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Assessment of genotoxicity induced by helminthes parasites in freshwater fishes of river Ganges

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Several parasites have been shown to induce genotoxicity in humans and fish are important intermediate hosts for completing the life cycle of many parasites, posing a huge economic loss worldwide through the ecosystem food chain. In the present study, we assessed the genotoxic potential of helminth *Rostellascaris* sp. through a benchmark of comet assay and micronucleus (MNi) tests on the hepatocytes, muscle, and whole blood of infected fish *Bagarius bagarius* (Hamilton) collected from different sites of the river Ganges. The percentage of the mean tail length of the comet was 10.28±0.36 in the reticulocytes of the infected fish which was significantly (P <0.05) longer compared to the control (2.86±0.12). Similarly, a significantly (P <0.05) higher DNA damage was observed in hepatocytes of parasite-infected fish (12.15±0.24) when compared to the control (3.024±0.013). A comparatively higher DNA damage was observed in the hepatocytes than the reticulocytes, indicative of tissue-specific DNA damage as hepatocytes are the biomarkers of metabolic functions prone toward biotic stress. A higher induction of MN was observed in infested fish (0.18±0.07) as compared to the control. Our results suggest that parasites contribute to the induction of cellular and DNA damage in fish during the progression of the host-parasite interaction.

Keywords: Comet assay, Host-parasite interaction, Micro Nucleus Test, Tissue specificity

Fishes are one of the common sources of protein in many regions of the world. Their habitat, water serves as an abiotic component in which they carry out their function like breeding and feeding that includes digestion and excretion[1]. The parasitology of fishes is a fast-growing topic for aquatic research, especially as a result of the increasing relevance of fish farming. The parasitic fauna linked with the catfish *Bagarius bagarius* (Sisoridae) may differ based on the excessive use of inorganic fertilizers and pesticides in cultivated fields, industrial effluent discharge, insufficient waste disposal, and other factors that might affect changes in the aquatic environment indirectly. Parasites have a distinct place in the animal kingdom because of their extraordinary adaptations and detrimental behaviours toward their hosts, and helminth parasites remain a serious public health problem, particularly in Asia, because they can only be transferred to humans through fish[2]. Freshwater fish offers an ultimate, intermediary, or paratenic host to complete the life cycle of numerous species of parasites[3,4]. Some parasites in fish aquaculture can be very harmful, resulting in substantial fish mortality and financial loss, whereas they exist in natural systems and act as a menace to the abundance and diversity of inhabitant fish species. It is critical for consumer concerns, particularly in areas where raw, half-cooked, or smoked fish is consumed. Parasites may affect the production of fish as well as the economic value of fish production. Therefore, a fish parasite remains a big challenge for the fish biologists and further, a disease caused by parasitic infections makes fish farming a high-risk investment. The survey of helminth parasites in freshwater fishes was undertaken to investigate the influence of the environmental parameters inside the body of the host. It has been reported that season, temperature, humidity and the age of the host all have an impact on the dynamics of helminths[5-7].

The helminthic infection causes cancer because the host is exposed to the parasite for several years, resulting in chronic inflammation of infected tissues, and some of the parasite's excretory-secretory metabolic products are highly immunogenic[8], causing genetic instability and malignant transformation[9]. The parasite's excretory-secretory (E/S) products are

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developmentally controlled and correspond with parasite movement through the host tissues; these products include proteases, which not only have substantial virulence potential but also operate as a tissue-damaging component[10]. It has been noted that parasites can damage the host's DNA either directly during infection or by inducing inflammatory responses that can cause DNA damage[9]. This is mediated by the production of free radicals such as reactive oxygen and nitrogen species, which may damage the host's DNA, resulting in genetic and epigenetic processes that regulate cell proliferation, influencing carcinogenesis[11-13]. The hepatocellular carcinoma caused by helminths is understood to have a distinctive consequence when induced by the trematodes, *Clonorchis* sp., while the carcinoma caused due to the organisms of zoonoses, particularly anisakid roundworms, is yet unknown[14]. Anisakid infections are common in marine fish hosts globally, but fewer host fishes from freshwater bodies harboring anisakid roundworms have also been reported. In view of the fact that the zoonotic significance of these worms remains a serious issue of public health, the present work will benefit immensely to mankind.

