Molecular Detection and Identification of Aspergillus fumigates from Goats in Waist Governorate of Iraq

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Abstract
A total of 100 samples of goats include 50 samples were collected from each tracheal swabs, and vaginal swabs from slaughter house and herds of goats in Waist province. During these studied, molds were isolated and identification depending on the cultural characteristic, microscopic feature and conventional Polymerase chain reaction (PCR). This study showed increase in the isolation of opportunistic molds with increase age of animals, which observation from this study on goats found highest percentage of molds in those with ages between 3-5 years, while decrease percentage in animals that ages less than six months. The results showed significant differences (P<0.01) in molds isolation with increase animals ages in goats. Through study found Aspergillus spp. were predominant species. The most isolate of opportunistic molds were from tracheal swabs at percentage 63.6%, were significantly different (P<0.01) than vaginal swabs. The results of molds isolation showed the A. fumigatus and A. niger, were more isolated at percentage 40.9% and 22.8% respectively, followed by A. terreus (18.2%), A. flavus (9.1%), Aspergillus spp (4.5%) respectively, also isolated Penicillium spp. at percentage (4.5%). Showed result Significant differences (P<0.01) were found in isolation of molds between tracheal swabs, and vaginal swabs. The results of the PCR amplification of the rRNA gene showed that, this gene was present in 9 samples out 22 positive samples which isolation from the goats with a PCR product size of approximated 380 bp, while 13 samples out 22 positive samples showed negative results for the presence of this gene as indicated by the absence of the PCR products. The conclusion of present investigation indicated Aspergillus fumigates the most dominant molds isolated from tracheal swabs in goats, and this may be indicating a high contamination and exposure to Aspergillus spores. Molecular methods have revealed the important diversity of Aspergillus species within these action Fumigati.

Keywords: A. fumigatus, Goats in Waist Governorate, Polymerase chain reaction (PCR), rRNA gene

1. Introduction
"Aspergillosis is a spectrum of diseases that may be caused by a number of Aspergillus species which are ubiquitous saprobes in nature, and aspergillosis are found worldwide. The genus Aspergillus includes over 185 species. Around 20 species have so far been reported as causative agents of opportunistic infections in man and animal. Among these, Aspergillus fumigatus is the most commonly isolated species, followed by Aspergillus flavus, Aspergillus niger and A. terreus". (Pattron, 2006 and Steinbach et al., 2012).

Aspergillus causes diseases of respiratory system by the inhalation of Aspergillus conidia. The clinical manifestations of pulmonary aspergillosis are many ranging from harmless saprophytic colonization to a cute invasive disease (Zhao et al, 2010 and Seyedmousavi et al, 2015). In immunocompromised led to invasive aspergillosis symptoms including fever, cough, dyspnea and hemoptysis. Hyphae invade the lumens and walls of blood vessels causing thrombosis, infarction, and necrosis from the lung. The disease may spread to the gastrointestinal tract, kidney, liver, brain or other organs producing abscesses and necrotic lesion (Radostits et al., 2007).

Aspergillus spp. are rapidly growing mould with septate hyphae. Many have highly colored colonies ranging from bluish –green through yellow to black due to the profuse production of pigment spores conidia (Fraga et al, 2008). Aspergillus spp. can causes disease in several ways. They can be the invasive cause of mycotoxicoses and are involved in allergic reactions in human (Brooks et al., 2007).

Aspergillus fumigates cause many diseases in domestic animals. In dog it causes a disease in mucous membranes, guttural pouch in horses, lung and air sac in poultry, and mastitis and abortion in bovine (Tell, 2005). Aspergillus species can be identified by the characteristic sporing head seen on microscopy and the strong color of the spores which is conferred on the colony. For example, A. fumigatus produces a deep blue-green colony, A. flavus light to yellow green, A. terreus cinnamon to beige or sandy brown, and A. niger dark brown to black. Various morphological features associated with the sporing head allow accurate identification (Ryan and Ray, 2004). Therefore the aim of present study to Molecular detection and identification of Aspergillus fumigates from goats.

2. Material and Methods
2.1. Samples Collection
A total of 100 samples in a ratio of 50 samples for each of tracheal, and vaginal swabs were collected from 100 goats in Waist Governorate of Iraq during six months.
goats, from slaughter house and herds of goats in Waist province.

2.2. Tracheal Swabs
Fifty samples of tracheal swabs have been collected from goats. The tracheal swabs were collected by sterile cotton swab after slaughter of animal and emptied of viscera, then made incision in the lower part of tracheal and inter the sterile cotton swab in the lumen of tracheal. These swabs were transferred to the laboratory for diagnosis after adding few drops of sterile distilled water. In the laboratory the swabs were directly inoculated on to plates of Sabouraud dextrose agar with chloramphenicol, and incubated duplicated culture at 30 °C for 2 weeks (Koneman and Roberts, 1985).

2.3. Vaginal Swabs
Fifty samples were taken from female animals (50 goats) by sterile cotton swab from vagina, and then transferred to the laboratory for diagnosis after adding few drops of sterile distilled water in the laboratory. The swab was directly inoculated onto plates of SDA with chloramphenicol, and duplicated cultures were incubated at 30 °C cultures were maintained for 2 week (Koneman and Roberts, 1985).

