observed for ampicillin and methicillin antibiotics. Hence, this indicates the presence of Multi Drug Resistance (MDR) gene in *E. coli* which was isolated from the fish (Fig. 4).

Isolation of DNA

E. coli isolated showed the highest count in the Muscle and intestine of the freshwater fish. Pathogens from Muscle and Intestine were isolated and inoculated into Luria Bertoni broth. This broth was used for bacterial DNA isolation which was further used for PCR amplification. Alka grover *et al.,* (2012) isolated DNA using phenol, chloroform and isoamyl alcohol method. Thus, the same protocol was followed in this study for bacterial DNA isolation. Then, agarose gel electrophoresis was performed.

Table.2 Zone of inhibition for *E. coli* strains

Pathogen	Name of the comb	Zone of Inhibition (mm)
E. coli	Ampicillin	Nil
E. coli	Methicillin	Nil

Figure.1 Fish sample (*Cirrhinus cirrhosis*)

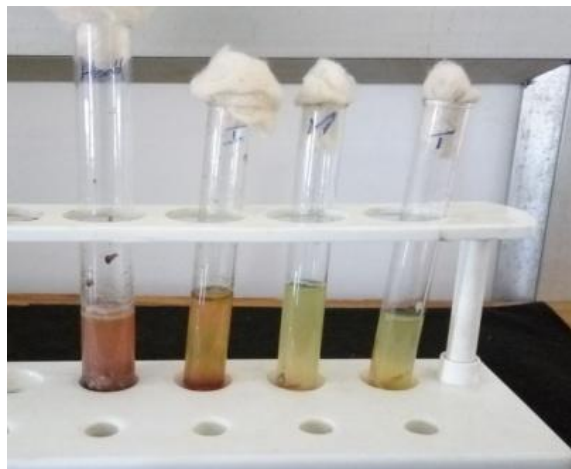**Figure.2** Isolation of pathogens from fish samples

Figure.3 MacConkey agar plate (*E. coli)*
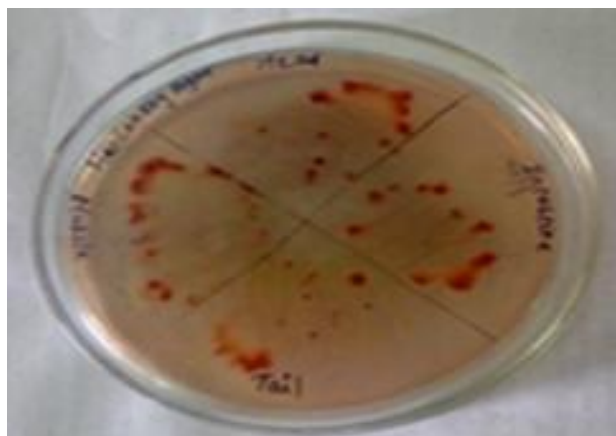
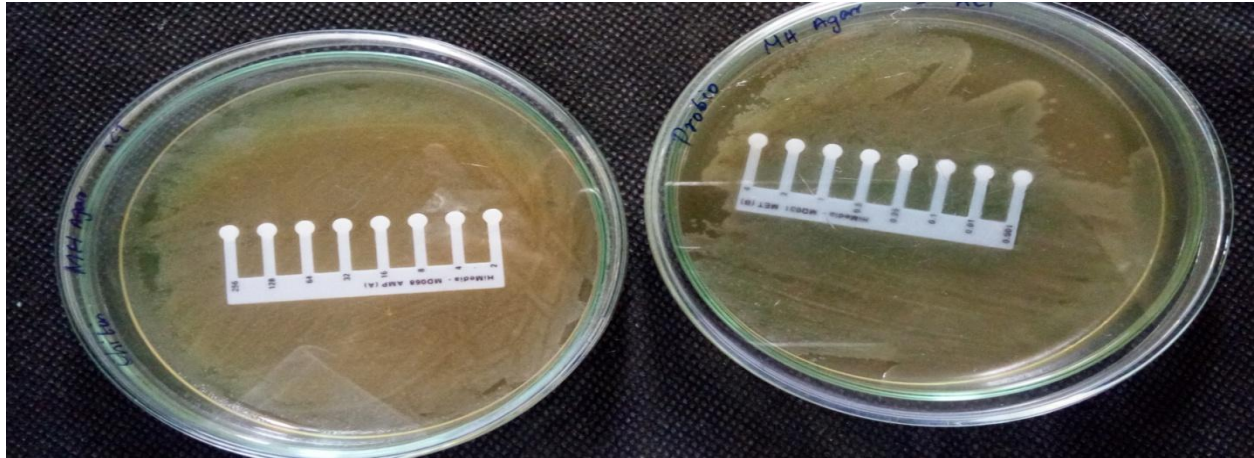**Figure.4** Zone of inhibition for *E. coli* strains using antibiotic combs

