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ISOLATION, GENETICAL IDENTIFICATION AND ANTIBIOTIC SENSITIVITY OF *SALMONELLA TYPHI* ISOLATED FROM MARINE EDIBLE FISH *RASTRELLIGER KANAGURTA*-A NOVEL STUDY

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ABSTRACT

Lack of information in view of presence of pathogenic *salmonella species* in shoaling and schooling marine edible fishes shall cause major adverse health consequences on public health across the globe. In the current study, out of 72 types of marine fishes, 12 edible fishes are identified for presence of pathogens including *Salmonella*. Numerous hazardous microorganisms are isolated from different body parts of fish. Biochemical characterization, 16*S rRNA* studies were performed for *Salmonella species* and confirmed the presence of *Salmonella typhi*. Optimization of media components for the growth of *salmonella typhi* was performed and genetic identification tests were carried out. Pathogenic evaluation was performed by O-acetyl content and Vi polysaccharide. Antibiotic sensitivity was performed with wide range of antibiotics and concludes that *Salmonella typhi* threshold levels in view of tolerance of antibiotics *i.e.,* Amoxyclav, Ceftriaxone, Cefotaxime, Cefepime and Aztreonam at a concentration of 20 µg/ml and highly sensitive at concentration of 30 µg/ml per 4 kg body mass index (BMI) of fish. *Salmonella typhi* threshold levels in view of tolerance of antibiotics *i.e.*, Meropenem, Imipenem, Cefixime, Oxacillin and Ofloxacin at a concentration of 30 µg/ml and sensitive at concentration of 40 µg/ml per 4 kg BMI of fish. For the first time, *Salmonella* species was isolated from marine edible fish from coastal region, Krishna district, Andhra Pradesh, India and its antibiotic sensitivity was performed. Further studies aims towards vaccine industry in view of public health.

Keywords: Marine edible fish, *Salmonella typhi*, Characterization, Optimization, Antibiotic activity.

1. INTRODUCTION

Water is categorized as essential source of human life. Most of the people don't have access to clean and pure water for consumption, which leads to mortality because of waterborne bacterial infections. In addition to water, marine fishes act as pathogen carriers for creating health emergency in humans across the globe [1]. As per 2019 study report conducted by Central Marine Fisheries Research Insititute (CMFRI), marine fish production in India has recorded a marginal rise of 2.1% in 2019 compared to previous year. Consumption of unprocessed marine water fishes also leads to different disorders.

For the first time, research was published on microbial flora of fish gut [2, 3]. In U.S, approximately 93.8 million people are alleged from illness of salmonellosis, out of which 80.3 million are affected by food borne transmission and approximately 155,000 deaths occurred each year [4]. In 48 states of U.S, *Salmonella* causes 1.35 million infections, 420 deaths and 26,500 hospitalizations annually, according to the CDC, 2019.

Salmonellosis, a bacterial origin food borne disease exists worldwide. Now a days, marine fish and fish products gained lot of importance because of presence of *Salmonella species* which impact human health and requires remedies. *Salmonella*, a rod-shaped gram negative bacteria belong to the family enterobacteriaceae is familiarly known human pathogen and its waterborne transmission has been well documented in various studies. *Salmonella* has been identified in foods [5], poultry and poultry feeds [6], shellfish [7], oyster [8] and shrimp [9]. In the 11 marine species, most frequently

reported bacteria were Cytophaga, Flavobacterium, Enterobacteriaceae, *Vibrio* and *Pseudomonas* [5, 7, 9]. The bacteria serologically contain lipopolysaccharide antigens O9, O12 and distinct polysaccharide capsular antigen Vi [10, 11].

O-acetyl content is the part of virulence polysaccharide, plays a major role in salmonellosis. Hence in the current study isolation, biochemical identification, 16*S rRNA* characterization and genetic identification was performed and antibiotic sensitivity test was done with different antibiotics at different concentrations.

