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Non-Selectivity of R-S Media for *Aeromonas hydrophila* and TCBS Media for *Vibrio* Species Isolated from Diseased *Oreochromis niloticus*

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Abstract

The current study was conducted to determine the bacterial pathogens incorporated in mass mortality observed in cultured *Oreochromis niloticus* farms at Kafrelsheikh province, Egypt, during the summer season of 2015. Moribund fish samples were collected from six infected farms. General signs of septicemia were dominant in the clinical and gross internal examination of diseased fish. The pathogenic bacteria were isolated on specific media then confirmed by polymerase chain reaction (PCR). Out of thirty isolates, nineteen *Aeromonas hydrophila*, seven *Vibrio cholera* and three *Vibrio alginolyticus* isolates were recovered and identified using PCR. Current study indicated non-selectivity of Rimler-Shotts media for selective isolation of *Aeromonas hydrophila* also non-selectivity of TCBS media for *Vibrio* spp. isolated from diseased *Oreochromis niloticus*.

Keywords: *Aeromonas*; *Vibrio alginolyticus; Vibrio cholera;* PCR.

Introduction

Fish is cheap delicious animal protein of high nutritive value; it represents a good substitute to red meet especially in developing countries. Expanding in global aquaculture help to provide the excessive demand by increased human population. Finfish production comprised 49.8 million tons, (99.2 billion USD), Egypt ranked as the 8th aquaculture producing country in 2014 [1], it is producing about 1.130.000 ton of finfish that representing 2.26% of global aquaculture of these species. Egypt also, ranked as the second global tilapia producer after China, it produces about 867557 tons in 2014 [2].

Economic losses from diseases outbreaks in global aquaculture estimated by 3 Billion USD in 1997 [3]. Bacterial diseases reported to be the main etiological agents responsible for severe economic losses in cultured fish, including infection with *Aeromonas* spp., *Pseudomonas* spp., *Vibrio* spp., *Streptococcus* spp. and *Enterococcus* spp. as recorded by Aboyadak et al. [4], Zhang et al. [5], Olugbojo and Ayoola [6], Darak and Barde [7], Thune et al. [8].

Bacterial fish diseases resulted in various clinical finding including skin hemorrhage, ascites and exophthalmia [9-11]. Enlarged congested internal organs including liver spleen and kidney, and intestinal hemorrhage with or without presence of ascetic fluid in abdominal cavity are the main internal lesions [12-14].

The genus *Vibrio* is a member of the family *Vibrio*naceae, it is a Gram negative non-spore forming comma shape rods. Thiosulphate citrate bile salt sucrose (TCBS) agar is highly selective for the isolation of *V. cholerae* and another *Vibrio* spp. The acidification of the medium resulting from the fermentation of sucrose by *Vibrio* makes bromthymol blue turns yellow.

The genus *Aeromonas* is belongs to the family Aeromonadaceae, it is a Gram negative facultative anaerobic cocco-bacilli. Rimler-Shotts (RS) agar used for selective isolation and identification of *Aeromonas hydrophila,* the organisms that ferment maltose are seen as yellow colonies.

Isolation and biochemical identification of pathogenic bacteria are laborious and time consuming, application of molecular methods such as the polymerase chain reaction (PCR), is an easy, less expensive, and more rapid means for diagnosis of such diseases [15,16].

The present work was conducted to determine the bacterial pathogens responsible for mass mortality in cultured tilapia farms at Kafrelsheikh province using selective media and polymerase chain reaction.

Materials and Methods

Study area

Samples were taken from six affected tilapia farms at Torombat seven, Alreiad district, Kafrelsheikh governorate northern Egypt. The affected farms complain increased mortality during the study period from March to August 2015.

Samples

Thirty live diseased Nile tilapias (*Oreochromis niloticus*) with obvious signs of septicemia were collected; five fish were taken from each farm. Diseased fish weight was ranged between 100-250 g and 16-24 cm in total body length. Each fish sample was packed alive in a separate sterile labeled plastic bag and transported in ice pox to lab.

