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Mycological and Molecular detection of some fungi causing diarrhea in sheep and goats

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ABSTRACT

Sheep and goat are important production sources of meat and milk in Egypt. Diarrhea is very common and the chronic form of it may lead to deaths, so the accurate diagnosis of fungal diseases causing diarrhea is a priority. In the current study, the fungal infections causing diarrhea in sheep and goats were investigated. Out of 200 examined samples; 27 yeast isolates (13.5%) and 112 mold isolates (56.00%) were recovered from the mycological examinations of the collected samples from diarrheic animals, contact workers and feed stuffs. Asperigillus flavus and Rhodotorula *sp.* were the most prevalent mould and yeast isolated from the examined samples, respectively. Asperigillus flavus and *Rhodotorula sp*. were molecularly identified using PCR tests. They showed clear bands at 305 bp molecular weight for *A. flavus* and 560bp for *Rhodotorula sp*. PCR is a useful method for diagnosing fungi that cause diarrhoea in sheep and goats in a direct and timely manner.

1. 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).

Sardińas *et al.* (2011) found that a QPCR assay could identify spore quantities equivalent to or greater than 10^6 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).

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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 Axéll *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 Sabaroud's dextrose agar (SDA) with chloramphenicol (50 mg/mL); to stay away from bacterial stainting; and incubated at 37 ^{0}C for 2 days (for yeast isolation) and the other plates were incubated at 25^{0}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 micro 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 to 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 50µ 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 Alcaíno *et al.* (2008). The primer pairs amplify a 305 bp fragments in *A. flavus* and 560 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

Mould species	Primer pairs	Sequence (5′→3′)	Amplicon size (bp)	References
A. flavus	F-1st nested-PCR	CAGCGAGTACATCACCTTGG		Sugita *et al.*, (2004)
	R-1st nested-PCR	CCATTGTTGAAAGTTTTAACTGATT		
	F-2nd nested-PCR	ACTACCGATTGAATGGCTCG	305 bp	
	R-2nd nested-PCR	TTCACTAGATCAGACAGAGT		
Rhodotorula sp.	crtR –F	CARACTGGKACDGCHGARGATT	560 bp	Alcaíno *et al.*, (2008)
	crtR –R	WGGDCCRATCATGAYRACTGG		

Table 2 PCR protocol for amplification conditions of PCR products

Amplified DNA	Initial denaturation	Actual cycles ˚C/second	Final extension
A. flavus 1st nested PCR	95°C for 5minutes	30 cycles of : Denaturation: 94˚45 Annealing: 60/60 Extension: 72/60	72˚C for 5 minutes
A. flavus 2nd nested PCR	95°C for 5minutes	25 cycles of : Denaturation: 94˚50 Annealing: 58/40 Extension: 72/45	72˚C for 8minutes
Rhodotorula sp.	95°C for 3 minutes	35 cycles of : Denaturation: 94/30 Annealing: 55/30 Extension: 72/180	72˚C for 10 minutes

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 gigantic cells (Fig. 1).

Aspergillus flavus (A. flavus) appeared smooth with various aerial developments; the shading progressed by maturing from yellow to yellowish green (Fig. 2).

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).

Table 3 Incidence of fungi isolated from all examined samples.

Type of sample	Animals						Workers			Feed stuffs					Total number in this study
	Sheep			Goats								Beet			
	ewe	ram	lamb	She	Male	kids	Buccal	Nasal	Hand	Maize	Hay	Roots	Leaves	Grasses	
Number of samples	40	20	10	16	9	5	16	12	12	28	14	4	6	8	200
yeast isolates	4	1	8	5	3	3	3	0	0	0	0	0	0	0	27
Total yeast isolates		13			11			3				0			13.5 %
Mould isolates	28	19	7	9	5	2	6	6	2	14	6	2	4	2	112
Total mould isolates		54			16			14				28			56 %

