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RESEARCH ARTICLE

Azoxystrobin-induced Oxidative Stress in Gills, Hematological Biomarkers and Histopathological Ailments in Fresh Water Fish

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ABSTRACT

Azoxystrobin (AZX), also known as Amisida, is a widely used medication for treating and managing fungal diseases in agriculture. The hazardous effects of azoxystrobin on non-target organisms, particularly fish have generated significant attention for its toxicological study since it penetrates in aquatic environment from several locations. The toxicity of azoxystrobin on experimental fish was investigated in this study using a variety of biomarkers including hematology, antioxidant enzymes, oxidative stress indicators, histopathological alterations in gills, and various morphological and nuclear changes in erythrocytes. Sixty-four (64) freshwater fish reared in aquaria containing water were randomly divided into four treatment groups (T0 and T1-T3). All the fish were exposed to AZX at different concentrations (60.0, 80.0, and 100 µg/L) to determine the harmful effects of AZX. All the fish except in control (untreated) group received the AZX treatment for the period of 42 days. Hematology results showed reduced erythrocyte and hemoglobin values and higher total and differential leukocyte values. The findings showed that treated fish had considerably higher levels of various oxidative stress biomarkers in gills while lower levels of total proteins and antioxidant enzymes (catalase, superoxide dismutase, peroxidase and reduced glutathione). This research exhibited that certain nuclear abnormalities (erythrocytes with condensed, lobed nuclei, and erythrocytes with micronuclei, and morphological alterations (erythrocytes with spindle shape, pear shape, and spherocyte) were significantly increased in the erythrocytes of the experimental fish. AZX induced microscopic abnormalities in gills of different treated fish. Our experimental study suggested that AZX disturbs physiological and biochemical markers at higher concentrations.

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INTRODUCTION

Fungicides, insecticides and herbicides (also defined as pesticides) are poisonous compounds with either chemical or biological origin those are dispersed in the environment to prevent, control, and/or reduce populations of fungi, weeds, insects, rodents, and other undesirable pests (Soliman *et al*., 2015; Hussain *et al*., 2019). Pesticides have a lot of potential to enhance the yield of crops, but their widespread usage has raised serious questions about their safety for both humans and the environment (Hussain *et al*., 2014; Namratha *et al*., 2021; Mujahid *et al*., 2021). Pesticides may be dispersed into the air, absorbed into the soil, or consumed by plants and animals. The fate of pesticides is determined by environmental factors as well as their chemical and physical properties including the solubility of these chemicals in water, air volatility, and soil mobility (Raffa and Chiampo, 2021).

Azoxystrobin (AZX) is a well-known fungicide with a broad-spectrum antifungal activity. It is a member of the strobilurins family of fungicides which are authorized for use in cultivation of soybeans, vegetables, cereals, rice, and fruits around the world (Han *et al*., 2014). It is recorded that insecticides and fungicides are extensively and persistently

employed in protection of agricultural products, veterinary practices to remove internal and external parasites and in public health management resulting in contamination of the environmental. Moreover, such kind of synthetic chemicals can remain in agricultural crops, air, soil or water and ultimately enter into aquatic environments via runoff. According to environmental evaluations, strobilurins have been found in surface water, groundwater, and paddy water globally. In paddy water, these fungicide concentrations can be as high as >100 mg/L, but they can only be found in water systems up to 10 mg/L in other places. As these fungicides are employed more frequently for crop protection and eventually find their way into water systems, aquatic biodiversity may thus be in danger (Sun *et al*., 2014; Garanzini and Menone, 2015; Edwards *et al*., 2016). For aquatic animals, strobilurins are thought to be relatively severely poisonous (Cui *et al*., 2017).

Earlier research demonstrated that AZX is extremely hazardous to freshwater fish leading to disruption of mitochondrial functions and different mechanisms involving regulation of cell growth and division (Olsvik *et al*., 2010). Pesticides induce deleterious impacts on multiple biological functions in aquatic and terrestrial organisms which can be screened using suitable biomarkers. Catalase, glutathione peroxidase and superoxide dismutase are the key enzymes that prevent the cellular organelles from free radicals produced during the oxidative stress (Hoseinifar *et al*., 2020). It has been estimated that the different blood parameters like erythrocyte count, leukocyte count, hematocrit, hemoglobin and some other blood parameters can be used to measure a response of exposed organisms to environmental stressors (Parrino *et al*., 2018). Fungicides in particular induce a physiological stress in aquatic life that results in the development of multiple genotoxic abnormalities in aquatic and terrestrial animals (Al-Emran *et al*., 2022). The aim of this study was to investigate the nuclear and morphological changes in red blood cells, histopathological changes, antioxidant enzyme concentrations, and characteristics associated with oxidative stress in the gills of experimental fish.

