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Article

Isolation, molecular identification and characterization of *Aeromonas hydrophila* from infected air-breathing catfish Magur (*Clarias batrachus*) cultured in Mymensingh, Bangladesh

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Abstract: The study was carried out from November, 2014 to February, 2015 with the objective of isolating *Aeromonas hydrophila* an important fish pathogen from infected air-breathing catfish Magur (*Clarias batrachus*) in Mymensingh district. Quantitative study of the isolated bacteria from infected *C. batrachus* was found variation of number in different organs. Total bacterial load was found to be 1.16 x 10^5 to 3.15 x 10^6 cfu/g in lesions, 2.14 x 10^8 to 4.17 x 10^9 cfu/g in liver, 1.90 x 10^7 to 5.12 x 10^8 cfu/g in spleen and 2.32 x 10^6 to 5.24 x 10^8 cfu/g in kidney of infected *C. batrachus* in Mymensingh district. The isolates were found to produce acid from arabinose, whereas acid and gas from different sugar media such as maltose, sucrose, and dextrose. Morover, they were capable to ferment glucose but resistant to vibriostatic agent 0129 test. Further identification of *A. hydrophila* was accomplished using PCR. The PCR products of desired 760 bp were obtained for *A. hydrophila*. The isolated *A. hydrophila* were 96% sensitive to Enrofloxacin followed by 88% to Ciprofloxacin and 76% to Levofloxacin. On the other way, 100% were resistant to the Ampicillin followed by 96% to Penicillin and 92% to Novobiocin. So far, this is the first molecular identification of *A. hydrophila* from farmed *C. batrachus* in Bangladesh. The present study will provide future research scopes on identification of pathogenicity island in chromosome and serotyping of all *A. hydrophila* isolates.

Keywords: *Clarias batrachus*; bacterial load; *Aeromonas hydrophila*; molecular identification

1. Introduction

Among the different air-breathing catfishes, Magur (*Clarias batrachus*) is very popular and highly valuable fish species in Bangladesh. It is not only recognized for its delicious taste and market value but it is also considered as a medicinal fish and traditionally remained a strike among the pregnant & lactating mothers, the elderly and children. It is prescribed prophylactically to the anemic & malnourished individuals as well as for the convalescent of the patients due to the nutritional superiority (Debnath, 2011). Generally, most of the catfishes are very hardy that can survive for quite a few hours outside the water due to presence of accessory respiratory organs (Khan *et al.*, 2003; Monir and Rahman, 2015). *C. batrachus* was abundantly available in open water of Bangladesh but presently, it is threatened due to over exploitation and various ecological changes in its natural habitat. Although, the appropriate breeding, nursing and rearing technology of fry and fingerlings of *C. batrachus* had been developed by Bangladesh Fisheries Research Institute (BFRI) in few years ago but unknown diseases of *C. batrachus* causes serious economic losses because of their high mortality.

By the increasing intensification of *C. batrachus* production particularly in greater Mymensingh region and lack of health management measures have lead to many disease problems of bacterial, fungal and parasitic origin. In most of the cases hemorrhages, septicemia, skin lesions are the common symptoms of the diseased fish. Skin of fish serves as organ of interaction with its environment and as the first site of attachment for different microorganisms (Noga, 2000). Attachment of microorganisms to fish skin often induces skin lesions which, irrespective of the size, results in colonization by many opportunistic pathogens, life-threatening osmotic stress, increased energy costs from locomotion due to impairment of mucus production, swimming imbalance, increased predation due to colour change and deficiency in oxygen uptake (Mohanty and Sahoo, 2007; Declercq *et al.*, 2003; Toranzo *et al*., 2005; Ahamad *et al*., 2013). Therefore, skin lesions adversely affect performance and productivity of the affected fish. As results, most of the fish farmers and various aquatic-drugs companies usually try to treat these infections using various antimicrobial chemicals as well as antibiotics but finally fail to stop mass mortalities and losses of *C. batrachus* due to development of resistance by the incriminated organisms.