Although, the utility of the Comet assay technique is basically helpful to detect DNA damage in cell suspensions of different fishes found in Gangetic plains in U.P., India[15,16]; the testing of the applicability of the comet assay technique on the impacts of genotoxic potentials of helminth parasites on the integrity of DNA in certain fishes of Ganga River has proved difficult. Breakage of DNA strands, genetic mutation, chromosomal abnormalities, and other genotoxic alterations are examples of significant genotoxic changes[12]. Parasitic worms infect billions of people worldwide, and infections, such as roundworms, flatworms, and food-borne liver flukes, are responsible for 15% of all cancers[17]. These infections, which include roundworms, flatworms, and food-borne liver flukes, cause particularly serious diseases in humans that are often overlooked. Due to their long-term survival in the host, helminth infections may induce chronic inflammation and genetic variability which has been related to DNA single-strand breaks and cancers caused by the oxidation of free radicals and nitrogen species created by the inflammatory process.

For the reason that most rivers are a sink for a variety of spills, urban residues, pesticides, and trace metals[18], the purpose of this study is to determine the effect of certain parasites on the genomic stability and physiology of fish harboring anisakid and other zoonotic worms caught at polluted sites along the river Ganga at Prayagraj. However, there is essentially little evidence of the genotoxic effects of helminthiasis caused by a group of helminth parasites. As a result, in the current investigation, helminth infections in freshwater fish were explored to see if there were any genotoxic consequences related to host-parasite interactions during the acute phase of infection.

Materials and Methods

The present study was piloted on randomly collected fish from the local fish market of Prayagraj, Uttar Pradesh. The collection of hydrobiological samples from different sites and selection of hosts and collection of parasites were accomplished at different sites on River Ganga. Helminthes worms and other parasites were obtained from freshwater fish and their diversity analysis was performed for initiation of the experiments.

Sample collection and Identification

After observing some freshwater fish species, it was found that more numbers of *Bagarius bagarius* were infected, therefore furthermore 75 freshwater catfish *B. bagarius* species were observed from the local fish market of Prayagraj from August 2019 to March 2020. The length of the fish was measured using a ruler scale and the fish’s sexes were revealed by visual examination of the urinogenital system for further experimentations.

Parasite dissection and examination

Prior to dissection, the fish were immobilized by cervical dislocation for easier handling. Using a surgical blade, a longitudinal slit was cut on the ventral surface from the anus to a position level with the pectoral fins, allowing the fish to be dissected through the abdomen. The gut and gallbladder were separated from the rest of the alimentary system and extended out. The tissue sections were kept in three separate petri dishes containing 0.6 percent saline[19]. The fat was removed from the intestine and then the intestine was dissected with a pointed scissor with the help of pointed forceps, and the intestine was finally spread with the help of a pointed brush and observed with naked eyes. The suspected specimen was collected in a separate petri dish containing lukewarm water and was observed under a microscope to confirm that it was a parasite further those parasites were separated into the cestode, trematode, and nematode. The prevalence of nematode parasite *Rostellascaris* sp. was found to be higher in the fish specimens.

Hematological parameters

Hematological parameters such as hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), mean platelet volume (MPV), red blood cells (RBC), red blood cell distribution width (RDW), platelet distribution width (PDW) and the ratio of platelets-large cell ratio (P-LCR) were analyzed. All of these variables were considered and studied from the whole blood of fish which were further evaluated under a Sysmex KX21 Hematology Analyzer.