MATERIALS AND METHODS

Acclimatization of fish: A total of 64 fish specimens were obtained for this study from the local fish hatchery located in district Bahawalpur, Punjab, Pakistan. All organisms were brought to the laboratory immediately after collection, where they were acclimated for a duration of 10 days in glass aquariums containing sterile water and equipped with suitable oxygenation systems. Fish were fed (commercial feed containing 35% crude protein) twice daily at a rate of 2.5% of body weight.

Fish handling: After acclimatization, 64 freshwater fish were blindly picked and placed in four experimental groups in glass tanks under controlled laboratory conditions and were exposed to various doses of AZX (60.0, 80.0, and 100 µg/L) in order to evaluate the pathophysiological effects. All test specimens (fish) in groups (T1-T3) were kept at varying concentrations of AXZ (60.0, 80.0, and 100 g/L) for a total duration of 42 days and the fish reared in group T0 were considered untreated/normal group. The concentrations/doses of azoxystrobin were calculated by consulting previous literature (Olsvik *et al*., 2010; Han *et al*., 2016; Liu *et al*., 2021). The entire experiment was conducted in the laboratory of the Department of Zoology (Government Sadiq College Women University of Bahawalpur) and Department of Pathology (Faculty of Veterinary and Animal Sciences) Islamia University of Bahawalpur.

Hematological studies: A sterile needle (hypodermic, 26 gauge) was used to draw blood from caudal veins of exposed fish that at days 14, 28, and 42 of the experiment. Several hematological indicators were measured accordingly (Islam *et al*., 2019a, 2019b). The erythrocyte count, hematocrit, hemoglobin, total, and differential leukocyte counts were measured (Mahmood *et al*., 2021).

Blood sampling and evaluation of erythrocytic abnormalities: On days 14, 28, and 42 of the experiment, blood samples (2mL) were taken from the caudal vein of experimental fish using hypodermic needle (26 gauge). Fresh blood from each test specimen was used to create blood films on glass slides (without the use of an anticoagulant). After drying, thin blood smears were preserved by using 100% ethanol and stained with Giems'a solution (Benjamin, 1978). A computer-assisted light microscope was used to analyze all blood films in order to spot erythrocytic nuclear and morphological abnormalities. A computer assisted light microscope was used to examine 1500 red blood cells from each test specimen (Saleh and Sarhan, 2007; Hussain *et al*., 2012).

Status of antioxidant enzymes and oxidative stress parameters in gills: Fish from the control and treatment groups were dissected on days 14, 28, and 42 of the trial. Gills tissues were immediately separated from each fish and were subjected to investigation of antioxidant enzymes (reduced glutathione (GSH), reactive oxygen species (ROS), catalase (CAT) and oxidative stress profile (thiobarbituric acid reactive substances) according to earlier procedure (Akram *et al*., 2021). The values of superoxide dismutase and peroxidase investigated according to published literature (Kakkar *et al*., 1984; Raza *et al*., 2022). The estimation of oxidative stress indices, ROS (Hayashi *et al*., 2007), GSH (Jollow *et al*., 1974; Akram *et al*., 2021), and TBARS (Iqbal *et al*., 1996; Raza *et al*., 2022) in the gills were evaluated. The determination of antioxidant enzymes such as POD, CAT (Chance and Maehly, 1955; Raza *et al*., 2022) and SOD (Kakkar *et al*., 1984; Raza *et al*., 2022) in gills of treated and control fish was done in accordance with the previously described techniques.

