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Mycological Identification of Some Fungi Isolated from Meat Products and Spices with Molecular Identification of Some *Penicillium* Isolates

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Abstract | This study was done to determine the fungal quality of meats products (luncheon, basterma, kofta and burger) and spices (used in meat processing) marketed in some groceries and supermarkets in Gharbia Governorate under various trade names and molecular (ITS) characterization for some *Penicillium* species. In 120 tested meat products and 33 spices samples, 9 mould genera were detected. The identified mould genera were *Aspergillus, Penicillium, Acremonium, Cladosporium, Geotrichum, Mucor, Claveolaria, Emericella Nudulans* and *Scorulopsis*. The main identified Penicillium species were; *P. citrinum, P. aurantigreum P. chrysogenum* were the most predominant species isolated from burger samples, while from kofta samples, *P. Paxilli and P. restrictum* were the most predominant isolated species. *P. citreognigrum* and *P. carneum* only detected in luncheon and basterma samples. In spices the main identified Penicillium species were; *P. aurantigreum, P. carneum, P. chrysogenum, P. citreognigrum, P. citrinum, P. crustosum, P. Paxilli, P. restrictum, P. implicatum* and *P.simplicissimum*. Three *Penicillium* isolates (*P. chrysogenum*, *P. carneum* and *P. restrictum*) from meat products and one *Penicillium* isolate (*P. crustosum*) from spices were subjected to PCR identification, and were sequenced in both directions. The obtained sequences were deposited under (Gene bank accession number: MK643347 *P. restrictum* isolate AYMA4, MK643348 *P. chrysogenum* isolate AYMA5, MK643349 *P. carneum* isolate AYMA6 and MK643350 *P. crustosum* isolate AYMA7. Sequences of *Penicillium* isolates were analysed with phylogenetic tree and percent identity. Phylogenetic analysis of *Penicillium* isolates using ITS sequence data grouped them into four clusters. PCR and gene sequence are easy and fast diagnostic methods. As conclusion meat products and spices highly contaminated with mould especially *Aspergillus* spp and *Penicillium* spp which may gain access during the manufacturing process leading to high economic losses and have a public health hazard due to the production of mycotoxins.

Keywords | Meat products, Spices, Moulds, *Penicillium*, PCR

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INTRODUCTION

Meat products are the most palatable of highly nutritive value foods for human being as they are important sources for protein, fat, essential amino acids, minerals, vitamins and other nutrients (Biesalski, 2005). On the other hand, they are considered as an ideal culture medium for growth of many organisms because of the high moisture, the high percentage of nitrogenous compounds, plentiful supply of minerals, some fermentable carbohydrates (glycogen) and of a favorable pH for most microorganisms (Alahakoon et al., 2015).

Moulds are common inhabitants of soil, water and may be dispersed through the air and water and by the activities of small animals, particularly insects (Rawat, 2015). Mould gaining access into meat product from meat, spices, and other ingredients, from environment, equipment, and handlers during processing affect the status of the products (Morshdy et al., 2015). Contamination of meat products

with different mould species considers a real hazard as it affecting the quality of these meat products by increasing the opportunity for its spoilage and deterioration (Alcaide-Molina et al., 2009).

The increase in the production of processed foods and high demand for meat is the major reason behind the rapid increase in spices consumption (Little et al., 2003). Spices are largely produced in countries where tropical climates are favorable to mycotoxin contamination. Furthermore, they are usually dried on the ground in the open air in poor hygienic conditions that even more promote growth of mold and production of mycotoxin (Martins et al., 2001).

The growth of some mould species is dangerous, because they may be produced mycotoxins (Empting, 2009). Mycotoxin; is one of the problems threatening human and animal health in food industry (Kaynarca et al., 2019). Studies have discussed their toxigenic, nephrotoxic, hepatotoxic, carcinogenic, immunosuppressive and mutagenic characteristics of mycotoxins (Da Rocha et al., 2014).

Molecular approaches have been applied as an alternative assay replacing cumbersome and time-consuming microbiological and chemical methods for the detection and identification of mould (Niessen, 2008). PCR-based methods for simultaneous detection of mould are useful tools to be used in food safety programs. These rapid and sensitive techniques allow taking corrective actions during food processing or storage for avoiding accumulation of mycotoxins in them (Rodríguez et al., 2017). Internal transcribed region of the DNA (ITS) are the best tool for the identification of fungi (Mirhendi et al., 2007). Therefore, this study was carried out to throw light on the mycological aspects of some meat products and spices with molecular characterisation of some *Penicillium* isolates existing in such products.

