INCIDENCE OF BROODER PNEUMONIA AND GENOTYPING *ASPERGILLUS FUMIGATUS* IN BROILER FARMS IN SULAIMNAIYAH

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ABSTRACT

Brooder pneumonia is aspergillosis occurs in chicks caused by *Aspergillus fumigatus* during the first-week post-hatching. It has not been confirmed yet that all *A. fumigatus* strains cause brooder pneumonia or it restricted to pathogenic strains. From an outbreak included 28 broiler farms in Sulaimaniyah province, 575 dead and sick chicks were randomly selected, diagnosed clinically, histopathologically, and by *post-mortem* isolation of *A. fumigatus*, among them 321 chicks were confirmed to be infected by aspergillosis with incidence rate of (57.5 %). Twenty eight clinical isolates of *A. fumigatus* along with 15 farm environment isolates represented all the farms were subjected to genotyping through $(CA)_n$ and $(CACCAC)_n$ short tandem microsatellite DNA repeats. All clinical and environmental isolates were highly polymorphic except two clinical isolates from two different farms which were with the same genotype. It could be concluded that all *A. fumigatus* strains are potential causative agents of brooder pneumonia.

Keywords: Aspergillosis, Chicks, Microsatellite DNA

حدوث ذات رئة الحضن والتنميط الوراثي ل *Aspergillus fumigatus* في حقول دجاج اللحم في محافظة السليمانية

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المستخلص

تعد ذات رئة الحضن في أفراخ الدجاج من أمراض الرشاشية المسبب بوساطة *Aspergillus fumigatus* في الأسبوع الأول بعد الفقس. لم يتم التأكد بعد فيما اذا كانت جميع عترات *A. fumigatus* تسبب ذات رئة الحضن أو ان المرض محدد بعترات ممرضة. من تفشي في 28 حقلا لدجاج اللحم مصابة في محافظة السليمانية جرى انتقاء 575 طيرا ميتا و مريضا بصورة عشوائية، اجرى تشخيصها سريريا وبالأختبارات المرضية النسيجية والعزل مابعد الموت ل *A. fumigatus*.. وقد تبين اصابة 321 فرخا منها بالرشاشيات بمعدل حدوث قدره (57.5%). جرى تنميط 28 عزلة سريرية و 15 عزلة من بيئة الحقل مثلت جميع الحقول من خلال تتابعات الدنا التابعية المتعاقبة الدقيقة القصيرة $(CA)_n$ و $(CACCAC)_n$. جميع العزلات السريرية والبيئية كانت عالية تعدد الأشكال باستثناء عزلتين سريريتين لحقلين مختلفتين امتلكتا نفس النمط الوراثي. تبين بأن جميع عترات *A. fumigatus* الممرضة والبيئية تعد مسببات كامنة لمرض ذات رئة الحضن.

