Genetic diversity and antifungal susceptibility of Aspergillus spp. isolates from avian farms in Guangxi, China

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Table of content

Genetic diversity and antifungal sensibility of *Aspergillus* spp. isolates from avian farms in Guangxi, China

Abstract

Fungi of the genus *Aspergillus* are moulds, which occur most frequently in soil, water and decaying vegetation. They sporulate abundantly and the spores are easily dispersed into the environment by air. As a result of this ubiquitous presence, animals and people are constantly exposed to *Aspergillus* spores. *Aspergillus fumigatus* and *A. flavus* are recognized as predominant causes of fungal diseases in humans and wide range of animals. Birds are much more sensitive that mammals and in avian farms, environmental conditions are favorable to the development of many fungal species, including *Aspergillus* spp. The objective of the present study was to assess the genetic diversity and antifungal susceptibility of *Aspergillus* isolates from avian farms in Guangxi, China. The first part of the experimental work related the evolution of fungal contamination in 3 avian farms near the city of Nanning and one farm (including a hatchery) near the city of Guilin. Pharyngeal swabs and air samples were collected during several weeks and 3 cycles of hatching were monitored. The average contamination level with *Aspergillus* spp. and Mucorales was significantly different according to the farms. The survey allowed to collect a total number of 188 *A. fumigatus* and 159 *A. flavus* isolates. The second part of the work was about the genetic diversity of *A. fumigatus* and *A. flavus.* For that purpose, the Multiple Locus Variable-number tandem-repeat (VNTR) Analysis was specifically developed and used. For *A. flavus*, 8 VNTR markers were selected and a multiplex reaction was designed. A total number of 91 *A. flavus* isolates, including 6 reference strains were typed with the panel of 8 VNTRs. This analysis yielded 78 different genotypes, which corresponds to a combined loci index of 0.993. Among all genotypes, 71 were only found once. The analysis of 188 *A. fumigatus* isolates using 10 VNTR markers led to the resolution of 142 distinct genotypes. Clusters of *A. flavus* or *A. fumigatus* isolates could be defined by using the graphing algorithm Minimum Spanning Tree. The third part of the experimental work was about the antifungal susceptibility of 177 *A. fumigatus* isolates collected in avian farms in China and France. Most of the isolates from China were susceptible to itraconazole with a Minimum Inhibitory Concentration (MIC) comprised between 0.38 and 0.75 µg/mL. Most of the isolates from birds and avian farms in France were susceptible to itraconazole with a MIC comprised between 0.19 and 1 µg/mL. MIC values of isolates collected in farms with antifungal chemoprophylaxis were not higher than those of isolates collected from birds that never received antifungal drugs before the sampling. Susceptibility testings demonstrated that 4 isolates should be considered as resistant to itraconazole: 2 isolates from avian farms in Guangxi, China and 2 isolates from avian farms in France. A modification of the *Cyp51A* sequence was identified in 11 isolates (3 azole-resistant and 8 azole-susceptible isolates). Twenty-one nucleotidic mutations were detected. Eleven of these mutations were silent and 10 yielded to amino acid substitutions. Seven of these substitutions had already been described whereas mutations A116R, E130D and Q131H were original.

Key-words:

- *Aspergillus fumigatus*,
- *Aspergillus flavus*,
- genotyping,
- antifungal susceptibility,
- avian farms,
- Guangxi, China.

Diversité génétique et sensibilité aux antifongiques d'isolats d'*Aspergillus* spp. provenant d'élevages aviaires du Guangxi en Chine

Résumé

Les champignons du genre *Aspergillus* sont des moisissures banales de l'environnement. Elles sont présentes dans le sol et sur des végétaux en décomposition. Les *Aspergillus* se propagent par l'intermédiaire de spores microscopiques en suspension dans l'air. L'Homme et les animaux sont exposés en permanence aux spores aspergillaires mais les défenses immunes empêchent leur développement dans l'organisme. Lorsque ces défenses sont amoindries, une aspergillose est possible. Dans ce cas, *Aspergillus fumigatus* et *A. flavus* sont le plus souvent incriminés. Les oiseaux sont beaucoup plus sensibles que les mammifères et l'environnement représenté par les élevages aviaires est propice à la prolifération des moisissures du genre *Aspergillus*. L'objectif de ce travail de thèse a été de caractériser la diversité génétique et la sensibilité aux antifongiques d'isolats d'*Aspergillus* provenant d'élevages aviaires dans la province du Guangxi en Chine. Une première enquête a été réalisée dans 3 élevages près de la ville de Nanning et dans un élevage (incluant un éclosoir) à proximité de la ville de Guilin. Des écouvillonnages pharyngés et des prélèvements d'air ont été réalisés pendant plusieurs semaines. Des prélèvements ont également été faits sur des œufs dans l'éclosoir. Cette enquête a montré que le niveau de contamination fongique dépendait du type d'élevage. De nombreux isolats fongiques ont pu être collectés : 188 isolats d'*A. fumigatus* et 159 isolats d'*A. flavus*. La seconde partie du travail expérimental a porté sur la caractérisation de la diversité génétique d'*A. fumigatus* et d'*A. flavus.* Pour cela, la technique MLVA (*multiple locus VNTR analysis*) a été utilisée. Pour *A. flavus*, 8 marqueurs VNTR (*variable-number tandem-repeat*) ont été sélectionnés et une réaction PCR multiplex a été mise au point. Au total, 91 isolats d'*A. flavus*, incluant 6 souches de référence, ont été caractérisées avec le panel des 8 marqueurs VNTR. Cette analyse a permis de définir 78 génotypes distincts et un index de discrimination de 0,993. L'analyse de 188 isolats d'*A. fumigatus* avec 10 marqueurs VNTR a permis de définir 142 génotypes distincts. Certains génotypes d'*A. flavus* ou d'*A. fumigatus* sont clairement regroupés dans le nuage de point généré par l'analyse MST (*minimum spanning tree*). La troisième partie du travail expérimental a porté sur la sensibilité aux antifongiques de 177 isolats d'*A. fumigatus*. Ces isolats ont été récupérés dans des élevages aviaires en Chine et en France. Les isolats de Chine sont pour la plupart sensibles avec des valeurs minimales inhibitrices (vis-à-vis de l'itraconazole) comprises entre 0,38 et 0,75 µg/mL. Les isolats de France sont pour la plupart sensibles avec des valeurs minimales inhibitrices (vis-à-vis de l'itraconazole) comprises entre 0.19 and 1 µg/mL. Quatre souches ont été considérées comme résistantes : 2 souches provenant de deux élevages en Chine et 2 souches provenant de deux élevages en France. Des mutations sur le gène *Cyp51A* ont été détectées pour 11 isolats (3 résistants et 8 sensibles). Vingt et une mutations nucléotidiques ont été identifiées. Onze de ces mutations sont silencieuses et 10 sont à l'origine d'un changement de la composition de la protéine. Sept substitutions ont déjà été décrites dans la littérature ; les mutations A116R, E130D et Q131H sont originales.

Mots-clés:

- *Aspergillus fumigatus*,
- *Aspergillus flavus*,
- typage moléculaire,
- sensibilité aux antifongiques,
- élevage aviaire,
- Guangxi, Chine.

中国广西家禽曲霉菌（*Aspergillus* spp）遗传多态性及对抗真菌药物敏感性分析

摘　要

曲霉属的真菌是主要存在于土壤，水以及腐败的营养物的一类霉菌。它们产生大量的孢子，这些孢子通过空气能很容易的扩散到环境中，由于这类孢子的普遍存在，人和动物不断的暴露于曲霉菌孢子中。烟曲霉和黄曲霉被认为是引起人和各类动物真菌性疾病的主要病原，相比哺乳动物而言，鸟类更易感。家禽养殖场的环境条件非常适合许多真菌，包括曲霉菌的生长于繁殖。本研究的主要目的是评估广西家禽养殖场分离的主要曲霉菌的基因多样性及对抗真菌药物的敏感性。实验第一部分主要对南宁的3个养殖场以及桂林的一个（包含一个孵化场）种鸡场真菌污染的情况进行调查。调查主要采集家禽咽部样品及空气样本，采样时间一般持续几周，同时对孵化场进行了三个孵化周期的监测。最终共收集到188份烟曲霉和159份黄曲霉。实验第二部分建立或应用多位点数目可变串联序列（VNTR）多态性对黄曲霉和烟曲霉进行遗传多样性分析。本研究筛选了8 个VNTR位点对黄曲霉遗传多样性进行分析，并设计双重反应。利用筛选到的8个多态性位点对91份黄曲霉分离株，其中包括6个参考株进行分型分析，结果共产生78种基因型，其中71种基因型仅出现一次，辛普森多样性指数为0.993. 对用10个VNTR多态性位点对188个广西烟曲霉分离株进行遗传多样性分析，结果共产生142种基因型。可使用图形算法最小生成树（MST）对黄曲霉或烟曲霉进行聚类分析。实验第三部分是对来自中国和法国的177个分离株进行抗真菌药物的敏感性分析。绝大部分中国和法国分离株对伊曲康唑敏感，其中中国分离株最小抑菌浓度在0.38～0.75微克/毫升，法国分离株的最小抑菌浓度在0.19～1微克/毫升。来自曾经用化学药物进行预防的养殖场的分离株的最小抑菌浓度并不比从未使用抗真菌药物的高。药敏试验显示4个分离株对伊曲康唑耐受：2株来自中国，2株来自法国。*Cyp51A* 基因测序分析显示有11株分离株发生基因变异（3株抗药株和8株药敏株），共检测到20个点突变，其中11个点突变不引起氨基酸改变，9个点突变导致氨基酸序列的改变。氨基酸突变种有6种突变是已经报导的，但A116R, E130D和Q131H三个位点的突变是新发现的。

关键词：

烟曲霉（*Aspergillus fumigatus*）
黄曲霉（*Aspergillus flavus*）
基因分型
抗真菌
养禽场
广西

List of abbreviations

ABPA allergic bronchopulmonary aspergillosis
AFLP amplified fragment length polymorphism
ANSES Agence Nationale de sécurité Sanitaire
A_w water activity
CBS Central Bureau vor Schimmelsculture
CFU colony forming unit
ENVA Ecole Nationale Vétérinaire d'Alfort
GM galactomannan
IA invasive aspergillosis
ITZ itraconazole
MIC minimum inhibitory concentration
MLP microsatellite length polymorphism
MLST multilocus sequence typing
MLVA multiple locus VNTR analysis
MST minimum spanning tree
PCR polymerase chain reaction
RAPD random amplified polymorphic DNA
RFLP restriction fragment length polymorphism
UPGMA unweighted pair group method with arithmetic mean
VNTR variable number tandem repeat

Abbreviations of amino acids

A alanine
D aspartic acid
E glutamic acid
F phenylalanine
H histidine
K lysine
M methionine
N asparagine
Q glutamine
R arginine
T threonine
V valine
Y tyrosine

Glossary

aflatoxins: Toxic metabolites (aflatoxins B1, B2, G1, G2) produced by certain fungi in or on food and feed, including peanuts, corn, and cottonseeds. The toxin was named "aflatoxin" because it was produced by some strains of *Aspergillus flavus*; *A. flavus* produces only aflatoxins B1, B2. Other fungal species are also producers, mainly in the *Aspergillus* section *Flavi*: *A. parasiticus, A. nomius*, *A. bombycis, A. arachidicola* and *A. minisclerotigenes* produce both aflatoxins B and G.

anamorph: the asexual ("imperfect") state in the life cycle of a fungus. Reproduction via mitosis not involving union of two nuclei as in karyogamy and meiosis.

Ascomycota (Ascomycetes): phylum of Fungi characterized by sexual stages composed of ascocarps containing asci with ascospores.

ascocarp (syn. **ascoma** pl. **ascomata):** an ascus-containing structure.

ascospore: a haploid sexual spore formed within an ascus following karyogamy and meiosis. Characteristic of the phylum Ascomycota or spore formed in a sac-like cell known as an ascus, the shape of which aids in identification of the fungus. Often eight (8) spores formed.

ascus (pl. **asci**): the sexual, sac-like, multicellular structure in which the haploid ascospores are formed after karyogamy and meiosis. Characteristic of the phylum Ascomycota.

asexual reproduction: spores (reproductive bodies of a fungus) are formed directly from the vegetative mycelium or from specialized hyphae.

aspergilloma: a fungus ball generally found in a preformed cavity of the lung. An aspergilloma is composed of a mass of *Aspergillus* species hyphae in a matrix of cell debris and fibrin and is suspended in an air space. Aspergilloma also may occur in a nasal sinus.

cleistothecium (pl. **cleistothecia**): an enclosed, typically rounded, sexual fruiting body (ascocarp) of the Ascomycetes that contains randomly dispersed asci within. Lacking an opening or ostiole, the cleistothecium must break open before the asci are liberated.

conidiophore: a specialized simple or branched hyphal stalk at the tip or side of a hypha, bearing (or consisting of) conidiogenous cells from which conidia are produced.

conidium (pl. **conidia**): a unicellular or multicellular, non-motile, specialized asexual reproductive propagule of a fungus. Conidia are released by detachment from the conidiogenous cell (not developed by cytoplasmic cleavage like sporangiospores in a sporangium). Often referred as "external asexual spores". There are many types of conidia according to the mode of conidiogenesis.

