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The Detection of *Aspergillus flavus* in the Milk by Molecular Method in Diyala Province

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Abstract: Since ancient times, milk and its derivatives have been one of the most popular foods. This value put the food hygienists in a real challenge to provide safe milk to consumers with maintaining its nutritional value. The consumption of milk was estimated to be every day for a lot of people as it was the source of many nutrient essential for human. The main objectives of the present study were to isolate the fungus of *Aspergillus flavus* from milk samples and detect aflatoxigenic *A. flavus.* Accordingly, a total of 100 samples of milk samples were collected randomly from location (Baqubah , Buhriz , Alkhalis , Bani sa'ad , Muadadiya and Hebheb) and different animals (cows, sheep and goat) from Diyala Province. A conventional polymerase chain reaction assay was applied for the confirmation of *A. flavus* by using published primers (ITS1 and ITS4), and the result revealed 15 samples of crud milk have toxigenic isolate of *Aspergillus flavus* which have size 600bp. sequence and phylogenetic analysis to determinate *A. flavus* strain and its origin also this was isolates seven strain and then resulted in single new strain gene , This was done by recorded new strain of *A. flavus* in Gene bank data base with accession number (MH213344) that isolated from milk in Iraq, in addition to antifungal sensitivity where done for 15 toxigenic samples and the result reveled that all of them are resistance to antifungal drugs (Nystatin, Amphotericin B, Fluconazole) that made them more virulent effective on human health.

Keywords: *Aspergillus flavus*, Milk, PCR, sequencing.

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Introduction:

Milk contamination by fungal through environment, tools, milk handlers and packaging materials. Molds and yeasts are identified as an essential reason of spoilage of various dairy products (1). Same fungi are identified by producing mycotoxins that are harmful to human health, these fungi are *Aspergillus*, *Fusarium* and *Penicillium* (2).

Mycotoxins have a multiplicity of adverse impact on both humans and animals when ingested (3). *Aspergillus flavus* and *Aspergillus Parasiticus* were the most filamentous fungi producing aflatoxin that affect in human and animals feed staff (4). The most toxic toxin reported was Aflatoxin B1 and known as potentially hepato-carcinogenic (5). Aflatoxin M1 is excreted through milk, feces and urine of animals following utilization of the AFB1 contaminated feeds (6).

The metabolite of Aflatoxin B1 was AFM1, it was reported to be present human and animals' milk when they were feed on grains that contained molds with AFB1. There was sufficient evidence that AFM1 less toxic than AFB1, but both toxins had potential hazardous on human health. Cell transformation, DNA damage, chromosomal anomalies and gene mutation were reported to be due to the

effect of both above-mentioned toxins(7).

Several studies on the use of PCR technology for the detection and diagnosis of fungi have been published Henson and French (1993)(8). Detection or identification of Aspergillus species can be performed by the use of molecular biological methods that included amplification of fungal DNA by polymerase chain reaction (PCR) techniques that was followed and in sometimes by sequencing of particular gen product to ensure the diagnosis of particular strain of fungi as in case of those producing mycotoxins and the genes associated with regulation of aflatoxin production as it was pointed that 25 were responsible in biosynthesis of aflatoxins(9,10).

Polymerase chain reaction techniques were reliable and sensitive methods that can be used to study the genomic relationships between pathogenic and nonpathogenic microbes(11). The good target for such relationship is the ribosomal gene in nuclear DNA and especially the Internal Transcribed Spacer (ITS) that can be used to study the phylogenetic relationship of different strains or isolates of a fungus (12).

Several authors have described PCR assays targeting ITS regions or genes involved in aflatoxin biosynthesis for detection of *Aspergillus* species(13-16). The ITS region of nuclear ribosomal DNA, including ITS1, ITS4 and the intervening 5.8S rRNA gene, has been widely used to study the variability in fungi at the species and sub-species levels (17,18).

The current study aimed to identification of aflatoxigenic *A. flavus* and phylogenetic analysis of detected strain and Estimate the susceptibility of *A. flavus* isolated strain to antifungal drugs.