Micronucleus genotoxicity assays test

Blood samples were taken from the caudal vein of the laboratory acclimatized fish specimens that were considered as control, a positive control treated with Cyclophosphamide for verification of the experimental procedures, and the parasite infested specimens collected from sampling sites were smeared differently all over the previously cleaned slides. The slides were allowed to air dry after 20 min of fixation in pure ethanol, and the smears were stained with a 6 percent Giemsa solution for 25 min[18]. The samples from each specimen were prepared on two slides, with a total of 2,000 erythrocyte cells evaluated under 100X magnification on each slide. Micronuclei are small, non-refractive, circular, or ovoid chromatin structures that exhibit the same staining and focusing pattern as the major nucleus. (MN), and its frequency was calculated as:

$$\text{MN\%} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \text{ X } 100$$

Comet Assay or alkaline single cell gel electrophoresis (SCGE)

Ample interest in the method of Comet Assay technology has been generated from its potential uses in biomonitoring and the ecological evaluation of sentinel organisms exposed to environmental pollutants. The fact that there is no clear link between the quantities of DNA damage generated by a given parasite or chemical and the biological consequence of that damage in itself complicates the interpretation of comet data. After Moller (2005) and Speit and Hartmann (2005), the analytical procedures were followed in a series of steps like- slide precoating, sample preparation, lysis, and electrophoresis, slide staining, and slide analysis in the agarose preparation process[20,21].

Image analysis

At least 50 comet images from each plate were examined to assess the cells. The analysis of comets at slide edges was avoided. Additional images were scored for the experimental repeats Individual comet images were evaluated using image analysis software for several parameters such as overall intensity (DNA content), tail length, percent DNA in tail, and tail moment. The experimental triplicates have been maintained during these laboratory procedures.

Analytical statistics

Statistical analysis of the observed data was performed using means or medians, depending on population distributions. For a single parameter, error bars generally indicate the variability across experiments, not the variability within slides. The mean difference in percent Tail DNA between the infected and control fish species was compared using a one-way analysis of variance (ANOVA). The Mann-Whitney test was used to compare the observed percent MN frequencies between affected species. Statistical significance was determined by p values of less than 0.05.

Results

Hematological tests are a useful tool for keeping track of fish health, hence these parameters were performed in view to analyze the general health and well-being of the parasite-infected fish population. All the relevant parameters like the Red Blood Cell (2. 10^6/µL), Haemoglobin (7.35 gm), and Packed Cell Volume (24.98 %) were in the normal range except for the increase in White Blood Cells (98.67 10^3/µL) as depicted in Fig. 1 and micronucleated red blood cells in *Rostellascaris* sp. infected fishes.

The freshwater catfish *B. bagarius*, infected with nematode helminth parasites *Rostellascaris* sp. revealed genotoxic effects with hepatocytes and reticulocytes displaying the most DNA damage as depicted with the observations of the MNi test and the comet assay. The comet assay, which used single-cell electrophoresis of reticulocytes and hepatocytes of fish, validated the DNA damage as a sign of genomic instability caused by a parasitic infestation. In the reticulocytes of infected fish, the mean tail length of the comet was 10.28453±0.356327 which was significantly (P <0.05) greater than the control

(2.853781±0.113889), as shown in Fig. 2. Similarly, a significantly higher (P <0.05) percentage of DNA damage was observed as represented by the increased tail length in hepatocytes of parasite-infected fish (12.14622±0.23746) when compared to the control (3.023645±0.0125496).

In addition, it was observed that the fish infected with nematode helminths parasites *Rostellascaris* sp. exhibited about 0.1746±0.03589 micronuclei formation in reticulocytes of parasite-infected fish collected from the different sites of Ganga River (Fig. 3) when compared to the control (0.039188±0.008816) and positive control (0.049197±0.022061). It is interesting to note that a comparatively higher DNA damage was observed in the hepatocyte cell when compared with the reticulocyte cells indicating tissue-specific DNA damage (Fig. 4). This may be attributed to the fact that hepatocyte cells are the target site of all metabolic functions of the body which is moiré prone to the wear and tear of the environmental, biotic, or abiotic stress.