Deuteromycota: artificial group of mitosporic fungi (also called "Fungi Imperfecti") for which sexual states remain unknown.

disseminated: (i) an infection distributed in multiple organ systems; (ii) scattering of conidia/spores upon release.

Etest: commercial tool for the determination of minimum inhibitory concentration (MIC); a plastic strip is precoated at the factory with a concentration gradient of an antimicrobial compound. The strip is then placed on a freshly seeded lawn of a fungus or bacterium. After incubation, an ellipical zone of inhibition appears if the

said microbe is susceptibile to the drug. The point at which the zone touches the strip is read as the endpoint titer expressed in µg/mL.

foot cell: (i) in *Aspergillus*, a hyphal cell at the base of a conidiophore; (ii) in *Fusarium*, the sharply angled, heel-like end at the base of the macroconidium where it attaches to the hypha.

genotype: the genetic constitution of an organism, which is modulated by the environment before being expressed as a phenotype.

galactomannan (**GM**)**:** polysaccharide, a major component of the cell wall of the mold *Aspergillus*. GM-EIA, galactomannan antigenemia enzyme immunoassay.

heterothallic: condition of sexual reproduction in which the interaction of two different thalli is necessary.

holomorph: the whole fungus in all its morphs and phases, i.e. the teleomorph (for the sexual form) and the anamorph (for the asexual form).

homothallic: condition in which sexual reproduction can occur without the interaction of two different thalli.

hypha (pl. **hyphae**): the microscopic, thread-like branching, tubular body of a filamentous fungus; it may be septate or aseptate; numerous hyphae develop to form a mycelium.

karyogamy: fusion of two sex nuclei after cell fusion (plasmogamy).

mating type: the factor determining wether a strain will or will not be able to mate with an other strain.

metula (pl. **metulae**)**:** branch of a conidiophore bearing phialides

mould (alternate spelling: **mold**): a filamentous fungus. They may be meiosporic (known sexual stage) or mitosporic (only asexual reproduction observed).

mycelium (pl. **mycelia**): a mass of hyphae, constituting the thallus of a fungus.

mycosis: a disease caused by the fungal invasion of tissues.

mycotoxin: a non-enzymatic secondary metabolite of fungal origin.

mycotoxicosis: disease due poisoning of animals and human by feed and food products contaminated by toxin-producing microfungi (there is no development of the fungus in tissues as seen in a “mycosis”)

opportunistic (of fungus, of infection): normally saprobic and usually common, but able to develop in a host rendered susceptible by predisposing factor(s), causing a disease.

phenotype: expressed (observable) traits or characteristics of an organism, regardless of whether or to what extent the traits are the result of genotype or environment, or of the interaction of both. It includes morphology, development, biochemical and physiological properties, behaviour of the organism.

phialide: a tube- or vase-shaped conidiogenous cell that gives rise to successive conidia from a fixed site without increasing in length as each conidium is produced (versus “annelloconidium”); a collarette may surround the phialide opening.

phialoconidium: a conidia-type produced by a phialide.

plasmogamy: fusion of two cells without karyogamy or a precursor to karyogamy.

polymorphism: genome segment (locus), within or outside a gene, in which alternate forms (alleles) are present. In population genetics, variation is polymorphic if all alleles are found at frequencies >1%. In clinical

genetics, a polymorphism refers to any genetic variation not known to be a direct cause of disease, in contrast with a mutation. However, the distinction between mutation and polymorphism in the latter sense may be rather fuzzy, as the path from genetic variation to disease can be sometimes very complex. In molecular epidemiology, metabolic and DNA repair gene polymorphisms are some of the markers (indicators) used to explore genetic susceptibility to develop a disease. They are considered under the hypothesis that they can affect the development of the disease only in the presence of an environmental risk factor.

saprobe (syn. **saprogen, saprotroph**)**:** an organism using dead organic matter as food (preferred term for fungi) (adj. **saprobic**, **saprogenic, saprotrophic**).

saprophyte: a plant feeding by external digestion of dead organic matter; commonly misapplied to fungi where "saprobe" is preferred.

sclerotium (pl. **sclerotia**): a resting resistant body, often rounded, composed of a hardened mass of hyphae with a dark rind., and not containing spores (must be differentiated from a "stroma" despite a similar aspect). When conditions are favorable, hyphae germinate and grow. Also linked with mycotoxin production in some fungal species.

sexual reproduction: zygote / spore formation follows the fusion of two haploid nuclei.

spore: a sexual reproductive propagule as seen in an ascus or on a basidium; or an asexual propagule developed in a sporangium by a process of cytoplasmic cleavage yielding sporangiospores ("endospore"); or an asexual propagule, usually caducous, produced by external process (see "conidium").

stroma (pl. **stromata**): mass of vegetative hyphae, sometimes sclerotium-like in aspect, in which fruiting bodies containing sexual spores are formed.

systemic mycosis: a fungal disease involving one of the deep tissues or organs of the body (compare with "disseminated").

teleomorph: the sexual reproductive state of a fungus; also termed the "perfect" state.

water activity: measure of water availability. Range from zero (absence of water) to 1.0 (pure water).

Most of the glossary is adapted from Hawksworth D.L., Kirk P.M., Sutton B.C., Pegler D.N. (1995). Ainswoth & Bisby's Dictionary of the Fungi. CAB International Wallingford, UK. pp 616

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Chapter I

Introduction and outline of the thesis

Fungi of the genus *Aspergillus* are moulds, which occur most frequently in soil, water and decaying vegetation. They sporulate abundantly and the spores are easily dispersed into the environment by air. As a result of this ubiquitous presence, animals and people are constantly exposed to *Aspergillus* spores. Several species have been described as animal and human pathogens and there are many clinical manifestations in which *Aspergillus* fungi play an important role.

Aspergillus species are recognized as major human pathogens. In immunocompromised patients, especially neutropenic individuals, they can cause invasive aspergillosis, which is a life-threatening disease. Pulmonary infections arise by local tissue invasion, and further disseminate to other deep-seated organs. Invasive aspergillosis is a growing problem in hospitals, because of the increase in the number of patients undergoing bone marrow or solid organ transplantations and because of therapy with corticosteroids. The main causes of nosocomial *Aspergillus* outbreaks are attributable to construction, renovation, demolition and excavation activities. Allergic bronchopulmonary aspergillosis (ABPA) is an uncommon but serious respiratory condition characterized by chronic airway inflammation and airway damage resulting from persistent colonization by and sensitization to *Aspergillus*. ABPA is a severe complication in children, adolescents and adults with cystic fibrosis.

In animals, aspergillosis has been described in invertebrates (especially in bees and coral cnidarians) (Rypien *et al*. 2008) as well as in warm-blooded vertebrates: mammals (like dogs, horses and cattle) and birds (a large number of species) (Tell 2005). Birds are much more susceptible to the infection than mammals. Acute aspergillosis generally occurs in young birds resulting in high morbidity and mortality. The chronic form is sporadic. It causes lesser mortality and generally affects older birds, especially breeders in poultry, presenting a compromised immune system due to poor husbandry conditions (Arné *et al.* 2011). *Aspergillus* species have been isolated from lesions in wild birds since the early 1800s (Mayer 1815). Major die-offs of free-ranging wild birds have been reported from waterfowl, gulls and corvids following dumping of mouldy waste grains in areas where birds feed (Adrian *et al.* 1978, Friend *et al.* 1999, Souza *et al.* 2005, Zinkl *et al.* 1977). Infection by *Aspergillus* is also found in birds of prey, penguins and parrots held in captivity (Alvarez-Perez *et al.* 2010**,** Redig *et al.* 2005). Incidence may be elevated in debilitated birds sheltered in wildlife centres and severely impair rehabilitation success (Balseiro *et al.* 2005, Xavier *et al.* 2007). Infection by *Aspergillus* species has been reported in almost all domesticated avian species and production types: layer cockerels (Throne-Steinlage *et al.* 2003), pullets in cages (Corkish *et al.* 1982), broiler breeders (Martin *et al.* 2007) and growers of chicken (Akan

et al. 2002, Zafra *et al.* 2008) or turkey poults (Cortes *et al.* 2005, Dyar *et al.* 1984, Olias *et al.* 2010, Singh *et al.* 2009), common duck breeders (Planel *et al.* 2001), goslings (Beytut *et al.* 2004, Okoye *et al.* 1989), great rheas (Copetti *et al.* 2003), ostriches (Perelman *et al.* 2002), Japanese quails (Olson *et al.* 1969) or pigeons (Tokarzewski *et al.* 2007). Beside direct losses related to mortality, feed conversion and growth rate in recovering birds remain poor. Indeed, airsacculitis is a major reason for carcass condemnation at slaughter inspection (d'Arc Moretti *et al.* 2010, Kunkle 2003, Lupo *et al.* 2006, Stuart *et al.* 1980).

Of all *Aspergillus* species, *A. fumigatus* is the organism most frequently isolated from animal and human infections. *A. flavus* is the second most common etiologic agent of aspergillosis (Krishnan *et al.* 2009, Pasqualotto 2008). Besides these two, other aspergilli like *A. niger*, *A. nidulans* and *A. terreus* can also be the causative agents of various forms of infections in animals and humans.

In addition, some *Aspergillus* species such as *A. flavus* are pathogenic for economically important crops (maize, peanuts) (Hedayati *et al.* 2007) and can produce mycotoxins, which are a serious problem as they have adverse effects on animal and human health (Bennett & Klich 2003). Aflatoxins, which are produced by some isolates from the *Aspergillus* section *Flavi* such as *A. flavus*, *A. parasiticus*, *A. nomius* and *A. caelatus*, are highly carcinogenic, mutagenic and teratogenic secondary metabolites. It is estimated that about 5 billion people suffer from uncontrolled exposure to aflatoxins worldwide and costly preventive measures are necessary to reduce the risk of aflatoxin-induced human or animal diseases (Khlangwiset & Wu 2010, Yin *et al.* 2008).

1. The genus *Aspergillus*

1.1. Ecology

Aspergillus is one of the oldest names used for a fungal genus. Micheli described the microscopic spore-bearing structure and found that it resembled the device used by the Roman catholic clergy to sprinkle holy water during a part of the liturgy called the asperges (from the Latin verb *aspergere*) (Micheli 1729). Thom and Church published the first major monograph on the genus in 1926 (Thom & Church 1926). Since then, the genus *Aspergillus* has become one of the best-known and most studied mould groups.

Aspergillus fungi grow abundantly as saprophytes on soil and decaying vegetation such as moldy hay, organic compost piles and leaf litter. They degrade complex organic and inorganic materials and recycle carbon, nitrogen and other important elements (Proscott *et al.* 1993).

The genus *Aspergillus* comprises more than 250 species (Pitt 1994, de Hoog *et al.* 2000，Klich 2009). This number is probably an underestimation, and conflicting reports exist on the actual number of accepted species owing to the complex taxonomy of the genus (Peterson *et al.* 2008). Approximately 40 species have been reported to cause mycoses (Klich 2006). *Aspergillus fumigatus* and *A. flavus* are most frequently incriminated but other species may also cause human or animal diseases: *A. niger*, *A. terreus,* (Dixon 1992), *A. glaucus*, *A. wentii* (Denning *et al.* 1998), *A. nidulans* (in horses) (Tell 2005) and *A. sidowii* (in corals) (Toledo-Hernández *et al*., 2008, Rypien *et al*. 2008).

1.2. Reproduction and morphological features

The genus *Aspergillus* includes species with a range of different reproductive modes. Some “mitosporic” species are only known to reproduce by asexual means; they represent the anamorphs of the fungi and have been traditionally classified in the fungal phylum the Deuteromycota, which encompasses fungi lacking a known sexual state (Taylor 1999). By contrast, “meiosporic” species can also reproduce by sexual means; they are the teleomorphs of the fungi, which have been classified in the Ascomycota phylum, according to the characteristics of the spores produced. Meiosporic *Aspergillus* species exhibit either heterothallic (obligate outbreeding) or homothallic (self-fertile) sexual breeding systems. The majority of accepted *Aspergillus* species are only known to reproduce by asexual means, while approximately one-third produce sexual stage. Those that exhibit sexual cycles are overwhelmingly homothallic (Kwon-Chung & Sugui 2009), although the main

aflatoxin-producing species *A. flavus* and *A. parasiticus* are heterothallic (Ramirez-Prado *et al.* 2008). The teleomorphic *Aspergillus* species are distributed among ten different genera (Geiser 2009).

In the asexual cycle, haploid hyphae form foot cells. The conidial heads are produced at the tip of the conidiophore (or stip), which arises perpendicularly from the foot cell in the vegetative hyphae. The tip of the conidiophore swells, forming a vesicle, which can be globose, hemispherical, elliptical, flask-shaped, or clavate and which produces conidiogenous cells (phialides) on the surface (figures 1 and 2). For some species, intermediate elements (called metulae) are present between the vesicle and the phialides.

Each phialide narrows at the apex into a conidium-producing tube. The nucleus (or nuclei) in the phialide divides, and daughter nuclei pass into the conidial tube. A septum is then laid between the phialide and the conidial tube to separate the first spore. The repeated formation of conidia results in a chain of spores, with the oldest at the tip and the youngest at the bottom (basipetal chain). Conidia are spherical to elliptical in shape, extremely hydrophobic, and easily airborne when they are mature.

