Isolation and molecular detection of the most significant fungi and their mycotoxins genes from infected Chickens in Iraq's Wasit Governorate

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DOI: https://doi.org/10.36077/kjas/2025/v17i2.12394

Received date: 10/6/2023

Accepted date: 2/8/2023

Abstract

Fungi cause economic losses to the poultry industry either due to their direct infectious nature or due to the production of mycotoxins. The aim of the study is to isolate the most important fungi that cause mycotoxin secretion.One hundred samples were collected from poultry farms in Wasit Governorate, Iraq. The samples were taken from 70 sick broiler chickens and 30 newly dead (liver, lung and air sacsfrom each bird) after clinical examination and post-mortem examination. The results of the present study showed the isolation and identification of (26) isolates as *Aspergillus spp.* It was observed that the most frequent mold isolates were *Asperigellius spp.* specifically *Aspergillus flavus* at a percentage of 8 (8%) followed by 5 (5%) for *A.fumigatus* and *A.terrus* while 4(4%) for *A.niger* and 3(3%) for *A.parasiticus* and1(1%) for other *Aspergillus spp*. Also showed the prevalence of mold isolates from different sites of poultry farms as it showed the highest incidence in lung and air sac with 19 (19%) and 15 (15%), respectively followed by liver with 5 (5%).In this study to investigate Gliotoxin gene was detected 4/5 (8%) in *A.fumigatus*, with PCR product of this gene was approximately 512 bp, While it was observed aflatoxin (aflR) gene was found 8/8 (%100) in *A.flavus*, with PCR product of this gene was approximately 588bp. In ours study lipase gene was detected by PCR in 5/5 (100%) samples of *A. terreus*. The PCR amplification of lipase gene was approximately 591bp.Conclusions, It has been concluded that the most common isolate was *A.flavus*and amplifying the ITS1 region of all *Aspergillus* isolates by PCR and the virulence gene responsible for mycotoxins such as gliotoxin and aflatoxin was revealed.

Keywords: Mycotoxins, Aflatoxin, *Aspergillus*, PCR.

Introduction

Mycotoxins are secondary metabolites produced by numerous Fungi, which are toxic to humans, animals and plants. Which causes huge economic losses in animal production (13). Ingestion, inhalation or assimilation through the skin may cause a variety of diseases and even death (9).Mycotoxins may have toxic effects ranging from short-term mucous membrane irritants to immune system suppression and cancer. Almost everything related to diseases caused by mycotoxins has to do with eating contaminated food (15). The main classes of mycotoxins are aflatoxins, which are produced by *A.flavus*and *A. paramiticus* and are important pathogens. Its effects range from acute death to chronic diseases such as tumors (4). Several factors affect the rate of mycotoxin contamination of poultry feed. Fungal species may contaminate poultry feed and its ingredients, and remain inactive until appropriate growth desires are available, such as high humidity and aerobic conditions (5). Under the desired conditions, the contaminated fungi begin to grow and produce various mycotoxins as by-products (10).The incidence of aflatoxins in chick formation results in economic loss through poor growth and feed change, increased mortality, leg effort, and carcass culling. Aflatoxins cause a wide range of metabolic changes and are associated with liver damage, decreased gastrointestinal enzyme activity, and immunosuppressants(20).Numerous reports of poultry feed contamination with Aflatoxin shave emphasize the importance of studies to identify alternatives to prevent fungal poisoning in the poultry industry (18).Therefore, the aims of this study werethe isolation and identification of the most important fungi found in infected chickens and feeding them by molecular diagnostics in Wasit Governorate, Iraq

Material and Methods

Samples collection

One hundred chickens were composed from15 diverse commercial broiler farms (1 - 40 Days) in Wasit Governorate were checked for fungal infection from (July - 2022 to December -2022). Following clinical evaluation and post-mortem inspection, samples were collected from 70 ill broiler chickens and 30 recently dead chickens (liver, lung, and air sacs from each dead bird). All examined organs were kept alone in a sterile condition. Plastic bags were kept in an ice box and transported to the laboratory for fungal examination.