Table 4 Incidence of Salmonella species in the examined samples

	Animals													
	Sheep						goats						Total	
	Ewe		Rams		lambs		She goat		Male goat		kids			
Samples	40		20		10		16		9		5		100	
Rhodotorula sp.	2	5 %	-	0 %	1	100 %	3	18.75 %	2	22.22 %	2	40 %	10	10 %
C. pseudotropicalis	-	0 %	1	5 %	2	200 %	1	6.25 %	-	0 %	-	0 %	4	4 %
C. tropicalis	1	2.5 %	-	0 %	-	0 %	-	0 %	1	11.11 %	-	0 %	2	2 %
Torulopsis	-	0 %	-	0 %	2	200 %	-	0 %	-	0 %	-	0 %	2	2 %
Geotrichum candidum	-	0 %	-	0 %	2	200 %	-	0 %	-	0 %	1	20 %	3	3 %
Saccharomyces	1	2.5 %	-	0 %	1	100 %	1	6.25 %	-	0 %	-	0 %	3	3 %

Table 5 Incidence of mold isolated from diarrheic animals

	Animals												Total	
	Sheep						Goats							
	Ewe		Rams		Lambs		She goat		Male goat		kids		Total	
	40		20		10		16		9		5		100	
A. flavus	11	27.5 %	4	20 %	4	40 %	4	25%	2	22.22%	1	20%	26	26.00%
A. niger	6	15 %	7	35 %	-	-	3	18.75 %	1	11.11%	-	-	17	17.00
A. fischeri	1	2.5 %	-	-	-	-	-	-	-	-	-	-	1	1 %
A.carbonarius	-	-	1	5 %	-	-	-	-	-	-	-	-	1	1 %
Penicillium expansum	-	-	-	-	1	10.00 %	-		-	-	-	-	1	1 %
Fusarium chlamydosporum	-	-	1	5	-	-	-		-	-	-	-	1	1 %
A. terrus	1	2.5 %	3	15 %	1	10 %	-	-	1	11.11%	-	-	6	6 %
Rhizopus sp.	4	10 %	2	10 %	1	10 %	1	6.25 %	1	11.11%	-	-	9	9 %
Mucor sp.	5	12.5%	1	5 %	-	-	1	6.25 %	-	-	1	20%	8	8 %

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 yeast infections in all analyzed samples (animals, personnel,

and feed stuffs) (13.5 %). This may be due to that the examined feed stuffs as well as the nasal and hand samples of the contact workers were completely free from yeast infections. The high recurrence of mould isolates concurs with Refai *et al.* (2010). Rhodotorula sp. and *A. flavus* were the predominant yeast and mould isolated from the samples, respectively. These findings are nearly identical to those of Jensen *et al.* (1992) and Sarfati *et al.* (1996), who stated that aspergillosis, candidiasis, and zygomycoses were the most common mycotic infections in ruminants. The respiratory and gastrointestinal tracts are portals for mycotic infections, according to Jensen *et al.* (1992), and the omasum is the main organ for infection. *Candida sp.* can induce gastro-intestinal candidiasis in animals, according to Refai *et al.* (2010). Hassan *et al.* (2010) discovered that yeasts isolated from sheep and goats with diarrhoea had higher rates than yeasts obtained from apparently healthy animals. Radostits *et al.*, (2000) declared that diarrhoea in adult ruminants was mostly associated with mycotic omasitis, rumenitis and enteritis. Donskey (2004) demonstrated that yeasts can infect animals' digestive tracts by exposing them to contaminated soils containing yeast pathogens and licking the perianal region of animals infected with anal candidiasis by other healthy animals, resulting in oral candidiasis and, eventually, gastrointestinal tract candidiasis. According to Tell (2005), asperigillus sp. causes gastroenteritis in ruminants. According to Abou-Elmagd *et al.* (2011), faeces from sheep and other ruminants contain more yeast than milk and rectal swabs, implying that the GIT serves as a significant reservoir for candida species.

Fungi are opportunistic pathogens with a variety of virulence characteristics that allow them to grow and establish infection within the host. Proteases (Hube, 1998) and phospholipases (Ibrahim *et al.*, 1995) are secreted by pathogenic fungi, and this could explain the diarrhoea of pathogenic fungal-infected animals.

As shown in Fig. 1, rhodotorula sp. was identified on SDA by the growth of carotenoid colors; however, it displayed budding of round, oval gigantic cells when stained with Gram's stain. This result is in accordance with some authors (Refai *et al.*, 2010). On the other side, *A. flavus* seemed smooth with various aerial developments; the shading changes from yellow to yellowish green by maturing (Fig. 2). These outcomes concurred for certain reports (Refai *et al.*, 2010 and Abd El-Tawab *et al.*, 2020).

