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Original Article

Identification, isolation and pathogenicity of *Aeromonas salmonicida* and histopathology of infected *Oncorhynchus mykiss* in Punjab and northern areas of Pakistan

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Abstract
Aeromonas salmonicida causes furunculosis in the *Oncorhynchus mykiss* (rainbow trout) and the effective control over this infection requires knowledge of the genetic variability and epidemiology of *A. salmonicida*. Pathogenic strains were isolated from kidneys, muscles, liver and spleen of rainbow trout from the provinces, Punjab, Khyber Pakhtunkhwa and Gilgit Baltistan, Pakistan. On the base of 16S rRNA sequencing, physiological and biochemical characterization, isolated bacterial strains were identified as *A. salmonicida* (NCBI Ref. ArS-Pak-19 [MW307221], ArS-Pak- GB1-19 [MW720959], ArS-Pak-MRE-19 [MW720960], ArS-Pak-SW2-19 [MW720961], ArS-SW1-Pak-19 [MW720962]). Isolated strains were resistant to antibiotics like sulfamethoxazole, penicillin, vanomycine, rifampicin and bacitracin but were extremely sensitive to spectinomycin, ciprofloxacin, ofloxacin, levofloxacin and nalidixic acid. To check out the pathogenicity, rainbow trout were experimentally infected with isolated strains. Experimental fishes showed the same symptoms as were recorded in naturally infected fish including jaw bleeding, intra-abdominal fluid, intestinal bleeding and gill filament anemia. After ten days of post-challenge study, histopathological analysis revealed that there were severe alterations in the spleen, liver and kidney of the infected fish. The present study provides further research foundation and for upcoming research on *A. salmonicida* disease, its control and epidemiology.

Keywords: 16S rRNA; *Aeromonas salmonicida*; biochemical characterization; histopathology; isolation

1 | INTRODUCTION

Aquaculture industry is globally vital but frequently undergoing heavy economic losses that threaten the growth and sustainability mostly due to uncontrolled microbial infections resulting in bulk mortalities (Kowalska *et al.* 2020; Lim and Hong 2020). The most important factors out of all the microbial infections are bacterial and viral (Choi *et al.* 2019). Recognition, treatment and prevention

of fish diseases are vital for the improvement of fish culture and subsequent increase in fish production (Waiho *et al.* 2021). *Aeromonas salmonicida* is a potential agent of furunculosis in various fish species (Crumlish and Austin 2020) such as *Salmo salar* (Yi *et al.* 2021), Chinese freshwater fishes (Lin *et al.* 2020), *Oncorhynchus mykiss* (Diao *et al.* 2020), *Psetta maxima* (Zhao *et al.* 2021), *Esox lucius*, *Petromyzon marinus*, *Carassius carassius* (Jin *et al.* 2020) and *Sebastes schlegelii* (Li *et al.* 2021). *Aeromonas salmonicida* is a notorious bacterial pathogen in cold water aquaculture worldwide (Hayatgheib *et al.* 2020). At present, furunculosis causes economic loss in rainbow trout farming and disease outbreaks become more frequent during stress periods with the increase of temperature from July to August (Uiuiu *et al.* 2021).

The rainbow trout, *O. mykiss*, is important globally, belongs to the salmonids and is native to the North drainage of the Pacific and now it is widely distributed all over the world (DIAS 2019). The farming of rainbow trout in Pakistan has a wide range and is currently receiving attention. It can be found in freshwater lakes, mountain streams, and clean water bodies (Yonar 2018; Hoseinifar *et al.* 2020; Rashidian *et al.* 2020). In Pakistan's northern regions, rainbow trout farming provides a source of income for the local population, but research on growth, nutrition and most importantly, disease and health management is lacking. Pakistan trout farming faces the furunculosis that leads to economic losses to the trout farmers in the province of Punjab, Gilgit Baltistan and Khyber Pakhtunkhwa, Pakistan as there is very little information regarding the pathogenicity of *A. salmonicida* and epidemiology in rainbow trout. The isolation and molecular identification and pathogenicity of causative agents were the core objectives of present study which also provides bases for future study of disease caused by *A. salmonicida* and prevention epidemiology (Akram *et al.* 2023).