Aeromonas spp. are ubiquitous inhabitants of aquatic ecosystems such as freshwater, coastal water, and sewage (Monfort and Baleux, 1990). They are increasingly being reported as important pathogens for humans and for lower vertebrates, including amphibians, reptiles and fish (Janda and Abbott, 1998). However, A*eromonas hydrophila* has been reported as a causative agent of outbreaks such as hemorrhagic septicemia and epizootic ulcerative syndrome (Rahman *et al*., 2002). It is the causative agent of MAS (motile *Aeromonas* septicemia) in both farmed and wild fishes. The disease is characterized by swollen abdomen, red mouth, hemorrhage in external surface and surrounding of the anus (Alain, 2009). *A. hydrophila* were frequently isolated from various lesions of epizootic ulcerative syndrome (EUS) of different fishes (Hasan, 2007). However, due to lack of proper diagnostic techniques and appropriate treatments, fish farmers are suffering from huge financial losses every year. Consistently, there is a serious lack of information on the pathogen associated with the diseases of farmed *C. batrachus* in Bangladesh. Therefore, there is an urgent need to give immediate attention to investigate diseases of *C. batrachus* and develop appropriate health management strategies. The objectives of this study were to isolate and identify the causative agent of disease from *C. batrachus* and evaluate their cultural sensitivity. The present study was designed with a view to achieve the following objectives:

a) Isolation and identification of *Aeromonas hydrophila* from diseased Magur (*C. batrachus).*
b) To determine the antimicrobial resistance patterns of the isolated bacteria.

2. Materials and Methods

2.1. Sample collection

A total of 200 moribund Magur (*Clarias batrachus*) presenting at least one or more of the clinical signs of haemorrhage on skin and fins, external and ulcerative skin lesions and abdominal distension were collected from ten commercial fish farms of Mymensingh district located in 24°38′3″N 90°16′4″E of Bangladesh during the period from November, 2014 to February, 2015. Minimum 20 moribund fish were collected by using scoop net from each affected fish farms. Most of the diseased fish were collected in a live condition and the samples were transported in well-aerated water to the Fish Disease and Health Management Laboratory of Bangladesh Fisheries Research Institute (BFRI), Mymensingh. The clinical signs and postmortem findings were recorded according to Rashid *et al.,* 2008; Ahmed *et al*., 2009.

2.2. Isolation of bacteria

Isolation of bacteria was carried out from the skin lesions and internal organs of the infetced *C. batrachus*. Lesions of skin were disinfected with 70% ethanol for removing the surface contaminants. Smears from the skin lesions and internal organs were aseptically inoculated on Tryptic Soya Broth (TSB) as described by Ferdowsy *et al*., 2011; Monir *et al*., 2016. The overnight enriched broth was streaked onto Aeromonas selective media and incubated at 37 °C for 24 hrs. Single colony was further sub-cultured until pure culture of bacteria was obtained. The isolated bacteria were identified according to their biochemical characteristics (Sabur, 2006; Ahammed *et al*., 2016).

2.3. Total viable count of bacteria

A drop of blood from collected moribund fish was dissolved on TSA plate for bacterial colony counting. Moreover, the fish samples were dissected immediately after further clinical examination. The portions of infected skin, liver, spleen and kidney were aseptically removed, weighed on an electric balance and kept in a sterilized pastel mortar for homogenizing. Each organ was homogenized with physiological buffered saline (PBS) in the ratio of 0.1 g of organ: 0.9 ml of PBS to make stock solution. Eight decimal dilutions were

prepared by transferring 0.1 ml from the earlier test tube to the next. These eight tubes were designated as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. Two samples of 0.1 ml from 10^{-2} and 10^{-3} in case of liver and kidney; 10^{-3} and 10^{-4} in case of spleen were transferred to Aeromonas selective medium to get only the colonies of *Aeromonas* spp (Ahammed *et al*., 2016).

Total bacterial load of each organ was calculated using the following formula used by Rashid *et al*. (2008).

$$\text{Total bacterial load} = \frac{\text{Avarage number of colonies on plates}}{\text{Dillution factor} \times \text{Volume plated}}$$

2.4. Identification of the isolated *Aeromonas* spp.

Identification of the isolated *Aeromonas* spp. was done based on detailed morphological, physiological and biochemical characterization. The isolated bacteria were sub-cultured onto TSA plates to obtain fresh 24 hours culture. They were then streaked onto the Aeromonas selective medium for preliminary identification of the genus *Aeromonas* and discarding the others. Colonies grown on the selective medium were sub-cultured again onto TSA plates and subjected to biochemical tests using commercially available media after autoclaving at 121°C for 15 minutes.