2.4. Morphology Diagnosis
The identification was done depended on the shape and color of the fungus on the plate, and examined under the microscope. For appearance of the fungus, small portion from the fungal growth was taken, mixed with one drop of lacto phenol cotton blue and covered with cover slip then examined under (40X) by the microscope (Chandler et al., 1980; Ellis, 1994).

2.5. DNA isolation from culture
Fungal strains were grown on Sabouraud agar at 28°C at least 5 days to produce a visible colony, and a tiny portion of the colony was transferred directly to the PCR tube, followed by the steps installed and attached with the QIAamp DNA mini kit (Qiagen, Germany) was used to complete the extraction, following the manufacturer's protocol from the step. DNA yield was stored at (-20° C) until use.

2.6. Determination of genomic DNA concentration and purity
The concentration and purity of the purified DNA were quantified by nanodrop instrument, by following the instruction of the manufacturer(Act Gene NAS99) Briefly, 3µl was aspirated using special tips (Aeroject tips 10µl) and inserted in specified socket in the machine, DNA was quantified by the refractive index using the wave length 260nm, 280nm. DNA concentration was calculated with the OD260nm. The purity was estimated with the OD260nm/OD280nm ratio, a ratio of ~1.8 was generally accepted as “pure” DNA, indicating a low degree of protein contamination.

2.7. Gel electrophoresis
DNA samples were electrophoresed by horizontal agarose gel electrophoresis according to (Sambrook et al., 2001) as follows: Agarose(Promega, USA) at a concentrations of 2% was prepared, the agarose solution was left to cool at 55°C, then(0.5µl) of ethidium bromide solution(Promega, USA) was added, Agarose solution poured into the taped plate. A comb was placed near one edge of the gel. The gel was left to harden until it became opaque; each of the comb and tape were removed gently. TBE buffer (1X) prepared was poured into the gel tank and the slab was placed horizontally in electrophoresis tank. About 3 microliters of loading buffer prepared was applied to each 7 µl of DNA sample wells were filled with the mixture by a micropipette, PCR products were directly applied. Power supply was set at (5 V/cm (70) for 1 hr) for genomic DNA and PCR products electrophoresis. When the electrophoresis was finished the gel was exposed to UV light using UV transilluminator and then photographed using digital camera.

2.8. Primer design.
The specific of oligonucleotide primer sequences "were designed based on the sequence data for the internal transcribed spacer (ITS) region of the rRNA gene .Species-specific primer pairs— AFUM1-AFUM2 The forward primers (primer 1 of each pair) were designed within the ITS1 region, and the reverse primers (primer 2) were designed from the ITS2 region". This primer sequences were taken from (Guizhen and Thomas, 2002). and synthesized in Alpha DNA® (Canada) were used in conventional PCR to detect the Aspergillus fumigates as shown in. (Table:1)
2.9. Polymerase chain reaction PCR

The specific of oligonucleotide primer sequences were used in conventional PCR to detect the presence of (ITS) region of the rRNA gene to detect the *Aspergillus fumigates*, the primers (AFUM1 and AFUM2) were diluted by adding nuclease free water according to the manufacturer instructions. The master mix contents were thawed at room temperature before use, and the PCR master mix was made on a separate biohazard safety cabinet with wearing hand gloves at all times to avoid contamination. For each reaction within each single pre-mixed PCR reaction tube, 2µl from each forward primer and reverse primer were added. Five microliter of DNA template was added for each reaction tube. Twelve and a half microliters of GoTaq® Green Master Mix(Promega, USA) was added for each reaction tube, the volume was completed to 25µl with Deionized Nuclease –Free as shown in table no.2, tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components. PCR mixture without DNA template(non-template negative control) were used as negative control. The tubes were placed on the PCR machine and the PCR program, with the right cycling conditions pre-installed, was started. Cleaver Scientific Thermal Cycler TC32/80 was used for all PCR amplification reactions. The PCR thermocycler program used with The PCR thermocycler program used with (ITS) region of the rRNA gene was designed on the basis of published paper as shown in table no. 3.

**Table (1): Primers sequences with their relevant product size**

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer name</th>
<th>Primer sequence  (5' → 3')</th>
<th>GenBank Accession No</th>
<th>Product Size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ITS) region of the rRNA gene</td>
<td>AFUM1</td>
<td>CGC CGA AGA CCC CAA CAT GAA</td>
<td>AF176662, AF078889</td>
<td>385</td>
</tr>
<tr>
<td></td>
<td>AFUM2</td>
<td>TAA AGT TGG GTG TCG GCT GGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.10. PCR Gel Electrophoresis

Electrophoresis was done as stated earlier. Five microliters of the 100bp DNA ladder(Promega, USA) were mixed with one microliter of blue/orange 6X loading dye(Promega, USA) and subjected to electrophoresis in a single lane. Served as marker during PCR products electrophoresis. The gel was exposed to UV using UV light transilluminator and then photographed using digital camera (Sony-Japan).

