**INTRODUCTION**

Small ruminants (sheep and goats) play a predominant role in the economy of million people (Thornton, 2010). Diarrhea is a common symptom in goats and sheep. It is caused by enteritis, an inflammation of the intestinal mucosa, characterized by abdominal pain, loose stools, increased stool mass and frequency of defecation, or stool fluidity (dehydration) containing 70-95 percent water, the chronic form of diarrhea may last for days or weeks and may culminate in death (Radostits et al., 2000). Fungal infections can occur in healthy animals but are more common as opportunistic infections in debilitated and immunocompromised hosts whose normal defense mechanisms are impaired. A fatal outcome is possible in these individuals, as fungal infection may remain undiagnosed (Randhawa, 2000).

Traditional diagnostic procedures for fungi are culture and galactomannan assays but they are not advised since they lack sensitivity and specificity, as well as being time-consuming and labor-intensive (Denning, 1998 and Latge, 1999). Real-time PCR has recently been characterized as a rapid, accurate, and sensitive analytical approach for identifying and quantifying mould, even down to the species level (Costa et al., 2002). In order to diagnose invasive aspergillosis in immune-compromised patients, a PCR assay was devised. The entire nucleotide sequences of the genes encoding the 18S rRNA of *A. nidulans*, *A. terreus*, *A. niger*, and *A. flavus* were elucidated and aligned to *A. fumigatus* and other clinically important prokaryotic and eukaryotic microorganisms Melchers et al. (1994).

Sardinas et al. (2011) found that a QPCR assay could identify spore quantities equivalent to or greater than 10⁶ spores/g in samples without prior incubation. Fungal pathogens can be detected through sequence analysis of internal transcribed spacer n/5.8S ribosomal DNA (rDNA) Consuelo et al., (2001).

Rapid yeast identification gives timely information for patient care, allowing for successful and early antifungal treatment. Traditional methods that rely on presumptive pathogen cultivation take a long time and need a lot of effort. The polymerase chain reaction is a modern, quick, and specific approach for detecting pathogenic yeast (Kurzai et al., 1999). In recent years, PCR-based approaches for detecting ribosomal RNA genes have become popular, and they are quite straightforward to use. Restriction analysis of variable internal transcribed spacer (ITS) sequences framing the more conservative 5.8S rRNA gene (rDNA) has proven to be the most useful, allowing for both species identification and isolate typing (Fernández et al., 1999). This method, which is based on a large database, has been presented for the rapid and routine detection of yeasts (Esteve-Zarzoso et al., 1999).
The current study was carried out to investigate most predominant fungi causing diarrhea in sheep and goats with special focus on molecular identification using PCR technique.

2. MATERIAL AND METHODS

2.1. Collection of samples:
Aggregate of one hundred (100) fecal swabs were gathered in sterile tubes; from 70 sheep and 30 goats (from different ages, sexes and/or breeds) experiencing diarrhea at Fayoum Governorate, Egypt. Buccal, skin and nasal samples were gotten from 40 contact workers as indicated by Axell et al., 1985 and Polzehl et al., 2005. A total number of 60 feed stuffs of the herd (Maize, Hay, Beet roots, Beet leaves and Grasses) were gathered in sterile, clean and dry plastic bags for mycological assessment (ISO 21527-2, 2008). The samples were inoculated onto sterile test tubes having 10 – 15 ml sterile saline. The samples were transported to the lab of Animal Health Research Institute, Dokki, Giza, Egypt under complete aseptic conditions.

2.2. Isolation of fungi:
Mycological examination was performed according to Cruickshank et al. (1975). The collected samples were inoculated into Sabouraud’s dextrose broth tubes (SDB) for 24-48 hours, and afterward moved to duplicate plates of Sabouraud’s dextrose agar (SDA) with chloramphenicol (50 mg/mL); to stay away from bacterial staining; and incubated at 37 °C for 2 days (for yeast isolation) and the other plates were incubated at 25°C for 5-7 days (for mould isolation). Negative plates were kept for at least two weeks before being discarded (Feingold & Baron, 1986). All positive mould cultures had their gross and microscopic morphological features assessed (Collins & Lyne, 1984).

2.3. Identification of the isolated fungi:
The morphological examination of the growing colonies as cultures growth appearance, rate of growth, color and texture of the colonies surface and reverse side were recorded as described previously (Lodder, 1970, Al- Dorry, 1980 and Finegold & Martin, 1982). Preliminary recognition was carried out using wet mount preparation by taking a small part of fungal colony between a glass slide with distilled water drop, then teased a part with two needles, then covered with a cover slide and examined microscopically. Cellophane tape technique was also used to identify fungi by removing a little piece of a young colony’s periphery and putting it on a clean glass slide with a drop of lactophenol cotton blue stain on it and microscopically examining it.