2 | METHODOLOGY

2.1 Sample collection

Four hundred live samples of Rainbow trout were observed from the trout hatcheries of Gilgit Baltistan, named as Kargah Trout Fish Hatchery and Farms, District Gilgit (*n* = 33; 35.91°N/74.26°W.), Sherquila Trout Fish Hatchery, District Ghizer (*n* = 67; 36.28°N/74.31°W), Trout Fish Farm, District Goru (*n* = 93; 35.92°N/74.31°W), Madyan Trout Fish Hatchery Swat, Khyber Pakhtunkhwa (*n* = 107; 35.14°N/72.54°W), Masoot Trout Fish Hatchery Kali Mitee, Murree, Punjab (*n* = 100; 33.91°N/73.40°W). Out of 400 fish individuals, 96 were separated, potentially infected by the bacterial pathogens on the basis of symptoms like skin lesions, bleeding and darkening of skin.

The live infected fish were packed, according the guidelines of UVAS ethical committee, into polythene bags, filled with oxygen and transported at Quality Operations Laboratory (QOL), UVAS, Lahore, for further processing. Samples of the diseased fishes from Swat, Khyber Pakhtunkhwa, were dissected at the spot and infected organs (muscles, liver, spleen and kidney) were preserved in nutrient broth for bacterial culture in ice boxes and transported to the laboratory.

2.2 Isolation of pathogenic strain and molecular identification

Fish samples were dissected in the laboratory to isolate bacteria from muscles, spleen, kidney and liver from the diseased fish and inoculated on MacConkey agar and Nutrient agar plates and incubated at 25°C for 72 h. Molecular identification of isolated bacteria was done by using standard procedures (Sughra *et al.* 2022). The genomic DNA of the pathogenic strain was extracted by bacterial DNA extraction kit (GenElute) with standard protocol (Jin *et al.* 2020). The extracted bacterial genomic DNA from the isolates was used for Polymerase Chain Reaction (PCR). PCR amplification was done by using universal primers (16S rRNA) 27F (5′- AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GATACCTTGTTACGACTT-3′) (Weisburg *et al.* 1991). The conditions for PCR, were as follow: denaturation was for 5 minutes at 94°C; 30 cycles of denaturation at 94°C for 30 seconds, annealing was carried out at 55°C for 30 seconds and the extension was carried out at 72°C for 10 minutes using 1% agarose gel. The product of the PCR, purified and further processed for gene sequencing by using ABI 3730. The sequences were submitted to NCBI for accession numbers were ArS-Pak-19 (MW307221), ArS-Pak- GB1-19 (MW720959), ArS-Pak-MRE-19 (MW720960), ArS-Pak-SW2-19 (MW720961), ArS-SW1-Pak-19 (MW720962) and evolutionary tree was made by using Molecular Evolutionary Genetic Analysis (MEGA; Sughra *et al.* 2022).

2.3 Biochemical testing of isolated bacteria

The identification of bacteria was carried out using common bacterial identification, physiological and biochemical criteria. The isolates were stained using Gram staining technique and examined under microscope (Jin *et al.* 2020) to observe bacterial morphology.

2.4 Antimicrobial sensitivity tests

After the confirmation of bacterial isolate, disc diffusion method was used to test the drug susceptibility according to the Jonasson *et al.* (2020). The isolates were replicated and incubated in nutrient broth for 72 h at 25 °C with continuous shaking. The bacterial concentration was calculated by using the formula: CFU (ml^{-1}) = Number of colonies × total dilution factor / volume of the culture plated per ml and adjusted at 1.0×10^{7} CFU ml^{-1} and 0.1 ml spread on the nutrient agar plates equally, after that the drug was added on the inoculated agar (Jin *et al.* 2020).

Susceptibility of bacterial strain was tested against 17 generic drugs.

2.5 Experimental pathogenicity study

Experimental pathogenicity test was carried out on three experimental groups (T1, T2 and T3) injected with three different doses of pathogenic bacterial counts (1×10^9, 1×10^8 and 1×10^7 CFU ml^{-1}) and a control group injected with same amount of saline water. Forty healthy rainbow trout (145 – 150 g, five in each treatment) were acclimatized for seven days in cemented rectangular tanks (0.9 m × 0.6 m × 0.7 m). After acclimatization fish were injected intraperitoneally with 0.1 ml of each bacterial concentration and observed for further 10 days to check the pathogenicity of bacteria on healthy fish through histopathological examination. At the end of the trial, three samples from each experimental group were dissected for fish organs (liver, spleen and kidney) embedded in paraffin, sectioned and stained utilizing 10% neutral buffered formalin to permanently fix them, hematoxylin and eosin was used to analyse the histopathology to analyse the histopathology and to check the pathogenicity of *A. salmonicida* on fish organs.