MATERIALS AND METHODS

Isolation and Identification of mould

Twenty five grams from each sample of 120 meat products and 33 spices (collected from supermarkets in Gharbia governorate, Egypt) were carefully and aseptically homogenized in a blender with 225 ml of sterile peptone water 0.1% to form a dilution of 1:10, from each tenth fold serial dilutions were accomplished up to 10^6. One ml of the previously prepared serial dilution was separately poured into duplicated Petri dishes carefully and mixed with Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Oxoid) then incubated at 25^0C for 5-7 days. The isolated colonies were picked up from the DRBC agar and cultured into slope Sabauroud dextrose agar (SDA) (Oxoid) and incubated at 25^0c for 5-7 days after then transferred to Malt Extract Agar (MEA) (Oxoid) and Czapek yeast agar (CYA) (Oxoid) plates for identification. Colony appearance, exudate production, pigmentation and reverse coloration were assessed and colony diameters were measured and recorded (Pitt and Hocking, 2009).

PCR amplification and sequencing

DNA extraction from four *penicillium* isolates using DNeasy plant Mini kit Qiagen Genomic as described by manufacturer manual of Qiagen, Germany. Cat. No. 69104. DNA samples were tested in 50 µl reaction volume in a 0.2 ml eppendorf tube, containing 25 µl PCR Masster Mix, 1 µl of each primer, 3 µl target DNA, complete to a final volume of 50 µl with sterile PCR water. Penicillium isolates were identified via ITS-PCR of rDNA region with ITS15´-TCCGTAGGTGAACCTGCGG-3´ and ITS25´-TCCTCCGCTTATTGATATGC-3´ (560 bp) (White et al., 1990). PCR amplification conditions for four *Penicillium* isolates *were*: 5 min initial denaturation step followed by 35 cycles at 95 °C for 30 sec, 35 cycles at 56^0C for 30 sec and 35 cycles for 72 °C for 1min and a final extension step at 72 °C for 10 min. Amplification products were electrophoresed in agarose gels (1.5% w/v) (Agarose, Sigma, USA) and stained with ethidium bromide using Gene Ruler 100bp DNA Ladder (H3 RTU, Cat. No. DM003-R500 Gene Direx, BIO-HELIX Co., LTD). The amplified fragments were purified using Gene Jet PCR purification kit; Fermentas (Cat no. KO701). Sequencing was performed by sequencing to the PCR product on GATC Company by use ABI 3730x1 DNA sequences by using forward and reverse primers. Sequences were deposited at gene bank and phylogenetic analysis was done by MEGA X (Kumar et al., 2018), while sequence divergence and identity percent by DNA star (Felsenstein, 1985) and (Tamura et al., 2004).

RESULTS

Aspergillus spp. was the most prevalent species in the examined samples. It constituted 24 (88.88%) in luncheon, 36 (85.71%) in kofta, 35 (85.36%) in basterma and 46 (58.23%) in burger followed by *Penicillium* spp. 3 (11.12%), 6 (14.29%), 3 (7.32%) and 12 (15.18%) respectively. *Mucor* spp.3 (7.32%) in basterma, 9 (11.42%) in burger, while *Geotrichum* spp. 6 (7.59%), *Cladosporium* spp, *Acremonium* spp. 3 (3.79%) detected in burger samples only. While in spices, the predominant mould genera isolated were *Aspergillus* spp. 86 (55.48%), *Penicillium* 24 (15.48%), *Mucor* spp 21 (13.55%), *Cladosporium* spp 9 (5.80%), *Acremonium* spp 3 (1.94%) *Scorulopsis* 6 (3.85%), *Claveolaria* 3 (1.94%) and *Emericella.nudulans* 3 (1.94%). Further identification to *Penicillium* isolates was done in meat products, the predominant *Penicillium* species isolated from burger samples were *P. citrinum* 6 (7.59%) followed by *P. aurantigreum* and *P. chrysogenum* 3 (3.795%). Where *P. Paxilli and P. restrictum* were the most isolated *Penicillium* species from kofta samples with incidence rate 3 (7.14%)

Table 1: Incidence of mould isolated from examined meat product samples.