Overall, it can be summarized that parasites infected reticulocytes of the fish specimen show a significantly higher percentage of micronuclei frequencies when compared with the normal control and positive control fish (Fig. 5). The observations of the micronuclei test reveal that the parasites are capable of causing not only the genotoxic but also the mutagenic effects on the red blood cell in the form of micronuclei formations that are end products of mutated fragments of the genetic material that remain in the subsequent mitotic divisions. In addition to the significant MNi, the cellular abnormalities including the blebbed and notched-like cells, etc. were also seen in the reticulocytes of the infected fish, which are certainly a topic of concern to be taken into consideration in the future studies. The 24-months collection of data on helminth species, including

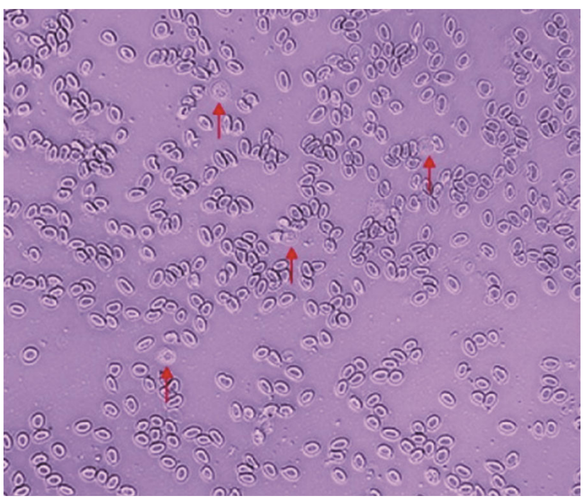

Fig. 1 — Prevalence of white blood cells (as represented with arrows) in *Rostellascaris* sp., parasite-infected fish *Bagarius bagarius* after hematological studies. [Bar represents 100 μ]

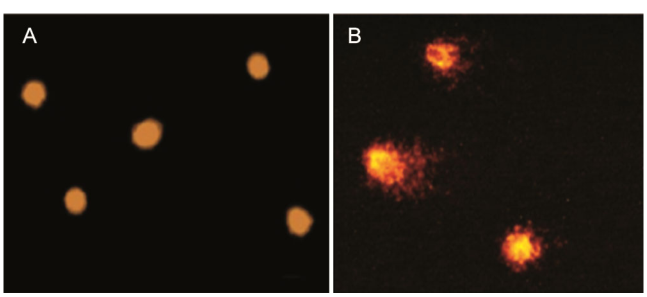

Fig. 2 — DNA damage caused by helminths parasites *Rostellascaris sp.*, seen via fluorescence microscopy using the Comet Assay in red blood cells of *B. bagarius*. Reticulocytes (Red blood cells) isolated from (A) normal fish; and (B) infected fish represented. The ethidium bromide-stained nuclei after electrophoresis show "comet tail" in exposed cells. [Bar represents 10 μ]

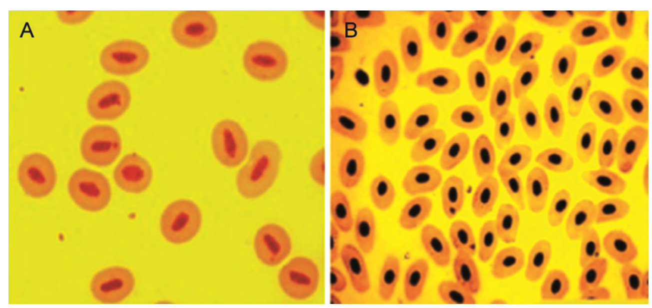

Fig. 3 — Cytological visualization of micronuclei induced by helminth parasites (*Rostellascaris sp.*) using Micronuclei Test in red blood cells of *B. bagarius*. Red blood cells isolated from (A) fish specimen showing no micronuclei; and (B) an infected fish specimen showing micronuclei [Bar represents 10 μ]

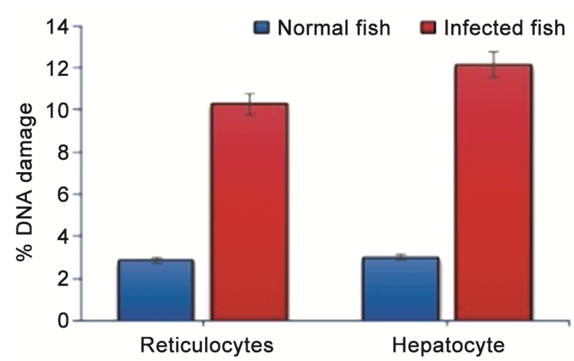

Fig. 4 — The % DNA damage (±S.E.) in erythrocytes and hepatocytes of catfish *B. bagarius* collected from River Ganges infected with helminth parasite *Rostellascaris* sp.