Materials and Methods:

Samples Collected:

Total of 100 samples were collected from crud milk of cow, sheep and goats in different districts of Diyala Province (Baqubah , Buhriz , Alkhalis, Bani sa'ad , Muadadiya and Hebheb). The isolates were identified according to their morphological and microscopic features. Fifteen samples of them diagnosed as *Aspergillus flavus*, after that detect *Aspergillus flavus* by molecular method.

Extraction of Genomic DNA:

Aspergillus flavus cultures were grown in 250 ml conical flasks containing 100 ml potato dextrose broth at room temperature (28±2) °C for (5-7) days. Mycelium was harvested by filtration and then freeze-dried. DNA was extracted from ground, freeze-dried mycelium following the method of Liu *et al.* (19). DNA extraction kit (Quick DNA ™ Fungal Miniprep Kit) was used and according to the procedure mentioned and advised by manufacturer.

Estimation of Genomic DNA Concentration:

Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quant Flour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

Primer Selection:

The primers for (ITS1- ITS4) gene of *A.flavus* were selected according to(20). The primers sequence of (ITS1-ITS4) gene and their size of product are show in (Table 1).

Table (1): The primers sequences of ITS gene of *A.flavus* and their product size

Name of primer	Sequence of primer	Size of product
Forward ITS1	TCCGTAGGTGAACCTGCGG	≈600bp
Reverse ITS4	TCCTCCGCTTATTGATATGC	

Single plex PCR Program to detect ITS:

Region gene of *A. flavus* isolates:

Compound	Concentration	Amount(µl)
Master Mix	X1	12.5
Forward primer	1µM	1
Reverse primer	1µM	1
Nuclease Free Water	–	8.5
DNA	10 ng\ µl	2
TOTAL		25

❖ Singleplex PCR Program to detect ITS gene of *A. flavus* isolate.

No.	Steps	Tem.	Time	No of Cycle
1	Initial Denaturation	95°C	5min	1
2	Denaturation	95°C	30sec	35
3	Annealing	55°C	30sec	
4	Extension	72°C	45sec	
5	Final extension	72°C	7min	1

PCR products were separated on 1.3% agarose gel in TAE buffer, stained with ethidium bromide and visualized under UV transillumination. The sizes of the digested products were determined by comparison with standard 100 bp molecular markers.

The nucleotide sequences were carried out commercially (Macrogencompany in South Korea for direct sequence by using the AB1 13730XL® Terminator v3.1 cycle, automated DNA. The results were received by email then analyzed using genius software.

National Center for Biotechnology Information (NCBI) offered the basic alignment search tool (BLAST) program to be used for homology searching (http://www.ncbi.nim. nih.gov/ GenBank/index.html). Accordingly, and through the Gene Bank, The isolated sequences were analysis by two way .Firstly ,by using Clustal Omega software the 8 sequence compared with the first option in gene bank Second way by 21randomly selected sequences of 5,8 ribosomal RNA gene, ITS complete sequence, and the sequence of large ribosomal RNA gene were used to genetically compare with sequence MH213344 , only one sample designated as was rerestarted in NCBI.

Bioinformatics software (Bio edit version 7.0.9) was used for multiple alignments of nucleic acid sequence. Neighbor joining method and cluster algorism program (Bio edit version 7.0.9) and MEGA 6 software through constriction phylogenetic tree were used to derived the homology matrix and phylogenetic tree. PHYLIP format was used to display the phylogenetic trees with bootstrap values.

Sensitivity Test:

The Fungal isolates of *A. flavus* strain were isolated in Laboratory of microbiology during the study (Diyala-Iraq) susceptibility of fungus by Disc diffusion method was used in this test, to determine the ability of *A. flavus* fungal resistance to antifungal. All 15 isolates *A. flavus* were refreshed by re cultivated from stock isolates on SDA at 30 ºC for (5 – 7) days.