Clinical examination

The clinical examination including observation of any external lesions was performed according to the method described by Noga [13].

Post mortem examination

The post mortem examination was performed according to the method described by Heil [17].

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Isolation and identification of the causative agent

Under complete aseptic condition, a small tissue pieces from heart, hepatopancreas, spleen, posterior kidney and from lesions in musculature were taken from each fish to a labeled test tube containing 10 ml sterile peptone water, after that the sample was homogenized at 3000 rounds per minute (rpm) for 1 min using homogenizer pro® USA. Test tubes were centrifuged at 500 rpm for 30 sec and one ml from supernatant was added to another test tube containing sterile tryptic soy broth then incubated at 33°C for 24 h. Rimler-Shotts media with novobiocin selective supplement (HiMedia, India), tryptic soy agar (Oxoid, England), *Pseudomonas* selective agar with CFC (Cetrimide – Fucidin – Cephaloridine) and glycerol supplement, (Lab M, United Kingdom), TCBS agar media (Oxiod, England) and Edwards media modified (Oxiod, England) were streaked from each sample then incubated at 33°C for 24h. Few morphologically similar colonies from isolates grown on each media were picked up and inoculated to trypticase soy broth, incubated at 33°C for 24 h then 0.5 ml from this broth was preserved on sterile 50% glycerol (biotechnology grade) one volume from overnight broth : one volume from 50 % glycerol, after that it was stored on - 80°C for - PCR identification.

Identification of collected strains using PCR

DNA extraction: Bacterial DNA extraction was performed by thermolysis based on destruction of bacterial cells using dry heat as described by Ahmed et al. [18]. Two hundred microliter of overnight cultured broth was mixed with 800 µl of nuclease free double deionized distilled water in eppendorf tube, after that the eppendorf tubes were placed in heat block for 5 min at 95°C followed by cool centrifugation for 2 min at 15000 rpm and 4°C, the supernatant was used as DNA template and stored at -20°C for PCR study.

Primers

Primers used for identification of recovered isolates shown in (table 1).

PCR procedures

PCR mixture for amplification of targeted DNA: The reaction volume for all performed PCR reaction was 25 µl, each reaction volum consists of 5 µl of 5X master mix (taql/high yield- Jena Bioscience, Jena, Germany, consists of DNA polymerase, dNTPs mixture, (NH4) SO4, MgCl2, Tween 20, Nonidet P-40, stabilizers) + 1.25 µl of each primer (forward and reverse) (20 pmol/µl), + 5 µl of extracted bacterial DNA + 12.5 µl of nuclease free double deionized distilled water. Amplification of targeted DNA gens was carried out in thermal cycler (T100TM Thermal cycler, BIORAD, USA).

A- Thermal cycle adjustment for amplification of genus *Aeromonas* and *Aeromonas hydrophyla* target DNA: Amplification of genus *Aeromonas* targeted DNA was performed according to the method described by Lee et al. [19], with an initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation each at 94°C for 1 min, annealing at 68°C for 30 sec and extension steps at 72°C for 45 secs, then final extension step at 72°C for 10 min.

Amplification of *Aeromonas hydrophila* targeted DNA (specific-16S rRNA gene) was done using the same primer described by Trankhan et al. [20] but with different PCR condition. The thermal cycler condition was adjusted as following: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation, at 94°C for 30 secs, annealing at 55.5°C for 30 sec and extension steps at 72°C for 30 secs, then one final extension step at 72°C for 10 min.

