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Mycotoxin production by fungi isolated from commercially prepared livestock feed in Kerala

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Abstract
The study was carried out to identify the common molds growing in the livestock feeds in Kerala. A total of 14 feed samples were analyzed for detecting the mycoflora.6 predominant fungus like *Pencillium* spp, *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Mucor* spp, *Rhizopus* spp and *Fusarium* spp were isolated. Among them *Penicillium* spp was the most prevalent fungi with an isolation frequency of 32%. Mycotoxin are secondary metabolites produced by fungi, which cause health hazards to animals and human beings. Mycotoxins are considered hazardous and there is a need for accurate detection of each toxin. Several screening methods for direct visual determination of mycotoxin production have been reported. These methods rely on using different types of coconut culture media, methylated β-cyclodextrin and ammonium hydroxide vapour tests. However our results showed that use of these techniques were not sufficiently sensitive for all *Aspergillus* species, *Fusarium* species and suggested a simple thin layer chromatography (TLC) as a sensitive and reliable technique for detection of Mycotoxin.

Keywords: Mycotoxin, Toxigenic, Aflatoxin, Coconut culture media

Introduction
Animal feed plays an important role in the production of quality milk, egg and meat for human consumption. If the quality of feed is reduced both animal and human health will be affected (Sivakumar *et al*., 2014) [13]. Agricultural products including cereals and oilseeds meals constitute a major component of poultry feed ingredients. Mold contamination is wide spread in tropical countries where poultry production and processing are expanding rapidly (Muhammad *et al*., 2010) [8].

The contamination of agriculture commodities with toxigenic fungi under favorable conditions may lead to Mycotoxin buildup reaching to injurious levels for farm animals and human health (Muhammad *et al*., 2010) [8]. Fungal and Mycotoxin contamination of food and poultry feeds can occurs at each step along the chain from grain production, storage and processing. Several physical factors including moisture, humidity, ambient temperature, storage time, pH and oxygen affect fungal growth and Mycotoxin production (Kana *et al*., 2013) [5]. A fungus proliferates after getting favorable conditions and secretes Mycotoxin but actual reason of Mycotoxin production is yet unknown (Shareef, 2010) [12].

The production of Mycotoxin is often species specific; therefore, identification of fungi is of prime importance (Krnjaja *et al*., 2009) [6]. Among different Mycotoxin, aflatoxins (AF) and ochratoxin A (OTA) are the most important contaminants of poultry feeds (Muhammad *et al*., 2010) [8]. Mycotoxin producing fungi such as *Aspergillus, Fusarium* and *Penicillium* are of concern because they pose a relatively greater toxic threat to animals and humans (Kana*et al.,* 2013) [5]. The effects of Mycotoxin on higher animals include hepatotoxicity, nephrotoxicity, immuno toxicity, oncogenes is and genotoxicity (Shareef. 2010) [12].

Not all strains of fungi are able to produce Mycotoxin, therefore, there is a need for screening for their toxin production abilities (Yazdani *et al.,* 2010) [14]. The main objective of the present study was to detect the mycoflora of feeds and to evaluate the ability of different strains to produce aflatoxin.

Materials and Method
Sample Collection
A total of 14 samples of feed mixtures, designed for poultry feeding (sample1-7) and cattle feeding (sample 8-14) were collected from local providers and cattle farms.

These samples were collected in sterial bottles and were immediately stored at 4 ºC until analysis.

Fungal Isolation and Identification

For each feed sample obtained, a six fold serial dilution of 1g of feed was carried out using 9ml of sterile distilled water and 1ml of appropriate dilution was aseptically plated, using pour plate technique, on to Sabouraud dextrose agar (SDA) supplemented with chloramphenicol (Davis *et al.,* 1980) [3]. The inoculated plates were incubated at room temperature for 3 to 7 days. Pure culture of the different colonies (based on morphology) was obtained by sub-culturing of the isolates on SDA plates. The fungal isolates were identified to the genus/species level based on macroscopic and microscopic characteristics of the isolates obtained from pure cultures. Such characteristics include; colour of obverse and reverse side of the culture plate, shape, texture and consistency of the growth, septation of hyphae, shape, size, texture and arrangement of the conidia etc (Carter and Cole 1991) [1]. The fungal isolates were sub-cultured on SDA slants, incubated at 27 ºC for 3 days and stored in refrigerator for future studies (Saleemi *et al.,*2010) [11].