2.4. Genotypic identification of fungi:
DNA extraction and purification:
DNA extraction was carried out using Qiagen extraction kit (Qiagen, Hilden, Germany) following the manufacturer’s guidelines.
DNA samples were evaluated in 50 µl reaction volume in a 0.2 ml. eppendorf tube, containing 25 µl PCR Master Mix, 1 µl of each primer, 3 µl target DNA, completed to a final volume of 50µl with sterile PCR water. DNA samples were evaluated in 50 µl reaction volume in a 0.2 ml. Eppendorf tube, containing 25 PCR Master Mix, 2 µl of each primer, 5 µl target DNA, completed to a final volume of 50 µl with sterile free DNase, RNase water.
For the amplification of the partial internal transcribed spacer (ITS) region, specific nested PCR primer pairs (Table 1) were utilized as described by Sugita et al. (2004) and Alcaino et al. (2008). The primer pairs amplify a 305 bp fragments in A. flavus and 500 bp fragments in Rhodotorula sp. The PCR mixture contained 25 µl of 2×Master mix (thermoscientific), 15 pmol of each primer, 3 µl of DNA template solution, and enough water up to the total reaction volume of 50µl. PCR was performed with the cycle condition Table (2) by a thermal cycler (Bio-RAD –S-1000).
One microliter of 1/100 diluted of the first PCR product was used as a template for the second (nested) PCR.
PCR products were analyzed for the presence of specific fragments of the expected length in a 1.5% agarose gel electrophoresis stained with ethidium bromide.

Table 1 primers used in PCR reactions

<table>
<thead>
<tr>
<th>Template</th>
<th>Primer pair</th>
<th>Sequence (5′→3′)</th>
<th>Annealing</th>
<th>Extension</th>
<th>Denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. sp.</td>
<td>R1-R2</td>
<td>CARACTGGKACDGCHGARGATT</td>
<td>55°C for 30′</td>
<td>72°C for 5 minutes</td>
<td>94°C for 2 minutes</td>
</tr>
<tr>
<td>R. sp.</td>
<td>F1-R2</td>
<td>CAGCGAGTACATCACCTTGG</td>
<td>55°C for 30′</td>
<td>72°C for 5 minutes</td>
<td>94°C for 2 minutes</td>
</tr>
</tbody>
</table>

Table 2 PCR protocol for amplification conditions of PCR products

<table>
<thead>
<tr>
<th>Amplified DNA</th>
<th>Initial denaturation</th>
<th>Actual cycles</th>
<th>Final denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus</td>
<td>95°C for 1 minute</td>
<td>25 cycles of:</td>
<td>72°C for 1 minute</td>
</tr>
<tr>
<td>R. sp.</td>
<td>95°C for 1 minute</td>
<td>10 cycles of:</td>
<td>72°C for 3 minutes</td>
</tr>
</tbody>
</table>

3. RESULTS
In the current study, mycological analysis of the samples from diarrheic animals, contact workers and feed stuffs revealed the isolation of 27 fungal isolates out of 200 that were positive for yeast in a total percentage of (13.5%) and 112 fungal isolates out of 200 that were positive for mould in a total percentage of (56.00%) as shown in Table 3.
As shown in Table 4, based on the mycological culture shape; Rhodotorula sp. was the most commonly isolated yeast from all diarrheic animal faeces (10.00 %), followed by C. pseudotropicalis (4.00 %), and C. tropicalis and Torulopsis were the least isolated yeasts (2.00 % for each). From all faecal samples, Geotrichum candidum and Saccharomyces were identified at the same rate (3.00 % for each). However, A. flavus was the most common mould identified from the same faecal samples, accounting for 26.00 %, followed by A. niger (17.00 %), and A. fischeri, A. Carbonarius, Penicillium expansum and Fusarium chlamydosporum, each accounting for 1.00 %; as shown in Table 5.
Rhodotorula sp. was identified on SDA by the growth of carotenoid colors that ranged from orange to red (light pink flat colonies); nevertheless, when stained with Gram’s stain, microscopically, it displayed budding of round, oval giantic cells (Fig. 1). Aspergillus flavus (A. flavus) appeared smooth with various aerial developments; the shading progressed by maturing gigantic cells (Fig. 1). The PCR assay was highly specific and sensitive for the detection of A. flavus, it showed clear bands at 305 bp molecular weight (Fig. 3). While Rhodotorula sp. strains showed the bands at 560bp molecular weight (Fig. 4).
4. DISCUSSION

