Research Note: Genetic analysis, pathology, and vectors of echinostomiasis, a zoonotic helminth infection in chickens in Bangladesh

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ABSTRACT Echinostomes (Trematoda: Echinostomatidae) are food-borne zoonotic flatworms that affect birds, animals and humans, and has been classified as neglected tropical diseases (**NTDs**) by the World Health Organization (**WHO**), which cause severe enteritis in poultry and hamper production. Here, we confirmed the species of echinostomes affecting chickens in Bangladesh along with their genetic analyses, pathology and vectors. We isolated and identified adult worms from chickens, cercariae from fresh water snails and metacerariae (**MC**) from some wild fishes. We recovered *Echinostoma revolutum* (10.3%) and *Hypoderaeum conoideum* (6.0%) from chickens. Zoonotic *E. revolutum* was confirmed by amplifying *nad1* gene and subsequent sequencing. Several mutations were detected in *nad1* gene and our isolates belonged to the Euro-Asian clade. We observed thickening of mucosal layer, hyperplasia of goblet cells, infiltration of eosinophils, lymphocytes and must cells in the infected intestine. About 5.3% snails were infected and the highest percentage of infection was found in *Lymnaea luteola* (12.1%). Echinostome infection in snails was the highest in November (9.6%) and lowest in February (3.1%) in Bangladesh. MC of echinostomes were identified from blue panchax (*Aplocheilus panchax*) and tank goby (*Glossogobius giuris*). In conclusion, echinostomiasis is a notable big problem in indigenous chickens in Bangladesh and people, especially, villagers are at risk.

Key words: echinostomiasis, zoonotic food-borne trematodes, genetic analysis, snail, fish

2022 Poultry Science 101:101682
https://doi.org/10.1016/j.psj.2021.101682

INTRODUCTION

Echinostomes (Trematoda: Echinostomatidae) are food-borne zoonotic, intestinal worms, which infect a wide range of vertebrates including humans (Anisuzzaman et al., 2005; Toledo et al., 2014). Echinostomiasis, particularly by *Echinostoma revolutum*, has been listed as neglected tropical diseases (**NTDs**) by the World Health Organization (**WHO**) under the subclass food-borne trematode infections. A total of 20 species belonging to nine genera of the family Echinostomatidae are responsible for the infection. Echinostomes are 10 to 22 mm long and up to 2.25-mm wide and are uniquely characterized by the presence of head-collar armed with variable number of spines, which is the major identifying feature for the family. Since the echinostomes are digenetic trematodes, they have a complex lifecycle and pass through different developmental stages. Eggs pass through feces and hatch in water releasing miracidium, the first larval stage (Toledo et al., 2014). The miracidium can swim in water and try to penetrate a suitable fresh water snail (**FWS**), the first intermediate host. Asexual development of echinostomes occurs in various species of FWSs belonging to several genera namely *Physa, Lymnaea, Helisoma, Bulinus*, and *Indoplanorbis* (Soulsby 1982). Within the molluscan vectors, miracidium ultimately develops to cercariae, another freely swimming larval stage. The cercariae may either encyst within the same snail or escape and enter into another snail, fishes, shrimps, or tadpoles to develop into metacercariae (**MC**), the infective stage for definitive and

Received October 18, 2021.
Accepted December 17, 2021.

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reservoir hosts (Sah et al., 2018). Definitive hosts or reservoirs become infected by ingesting infected snails, fishes, shrimps, and tadpoles containing viable MC (Toledo et al., 2014; Sah et al., 2018).

Echinostomes are distributed worldwide but it is mainly endemic in Southeast Asia, the Far East, India, Bangladesh, Europe, and North America (Anisuzzaman et al., 2005; Mohanta et al., 2019). They cause damage to the intestinal mucosa and induce extensive intestinal and duodenal erosions, and catarrhal inflammation. Peripheral eosinophilia, profuse watery diarrhea, anemia, edema, malnutrition, or intestinal perforations are usually present and heavy worm burdens can cause death (Soulsby, 1982). However, echinostomiasis in chickens along with its genetic analysis, pathology, epidemiologic aspects, and vectors are yet to be addressed in Bangladesh. Here, we validate *E. revolutum* by employing molecular tools in indigenous chickens in Bangladesh as well as its genetic analysis, epidemiology, pathology, and vectors.