Evaluation of histopathological alterations in gills: At days 14, 28, and 42 of the trial, five test specimens (fish) were blindly picked from each treatment, weighed before killing and dissected. Clove oil (4.6 mg/L) was used to anaesthetize all of the experimental fish prior to sampling (Islam *et al*., 2019a, 2019b). Gills were promptly removed after dissection and stored in 10% formaldehyde solution. All of the retrieved gills were processed using Hematoxylin and Eosin staining techniques to examine histological alterations using light microscope (Nikon Eclipse 80i, Nikon Co., and Tokyo, Japan) was used to examine the gill tissue sections (Hussain *et al*., 2019).

Statistical analysis: All of the obtained data was normally distributed. The IBM SPSS statistical tool (version no. 20) was used to determine the significant difference applying one-way analysis of variance in mean values (mean SE) of data on different study variables in control and experimental groups using a Tukey's test with a significance level of 0.05.

RESULTS

Hematology: Various hematological biomarkers of *Labeo rohita* reared in untreated fresh water and water containing azoxystrobin indicated that RBC count, hematocrit, and hemoglobin values reduced significantly in experimental fish reared in water having high concentration of AZX (80 µg/L) at day 42, and AZX (100 µg/L) at days 28 and 42 of the study (Fig. 1). The lymphocyte counts significantly decreased in *L. rohita* exposed to AZX (80 and 100 µg/L) at days 28 and 42 of the study. The results on hematological measurements showed significantly increased WBC counts and neutrophil population (Fig. 1) at days 28 and 42 of trial in *L. rohita* exposed to AZX (80 and 100 µg/L)

Nuclear and morphological alterations: The results of our research showed that in comparison to untreated normal fish, the fish exposed to increasing concentrations of AZX had significantly higher frequencies of several morphological and nuclear abnormalities in their erythrocytes. At days 28 and 42 of the current investigation, fish that were exposed to AZX exhibited significantly high prevalence of many morphological abnormalities in their erythrocytes (Fig. 2). In comparison to the control group, fish subjected to AZX (80 µg/L) at day 42 and AZX (100 µg/L) at days 28 and 42 of the study ((Fig. 2) had a significantly higher percentages of erythrocytes different nuclear ailments (lobed nuclei, blebbed nuclei, vacuolated nuclei, and notched nuclei). Fish subjected to AZX (80 and 100 µg/L) on day 42 of the current study had considerably greater percentages of erythrocytes with condensed nuclei and micronuclei compared to untreated control fish. *L. rohita* treated to AZX (80 and 100 µg/L) at 28 and 42 days of the experimental study showed an increased percentile rate of erythrocytes with morphology of pear shape and spherocytes (Fig. 3).

Status of antioxidant and oxidative stress parameters in gills: When fish were treated to AZX (80 µg/L) and AZX (100 µg/L) at 28 and 42 days of the study, the levels of ROS in isolated gill tissues were noticeably elevated. While at days 28 and 42 of the trail, exposure to AZX (80 and 100 µg/L) considerably enhanced the amounts of TBARS in gills of exposed fish (Fig. 4). At days 28 and 42 of the present research, isolated gills of fish exposed to AZX (80 and 100 µg/L) had lower GSH and total protein levels (Fig. 4). In the recent experiment, fish exposed to AZX (80 µg/L) at day 42 and AZX (100 µg/L) at days 28 and 42 had significantly lower amounts of antioxidant enzymes like, CAT, POD and SOD in gills of exposed fish.

Histopathological alterations: Results on histopathology level showed different microscopic alteration in gills of experimental fish including necrosis of lamellar cells of secondary lamellae and degeneration of primary and secondary lamellae in fish exposed to AZX (100 µg/L) at day 42th of trial. Various microscopic alterations like aneurysm, necrosis of lamellar cells (primary and secondary) and disorganization of cartilaginous core in gills of fish reared at AZX (100 µg/L) were observed at day 42th of trial (Fig. 5).

DISCUSSION

The present study shows concentration and duration of exposure dependent hematological markers, erythrocytic nuclear and morphological irregularities, histopathological alterations, and status of antioxidant enzymes and oxidative stress parameters in gills of experimental fish.