2. MATERIAL AND METHODS

2.1. Selection and sample collection

Current study geography states that the location of the city is at an Altitude (feet) 55, Lat (DMS) 16° 11' 15N, Long (DMS) 81° 8' 20E and Altitude (meters) 16, corresponding to the latitude 16.18756 and longitude 81.1390 in an arid zone of area about 70,000 Sq.km. Out of 72 fishes, 12 edible marine fishes were collected from the commercial fish market, Machilipatnam, Krishna District, Andhra Pradesh. Whole marine fish as a source of sample was collected in winter season (between October to December, 2018). Surface sanitization was performed with 70% IPA before dissecting the fishes, and washed by using running sterile distilled water for 20 minutes to eradicate the surface microbial load. For sample collection, different body parts like gut, intestine, muscle, tail muscle, gills, mouth, analpin, oesophagus, spinal cord and kidney were collected. Approximately 0.5 to 1.0 gm of sample from different parts were suspended in normal saline and grounded by using pestle and mortar for 15-20 minutes by using sterile scalpel (table 1).

Table 1: Name of the fish and sample source

S. No	Name of the fish		Source of sample
	Local name	Scientific name	
1	Sardines	*Sardina pilchardus*	Guts, Intestine, Muscle, Tail muscle, Gills, Mouth, Analpin, Esophagus, Spinal cord, Kidney & Near urinary bladder
2	Indian Mackerel	*Rastrelliger kanagurta*	
3	Tuna	*Thunnus thynnus*	
4	Ribbon fish	*Lepturacanthus savala*	
5	Seer fish	*Scomberomorus commerson*	
6	Barracuda	*Sphyraena barracuda*	
7	Marlin	*Makaira nigricans*	
8	Black Pomfret	*Parastromateus niger*	
9	Cat fish	*Clarias gariepinus*	
10	Croakers	*Micropogonias undulates*	
11	Flat fish	*Psettodes erumei*	
12	Thread fin	*Eleutheronema tetradactylum*	

2.2. Isolation and Identification of *Salmonella*

Approximately 132 no's of samples were collected from the selected fishes and 10 fold serial dilution was performed by using normal saline. The diluted samples were streaked on nutrient agar and incubated at 37°C for overnight. After incubation, the presumptive colonies were sub cultured into XLD agar to get the pure culture of *Salmonella spp*. Phylogenetic analysis was performed along with morphological and biochemical characterization to identify the isolates.

2.3. PCR amplification

16*SrRNA* gene sequencing was used to confirm the *Salmonella* at species level. The full length of 16*SrRNA* gene was amplified using thermal cycler with universal 16*S rRNA* primer set (Forward primer, 5'GGAACTGAGACACGGTCCAG3' & Reverse primer, 5'CCAGGTAAGGTTCTTCGCGT3'). PCR reaction volume was set for analytical grade (10µl) for identification at different annealing temperatures ranges from 50°C to 65°C. Later volume was set for preparative grade (50µl).

Analytical grade reaction contained 1µL (40ng) of genomic DNA, 1µL (10 pmol) of forward primer, 1µL (10 pmol) of reverse primer, 5 µL of 2X Master Mix and 2µL of nuclease free water. Thermal cycler conditions were maintained as 5 minute at 95°C for initial denaturation, 30 cycles of 1 minute each at 95°C for denaturation, 1 minute at 60°C for annealing and 1 minute at 72°C for extension and final extension at 72°C for 10 minute. PCR product was run on 1% agarose gel against 100 bp DNA ladder [12].

2.4. Genetic identification

Genetical identification of *Salmonella typhi* was confirmed with gene specific primers for fliC gene. Forward Primer 5'ACTGCTAAAACCACTACT3' and reverse primer 5'TTAAGCCAGTAAAGAGAG3'. Analytical reaction contained 1µL (40ng) of genomic DNA, 1µL (10 pmol) of forward primer, 1µL (10 pmol) of reverse primer, 5 µL of 2X Master Mix and 2µL of nuclease free water. Thermal cycler condi-tions were maintained as 5 minute at 95°C for initial denaturation, 35 cycles of 1 minute each at 95°C for denaturation, 1 minute at 60°C for annealing and 1 minute at 72°C for extension and final extension at 72°C for 15 minute. PCR product was run on 1.2% agarose gel against 100 bp DNA ladder [12].