Fungal culture examination

The surface of the organs was burned with a hot spoon, and then a sterile ring was inserted through the burned part of the organs. The rings were inoculated in modified Sabouraud dextrose broth (Oxoid, England) under aseptic conditions and incubated aerobically at 25 °C for 7 days. Two episodes were taken, the first being planned on Sabouraud's dextrose agar (Oxoid, England).

Identification of the isolates

Isolated fungi were identified on the basis of their general morphology and cultural description accordingly (17).

Molecular Detection

Fungal DNA Extraction

Using the fungal/yeast DNA extraction mini-kit(Favorgen, Taiwan)fungalgenomic DNA was isolated from the isolates as follows, according to the manufacturer's instructions.

Primers sequences.

PCR was practical by universal fungus-specific primer pairs using the forward primer (ITS1) and the reverse primer (ITS4) It was used to determine the genetic traits of Aspergillus by amplifyingg,all Aspergillus isolates tested, providing a single PCR product of approximately 600 base pairs.Then design primers genes for virulence factors and those responsible for the secretion of mycotoxins. The sequences of this primer were taken based on the method adopted by Bok *et al.*(7).It is manufactured in Alpha DNA® (Canada) and used in conventional PCR (Table: 1).

PCR amplification of DNA involves three stages with alternating temperatures accordingto White *et al* (22).as shown in (Tables 2 and 3)

Statistical Analysis

By means of SPSS V 25.0 (IBM, USA)for Windows, data were entered and analyzed. Inferential statistics (Chi-Square test) and evocative statistics (frequencies, mean standard deviation and complementary tables and graph) were applied. a Statistically significant 1 P-value ≤ 0

Table 1. PCR detection gene primers with their nucleotide sequence and product size for *Aspergillus spp*.

Fungal isolation	Type of gene		Sequence(5'-3')	PCR product size
Aspergillus spp.	ITS	F	TCCGTAGGTCCTGCHG	600bp
		R	TCCTCCGCTTATTGATATGC	
A.flavus	Aflatoxin)	F	TTGATTCAACTCGGCGACCA	588bp
		R	TCGTCATCAGGTTGCACGAA	
A. fumigates	Gliotoxin	F	GCGACCCTCCGATCTTGTAG	512bp
		R	GTTCCCGGGCAAATGAG	
A. terreus	lipase	F	TGAAGGAGTTTCTCGCCGAC	591bp
		R	CCGCATTCCCCTTTCTCGAT	

Table 2. Standard PCR master mix protocol

Components	Volume/ml
PCRGreenmastermix	12.5µl
Reveresprimer(10pmol)	2µl
Forwardprimer(10pmol)	2µl
PCR water	3.5µl
DNAtemplate	5µl
Totalvolume	25µL

Table 3. PCR thermo-cycler conditions protocol

PCRcycle	**Repeat**	**Temp**	**Time**
Initial denaturation	1	95˚C	5min
Denaturation	34	95˚C	30sec
Annealing		58˚C	30sec
Extension		72˚C	1min
Finalextension	1	72˚C	5min
Hold	-	4˚C	5min

Results

Identification of the fungi

In this study, one hundred samples were collected from poultry farms in Wasit Governorate, Iraq. 39 (39%) samples were positive for the growth of fungi, which were classified into (26) isolates identified as *Aspergillus spp*. and (13)isolates identified as other molds, It was observed the most frequent molds isolates were *Asperigelliusspp*. specifically *A.flavus*at a percentage 8 (8%) followed by 5 (5%) for *A.fumigatus* and *A.terrus while* 4(4%) for *A.niger*and 3(3%) for*A.parasiticus*and1(1%) for *Aspergillus spp*. On the other hand, other fungi were isolated such as 1(1%)for*Penicillumrubrum, Penicillum spp.*, *Alternaria alternate*, *Mucorcircinelloides*, *Mucorspp,Rizopusspp,Cladosporium, Fusariumsolani,Fusarium spp*.respectively and 2(2%) *for Mucorhiemalis* and*Alternerria spp*. respectively.This study showed the prevalence of mold isolates from different sites of poultry farms as it showed the highest incidence in lung

and air sac with 19 (19%) and 15 (15%) respectively followed by liver with 5 (5%) (Table 4). In the present study showed that the number of isolated fungi in infected chickens increases in the first and second week, while the isolation of the fungi decreases in the third, fourth and fifth week (Table 5).