2.5. Motility test

The young and actively growing culture of the bacteria were collected from 24 h culture at 30°C for motility test. A single bacterial colony was mixed with 3 ml of PBS. A drop of the suspension was taken on clean glass slide, covered with cover slip and placed under compound microscope. Bacterial motility was observed in a LCD monitor screen, adjusted with the microscope (OLYMPUS, Model CHS, Japan).

2.6. Physiological characterization of the isolated bacteria

Physiological characters were observed to growth of each isolate at temperature of 4°C, 37°C and 40°C. Growth of each isolate was also observed in different concentrations of NaCl as 0%, 1%, 2%, 3%, 3.5% and 4%.

2.7. Biochemical characterization of the isolated bactreia

Different biochemical tests were conducted to evaluate the biochemical characteristic of the isolated bacteria. The biochemical tests were oxidase, catalase, oxidative-fermentative test, 0/129 test, esculin hydrolysis test, acid and gas production from sugars: Glucose, Lactose, Sucrose, Mannitol; methyl-red (MR) test, Voges-Proskauer (VP) test, indole and H_2S production, decarboxylase test and citrate utilization test.

2.8. Molecular dectection of *Aeromonas hydrophila*

The genomic DNA was isolated as per the protocol described by Swaminathan *et al*. (2004); Borty *et al*. (2016). The isolates bacteria were sub-cultured onto TSA plates to obtain fresh 24 hours culture. A single colony from the sub-cultured was inoculated in 10 ml of Tryptic Soya Broth (TSB) and incubated at 29°C for 24 hours. The fresh culture was centrifuged at 5000 rpm for 10 minutes. Then four hundred microlitre of solution I (50mM Tris.HCl pH-8.0, 50mM EDTA pH-8.0, 25% sucrose, 1 mg lysozyme), was added to the washed cell pellet and porperly mixed and incubated at 37°C for 15 minutes. Thereafter 400 ml of solution II (10mM Tris. HCl pH 8.0, 5mM EDTA pH-8.0, 1% SDS, 40µg Proteinase K) was added to the cells and incubated at 55 ^{o}C for three hours. Then the suspension was centrifuged at 6000 rpm for 10 minutes. The aqueous layer from the top was removed carefully to avoid any protein debris and transferred to a fresh microfuge tube. Double amount of chilled ethanol was added to aqueous phase so as to precipitate the DNA. The DNA was pellted by centrifugation at 12000 rpm for 10 minutes. The pellet, washed with 70% ethanol was dried and dissolved in 100 µl of TE buffer (pH 7.6). Primers used for the amplification of DNA as shown in Table 1. PCR was done as per the method described previously by Cascon *et al*. (1996); Borty *et al*. (2016). Amplification was performed with a DNA thermal cycler (Mastercyclear, Eppendorf, Humburg, Germany) with some modifications as follows: The reaction mixture consisted of 1µl of Taq polymerase (1unit), 5 µl of 10X PCR amplification buffer (100 mM Tris-Hcl, 25 mM MgCl2, 500mM KCl, pH-8.3), 3 µl of deoxynucleoside triphosphate (100µM), 0.5 µl of each primer (100 pmoles) and double distilled water upto a final volume of 50 µl. A total of 40 PCR cycles were run under the following conditions: Initial denaturation at 94^{0}C for 4 minutes, denaturation at 94 ^{0}C for 1 minute, primer annealing at 65 ^{0}C for 1 minute, DNA extension at 72 ^{0}C for 1.5 minutes and final extension at 72 ^{0}C for 5 minutes. The DNA concentration was estimated by visual comparison with the standard DNA size markers after electrophoresis through 0.8% agarose TAE (tris-acetate EDTA) gels stained with 0.5 mg ml-l ethidium bromide (Sigma Chemicals Co.).