Isolated mould	Luncheon		Kofta		Basterma		Burger		Spices	
	No	%	No	%	No	%	No	%	No	%
Aspergillus spp.	24	88.88	36	85.71	35	85.36	46	58.23	86	55.48
Acremonium spp.	-	-	-	-	-	-	3	3.79	3	1.94
Cladosporium spp.	-	-	-	-	-	-	3	3.79	9	5.80
Geotrichum spp.	-	-	-	-	-	-	6	7.59	-	-
Penicillium spp.										
P. aurantigreum	-	-	-	-	-	-	3	3.795	6	3.87
P. carneum	-	-	-	-	3	7.32	-	-	-	-
P. chrysogenum	-	-	-	-	-	-	3	3.795	3	1.94
P. citreognigrum	3	11.12	-	-	-	-	-	-	-	-
P. citrinum	-	-	-	-	-	-	6	7.59	3	1.94
P. crustosum	-	-	-	-	-	-	-	-	6	3.87
P. Paxilli	-	-	3	7.145	-	-	-	-	-	-
P. restrictum	-	-	3	7.145	-	-	-	-	-	-
P.implicatum	-	-	-	-	-	-	-	-	3	1.94
P.simplicissimum	-	-	-	-	-	-	-	-	3	1.94
Total of *Penicillium* spp	3	11.12	6	14.29	3	7.32	12	15.18	24	15.48
Mucor spp	-	-	-	-	3	7.32	9	11.42	21	13.55
Claveolaria	-	-	-	-	-	-	-	-	3	1.94
Emericella. Nudulans	-	-	-	-	-	-	-	-	3	1.94
Scorulopsis	-	-	-	-	-	-	-	-	6	3.85
Total	27	100	42	100	41	100	79	100	155	100

for both isolates. *P. citreognigrum 3* (11.12%) isolated from luncheon While *P. carneum* 3 (7.32%) from basterma, While *in* spices the isolated *Penicillium* species were 6 (3.87%), 3(1.94%), 3 (1.94%), 6 (3.87%), 3 (1.94%), 3 (1.94%) for *P. aurantigreum, P. chrysogenum, P. citrinum, P. crustosum, P. implicatum, P. simplicssimum,* respectively. (Table 1).

Three *Penicillium* isolates (*P. chrysogenum*, *P. carneum* and *P. restrictum*) from meat products and one *Penicillium* isolate (*P. crustosum*) from spices were positive on agarose gel electrophoresis (Figure 1) and the strains were successfully amplified and sequenced. The obtained sequences were deposited at NCBI under accession No. (Gene bank accession number: MK643347 *P. restrictum* isolate AYMA4, MK643348 *P. chrysogenum* isolate AYMA5, MK643349 *P. carneum* isolate AYMA6 and MK643350 *P. crustosum* isolate AYMA7. *Penicillium* isolates compared with published strains in Gene Bank through phylogenetic tree (Figure 2) and percent identity (Figure 3).

DISCUSSION

The presence of moulds may cause spoilage of meat products by breaking down their components and liberating different acids and gas with subsequent change of their odour and flavor. Some moulds are capable of producing toxic metabolites known as mycotoxins such as aflatoxins which may cause carcinogenic effect (Pitt and Hocking, 2009).