Various PCR methods have been utilized for detection of aspergillus DNA in clinical specimens over the years, including conventional PCR, competitive PCR, nested PCR, quantitative PCR (qPCR), real-time qPCR, PCR-enzyme linked immune sorbent assay (PCR-ELISA) and real-time PCR (Hadrich *et al.*, 2011). *A. flavus* showed clear bands at 305 bp molecular weight and rhodotorula sp. showed the bands at 560bp molecular weight. In previous research, PCR analysis has been developed for the detection of aspergillus in a wide variety of clinical samples such as bronchoalveolar lavage fluid (White *et al.*, 2010), soil (Hong *et* al., 2010) and indoor environment (Udagawa *et al.*, 1996) also is providing a good alternative to the time-consuming isolation test normally used in laboratory routine.

5. CONCLUSION

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

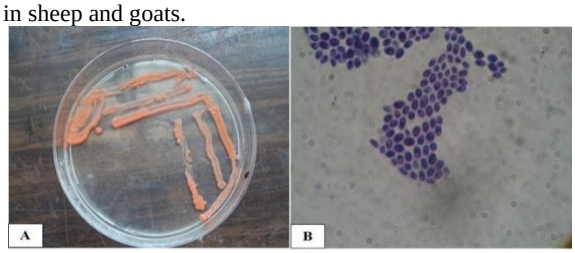

Figure 1 (**A):** *Rhodotorula* species on SDA produce cartenoid pigment. **(B):** *Rhodotorula* species showing round to ovoid cells, single or in clusters, stained by Gram´s stain.

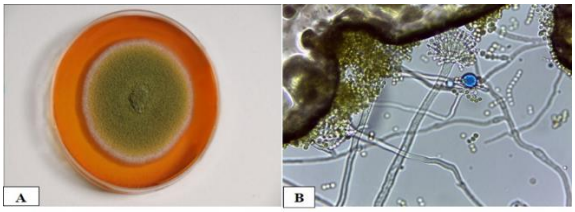

Figure 2 (A): A colony of *A. flavus* on SDA at 25 ºC, one week old.
(B): Typical head of *A. flavus*, stained by lactophenol cotton stain (40X).

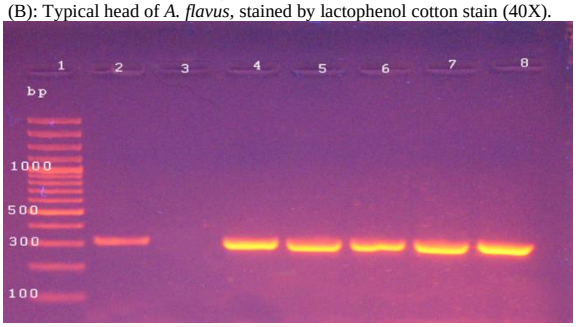

Figure 3 Agarose gel electrophoresis for PCR products representing amplification of 300 bp of inter-transcribed spacer (ITS) gene in *Asperigillus flavus.* Lane 1: 100 bp DNA ladder, Lane2: positive control, Lane 3: negative control, Lane 4-8: s isolates

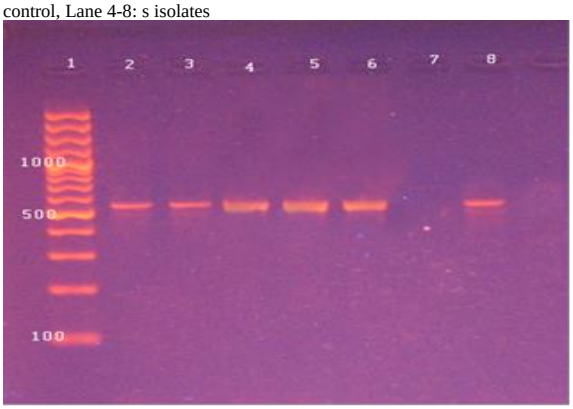

Figure 4 Ethidium bromide-stained Agarose gel electrophoresis for PCR assay of *Rhodotorula sp.* strains. The position corresponding to crtR PCR amplicons was at 560bp. Lane 1: 100 bp DNA ladder, Lane2 -6 isolates, Lane 7: negative control, Lane 8: positive control.

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