The Role Of Bad Hygienic Environment Of Dairy Animals And Their Milk In The Epidemiology Of *Aspergillus Fumigatus* Pulmonary Infection Among Inhabitant Children

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ABSTRACT

This study was designed according to the observation of many pulmonary infection cases not responding to antibiotics among children inhabitant in close to bad hygienic environment of animal's yards inside their farmer's owner houses. The observed region was in an area of 16 km^2 including 3 villages belonging to El-Fateh center of Assiut governorate and called Arab El-Atawla, Arab Motair and Beni Mor. There was an expectation of transmissible fungal infection between environment and children cases. Therefore, many samples were collected from 20 farmer's houses and represented totally 143 samples distributed as, 80 environmental samples from air and water, 48 milk samples from dairy animals and 15 clinical samples of sputum from the children cases. All these samples were subjected to fungal culture, DNA extraction and PCR protocol that yielded high frequencies of *Aspergillus fumigatus* in 80% of air, 55% of pail water, 55% of driven well water, 10% of tap water, 16.67% of milk and 53.33% of sputum. The statistical analysis results proved a significant correlation between its frequency in sputum and their frequencies in each of air, pail water and driven water samples. Furthermore, the obtained results viewed *A. fumigatus* epidemiologic infections among the 3 villages with frequencies in 37.04% of samples from Arab El-Atawla, 47.28% of samples from Arab Motair and 29.41% of samples from Beni Mor. It can be concluded that the bad hygienic environment enabling endemic exposure of children to conidiospores which played the main role of their infection with pulmonary aspergillosis.

INTRODUCTION

The genus Aspergillus includes over 185 species. Around 20 species have been reported as causative agents of opportunistic infections in man. Among these, *Aspergillus fumigatus* is the most commonly isolated species, followed by *A. flavus* and *A. niger*. These 3 make up 95% of all aspergillosis infections.

A. fumigatus is a filamentous fungus which causes several pulmonary conditions, including allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis *(1)*. These conditions are usually acquired by inhalation of fungal conidiospores, which exist in the air at concentrations 1.5-2/m^3 *(2)*, and are small enough (2-3 µm in diameter) to reach the alveolar spaces of the lungs. Pulmonary aspergillosis occurs when colonize the respiratory tract of patients with compromised airways *(3)*. The fungal spores trigger an asthma like allergic reaction, reducing the efficiency of the air passages in the lungs and causes coughing, wheezing and shortness of breath. The most common complication of Aspergillus colonization is allergic bronchopulmonary aspergillosis, in children.

This fungal species is a common inhabitant of soil, where it colonizes organic debris and releases a high number of conidia to the atmosphere. Due to its presence in the atmosphere, outbreaks of nosocomial aspergillosis have often been associated with an increase of its airborne spore's concentration *(4)*.

As early as 1985, Aspergillus species and other filamentous fungi were shown to inhabit water distribution systems that deliver water to

hospitals in Europe and USA *(5)*. Recently, there has been an increase in data supporting water as a potential source of filamentous fungi especially *A. fumigatus (6)*.

Excessive moisture and water (dampness) indoors due to water intrusion or leaking are the key factors leading to fungal growth indoors. Indoor molds pose population health issues and their accompanied with dampness were related to 50% increases in asthma and 60% increases in upper respiratory disease. Harvard and Canadian studies showed that 10% of the residential buildings had dampness problems to a degree, which could result in population health effects. The Canadian Federal and Provincial Committee on Environmental and Occupational Health recommended that exposure to indoor mold be minimized, and recognized there is a relationship between the population health effects of mold and dampness and the existence of risk groups. It led to a 1990 National Building Code, which requires mechanical ventilation in residences and applies to most Canadians *(7)*.

Detailed epidemiological studies of *A. fumigatus* are hampered by difficulties in characterizing isolates. The prevalence of its spores in the air and the similar colonial morphologies of many isolates can cause problems in laboratories where the fungus is being studied. For these reasons, it would be useful to have a quick and easy means of distinguishing between fungal isolates that are morphologically identical. Thus, the obtained fungal isolates of the present study were identified using PCR protocol that was done in National Agricultural Research Centre, Kyushu Okinawa Region, Kumamoto, Japan.

