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Original Article

Quantitative analysis of a fish pond for coliform bacterial content

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Abstract
A totalof seventy five water samples were collected from three different levels;level 1(surface), level 2 (sub-surface), and level 3(bottom) of a fish pond and subjected to enumeration and isolation of colony forming units of coliform bacteria by growing on EMB agar. Highest number of CFUs/ml was obtained in the samples collected from level 3. Following pure culturing, each isolate was processed for colonial characteristics, biochemical characterization and staining reactions. These isolates exhibited gamma and beta hemolysis but none of them was found resistant against polymyxin B contrary to erythromycin. These isolates were identified as *Klebsiella and Citrobacter*and the latter genus dominant.
Key words:*Enterobacteriacea,Klebsiella, Citrobacter,*coliforms, polymyxin, erythromycin,fish pond

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INTRODUCTION

Water pollution is one of the main causes which inhibit the proper growth of fish and ultimately decreases fish production (Benchalgo *et al.*, 2014).The water sources are mostly contaminated with fecal wastes(Ponce-Terashima *et al.*, 2014), which may cause various gastrointestinal diseases like diarrhea (Gruber *et al.*, 2014) and even death (Atlas and Bertha, 1997). Evaluation of water quality has been conducted for a long time by measuring fecal coliforms such as *Escherichia coli* and*Salmonella* spp., etc. (Abdelzaher *et al.*, 2010) that may cause water-borne diseases (Schets and de Roda Husman, 2014).

Bacterial as well as coliform count has been reported for pond water (Gogoi and Sharma, 2013).Fecal coliforms like *Escherichia coli* are markers of water quality(Leclerc *et al.*, 2001).They have long been considered as an indicator of fecal contamination (McLellan and Eren, 2014). *E. coli*, member of the family *Enterobacteriaceae* is the most sensitive indicator of fecal pollution (Edberg *et al.*, 2000)and is widely distributed in the intestines of humans and warm-blooded animals (Parveen *et al.*, 1999).*Klebsiella*isanothergenus of family *Enterobacteriaceae*. It is an important cause of nosocomial infections (Li *et al.*, 2014).Another group of the family *Enterobacteriaceae*is *Citrobacter* , causative agent of infections in the urinary tract, blood, superficial wounds, skin, peritoneum and several other normally sterile sites (Gupta *et al.*, 2003a).

Rivers, beaches and ambient water may get contaminated with human waste (Yamahara *et al.*, 2007; Gomi *et al.*, 2014). Swimming at beaches polluted with human waste can cause diarrhea and eye, ear, skin and respiratory infections, and even hepatitis and meningitis. However, some studies were shown that fecal bacteria cannot be always correlated with pathogens in wastewater (Thompson *et al.*, 2003)or environmental waters (McQuaig *et al.*, 2006) which may reach groundwater and surface water via runoff (Ying *et al.*, 2002).

Waterfowl are major excreters of fecal coliforms and *Streptococci* (Ashbolt *et al.*, 2001).They harbour bacteria in their intestinal tract that are potential human pathogens like *Salmonella* and *Campylobacter* causing gastroenteritis(Timbury *et al.*, 2002; Abulreesh *et al.*, 2004).These organisms have also been found in the intestinal tract of ducks and geese (Refsum *et al.*, 2002). It is known that fecal bacteria may contaminate fish (Cam *et al.*, 2007) reflecting the bacteriological conditions of water (Bisht *et al.*, 2014). Manure from livestock production is directly consumed by fish (Little

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and Edwards, 1999; Ahmed *et al.*, 2011) as it contains almost all the essential nutrients (Jana *et al.*, 2001), which stimulate the growth of planktons(Kadri and Emmanuel, 2003). Fish farming systems may introduce antimicrobials, their residues, and antimicrobial-resistant bacteria into fish ponds through animal manure (Petersen *et al.*, 2002).Antimicrobial resistance has been emphasized in traditional fish farming systems (Schmidt *et al.*, 2000); as residues of antimicrobials have been found in the sediments of marine fish farms too (Bjorklund *et al.*, 1990). This communication reports coliform content of University of the Punjab, Research Fish Farm.

MATERIALS AND METHODS

Seventy five samples were collected from the fish pond at The University of Punjab, near Zoology Department. Twenty five samples were collected randomly from level 1, level 2 and level 3 *i.e* fromsurface, sub surface and bottom zonationsof the pond, respectively. The samples were processed within 1 hr of collection and 0.1ml of the original sample was spread over prepared EMB agar plates. Similarly a 100-fold dilution of each sample was also spread in the same way with subsequent incubation at 37°C for 48 hrs. The count was expressed as colony forming units (CFUs/ml). Well separated colonies were purified by alternative streaking on EMB agar and nutrient agar for five times. Mature colonies were observed for variouscharacteristics like elevation, shape, color, size, consistency, elevation, motility and optical nature. Furthermore, each isolate was processed for Gram's and endospore staining in addition to various biochemical tests viz: catalase, indole, citrate utilization, oxidase, methyl red andVogesProskaurtests I & II andpathogenicity. The isolates were also assessed for drug resistance(Pelczar *et al.*, 1986; Benson, 2001).