الكلمات المفتاحية: الرشاشيات، صيصان، الدنا التابعي الدقيق

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INTRODUCTION

The poultry industry is one of the main pillars for providing food to the world's population (1) Broiler farms are regarded as the primary target for many infectious diseases, which cause a severe problem, as they are difficult to control due to intense farming and higher germ load in the farm environment. Among the infectious diseases, fungal diseases have their importance and seem to be one of the significant obstacles for the poultry farmers in the form of high morbidity, mortality, and production losses (4). Study finding by Olias (20) reported that fungal pneumonia might represent a high-risk factor for the white stork population in Germany. Fungal infection by *Aspergillus* species is called aspergillosis. It forms a significant cause of morbidity and mortality among certain species of captive and free-ranging chickens (6, 23). In chicks, respiratory aspergillosis almost always causes brooder pneumonia, which is considered a primary cause of morbidity caused by *A. fumigatus* (23). *A. fumigatus* is a filamentous fungus that thrives on decaying vegetation and organic debris. It releases large amounts of asexual spores (conidia), which are dispersed by air. Inhalation of airborne conidia is the principal mode of exposure. Once conidia are inhaled, they will deposit deep in the respiratory tract (8, 22). Aspergillosis in young chicks and pullets is commonly associated with overwhelming exposure to large numbers of conidia from heavily contaminated feed, litter, or the hatchery environment (13). In brooding age, aspergillosis occurs as an acute form with high morbidity and mortality, but also tends to be chronic at older ages. Clinical signs such as dyspnea, gasping, cyanosis of un-feathered skin, and hyperemia are usually associated with the disease. However, affected birds typically do not produce respiratory noise associated with other respiratory problems (21). Lesions are commonly confined to lungs and air sacs, although oral mucosa, trachea, and eyes may be affected. Typical lesions are fungal nodules or plaques within the lungs and on the air sacs, while syrinx may also be affected occasionally (21). Currently, there is poor knowledge whether any individual *A. fumigatus* has the opportunity to cause aspergillosis in chicks. Although David (10) has referred to the virulence of *A. fumigatus* to involve networks of genes that have likely evolved to support the organism in its primary ecological niche, the fingerprinting methods are with high discriminatory power. They so could be applied to investigate the genetic and epidemiological relationships between environmental and clinical isolates. Indeed, Interlaboratory reproducibility and objective interpretation of the fingerprinting data are highly desirable in the differentiation between pathogenic and environmental strains (12). Typing methods used in this area which are based on DNA short tandem repeats (STRs) such as microsatellite length polymorphism (5,11,12, 25). Several molecular typing studies have referred to variability among avian isolates and multiple genotypes recovered from healthy and diseased birds, such as random amplified polymorphic DNA, restriction fragment length polymorphism, and amplified fragment length polymorphism analyses. Among them, short tandem repeats (STR) typing is claimed to be less complicated, reproducible, and any technical complications associated with microsatellite typing can be appropriately addressed. However, these findings are not definitive to distinguish sharply between pathogenic and nonpathogenic *A. fumigatus* (2,18). Hence, this study was aimed to determine the incidence rate of brooder pneumonia among boiler farms spread by the disease in Sulaimaniyah province, Kurdistan region, Iraq, and to investigate the ability to cause disease by clinical isolates of *A. fumigatus* in comparison to environmental ones using microsatellite DNA genotyping.

MATERIAL AND METHODS

Histopathology and isolation of *A. fumigatus*

A total of 575 chicks, were selected randomly from 28 farms hit by brooder pneumonia in the period between Feb 2017 and July 2018, submitted for post-mortem examination systematically following a standard protocol and isolation of *A. fumigatus* (9). Necropsies from lung, trachea, and liver were collected and preserved in 10% formaldehyde solution (Cell path Newtown, UK). After proper fixation, paraffin-embedded tissue sections (4–6 μm) were prepared and stained by routine

hematoxylin and eosin (H&E) stain (Abcam Burlingame, USA) for microscopic examination (3). Duplicate sections were stained with Periodic Acidic Schiff (PAS) stain (BioVison, Zurich, Switzerland) for demonstration of fungal hyphae in the tissues (19). A block section of the necropsy nodule was obtained aseptically, crushed, and cultured on streptomycin and cycloheximide supplemented potato dextrose agar (PDA) and sabaroud dextrose agar (SDA), incubated for 4-5 days at 28 °C, and then *A. fumigatus* was identified microscopically and by cultural characteristics (16).

Molecular analysis

DNA was extracted from 28 clinical and 15 environmental new pure *A. fumigatus* colonies by using a kit (GeNet bio, Chungcheongnam, Korea), and then stored at freezing (-20 °C). Extracted DNA was amplified by PCR using a thermocycler (Prime Staffordshire UK) through a reaction procedure of a 20-µl total volume master mix, containing 10 µl DNA sample, 1 µl forward primer, 1 µl reverse primer, 5µl DNA template, and 3 µl nuclear-free water. The PCR was programmed based on the instruction of the Mastermix 2x Easy Taq^{R} PCR SuperMix (TRAN Chungcheongnam Korea); denaturation 94 °C (5 min), annealing 58 °C (1 min), extension 72 °C (1 min), and a final extension at 72 °C (5 min). Two previously designed primer sets (5) were used to amplify two loci of short tandem repeats (STR), STRC and STRA. The sequences of the STRC primers were forward 5′-GCCTACGATGACCGAAATGA-3′ and reverse 5′-CTGTTTTGAGAAGCGGATGG-3′, while the STRA primers were forward 5′-CGAAGCTCTCCCCTGCAAATC-3′ and reverse 5′-GATGCCGCTGGTGGTGTTGT-3′. PCR products were electrophorized on 1% agarose gel (Macroge Seoul Korea), and the amplification bands (amplicons) were visualized inside a transilluminator (SYNGENE London UK) (Figure 1).