This study aimed to investigate the prominent fungi inside the noticed bad hygienic environment of dairy animal housing that may be suspected to be the cause of pulmonary aspergillosis in children live beside animal enclosures.

MATERIALS AND METHODS

This study was conducted in 20 farmer houses distributed in 3 villages of El-Fateh center located east north of Assiut city. The villages called Arab El-Atawla, Arab Motair and Beni Mor whom being in an area of about 16 Km^2. Each farmer house (of an average area 100 m^2) included one animal yard (of an average area 8 m^2). The 20 farmer houses were selected as 7 from Arab El-Atawla, 7 from Arab Motair and 6 from Beni Mor. The selection was based on the presence of moist cough of pulmonary infections affecting the inhabitant children (aged 3-7 years) whom not responding to antibiotic. The adults were excluded from the study to avoid the role of smoking and bronchial asthma cases.

Sampling: A total of 143 samples were taken in 3 forms as environmental, milk and clinical samples:

I) Environmental samples:

1. **Air samples:** Twenty air samples were taken as one sample from each yard. The aerobiological survey was conducted using sedimentation method recommended by **APHA** *(8)*, in which a new paper towel was placed at the location where the air is to be sampled and then a Petri plate (containing Sabouraud's dextrose agar) was set horizontally on a flat surface and exposed to the air by removing its cover for 3 min.
2. **Pail water samples:** Twenty pail water samples were taken as one sample from each yard. The pail water was used for drinking animals and this water was usually obtained from driven wells. Each sample was of 500 ml in a sterile glass bottle.
3. **Driven well water samples:** Twenty driven well water samples were taken as one sample from each house. Driven wells were opened for 5 to 10 min just before taking the samples to rinse any accumulated dust and dirt from them. Each sample was 500 ml in a sterile glass bottle.
4. **Tap water samples:** Twenty tap water samples were taken as one sample from each house. Taps were opened for 5 to 10 min just before taking the samples to rinse any accumulated dust and dirt. Each sample was of 500 ml in a sterile glass bottle.

II) Milk samples: Forty eight buffalo's milk samples were taken according to the

intensity of dairy animals and their milk production. Each milk sample was taken after milking from its milk pail. The taken samples were distributed as 21 samples from 7 houses in Arab El-Atawla village (3 samples per house), 21 samples from 7 houses in Arab Motair village (3 samples per house) and 6 samples from 6 houses in Beni Mor village (one sample per house). Each sample was 500 ml in a sterile glass bottle.

III) **Clinical samples:** fifteen sputum samples were taken from children cases and distributed as 5 samples from 7 houses in Arab El-Atawla village, 6 samples from 7 houses in Arab Motair village and 4 samples from 6 houses in Beni Mor village. Each sample was taken using sterile swab.

- Culture and identification

All samples were grown over Sabouraud's dextrose agar (Oxoid) supplemented with chloramphenicol (5 mg/ml) to inhibit bacterial growth. All plates were incubated at 27±2°C for 7 days and examined for the presence of filamentous fungi every 24 h. Fungal isolates were identified by macroscopic and microscopic characteristics according to **Bally and Scott** *(9)*. After cultivation and identification, subcultures were made from every CFU to obtain a pure culture. Fungal isolates were stored at –20°C until PCR analysis.

DNA extraction: (this part was done in National Agricultural Research Centre, Kyushu Okinawa Region, Kumamoto, Japan)

DNA extraction was carried out according to **Griffiths *et al.* *(10)***. One cm^2 area of culture was removed from the agar, added to 0.9% sterile saline (Oxoid) and vortexed. 10 µL of each dilution of conidia was added to 360 µL AL buffer (Qiagen) and 20 µL proteinase K at a concentration of 20 mg ml-1 (Qiagen) from which DNA was to be extracted. Beads were then added and shaken in a Mini-Bead Beater-8 (Biospec) for 3 min on the 'homogenize' setting. DNA extraction was followed by the addition of 20 µL proteinase K at a concentration of 20 mg ml-1 (Qiagen) and incubation for 2 h at 55°C. DNA purification using the QIAamp DNA Mini kit (Qiagen) was carried out following the tissue protocol with the amendment of using a 100 µL elution volume.