In this method, the spore's *A. flavus* were obtained from pure mature culture at one week of cultivation by adding 5ml of sterile distilled water in each plate and adding of tween 80. The spores then harvested and separated from other parts by using sterile gauze , followed by a series of dilutions on the suspension using sterile distilled water and then the suspension was ready to use by taking 0.2 ml of suspension and placed on the surface of dishes Spread evenly and left the dishes without stirring so that the drop of suspension would be absorbed by the media after wards spread antifungal disks on the surface of media and cultivate on SDA (27-30) ºC for (5-7) days and observed in day 3 , 5 and 7 to observe the fungal growth(21).

Results and Discussion:

Molecular Detection of *A . flavus*:

In the present study and for the molecular biological purposes, the DNA from suspected *A. flavus* of 15 milk samples was extracted by the use of commercial DNA kit extraction. This was followed by subjecting the extracted DNA to PCR using ITS1 forward primer and ITS4 reverse primer. The extracted DNA appeared in different concentration according to amount of cultured fungi (Table 2).

Table (2): Concentration of extracted DNA in (ng/µl) from 15 cultured fungal samples of milk.

Sample	Conc.ng/µl
01	12
02	13
03	8
04	14
05	6.9
06	9.2
07	8.1
08	8.2
09	16
10	16
11	12
12	60
13	38
14	46
15	41

PCR amplification:

The internal transcribed spacer gene was used to prepare the oligonucleotide primer sequences from DNA of the ribosome. All the amplified cDNA by using conventional PCR showed identical mobility on 1% agarose gel. All 15 positive samples generated specific DNA band of 600bp (Figure 1).

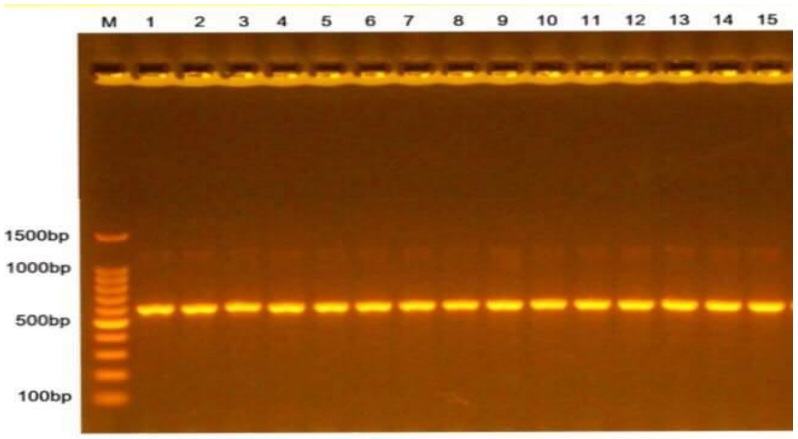

Figure (1): The PCR of screened15different fungal milk samples. The highly conserved region of (ITS) regions of the ribosomal DNA, primers pair ITS1and ITS4 produced the target band of 600bp, Lane no. 1-15 are fungal samples; M: 100bp marker, Voltage5 volt, for 2 hours.

The PCR was one of the easiest techniques for the detection of any microorganism in samples. Thus, we observed that all 15 *A. flavus* isolates reacted positively with the primer set (Figure 1). The resulted DNA PCR fragment of Aspergillus was found to be of 600bp and estimated according to DNA ladder that electrophoresed on the same gel and under the same conditions. These results are consistent with those of other studies of (22,20,23) who showed a high sensitivity and specificity by using PCR amplification of genomic DNA extracted from *A. flavus* isolates with ITS1 and ITS4 primers resulted in the amplification and predicated the target band.

Data Analysis:

Analysis of sequences and confirmation of microorganism's homogenic data using rRNA database (NCBI) after amplification of Fungi's RNA ribosomal. All processes including Fungi DNA extraction, PCR amplification, sequencing, and assembly. For Fungi, Analysis on ITS region (18S prior to update); length greater than 500 bp guaranteed.