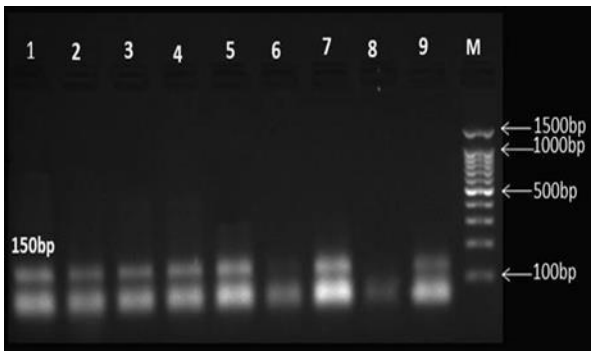

Figure 1. Gel electrophoresis of PCR-amplified *A. fumigatus* isolates lane M; ladder, lane 1-5 *A. fumigatus* isolates amplified by STRC, lane 6-9 amplified by STRA

Interpretation of genotypic diversity

The amplicons were sequenced (Macrogen, Seoul Korea), submitted to the GenBank database (https://www.ncbi.nlm.nih.gov), and given accession numbers. The STR genotypes of sequences were determined through the number of $(CA)_n$ and $(CACCAC)_n$ tandem repeats using the REPFIND program (7). The sequences of the STRs were analyzed for phylogenetic relationships among them by constructing a dendrogram (MEGA-X). STR genotypes were blotted against the phylogenetic dendrogram of their amplified sequences to determine the genotype diversity in relation phylogenetic relationship (17).

RESULTS AND DISCUSSION

The lung slides stained by H&E of the infected birds showed severe and diffuse accumulation of cellular necrotic debris together with inflammatory cells in the parabronchiolar area with centrally caseated eosinophilic granuloma and blood vessels congestion (Figure.2a).Caseation necrosis mixed with dead tissue debris was observable within the lumen of bronchioles (Figure. 2b) and the center of the eosinophilic caseation necrosis was surrounded by diffuse infiltration of mononuclear inflammatory cells, mainly macrophages, in the form of chronic granuloma (Figure. 2c). Histopathologically, lesions of granulomatous nodules are commonly confined to lungs, air sacs, and visceral organs. The nodules in the lung parenchyma have a central core of caseation necrosis in which the organisms are found surrounded by a broad zone of epithelioid granulation tissue. The signs were abundant while a previously study detected

granulomatous nodules in lung and air sacs only (9).Some of the slides stained by PAS showed invasion of fungal hyphae with conidial heads with the characteristics of *Aspergillus* spp. (Figure.2d).

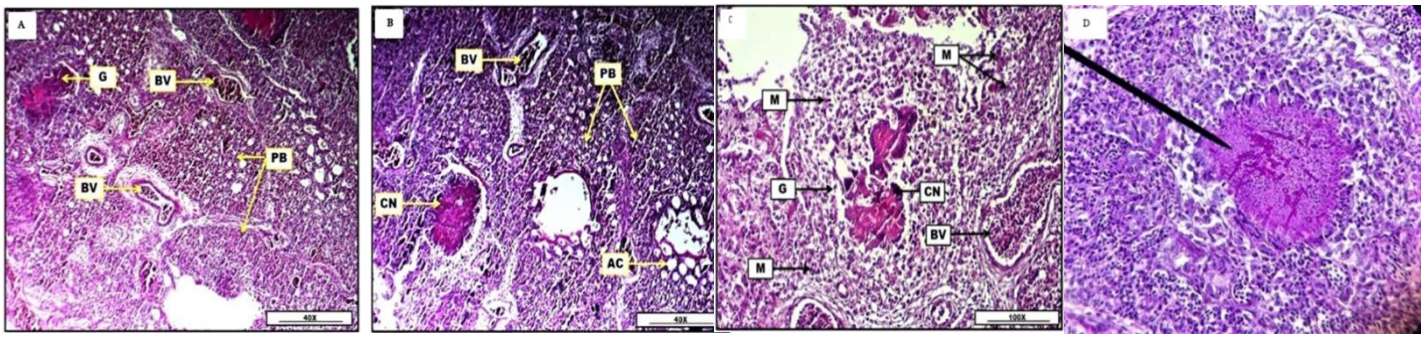

Figure 2. Photomicrograph of chick lungs with brooder pneumonia. A: (G) granuloma, (BV) blood vessel congestion, (PB) parabrochiolar area. B: (CN) caseation necrosis, (AC) air capillaries. C: (M) macrophage, (G) granuloma, (CN) caseation necrosis, (BV) blood vessel congestion. D: invasion of lung tissue by hyphae of *A. fumigatus*