The isolation frequency (Fr) of species was calculated according to Ifeanyi *et al,* 2010 as follows:

$$Fr(\%) = \frac{Number\ of\ samples\ with\ a\ species\ of\ genus}{Total\ number\ of\ isolates} \times 100$$

Aflatoxin Detection Methods

- **Coconut Milk Agar Medium**

In CMA preparation, desiccated coconut was obtained locally(protein 0.65g,carbohydrate 1.05g,fat 7.5g/50ml based on company report).100g desiccated coconut were mixed with 600ml hot water in a separation funnel.350ml clear extract were slowly separated from the bottom of the funnel. pH of the media was adjusted to 6.8 with 2N NaOH. Agar (15g/l) was added to this and the mixture heated to boiling and autoclaved for 15 min for 121 ºC, and poured into sterile plates. The plate center was inoculated with fungal culture and incubated in the dark at 28 ºC the presence or absence of a fluorescent ring in the agar surrounding the colonies under UV light after 7 days incubation was noted and the results were scored as positive or negative.

- **Ammonia Vapour Test**

The fungal isolates were grown on YES agar as single colonies in the center of plate and incubated in the dark at 28 ºC (Kumar *et al.,* 2007) [27]. After 3 days, a set of plates were inverted over 2ml of ammonium hydroxide. This was repeated with another set after 7 days. A change in colour of the culture medium was used to determine the toxicity or otherwise of isolates. After ten minutes, the underside of aflatoxin producing isolates turned into pink to red colour. But no colour change occurred in the non-toxic isolates.

- **Methyl - B - Cyclodextrin Test**

Based on Fente *et al* (2001), YES agar was used as the culture medium. 3% (W\V) methylated -β- cyclodexdrin were added to autoclaved YES Agar in a petri dish. The plate center was inoculated with fungal culture. Plates were incubated in dark at 28 ºC the presence or absence of a fluorescence ring in the agar surrounding the colonies observed under UV light was scored as positive or negative.

Result and Discussion

Isolation of Fungal Strains

The results obtained in the present study showed high degree of fungal contamination of animal feeds. This renders them unfit for animal consumption and significantly lowers the value of grains as an animal feed and as an export commodity. Fungal infection percentage was found to be higher in poultry feed (sample 8-14) compounds followed by cattle feed (sample 1-7). The high prevalence rate of fungal species seen in this study supports Cheesbroughi (2000) [2], which states that tropical countries are more prone to fungal and microbial contaminations of poultry feed raw materials.

Table 1: Number of isolates obtained from feed samples

List Of Samples Collected	Number Of Isolates Obtained From Each Sample
Sample 1	2
Sample 2	2
Sample 3	2
Sample 4	2
Sample 5	2
Sample 6	No Growth
Sample 7	2
Sample 8	2
Sample 9	3
Sample 10	3
Sample 11	2
Sample 12	3
Sample 13	No Growth
Sample 14	No Growth

During the study 3 samples showed no growth. A possible reason for no fungal contamination in some commercially prepared feeds may be due to inclusion of antifungal agents by the producers to prevent fungal growth during prolonged and varied storage conditions at farms.

Identification of Fungal Isolates

Growth, morphological characteristics and colony colorations were considered for fungal identification. The results of the fungal identification studies are presented in Table 2. The results showed that, almost all the feed samples were infected with *Pencillium* spp, *Aspergillus* spp, *Mucor* spp, *Rhizopus* spp and *Fusarium* spp. The occurrence of *Aspergillus* spp, *Fusarium* spp and *Penicillium* spp could be as a result of their high pathogenicity as reported by researchers elsewhere (Pitt *et al*., 1994).

Table 2: Fungal isolates found in feed samples

Feed Sample	Species Identified
Sample 1	*Mucor*(M_1), *Penicillium*(P_1)
Sample 2	*Rhizopus*(R_1), *Penicillium*(P_2)
Sample 3	*Mucor*(M_2), *Aspergillus niger*(A_1)
Sample 4	*Aspergillus niger*(A_2), *Penicillium*(P_3)
Sample 5	*Pencillium*(P_4), *Yeast*
Sample 6	No Growth
Sample 7	*Pencillium*(P_5), *Aspergillus fumigatus*(Af_1)
Sample 8	*Aspergillus niger*(A_3), *Aspergillus fumigatus*(Af_2)
Sample 9	*Fusarium*(F_1), *Penicillium* (P_6), *Yeast*
Sample 10	*Aspergillus niger*(A_4), *Aspergillus flavus*(Afl_1), *Penicillium* (P_7)
Sample 11	*Aspergillus niger*(A_5), *Rhizopus*(R_2)
Sample 12	*Penicillium* (P_8),), *Aspergillus flavus*(Afl_2), *Yeast*
Sample 13	No Growth
Sample 14	No Growth