Various hematological biomarkers such as erythrocyte counts, lymphocyte counts, hemoglobin and hematocrit values significantly reduced in *Labeo rohita* reared in azoxystrobin. It has been noted that hematological parameters are reliable and useful markers of physiological stresses in a variety of terrestrial and aquatic species (Mujahid *et al*., 2021; Jabeen *et al*., 2021). The decreased hematological findings in this study might be the result of stress on hematopoietic tissues, lysis of red blood cells, and oxidation of hemoglobin (Gul *et al*., 2020). In current study, increased leukocyte counts and neutrophil counts in *L. rohita* exposed to AZX can be related to activation of immune responses and induction of injurious stimuli in multiple visceral tissues. Previous studies have also reported lower hematological biomarkers including erythrocyte counts, concentration of hemoglobin, hematocrit and lymphocytes in various species of fish such as *Australoheros facetus* (Crupkin *et al*., 2021) and *Salmo salar* (Olsvik *et al*., 2010). Moreover, previous studies have recorded higher values of WBC counts due to increased concentrations of various fungicides in *Oreochromis mossambicus* (Ghane *et al*., 2017), *Oncorhynchus mykiss* (Li *et al*., 2013). The hematological abnormalities measured in our study may also be due to inflammatory responses in vascular channels leading to formation of free radicals, destruction of red blood cells or poor efficiency of hematopoietic organs and disruption of process of osmoregulation in gills resulting in an inadequate delivery of O_2 to blood-forming organs in *L. rohita*. Studies have shown that hematological cells (leukocytes) may decrease or increase in certain toxicological conditions (Banaee *et al*., 2008; Hussain *et al*., 2011; Akram *et al*., 2021) and diseases. Significantly lower values of lymphocytes in AZX treated fish in this study might be related to abnormal hematological functions (kidneys and spleen) and are suggestive of immune system deficiency.

Nuclear and morphological abnormalities are considered as useful and valid biomarkers which provide a true picture of several naturally occurring and synthetically produced toxins responsible causing genetic changes in fish and other avian red blood cells (Raza *et al*., 2022). It has been demonstrated that by examining the morphological and genetic changes of erythrocytes, oxidative stress should be detected with great accuracy (Yamin *et al*., 2020). According to the findings of the present study, exposed fish had expressively higher frequencies of different morphological and nuclear variations related to red blood cells, including erythrocytes

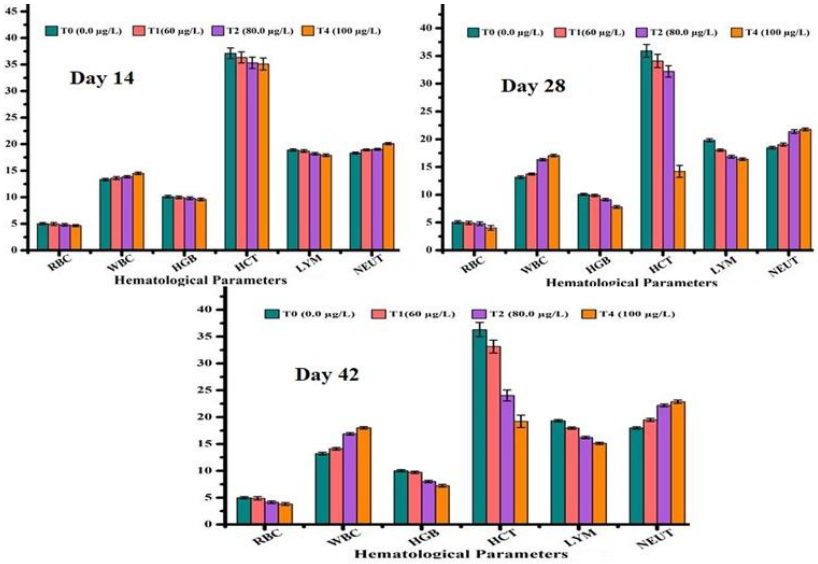

Fig. 1: Photograph showing comparison of different hematological alterations such as RBC (10^9/mm^3), WBC (10^6/mm^3), HGB (g/dL), HCT (%), LYM (%) and NEU (%) in *Labeo rohita* exposed to various concentrations (60.0, 80.0 and 100µg/L) of AZX.