Lesions of liver samples characterized by severe vascular congestion within the hepatic major blood vessels and the sinusoidal capillaries (Figure. 3a). Evidenced by the presence of nucleated erythrocytes and accumulation of lipid vacuoles within the cytoplasm of hepatocytes (Figure. 3b).

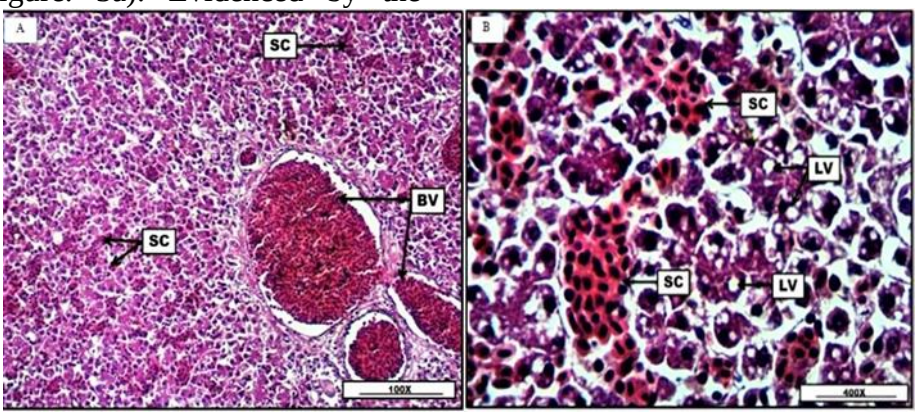

Figure 3. Photomicrograph of liver of chick infected with brooder pneumonia. A: (SC) sinosoidal capillariy, (BV) blood vessle congestion. B: (SC) sinosoidal capillariy, (LV) cytoplasm of hepatocyte

The trachea showed inflammatory conditions evident by the accumulation of tissue debris mixed with sloughed endothelium and blood cells in the lumen. Infiltration of inflammatory cells in the tracheal mucosa was evident (Figure.4a). Diffuse infiltration of inflammatory cells was seen in the lamina propria of tracheal mucosa in addition to edema in the submucosa (Figure. 4b).

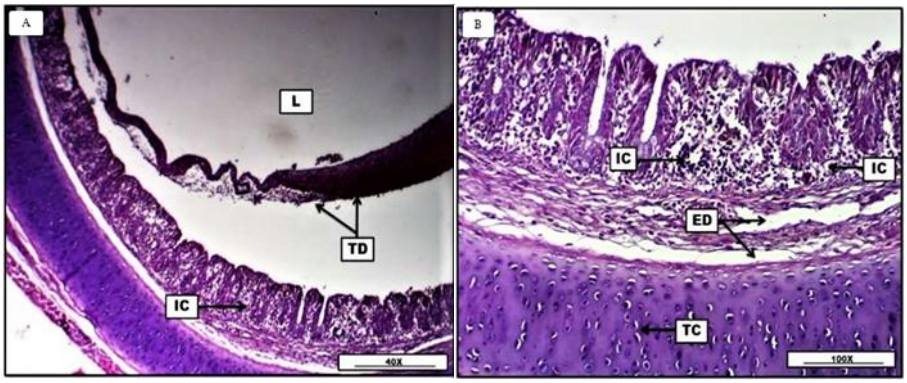

Figure 4. Photomicrograph of Trachea of chick infected with brooder pneumonia. A: (L) lumen of trachea, (TD) tissue dibresi, (IC) inflamtory cells. B: (IC) inflamatory cells, (TC) tracheal hayline cartilage, (ED) edema