MATERIALS AND METHODS

Sampling

We randomly selected, collected and examined 200 nondescriptive, indigenous chickens. We collected and examine a total of 20,000 FWSs from different habitats. Also, we examined seven species of small wild fishes such as zebra fish (*Danio rerio*), elongate glass-perchlet (*Chanda nama*), Indian glassy fish (*Parambassis ranga*), highfin glassy perchlet *(Parambasis lala),* blue panchax (*Aplocheilus panchax*), tank goby (*Glossogobius giuris*), and annandale loach (*Lepidocephalichthys annandalei*), and one crustacean species kuncho river prawn (*Macrobrachium lamarrei*).

Ethical Approval

The chickens handling and care were approved by the Animal Welfare and Experimentation Ethics Committee of Bangladesh Agricultural University, Mymensingh with the approval number of AWEEC/BAU/2018 (25).

Postmortem Examination of Chickens, Parasite Collection, and Identifications

Chickens were collected from local markets in the Mymensingh District, Mymesingh, Bangladesh, and euthanized and digestive tract was collected. The alimentary tracts were collected, opened along the long axis and the mucosal surface was carefully examined to detect helminths. Mucosal surface was gently washed with PBS in a jar. Washings were suspended and supernatant was discarded. The procedure was continued to clean up the washing. Adult worms were collected and identified by preparing permanent slides following the keys and description given by Soulsby (1982).

Gross and Histopathology in Chickens

Gross changes induced by echinostomes at the site of attachment were recorded carefully. Tissues were collected and preserved in 4% paraformaldehyde (Sigma, Gillingham, UK) added with 0.01% glutaraldehyde (Sigma). Histological sections were stained with Haematoxylin and Eosin (**H&E**) and examined by at least 2 blind investigators. Tissues from the noninfected, age, and sex matched chickens were used as a control. Three slides from each sample and 3 foci from each slide were evaluated.

Collection of Snails, Shedding, and Identification of Cercariae

We collected 7 species of FWS, namely *Lymnaea luteola, L. auricularia, Indoplanorbis exutus, Physa acuta, Vivipara vivipara, Brotia* spp., and *Thiara* spp. and snail habitats were recorded. Shedding of cercariae was induced by exposing snails to light as described previously (Frahm et al., 2019). The cercarae were examined under a light microscope by adding 2% iodine solution. Additionally, to detect other developmental stages, snails were crushed and examined.

Fish Sampling, Processing, and Recovery of MC

We used pooled sample where each sample consisted of 100 g of fishes of the same species. Visceral organs of each fish were removed carefully and chopped and blended adding artificial gastric juice containing 0.3% of pepsin (LOBA Chemie, Mumbai, India) and digested at 37°C overnight under vigorous stirring. Digested fishes were examined under a microscope (Labomed, Los Angeles, CA) using a 10 X objective. Each sample was examined at least in triplicate as described previously (Labony et al., 2020).

DNA Extraction, PCR, and Sequencing

Genomic DNA of Adult flukes, cercariae or MC of echinostome was extracted using QIAamp DNA Mini Kit (Qiagen, Germany). To confirm the species, partial fragment of the mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1 (**nad1**) was amplified using the primers (NDJ11: 5′-AGATT CGTAAGGGGCCTAATA-3′ and NDJ2A: 5′-CTTCA GCCTCAGCATAAT-3′). PCR amplifications were performed in a total volume of 25 μl reactions using One Taq Quick-Load 2X Master Mix (New England BioLabs Inc., UK), 10 pmol of each PCR primer, and 50 ng of genomic DNA. The PCR thermo cycling profile comprised initial denaturation at 95°C for 5 min, followed by 35 cycles (30 s denaturation at 94°C, 20 s primer annealing at 48°C, and 45 s at 72°C for primer extension), with a final extension step of 4 min at 72°C and the PCR product was subjected to agarose electrophoresis

(Georgieva et al., 2014). PCR products of the *nad1* gene were purified and the purified PCR products were subjected to sequencing in both directions with the same primers and edited using BioEdit 7.2 software. Obtained sequences were deposited to the GenBank under the accession numbers: MZ824394, MZ833485, MZ888984, and MZ913259.