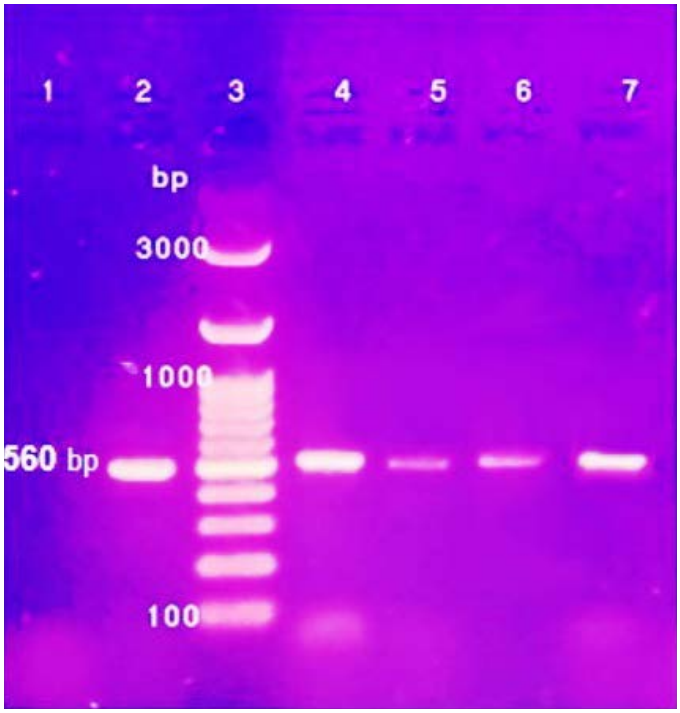

Figure 1: PCR products of ITS1-ITS2 regions from representative clinical isolates of *Penicillin species* Lane: 1 control Negative; Lane 2: control positive; Lane 3: 100 bp DNA ladder; Lane 4: *P. restrictum*; Lane 5: *P. chrysogenum*; Lane 6: *P. carneum* and Lane 7: *P. crustosum*.

In recent years, there has also been a steady increase in the production and consumption of processed meat products worldwide because of their high nutritive value and convenience (Rajic et al., 2007).

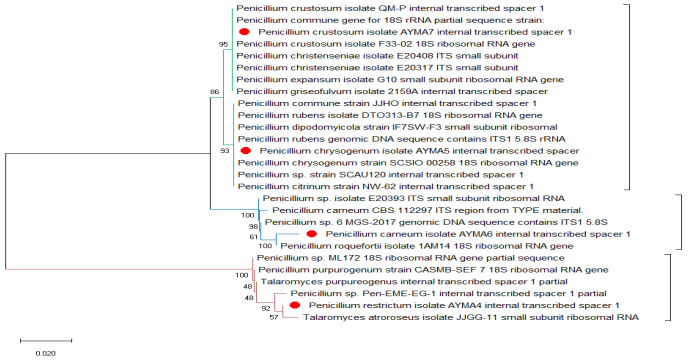

Figure 2: Phylogenetic tree of nucleotide sequences of ITS of different *penicillium* spp.

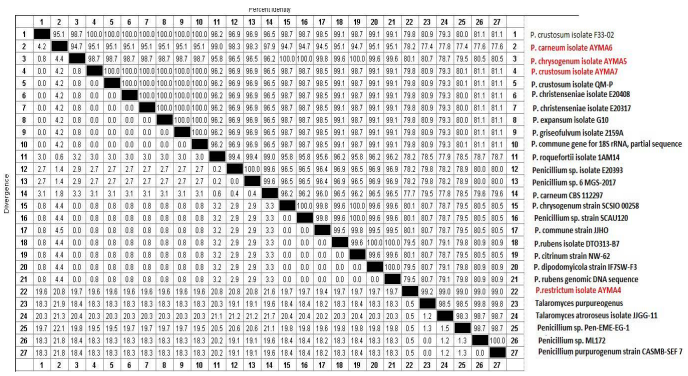

Figure 3: Nucleotides identity of ITS sequences of different *penicillium* spp.

The results of mould incidence in meat products (Table 1) agreed with the results reported by (Shaltout and Salem, 2000; Brr et al., 2004; Zohri et al., 2014; Morshdy et al., 2015) who determined that *Aspergillus* spp. and *Penicillium* spp. were the most predominant mould species isolated from meat products samples. Occurrence of various types of molds in meat products may be attributed to the condition of the environment at which the meat products prepared, beside manufacturing rooms, stores, refrigerators, which are very suitable for the development of mold on meat and inside the meat products.

Spices have been used in many industries. They are commonly heavily contaminated with moulds. The most frequent fungal contaminants of spices are species from the genera *Aspergillus* and *Penicillium*. (Kocic´-Tanackov et al., 2007). Data presented in Table 1 revealed the dominant of *Aspergillus* and *Penicillium* spp. in all examined spices samples was in correct manner with the results of (Mandeel, 2005; Farghaly, 2006; Bokhari, 2007; Hashem and Alamri, 2010) who reported that *Aspergillus* and *Penicillium* spp. were the main components of spices.