2.5. Media optimization

In order to determine the growth profile of the organism, the isolated organism was cultured on soyabean casein digest medium and the growth curve was plotted against time [13]. Average of the data obtained from three experiments conducted independently in triplicates was considered for statistical significance, identification of extra cellular O-acetyl content by Hestrin method [14] and subsequently the O-acetyl content was converted to Virulence polysaccharide (ViP) by using conversion factor 0.294.

In order to optimize the process for maximum production of polysaccharide in fermentation process, the critical physical parameters (Temperature, RPM, pH and aeration) and nutritional parameters (mainly different carbon sources) were selected based on literature [15] and few uni-dimensional and statistical approaches were followed to develop a optimized medium for fermentation [13]

2.6. Radial immunodiffusion (RID)

Post optimization of medium, 5 ml of culture broth was centrifuged at 10000xg for 15 min at 4°C. Supernatant was used to confirm the Vi antigen concentration by single radial immunodiffusion (SRD) in which antibody was incorporated into molten agarose and poured into a petridish. After making wells in the agarose gel, known concentrations of standard antigen and test antigens were added to identify the specificity of test antigen towards the antibody, and its potentiality as vaccine. A single radial immunodiffusion (SRD) assay was used to measure Vi antigen content in fermentation broth. As the immunoassay is specific for Vi antigen, interference of other components is totally nullified during quantification.

2.7. Antibiotic sensitivity test

The isolated strains of *Salmonella spp* were tested for its antibiotic sensitivity with different concentrations of antibiotics *i.e.,* Amoxyclav, Cefixime, Ceftriaxone, Cefotaxime, Ofloxacin, Cefepime, Oxacillin, Mero-penem, Aztreonam, Imipenem ranging from 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml.

3. RESULTS AND DISCUSSION

3.1. Isolation of micro organisms

Out of 12 edible marine fishes, numerous micro organisms are identified from different body parts of fish. Out of all those, organism which causes Salmonellosis is concentrated in this study. *Salmonella* was isolated from Gut and gills of *Rastrelliger kanagurta*. Other organisms are also isolated and count was populated in table 2.

Table 2: Isolated microorganisms from marine fishes

S. No	Bacterial species*	Number of organisms isolated
1	*Salmonella*	2
2	*Escherichia*	81
3	*Pseudomonas*	63
4	*Staphylococcus*	48

** Results are based on biochemical characterization*

3.2. Phylogenetic analysis

The phylogenetic data described below was obtained by alignment and phylogenetic analysis of the bacterial sequences. The nucleotide sequences of 16*SrRNA* was aligned by using the CLUSTAL V computer program [16]. A neighbor-joining analysis [17] was used to reconstruct phylogenetic trees with the MEGA software (version 4.02) [18] (fig. 1).

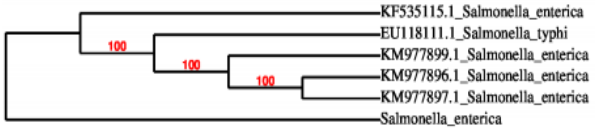

Fig.1: Phylogenetic analysis of *Salmonella typhi*

3.3. Characterization of isolated microbes

Morphological and biochemical characterizations were performed to identify the isolates and tests performed

to identify the organisms are summarized in the table 2. By the Gram Staining, Motility, Catalase, Oxidase, Nutrient Agar, Mac Conkey Agar, TCBS Agar, Glucose fermentation, Lactose fermentation, Sucrose fermentation, Mannitol fermentation, Indole, Methyl Red, Voges Proskaeur, Citrate Utilization, Urease, TSI, the organisms isolated were identified as *Salmonella, Eschereschia, Staphyloccocus and Pseudomonas spp* were characterized by standard biochemical methods.

Even though, *E.coli, Staphylococcus, Pseudomonas spp* were also predominant in nature, *Salmonella spp* was the organism selected for the isolation and characterization of metabolites produced like Vi polysaccharide and proteins have lot of importance in vaccine industry too.