B- Thermal cycle adjustment for amplification of genus *Vibrio*, *Vibrio cholera*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* target DNA: Thermal cycler adjustment for amplification of genus *Vibrio* and mentioned *Vibrio* species targeted DNA were the same, it performed according to the method described by Wei et al. [15] except, reaction for each species was performed separately (not multiplex). Amplification starts with an initial denaturation at 94°C for 3 min, followed by 30 cycles of (denaturation at 94°C for 30s, annealing at 60°C for 30s, and extension step at 72°C for 120 s), finishing with a final extension step at 72°C for 10 min then storage at 4°C.

Table 1: Primers used for detection of Genus *Aeromonas* and *Aeromonas hydrophila*, Genus *Vibrio*, *Vibrio cholera*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus*.

Target bacteria	Primer Name	Oligonucleotide Sequence (5´-3´)	Size (Bp)
Genus Aeromonas	AER-F	CTA CTT TTG CCG GCG AGC GG	953 bp
	AER-R	TGA TTC CCG AAG GCA CTC CC	
A. hydrophila	16SrRNA-F	GGC CTT GCG CGA TTG TAT AT	103 bp
	16SrRNA-R	GTG GCG GAT CAT CTT CTC AGA	
Genus *Vibrio*	16SrRNA-F	CCT GGT AGT CCA CGC CGT AA	168 bp
	16SrRNA-R	CGA ATT AAA CCA CAT GCT CCA	
V. cholera	OmpW-F	CAC CAA GAA GGT GAC TTT ATT GTG	427 bp
	OmpW-R	CGT TAG CAG CAA GTC CCC AT	
V. parahaemolyticus	Collagenase-F	GAA AGT TGA ACA TCA TCA GCA CGA	271 bp
	Collagenase-R	GGT CAG AAT CAA ACG CCG	
V. alginolyticus	GyrB-F	GAG AAC CCG ACA GAA GCG AAG	337 bp
	GyrB-R	CCT AGT GCG GTG ATC AGT GTT G	

DNA assay

DNA was assayed by agarose gel-electrophoresis, 5 µl from the PCR products were loaded in 1.5% agarose 0.6 µg/ml ethidium bromide in 1X tris acetate EDTA buffer using gel electrophoresis apparatus (SCIEPLAS, UK). DNA bands were visualized using UV transilluminator (Winpact Scientific, USA).

Results and Discussion

Bacterial infections represent the major cause of economic losses in fish farms [21]. The recorded mortality in affected farms was 8% to 15% during 2 weeks after disease onset (appearance of clinical signs as daily mortality was 0.5% to 1%) which considered direct economic losses due to bacterial infection.

General signs of septicemia including presence of hemorrhagic patches on the skin and at the base of pectoral fin together with scale desquamation and skin ulceration were the most common observed signs (Figure 1). Increase the abdomen size (ascites), (Figure 1) and exophthalmia was occasionally observed in diseased fish. Congestion of internal organs with enlarged liver and enlarged gall bladder (Figure 1), congested stomach walls and intestine was the dominant postmortem lesion (Figure 1). Similar clinical and post mortem finding was observed by Asaad [22], Zakaria [23], Laith and Najiah [24] and Zhang et al. [5] in naturally infected fish with *Aeromonas hydrophyla*. The same clinical finding was also observed in naturally infected *Oreochromis niloticus* with different *Vibrio* species as described by Chen et al. [25] and Okasha et al. [26].

Isolated bacterial pathogens express their effect through different virulence factors. *Aeromonas hydrophila* release a variety of virulence factors which are important for their pathogenicity [27], these virulence factors including outer membrane protein, extracellular products (cytotoxins and proteases) and enterotoxins [12,28]. Pathogenic *Vibrio* species produce a wide variety of proteases and extracellular enzymes

that capable of causing tissue and cell damage in infected fish, purified *Vibrio* proteinases are toxic to fish [8], *Vibrio cholera* is a highly virulent fish pathogen, [29], it has the ability to bind collagen, fibrinogen, gelatin, and fibronectin and have specific surface receptors for connective tissue [30]. Haemolysins and toxic proteases produced by *Vibrio alginolyticus* play great role in pathogenicity [31].