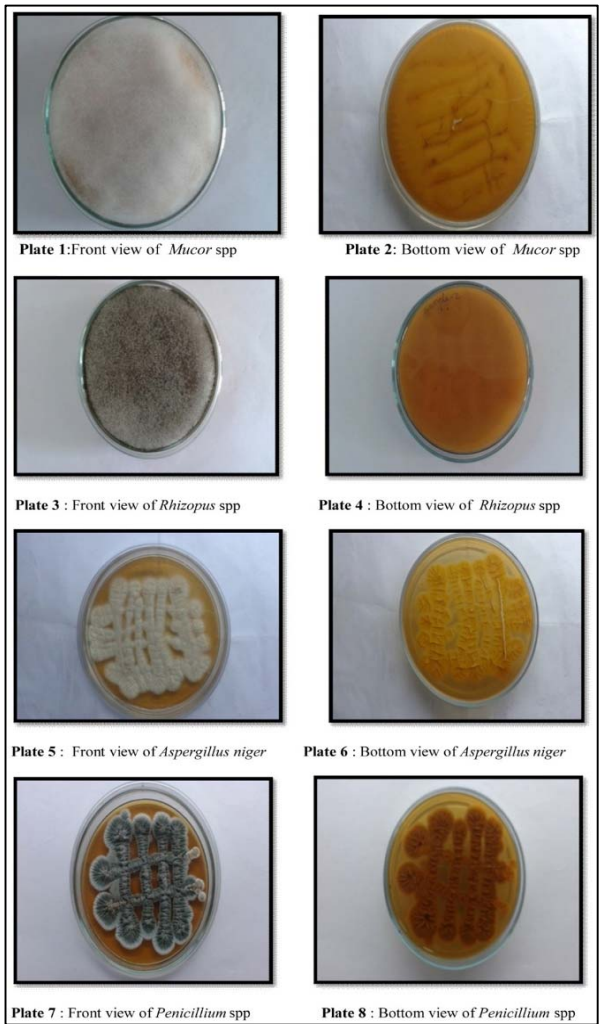
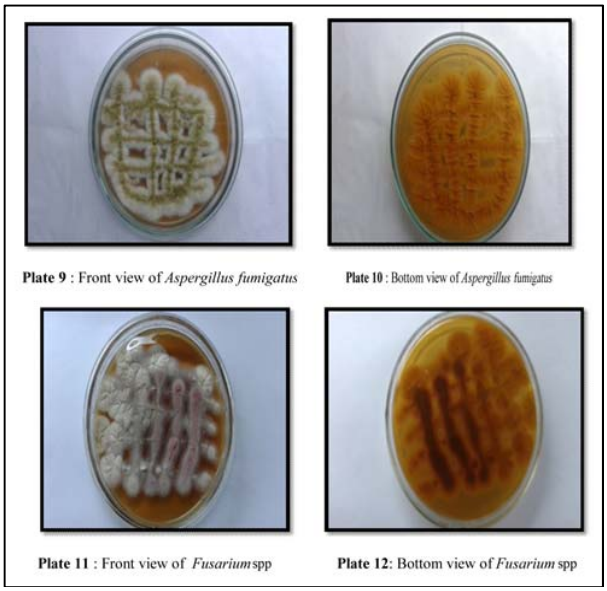