3 | RESULTS

3.1 Biochemical and morphological characteristics

Fish showed infestation of bacteria with diseases symptoms including swollen belly, bleeding surfaces and pale gills (Figure 1). Out of 96 diseases fish, 68 (17% of the total specimens) were infected with the pathogenic bacteria. Isolated bacteria were cultured on the nutrient agar which resulted in yellowish colour colonies of 1 mm in size (Figure 2). Gram staining indicated strain as gram negative, non-motile and rods appearance. On the bases of biochemical and physiological tests the isolated bacterium was identified as *A. salmonicida* (Table 1).

3.2 Tests of antibiotics susceptibility

The tests of antibiotics susceptibility of the isolated strains of *A. salmonicida* showed that it was extremely sensitive to ofloxacin, ciprofloxacin, spectinomycin, lomefloxacin and nalidixic acid. These were reasonably sensitive to tetracycline, kanamycin, erythromycin, streptomycin, neomycin, tobramycin and gentamicin (Table 2). These strains were resistant to penicillin, vancomycin, compound sulfamethoxazole, rifampicin and bacitracin (Table 2).

3.3 Pathogenicity results

After the confirmation of species, a pathogenicity test was conducted for all five isolates to confirm the bacteria as pathogenic organisms and the most virulent was ArS-Pak-19. The pathogenicity experiments of isolated strains of *A. salmonicida* showed that these were strongly virulent in rainbow trout (Table 3). The Figure 3 showed a curve which indicates mortality of the fish with different concentration of vaccines.

TABLE 1 Biochemical characterization of isolated strains.

Test	Result
L-serine	-
D(+)-sucrose	-
Acetate	-
Oxidase	+
L-arabinose	+
Tartaric acid	-
L-rhamnose monohydrate	-
6% NaCl growth	-
Raffinose	-
D-xylose	-
Mucic acid	-
Trehalose	+
D(+)-cellobiose	-
L-alanine	-
Citric acid	-
1% NaCl growth	+
0% NaCl growth	+
Phenylalanine deaminase	-
O. decarboxylase	-
Nitrate reduction	+
Kinesis	-
Maltose	+
Sorbitol	-
D-glucose acid	+
D(-)-salicin	-
Lysine decarboxylase	-
APG	-
Galactose	+
Melibiose	-
Hydrogen sulfide production	-
D-mannitol	+
Voges-Proskaure.	-
Lactose	-
Methyl red	+
Indole substrate	-

3.4 Histopathology

In inoculation experiments, after 10 days experimental fish were microscopically biopsies and compared the pathological changes in healthy and infected tissues. There were remarked pathological changes occurred after infestation of *A. salmonicida* in liver (Figures 4A and 4B), kidney (Figures 4C and 4D) and spleen (Figure 4E and 4F) of the artificially infected fish.

The most pathogenic strain was ArS-Pak-19 inoculated in fish was isolated from tissues of the artificially infected fish. The morphology of the colony and other characteristics of the isolated strain were the same as ArS-Pak-19. In the pathogenicity test the infected fish had the same symptoms as in natural infection. The key symp-

toms observed were bleeding from the fin base, ascites, jaws bleeding and enteritis. According to third and fourth Koch's postulates, the pathogen isolated from the host and inoculated again in the healthy organism will cause the same symptoms.

Finally, it was concluded that the isolated strain was *A. salmonicida* a pathogenic bacteria that causes severe losses to the salmon industry in Pakistan. In this study 400 diseased fish was collected and 68 were infected with *A. salmonicida* which cause 17% death of salmon in Pakistan salmon industry.