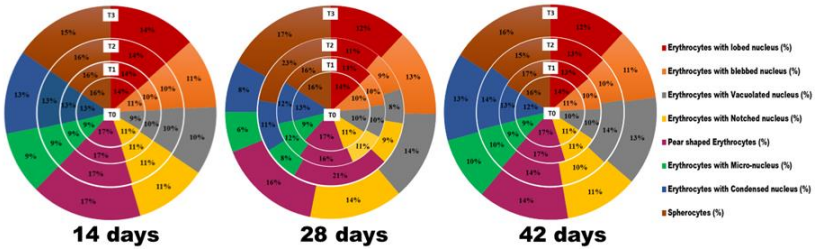

Fig. 2: Photograph showing comparison in percentile rate of morphological and nuclear alterations in red blood cells of *Labeo rohita* exposed to various concentrations (60.0, 80.0 and 100µg/L) of AZX.

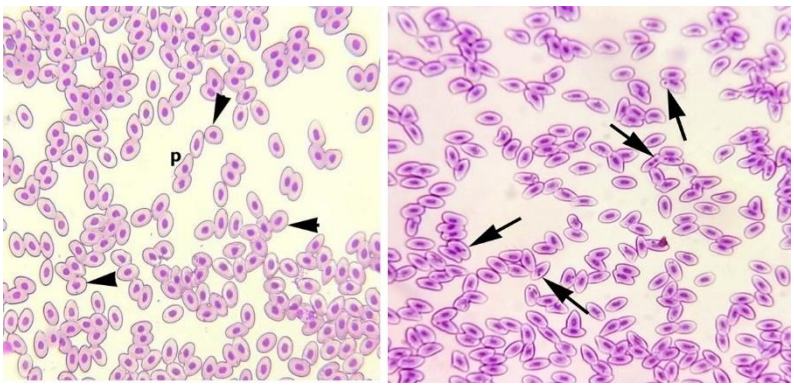

Fig. 3: Photomicrograph of thin blood smear of fish showing occurrence of micronuclei (arrows), pear shape erythrocyte and spherocytes (arrow heads). Field Stain A and B. 1000X

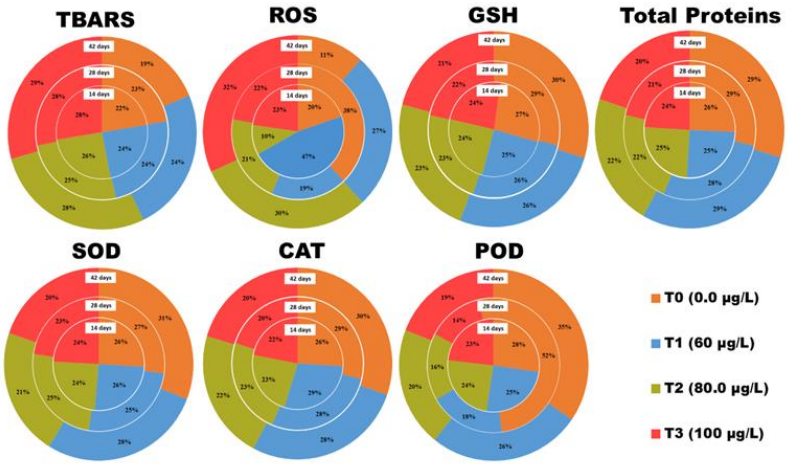

Fig. 4: Photograph showing comparison of different oxidative stress and antioxidant enzymes in gills of fish exposed to various concentrations (60.0, 80.0 and 100µg/L) of AZX.

with blebbed nuclei, lobed nuclei, notched nuclei, vacuolated nuclei, condensed nuclei, micronuclei and bi-nucleated red blood cells, as well as different morphological changes like spherocytes and pear-shaped RBCs. It is well established that the erythrocytes of aquatic and terrestrial animals are valuable indicators of artificial and natural stress as well as exposed to different toxicants (Hussain *et al*., 2014; Faheem *et al*., 2021). The higher percentage of erythrocytes in our study with nuclear ailments like notched nuclei, micronuclei and lobed nuclei