Molecular detection

In this study, PCR was used to confirm filamentous fungi using specific global primer pairs (ITS1 and ITS4) that were used to determine the genetic characteristics of *Aspergillus* species. They were capable ofsuccessfullyintensifyingthe ITS1 region of all *Aspergillus* isolates, given thata single PCR product of ~600 bp.For all isolates tested as described in (Fig. 1).

Detection of Virulence gene to *Aspergillus* spp.

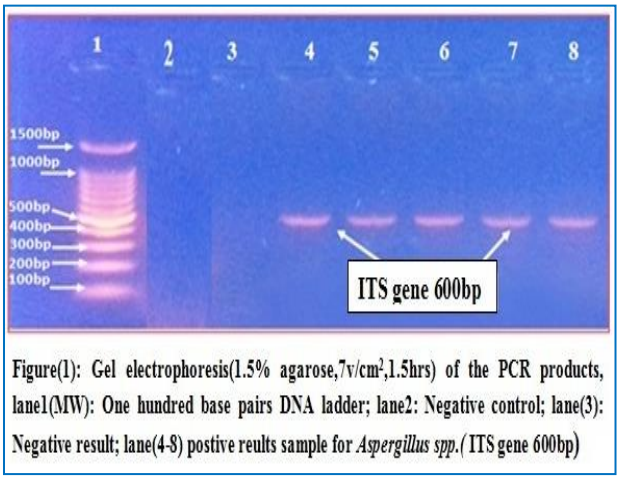
In this study to investigate the Gliotoxin gene was detected 4/5 (8%) in *A.fumigatus*, with PCR product of this gene was roughly512 bp. as shown in (Fig2).While it was observed aflatoxin (aflR) gene was found 8/8 (%100) in *A.flavus*, with PCR product of this gene was nearly588bp. As shown in (Fig. 3). In our study lipase gene was detected by PCR in 5/5 (100%) samples isolation of*A. terreus*. The PCR amplification of the lipase gene was approximately 591bp. as shown in (Fig4)

Table 4. Prevalencemolds isolated from different organs of broiler chickens

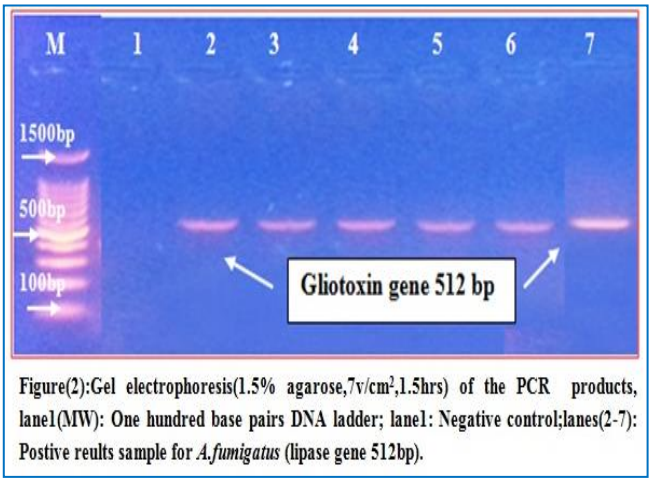
Type of moulds	Type of isolates			
	Liver	Lung	Air sac	Total isolates
Aspergillus flavus	1	5	2	8(8%)
Aspergillusfumigatus	0	3	2	5(5%)
Aspergillus terrus	1	2	2	5(5%)
Aspergillusniger	1	2	1	4(4%)
Aspergillusparasiticus	0	2	1	3(3%)
Aspergillus spp	0	1	0	1(1%)
Penicillumrubrum	0	1	0	1(1%)
Penicillumspp	0	0	1	1(1%)
Alternariaalternata	0	0	1	1(1%)
Mucorcircinelloides	0	1	0	1(1%)
Mucorhiemalis	0	1	1	2(2%)
Mucorspp	0	0	1	1(1%)
Rizopusspp	1	0	0	1(1%)
Alternerriaspp	1	0	1	2(2%)
Cladosporium	0	1	0	1(1%)
Fusarium solani	0	0	1	1(1%)
Fusariumspp	0	0	1	1(1%)
Total	5(5%)	19(19%)	15(15%)	39(39%)