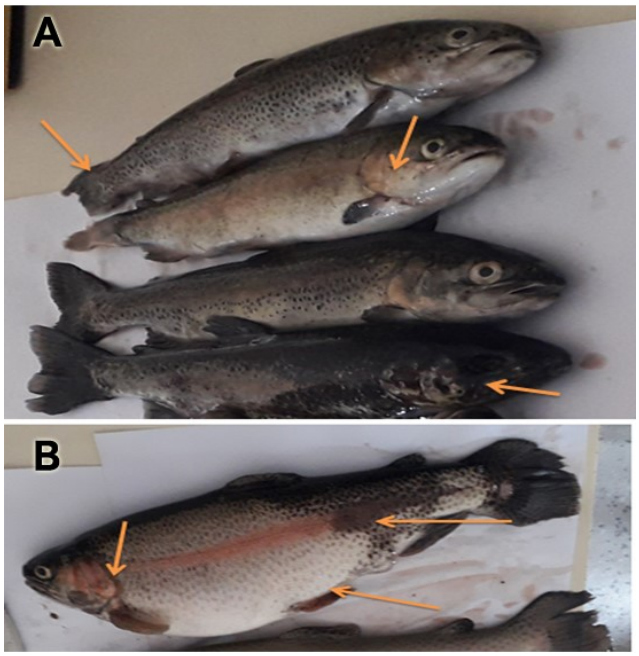

FIGURE 1 Infected rainbow trout showing tail rot, bleeding from body surface and sever ulcer around eye (A) and gill anemia, body furuncles and abdominal ascites (B).

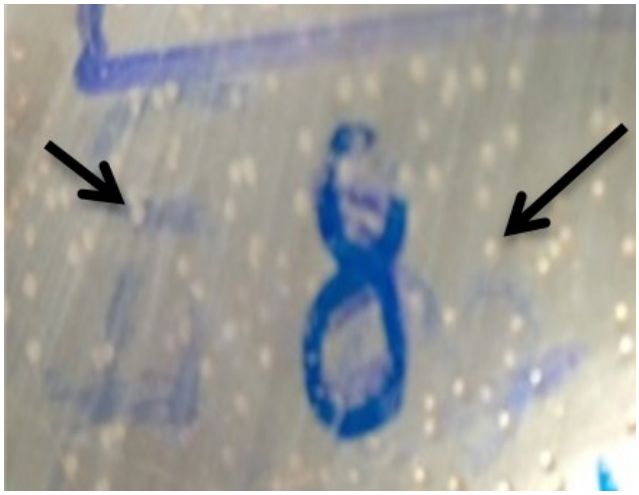

FIGURE 2 Colonies of *Aeromonas salmonicida*.

TABLE 2 Antibiotics sensitivity of the isolated and identified strains as *Aeromonas salmonicida*.

Antibiotics	Zone of inhibition (mm) (*n* = 3; mean ± SD)	Result
Sulfamethoxazole	18.65 ± 1.52	+2
Penicillin	5.00	-1
Neomycin	20.55 ± 1.50	+2
Erythromycin	22.30 ± 1.51	+2
Ofloxacin	33.57 ± 2.07	+3
Ciprofloxacin	32.57 ± 2.06	+3
Vanomycine	5.00	-1
Kanamycin	24.65 ± 0.58	+2
Nalidixic	37.64 ± 2.06	+3
Lemofloxacin	32.64 ± 2.05	+3
Tetracycline	24.00 ± 1.00	+2
Spectinomycin	30.64 ± 0.07	+3
Bacitracin	5.00	-1
Rifampicin	13.64 ± 0.57	+1
Tobramycin	19.98 ± 1.00	+2
Gentamycin	19.99 ± 1.00	+2

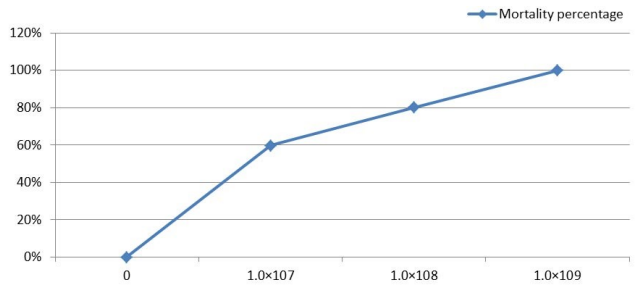

FIGURE 3 Percentage mortality and various concentrations of the vaccines.