2.9. Determination of antibiogram of *Aeromonas hydrophila*

Antibiotic susceptibility of the bacteria isolates was determined using the disc diffusion method as described by Finegold and Martin (1982). Stock cultures of the bacterial strains were grown in TSA for 24 h at 37 ^{o}C. Then colonies of each of the isolate were adjusted to 0.5 McFarland's turbidity standard (equivalent to 1x10^{8} colony forming unit/ml) in sterile phosphate buffered saline (PBS) and the bacterial suspension was spread onto Mueller–Hinton agar (Oxoid). Antibiotic-impregnated discs were kept on the solid medium and the plates were incubated at 37 ^{o}C for 24 h. Zones of inhibition formed around the discs were measured and antibiotic sensitivity was assayed from the length of the diameter of the zones (in mm). The zone radius was actually scaled from the centre of the antibiotic disc to the end of the clear zone where bacteria could be seen growing. The antibiotics, their codes and concentrations were as follows: ampicillin (10 µg), ciprofloxacin (5 µg), enrofloxacin (10 µg), gentamicin (10 µg), oxytetracycline (10 µg), penicillin (10 µg), levofloxacin (5 µg), azithromycin (10 µg), tetracycline (30 µg) and novobiocin (5 µg). Tested bacterial strains were classified into three categories: sensitive, intermediate, and resistant and depending on the diameters of inhibition zones and standards supplied by Himedia Laboratories and comparing with other related references (Table 2). All tests were carried out in Fish Diseases and Health Management Laboratory of Bangladesh Fisheries Research Institute (BFRI), Mymensingh.

3. Results

3.1. Diseased signs

The infected *C. batrachus* were observed usually hemorrhage at the base of fin and edge of head, ulcerative skin lesions on body, body and tail erosion (Figure 1). Internally, hemorrhagic and grossly enlarged liver, spleen, kidney and the presence of congested blood vessels were also observed.

Figure 1. Erosive skin lesions in naturally-infected *C. batrachus* (arrows).

3.3. Morphological, physiological and biochemical test results of isolates

The isolated *A. hydrophila* from infected *C. batrachus* was identified based on the morphological, physiological, conventional, and biochemical characteristics. Morphologically the isolated colonies showed yellowish opaque, round, convex, smooth edged, and semi-translucent colonies on TSA plates. They were gram-negative, rod-shaped motile bacteria, positive for oxidase and catalase test. The isolated were found to produce acid from arabinose, whereas acid and gas from different sugar media such as maltose, sucrose, and dextrose. Moreover, they were capable to ferment glucose but resistant to vibriostatic agent 0129 test. Consequently, the isolates showed positive growth at 37 °C with the optimum at 24 °C but no growth was found at 4 °C and 40 °C. Furthermore, they were capable of growing in 0-1% NaCl, however, no growth was noted in 2-4% NaCl media (Table 3).

3.2. Bacterial load in skin lesions and infected internal organs

Load of bacteria in skin lesions, liver, spleen and kidney of infected *C. batrachus* were calculated and then observed to have a variation. Total bacterial load was found to be 1.16 x 10^{5} to 3.15 x 10^{6} cfu/g in lesions, 2.14 x 10^{8} to 4.17 x 10^{9} cfu/g in liver, 1.90 x 10^{7} to 5.12 x 10^{8} cfu/g in spleen and 2.32 x 10^{6} to 5.24 x 10^{8} cfu/g in kidney.

Table 1. Primers used for *Aeromonas hydrophila* genome detection.

Primers	Sequences (5′ – 3′)	Amplicon Size (bp)	Reference
Forward Primer	5′-AACCTGGTTCCGCTCAAGCCGTTG- 3′	760	Cascon *et al*., 1996; Borty *et al*. 2016
Reverse Primer	5′-TTGCCTCGCCTCGGCCCAGCAGCT- 3′		

Table 2. Interpretation standards for disc diffusion susceptibility testing for *Aeromonas hydrophila* (CLSI 2012).