Genetic Data Analysis and Phylogenetic Study

Sequences were searched using BLAST and sequences with higher identity (~x223C98%) were chosen. Sequences were aligned using CLASTALW for pair-wise comparisons with previously published sequences. Phylogenetic analysis was performed using neighbor joining (**NJ**), maximum likelihood (**ML**), and minimum parsimony (**MP**) methods, based on the Tamura-Nei model where *nad*1 gene of *E. robustum* was used as an outgroup. Confidence limits were assessed using the bootstrap procedure (1,000 replicates) and other settings were obtained using the default values in MEGA X. A 50% cut-off value was implemented for the consensus tree.

Statistical Analysis

Statistical analysis was performed using z-tests and $P < 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Echinostomiasis in Indigenous Chickens and Confirmation of Species

During our study, 30 (15%) chickens were found infected with echinostomes (data not shown). On the basis of morphological and morphometric analysis, we identified 2o species of echinostomes such as *E. revolutum* and *H. conoideum* (Figure 1A). Morphologically, *E. revolutum* were 9.5 to 20 mm long and 2.0 to 2.5 mm wide, and characterized by the presence of a strong head-collar bearing 37 spines and 2 groups of corner spines at both right and left corners of the head collar. They have well-developed oral and ventral suckers, and ventral suckers at the level of cecal bifurcation. Testes are tandem, slightly lobulated, and nearly at the equatorial zone. In contrast, *H. conoideum* was relatively smaller (4.5−11.5 mm long × 1.75−2.2 mm wide) and with very weakly developed head-collar that bear 2 corner spines at either sides (Figure 1A). Prevalence of *E. revolutum* (10.3%) was higher than *H. conoideum* (6.0%) (data not shown) and each infected chicken harbored 1 to 25 flukes. In a previous report, a very high prevalence (~x223C50%) of *E. revulotum* had been recorded from domestic ducks reared in Bangladesh (Anisuzzaman et al., 2005). The parasite maintains aquatic lifecycle, therefore, higher infections in ducks is quite logical. However in Bangladesh, backyard poultry