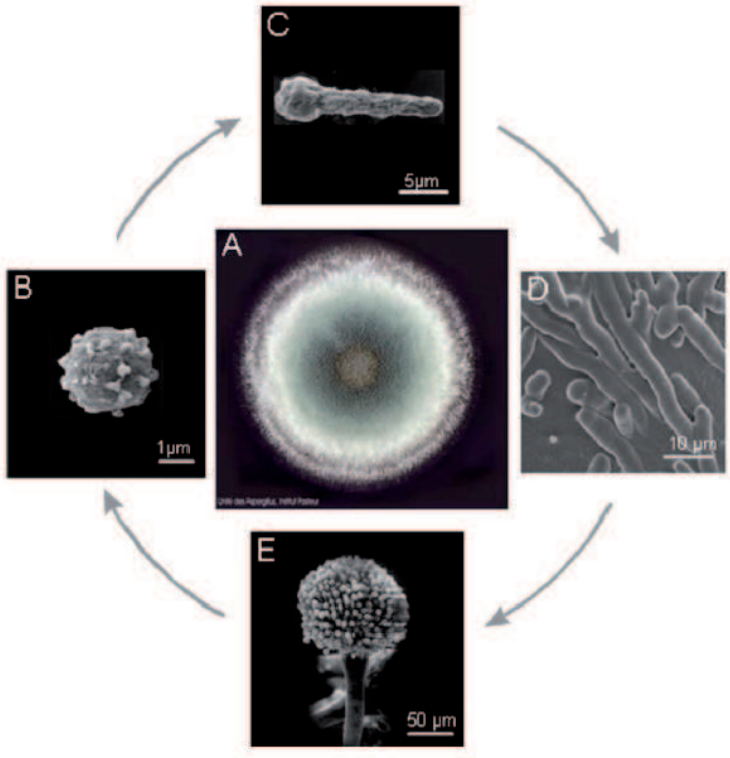


Figure 1. The asexual life cycle of fungi of the genus *Aspergillus*.
(A) macrospical aspect of a colony, (B) airborne asexual spore (phialoconidia), (C) germinative tube from a swollen condia, (D) vegetative hyphae, (E) conidial head (from Desoutter 2008)

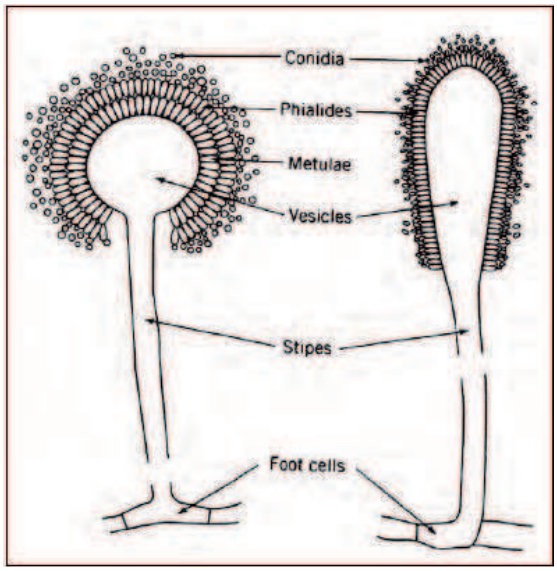


Figure 2. Conidial heads characteristic of the genus *Aspergillus*

Once these spores are released, they can grow to become haploid mycelia (Bennett 1992). If the mycelia do not come into contact with other haploid mycelia, foot cells are grown to start further asexual reproduction. If the mycelia do come into contact with other haploid mycelia, sexual reproduction may occur for some species. Conidial growth can occur from below 22°C to 55°C, while conidia can survive temperatures as high as 70°C.

During their asexual cycle, various *Aspergillus* species (e.g. *A. flavus*, *A. niger*, etc.) produce sclerotia. These spherical resting bodies composed of a mass of hyphae can germinate, and have an important role in overwintering and production of mycotoxins (Geiser *et al.* 2000, Nesci *et al.* 2007).

In the sexual cycle, haploid hyphae connect in a process called plasmogamy. Once the plasma membranes fuse, they are said to be in a dikaryon stage (n+n). The two nuclei fuse in a process called karyogamy, which occurs while the cell membrane shapes into an ascus, an elongated, oval shaped cell. This diploid ascus meiotically divides, forming eight spores referred to as ascospores. Ascospores are released from the dikaryon ascocarp into the air. If they survive, the ascospores grow into haploid mycelia and the cycle continues (Freeman *et al.* 2005).

1.3. Identification of *Aspergillus* species

Morphological identification is based on the description of the colonies and of the conidial heads.

Aspergillus species grow well on a variety of conventional media, such as Sabouraud dextrose agar. The most useful media for identification are Czapek agar and malt extract agar supplemented with antibiotics. Most

pathogenic species reach optimal growth at a temperature of 30 to 37°C but sporulation may be more abundant at 30°C. Conidial heads are usually produced within 48 to 72 hours. The color, shape, and size of the conidial heads, which are composed of the vesicle, phialides and conidial chains, are the identifying feature of the various species. The conidial heads can be bluish green or green and have a long and short, columnar shape as in *A. fumigatus* and *A. nidulans*. In all pathogenic species, the conidiophores are unbranched, mostly nonseptate and slightly pigmented or colourless. In a few species, however, one or two septa may rarely be found in the conidiophores (that can be smooth or rough). In most species, the width of conidiophores is larger than that of the hyphae, and the conidiophore wall is thicker than that of the hyphae. The conidia may be smooth, as in *A. terreus*, or echinulate, as in *A. niger*. The colour in the wall of the conidia varies from bright to dark and determines the color of the conidial head.

1.4. *Aspergillus fumigatus*

Aspergillus fumigatus belongs to the *Aspergillus* subgenus *Fumigati*, section *Fumigati* (Gams 1985). This section includes 9 anamorphic species and 24 species able to reproduce sexually (*Neosartorya* spp.). Sexual reproduction of *A. fumigatus* was described in 2009 (O'Gorman *et al.* 2009).

Colonies on Czapek and malt extract agar are usually spreading, velvety, blue-green to dark green (figure 3).

Figure 3. Macroscopical aspect of *Aspergillus fumigatus* (Parasitologie, ENVA)

Conidial heads are columnar with short, smooth-walled, 300 µm long conidiophores (figures 4 and 5). Vesicles are broadly clavate, green in upper part, usually fertile on the upper half only, 20-20 µm in diameter. They bear only phialides. Phialides are often pigmented green, 6-8 x 2-3 µm. Conidia are green, rough-walled to echinulate, subglobose to globose, 2.5-3.0 µm in diameter.

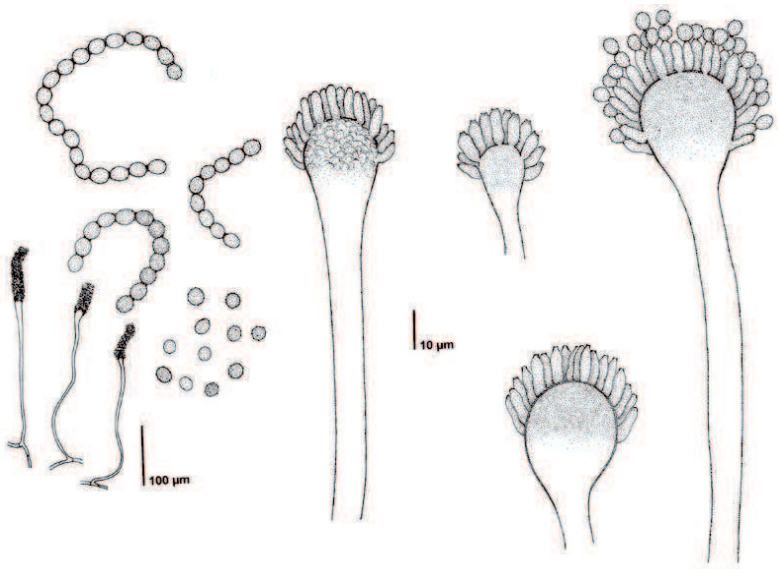


Figure 4. Microscopical aspect of *Aspergillus fumigatus* (de Hoog & Guarro 2000)

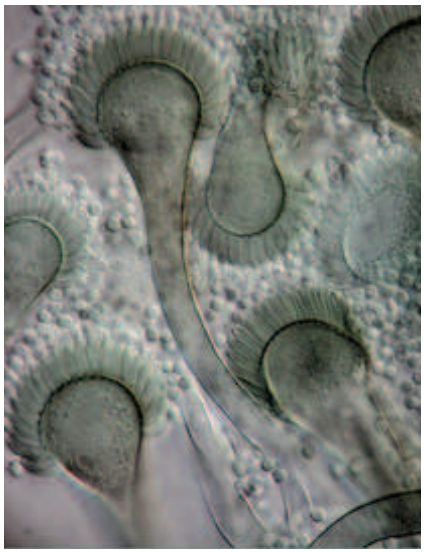


Figure 5. Microscopical aspect of *Aspergillus fumigatus* (Latgé & Steinbach 2009)

The sequencing of *A. fumigatus* (Af293) has been completed. The definite assembly indicates that the *A. fumigatus* genome is 29.4 Mb in size and consists of 8 chromosomes and 9926 predicted genes. The mean gene length is 1431 bp (Nierman *et al.* 2005).

Aspergillus fumigatus has the remarkable ability to survive extended periods of adverse environmental conditions. Because of a wide range of water activity (a_w) minimal values (0.85-0.94), *A. fumigatus* is considered as a hydrophilic but also a slightly xerophilic fungal species (eamlabs.com). The conidia exhibit resistance to a

variety of environmental stresses, including desiccation, extreme temperatures, and osmotic or oxidative stress. When favorable conditions appear, these metabolically inactive cell types will reactivate the cell biochemical and genetic machineries.

The taxonomy of *A. fumigatus* and related species was recently revised (Samson 2007). Based on molecular, morphological and physiological characters, *A. fumigatus sensu lato* is now divided into five taxa: *A. fumigatus sensu stricto*, *A. lentulus*, *A. fumigatiaffinis*, *A. novofumigatus* and *A. viridinutans*. The species are macroscopically similar. However, strains of *A. lentulus*, *A. fumigatiaffinis*, *A. novofumigatus* and *A. viridinutans* usually show less sporulation compared to typical *A. fumigatus* isolates. According to the species, there may be some differences in the width of the conidiophore and the shape and size of the vesicle. Most of the vesicles of *A. fumigatus sensu stricto* isolates are wider than 22 µm, while in the other taxa, the vesicles are narrower.

Recent molecular studies revealed that several clinical and soil isolates previously identified as *A. fumigatus* belong to another species (especially *A. lentulus*) which may exhibit altered antifungal susceptibility profiles against several antifungal drugs compared to *A. fumigatus* (Balajee et al. 2006, Mellado et al. 2006). To our knowledge, this kind of misidentification has never been reported with isolates coming from avian farms or from lesions of aspergillosis in birds.

The discovery of a sexual cycle in *A. fumigatus* provides insights into the biology and evolution of the species. It helps explain the presence of diverse genotypes despite predominantly clonal reproduction, conservation of sex-related genes (Galagan *et al.* 2005) and aspects of genome evolution. In 2009, O'Gorman *et al*. set up crosses with 12 confirmed *A. fumigatus sensu stricto* isolates in all possible combinations of opposite mating types on a range of growth media at different temperatures to determine whether it was possible to induce sexual reproduction *in vitro*. After 6 months of incubation, mature cleistothecia were detected on pairings grown on Parafilm-sealed Oatmeal agar plates at 30 °C in the dark. The light-yellow cleistothecia (150–500 µm in diameter) formed small clusters, mainly along the junction where hyphae of the parental isolates came into contact (figure 6). There was marked variation in the numbers of cleistothecia produced between different pairings. When cleistothecia were squashed, numerous yellowish-white to greenish-white lenticular ascospores (4-5 µm in diameter) with two equatorial crests were observed, together with occasional intact asci (figure 6). The teleomorph of *A. fumigatus* was assigned to the genus *Neosartorya* on the basis of phylogenetic relatedness and morphology of the cleistothecia and ascospores, and named *Neosartorya fumigata* O'Gorman, Fuller & Dyer sp. nov.