4 | DISCUSSION

Aeromonas salmonicida is pathogenic bacteria which cause furunculosis in rainbow trout (*Oncorhynchus mykiss*). Pathogenic strains were isolated from kidney, muscles, liver and spleen of rainbow trout from the province of Punjab, Khyber Pakhtunkhwa and Gilgit Baltistan, Pakistan. Molecular identification of the bacterial strains with 16S rRNA was confirmed as *A. salmonicida*. According to the first two postulates of Koch, the pathogen is found in diseased samples and was isolated by using 16S rRNA (Finlay 2020). These isolates were all tentatively equated with *A. salmonicida* isolated by Martin-Carnahan and Joseph (2005). They produced smooth convex colonies on TSA and comprised of gram-negative rod-shaped bacteria that produced catalase, b-galactosidase, gelatinase, indole and oxidase, but not tryptophan deaminase or urease, fermented glucose, mannose, rhamnose, melibiose and sucrose; they did not utilize sodium citrate and were positive for the Voges–Proskauer reaction (Zdanowicz *et al.* 2020) and all results were correlate with present findings.

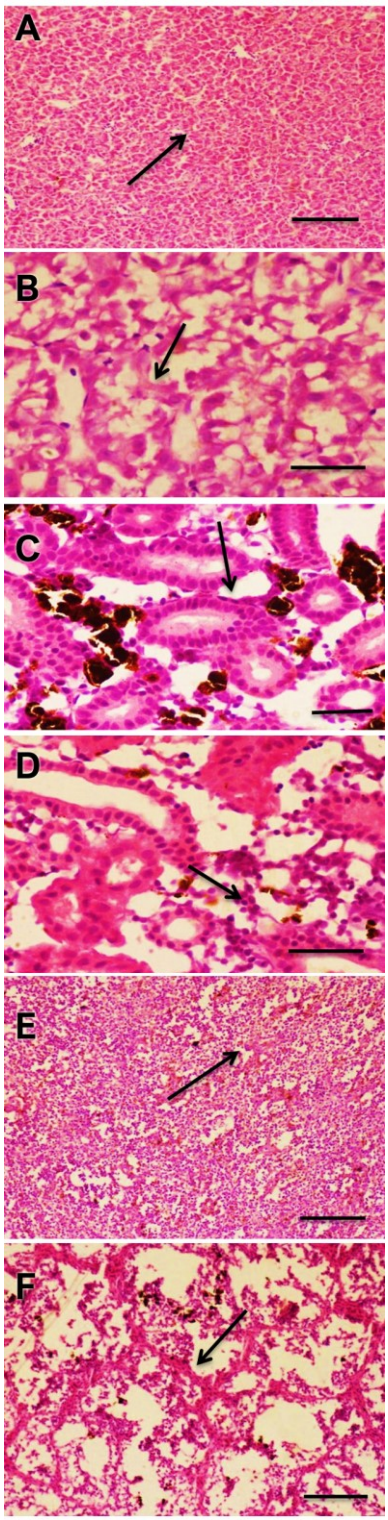

FIGURE 4 (A) Normal hepatic parenchyma. No tissue changes were shown in the liver of the control group; (B) cellular swelling and hydropic degeneration in hepatocytes of hepatic cords; (C) normal kidney no tissue changes are shown in the kidney of the control group; (D) peritubular infiltration of inflammatory cells; (E) normal spleen no tissue changes are shown in the spleen of the control group; and (F) some degenerative changes are seen and even some cells are disintegrating (400×).

Due to multiple antibiotics in various *Aeromonas* species, such as *A. veronii*, *A. hydrophila*, *A. salmonicida*, *A. caviae* and *A. sorbia*, it has become a major challenge for aquaculture industry all over the world (Zdanowicz *et al.* 2020). Piotrowska (2017) reported that this fast increase in the antibiotic resistance is due to transfer of antibiotic resistant genetic elements, such as transposons, plasmids, class one integrons, IS elements and gene cassettes, from one organism to another by physical contacts of the cell. Several mobile elements possess antibiotic resistant characters due to which antibiotic resistance increases in the aquaculture. In the past decade, probiotics were extensively used specially in China (50,000 T) that leads to great antibiotic resistance in pathogenic bacterial strains (Fečkaninová *et al.* 2017).