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MH593840.1    TCTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA    60
1             ------------------------------------------------------------    0
2             ------------------------------------------------------------    0
3             ------------------------------------------------------------    0
4             ------------------------------------------------------------    0
5             ------------------------------------------------------------    0
6             ------------------------------------------------------------    0
7             ------------------------------------------------------------    0
8             ------------------------------------------------------------    0

MH593840.1    GGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC    120
1             ----------------------CTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC    36
2             ----------------------CTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC    36
3             ----------------------CTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC    36
4             ----------------------CTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC    36
5             ----------------------CTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC    36
6             ----------------------CTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC    36
7             ----------------------CTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC    36
8             ----------------------CTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTAC    36
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MH593840.1    CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGGCTCTCAGCCCCGGGCCCG  180
1             CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCG   96
2             CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCG   96
3             CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCG   96
4             CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCG   96
5             CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCG   96
6             CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCG   96
7             CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCG   96
8             CTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCG   96
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MH593840.1    CGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCG  240
1             CGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCG  156
2             CGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCG  156
3             CGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCG  156
4             CGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCG  156
5             CGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCG  156
6             CGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCG  156
7             CGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCG  156
8             CGCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCG  156
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MH593840.1    CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC  300
1             CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC  216
2             CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC  216
3             CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC  216
4             CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC  216
5             CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC  216
6             CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC  216
7             CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC  216
8             CAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC  216
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MH593840.1    GAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCAC  360
1             GAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCAC  276
2             GAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCAC  276
3             GAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCAC  276
4             GAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCAC  276
5             GAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCAC  276
6             GAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCAC  276
7             GAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCAC  276
8             GAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCAC  276
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MH593840.1    ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG  420
1             ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG  336
2             ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG  336
3             ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG  336
4             ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG  336
5             ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG  336
6             ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG  336
7             ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG  336
8             ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG  336
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MH593840.1    CACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG  480
1             CACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG  396
2             CACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG  396
3             CACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG  396
4             CACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG  396
5             CACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG  396
6             CACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG  396
7             CACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG  396
8             CACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCG  396
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MH593840.1    GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC   540
1             GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC   456
2             GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC   456
3             GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC   456
4             GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC   456
5             GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC   456
6             GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC   456
7             GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC   456
8             GCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC   456
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MH593840.1    CGGCGCTTGCCGAACGCAAATCAATCTTTCCAGGTGACCTCGATCAGTACGTACCG   596
1             CGGCGCTTGCCGAACGCAAATCAATCT----------------------------   483
2             CGGCGCTTGCCGAACGCAAATCAATCT----------------------------   483
3             CGGCGCTTGCCGAACGCAAATCAATCT----------------------------   483
4             CGGCGCTTGCCGAACGCAAATCAATCT----------------------------   483
5             CGGCGCTTGCCGAACGCAAATCAATCT----------------------------   483
6             CGGCGCTTGCCGAACGCAAATCAATCT----------------------------   483
7             CGGCGCTTGCCGAACGCAAATCAATCT----------------------------   483
8             CGGCGCTTGCCGAACGCAAATCAATCT----------------------------   483
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Figure (2): Identical in the nucleotide sequences of *Aspergillus flavus* isolates belonging to the 8 samples according to accession number MH593840.1.

The nucleotide sequences obtained in this study belong to the aflatoxigenic Aspergillus of *A. flavus* according to BLAST search of the GenBank database. These 8 sequences were differenced from MH593840.1 by source of isolated and location, However, the sequences of the *A. flavus* isolates from milk were identical with isolate sequences of *A. flavus* that available in the GenBank database (access no.MH593840.1) as shown in (Figure 2).