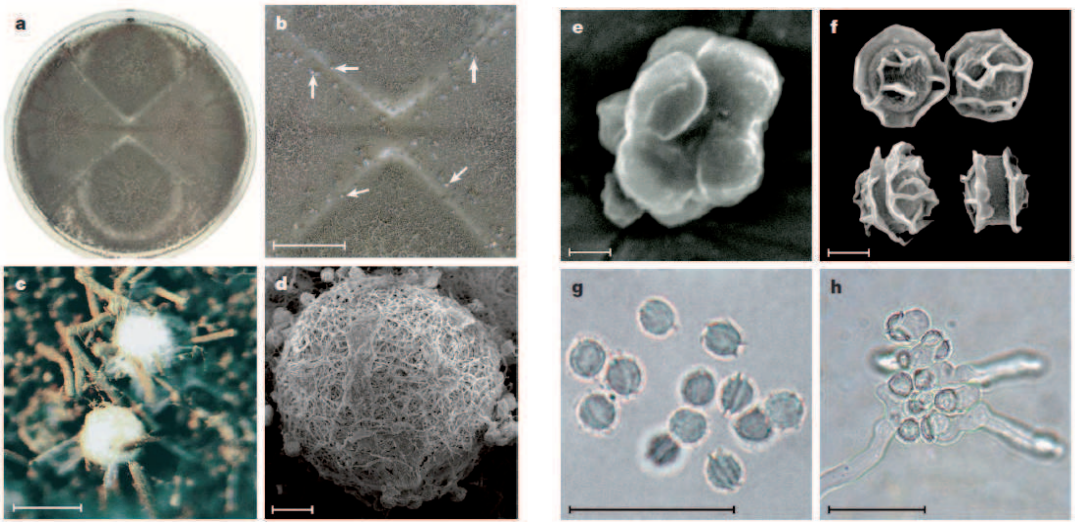


Figure 6. *Neosartorya fumigata* sp. nov.

a: paired culture incubated for 6 months at 30 °C on Oatmeal agar medium. b: cleistothecia (arrows) along junctions of intersecting colonies of opposite mating type. Scale bar 1 cm. c: cleistothecia among chains of conidia. Scale bar 400 µm. d: scanning electron micrograph of a cleistothecium. Scale bar 100 µm. e, f: scanning electron micrograph of an eight-spored ascus (e) and ascospores (f). Scale bars 2 µm. g, h: ascospores ungerminated (g) and germinating after exposure to 70 °C for 60 min (h). Scale bars 20 µm (O'Gorman *et al*. 2009).

1.5. *Aspergillus flavus*

Aspergillus flavus was described by Link in 1809. This species belongs to the *Aspergillus* subgenus *Flavi*, section *Flavi* (Gams 1985, Raper & Fennell 1965). Morphology, determination of ubiquinone systems and sequencing of the ITS region allowed Rogo *et al.* (2002) to describe 18 species and 3 varieties within the section *Flavi* which are distributed among 3 main clades: the "*A. flavus*" clade (*A. flavus*, *A. flavus* var. *columnaris*, *A. oryzae, A. parasiticus*, *A. sojae*, *A. toxicarius*, *A. subolivaceus*, *A. thomii*, *A. terricola* var. *americanus*), the "*A. tamarii*" clade (*A. tamarii*, *A. flavofurcatis, A. terricola, A. terricola* var. *indicus, A. coremiiformis*, *A. caelatus*), the "*A. alliaceus*" clade (anamorphs of *Petromyces alliaceus* and *P. albertensi*, *A. lanosus*), and 3 other species forming separate lineages (*A. nomius*, *A. avenaceus*, *A. leporis*). Species identification within *A. flavus* group remains difficult due to overlapping morphological and biochemical characteristics, including the mycotoxin production. *Aspergillus flavus* colonies reach 3-7 cm diameter in 10 days at 24-26°C on Czapek medium and 6-7 cm on malt extract medium. Their color is olive to lime green with a cream reverse. Texture is woolly to cottony to somewhat granular (figure 7). Sclerotia, when present, are dark brown and can be of two different types (S and L) according to their size (small or large, < or > 400µm diameter).

Figure 7. Macroscopical aspect of *Aspergillus flavus* (Parasitologie, ENVA)

Conidial heads are typically radiate, splitting into several poorly defined columns, most commonly 300 to 400 µm (figures 8 and 9). Conidiophores are uncoloured, coarsely roughened, usually less than 1 mm in length but in occasional strains (particularly those long maintained in laboratory cultures) up to 2.0 to 2.5 mm. The vesicles are elongate when young, later becoming subglobose or globose, varying from 10 to 65 µm in diameter but most commonly 25 to 45 µm. Phialides are either alone or associated with metulae with the two conditions rarely occurring in the same head. Phialides are sometimes elliptical when first formed, infrequently remaining so and then measuring about 4.5 to 5.5 µm by 3.5 to 4.5 µm. Conidia are smooth to very finely roughened, globose to subglobose, 3 - 6 µm in diameter.

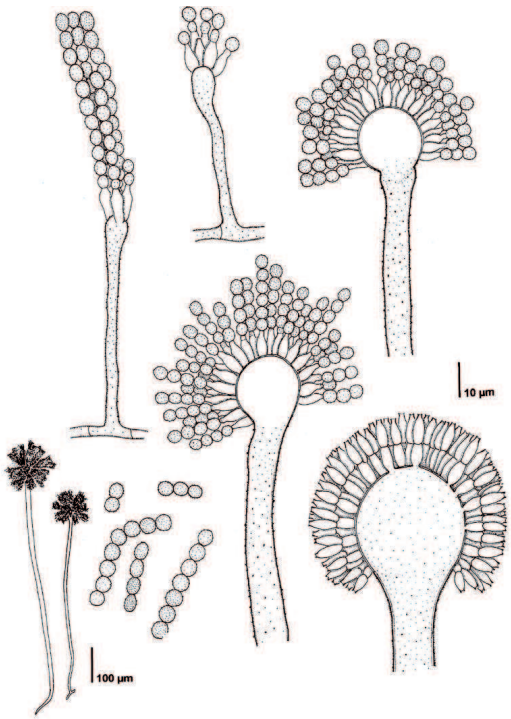


Figure 8. Microscopical aspect of *Aspergillus flavus* (de Hoog & Guarro 2000)

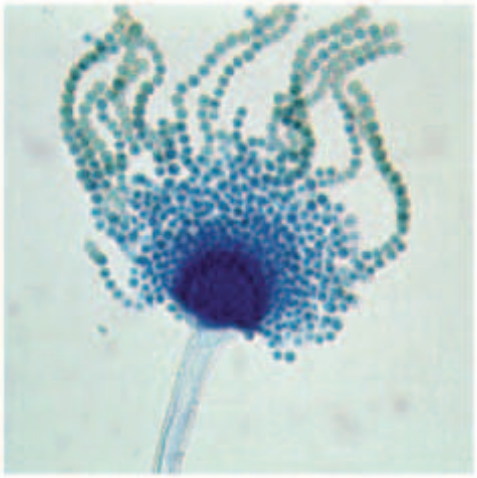


Figure 9. Microscopical aspect of *Aspergillus flavus* (Hedayati *et al.* 2007)

The sequencing of *A. flavus* (NRRL 3357) is nearing completion. The estimated genome size is just under 40Mb and consists of 8 chromosomes and 13,487 predicted genes. The mean gene length is 1,485 bp (Payne *et al.* 2006; http://www.aspergillusflavus.org/genomics/). *Aspergillus flavus* is genetically almost identical to *A. oryzae* (Chang & Ehrlich 2010, Machida *et al.* 2005).

Sexual reproduction is known for *A. flavus* and the teleomorph is *Petromyces flavus* Horn, Carbone & Moore, sp. nov. Ascocarps containing asci with usually 8 ascospores are produced within dark brown stromata that are similar to sclerotia. It is closely related to *P. parasiticus*, the teleomorph of *A. parasiticus* (Horn *et al*. 2009).

Aspergillus flavus is worldwide in its distribution but found mainly in tropical and subtropical regions. Atmosphere composition has a great impact on mould growth, with humidity being the most important variable (Gibson 1994). *Aspergillus flavus* is a xerophilic species with water activity (a_w) with minimal values of 0.78-0.80 (eamlabs.com), but it grows better between 0.86 and 0.96 a_w (Vujanovic *et al.* 2001). Unlike most fungi, *A. flavus* is favored by hot dry conditions. The optimum temperature for *A. flavus* to grow is 37°C, but fungal growth is observed at temperatures ranging from 12 to 48°C. Because of its toxin production, occurrences on food have received particular attention. In soil, the distribution does not seem to be limited by pH or soil depth. *Aspergillus flavus* was frequently isolated from samples taken at 45 cm depth and colonized hair baits exposed to soil in the range between saturation and -140 bars. It has a relatively high competitive ability. *A. flavus* has been found in the rhizosphere of wheat. Many isolations originate from seeds immediately

after harvest or after storage; the fungus was found in thorough investigations on groundnuts in various groundnut-producing countries. As to be expected from its frequent colonization of seeds, it has also been found in flour and dough products.

Aspergillus flavus has been particularly prevalent in the air of some tropical countries (Gupta *et al.* 1993, Adhikari *et al.* 2004). Climatic conditions markedly influence the prevalence of *A. flavus* in outdoor air. In Barcelona *A. flavus* and *A. niger* were the most frequent airborne aspergilli (Calvo *et al.* 1980) whereas in Madrid *A. fumigatus* was the most prevalent species (54%) (Guinea *et al.* 2005). Comparing *Aspergillus* species in the air in London, Paris, Lyon and Marseille, Mallea *et al*. (1972) showed that *A. glaucus* and *A. versicolor* group predominated in southern France. On the other hand, *A. fumigatus* represented more than 35% of the isolates in Paris and London, whereas *A. glaucus* group never exceeded 20% (Mallea *et al.* 1972). In Brussels, *A. fumigatus* was the most common whereas *A. flavus* represented only 1% of isolates (Vanbreuseghem & Nolard 1985).

1.6. Production of mycotoxins

Mycotoxins are low-molecular-weight natural products produced as secondary metabolites by filamentous fungi. These metabolites constitute a toxigenically and chemically heterogeneous assemblage. Over 300 mycotoxins are known and three genera of moulds – *Aspergillus*, *Penicillium* and *Fusarium* – are the main producers. Most *Aspergillus* species are able to produce mycotoxins that could be released in the tissues during the invasive process participating in the virulence of the fungus (e.g. gliotoxin or verruculogen produced by *A. fumigatus*) (Latgé 1999) or in mouldy crops or aliments (e.g. aflatoxins produced by *A. flavus* and *A. parasiticus*) (Yu *et al.* 2004).

The aflatoxins B1, B2, G1 and G2 are the major four toxins amongst at least 16 structurally related toxins. Aflatoxin B1 is particularly important, since it is the most toxic and potent hepatocarcinogenic natural compound ever characterized (Bennett & Klich 2003). In 1960, the aflatoxins were isolated and first characterized after the death of more than 100,000 turkey poults ("turkey X disease") was traced to the consumption of a mould-contaminated peanut meal in UK (Blount 1961, Forgacs 1962).

The diseases caused by aflatoxin consumption are called aflatoxicoses. Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune suppression, and other pathological conditions. The liver is the

primary target organ, with liver damage occurring when poultry, fish, rodents, and nonhuman primates are fed aflatoxin B1. There are substantial differences in species susceptibility. Moreover, within a given species, the magnitude of the response is influenced by age, sex, weight, diet, exposure to infectious agents (for example the HBV), the presence of other mycotoxins and pharmacologically active substances. Thousands of studies on aflatoxin toxicity have been conducted, mostly concerning laboratory models or agriculturally important species (Klangwiset & Wu 2010).

2. Molecular typing of *Aspergillus* species

Understanding pathogen distribution and relatedness is essential for determining the epidemiology of infections in hospitals or avian farms and aiding in the design of rational pathogen control methods. Typing techniques may also give a deeper understanding of the colonization pattern of patients or animals. The genetic diversity in patients or animals with aspergillosis can also be investigated to characterize the dissemination routes of isolates from the respiratory tract to the lung tissue and other organs. Several genotypic studies have been used to distinguish between individual *Aspergillus* isolates (de Valk *et al.* 2008, Hadrich *et al.* 2011).

2.1. Random amplified polymorphic DNA (RAPD)

RAPD is based on a single primer (8 to 20 base pairs (bp) long) in combination with low annealing temperatures during the PCR reaction. Under such conditions a primer can anneal to multiple positions on the genome in an aspecific fashion and a number of arbitrary fragments are generated. Following amplification, the resulting fragments are separated by agarose gel electrophoresis. The number and sizes of the obtained fragments can be determined by the number and location of annealing sites. Unrelated isolates usually yield different banding patterns.

Aufauvre-Brown *et al*. (1992) tested a total of 44 different primers on three different *A. fumigatus* isolates. An often used primer is R108, which also seems suitable for other *Aspergillus* species, including *A. flavus* (Baddley 2003, Diaz-Guerra *et al*. 2000). Several other primers suitable for typing *Aspergillus* species have been described (Heinemann *et al.* 2004, Myoken *et al.* 2003). The use of RAPD allowed to distinguish species of the *A. flavus* group. Batista *et al*. (2008) used six random primers giving a RAPD profile with very different products for each *A. flavus* strain, providing evidence of its high genetic diversity. The primer OPW-04 revealed low intraspecific variability and high interspecific variability. Among some strains previously classified as *A. flavus*, one was reclassified as *A. oryzae*, one as *A. parasiticus*, and two as *A. tamarii*. But also one strain previously identified as *A. parasiticus* was reclassified as *A. flavus*. The major problem in RAPD typing is a lack of reproducibility of patterns (variation of number, size, and intensity of bands) and the interlaboratory reproducibility.

2.2. Restriction fragment length polymorphism (RFLP) and related methods

RFLP methods are based on the fragmentation of a sample of DNA by a restriction enzyme that can recognize and cut DNA wherever a specific short sequence occurs. The resulting DNA fragments are further separated by length by agarose gel electrophoresis and transferred to a membrane via the Southern blot procedure. Hybridization of the membrane to a labelled DNA probe determines the length of the fragments that are complementary to the probe. Each profile may be considered as an allele and can be used in genetic analysis.