Statistical analysis

The data were analyzed by one way ANOVA using Microsoft Excel 2010.

RESULTS

Significant ($P<0.05$) higher coliforms' density was found in sample collected from level 3 than those of levels 1 and 2 with the mean values of 370,700 and 1050×10^2 CFUs/ml of the pond water, respectively (Fig 1).

Colonies obtained of the isolates for samples collected from different levels were round, butterious and opaque. Isolates were identified as *Citrobacter* and *Klebsiella.* All species of *Citrobacter*were found oxidase and VogesProskaur -ve, whereas +ve for methyl red, catalase, indole and citrate. All the isolates of *Klebsiella* were +ve for citrate, catalase and VogesProskaur, giving –ve response for oxidase, methyl red and indole tests.

Table I: Colonial and biochemical characteristics of coliform bacteria isolated from egg shell surface

Sr. No.	Genus	Size(mm)/ Color	Elevation/ Motility	MR/ Citrate	Indole/ Oxidase	Catalase	VP-I/ II	Hemolysis	Antibiotic sensitivity test **(D= mm)**	
									PB	**E**
1	*Citrobacter*	3/ Purple	Raised/+	+/+	+/-	+	-/-	γ	S	I
2	*Citrobacter*	2-3/ Blue	Raised/-	+/+	+/-	+	-/-	β	S	S
3	*Citrobacter*	1.5/ Purple	Droplike/+	+/+	+/-	+	-/-	γ	S	I
4	*Klebsiella*	3.5/ Orangish pink	Droplike/-	-/+	-/-	+	+/+	γ	I	R
5	*Klebsiella*	4/ Light purple	Droplike/-	-/+	-/-	+	+/+	B	S	S
6	*Klebsiella*	6/Pink	Droplike/-	-/+	-/-	+	+/+	B	I	R

PB: Polymyxin B with disk potency of 300µg and E: Erythromycin with disk potency of 15µg (Kirby-Bauer method).

Gamma hemolysis was shown by the isolates 1, 2 and 3, whereas all other isolates gave beta hemolysis when grown on blood agar. None of the isolates showed resistance to polymyxin B, and only isolate 4 and 6 showed intermediate behavior. In case of erythromycin, the isolates 4 and 6 were found resistant, 1 and 3 gave intermediate response, whereas 2 and 5 were found sensitive (Table I). Contents of the culturable coliforms increased progressively from surface towards bottom of the fish pond (Fig. 1).*Citrobacter*was found dominant over *Klebsiella* at each level (Fig. 2).

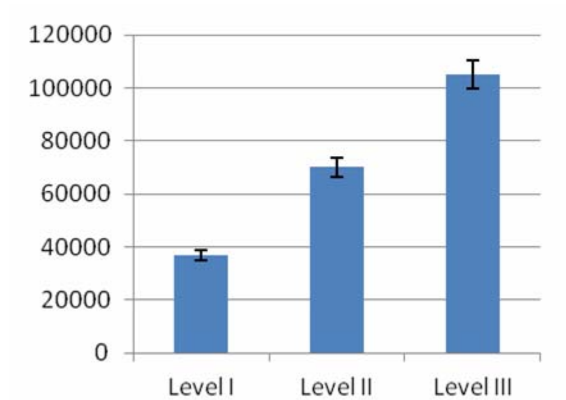

Figure 1 Number of CFUs /ml of original solution at different levels of the fish pond

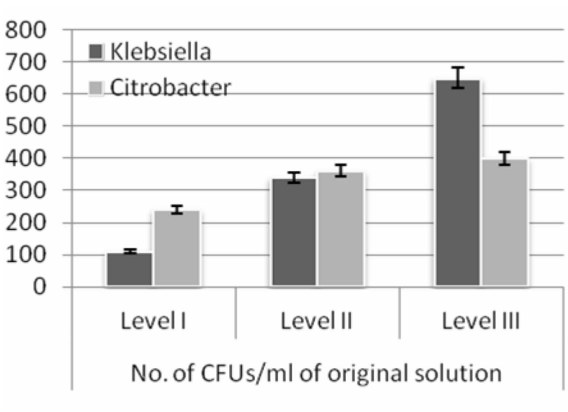

Figure 2 Number of CFUs /ml of original solution of isolates at different levels of pond

DISCUSSION

In the present study, lowest number of CFUs/ml of original solution was found at level 1 as compared to levels 2 and 3 of the pond. The level 3 contained highest number of coliform(Fig 1). As coliforms are facultative anaerobes, so it may be assumed that their number should be less at bottom of water body than its surface or sub surface part. But the reason for the highest number of coliforms at bottom of the pond in our experiment may be shallowness of pond.Kroger and Noll (1969) found *E. coli* and Gram's negative rod shaped bacteria in tube well water due to the low depth of water source.