Out of thirty sample twenty-two isolate grow on R-S media producing yellow colonies which is specific for *Aeromonas hydrophyla* (Figure 1) while on TCBS media fourteen isolate grow giving yellow colonies (Figure 1) specific for genus *Vibrio* and none of the thirty isolates growth over *Pseudomonas* selective agar or modified Edwards media. Only one sample not grows on any of previously mentioned specific media.

Results of growth on specific media was controversial, as 7 bacterial isolates grow on both R-S and TCBS media in the same time that means either presence of mixed infection between *Aeromonas* and *Vibrio* in the same sample or ability of some strains to grow on both media in the same time, and to determine any of these possibilities is true all the recovered yellow colonies grow on Rimler-Shotts media were subjected to PCR identification for genus *Aeromonas* and for genus *Vibrio*, and so for all isolates recovered on TCBS were subjected to PCR identification for genus *Vibrio* and for genus *Aeromonas*.

PCR identification results become more obvious, out of twenty-two isolate grow on Rimler-Shotts media nineteen isolate was positive for genus *Aeromonas* and all of them are *Aeromonas hydrophyla*, and three isolates were positive for genus *Vibrio* (Table 2) after that these three-*Vibrio* species were identified by PCR as two *Vibrio alginolyticus* isolates and one *Vibrio cholera* isolates.

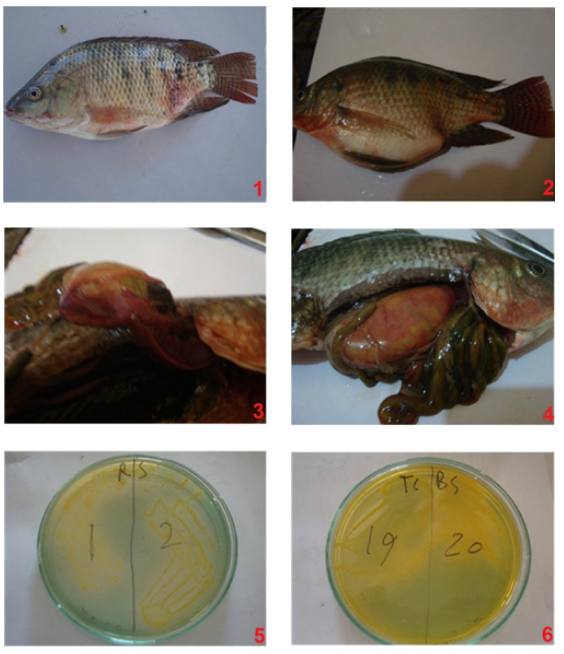

Figure 1: (1) Naturally infected *Oreochromis niloticus* showing scale desquamation, skin ulceration and hemorrhage. (2) Naturally infected *Oreochromis niloticus* showing abdominal distention with presence of hemorrhagic patches at caudal peduncle. (3) Naturally infected *Oreochromis niloticus* showing inflamed and severely congested hepatopancreas with presence of hemorrhagic patches. (4) Naturally infected *Oreochromis niloticus* showing inflamed and congested stomach and intestine. (5) R-S media with novobiocin selective supplement showing characteristic yellow colonies of *Aeromonas hydrophyla*. (6) TCBS media showing yellow colonies of *Vibrio cholera* and *Vibrio alginolyticus*.

Table 2: Number of clinically diseased fish samples and recovered bacterial isolates.