Different restriction enzyme and probe combinations have been analyzed for discrimination between isolates from several *Aspergillus* species. Girardin *et al*. (1993) looked for multicopy elements in the *A. fumigatus* genome and tested them in combination with the restriction enzyme *EcoRI* for their utility as an effective typing technique. This probe, called Afut1, possesses specific characteristics of a retrotransposon-like element and has been used in several RFLP studies (Lasker *et al.* 2002, Verweij *et al.* 1996). McAlpin *et al*. (1995) constructed a species specific probe for *A. flavus* named pAF28. James *et al*. (2000) demonstrated that DNA fingerprinting with the pAF28 repetitive probe is a highly reproducible and discriminatory method for tracing the transmission of human cases of *A. flavus* infection expanding its utility beyond the agricultural purpose for which it was developed. To discriminate *A. flavus* isolates from outbreak and non-outbreak related isolates, a RFLP analysis of *SmaI*-digested DNA using RAPD generated probes was performed (Buffington *et al.* 1994).

Geiser *et al*. (2000) performed a PCR–RFLP analysis of 11 nuclear genes and concluded that a collection of *A. flavus*, *A. parasiticus*, and *A. oryzae* strains could be divided into two clades (groups I and II) with group I comprising isolates of *A. oryzae*. The PCR–RFLP with Hae-III enzyme has been used to analyze the genotypic differences existing among *A. flavus* strains causing ocular infections. This method showed the same profile of bands for all isolates. For *A. flavus* complex, the RFLP techniques are generally suitable for discriminating among different species, but has insufficient discriminatory power to distinguish among unrelated isolates within a species.

In amplified fragment length polymorphism (AFLP)-analysis, first described by Vos *et al*. (1995), genomic DNA is usually cut with two restriction enzymes, one with an average cutting frequency and a second one with a higher cutting frequency. Synthetic double stranded DNA fragments are ligated to the obtained sticky ends to serve as primer binding sites in a successive PCR reaction. The ligated fragments are subsequently amplified in a PCR reaction using stringent PCR annealing temperatures. The number of fragments that will be generated can be modulated by extending the amplification primer(s) at the 3’ site with one or more selective nucleotides.

The addition of each nucleotide reduces the number of amplified fragments by a factor four. The PCR primer that spans the average-frequency restriction site is labelled. At first the primers were radioactive labelled, a major improvement is the switching from radioactive to fluorescently labelled primers for detection of fragments on a high-resolution electrophoresis platforms. A highly informative complex DNA banding pattern of 50 to 500 bp is obtained. Variations between different isolates originate by differences in the number and location of restriction enzyme recognition sites in the genome. Warris *et al*. (2003) investigated a collection of 96 clinical and environmental *A. fumigatus* isolates using the restriction enzymes *EcoRI* and *MseI* with three selective nucleotides. The different isolates could be separated from each other. With the same enzyme combination but with other selective nucleotides, the genetic diversity of *Aspergillus* section *Flavi* was examined (Montiel *et al.* 2003). This study revealed a clear separation of *A. flavus* from *A. parasiticus* but no genotypic differences between aflatoxigenic and non-aflatoxigenic producers could be detected.

2.3. Multilocus sequence typing (MLST)

MLST compares the nucleotide sequence of internal 400-500 bp regions of a series of genes (typically 6 to 10), which are present in all isolates of a particular species. For all unique sequences an allele number will be assigned in order of discovery. Each isolate is defined by the alleles at each of the sequenced housekeeping loci, which together comprise the allelic profile or sequence type (ST). Relationships among isolates are apparent by comparisons of allelic profiles: closely related isolates have identical STs, or STs that differ at only a few loci, whereas unrelated isolates have unrelated STs. The MLST built further on the success of MLEE (multilocus enzyme electrophoresis) by targeting the variation present in multiple housekeeping loci. MLST is increasingly applied as a routine typing tool that enables international comparison of isolates. MLST data have also been exploited in evolutionary and population analyses that estimate recombination and mutation rates and investigate evolutionary relationships among bacteria that are classified as belonging to the same genus. In the *Aspergillus* section *Fumigati*, the MLST method has led to the description of new pathogenic species, among which is *A. lentulus* (Balajee *et al.* 2005).

Bain *et al*. (2007) described a MLST scheme for *A. fumigatus*, where seven genes were selected to genotype a panel of 100 clinical and environmental *A. fumigatus* isolates. Among the 100 isolates analyzed, 30 STs were distinguished.

In 2008, Peterson *et al.* analyzed 460 *Aspergillus* isolates by DNA sequencing of 4 loci (the beta-tubulin,

calmodulin, ITS, and LSU rDNA and RNA polymerase II). Twelve lineages were observed. The *A. flavus* lineage included the ex-type cultures of *A. oryzae*, *A. flavus* var *columnaris*, and *A. thomii*. One species often held to be synonymous with *A. flavus*, *A. subolivaceus*, was supported as a separate lineage. The *A. parasiticus* lineage included the type strain of *A. terricola* var. *americana* and an isolate of *A. sojae*. The *A. tamarii* lineage included the type isolates of *A. flavofurcatis* and *A. terricola* (Peterson *et al.* 2008).

2.4. Microsatellite length polymorphism (MLP)

Microsatellites are short repetitive sequences that are abundantly present in the genomes of eukaryotic organisms. Different isolates can be distinguished from each other based on differences in repeat numbers. Microsatellite markers can be amplified by PCR using primers based on their flanking sequences. If one of the primers is fluorescently labelled, they can be sized very accurately using high-resolution electrophoresis platforms. The number of repeats in each marker can be deducted from the sizes of the fragments. All repeat numbers of the analyzed markers form a genotype for each individual isolate. These genotypes are easily compared to each other. Microsatellite based typing schemes yield unambiguous typing- and highly portable data.

Bart-Delabesse *et al*. (1998) were the first authors to use microsatellite markers for *A. fumigatus*. Four different dinucleotide markers were designed to discriminate between unrelated isolates. With this panel of markers, some 30.000 different genotypes can be identified. In 2005, de Valk *et al*. described another microsatellite panel called STR*Af* (Short Tandem Repeats for *A. fumigatus*). By expanding the number of markers to 9, an overwhelmingly large number of different genotypes can be distinguished. Since their first description in 1998, microsatellite markers were very frequently used for determining the epidemiology of infections in hospitals. They were also used for the characterization of isolates collected from birds and their environment. In order to elucidate the epidemiology of the different forms of avian aspergillosis in turkey farms, Fulleringer *et al*. (2003) collected 114 *A. fumigatus* isolates from sacrificed turkeys and 134 isolates from air samples. The isolates were further genotyped by microsatellite polymorphism marker analysis. Air sampling confirmed the huge diversity of *A. fumigatus* populations. Whereas older animals harbored several combinations of genotypes, 1-day-old chicks carried a unique genotype, suggesting a unique source of contamination.

Olias *et al*. (2011) reported outbreaks of invasive aspergillosis at white stork (*Ciconia ciconia*) nesting sites in Germany. Sixty-one clinical and environmental *A. fumigatus* isolates from six affected nesting sites were

genotyped by microsatellite analysis using the STRA*f* panel. The isolates showed a remarkable high genomic diversity and, contrary to the initial hypothesis, clinical and environmental isolates did not cluster significantly.

In 2011, Van Waeyenberghe *et al*. used microsatellite typing analyse 65 clinical avian isolates and 23 environmental isolates of *A. fumigatus*. The 78 genotypes that were obtained were compared with a database containing genotypes of 2514 isolates from human clinical samples and from the environment. There appeared to be no specific association between the observed genotypes and the origin of the isolates (environment, human or bird). Eight genotypes obtained from isolates of diseased birds were also found in clinical samples.

In 2012, Burco *et al*. tried to understand the source of avian aspergillosis in seabirds undergoing rehabilitation at selected northern California aquatic bird rehabilitation centers. Air, surface and water sampling was performed in three such centers and selected natural seabird loafing sites. A total of 37 *A. fumigatus* isolates from birds with confirmed aspergillosis and 42 isolates from environmental samples were identified and subsequently typed using an eight-locus microsatellite panel. Results of the study demonstrated the presence of five clonal groups, 13 genotypically related clusters, and 59 distinct genotypes. Six of the 13 genotypically related clusters contained matching genotypes between clinical isolates and local environmental isolates from the rehabilitation center in which these birds were housed.

For *A. flavus* and *A. parasiticus* seven polymorphic microsatellite loci have been characterized by Tran Dinh *et al*. (2000). Searches for microsatellite motifs were performed on genomic sequences of *Aspergillus* section *Flavi*. These 7 markers are tested on 20 *A. flavus* and 15 *A. parasiticus* isolates and yielded 2 to 11 alleles for *A. flavus* and 1 to 9 alleles for *A. parasiticus*.

Hadrich *et al.* (2010) identified microsatellite markers for *A. flavus*. Sixty-three *A. flavus* isolates (48 from Sfax, Tunisia and 15 from Marseille, France) were analyzed. The combination of all 12 markers yielded 35 different haplotypes with a 0.970 discrimination index. A 5 markers combination (AFLA1, AFLA3, AFLA7, AFM3, and AFM7) yielded 27 different alleles with a 0.952 index. Isolates from Tunisia and France (Marseille) displayed distinct haplotypes, indicating a highly significant geographical structuring in *A. flavus*. The typing of clinical and environmental *A. flavus* isolates in a hematology unit in Tunisia provided insights into its hospital epidemiology. From a heterogeneous genetic background, a cluster indicative of a clonal propagation episode within the unit could be identified. In two patients with invasive aspergillosis, the same genotype was found in clinical and environmental isolates, indicating hospital acquired colonization and infection (Hadrich *et al.* 2010).

For *A. niger,* six polymorphic loci were developed to analyze 28 isolates. These markers displayed 6 to 13 alleles,

which demonstrated diversity within *A. niger* (Esteban *et al.* 2005). For *Emericella nidulans* (teleomorph of *A. nidulans*) 5 to 12 alleles were found with 7 microsatellite loci on a collection of 44 isolates (Hosid *et al.* 2005).

2.5. Multiple locus VNTR analysis (MLVA)

The Multiple Locus Variable-number tandem-repeat Analysis (MLVA) is based on polymorphism of tandemly repeated genomic sequences called VNTR (Variable-Number Tandem-Repeats). VNTRs are classically separated into microsatellites (up to 8 bp) and minisatellites (9 bp and more) (Vergnaud *et al.* 2000). The MLVA technique has been used for the genotyping of many bacterial pathogens as well as the opportunistic yeast *Candida glabrata* (Grenouillet *et al.* 2007). For these pathogens, MLVA technique allowed to resolve closely related microbial isolates for investigation of disease outbreaks and provided information for establishing phylogenetic patterns among isolates. The MLVA technique can be performed with simple electrophoretic equipment. In 2010, Thierry *et al*. selected 10 VNTR markers located on 4 different chromosomes (1, 5, 6 and 8) of *A. fumigatus*. These markers were tested with 57 unrelated isolates from different hosts or their environment (53 isolates from avian species in France, China or Morocco, 3 isolates from humans collected at CHU Henri Mondor hospital in Créteil, France and the reference strain CBS 144.89). The Simpson index for individual markers ranged from 0.5771 to 0.8530. A combined loci index calculated with all the markers yielded an index of 0.9994. In a second step, the panel of 10 markers was used in different epidemiological situations and tested on 277 isolates, including 62 isolates from birds in Guangxi province in China, 95 isolates collected in two duck farms in France and 120 environmental isolates from a turkey hatchery in France. MLVA displayed excellent discriminatory power. The method showed a good reproducibility. The minimum spanning tree (MST) analysis revealed an interesting clustering with a clear separation between isolates according to their geographic origin rather than their respective hosts (Thierry *et al.* 2010).

The performance of typing techniques can be compared to each other with respect to their practical feasibility. Specific advantages and disadvantages are evaluated in terms of applicability, ease of use, exchangeability and reproducibility within a laboratory. Table I gives an overview of the different aspects for the typing techniques discussed before.

Table I. Characteristics of molecular techniques used for the typing of *Aspergillus* species

Techniques	Discriminatory power	Reproducibility	Exchangeability	Ease of use	Species specific	Interpretation
Random amplified polymorphic DNA	Limited	Limited	Bad	Easy	No	Moderate
Restriction fragment length polymorphism	High	Good	Bad	Difficult	Probe dept	Difficult
Multilocus sequence typing	Limited	Excellent	Excellent	Moderate	Yes	Easy
Microsatellite length polymorphism	High	Excellent	Good	Moderate	Yes	Easy
Multiple locus VNTR analysis	High	High	Good	Easy	Yes	Easy

3. Aspergillosis

3.1. Virulence factors

The importance of *A. fumigatus* (and other opportunistic *Aspergillus* species) infections is reflected by a number of reviews that have been published in the last few years concerning its biology and pathology and the great effort being made to identify virulence factors.