Table 5. Number of positive fungi isolation relation with age in broiler chickens

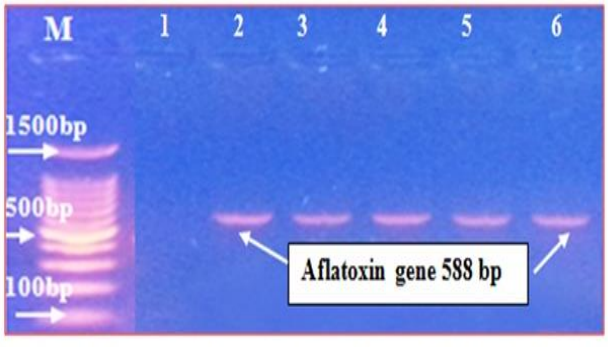
Type of moulds	Age of broiler chicken					
	First week	Second week	Third week	Fourth week	Fifth week	Total isolates
Aspergillus flavus	1	3	2	2	0	8(8%)
Aspergillus fumigatus	1	2	1	0	1	5(5%)
Aspergillus terrus	1	1	3	0	0	5(5%)
Aspergillusniger	2	2	0	0	0	4(4%)
Aspergillusparasiticus	1	2	0	0	0	3(3%)
Aspergillus spp	1	0	0	0	0	1(1%)
Penicillumrubrum	0	1	0	0	0	1(1%)
Penicillumspp	0	0	1	0	0	1(1%)
Alternariaalternata	0	1	0	0	0	1(1%)
Mucorcircinelloides	0	1	0	0	0	1(1%)
Mucorhiemalis	0	1	0	1	0	2(2%)
Mucorspp	1	0	0	0	0	1(1%)
Rizopusspp	0	1	0	0	0	1(1%)
Alternerriaspp	1	1	0	0	0	2(2%)
Cladosporium	0	1	0	0	0	1(1%)
Fusarium solani	0	1	0	0	0	1(1%)
Fusariumspp	1	0	0	0	0	1(1%)
Total	10(10%)	18(18%)	7(7%)	3(3%)	1(1%)	39(39%)



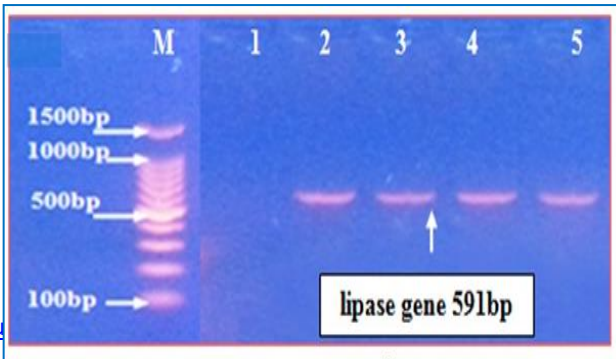
Figure(1): Gel electrophoresis(1.5% agarose,7v/cm^2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane2: Negative control; lane(3): Negative result; lane(4-8) postive reults sample for *Aspergillus spp.*(ITS gene 600bp)



Figure(2):Gel electrophoresis(1.5% agarose,7v/cm^2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane1: Negative control;lanes(2-7): Postive reults sample for *A.fumigatus* (lipase gene 512bp).