Aeromonas salmonicida is a major pathogen of cold water fishes and has been detected in many states of the world. *A. salmonicida* subspecies perforate the fish by the contusions on the skin or gills that propagate it to the intestinal epithelia of fish (Coscelli *et al.* 2014), through promulgation of tissues causing hemorrhage, sepsis and furuncles (Austin and Austin 2007; Coscelli *et al.* 2014). Sometimes, fish affected by *A. salmonicida* do not show the furunculosis disease (Noga 2010). It causes lurcative losses and has been reported to infect a variety of fish species (Wiklund and Dalsgaard 1998). *Aeromonas salmonicida* causes furunculosis disease in salmonids and ulcer disease in cyprinids and marine flatfish (Austin and Austin 2016). It causes sepsis, contusions, intestine infection, and spleen augmentation. When the disease propagates, ulcers seemed on the skin and muscle in salmonid fish (Austin and Austin 2007). It is commonly accepted that fish may espouse to disseminate the pathogenicity by becoming carrier of *A. salmonicida* (Austin and Austin 2007). Temperature influence the contamination of *A. salmonicida*. High temperatures deteriorate the functional protein and plasmid (Ishiguro *et al.* 1981; Stuber *et al.* 2003). *Aeromonas salmonicida* cause infection in cold water at low temperatures (Wiklund 1995) and the immunoglobulin retort against the infection that simulate the fatality by following provocation (Hrubec *et al.* 1996). In a study, the growth of *A. salmonicida* was observed higher at 4°C than at 15°C (Wiklund and Dalsgaard 1998). The present study reports, for the first time, an episode of furunculosis that causes mortalities in cultured sea bass. Biochemical characteristics provide the detailed description of bacterial isolate obtained from sea bass. The phenotypic characteristics of strains of bacteria isolated from sea bass showed similarity to the subspecies of *A. salmonicida* observed in present study. To detect the pathogenicity of *A. salmonicida* in fishes, bacterial isolate SC18032201 was cultured and inoculated intraperitoneally in two groups of Chinese perch. Fatality was observed daily for 30 days. Twelve deceased fishes displayed the pathological changes such as hemorrhage, necrosis, de-

generation of tissues and gills. Since, *A. salmonicida* has a very uniform group, it is very difficult to separate the subspecies on the basis of 16S rRNA (Benagli *et al.* 2012; Gulla *et al.* 2016). The study of Byers *et al.* (2002) also suggested that PCR technology can be used to replace the biochemical testing currently used to confirm the identity of *A. salmonicida* isolates cultured from both overtly and covertly infected salmonids. However, recent researches recommended vapA technology for species identification and sequence analysis of the 16S rRNA gene of *A. salmonicida* describe the confirmation about the genus *Aeromonas* and it does not provide any information about the species of *Aeromonas* (Figueras *et al.* 2011). The isolated strains were then compared with the reference strain of *A. salmonicida* after submitting it to the gene bank and it showed 99 – 100% identicalness. The phylogenetic tree clustered all the strain of *A. salmonicida*. Further studies, including a high number of strains, must be conducted to determine bacterial recognition and its pathogenicity. The identification of multiple strains of *Aeromonas* is helpful for the preparation of vaccine that can be effective for the control of the furunculosis in many fish to escape the appearance of new episodes in an affected farm.

TABLE 3 Mortality of *Oncorhynchus mykiss* infected with ArS-Pak-19 strains.

Experimental group	Number of fish	Bacterial inoculum (CFU ml^{-1})	Mortality	Mortality %
Control	10	0	0	0%
T1	10	1.0×10^{9}	10	100%
T2	10	1.0×10^{8}	08	80%
T3	10	1.0×10^{7}	06	60%

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CONFLICT OF INTEREST

The authors have no conflict of interest regarding this research work.

AUTHORS' CONTRIBUTION

Conceptualization and methodology: MA MH and ST; validation: FA and IA; software: SK, SZ and MA; data curation and analysis: MA, NM, MN and AK; writing original draft: AS, MT and MA; review and edit: MH, FA, MA and IA; resources: IH, KJ and FS. All the authors have read and agreed to publish this version of the manuscript.

DATA AVAILABILITY STATEMENT

The NCBI accession numbers for the research findings are MW307221, MW720959, MW720960, MW720961, and MW720962.

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