Virulence represents the ability of a pathogen to invade the host, overcome its natural defences and proliferate subsequently in the organism. The small size of the conidia (2-3 µm in diameter) allows their dispersal throughout the entire respiratory tract of hosts. In addition, *A. fumigatus* is a thermophilic fungus able to grow at 55°C (Beffa *et al*. 1998, Ryckeboer *et al*. 2003) and germinate efficiently at 37-40°C (Araujo & Rodrigues 2004), an essential ability to thrive in decaying organic matter and to be a mammalian or avian pathogen. It has been hypothesized that such physiological versatility contributes to its status as the predominant fungal pathogen (Hensel *et al*. 1998). It has been shown that *CgrA* gene (ortholog of a yeast nucleolar protein that functions in ribosome synthesis) is implicated in virulence of *A. fumigatus*. *CgrA* is necessary for growth at 37°C. Indeed, the Δ*cgrA* mutant has the same growth rate as the wild type at room temperature. However, when the cultures were incubated at 37°C the Δ*cgrA* mutant was unable to increase its growth rate and germinate later and lesser than the wild type. Moreover, the Δ*cgr*A mutant is hypovirulent in immunosuppressed mice and is associated with smaller fungal colonies in lung tissue (Bhabhra *et al*. 2004). The *rhbA* gene in *A. fumigatus* plays a role in a nitrogen-regulated signaling pathway (Panepinto *et al*. 2002). The ΔrhbA mutant grows as well as the wild type on solid medium containing the rich nitrogen source ammonium, but the growth is impaired on medium containing poor nitrogen sources. When the gene is deleted, the mutant is hypovirulent compared to the wild type in a mouse model of invasive aspergillosis (Panepinto *et al*. 2002). The decreased virulence of ΔrhbA mutant suggests that versatility in nitrogen utilization contributes to the growth of *A. fumigatus in vivo*. Many other genes and molecules involved in process such as cell wall composition and maintenance, resistance to the immune response, signaling, metabolism regulation and response to stress conditions, allergens and toxins have been shown to be related to *A. fumigatus* virulence (Abad *et al*. 2010).

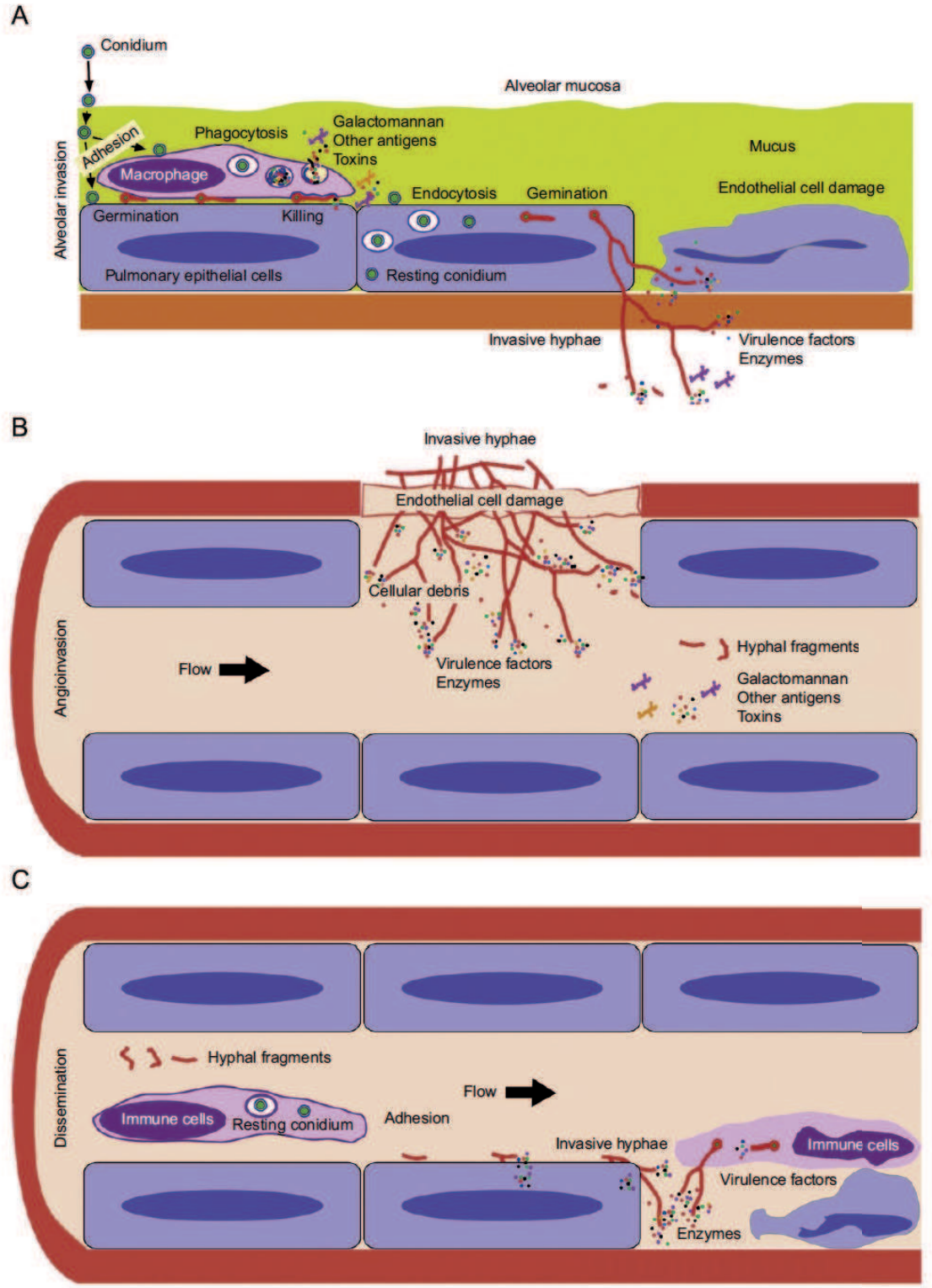


Figure 10. Model of invasive aspergillosis development (from Abad *et al*. 2010).
(A) First step of colonization and invasion of pulmonary epithelium; (B) Invasion of blood capillaries and haematogenous dissemination of hyphal fragments, galactomannan and other molecules; (C) Dissemination and first step of invasion of deep organs.

The fungus secretes various enzymes like proteases and toxic secondary metabolites (Latgé, 1999, 2001; Tekaia & Latgé, 2005). Gliotoxin is a highly immunosuppressive mycotoxin produced by various isolates of *A. fumigatus.* Concentrations exceeding 20 µg/g and 70 µg/g have been detected in poultry feedstuffs (Pena *et al.*, 2010) and in tissues obtained from turkeys with airsacculitis (Richard *et al.*, 1996), respectively. Turkey blood peripheral lymphocytes, when exposed to high levels of gliotoxin either died or exhibited a lower lymphoblastogenic response (Richard *et al.*, 1994). Considerable amounts of gliotoxin were found in lungs of turkeys just four days after experimental inoculation of *A. fumigatus* (Richard & DeBey, 1995). However the distinction of true virulence factors (Latgé, 2001) remains uncertain because either environmental or clinical isolates seem to be able to induce an aspergillosis in susceptible hosts (Tekaia & Latgé, 2005; Van Waeyenberghe *et al.*, 2011). In experimental conditions, intra-air sac inoculation of turkeys with mammalian, avian or environmental *A. fumigatus* isolates induced mortality and lesions in all groups but one (Peden & Rhoades, 1992). The species *A. fumigatus* might express virulence without requiring specific and unique fungal determinants. Indeed, the analysis of the genome of *A. fumigatus* suggests that its primary ecological niche is in plants and that opportunistic infections of animal hosts are a dead end for this fungal species (Kunkle, 2003; Tekaia & Latgé, 2005).

3.2. Immunity and host susceptibility

In mammals, the respiratory epithelium is a ciliated epithelium, covered with a layer of mucus, whose function is to eliminate inhaled particles. The mucociliary clearance will prevent the implantation of aspergillus spores. Therefore, the patients with altered airway epithelium, especially those with cystic fibrosis, bronchiectasis or chronic obstructive pulmonary disease, are more frequently colonized by *A. fumigatus*.

When *Aspergillus* fungi become established in the respiratory tract, the body responds by a non-specific immune inflammatory response. *Aspergillus* conidia are phagocytosed and killed by macrophages, while the neutrophils are active against hyphae, too large to be phagocytosed by macrophages. Activation of neutrophils leads their degranulation. The released oxygen derivatives then play a major role in eliminating *Aspergillus* hyphae by neutrophils (figure 10).

The recruitment of these cells is via the action of proinflammatory cytokines and other mediators, including the GM-CSF (granulocyte macrophage colony stimulating factor), TNF-α and IL-8. The most significant data on the

involvement of specific immune system during infection aspergillosis have been obtained from the mouse model. It was reported that such mice resistant to aspergillosis showed a Th1 cytokine profile (interferon-γ and IL-12) while the mice that developed invasive aspergillosis had a Th2 profile (IL-4 and IL-10) (Park & Mehrad 2009).

Cytokine production by airway epithelium in response to aspergillosis aggression is not well known. Indeed, most studies were performed with alveolar macrophages. However, on an alveolar cell line, there was a dose-dependent production of proinflammatory cytokines, IL-6, IL-8 and MCP-1 (monocyte chemotactic protein 1) after exposure to proteases *A. fumigatus* (Park & Mehrad 2009). These results were confirmed in primary culture of human nasal epithelial cells (HNEC) with *A. fumigatus* filtrates and other fungi in the environment. The production of these cytokines by airway epithelium is regulated by the activity of different receptors, including PAR (protease activated receptor) and TLR (toll like receptor).

Both host and fungus characteristics explain the particular susceptibility of birds to *A. fumigatus* infection. Aspergillosis is primarily an infection of the respiratory tract (Fedde, 1998; Kunkle, 2003). Birds placed in environments contaminated with aerosolized conidia may show significant pathology after only a short duration of exposure. Anatomy and physiology of the avian lung-air sac system is strikingly different from that of the bronchoalveolar lung of mammals. Nine air sacs function to move air through the lungs' gas exchange surface (Brown *et al.*, 1997; Fedde, 1998; Reese *et al.*, 2006). Upper respiratory clearance mechanisms rely on mucous-covered epithelial cells possessing cilia and lining the trachea, the primary bronchi and the roots of the secondary bronchi (Fedde, 1998; Reese *et al.*, 2006). The epithelium of the upper airway presents also a highly lytic activity (Nganpiep & Maina, 2002). When unanesthesized chickens (Corbanie *et al.*, 2006) or anesthesized pigeons (Tell *et al.*, 2006) were exposed to aerosolized populations of various size fluorescent microspheres, particles with a diameter of 3 µm or less were found throughout the respiratory tract. On the contrary, bigger spheres were confined to upper airways where mucociliary-dependent clearance might occur (Corbanie *et al.*, 2006; Tell *et al.*, 2006). Therefore, *A. fumigatus* conidia are small enough, 2-3 µm in diameter, to bypass initial physical barriers and disseminate deeply in the respiratory system. The gas pathway through lungs accounts for the susceptibility of the caudal air sacs to pathogen infections including mycosis, compared to the cranial air sacs (Fedde, 1998; Reese *et al.*, 2006). The larger diameter of *A. flavus* conidia (3.5-4.5 µm) may explain their lower pathogenicity when compared to *A. fumigatus* in experimental infections (Richard *et al.*, 1981). In a recent study (Féménia *et al.*, 2009), the effects of *Aspergillus* conidia on human respiratory cell apoptosis was

evaluated. *Aspergillus fumigatus* and *A. flavus* conidia inhibited cellular apoptosis while *A. nidulans*, *A. niger* and *A. oryzae* conidia did not. However, there were no differences in the inhibition of apoptosis by *A. fumigatus* conidia from either human, avian or environmental isolates.

The avian lung-associated immune system includes a bronchus-associated lymphoid tissue (BALT) localized at the junctions of primary and secondary bronchi and at the ostia to the air sacs, an interstitial immune system combining lymphocytes and macrophages and a phagocyte system (Klika *et al.*, 1996; Reese *et al.*, 2006). The latter should provide an immediate front line defence of the extensive gas-exchange surface area as observed in mammals (Brown *et al.*, 1997; Nganpiep & Maina, 2002). Lavages of the normal steady state avian respiratory system yield very few resident phagocytes that gather in clusters at the entrance to air capillaries (Klika *et al.*, 1996; Toth, 2000). Avian air sacs are particularly prone to contamination because they are submitted to an airflow that favours particle deposition. They have no available macrophages to remove foreign items and have an epithelial surface nearly devoid of a mucociliary transport mechanism (Brown *et al.*, 1997). By contrast, access to blood-gas barrier tissue is protected by an extensive phagocytic epithelium (Nganpiep & Maina, 2002). Furthermore, although rather refractory to elicitation by inert stimulants like nonviable *A. fumigatus* conidia (Kunkle & Rimler, 1998), the avian respiratory system responds efficiently to invasion by pathogens with a rapid influx of heterophils and macrophages from the sub-epithelial compartment and pulmonary vasculature (Nganpiep & Maina, 2002; Toth, 2000). During the acute phase response, kinetics of migration suggests that heterophils egress earlier and in larger number from the tissues to the lumen of the lungs. Those primary polymorphonuclear leukocytes are vital cellular components of innate immunity and function by killing the pathogens following phagocytosis (Kunkle & Rimler, 1998). Classical avian macrophage properties include chemotaxis, phagocytosis, pathogen elimination and cytokine production (Harmon, 1998). Many macrophages of turkeys exposed to *A. fumigatus* by aerosol 45 min earlier had conidia attached to them or had ingested one or more conidia (O'Meara & Chute, 1959). The ability of birds to respond to fungal antigens by organising a good and lasting cell-mediated response could be a determinant in infection resolution which means that one of the key events in the establishment of aspergillosis may be the resistance to phagocytosis and its slow killing *in vivo* (Latgé, 2001). Finally, if the avian respiratory system appears to lack any of the clearance mechanisms found in mammals, an effective resolution of infection largely depends on a precocious and strong recruitment of activated phagocytes (Brown *et al.*, 1997; Toth, 2000).