The PAS staining revealed fungal hyphae in lungs only whereas another study reported the presence of fungal hyphae in lung, abdominal air sac, and kidney (18). The hyphal growth invasion of more organs might be age-related that aspergillosis spreads with age; therefor the age of chicks is influential during sampling. However, the histopathological changes were similar to that of an aspergillosis outbreak studied previously (15). Infected birds were ultimately die; in this study death was up to the age of 16 days which confirms an earlier claim that all infected birds would die within 27 days (15). Among the selected birds, 321 were diagnosed as aspergillosis cby clinical, histopathological, and microbiologically with an incidence rate of (57.5%) among the selected birds. Out of the 43 clinical and environmental isolates, seven were positive for amplification of both STR C and A; six clinical, and one environmental. Twenty two clinical and 13 environmental isolates failed to amplify STR A loci. The seven isolates positive for the the two loci revealed six genotypes. Two clinical isolates that were isolated from different broiler farms shared the same genotype (G4), while the others were different (Table 1).

Table 1. Six genotypes for 7 *A. fumigatus* isolates by using two STRs loci with CA and CACCAC repetitives

Isolates	Source	STR C		STR A		Genotype	Genotype symbol
		CA	CACCAC	CA	CACCAC		
AF5	Clinical	0	0	30	0	0,0/30,0	G1
AF6	Clinical	20	0	0	0	20,0/0,0	G2
AF20	Environmental	0	0	18	0	0,0/18,0	G3
AF21	Clinical	0	0	0	0	0,0/0,0	G4
AF38	Clinical	31	5	0	0	31,5/0,0	G5
AF45	Clinical	25	0	28	0	25,0/28,0	G6
AF59	Clinical	0	0	0	0	0,0/0,0	G4

AF 5,6,20,21,38,45,59 *Aspergillus fumigatus* isolates were amplified by both (A and C) primerSTR Short Tandem Repeat, STRC Short tandem repeat amplified by primer C, STRA short tandem repeat amplified by primer A, G1-G6 Genotype 1- Genotype 6

Accession numbers for 21 sequences of STR C and the 22 of STR A were assigned by GenBank. When the genotypes represented by the two repetitions CA and CACCAC of the STR C were blotted againsed the sequences of the loci the sequences distributed on eight clusters, three of which were mixed of environmental and clinical despite that their genotypes were diverse. The remaining five clusters distributed on one pure environmental and four pure clinical (Figure.5). Seven diverse clusters were obtained by genotype-sequence blotting for STR A, two of which were mixed of both clinical and environmental isolates, whereas the remaining five were divided into two pure environmental and three pure clinical clusters(Figure.6). The genotypes regarding the two repetitions through the loci were diverse.

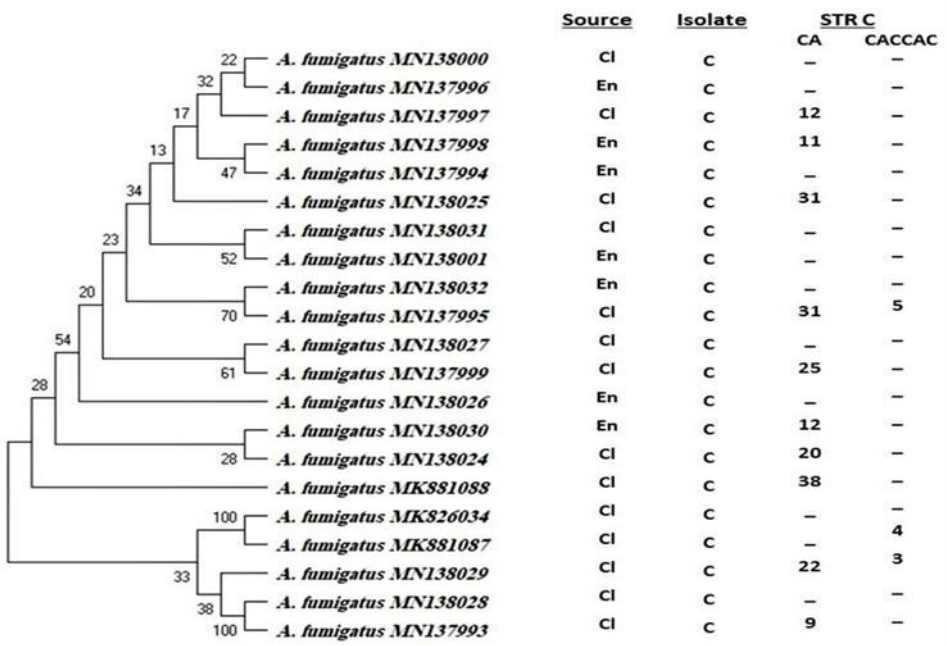

Figure 5. Dendrogram of the microsatellite analysis of *A. fumigatus* isolates isolated from broiler farms by short tandem repeat (STR C) of 14 clinical (Cl) and 7 environmental (En).