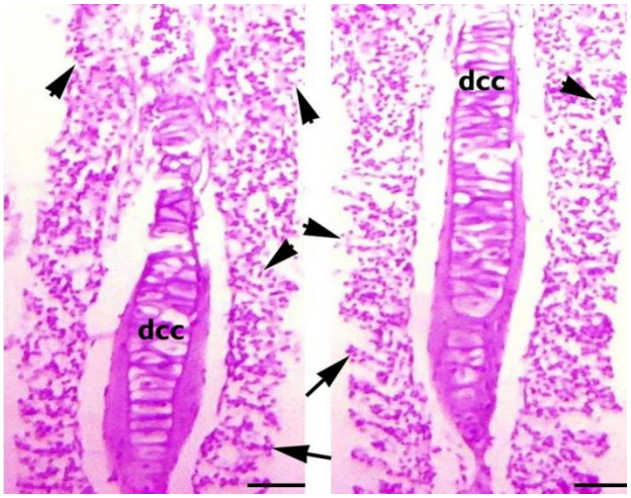

Fig. 5: Photomicrograph of gills of fish showing occurrence of histological ailments like necrosis of lamillar epithelial cells of secondary lamillae (arrows), degeneration of and necrosis of epithelium of primary and secondary (arrow heads) and disruption of cartilaginous core. 400X

might be attributed to increased production of free radicals leading to activation of caspase-activated DNase, cleavage of different cytoskeletal proteins (vimentin, fodrin, and gelsolin) and damage to normal functioning of mitochondrion (Ahmad *et al*., 2021). As a result of oxidative stress brought on by several pesticides, significant morphological and nuclear abnormalities have also been seen in the erythrocytes of several fish species in earlier studies (Naqvi *et al*., 2016; Crupkin *et al*., 2021; Al-Emran *et al*., 2022).

The quantity of several antioxidant enzymes like POD, CAT, SOD, and GSH as well as oxidative stress (TBARS and ROS) variables are well-known indicators of inflammatory reactions and are helpful tools for monitoring tissues from free radical harm (Namratha *et al*., 2021; Kiran *et al.,* 2022). Examining these factors is a standard practice when assessing the toxicological impacts of various environmentally risky and synthetically generated substances (Lee *et al*., 2019; Naseer *et al*., 2020). Our study found that experimental fish exposed to AZX at various dosages had significantly greater levels of oxidative stress markers in their gills, including reactive oxygen species (ROS) and thiobarbituric reactive substance. According to published studies, target and non-target animals exposed to different toxins quickly develop reactive oxygen species (Akram *et al*., 2021). The creation of reactive oxygen species (ROS) initiates the lipid peroxidation process, which finally results in abnormalities in the cellular membranes of various cells in exposed tissues, severely harming those tissues and generating thiobarbituric reactive material (Raza *et al*., 2022). Previous studies have shown that AZX is the main threshold for increased ROS and TBARS formation in *Australoheros facetus* (Crupkin *et al*., 2021). According to our research, the increased levels of oxidative stress parameters in exposed fish raised in water with different amounts of AZX may be caused by the depletion and inappropriate balance of antioxidant enzymes. The increased levels of oxidative stress markers might also be brought on by tissue damage and abnormal oxidative phosphorylation pathways (Akram *et al*., 2021).

Our research revealed that experimental fish given higher dosages of AZX had significantly lower levels of total proteins, GSH, POD, CAT, and SOD. The same pattern was previously noticed in *Australoheros facetus* (Crupkin *et al*., 2021). Recent research suggests that tissue dysfunction and increased energy consumption to resist oxidative stress may be to blame for the reduced levels of GSH and total proteins. Various histological abnormalities, such as epithelial necrosis, telangiectasia at the terminals of secondary lamellae, raising of lamellar epithelium, hypertrophy of chloride cells, and fusion of some secondary lamellae of various fish species like *Oreochromis niloticus* (Gaffaar *et al*., 2010) and Rainbow trout (Boran *et al*., 2012) due to various fungicides, have been found in gills in earlier investigations.

Conclusions: The results of this trial highlighted that the fish treated with the maximum concentrations of (80 µg/L and 100 µg/L) demonstrated substantial alterations in hematology, morphological and nuclear ailments in erythrocytes and oxidative stress in *Labeo rohita*. In addition, AXZ adverse actions on different tissues of fish may have unintended ramifications for the well-being of different aquatic animals in their natural environments. These results indicate serious concerns that AXZ exposure might be harmful to aquatic species even at very low doses.

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Authors contribution: SAHK, RI and RH designed the experiment. SAHK conducted the research and collected the data. RI, AAAD and RH analysed that data. SAHK and RI prepared the paper. MA and FL helped in interpretation of results. GAR and NB helped in statistical analysis, writing and organization of manuscript.

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