3.4. Molecular and genetic identification

Molecular identification of *Salmonella species* was performed by universal primers based polymerase chain reaction principle. Only two isolates out of 132 isolates were amplified through PCR analysis with and predicted at ~1500 bp. No amplification reaction was performed for remaining isolates. Two isolated samples were sent for sequencing to Yaazh Xenomics, Chennai, India. The sequenced isolate (Machilipatnam) was deposited in NCBI GenBank (Accession No. MK484110) [19] (fig.2 & 3). The 16*SrRNA* gene of *Salmonella typhi* was 1425 nucleotides and compared with other reported *Salmonella spp* at nucleotide level. Our present study isolate (MK484110-AP-India) clustered along with EU118111-Iran, AE014613-USA, CP003278-India, NR 074799-USA, CP002099-USA, AL513382-UK, LT905143-UK, CP029958-Brazil, CP029646-Brazil, CP030936Zhe-jiang China as a separate clade (Clade 1). LT906560-USA, LT905142-Philippines, CP033348-Hubei China, CP034705-Rwanda-Rulindo, CP034711-Rwanda-Rulin-do into a separate clade. Results of amplification of fliC gene were compared with Song *et al* [22] (fig. 4).

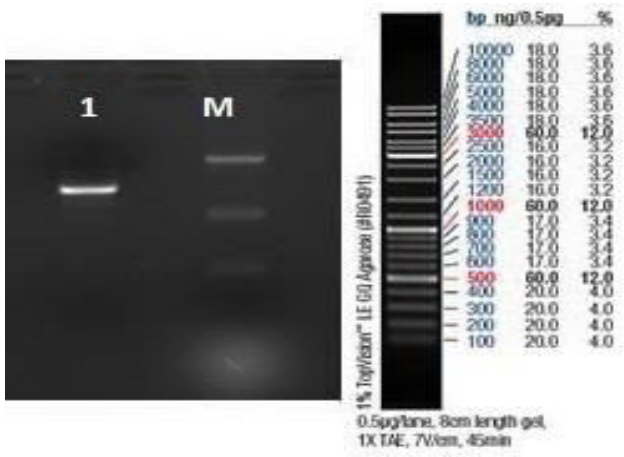

Fig. 2: 16*SrRNA* sequencing for 1425 base pairs. Lane-1: Gene of interest, M: Marker gene (500 bp, 1000 bp and 3000 bp)

5'GCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGAC
GGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACG
GTGGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCT
TGCCATCAGATGTGCCCAGATGGGATTAGCTTGTTGGTGAGGTAACGGCTCACCA
AGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGC
AAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTAC
TTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGACGTTACCCG
CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTC
GGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTTG
AGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATC
TGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGT
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCTACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTT
AAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC
TTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGA
ACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGA
ACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGT
CATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAG
AGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGAT
TGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGA
ATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGG
AGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGGGGCGCTTACCACTTT
GTGATTCATGA3'

Fig. 3: 16*S rRNA* gene partial sequence of isolated *Salmonella typhi* and GenBank Accession No. MK484110

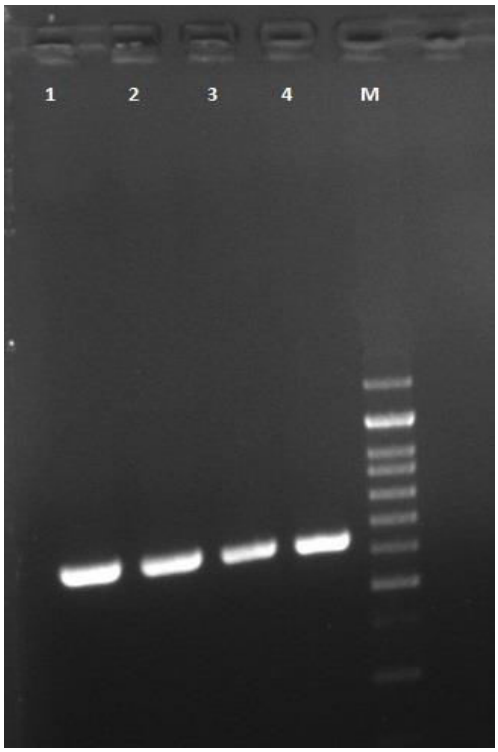

Fig.4: Genetic identification of fliC gene of *Salmonella typhi*. Lane1, 2, 3 & 4: Amplicon of fliC gene (458 bp). M: Marker (100 bp to 1000 bp)

3.5. Optimization of nutrients for the growth of *Salmonella typhi*

After isolation and identification, organism was cultured in SCDM. Out of different carbon sources, maximum production of O-acetyl content and ViP was observed in the media which is having glucose and sucrose rather than lactose and maltose.