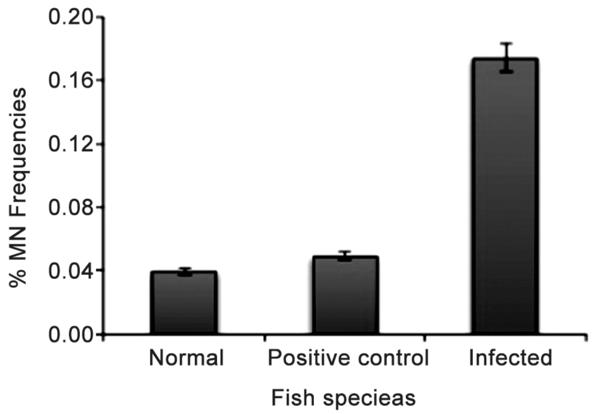

Fig. 5 — The % MN frequencies in *Rostellascaris* sp. parasites infected erythrocytes of cat fish *B. bagarius* collected from River Ganges. Mean percentage of micronucleus frequencies (±SE) in erythrocytes of *B. bagarius* (n = 2000) exposed to parasites infestation. Values differ significantly (P <0.05) between normal and parasite-infected fish. No. of cells observed Control, Positive control, and treated are 10080, 10111 and 10090, respectively in different five fishes.

several worms that have the potential to induce DNA damage, mutagenicity, and cellular oxidative stress facilitated the systematic inventorization of pathogenic constituents in the area of study.

Discussion

The fisheries sector is important for the Indian economy as it provides better employment opportunities, and has been a source of nutritional food that could earn foreign exchange and lead to the development of the nation. In India, Prayagraj is located on the Ganga River's western bank and is one of the major water resources for fish catching, which also has a wide variety of fish diversity. In terms of livelihood and economic development of people living around, freshwater fisheries are of eminent significance. Fish is taken as a part of the diet since long ago, as it is one of the most significant sources of animal protein, has a high nutritional value, is low in cholesterol, and is readily available to people. According to Geetarani *et al.*[22], parasite infection prevalence and severity differ depending on the age or duration of the host. Parasites are excellent colonists, expanding their ranges when afflicted animals move about in the ecosystem and can be easily detected through the proper examination. The parasite load in the gastrointestinal system may be determined by examining fresh fish feces[23]. The severity of the pathological changes under influence of genetic alterations could correlate with the elements of host specificity, duration of infection, the parasite burden, and the host's sensitivity. Genetic damage caused by helminths exerts a heavy socio-economic toll in India as well as in South-East Asia. The people are at great risk mainly due to the unawareness of the ill effects caused by this major public and clinical health problem amongst the population. In addition, the polluted waters have contributed to the autogenic characteristics of the life cycle of some anisakid worms and nematode parasites such as *Rostellascaris* sp. in freshwaters of the River Ganges[24]. The investigations would thus have a unique significance from the point of view of multifaceted analyses, particularly because of taxonomic relevance, as a component of basic research, as well as the zoonotic significance of human health concerns having clinical significance. The present study will help in imparting skills such as tools of species diversity analysis and to attain achievable targets in basic research.

These parasites have the potential to inflict significant economic damage and are one of the most common causes of mass fish death; thus, suitable safeguards should be practiced to preserve the high value of economic resources such as fish and aquaculture[25,26]. Fish are a large group of species that carry a variety of parasites, including meta-cercariae[27,28], another example is the fluke that adheres to the human esophagus and causes harm if it is unintentionally swallowed raw or semi-cooked fish[29]. Several studies already supported the view of protecting the fish population from harmful parasites. As previously anticipated, parasites have a distinct place in the animal kingdom due to their astonishing adaptations and detrimental behaviors toward hosts, and helminth parasites remain a serious public health problem, particularly in Asia, because they can only be transferred to humans through fish[2].