3.3. Human aspergillosis

The first human case of aspergillosis was described in 1842 by Bennett in Edinburgh (Bennett 1842). The first description of invasive aspergillosis as an opportunistic infection was made in 1953 at the necropsy of a window cleaner (Rankin 1953). Human infections caused by members of the genus *Aspergillus* are now considered as a major health problem worldwide. Immunocompromised individuals are at higher risk of succumbing to aspergillosis, a condition that encompasses a variety of diseases caused by members of the genus *Aspergillus*, including invasive aspergillosis, pulmonary aspergilloma, allergic bronchopulmonary aspergillosis and others (Stevens 2000) (table II).

Aspergillus fumigatus is the first agent that causes human aspergillosis; *A. flavus* is the second leading cause of aspergillosis in humans and the leading causative agent of chronic sinonasal infection in immunocompetent patients (Stevens 2000). Some evidence has been presented that *A. flavus* has more invasive potential, certainly for tissues of the upper airways.

Table II. Clinical forms of human aspergillosis

Hypersensitivity syndromes
- extrinsic asthma
- extrinsic allergic alveolitis
- allergic bronchopumonary aspergillosis
Local infections
- aspergilloma
- otomycosis
- keratitis
- subcutaneous mycetoma
Invasive infection
- pulmonary
• invasive pulmonary aspergillosis
• chronic necrotizing aspergillosis
- disseminated

Invasive aspergillosis (IA) is the clinical form observed in immunocompromised patients (figure 11). During this invasive infection, *Aspergillus* crosses through the epithelial barrier, with the possibility of embols and fungal spread (figure 10). The incidence of IA increased from 4 to 12% between 1990 and 1998 in patients with allogeneic bone marrow transplantation, and 0 to 5% in patients with autologous bone marrow transplantation. Lung disease is present in at least 90% of cases with a frequent dissemination. The main

organs affected are, in order of decreasing frequency, lung (95%), brain (10-18%), gastrointestinal tract, liver, kidneys and heart. Main risk factors of IA are deep neutropenia (<500/mm^3) and prolonged (>2 weeks) corticosteroid therapy.

Aspergilloma is a particular clinical form in which the fungus grows by forming a fungus ball in a pre-existing lung cavity. There is no tissue invasion. These cavities are often sequels following a tuberculosis, sarcoidosis or an idiopathic pulmonary fibrosis.

Allergic bronchopulmonary aspergillosis (ABPA) occurs on chronic pulmonary, first in asthmatics, but also in patients with COPD, cystic fibrosis and bronchectiasis. *A. fumigatus* is the most frequently agent isolated from airway specimens. This disease is due to fungal colonization of the airway epithelium without tissue invasion. The pathogenesis of ABPA is based on mechanisms of hypersensitivity (figure 11). Indeed, the presence of IgE demonstrates the development of a type I hypersensitivity response and the presence of precipitines in type III hypersensitivity reaction. Many antigens from *A. fumigatus* were identified as involved in these phenomena, including Aspf 1,2,3,4 and 6. This immunoglobulins elevation is also accompanied by an increased number of eosinophils. An inflammatory response is also associated with the *A. fumigatus* implantation in the airway epithelium, resulting in macrophage activation and recruitment of neutrophils. Clinically, asthma is almost constant, sometimes steroid-dependent. Indeed, the installation of an ABPA can lead to irreversible pulmonary fibrosis in the absence of treatment.

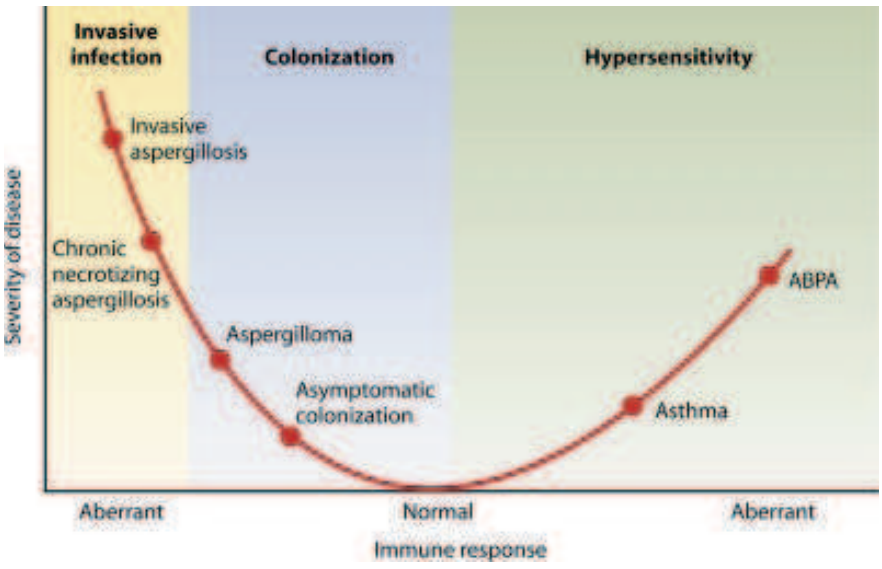


Figure 11. Diagrammatic representation of diseases attributed to *Aspergillus* species as a function of the host's immune response (Park & Mehrad 2009)

3.4. Avian aspergillosis

The first description of aspergillosis in animals was provided by Mayer in 1815, who observed the infection in the air sacs and lungs of a jackdaw (*Corvus monedula*). Any avian species, wild or domesticated, is considered susceptible to *Aspergillus* infection (Arné *et al.* 2011, Olias *et al.* 2010, Thomas *et al.* 2007). However, turkey poults have been reported to be particularly susceptible with considerable economic damage due to mortality, decreased weight gain, or condemnations at slaughter. The lung and air sacs are predominantly affected with lesions consisting of granulomas and whitish, caseous plaques (figures 12 and 13).

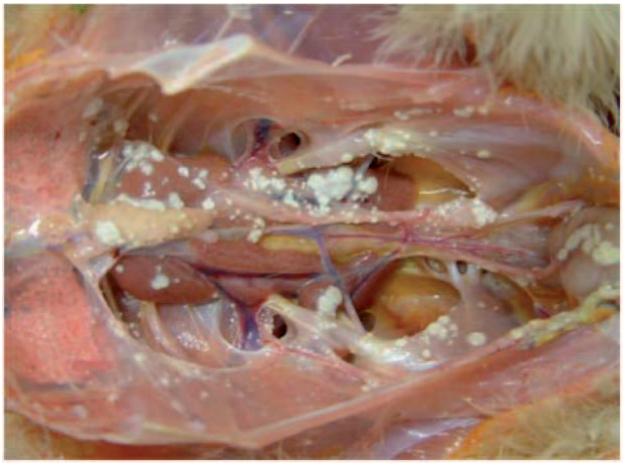


Figure 12. Aspergillosis in a cockerel. Diffuse lesions in the air sacs and on parietal and visceral serosae, with foci in varying size protruding from the surface of the affected organ, white in colour and dry in texture (Cacciuttolo *et al.*, 2009)

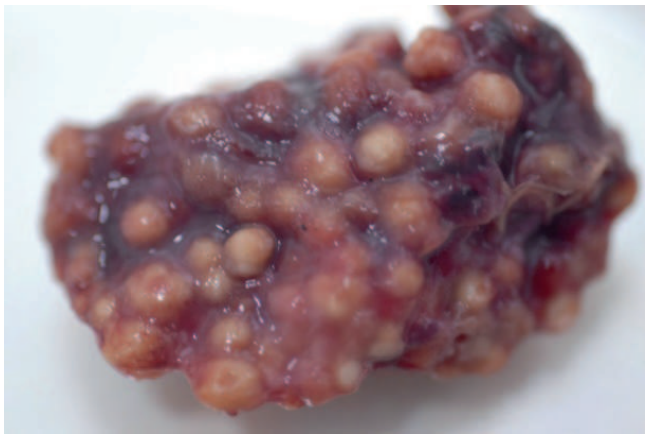


Figure 13. Aspergillosis in a duck. Multiple nodular lesions in the lung tissue (UMR BIPAR, ENVA)

Avian aspergillosis is common and could be classified into two main forms: acute or chronic disease. The acute presentation is usually a flock problem with juveniles that are exposed to high spore levels, progressing rapidly and causing death within 48 hours. In many cases it is severe and includes dyspnea, with high morbidity and mortality rates. The acute form is commonly called “brooder pneumonia”. The main route of dissemination of the fungi is through the respiratory system with only minor spread by blood circulation. The chronic presentation usually appears in individual adults that have been exposed to low spore levels but are immunosuppressed due to stress, disease, antibiotic or steroidal therapy. The bird becomes unable to effectively eliminate a normal inhalation of *Aspergillus* spores, which colonize local tissue and can cause focal and/or disseminated form of aspergillosis. The most frequent site of chronic focal form of the disease on marine avian is the trachea while the disseminated form generally starts in the lungs and air sacs and spreads to other tissues like bones, liver, spleen and kidney. The chronic presentation progresses slowly for weeks or months, with low rates of morbidity and mortality (Abundis-Santamaria *et al.* 2005, Kearns *et al.* 2003).

Aspergillus fumigatus has been reported to penetrate egg shells and infect embryos (figure 14).

Figure 14. Eggs contaminated by *Aspergillus fumigatus* (American Association of Avian Pathologists)

3.5. Treatment of fungal infections and emergence of azole resistance in *Aspergillus fumigatus*

Currently, five classes of antifungal agents are used orally or intravenously for the treatment of fungal infections (including aspergillosis) in humans or animals: polyenes, pyrimidine analogues, allylamines, azoles and the echinocandins. Benzimidazole, the first azole, was discovered in 1944 and demonstrated to be active against yeasts and several bacterial species. Approximately 100 derivatives of this compound were

subsequently synthesised (Denning & Hope 2010). In 1970, the tritylimidazole derivative clotrimazole was discovered. A few years later, phenethyl imidazole derivatives yielded miconazole, econazole and ketoconazole (the first orally bioavailable imidazole). Ketoconazole is still frequently used in veterinary medicine. Subsequently, many azoles for agricultural, veterinary and human use were developed. Modifications of the active ring produced the triazoles, and members of this class in current clinical use include fluconazole, itraconazole, voriconazole and posaconazole. Itraconazole was the first orally bioavailable agent with anti-*Aspergillus* activity (Denning *et al.* 1989). Human metabolism of itraconazole produces a metabolite, hydroxyitraconazole, which has comparable antifungal activity to the parent. The antifungal target for the azoles is the enzyme 14-α demethylase. The common imidazole or triazole ring interacts with the haem moiety of this enzyme. The side chain orientates the active site of the molecule to the haem moiety within the target protein. The interaction of the side chain within the binding pocket is responsible for the differential antifungal activity that is characteristic of these compounds. Inhibition of 14-α demethylase disrupts the synthesis of sterols that are required for normal fungal membrane function.

About half of patients with invasive aspergillosis recover if they are given triazoles. Worryingly, however, strains of *A. fumigatus* with resistance to several triazoles have been isolated from some patients in European countries, USA, Japan and China. If multi-azole resistant strains of *A. fumigatus* become common, they could have a serious impact on the management of invasive aspergillosis. Recently several investigations were made to estimate the prevalence and development of azole resistance in *A. fumigatus*.

A lot of work has been done at the Radboud University Nijmegen Medical Center in the Netherlands. Researchers from this Institute examined 1,908 *A. fumigatus* isolates that had been collected from 1,219 patients over a 14-year period (Snelders *et al*. 2008). Of these, the isolates from 32 patients grew in the presence of itraconazole. All the itraconazole-resistant isolates (which also had increased resistance to voriconazole, ravuconazole, and posaconazole) were collected after 1999; the annual prevalence of itraconazole-resistant isolates ranged from 1.7% to 6%. The researchers then sequenced the *Cyp51A* gene in each resistant isolate. Thirty had a genetic alteration represented as TR/L98H. This dominant resistance mechanism consisted of a single amino acid change in the *cyp51A* gene and an alteration in the gene's promoter region (the region that controls how much protein is made from a gene). Snelders *et al*. also analyzed *A. fumigatus* isolates from patients admitted to 28 other hospitals in the Netherlands. Itraconazole resistance

was present in isolates from 13 patients (out of 101 patients) from nine hospitals; the TR/L98H genetic alteration was present in 69% of the itraconazole-resistant isolates. Finally, itraconazole resistance was present in six isolates from four other countries (out of 317 isolates from six countries); only one Norwegian isolate had the TR/L98H genetic alteration.

Other investigations indicated that azole resistance is emerging in *A. fumigatus* and may already be more prevalent than generally thought. Given the dominance of the TR/L98H genetic alteration in the azole-resistant clinical isolates, Howard *et al*. (2009) suggested that *A. fumigatus* isolates harboring this alteration might be present and spreading in the environment rather than being selected for during azole treatment of patients. Why azole resistance should develop in *A. fumigatus* in the environment is unclear but might be caused by the use of azole-containing fungicides. Further studies are now urgently needed to find out if this is the case, to measure the international prevalence and spread of *A. fumigatus* isolates harboring the TR/L98H genetic alteration, and, most importantly, to develop alternative treatments for patients with azole-resistant aspergillosis.

Azole derivatives are frequently used in avian farms. Surprisingly, the hypothesis that the use of such drugs in farms may lead to resistance in *A. fumigatus* has never been suggested.