Number of affected farms	Number of clinically diseased fish samples	Number of recovered bacterial isolates
6	30	30
No. of isolates grown on R-S media	**No. of isolates grown on R-S, identified as genus *Aeromonas* by PCR**	**No. of isolates grown on R-S, identified as genus *Vibrio* by PCR**
22	19	3
No. of isolates grown on TCBS media	**No. of isolates grown on TCBS, identified as genus *Vibrio* by PCR**	**No. of isolates grown on TCBS, identified as genus *Aeromonas* by PCR**
14	10	4

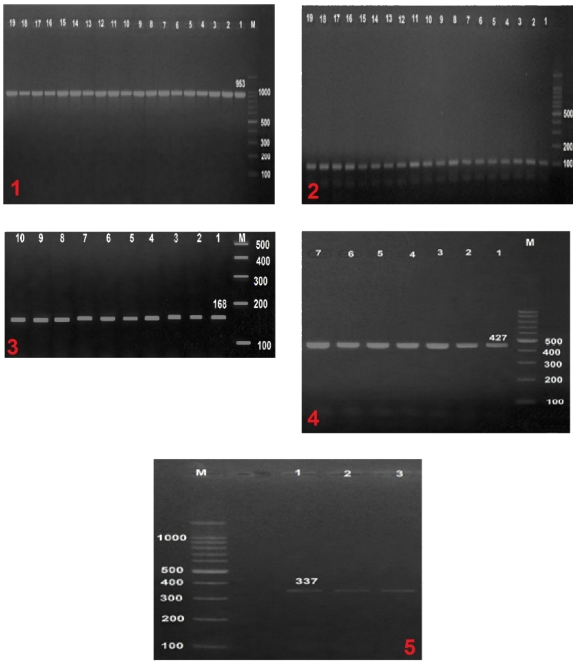

Figure 2: (1) Agarose gel electrophoresis of PCR product showing specific genus *Aeromonas* bands at 953 bp. (2) Agarose gel electrophoresis of PCR product showing specific *Aeromonas hydrophyla* bands at 103 bp. (3) Agarose gel electrophoresis of PCR product showing specific genus vibrio bands at 168 bp. (4) Agarose gel electrophoresis of PCR product showing specific *Vibrio cholere* bands at 427 bp. (5) Agarose gel electrophoresis of PCR product showing specific *Vibrio alginolyticus* bands at 337 bp.

PCR identification for fourteen isolates grown on TCBS indicating presence of only ten isolates were positive for genus *Vibrio*, from them seven isolates were *Vibrio cholera*, and three was *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. While the remaining four isolates identified as *Aeromonas hydrophyla* (Table 2).

PCR is a rapid and specific molecular technique provide a highly accurate results, amplification of 16S rRNA gene fragment indicated presence of nineteen *Aeromonas* sp. Isolate that characterized by appearance of specific bands at 953 bp, all these isolates were further identified by PCR as *Aeromonas hydrophyla* as characteristic bands appeard at 103 bp (Figure 2). Other researches indicating identification of such genus and species using the same primers design including Lee et al. [19] and Zakaria [23] for genus areomonas and Trankhan et al. [20] and Aboyadak et al. [4] for *Aeromonas hydrophyla*.

Agar gel electrophoresis of PCR product for detection genus *Vibrio*

and its different species revealed detection of ten isolates related to genus *Vibrio* at 168 bp by amplification of 16S rRNA gene (Figure 2), seven isolates were identified as *Vibrio cholera* at 427 bp through amplification of OmpW gene (Figure 2) and three isolates were identified as *Vibrio alginolyticus* at 337 bp through amplification of GyrB gene (Figure 2). In current research, no isolates were identified as *Vibrio parahaemolyticus*. Wei et al. [32] and Wei et al. [15] recorded the same results using the same primers but different thermal cycler conditions.

PCR results indicated the ability of 4 *Aeromonas hydrophila* isolates to grow on TCBS producing yellow colonies which was in complete agreement with Buller [33], Bridson [34] and Dworkin et al. [35], they indicated the ability of some *Aeromonas hydrophila* strains to grow on TCBS producing small yellow colonies. The PCR results also assert the ability of three *Vibrio* isolates to grow on Rimler-Shotts media, the three isolates were PCR negative for genus aeromonas and positive for genus *Vibrio*, one of them identified as *Vibrio cholera* and other two identified as *Vibrio alginolyticus*. Davis and Sizemore [36] and Arcos et al. [37] reported the ability of *Vibrio alginolyticus* to grow on Rimler-Shotts media. The current research results indicated non-selectivity of both Rimler-Shotts and TCBS media for isolation *Aeromonas* and *Vibrio* species from diseased fish and proved the inevitable need for other diagnostic techniques as PCR.