O-acetyl content is one of the active components of Virulence polysaccharide. Hence in order to know the production quantity of Virulence polysaccharide in fermentation process O-acetyl content titre is also important to evaluate. Formula for calculation of ViP content was mentioned below.

ViP = O-acetyl concentration X 0.294.

3.6. Determination of growth curve, O-acetyl content and ViP evaluation

Post-optimization of the media ingredients, for over expression of O-acetyl content, time of hours for higher production of O-acetyl content and ViP were performed and the results are tabulated in table 4. Based on the results obtained, it was concluded that at 24 hrs maximum production of ViP was observed and the same was in comparison with the results at 28 hrs. Hence optimized time for fermentation is 24 hrs (fig.5).

Table 3: Media optimization for the growth of *Salmonella typhi*

S. No	Glucose (%)	Sucrose (%)	Lactose (%)	Maltose (%)	O-Acetyl content (µm/ml)	ViP (mg/mL)
1	1	0.5	1	0.5	6.2	1.8228
2	1	1	0.5	1	7.1	2.0874
3	0.5	1	1	0.5	4.2	1.2348
4	1	0.5	1	1	3.8	1.1172
5	1	1	0.5	1	5.9	1.7346
6	1	1	1	0.5	6.9	2.0286
7	0.5	1	1	1	4.9	1.4406
8	0.5	0.5	1	1	3.8	1.1172
9	0.5	0.5	0.5	1	2.7	0.7938
10	1	0.5	0.5	0.5	1.9	0.5586
11	0.5	1	0.5	0.5	2.2	0.6468
12	0.5	0.5	0.5	0.5	1.5	0.441

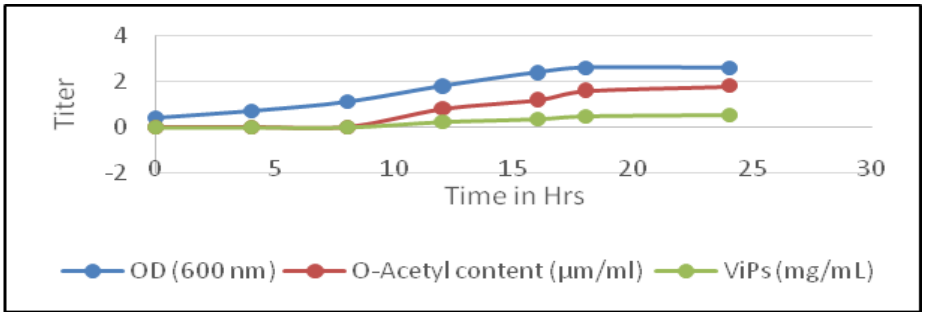

Fig. 5: Growth curve verses titre of O-acetyl content and ViP content

Table 4: O-Acetyl content and ViP content

S. No	Hrs*	OD (600 nm)	O-Acetyl content (µm/ml)	ViP's (mg/mL)
1	0	0.4	ND	ND
2	4	0.7	ND	ND
3	8	1.1	ND	ND
4	12	1.8	0.8	0.23
5	16	2.4	1.2	0.35
6	18	2.6	1.6	0.47
7	24	2.6	1.8	0.52
8	28	2.7	1.8	0.52
9	32	2.8	1.7	0.50

** Data is average of three independent experiments, ND = Not detected*

3.7. Antibiotic sensitivity test

The zone of inhibition was visible after 18-24 hours. The zone of inhibition will confirm the sensitivity of the organism to that particular antibiotic. Based on the sensitivity, minimum inhibitory concentration can be determined using MIC strips, which helps in setting a dose specification for treatment of infection. (MIC: The MIC is the lowest concentration of an antimicrobial agent that visually inhibits growth of a microorganism under defined experimental conditions). The diameter of the inhibition was measured in millimetres (mm). Apart from all antibiotics, Amoxyclav, Ceftriaxone, Cefotaxime and cefepime shows high zone of inhibition at a concentration of 30 µg/ml (fig. 6). Other antibiotics *i.e.,* Cefixime, Ofloxacin, Oxacillin, Meropenem and Imipenem which shows zone of inhibition at a concentration of 40 µg/ml. But zone of inhibition using 30 µg/ml and 40µg/ml is same (table 5).