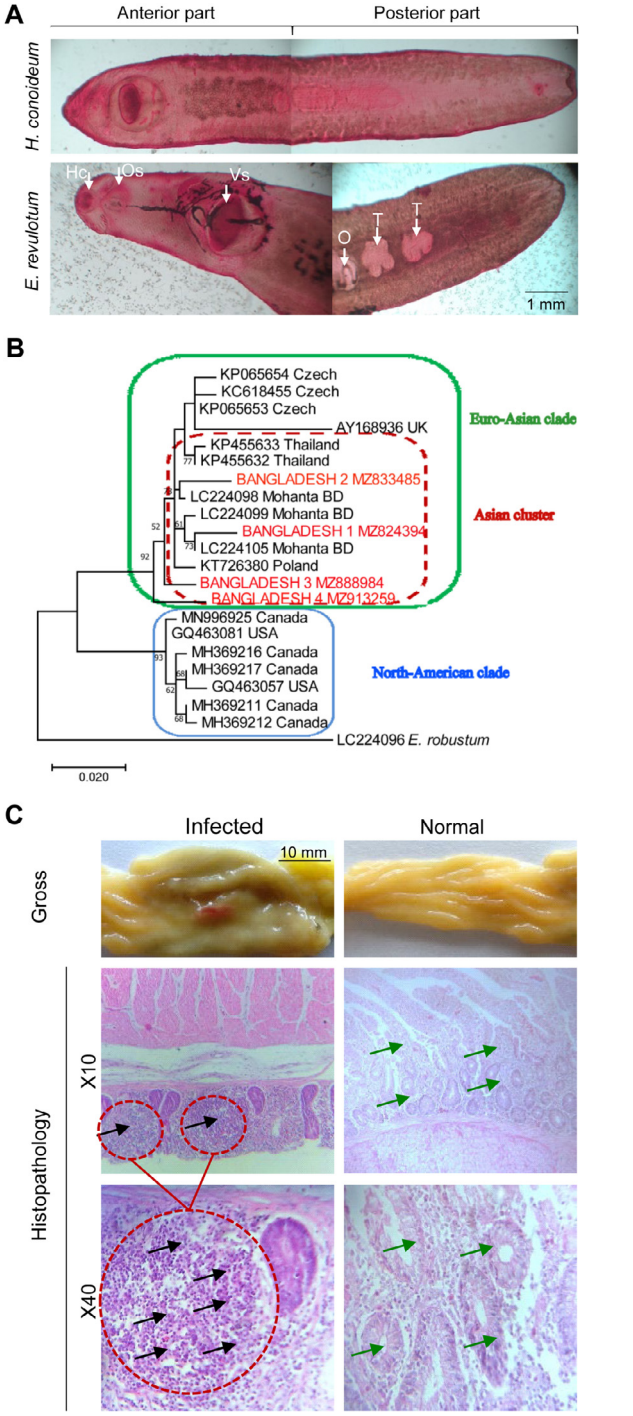

Figure 1. Echinostomiasis in indigenous chickens and its genetic analysis. (A) Morphological characterization of *E. revolutum* and *H. conoideum*. (B) Phylogenetic relationships of echinostomes based on the mitochondrial gene *nad*1 inferred from Maximum Likelihood (ML) tree. The newly sequenced isolates are colored in red. The scale bar indicates the expected number of substitutions per site. (C) Gross changes induced by echinostomes at the site of attachment were recorded carefully. Black arrows and red circles indicate infiltration in the infected part of intestines. Green arrows indicate normal structures of controls. Abbreviations: Hc, head collar; O, overy; Os, oral sucker; T, testes; Vs, ventral sucker.

is more common than the ducks, and therefore, as a reservoir of echinostomiaisis, chickens are more important and are assumed to play critical roles at least in zoonotic transmission cycle.

We isolated genomic DNA from tentatively identified adult flukes and PCR was performed, which revealed amplicon of expected size (~x223C475 bp) in all isolates, indicating the presence of *E. revulotum*. To ascertain our proof, we sequenced PCR-products and retrieved sequences having 479 bp and by BLAST search, we found identical sequences of *E. revolutum* having higher identity (up to 99.1%; accession numbers: LC224105.1), confirming the species. We aligned each *nad1* sequence of *E. revolutum* retrieved in the present study with the reference gene (accession numbers: LC224105.1) and single nucleotide polymorphisms resulting from the substitutions of nucleotides were detected at the positions 44, 97, 137, 147, 159, 169, 171, 178, 244, 314, and 328. We identified 6 transversion and 5 transition mutations in the sequences retrieved (data not shown). The reference sequence was an isolate from ducks and sequences reported here are from chickens, therefore, mutation is quite expected.

To determine genetic diversity, we constructed phylogenetic trees using our data set and other sequences deposited to the GenBank from different countries of Europe, America and Asia having identity ranging from 88.9 ton99.16% and a *nad*1 sequence of *E. robustum* was used as out group by employing 3 well-accepted methods (e.g., NJ, ML, and MP), which revealed similar results. However, for the convenience of the study, only the ML-dendrogram generated with 1,000 replicates had been presented and described. The ML-dendrogram showed 2 distinct clade supported by very strong bootstrap, which are Euro-Asian clade and the North American clade. However, within the Euro-Asian clade, there were 2 subgroups or clusters such as Asian cluster and European cluster, with a very high homology (Figure 1B), which conforms to the observations reported by Mohanta et al. (2019). These findings also imply that *E. revolutum* prevalent in Bangladesh have close evolutionary relationship with the European isolates.

Organ of Predilection of Echinostomes and Pathological Changes Induced in Chickens

During examinations, the flukes were detected both in small (4.5%) and large (12.5%) intestines; however, they were most commonly found in ceca (data not shown). They induced enteritis characterized by the presence of excessive mucus. However, in some cases hemorrhagic spots were detected at the site of attachment of the flukes. To reveal the participation of inflammatory cells, we did routine histopathological analysis. We observed thickening of mucosal layer, hyperplasia of goblet cells, infiltration of eosinophils, lymphocytes and must cells. Atrophy of the villi was a common feature and in some cases hemorrhages were noticed (Figure 1C). *E. revulotum* have strong oral and ventral suckers, which are utilized to attach themselves firmly to the intestinal mucosa. Additionally, the cuticle of the worms is spiny (Soulsby, 1982), which give continuous mechanical insults. Adult parasites can survive for years and feed on soft mucosal tissues, leading to the development of inflammations, which in turns elicit thickening of mucosal layer of the intestine, proliferation of goblet cells and excessive mucus production (Songsri et al., 2016). The flukes eat off the mucosal tissues, therefore, petechial hemorrhages are not unexpected. Infiltration of eosinophils is a common feature in helminth infection (Anisuzzaman et al., 2005, 2020) and in ectoparasitic infestations (Anisuzzaman et al., 2014).