Figure(3): Gel electrophoresis(1.5% agarose,7v/cm^2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane1: Negative control; lanes(2-6): Postive reults sample for *A.flavus* (aflatoxin gene 588bp).



Figure(4): Gel electrophoresis(1.5% agarose,7v/cm^2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane1: Negative control; lanes(2-5): Postive reults sample for *A. terreus* (lipase gene 591bp).

Discussion

Fungal infection of chickens is one of the most dangerous efforts that cause high economic losses, not only high rates of disease and mortality in young chickens, but they are also pioneers in causing immunosuppression in birds and reducing their level of confrontation with various viruses. Bacterial diseases and increase their mortality rate. On the other hand, an increase in it can be a fungal infection. Expected due to the widespread use of antibiotic preparations in the treatment of many diseases in addition to the extensive use of antibiotics as feed

Additives, fortifier fungalproblems if this It is intended to study fungi examination in fattening farms.In the current studyshownmost recurrent molds isolates were *Asperigelliu*sspp. Specific*Aspergillus flavus*at percentage 8 (8%) followed by 5 (5%) for *A.fumigatus*and*A.terrus*while 4(4%) for *A.niger*and 3(3%) for*A.parasiticus*and 1(1%) for *Aspergillus spp*. This result is consistent with Byrd *et a*l. (6), Klich(11),and Pscin*et al.* (16).Thisestablishes that the most main species isolated from poultry samples belong to the genus *Aspergillus***.** Molecular categorization of *Aspergillus*spp, approved out using PCR and sequencing of PCR products. *Aspergillus*spp, PCR recognition systems are based on the use of target 18S (cDNA). However, sequences in these regions are sealed across a wide range of fungi. So it is difficult to use them to identify *Aspergillus* spp. The ITS region contains variants that allow sequence discovery of*Aspergillus* species, so the region presents a potential model for the identification of diverse*Aspergillus*spp species. Whichever withdesigned raw materials from this region for different types of spp. or amplify region sequences using primers to amplify the ITS region(8). With regard to *Aspergillus* species, primers used to determine the polymerase chain reaction (PCR) are used to amplify the ITS region(12). A product with a strength of 600 bp and positivity was amplified by the polymerase chain reaction assay confirming the results of conventional methods.In this study, the fungal gene where the virulence factor for the production of many toxins is considered to be the presence of aflatoxin gene was detected in each of the *A. flavus*samples by polymerase chain reaction, and it was compatible withMirhendi*et al*(14).The presence of the lipase enzyme was consistent withDaejeon and Tsygankova(2) who observed that extracellular lipase was found to be produced in large quantities by A. Terrie. The Glyotoxin gene was an immune virulence factor as reported in a previous study by Sethi*et al.* (21)and this gene was discovered to be the most important viral factor that can escape immune reactions and damage the host.This study showed the prevalence of mold isolates from different sites of poultry farms as it showed the highest incidence in lung and air sac with 19 (19%) and 15 (15%)

respectively.These results came according to what was obtained previously(1). Lung and air sac as part of the respiratory tract,this system may be in direct contact with the environment of the external system has resulted in germs easily entering the respiratory tract through inhalation and the available environmental conditions of temperature and humidity make this system more susceptible to infection of the respiratory tract fungal infection (19). The number of fungi isolated in infected chickens also increased in the first and second weeks of this study in agreement with Ashraf *et al.*(3).

Conclusion

The current study showed that the most common isolate was*Aspergillus flavus,* which showed the highest incidence in lung and airsac.The increase in the number of isolates was observed in infected chickens during the first and second weeks of ageand it succeeded in amplifying the ITS1 region of all *Aspergillus* isolates by polymerase chain reaction, and the virulence gene responsible for mycotoxins such as gliotoxin and aflatoxin was revealed.

Acknowledgment

The author is grateful to all the veterinary staff at Wasit University for their help and support**.**

Conflict of interest

The authors declare no conflict of interest.

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