PCR protocol: (this part was done in National Agricultural Research Centre, Kyushu Okinawa Region, Kumamoto, Japan)

The 25 µL PCR mixture contained 1X Taqman universal PCR Master Mix (Applied Biosystems), 500 nM each primer, 100 nM probe and 7 µL DNA. DNA was amplified in an ABI Prism 7900HT sequence detector (Applied Biosystems) in optical 384-well plates. Cycling conditions were 95°C for 10 min, followed by 40 amplification cycles of 15 s of denaturation at 95°C and one min of hybridization and elongation at 60°C. The primers were 28S-466 (5'-CTC GGA ATG TAT CAC CTC TCG G-3') and 28S-533 (5'-TCC TCG GTC CAG GCA GG-3'), and the Taqman probe was 28S-490 (5'-6-carboxyfluorescein-TGT CTT ATA GCC GAG GGT GCA ATG CG-3'-6-carboxy tetramethylrhodamine) *(11)*. The threshold cycle (Ct) value, which is inversely proportional to the log of the amount of target DNA initially present, was calculated using SDS software version 2.0 (Applied Biosystems).

Statistical analysis

The statistical analysis that represented in the correlation matrix of quantitative data was done with using SPSS version 11.0.

RESULTS

Table 1. Frequency of the positive samples for *A. fumigatus*

Samples		Arab El-Atawla		Arab Motair		Beni Mor		Sum	
		+ve (total)	%	+ve (total)	%	+ve (total)	%	+ve (total)	%
Air		6 (7)	85.71	6 (7)	85.71	4 (6)	66.67	16 (20)	80.00
Water	Pail	4 (7)	57.14	5 (7)	71.43	2 (6)	33.33	11 (20)	55.00
	Driven well	4 (7)	57.14	5 (7)	71.43	2 (6)	33.33	11 (20)	55.00
	Tap	0 (7)	0.00	2 (7)	28.57	0 (6)	0.00	2 (20)	10.00
Milk		3 (21)	14.29	4 (21)	19.05	1 (6)	16.67	8 (48)	16.67
Sputum		3 (5)	60.00	4 (6)	66.67	1 (4)	25.00	8 (15)	53.33
Total		20 (54)	37.04	26 (55)	47.28	10 (34)	29.41	56 (143)	39.16

Table 2. Correlation matrix of quantitative data measuring the frequencies of *A. fumigatus* among the examined samples.

Samples		Air	Water			Milk	Sputum	Total
			Pail	Driven well	Tap			
Air		1.0000						
Water	Pail	0.9286	1.0000					
	Driven well	0.9286	1.0000**	1.0000				
	Tap	0.5002	0.7858	0.7858	1.0000			
Milk		0.0000	0.3710	0.3710	0.8659	1.0000		
Sputum		0.9881*	0.9727*	0.9727*	0.6229	0.1486	1.0000	
Total		0.8205	0.9730*	0.9730*	0.9031	0.5689	0.8975	1.0000

**Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed)

Table 3. Correlation matrix of quantitative data measuring the frequencies of *A. fumigatus* among 3 villages.

Villages	Arab El-Atawla	Arab Motair	Beni Mor	Sum
Arab El-Atawla	1.0000			
Arab Motair	0.9572**	1.0000		
Beni Mor	0.9098**	0.8176*	1.0000	
Sum	0.9956**	0.9682**	0.9243**	1.0000

**Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed)

DISCUSSION

Through the activities of veterinary community service in Assiut villages, it was noticed a bad habit reflecting the degree of bad hygienic environment inside their animal's yards, in which, the farmers leave the manure to be accumulated on the ground and cover it with dust and hay layers with repetition for many times till about 2 months leading to sinking of animals legs in these layers. This bad habit return to many reasons: 1) the farmers aim to

collect this manure as a fertilizer, 2) they hinder the movement of animals to render them quicker for fattening as they think, 3) they believe the animals will be more resistant to diseases when live in unhygienic environment.

To come into question, what induced the authors to do this study? The answer was summarized in our observation to bad hygienic environment of dairy animals housing plus presence of pulmonary infection in inhabitant children without responding to antibiotics. This observation forwarded us to study the probability of transmissible fungal infection. Thus, many samples from these environments and from the children cases were collected for fungal examination.