Sl. No.	Name of Antimicrobial agent	Disc concentration	Interpretation of results (zone in diameter in mm)		
			R	I	S
1	Ciprofloxacin	5 µg	≥ 16	12 – 15	≤ 17
2	Enrofloxacin	5 µg	≥ 18	13 – 20	≤ 21
3	Livofloxacin	5 µg	≥ 22	17 – 21	≤ 23
4	Gentamycin	10 µg	≥ 14	10 – 13	≤ 15
5	Azithromycin	15 µg	≥ 16	11 – 15	≤ 17
6	Tetracyclin	30 µg	≥ 14	09 – 13	≤ 15
7	Oxytetracyclin	10 µg	≥ 15	11 – 14	≤ 16
8	Novobiocin	5 µg	≥ 17	14 – 17	≤ 18
9	Apicilin	10 µg	≥ 22	16 – 21	≤ 23
10	Penicillin	10 µg	≥ 14	10 – 13	≤ 15

Sl = Serial, No. = Number, µg = Microgram, mm = Millimeter, S = Susceptible, I = intermediately resistant, R = Resistant, ≥ = Greater than or equal to, ≤ = Less than or equal to.

Table 3. Results of biochemical characteristic of isolated bacteria.

Characters	Characterization by Ahammed *et al*. (2016)	Characterization by Sabur (2006)	Present result
Gram stain	-	-	-
Shape	Rod	Rod	Rod
Motility	+	+	+
0129	ND	ND	-
Oxidase	+	+	+
Catalase	+	+	
OF test	F	F	F
Glucose	+	+	+
Lactose	+	+	+
Sucrose	+	+	+
Maltose	+	+	+
Manitol	+	+	-
Inositol	-	-	-
Sorbitol	-	-	-
Arabinose	ND	ND	+
Rhamnose	-	-	-
Esculin hydrolysis	+	+	+
Methyl-red test	-	-	-
Voges-Proskaur	+	+	+
Indole	+	+	+
H_2S production	+	+	-
Arginine decomposition	+	+	+
Lysine decarboxilation	-	-	-
Ornithine decarboxilation	-	-	-
Citrate utilization	+	+	+
TSI	ND	ND	'K' in slants but 'A' in butt
Growth at: 4°C	-	-	-
5°C	+	+	+
37°C	+	+	+
40°C	-	-	-

+: Negative; -: Positive; F: Fermentative; K: Alkaline; A: Acid, ND: Not done

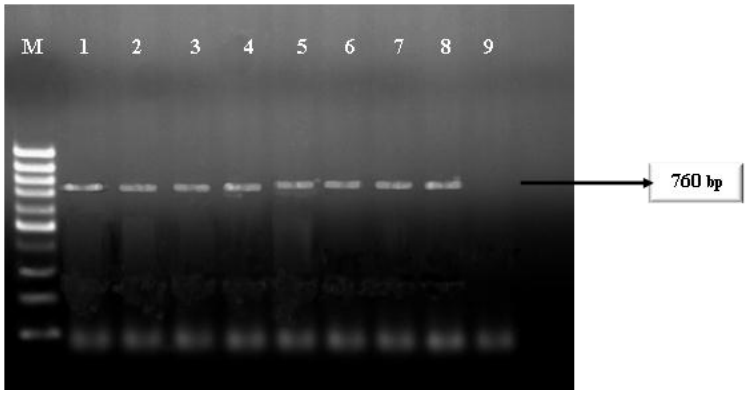

Figure 2. Agarose gel electrophoresis of PCR amplification generated by *Aeromonas hydrophila*. Lanes: (M) 100 bp DNA marker; (1-7) positive samples from field; (8) positive control; (9) negative control.

Table 4. Antibiogram profile of isolated *Aeromonas hydrophila* (n=25).

Antibiotics	No. (%)		
	Sensitive	Intermediate	Resistant
Ciprofloxacin (5µg)	22 (88)	3 (12)	0 (0)
Enrofloxacin (5µg)	24 (96)	1 (4)	0 (0)
Levofloxacin (5µg)	19 (76)	6 (24)	0 (0)
Gentamicin (10µg)	17 (68)	8 (32)	0 (0)
Azithromycin (15µg)	15 (60)	10 (40)	3 (12)
Tetracycline (30µg)	5 (20)	12 (48)	8 (32)
Oxytetracycline (10µg)	1 (4)	8 (40)	16 (56)
Novobiocin (5µg)	0	2 (8)	23 (92)
Ampicilin (10µg)	0	0	25 (100)
Penicillin	0	1 (4)	24 (96)

3.4. Molecular identification of *Aeromonas hydrophila* by PCR

PCR products of desired size 760 bp were obtained in reaction mixture containing genomic DNA of the targeted organisms, *A. hydrophila* wheares no product was detected in control (Figure 2).