Fig 1: Macroscopic View of Fungal Isolates on Sda Plates



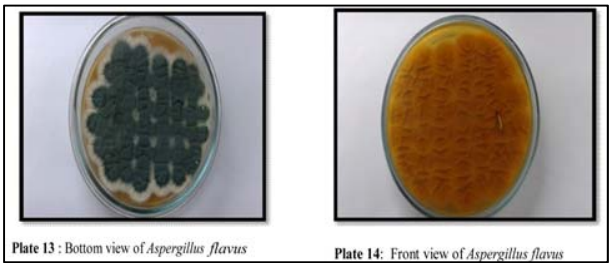
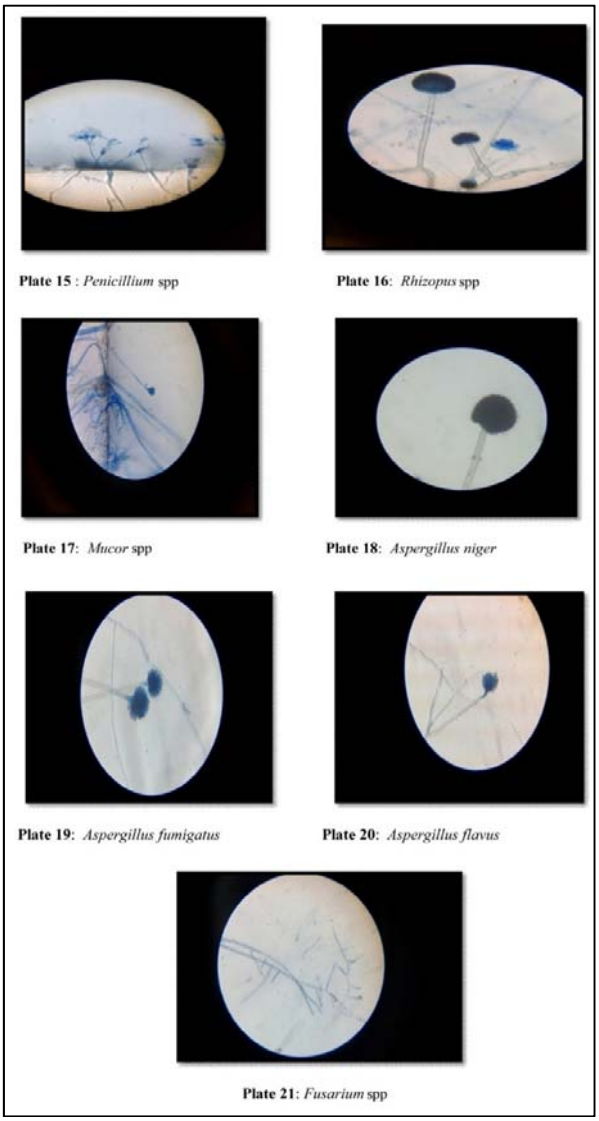


Fig 2: Microscopic View of Fungal Isolates



Isolation Frequency

Table 3 shows the overall frequency of isolation of different fungal species from commercial feeds sold in Kerela. *Penicillium* spp shows highest prevalence rate (32%) followed by *Aspergillus niger* (20%). *Aspergillus flavus*, *Aspergillus fumigatus*, *Mucor* spp and *Rhizopus* spp showed 8% of prevalence whereas *Fusarium* spp (4%) showed least occurrence. It was also found that 12% of isolates were *Yeast.*

Table 3: Isolation frequency of fungal isolates

Fungal Isolates	Number Of Isolates N = 14	Isolation Frequency (%) N=14
Mucor spp	2	8
Rhizopus spp	2	8
Penicillium spp	8	32
Aspergillus niger	5	20
Aspergillus fumigatus	2	8
Aspergillus flavus	2	8
Yeast	3	12
Fusarium spp	1	4
Total	25	100

Among the *Aspergilli* isolated from feed samples, *Aspergillus fumigatus* were the predominant species followed by *Aspergillus flavus.* These results are similar to some reports describing *Aspergillus niger* as the most predominant followed by *Aspergillus flavus* (Saleemi *et al.*, 2010) [11].

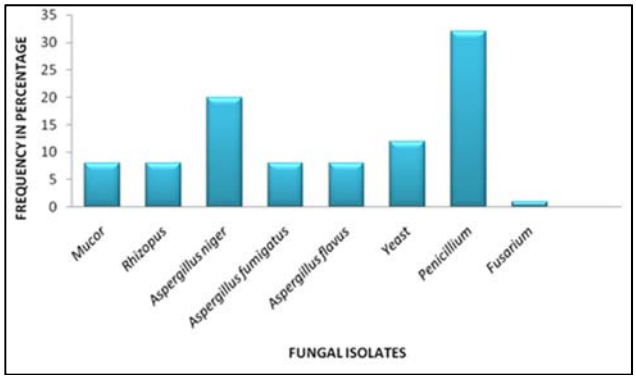


Fig 3: Percentage of isolated fungal species from livestock feeds

Detection of Aflatoxin Producing Strains

Presence of more than one genera and species of fungi in the animal feeds tested increases the risk of multiple Mycotoxins. In order to assure the quality of food and feeds used for human and animal consumption, feed have to be checked for the presence of toxicogenic molds and Mycotoxins. Isolates able to produce Mycotoxin were given in Table 4.