The large numbers of species which constitute the genus *Penicillium* occupy a wide spectrum of habitats in our environment. As a consequence, many have become economically important in either harmful or useful roles. Some species cause deterioration of wide range of stored products (Frisvad and Samson, 2004). Some species of genus *Penicillium* might induce pulmonary infection, external otomycosis, mycotic keratitis and endocarditis (Birera et al., 1998).

Identification of *Penicillium* species is not easy. Many common species look like to the uninitiated. At the same time, there is a great deal of variability within the species. In recent years, molecular approaches have been used increasingly in identification and phylogenetic classification of filamentous fungi and their application has led to the reconsideration of several genera (Bruns et al., 1991).

Three *Penicillium* isolates (*P. chrysogenum*, *P. carneum* and *P. restrictum*) from meat products were examined by molecular methods polymerase chain reaction (PCR) with using ITS primer. PCR products of isolated strains were positive on agarose gel electrophoresis of PCR amplification products (Figure 1). Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species by PCR analysis (Martin and Rygiewicz, 2005). In the genus *Penicillium*, the region spanning of the nuclear ribosomal internal transcribed spacer has been investigated to clarify the subdivision within the genus and to evaluate phylogenetic relationship of some species. However, the ribosomal DNA gene has too few informative differences to reveal the phylogeny of *Penicillium* (Samson et al., 2004).

Phylogenetic analysis showed that *P. restrictum* isolate AYMA4 accession no. (MK643347) was isolated but in different branches, away from *P. chrysogenum* isolate AYMA5, *P. carneum* isolate AYMA6, *P. crustosum* isolate AYMA7 but remained in the same cluster. While *P. restrictum* isolate AYMA4 accession no. (MK643347) still in the same group of *Talaromyces atroroseus* isolate JJGG-11 and *Penicillium* sp. Pen-EME-EG-1. While *P. chrysogenum* isolate AYMA5 and *P. crustosum* isolate AYMA7 had common ancestor. *P. chrysogenum* isolate AYMA5 still in the same group of *Penicillium chrysogenum* strain SCSIO 00258 and *Penicillium rubens* isolate DTO313-B7. *P. carneum* isolate AYMA6 was isolated but in different branches, away from *P. chrysogenum* isolate AYMA5 and *P. crustosum* isolate AYMA7 but remained in the same cluster and still in the same group of *Penicillium roquefortii*1AM14 (Figure 2).

The sequence similarity of *P. restrictum* isolate AYMA4 for the strains of *P. chrysogenum* isolate AYMA5, *P. carneum* isolate AYMA6, *P. crustosum* isolate AYMA7, *Talaromyces*

purpureogenus, *Talaromyces atroroseus* isolate JJGG-11 and *Penicillium* sp. Pen-EME-EG-1 were 80.1%, 78.2%, 79.8%, 99.2%, 99% and 99%. while the sequence similarity of *P. chrysogenum* isolate AYMA5 for *P. carneum* isolate AYMA6 and *P. crustosum* isolate AYMA7, *Penicillium chrysogenum* strain SCSIO 00258 and *Penicillium rubens* isolate DTO313-B7 were 94.7%, 98.7%, 100% and 99.6%. On the other hand, the sequence similarity of *P. carneum* isolate AYMA6 for *P. crustosum* isolate AYMA7and *Penicillium roquefortii* isolate 1AM14 were 95.1% and 99%. While the sequence similarity of *P. crustosum* isolate AYMA7 for *Penicillium crustosum* isolate F33-02 18S, *Penicillium christenseniae* isolate E20408, *Penicillium expansum* isolate G10, *Penicillium griseofulvum* isolate 2159A were 100% (Figure 3).

CONCLUSION

Meat products and spices highly contaminated with *Aspergillus* spp. and *Penicillium* spp. which may gain access during the manufacturing process leading to high economic losses and have a public health hazard due to the production of mycotoxins. The result demonstrates the fact that the unhygienic and poor sanitary conditions under which meat products and spices are handled and processed are not acceptable from sanitary point of view. It has further evidence that the undesirable level of contamination which might have acquired from the environment and agents. and to obtain wholesome safe and sound meat products, the principles Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Point (HACCP) must be adopted.

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AUTHORS CONTRIBUTION

All authors contributed equally.

CONFLICT OF INTEREST

No conflict of interest declared.

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