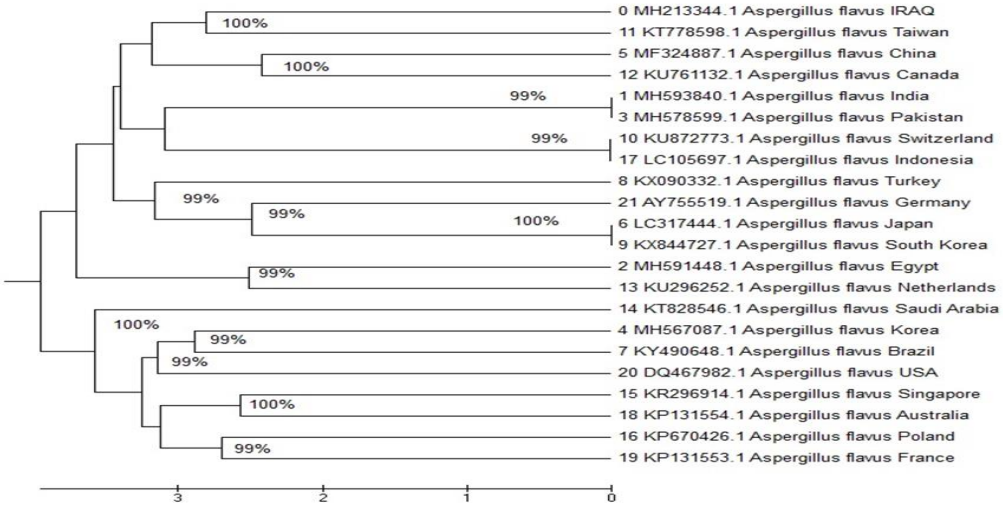

Figure (3): Phylogenetic relationships determined of the highly conserved region of (ITS) regions from the DNA of ribosome.

The phylogenetic trees were generated using tree top phylogenetic tree predication program (Gene Bee-Molecular Biology Server) and displayed in PHYLIP format with bootstrap values. Iraqi strain MH213344.1 isolate from milk at Diyala Province as a local isolate in Iraq compared with 21 of available sequences of fungi strains from the GenBank.

Table (2): Gene Bank Accession Numbers of Sequence 18S rRNA Genes of Fungus used for phylogenetic analysis.

	Accession	gene	country	Source
1.	ID: MH593840.1	18S ribosomal RNA gene	India	Aspergillus flavus
2.	ID: MH591448.1	18S ribosomal RNA gene	Egypt	Aspergillus flavus
3.	ID: MH578599.1	18S ribosomal RNA gene	Pakistan	Aspergillus flavus
4.	ID: MH567087.1	18S ribosomal RNA gene	Korea	Aspergillus flavus
5.	ID: MF324887.1	18S ribosomal RNA gene	China	Aspergillus flavus
6.	ID: LC317444.1	18S ribosomal RNA gene	Japan	Aspergillus flavus
7.	ID: KY490648.1	18S ribosomal RNA gene	Brazil	Aspergillus flavus
8.	ID: KX090332.1	18S ribosomal RNA gene	Turkey	Aspergillus flavus
9.	ID: KX844727.1	18S ribosomal RNA gene	South Korea	Aspergillus flavus
10.	ID: KU872773.1	18S ribosomal RNA gene	Switzerland	Aspergillus flavus
11.	ID: KT778598.1	18S ribosomal RNA gene	Taiwan	Aspergillus flavus
12.	ID: KU761132.1	18S ribosomal RNA gene	Canada	Aspergillus flavus
13.	ID: KU296252.1	18S ribosomal RNA gene	Netherlands	Aspergillus flavus
14.	ID: KT828546.1	18S ribosomal RNA gene	SaudiArabia	Aspergillus flavus
15.	ID: KR296914.1	18S ribosomal RNA gene	Singapore	Aspergillus flavus
16.	ID: KP670426.1	18S ribosomal RNA gene	Poland	Aspergillus flavus
17.	ID: LC105697.1	18S ribosomal RNA gene	Indonesia	Aspergillus flavus
18.	ID: KP131554.1	18S ribosomal RNA gene	Australia	Aspergillus flavus
19.	ID: KP131553.1	18S ribosomal RNA gene	France	Aspergillus flavus
20.	ID: DQ467982.1	18S ribosomal RNA gene	USA	Aspergillus flavus
21.	ID: AY755519.1	18S ribosomal RNA gene	Germany	Aspergillus flavus

