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Isolation and Identification of *Aspergillus fumigatus* from Brooder Pneumonia Affected Broiler Chicken

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Abstract
Brooder pneumonia is a common respiratory problem during brooding period of broiler chicken in high humid region. It is caused by fungal genus *Aspergillus*, specially *Aspergillus fumigatus*. The present study was carried out the isolation and identification of *Aspergillus fumigatus* from brooder pneumonia affected broiler chicken at 10 days old. The characteristic grayish colors cottony colony of *A. fumigatus* was isolated by culture the yellowish spherical caseous granulomatous lungs nodules in potato dextrose agar media. In histopathological examination the characteristic eosinophilic hyphae of *A. fumigatus* was demonstrated in the coagulative necrotic center of granulomatous nodules of lungs by routine Hematoxylin and Eosin staining procedure. Finally, the 26S gene of *A. fumigatus* was identified by amplification of 401-bp fragment by PCR test.

1. Introduction

Brooder pneumonia, a fungal disease of respiratory system of poultry and mostly caused by the spore of *Aspergillus fumigatus*. But other species of *Aspergillus* may involve in brooder pneumonia like *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus glaucus* and *Aspergillus nidulans* [1]. The fungal species of *A. fumigatus* predominant in brooder pneumonia might be the spore of *A. fumigatus* is smaller than others [2]. *A. fumigatus* is a ubiquitous saprophyte and opportunistic, DNA and spore forming fungus under Trichocomaceae family [3, 4]. It causes significant economic losses of poultry industries by increase mortality in both immunocompetent and immunodepressed birds [5, 6]. Brooder pneumonia called due to specially occurred in brooding age of broiler as an acute form with high mortality and have tendency of chronic form in older age of immunodepressed birds [7]. Inhalation of fungal spore is the main way of transmission in poultry [8]. Clinically the affected chicks shows dyspnea, gasping and lesions are mostly confined in lungs and air sacs, although oral organs like mucosa, trachea and eyes may be affected. Typical lesions are fungal nodules or plaques developed within the lungs and on the air sacs [9].

A. fumigatus has been first isolated from lung lesions of wild birds since the early 1800s [10]. It can grow and isolated from potato dextrose agar media at 25-27°C temperature and shows characteristic grayish color cottony colony [11, 12]. In histopathological study on lung nodules the organism shows eosinophilic hyphae in granulomatous lesion of lung by Hematoxylin and Eosin (H&E) staining technique [13, 14]. The confirmatory identification of *A. fumigatus* can be done by molecular techniques like polymerase chain reaction (PCR) test by amplification of 401-bp fragment of 26S gene [15].

The objectives of this study were isolation and identification of *A. fumigatus* from brooder pneumonia affected broiler chicken through mycological culture, histopathological examination and molecular techniques. Additionally, this study will try to confirm the etiology of brooder pneumonia and its diagnostic techniques.

2. Materials & Methods

2.1. Sample Collection and Processing

The study was conducted on 4 dead chicks of cob-500 broiler strain with brooder pneumonia clinical history at 10 days old from Bogra district of Bangladesh. Collection of lung samples after postmortem was divided into two parts. One part was kept for direct fungal culture test and another part was fixed in 10% formaldehyde solution for histopathological examination.

2.2. Culture of *Aspergillus fumigatus*

Preparation of potato dextrose agar media (PDA) with chloramphenicol according to manufacturer directions (HIMEDIA®, India). An inoculum was prepared from lung nodules and directly streaked on PDA media, then incubated at 25±2°C temperature for 48 hours. Observe the growth and colony morphology of *Aspergillus fumigatus* after 7 days of incubation [16].

2.3. Histopathology

The collected lung samples were prepared for histopathological examination by routine Hematoxylin and Eosin (H&E) staining procedure according to [17] guideline. Finally the histopathological slide examined under a 40× power objectives of light microscope.

2.4. Detection of *Aspergillus fumigatus* by PCR

The DNA of *A. fumigatus* was extracted from grinded nodules of *A. fumigatus* suspected lung by using QIAamp® DNA Mini Kit (Germany) according to manufacturer instructions. Then the extracted DNA was subjected to PCR for the detection of *A. fumigatus* targeting the 26S gene (401-bp) by using the primers suggested by [15] (Table 1). For preparation of master mix each PCR contained 10µl of 10× buffer, 15µl of glycerol, 2.5mM each dATP, dCTP, dGTP and dTTP, 2.5 U of Amplitaq and 5µl of each primer (132µl/ml). The PCR reaction was carried out by thermocycler (eppendorf®, USA) with thermal profile programme: initial denaturation with 1 cycle of 5 min at 94°C, for annealing 1 min 20s at 56°C, for primer extension 2 min at 72°C, then 31 cycles of 40s for denaturation, for annealing 80s and then 2 min of extension with a 3 min extension step in the final cycle. Then the PCR products were kept for analyzed by 2.00% agarose stained with ethidium bromide gel electrophoresis and DNA visualized by UV Solo Transilluminator.