The summarized results in Table 1, declared the high frequencies of *A. fumigatus* in the examined samples as 80% of air, 55% of pail water, 55% of driven well water, 10% of tap water, 16.67% of milk and also 53.33% of sputum of children cases. As the air plays a crucial role in the spread of Aspergillus species in the environment and into the patients, the statistical analysis results presented a significant correlation between *A. fumigatus* frequency in sputum and in the examined air (Table 2). With regards to the examined 3 types of water, they contained *A. fumigatus* as a fungal contamination from air into water ***(12)***. Furthermore, many milk samples were incorporated in this investigation to evaluate the degree of contamination inside these environments; in addition, this milk was observed to be consumed directly without heat treatment.

The factors explaining the role of the examined bad hygienic environment are: 1) hay layers that cover the manure are heavily contaminated with Aspergillus spores, 2) the conidial air counts were significantly higher in wet areas (like animal's yards) compared to dry areas ***(6)***, 3) bad ventilation ***(4)*** 4) surface water is heavily contaminated that has contact with ambient air, leading to fungal contamination from air into water ***(12)***, 5) wet route of transmission may be either directly by ingestion, aspiration, inoculation of vulnerable skin and wounds or indirectly by aerosolization leading to inhalation of secondarily airborne Aspergillus conidia ***(13)***.

The epidemiology of *A. fumigatus* infection among the 3 villages was noticed through the correlation matrix of quantitative data measuring its frequencies (Table 3). Conceptually, primary infection by endemic fungi during infancy is reminiscent of the infantile form of pulmonary pneumocystosis, which is associated with young age, malnutrition, and endemic exposure ***(14)***.

Finally, the bad hygienic environment of animals housing creating unhygienic conditions around the inhabitant children whom may playing inside the yard and sometimes with manure; so, it played the main role in their infection with pulmonary aspergillosis

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الملخص العربي

دور البيئة الصحية السيئة للحيوانات الحلابة وألبانها في وبائية الأصابة الرئوية بفطر الاسبرجلس فيرميجاتس بين الأطفال المقيمين

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صممت هذه الدراسة طبقا لمشاهدة العديد من حالات الأصابة الرئوية بين الأطفال الغير مستجيبين للمضادات الحيوية وكانوا مقيمين بجانب بيئات صحية سيئة لحظائر الحيوانات داخل بيوت الفلاحين. وكانت المنطقة الملاحظة على مساحة ١٦ كيلومتر تشمل ٣ قرى تابعة لمركز الفتح بمحافظة أسيوط وتسمى عرب الأطاولة وعرب العوامر وبني مر. وكان هناك توقع بإنتقال العدوى الفطرية بين البيئة وحالات الأصابة الرئوية. ولذلك تم جمع العديد من العينات من ٢٠ بيت للفلاحين ومثلت بإجمالي عدد ١٤٣ عينة موزعة كالآتي: ٨٠ عينة بيئية من الهواء والماء، ٤٨ عينة لبن من الحيوانات الحلابة و١٥ عينة إكلينيكية من لعاب حالات الأطفال. وخضعت كل هذه العينات للزرع الفطري ثم استخلاص DNA وتطبيق PCR، حيث أظهرت النتائج نسب عالية من فطر *Aspergillus fumigatus* في ٨٠% من الهواء، ٥٥% من ماء الأواني، ٥٥% من ماء الطلمبات، ١٠% من ماء الحنفية، ١٦,٦٧% من اللبن و٥٣,٣٣% من البلغم. وأظهرت نتائج التحليل الإحصائي وجود إرتباط معنوي بين نسبتها في اللعاب ونسبتها في كل من الهواء وماء الأواني وماء الطلمبات. بالإضافة إلى أن الأصابات الوبائية بين القرى كانت متمثلة بنسب وجود الفطر في ٣٧,٠٤% من عينات قرية عرب الأطاولة و47.28% من عينات قرية عرب مطير و٢٩,٤١% من عينات قرية بني مر. ونستخلص أن البيئة الصحية السيئة المسببة للتعرض المستوطن للأطفال للبوغيات تلعب الدور الرئيسي في العدوى الرئوية لمرض aspergillosis.