Vector Snails of Echinostomes in Bangladesh

We recovered echinostome cercariae (**EC**) from 1,090 (5.3%) snails (data not shown) belonging to 6 species such as *P. acuta, Thiara* spp., *L. auricularia, L. luteola, I. exustus*, and *V. vivipara*, and ECs but not in *Brotia* spp. (Figures 2A and 2B), suggesting that those FWS acted as the first intermediate hosts of echinostomes in Bangladesh. Initially, EC were morphologically identified by the presence of their head collar (Figure 2A). Additionally, we detected MC of echinostome in *L. auricularia, L. luteola,* and *I. exustus.* Developmental stages were further validated by PCR. FWS belonging to several genera such as *Physa, Lymnaea, Helisoma*, and *Bulinus* act as the intermediate hosts of echinostomes in different countries (Soulsby 1982). The highest percentage of infection was found in *L. luteola* (12.1%) and the lowest infection was found in *V. vivipara* (0.1%) (Figure 2B), indicating that lymnaeid snails are the major intermediate hosts of echinostomes in Bangladesh and plays a vital role in the survival and existence of echinostomes in the country. We detected relatively higher infection rate of EC in snails collected from canals, rice fields and rivers (Figure 2C). These water bodies are the principal habitat of lymnaeid snails, which are the main vector of echinostomes in Bangladesh. Therefore, it is quite pertinent that the rice fields, river and canals are the main niches to get echinostomes infected snails.

Our study uncovered that the seasons of the year had profound impacts on the infection rate in snails by the developmental stages of echinostomes. We found the highest infection in September followed by October and November but the infection rate was least in the colder month such as January and February (Figure 2D), suggesting that in these 3 m, chickens are more likely to get infection and warrant restriction of movement especially near the snail habitats. The entire processes for development from miracidium to cercariae require 6 to 8 wk (Soulsby, 1982), therefore, it is quite pertinent that infection rate in FWS would be relatively higher in September−November. In January and February ambient temperature may fall even below 10°C, when the FWSs become less active and even they may undergo hibernation, and (Labony et al., 2020). In the hot weather, snails also become relatively inactive. Besides, scorching heat of the summer may also have adverse effect on the