3.5. Susceptibility to antimicrobial agents

The isolated bacterial samples were tested against ten commercially available antibiotics and the results of their sensitivity are presented in Table 4. In this study, the isolated *A. hydrophila* were 96% sensitive to Enrofloxacin followed by 88% to Ciprofloxacin and 76% to Levofloxacin. On the other way, 100% were resistant to the Ampicillin followed by 96% to Penicillin and 92% to Novobiocin.

4. Discussion

Magur (*C. batrachus*) is considered as a high valued species in Bangladesh. Although, *C. batrachus* is a hardy fish but production of it's in the cultured ponds has been affected by various factors including diseases caused by bacterial and fungal pathogens. However, the clinical symptoms and post mortem findings of the infected *C. batrachus* in this study were hemorrhage at the base of fin and edge of head, ulcerative skin lesions on body, body and tail erosion which are more or less similar with the findings of Khalil *et al*. (2010); Mastan (2013); Ahammed *et al*. (2016). Congested liver and internal organs were also observed in the infected fishes by Ahmed and Shoreit (2001); Gamal *et al.* (2002). Total bacterial load was found to be 1.16×10^5 to 3.15×10^6 cfu/g in lesions, 2.14×10^8 to 4.17×10^9 cfu/g in liver, 1.90×10^7 to 5.12×10^8 cfu/g in spleen and 2.32×10^6 to 5.24×10^8 cfu/g in kidney of infected Shing which finding is similar with this study. Rahman and Chowdhury (1996) isolated *A. hydrophila* from kidney of carp fishes, total load of bacteria varied in the kidney of different sampled fishes were 2.6×10^5 to 1.7×10^6 cfu/g. Here the variations might be due to different fish species and seasonal variations that influences the disease incidence.

The results of phenotypic characterization of the *Aeromonas* species showed that the isolates were motile species and this finding further suggests that the lesions resulted from motile *Aeromonas* septicaemia (Noga, 2000). In the present study, the isolates were gram negative, produced acid and gas from glucose, resistant to

vibriostatic agent 0129 and hydrolyzed esculin which were almost similar to the findings reported by many investigators (Anyanwu *et al*., 2014; Monir *et al*., 2016). For the molecular detection of the bacterial causative agent of *C. batrachus* diseases PCR was done by using gene specific primer according to Cascon *et al*., 1996 ; Ahammed *et al*., 2016. Thune *et al.* (1093) also found focal necrosis haemorrrhages in the liver tissue, atrophy of the renal tubule in kidney and villi missing in intestine from the naturally infected shing fish by *Aeromonas hydrophila* in a mixed infection with the *Aphanomyces invadans* elicited EUS disease. Monir *et al*. (2016) reported that *A. hydrophila, A. salmonicida* and *A. sobria* were isolated frequently from the skin lesions of *Heteropneustes fossilis* but *Aeromonas* spp. appeared to be the main pathogen in the diseased fishes. However, available information suggests that in the aquatic environment, *A. hydrophila* should be considered as opportunistic bacteria with potential to become pathogenic for fish under stressful conditions. Furthermore, the antibiogram profile of different antibiotics against *Aeromonas hydrophila* was found more or less similar to those that previously reported by Borty *et al*. (2016); Monir *et al*. 2016. From this study, farmers of *C. batrachus* will be benefited for controlling skin lessons diseases by the administration of specific theraputents. However, this study will provide for future research scopes on identification of pathogenicity island in chromosome and serotyping of all *A. hydrophila* isolates.

5. Conclusions

The present study was conducted to identify the *Aeromonas hydrophila* from skin lesions of air-breathing catfish Magur (*C. batrachus*). This skin lesions could be a major cause of considerable economic loss to *C. batrachus* farmers particualrly in greater Mymensingh region. In addition, *Aeromonas* isolates from the skin lesions have developed multidrug resistance against avaialble commercial different antibiotics probably due to indiscriminate or abuse in aquaculture. However, isolation and identification of causative agent and determination of the antimicrobial profile of bacterial agents associated with skin lesions is very improtant for effective antimicrobial treatment. Therefore, attention should be drawn to maintain appropriate ambient for culturing *C. batrachus* to avoid common diseases.

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Conflict of interest

None to declare.

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