Table 4: Analysis of toxin production by fungal isolates with different detection techniques

Isolates	Cam	Ammonium Test	Yes +B Cyd
Mucor spp (M_1,M_2)	–	–	–
Rhizopus spp (R_1,R_2)	–	–	–
Aspergillus niger(A_1,A_2,A_4)	+	+	+
Aspergillus flavus(Afl_1,Afl_2)	–	–	+
Aspergillus fumigatus(AF_1)	+	+	+
Aspergillus fumigatus(AF_2)	+		+
Penicillium spp (P_1,P_3,P_6)	+	+	+
Fusarium spp (F_1)	–	–	+
Aspergillus niger(A_3,A_5)	–	–	–
Penicillium spp (P_2,P_4,P_5,P_7,P_8)	–	–	–

Out of five isolates of *Aspergillus niger,* three were found toxigenic capable of producing Mycotoxin. Three isolates of *Penicillium* spp and one *Aspergillus fumigatus* (AF_1) also showed Mycotoxin production. Whereas the Mycotoxin production of *Aspergillus fumigatus* (AF_2 and AF_1) and *Fusarium* spp have to be confirmed by further detection methods like TLC.

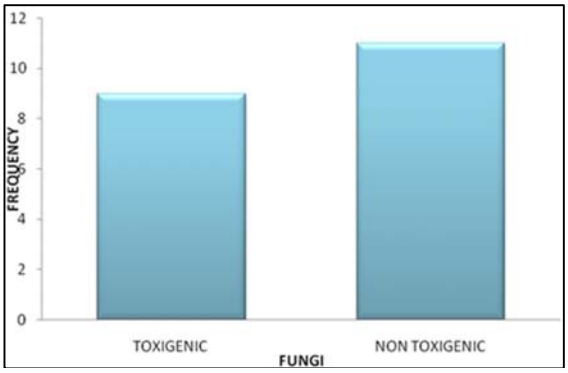


Fig 4: The frequency of toxigenic and non-toxigenic fungi from feeds

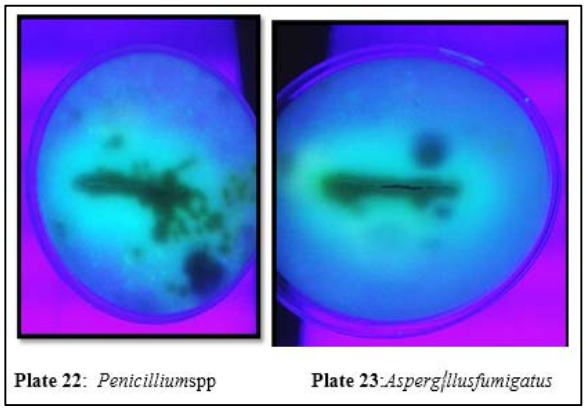


Fig 5: Mycotoxin Detection on Coconut Agar Medium

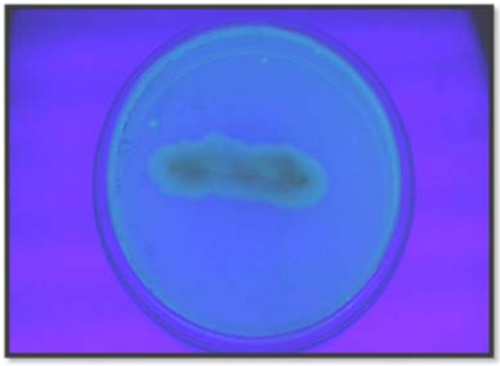


Plate 24: *Aspergillus niger*

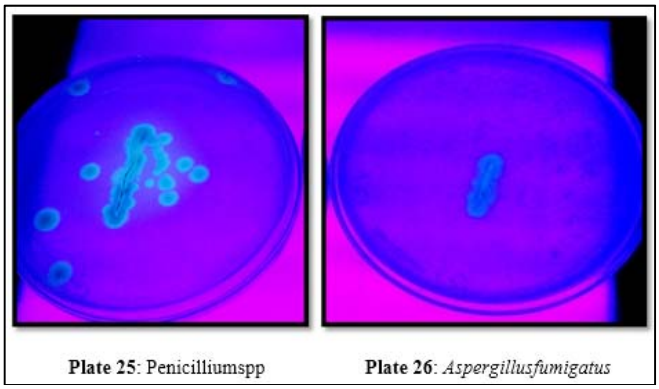


Fig 6: Mycotoxin Detection on Yeast Extract Sucrose Agar

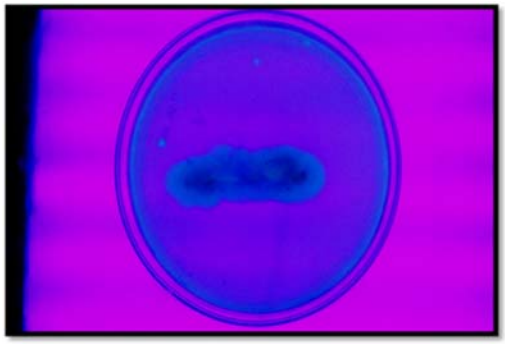


Plate 27: *Aspergillus niger*

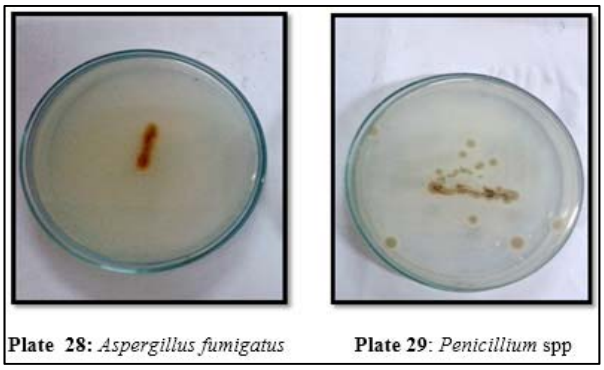


Plate 28: *Aspergillus fumigatus* **Plate 29**: *Penicillium* spp

Fig 7: Mycotoxin Detection By Ammonium Vapour Test

Plate 30: *Aspergillus niger*