2.11. Statistical Analysis

"In order to determine the statistical significances among different variables SPSS program (Statistical program for social sciences)" version 11, was used. Chi-square and RLSD by ANOVA test (One-Way Analysis of Variance) were applied.
3. RESULTS AND DISCUSSION
3.1 Age of Goats
This study showed increases of molds isolation in goats with the increase age of animals. Highest percentages were in age between 3-5 years. While less percentage were less than six months (Table 4). Statistical analysis showed significant differences ($P<0.01$) in molds isolation with increases in animals ages. In relation of occurrence of opportunistic mold with age, the present study revealed increase of mold isolation from animals with the increase of animals’ age. The highest percentage was with age more than 6 years compares with one year with significant differences ($P<0.01$). These results agree with Al-Maadidhi (2008) who studied the fungal type infection in reproductive system in ewes, he found that the percentage of infection raise with increase in the age animal. Other studies showed that the percentage of infection increase of with increase in animals age. Wiserman et al., (1984) they found that the percentage of systemic mycotic infection increased with increasing of animal ages. Samaka (2000) also found that the animals that ages more than 6 years the percentage of infection was 48.8 % whereas less percentage in animals that ages lower than 5 years. The causes may be due to the animals in this ages have the increase chance to environment contact and also the animals are sexually active in this age which may be contamination through coating, parturition and abortion with other microorganism.

Table (4): Number positive samples in relation with age in goats

<table>
<thead>
<tr>
<th>Age</th>
<th>Less than 6 months</th>
<th>1 year</th>
<th>2 years</th>
<th>3 years</th>
<th>4 years</th>
<th>5 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracheal</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Vaginal</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total positive</td>
<td>%0</td>
<td>%9.1d</td>
<td>%13.6cd</td>
<td>%13.6cd</td>
<td>%27.3bc</td>
<td>%36.4 ab</td>
</tr>
</tbody>
</table>

3.2. Morphology Diagnosis
The results of molds isolation from goats showed the total positive samples in goats 22(22%) out of 100 samples. Also, through this study the most isolate of opportunistic molds were from tracheal swabs 14(63.6%) out of 22 positive samples, the tracheal swabs were significant differences ($P<0.01$) than vaginal Swabs. The most common isolates were Asperigellius spp. particular $A.fumigatus$ at percentage (40.9 %) followed by $A.niger$ 22.8%, $A.terrus$ 18.2%, $A.flavus$ 9.1%, Aspergillus spp. and Penicillium spp. 4.5% respectively (Table 5 and Figures 1). These results agreed with Samaka (2000), who notes Aspergillus spp. isolates more frequently from tracheal swabs in cattle and sheep, in cattle he found $A.fumigatus$ more frequent in percentage 42% followed by $A.niger$ 26.3% , while in sheep also found $A.fumigatus$ more frequent in percentage of 33.3% . Other studies found that Aspergillus fumigatus more frequent than other molds, when mastitis milk of ewe was examined (Al-kubaysi, 2000). Whereas Ali and Khan (2006) observed the mycotic abortion is an important reproduction problem in cattle and buffaloes.

They found that the chief fungus associated with mycotic abortion was Aspergillus fumigatus which has been recorded from over 60% of cases, also notes no clinical symptoms have been observed in the dam either before or after abortion, but clinical diagnosis can be made on the pathological appearance of placenta and particularly the cotyledons and also on the presence of foetal skin lesions. Through our result the highest percentage of isolation of opportunistic systemic fungi showed in goats were from tracheal at percentage 63.6%. The tracheal were significant differences ($P<0.01$) than vaginal swabs.

The tracheal swabs as a part of respiratory system may be this system which contact directly with external system environment led to easily entry of the spores to the respiratory system by inhalation and available environment stander from temperature and moisture make from this system more exposure to the mycotic infection (Brook et al., 2007).

Table (5): Number and percentage of molds isolates in goats samples.
3.3. Molecular diagnosis

The specific of oligonucleotide primer sequences were used in conventional PCR to detect the presence of (ITS) region of the rRNA gene for *Aspergillus fumigatus*. The results of the PCR amplification of the rRNA gene showed that, this gene was present in 9 samples out 22 positive samples which isolation from the goats with a PCR product size of approximated 380 bp, while 13 samples out 22 positive samples showed negative results for the presence of this gene as indicated by the absence of the PCR products in their relevant lanes. (Figure. 2).

For many purposes, this rapid method has become routine for the amplification of DNA from cultures of *Aspergillus* species." The molds were much less amenable to direct amplification, perhaps because of more intractable cell walls, abundant endogenous nucleases, inhibitors of the PCR, or other factors. There was a tendency for younger mold cultures to be more PCR positive than older cultures".

4. Conclusion

Conclusion of present investigation indicated *Aspergillus fumigatus* the most dominant molds isolated from tracheal swabs in goats, and this may be indicating a high contamination and exposure to *Aspergillus* spores. Molecular methods have revealed the important diversity of *Aspergillus* species within these action *Fumigati*.

References