Current work refered the similarity between different isolated bacteria colonies color on specific media (R-S and TCBS) to the biochemical characters of each recovered bacterial species. On R-S media *Aeromonas hydrophila* ferment maltose that increase the pH to acidic side that subsequently turns bromthymol blue to yellow color, *Vibrio cholera* and *Vibrio alginolyticus* also ferment maltose producing similar change in media color to yellow, but the role of antibiotic substance (novobiocin) present in R-S media is to prevent the growth of any microorganisms other than aeromonas, but if *Vibrio cholera* and *Vibrio alginolyticus* resist novobiocin they can grow producing yellow color colonies similar to that of *Aeromonas hydrophila*, Rahim and Aziz [38] recorded resistance of 82% of toxogenic and 75% of non toxogenic *Vibrio cholera* strains isolated from water to novobiocin, and this support our point of view. On the other hand, *Vibrio cholera* and *Vibrio alginolyticus* can grow on TCBS giving yellow colonies as they ferment sucrose producing acid that decrease pH which makes bromthymol blue turn yellow color, this media depends on the presence of ox bile in inhibition of another bacteria growth. Current research documents the ability of some strains of *Aeromonas hydrophila* to grow on TCBS media giving yellow colonies, this can be explained by the ability of such microorganism to grow in the presence of bile and this suspect is supported by the ability of *Aeromonas hydrophila* to induce enteritis, moreover *Aeromonas hydrophila* has the ability to ferment sucrose as mentioned by Cipriano et al. [9] the produced acid shifts the pH to acidic side developing yellow color on TCBS media.

Thirty bacterial isolates were recovered from diseased fish samples (Table 3), *Aeromonas hydrophyla* represent 63.3% of total isolated pathogens, it considered one of the most important bacterial pathogens affecting cultured freshwater fish particularly tilapia inducing motile *Aeromonas septicemia* [9,39]. Ten *Vibrio* isolates represent 33.3% of isolated bacterial pathogens, 23.3% of them were identified as *Vibrio cholera* and 10% were *Vibrio alginolyticus*. *Vibrio* spp, which are considered a significant problem affecting aquaculture worldwide [40,41]. *Vibrio alginolyticus* and *Vibrio cholerae* are major pathogens in Chinese aquatic products [15]. Senderovich et al. [42] isolated *Vibrio cholera* from *Oreochromis aureus*; Rehulka et al. [43] isolated pathogenic *Vibrio cholerae* non-O1/non-O139 from moribund freshwater fish, and proved its ability to induce disease condition through experimental infection of common carp, infected carb developed typical signs of *Vibrio*sis. Unidentified pathogenic bacteria represent 3.3% (one out of 30 isolates).

Table 3: Total number and percent of each recovered bacterial isolates.

Recovered bacteria	Number of isolates	Percent (%)
Aeromonas hydrophila	19	63.33
Vibrio angiolyticus	3	10
Vibrio cholera	7	23.33
Other bacteria (not identified)	1	3.33

Conclusion

In conclusion, the disease condition affecting studied farms was induced by Gram negative bacterial pathogens including *Aeromonas hydrophila, Vibrio angiolyticus and Vibrio cholera* that represent (96.6%) of total isolates. Isolation and identification of bacterial fish pathogens depending on culture characters on specific media is inadequate as current research proves non-selectivity of R-S to *Aeromonas hydrophyla* and TCBS media for *Vibrio* sp. Isolated from fish, so polymerase chain reaction identification is inevitable.

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