All other antibiotics *i.e,* Cefixime, Ofloxacin, Oxacillin, Meropenem and Imipenem require more concentration when compared with the other antibiotics. In view of antibiotic threshold, low concentrations of antibiotics are preferred in view of safety towards human health. Hence, for washing of edible fish as a part of processing, those four antibiotics can be considered, however methods for minimizing their footprints need to be devised to avoid antibiotic resistance

Table 5: Antibiotic sensitivity of *salmonella* by different antibiotics at different concentrations

S. No	Name of antibiotic	Concentration (µg/ml)	Zone of inhibition (mm)*
1	Amoxyclav (AMC 5)	10	2
		20	6
		30	34
2	Cefepime (CPM 30)	10	4
		20	7
		30	32.5
3	Ceftriaxone (CTR30)	10	3
		20	6
		30	36.5
4	Cefotaxime (CTX 30)	10	3
		20	7
		30	35

* Results are analyzed in triplicates

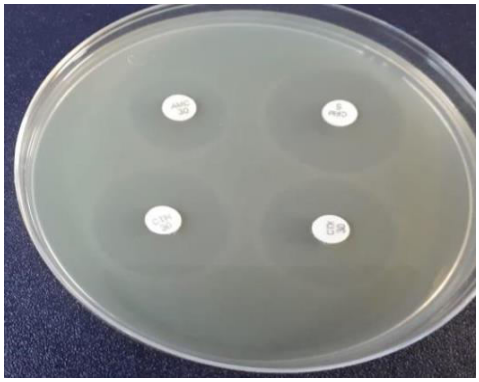

Fig. 6: Antibiotic sensitivity by different types of antibiotics i.e., Amoxyclav (AMC), Ceftria-xone (CTR), Cefotaxime (CTX) and Cefepime (CPM)

Different types of microorganisms *i.e. Escherichia coli, Salmonella typhi,* and *Vibrio cholerae* are identified from tap water and bottled water which will impact the public health directly or indirectly [20]. Marine fish consumption is increasing year by year across the globe. Hence this study conducted to know the presence of *Salmonella species* in marine edible fish *Rastrelliger kanagurta,* with respect to impact on public health and also to use the *Salmonella typhi* in vaccine industry [21].

In the current study, microorganisms are isolated from different parts of marine edible fish and confirmed the presence of *Salmonella spp* by 16*SrRNA*. Presence of *salmonella typhi* in *Rastrelliger kanagurta* was confirmed by

genetic identification and 458 base pairs of fliC gene amplification was observed [22]. The virulence polysaccharide (ViP) leads to salmonellosis was identified with respect to O-acetyl content.

Even *Salmonella* present in the fish does not have impact on host, because of unfavourable temperature conditions. If the fish meat is consumed without proper processing, it leads to different disorders. Hence eradication of *salmonella* during fish processing also needs lot of attention of researchers. In this view, antibiotic sensitivity test was performed to know the threshold levels of bacteria against different concentrations of different antibiotics. Out of all these antibiotics, Cefotaxime (CTX30), Ceftriaxone (CTR 30), Cefepime (CPM 30), Azetreonam (AZT 30) and Amoxyclav (AMC 5) are more effective even used at low concentration (30 µg/mL) when compared to Cefixime (CFM 40), Ofloxacin (OFL 40), Imipenem (IMI 40), Meropenem (MER 40) and Oxacillin (OXA 40) which are used at high concentration (40 µg/mL). Hence it will be useful to know the further step(s) of processing of fish for human consumption without any adverse health consequences. There is no potential breach on human health because of these lower concentrations of antibiotics. Hence *Salmonella typhi* present in marine edible fish *Rastrelliger kanagurta* shall be eradicated with lower concentration of antibiotics *i.e.,* Cefotaxime, Ceftriaxone, Cefepime, Azetreonam and Amoxyclav devoid of side effects during processing of fish. Further, studies shall be extended to vaccine manufacturing industries by applying Virulence polysaccharide (ViP) as a vaccine.

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Conflict of interest

Authors do not have conflict of interest in view of work carried out and results reported.

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