Aspergillus flavus that isolated from crud milk samples and recorded in the National Center Biotechnology Information (NCBI) was compared with isolates from different source that were under sequence(ID:MH593840.1 **,** ID:MH591448.1 **,** ID:MH578599.1 **,** ID:MH567087.1 **,** ID:MF324887.1 **,** ID:LC317444.1 **,** ID:KY490648.1 **,** ID:KX090332.1 , ID: KX844727.1**,** ID:KU872773.1 , ID:KT778598.1, ID: KU761132.1 , ID: KU296252.1, ID: KT828546.1 , ID: KR296914.1, ID: KP670426.1, ID: LC105697.1, ID: KP131554.1, ID: KP131553.1, ID: DQ467982.1 ID: AY755519.1, **)** respectively. This comparison showed compatibility the highest identity (>100%) and expect 0.0 with gene bank.

The genetic dimension between Iraq and the isolates of the world is detailed according to the Phylogenetic tree (Figure 3) and the comparison (Table 2). hierarchical cluster analysis determine the following clusters: large Cluster divided into several neck: first root the Taiwan : Iraq the genetic dimension was by 100% it is closest to Taiwan "ID: KT778598.1" according to(24) KT778598.1 strain was isolate from patient infected with bilateral otitis externa , infected occur because increase temperature under the condition of global warming and other risk factor such as insufficient hygiene , increased humidity in the ear channel by increased exposure to water and injuries may also play role in infected.

Tzean *et al.*, (25) revealed that *Aspergillus spp*. is most common soil fungi in Taiwan and as a result of commercial exchanges between Iraq and Taiwan that officially (known as the Republic of China), therefore, transmission strain to Iraq also the appropriate climate in Iraq played an important role in increasing the spread

of this strain. *Aspergillus spp.*, might be more common under moist tropical conditions in contrast to temperate regions. As many other *Aspergillus species*, *Aspergillus flavus* is found more frequently in tropical/ subtropical environments than in temperate ones(26).

Sensitivity analysis:

The results of this study revealed that in disc diffusion method, there was no inhibitory zone observed indicating that *Aspergillus flavus* was resistant to antifungal drugs Amphotericin B20mg, Fluconazole 10 mg and Nystatin 100mg (Figure 4).

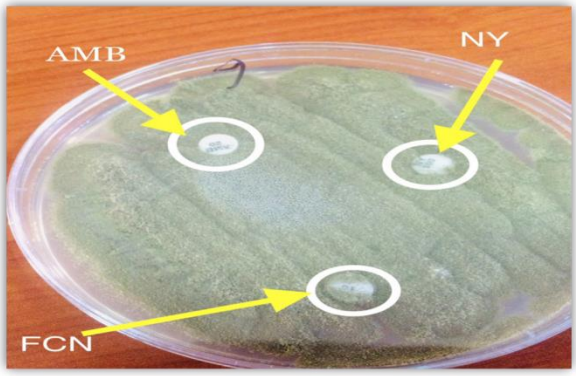

Figure (4): Culture showed resistance *Aspergillus flavus* to antifungal sensitivity test to many antifungal drugs.

There were many drugs available to treat fungal infection like amphotericin B (AmB), group of azole and Nystatin. Although the true rate of AmB resistance was unknown, some investigators have reported isolates of *A. flavus* resistant to AmB in vitro (27-30). Amphotericin B was considered to be an antifungal that rarely induced resistance in fungi compared to other antifungals such as the azoles. Most of the fungal strains resistant to polyene antibiotics have been obtained using mutagenic agents (31).

The results of the present study showed no response observed to the used antifungal drugs. Preliminary report has documented a steady increase in AmB resistance in vitro amongst Aspergillus isolates recovered since (29). This was in agreement with the findings of the present study that revealed resistance of *A. flavus* to AMB. Some investigators have hypothesized that the extensive use of AmB against fungal infections had led to the emergence of less susceptible species, such as *A. terreus* and *A. flavus* (32,33) found that the proportion of *Aspergillus spp*. resistant to antifungals (especially AmB) was much higher amongst isolates recovered from cancer patients with prior exposure to AmB.

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