Table 1. *Sequence of primers for the detection of A. fumigatus by PCR.*

Name of primer	Sequence	Amplification size	Reference
Af1	5'-CCTTGGCTAGATITGTTGGC-3'	401-bp	[15]
Af2	5'-CCAACTCCCCTCAGCCAACT-3'		

3. Results & Discussion

The postmortem lesion of dead birds was white to yellowish spherical caseous granulomatous nodules of various sizes in whole lungs (Figure 1). Similar observations were demonstrated by [18] who described congestion in lungs with grayish white nodules and frothy exudates in *A. fumigatus* affected commercial poultry in Pakistan. [7] also revealed similar results of yellow, green or white granulomatous foci in lungs of poultry affected with chronic aspergillosis. Acute infection of *A. fumigatus* results numerous miliary granulomatous foci in lung parenchyma [19].

A. fumigatus was demonstrated by culture the samples collected from suspected grinded lung nodules into Potato dextrose agar (PDA) media at 25±2°C temperature for 48 hours. The characteristic grayish color cottony colony of *A. fumigatus* was found (Figure 2). The findings of mycological culture were similar with the findings of [11] who isolated white to green *A. fumigatus* from lung lesions of ostrich. *A. fumigatus* was also isolated by [5] from lung tissue of broiler chicken by PDA media.

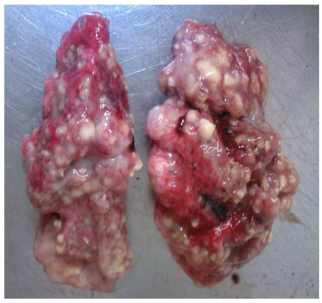

Figure 1. *White to yellowish spherical caseous granulomatous nodules of various sizes in whole lungs.*

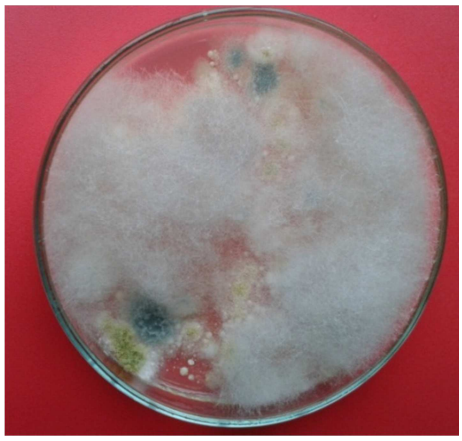

Figure 2. *Grayish color cottony colony of A. fumigatus in Potato dextrose agar media after 48 hours of incubation.*

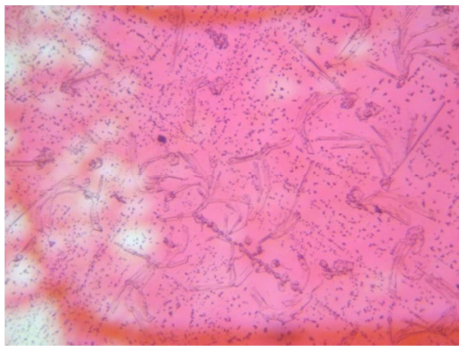

Figure 3. *Eosinophilic hyphae with frequent septation of A. fumigatus in histopathological slide of lung lesion. H&E stain, 40×.*

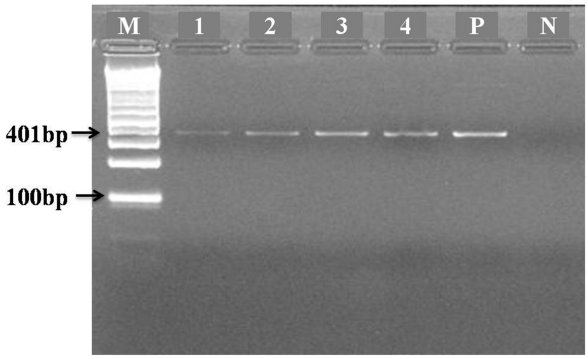

Figure 4. *Image showing the PCR product of A. fumigatus with specific primer. Lane M: 1.5-kb DNA ladder marker, Lane 1 to 4: mycological culture positive chicken, Lane P: positive control (previously confirmed in laboratory), Lane N: negative control (no DNA).*

Histopathologically, after routine Hematoxylin and Eosin (H&S) staining procedure the characteristic eosinophilic hyphae with frequent septation of *A. fumigatus* was present in the coagulative necrotic center of granulomatous nodules of lungs (Figure 3). The similar alteration in histopathological study was revealed by [5], who observed branching septate hyphae of *A. fumigatus* in lung samples by H&E stain in commercial poultry. By H&E staining the septate with dichotomously branching enclosed by palisade of radically arranged foreign body giant cells were shown in lung of broiler breeder chicken [20, 21, 22].

For detection of *A. fumigatus* the PCR test were performed for the all (n=4) samples confirmed in histopathology and mycological culture. The successful amplification of 401-bp fragment of 26S gene of *A. fumigatus* by PCR shown in Figure 4. Similar findings were reported by [15], who confirmed the identification of *A. fumigatus* by PCR test with same primers and thermal profile.

4. Conclusions

From the present study it may be concluded that *A. fumigatus* could be isolated and identified by observation of white to yellowish spherical caseous granulomatous nodules in lungs by necropsy, characteristic grayish color cottony colony in PDA media, eosinophilic hyphae in histopathological examination of lung nodules and finally detection of 401-bp amplification band size in PCR test.

Conflict of Interest

The authors declare that there is no conflict of interest.

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