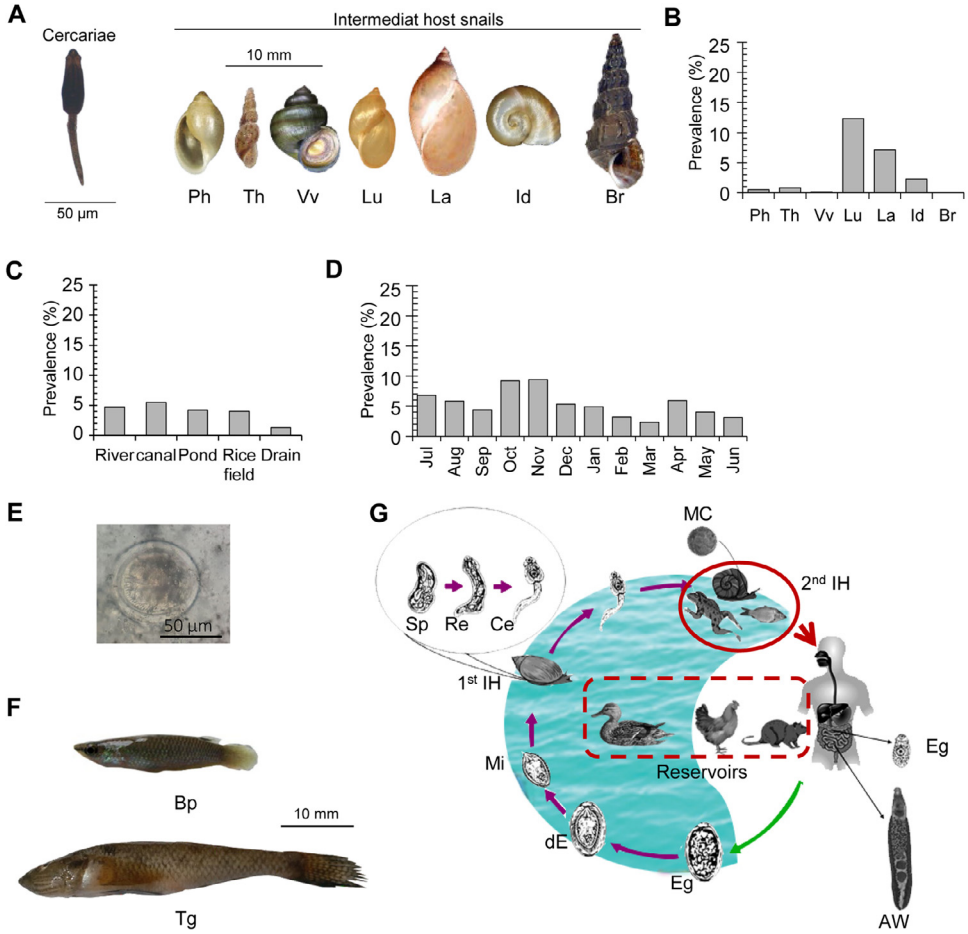

Figure 2. Vectors of echinostomes, and their spatial and temporal distributions. (A) Detection of echinostome cercariae from snails. Of the snails examined, we isolated cercariae of echinostomes from six species of snails such as *P. acuta, Thiara* spp, *L. auricularia, L. luteola, I. exustus*, and *V. vivipara*. Echinostome cercariae were morphologically identified by the presence of their head collar. (B) Vector snail preference and diversity of echinostomes in Bangladesh. (C) Niche-wise distribution of the infected vector snails. (D) Temporal distributions of echinostome infections in snails. (E) MC of echinostomes. (F) MCs of echinostomes were detected in blue panchax (Bp) and Tank goby (Tg). (G) Schematic presentation of lifecycle of echinostomes. Abbreviations: Aw, adult worm; Ce, Cercaria; eg, egg; dE, developed egg; IH, intermediate hosts; MC, metacercaria; Mi, miracidium; Re, redia; Sp, sporocyst.

development of echinostomes in snails as it is reflected lower rate of infections in snails in summer months.

Unlike schistosome, EC cannot cause infection directly in definitive hosts. Cercariae need to be encysted in the second intermediate hosts to develop MC, the infective stage for definitive hosts. Cercariae can be encysted within the same FWS in which they develop or can be released from the snail and after being released they swim in water by utilizing glycogen deposited in their tail and seek for suitable second intermediate hosts. They can enter into another snail of the same species or different species where they develop into MC (Soulsby, 1982).

MC of Echinostomes in Wild Fishes

To reveal the roles of wild fishes as the second intermediate hosts in the completion of lifecycle of echinostomes in Bangladesh, we collect and examined seven species of fishes, which share the common niches of FWS vectors. We isolated and identified MC of echinostomes from blue panchax (*A. panchax*) and tank goby (*G. giuris*) (Figures 2E and 2F), suggesting that they contribute to lifecycle of echinostome as the second intermediate hosts. Blue panchax lives in shallow water bodies like drains, canals, ponds, and paddy fields and is a surface-dwelling fish. Moreover, as blue panchax is not a food fish, very often the fishermen discard the species after netting; therefore, it is an easy prey to poultry. Tank goby also lives in shallow water and is very popular throughout the country. In a previous study, we recovered MC of echinostomes from fresh water gar fish (Labony et al 2020), which is also very popular among people in Bangladesh; therefore, people are at risk to the infection. Thus, utilizing chickens, FWS, and fishes, zoonotic echinostomes are able to complete their entire lifecycle in this endemic country (Figure 2G).

Taken together, prevalence of echinostomes in indigenous chickens is still high in Bangladesh, and our isolates belong to the Euro-Asian clade. Wild fishes carry infective MC stage for reservoirs and definitive hosts, including humans, and people of the country are at risk to the infection.

ACKNOWLEDGMENTS

The research works were funded by Bangladesh Agricultural University Research System (**BAURES**), Bangladesh Agricultural University, Mymensingh; Ministry of Education and Ministry of Science and Technology, The Government of Bangladesh, and University Grants Commission (**UGC**), Bangladesh. The authors gratefully acknowledge the Equipment Subsidies granted by Alexander von Humboldt, Germany.

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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