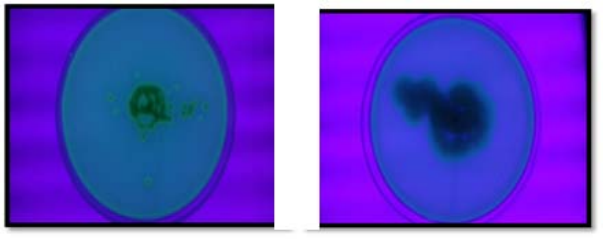


Plate 31: Aspergillusflavus **Plate 32**: *Aspergillusfumigatus*

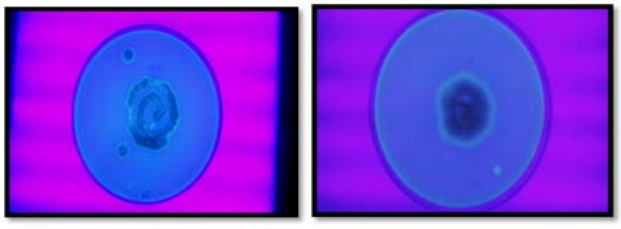


Plate 33: Penicilliumspp **Plate 34**: *Fusarium*spp

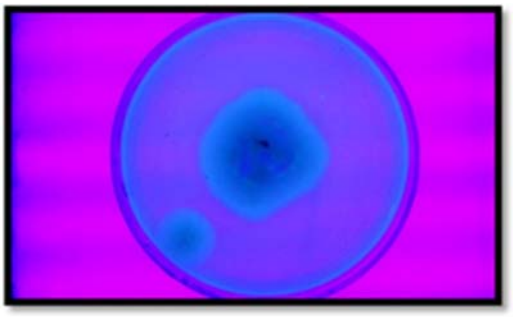


Plate 35: *Aspergillus niger*

Discussion

- Feeds and feedstuffs are excellent media for the growth of fungi and so, a very high standard of hygiene is necessary to avoid feed contamination.
- In the study area, fungal contamination was present in a high proportion of the feed samples (78.5%) (Table 1). Similar findings were also reported from many parts of the world (Saleemi *et al.,* 2010) [11].
- Different genera of contaminating fungi in the present study ranked according to their isolation frequency were *Penicillium* spp (32%) followed by *Aspergillus niger* (20%), *Aspergillus flavus* (8%), *Aspergillus fumigatus* (8%), *Mucor* spp (8%) and *Rhizopus* spp (8%) and the least *Fusarium* spp (4%). This finding is in agreement with that of Pacin *et al.*, (2003).
- The high occurrence of fungal species of public health concern may indicate obvious health hazard in terms of direct consumption of fungal contaminated feed or their toxins by farmed animal and subsequent public health problem.
- Due to this fact, regular microbiological and also mycotoxicological analysis should be necessary for determination of quality and safety of livestock feeds.

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