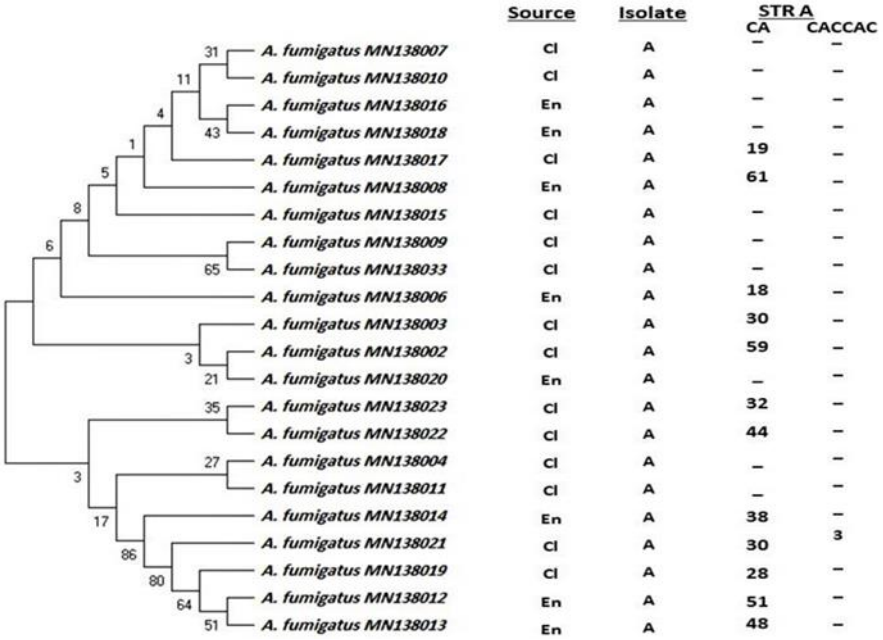

Figure 6. Dendrogram of the microsatellite analysis *A. fumigatus* isolates isolated from broiler farms by short tandem repeat (STR A) of 14 clinical (Cl) and 8 environmental (En).

However, microvariations were detected either at zero or less than two the findings that were suggested earlier that isolates with no more than two repeat units for a single locus regarded as microvariants and thus genetically related, while those with three or more repeat unit differences were considered unrelated (14). The genotype is determined in one environmental isolate only (Table 1), in this case it is very difficult to interprete the diversity by genotyping between isolates of clinical and environmental sources. On the other hand, The isolation of two clinical isolates from two different farms with the same genotype indicates a genotypic relationship, albeit weak, among clinical isolates. This relationship of two isolates belong to different farms is the first indication of the presence of pathogenic strains that can be distinguished from environmental strains. This finding was reffered to in the past with some confusion when it was reffered to genetic diversity between pathogenic and environmental strains, as well as among the pathogenic themselves (24). Indeed, we demonstrated a genetic diversity among the

clinical isolates based on the outbreak site which confirms what was reffered about the high genetic diversity among *A. fumigatus* isolates at each outbreak site also (20). The blotting of genotypes for each locus to the locus phylogenetic diversity indicated that genotyping is not of significant value in differentiation between pathogenic and environmental isolates. The phylogenetic relationship among loci revealed pure pathogenic, environmental, and mixed clusters with no apparent differences in number. If we take into consideration that the number of environmental isolates was lower than clinical, it is logical that mixed clusters become low. These findings are contarary to what was reported from that there is no marked phylogenetic cluster relationship between pathogenic and environmental isolates of *A. fumigatus* (24). Unfortunately, all isolates, except seven of them, were failed to amplify with the two loci simultaneously, which might be related to deletions of one or both loci in more isolates or due to technical error(s), as genotyping needs more microsatellite markers to give an accurate and realistic picture for genotyping. However, obtaining the same genotypes for two loci of two clinical isolates of different farms gives a good impression about the necessity of extensive research in giving priority to genetic methods to discriminate pathogenic from nonpathogenic *A. fumigatus*.

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