Figure.5 Lane 2: DNA; Lane1: 500 Base Pair Ladder
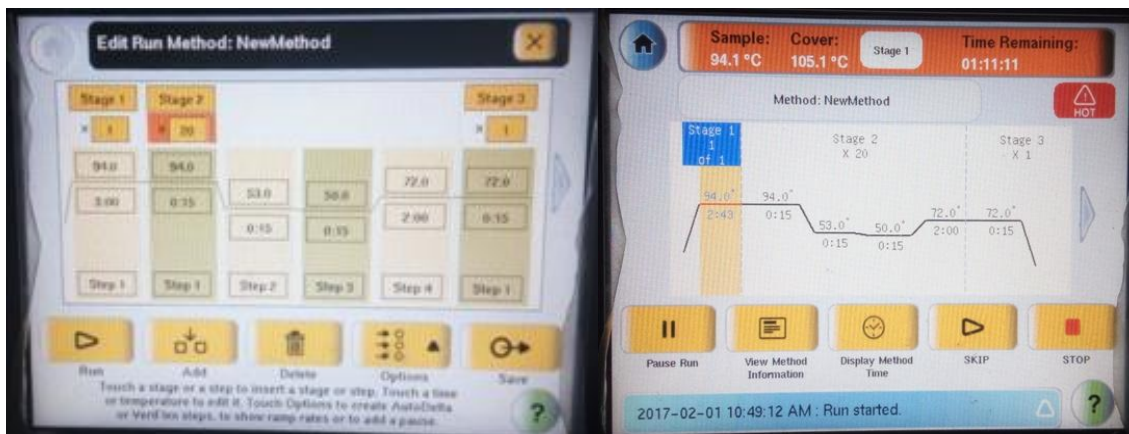

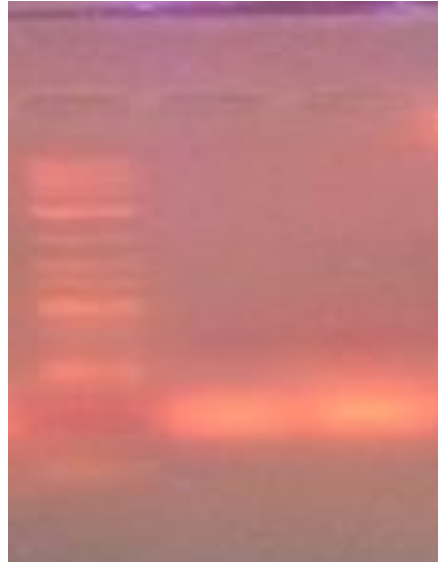**Figure.6** PCR setup   **Figure.7** PCR initialization

Figure.8 Lane 1: Amplified PCR gene – base pair marker;
Lane 2&3: *E. coli* 356kbs

Sebastião *et al.,* (2015) used direct colony PCR combined with 16S rRNA gene sequencing constitutes an efficient alternative for diagnosing bacterial fish diseases, with decreased cost and time compared with the classical methods used in Brazil, such as isolation, biochemical tests, and conventional PCR.

Agarose gel electrophoresis

0.9% agarose was used for agarose gel preparation. DNA sample was injected into the wells of agarose gel and electrophoresis was performed for 2 hours. As a result of agarose gel electrophoresis, clear bands were obtained for the DNA isolated from *E. coli.*

PCR amplification of SHV gene

Mohini joshi *et al.,* (2010) used the specific primers for amplification of MDR gene present in Gram- negative bacteria. Since *E. coli* is a gram negative bacteria, the same protocol was used in this study for PCR amplification of SHV gene.

Agarose gel electrophoresis was performed for PCR amplified gene using markers. Lane 1 shows the base pair marker and lane 2 shows the amplified *E. coli* 356. From the PCR result, it was obvious that a clear single band indicates the presence of MDR gene (Figs. 5-8).

In conclusion the fish pathogen specifically *E. coli* was isolated and screened for the MDR pattern. Various identifications tests for *E. coli* were performed to confirm that the isolated pathogen was *E. coli*. Among the 7 isolates one *E. coli* was confirmed as MDR. The isolated *E. coli* was stored on 1% nutrient agar slants and stored at $4^{°}$C for further studies. From the PCR result, it was proven that a clear single band indicates the presence of SHV gene in fresh water fish.

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