Fungi are one of the most common cause of diarrhea in sheep and goats; they may go unnoticed, causing economic losses and perhaps having zoonotic potential. Despite the numerous difficulties caused by fungal diseases in sheep and goats, little studies have been under-taken on them. In Egypt, sheep and goat are important production sources of meat and milk (Ayoub et al. 2020). Diarrhea in goat and sheep is very common and its chronic form may lead to death (Shabana et al., 2017). The accurate diagnosis of fungal diseases causing diarrhea is a priority. Results of the current study revealed 27, 112 yeast and mould isolates in a total percentage of (13.5% & 56.00%), respectively from the mycological examinations of 200 samples from diarrheic animals, contact workers and feed stuffs. Mould infections were more common (56 %) than yeasts infections in all analyzed samples (animals, personnel, stuffs. Mould infections were more common (56 %) than yeasts infections in all analyzed samples (animals, personnel, feed stuffs).

Table 4 Incidence of Salmonella species in the examined samples

<table>
<thead>
<tr>
<th>Animals</th>
<th>Sheep</th>
<th>Goats</th>
<th>Workers</th>
<th>Feed stuffs</th>
<th>Bees</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ewe</td>
<td>Rams</td>
<td>lambs</td>
<td>She goat</td>
<td>Male goat</td>
</tr>
<tr>
<td>Samples</td>
<td>40</td>
<td>20</td>
<td>10</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>Rhodotorula sp.</td>
<td>2</td>
<td>5 %</td>
<td>-</td>
<td>0 %</td>
<td>1</td>
</tr>
<tr>
<td>C. pseudotropicalis</td>
<td>-</td>
<td>0 %</td>
<td>1</td>
<td>5 %</td>
<td>2</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>1</td>
<td>2.5 %</td>
<td>-</td>
<td>0 %</td>
<td>-</td>
</tr>
<tr>
<td>Torulopsis</td>
<td>-</td>
<td>0 %</td>
<td>-</td>
<td>0 %</td>
<td>2</td>
</tr>
<tr>
<td>Geotrichum candidum</td>
<td>-</td>
<td>0 %</td>
<td>-</td>
<td>0 %</td>
<td>2</td>
</tr>
<tr>
<td>Saccharomyces</td>
<td>1</td>
<td>2.5 %</td>
<td>-</td>
<td>0 %</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5 Incidence of mold isolated from diarrheic animals

<table>
<thead>
<tr>
<th>Animals</th>
<th>Sheep</th>
<th>Goats</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ewe</td>
<td>40</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>A. flavus</td>
<td>11</td>
<td>27.5 %</td>
<td>4</td>
</tr>
<tr>
<td>A. niger</td>
<td>6</td>
<td>15 %</td>
<td>7</td>
</tr>
<tr>
<td>A. fischeri</td>
<td>1</td>
<td>2.5 %</td>
<td>-</td>
</tr>
<tr>
<td>A. carbonarius</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Penicillium expansum</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fusarium chlamydosporum</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>A. terreus</td>
<td>1</td>
<td>2.5 %</td>
<td>3</td>
</tr>
<tr>
<td>Rhizopus sp.</td>
<td>4</td>
<td>10 %</td>
<td>2</td>
</tr>
<tr>
<td>Mucor sp.</td>
<td>5</td>
<td>12.5 %</td>
<td>1</td>
</tr>
</tbody>
</table>

40 (2) : 78-83
mould and yeast isolated from the diarrheic animals, contact workers and feed stuffs. PCR assays were used to molecularly identify A. flavus and rhodotorula sp. They showed clear bands at 305 bp molecular weight for A. flavus and 560bp for rhodotorula sp. PCR is a valuable tool for direct and rapid diagnosis of fungi associated with diarrhea in sheep and goats.

Figure 1 (A): Rhodotorula species on SDA produce carotenoid pigment. (B): Rhodotorula species showing round to ovoid cells, single or in clusters, stained by Gram’s stain.

Figure 2 (A): A colony of A. flavus on SDA at 25 ºC, one week old. (B): Typical head of A. flavus, stained by lactophenol cotton stain (40X).

Figure 3 Ethidium bromide-stained Agarose gel electrophoresis for PCR products representing amplification of 300 bp of inter-transcribed spacer (ITS) gene in Aspergillus flavus. Lane 1: 100 bp DNA ladder, Lane 2: positive control, Lane 3: negative control. Lane 4-8: s isolates

5. CONCLUSION
According to the current investigation, it could be concluded that A. flavus and rhodotorula sp. were the most prevalent

6. REFERENCES


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