Another reason for the highest count at bottom may be that fecal coliforms, after settling down might had encountered a favorable environment for reproduction (Davies *et al.*, 1995). The sediment's surface area and nutrient content promote the growth of bacteria (Sherer *et al.*, 1988). Fecal coliforms can survive for up to 60 days in freshwater sediments (Davies *et al.*, 1995) and can persist for a long time under hot and dry summer range conditions (Okafo *et al.*, 2003).

As samples were collected following a rainy day, so it may be another reason. For the higher counts of coliform Qureshi and Dutka (1979) had declared that coliform population increases during monsoon months due to rain water. Kistemann *et al.* (2002) observed that in case of rainfall, the microbial loads of runoff water may suddenly increase and reach the lakes very quickly. As the sampling was done in summer, so it provided favorable condition for multiplication and growth of coliforms.As Badge and Varma (1982) had reported that multiplication of coliform is retarded at low temperature.

Different isolates obtained in the present study belonged to genus *Citrobacter*and *Klebsiella*. Although these genera are generally thought as non-fecal coliforms but have been reported from a treated sewage effluent with multiple drug resistance too (Silva *et al.*, 2006). *Citrobacter*was found as the most dominant species in the present study (Fig 2).Daboor (2008) found *E.coli* as the most dominant bacterial species in most samples of fish farm. In another study *Salmonella* has been isolated from tannery polluted fish pond with higher number of total coliforms as compared to fresh water (Begum *et al.*, 2007).

Birds visiting the ponds may be a source of fecal contamination. In fact magnitude and diversity of microbial load of a water bodies from many factors (Jones, 2002). Generally, it is believed that fecal coliforms are present only in feces of warm-blooded animals but it may be speculated that the two genera *i.e.,Klebsiella* and *Citrobacter*isolated in the present study may be present in feces of cold-blooded fish inhabiting the fish pond; as several coliforms including fecal *Citrobater* , *Enterobacter* and *Klebsiella* have been isolated by Harwood *et al.* (1999) from the fecal matter of fresh water turtle which is also a cold blooded animal.

Another source of contamination of pond may be the water entering the pond through water pipeline. A study done by Kirmeyer *et al.*(1999) showed that even post-treatment contamination may be detected in surrounding distribution system of pipelines. *Enterobacter* and *Klebsiella*have been found to multiply in the water mains and storage tanks under favourable environment (Edberg *et al.*, 2000).

Polymyxin B showed excellent activity against *Citrobacter* (Table I).Comparable results have been documented in previous studies (Parchuri *et al.*, 2005; Gales *et al.*, 2006).Polymyxin B is considered as final treatment of many infections caused bymulti drug resistant (MDR) Gram-negative bacteria, such as *Pseudomonas*, *Acinetobacter*and *Klebsiella*(Zavascki *et al.*, 2007).

Klebsiella has been declared as ESBLs (extended spectrum beta- lactamase producer) thus as multidrug resistant (Polishko *et al.*, 2011), and had been found resistant against many drugs (Gundogan *et al.*, 2011) owing to a group of enzymes known as KPC (Miriagou *et al.*, 2003) for inducing resistance in several pathogens(Smith *et al.*, 2003; Bratu *et al.*, 2005). Another enzyme NDM-1 carbapenemasehas been held responsible to induce resistance in*Enterobacteriacea* against several groups of antibiotics in Pakistan, India and UK (Kumarasamy *et al.*, 2010). Perhaps this is the reason that *Klebsiella* is β-hemolytic(Li *et al.*, 2014).Erythromycin has been found as most effective against many Gram's negative bacilli like *Klebsiella* and *E.coli*(Lorian and Sabath, 1970) as was also found in present study. Less resistant and sensitive response of *Citrobacter*was observed against erythromycin as compared to findings of Fass(1993), where none of the three drugs tested including erythromycin were found active against members of the family *Enterobacteriaceae* or nonfermentative gram-negative bacilli. *Citrobacter* has been reported as infectious agent in immunosuppressed host being opportunistic pathogen (Gupta *et al.*, 2003b).Such pathogens in water may cause infections in fish too (Sanaa, 2009; Tetlock *et al.*, 2012). Introduction of antimicrobial-resistant bacteria from animal manure seems to favor prevalence of antimicrobial-resistant bacteria in the pond environment (Petersen *et al.*, 2002).

The present study suggests that fish reared in ponds receiving waste water passes danger of contaminated fish flesh with microorganisms pathogenic to humans as observed by Mandal *et al.*(2009). Fish ponds should have a proper boundary to check the approach of tetrapods. A proper netting above the pond may be used to avoid fecal contamination of visiting birds. Sterilization of feed/manure may minimize the chances of contamination. Equipping of ponds with emergency shades will certainly reduce the risks of introduction of pathogens via rain. Disinfection of pond water at regular time intervals and sanitization of water supply as well as drain will assure the decrease in level of contamination. Such practices must be considered compulsory in order to increase fish productivity and avoid post-harvest infections to the consumers.

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