Parasitic illness is the greatest serious hazard to the global fishing sector, particularly in the tropics[30]. Fish parasites are intimately linked to human health, and there is a widespread problem in India with the under-reporting of fish parasites owing to a lack of interest[31]. Acanthocephalans are a harmful group of parasites that are commonly found in the guts of freshwater Channidae fishes, young fishes being more sensitive to the tail area, prone to metacercarial infection, and therefore should be avoided for human consumption[32].

Helminth parasites frequently cause harm to the environment of their microhabitat within the host body. When parasites pierce numerous organs of the digestive system to obtain food from the host's body, the movement of the parasites disrupts the host's physiological systems. The cluster of parasites stops the fluid channel in the host's body, resulting in a lack of nourishment, the occurrence of sores, ulcers, and eventually death of the host[33]. Similar to trematodes and cestodes, the larvae of several nematode species travel widely through fish tissues, causing harm mostly by direct cellular destruction and hemorrhage in the passage through fibrous capsules.

Several endo-parasitic tapeworms (cestodes), such as *Diphlyllo bothrium*, have a larval stage that can be lethal to fish and infective to humans; cestodes infect the alimentary canal, muscle, or other internal organs, resulting in a bloated belly, overall wasting, and troublesome swimming[34]. Roundworms, also known as nematodes, may be found in fish and have a complicated indirect life cycle, with fish serving as both intermediate and ultimate hosts; the developmental stages of nematodes contain an egg, four larval stages, and adults, which are primarily located in the digestive tract, swim bladder, and body cavity[35]. The population dynamics studies revealed a tissue-specific infestation pattern and prevalence of different helminth parasites in freshwater fishes *Channa punctatus* and *Heteropneustes fossilis* in Moradabad, Uttar Pradesh, India[36]. The gastrointestinal parasites of the fish *Tilapia zilli*, *Auchenoglanis occidentalis*, *Clarias garipinus*, and *Mormyrus rume* can be employed as heavy metal contamination bioindicators in the aquatic environment of the Chanchaga River[37]. Although several reports have revealed the status of environmental pollution through several parasitic bio-indicators across the world[38-41], still a lot of work needs to be done regarding freshwater river pollution and the use of parasites as potential bioindicators and genotoxic agents as well, keeping in mind their huge utility in terms of habitat and ecological niche of many organisms.

Tenia solium cysticerci secrete has been identified as a factor involved in the development of micronuclei in cultured human lymphocytes[9,11]. In recent times, many helminth parasites have been identified as relevant sources of human carcinogens and several remedial strategies are still under shape[42,43]. While the role of the helminth parasite as a contributing factor in global cancer is small, the growing trend of helminth parasite zoonosis makes it a relevant component to investigate for future genotoxicity studies as well[17,44,45]. In the present investigation, a considerable number of parasitic infections in the experimental fish host, particularly endoparasite helminths, were found to be causing genotoxic effects, giving rise to several losses at the cellular, physiological and molecular levels. As a result, the risk of future disease development leading to malignant transformations cannot be completely ruled out. This remains to be addressed in the future studies of genotoxicity induced by parasites not only in fish host but also in the humans through the ecosystem.

Conclusion

Cellular and DNA damage in fish owing to parasitic infestations decrease the fish productivity and might impose serious effects in humans through food chain. In the present study, the genotoxic potential of helminth *Rostellascaris* sp. on the hepatocytes, muscle, and whole blood of infected fish *B. bagarius* collected from different sites of the river Ganges was analysed by using comet assay and micronucleus tests. A comparatively higher DNA damage was detected in the hepatocytes than the reticulocytes, suggestive of tissue-specific DNA damage; and also a higher induction of micronuclei found in infested fish as compared to the control. These outcomes are suggestive of the fact that parasites cause the induction of cellular and DNA damage in a tissue-dependent manner in fish during the progression of the host-parasite interaction. In the future, the inferences of the study may be utilized for setting up severe regulatory biocontrol measures for the eradication of these parasites from specific hosts with a broader perspective of combating environmental contamination and pollution.

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Conflict of Interest

Authors declare no competing interests

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