4. Importance of poultry in China and Guangxi

4.1. Geographical context

People's Republic of China is situated in the eastern part of Asia, on the west coast of the Pacific Ocean, in the southeastern part of the Eurasian continent, bordering the East China Sea, Korea Bay, Yellow Sea, and South China Sea, between North Korea and Vietnam. Its border countries include Afghanistan, Bhutan, Burma, India, Kazakhstan, North Korea, Kyrgyzstan, Laos, Mongolia, Nepal, Pakistan, Russia, Tajikistan, and Vietnam. China is the most populous country in the world with more than 1.347 billion persons unevenly distributed on a mainland of 9.6 millions km^2 at the end of 2011. In the last census, the proportion of urban population slightly overcomes 50% for the first time following a continuous expansion of at an average rate of 10% annually during the last decade due to massive internal migrations from the countryside to urban locations (National Bureau of Statistics of China, www.stats.gov.cn/). The impact of both urban development and reduction of population on cropland represents a major problem regarding national food security in the near future. With less than 12% of arable land (including land under temporary crops, temporary meadows for mowing or for pasture, land under market or kitchen gardens, and land temporarily fallow) each hectare must be able to feed 10 Chinese inhabitants. Despite these constraints, China remains surprisingly self-sufficient in food and even is a major net exporter of many food products (FAO, World Bank, http://data.worldbank. org/indicator/AG.LND.ARBL.zs).

Climatic zones of China

Three climatic zones can be distinguished on the vast Chinese territory due to the effects of surrounding atmosphere and topography: Qinghai-Tibet Alpine Zone, Northwest Arid and Semi-Arid Zone and East Monsoon Zone (figures 15 and 16). The latter covers 45% of the land and is characterized by important seasonally rainfalls and high solar radiation, which gives appropriate conditions for plant growth and forest development. From south to north, eastern China can be divided in a tropical zone, a subtropical zone and finally, a temperate zone. The Northwest arid and semiarid area (30% of total land) is located in inner Eurasia and influenced by a continental climate. Steppe and desert dominate the landscape with precipitation decreasing gradually from east to west from 400 mm to less than 100 mm per year. Qinghai-Tibet alpine zone (25% of total land) is an area of natural grassland and alpine forests with very few disseminated arable land patches. Solar radiation is plentiful, temperatures are low and air is rarefied due to altitude with dramatic vertical variations.

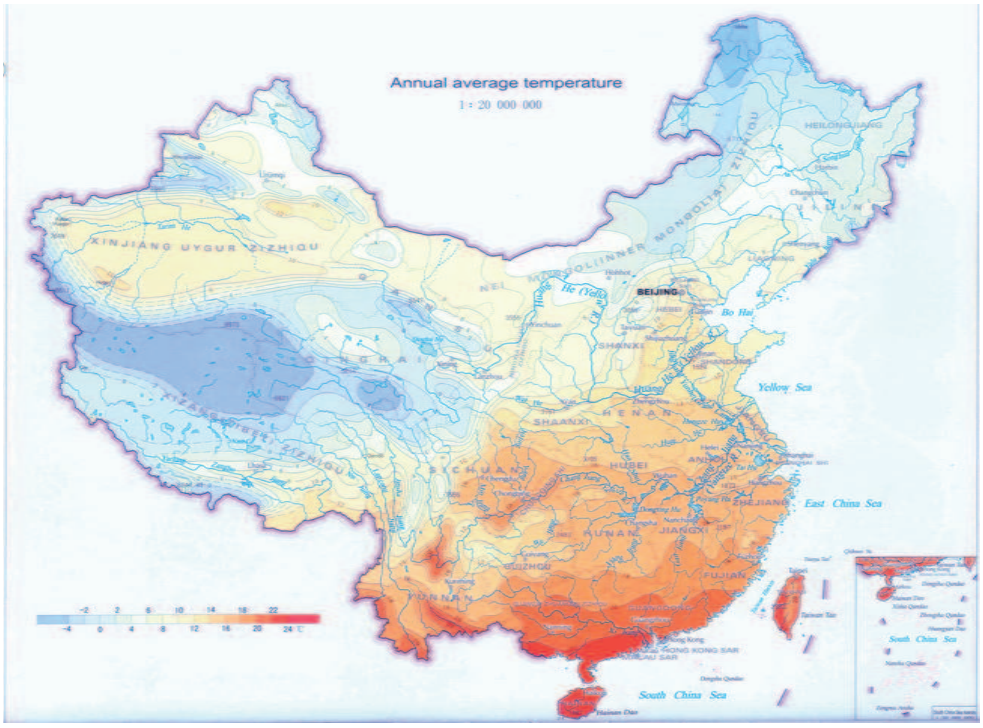


Figure 15. Annual average temperatures in China (http://www.chinamaps.org/)

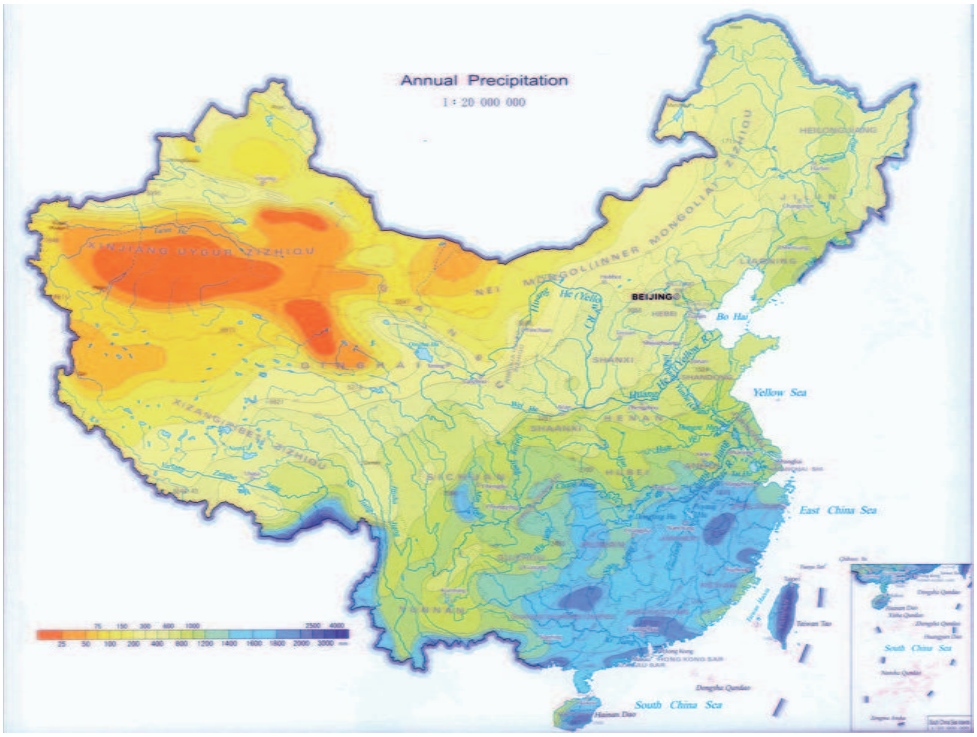


Figure 16. Annual average precipitations in China (http://www.chinamaps.org/)

Agro-ecological zones

Landform, climate, soil and land cover variations (figure 17) allow to define agro-ecological zones with specific potentials and constraint for land use. Ecological and topographical conditions, including water availability as the prominent factor, design a clear agriculture zonation (figure 18) between eastern China where crops predominate and western territories devoted to pastoralism with a transitional area called the semi pastoral belt (Verburg & van Keulen 1999, Zizhi & Degang 2006).

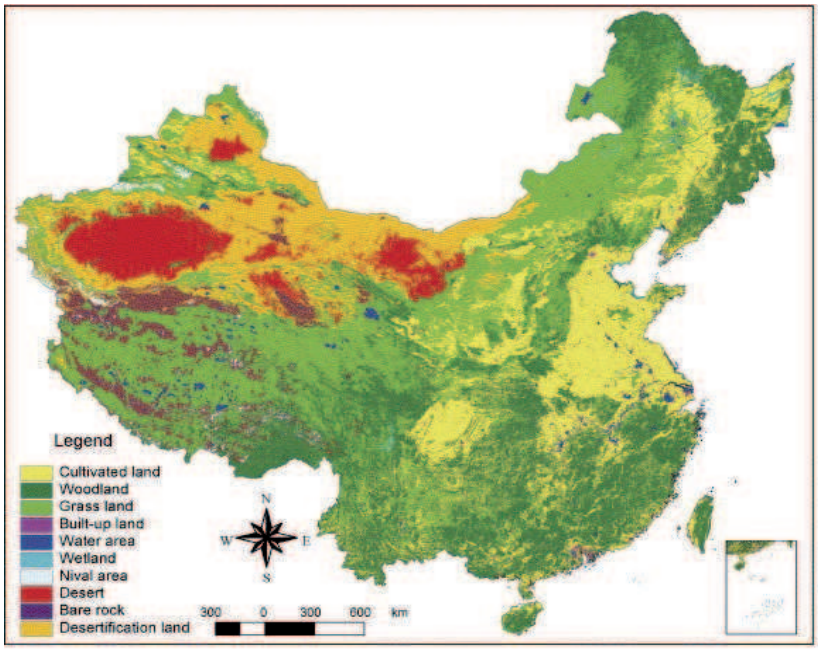


Figure 17. Land cover map in China in 2000 (Yue *et al.* 2007*)*

Figure 18. Map of China with division into pastoral and agricultural regions (Verburg & van Keulen, 1999)

Presentation of Guangxi

Guangxi Zhuang Autonomous Region is located in southern China and bordered with four Chinese provinces (Yunnan, Guizhou, Hunan and Guangdong), Vietnam and the Gulf of Tonkin (figure 19). Almost 52 millions inhabitants live in a territory of 237 000 km^2 (Bureau of Statistics of Guangxi Zhuang Autonomous Region, 2012). Its administrative status like the four other National Autonomous Regions is due to the presence of a particular minority ethnic group, represented by a high Zhuang population in Guangxi. This mountainous region belongs to the south tropical humid monsoon zone and therefore benefits from very important rainfalls and abundant heat resources. Climate is particularly favourable to agriculture and especially for production of rice, wheat, maize, sweet potatoes, peanut and many fruits.

Figure 19. Administrative divisions of the People's Republic of China. Guangxi Zhuang autonomous region is surrounded by a blue circle (Wikipedia http://en.wikipedia.org/wiki/China)

4.2. Chinese market of animal products

Meat consumption

Farm animal sector plays a considerable role in Chinese economy. During the so-called "livestock revolution" both consumption and production of meat, milk and eggs increased at rates well-above those of developing World in general and even faster than some developed countries. Therefore, the Chinese meat market has experienced a vigorous growth in the past two decades due to consumers' demand continuous development which is driven by demography, regular improved incomes, urbanisation and changes in diet habits. Per capita consumption increased from less than 9kg in 1978 to almost 57kg in 2009 with great differences according to meat type (table III). Poultry is raised throughout China on private farms and constitutes, together with fish and pork, the main source of dietary protein for Chinese people. However, significant consumption gaps of livestock products still exist between rural and urban areas, between the poor and the rich and among the provinces (higher consumption in wealthier regions such as Guangdong, Hainan, Fujian, Zhejiang, Shangai and Jiangsu). If pork is still the major meat consumed in both urban and rural areas, urban residents consume a higher proportion of poultry meat, poultry eggs and aquatic products. Central and northeast China (Shandong, Henan, Hebei, Anhui, Tianjin and Kianing) inhabitants traditionally eat more poultry eggs (Xin *et al*., 2005).

Table III. Distribution of individual meat consumption in China (China Meat Association)

Meat	Per capita meat consumption (in kg) in 2009	Percentage share of total meat consumption
Pork	36.7	65%
Poultry	12.0	21%
Beef	4.8	9%
Mutton	2.9	5%

Livestock production

China is now the largest producer of meat with 79.6×10^6 tons in 2011 and ranks first and second respectively for pork and poultry production, respectively. Consequently, pork represents by far the most important tonnage (50.5×10^6 tons), followed by poultry (27.6×10^6 tons), beef (6.48×10^6 tons) and mutton (3.9×10^6 tons). China is also a major World actor in milk (third rank with 36.6×10^6 tons), egg (first rank with 28.1×10^6 tons) (Bureau of Statistics of Guangxi Zhuang Autonomous Region, 2012).

The pig number has grown dramatically from 90 millions hogs in 1952 to more than 450 millions in 2011 accounting for half of world herd. Highest concentrations of pigs are found in Sichuan, Henan, Hunan and Shandong Provinces.

Sheep and goats are China's most important grazing animals. The herbivorous are generally bred in the semiarid steppes and deserts in the northern, western, and northwestern parts of the country. Sheep are traditionally raised by pastoral herders often belonging to ethnic minorities, in the semiarid lands of Xinjiang, Inner Mongolia, Gansu, and Sichuan. The national sheep herd has increased from $36.9x10^6$ in 1952 to nearly $133.2x10^6$ in 2001. Similarly, goats are primarily found in semiarid areas and are kept both for their milk and meat products. The economic interest of this latter species explains the development of the herd from $24.9x10^6$ in 1952 to $157.4x10^6$ of animals in 2001. Sheep and goats together are $288x10^6$ in 2001, mainly located in Shandong, Inner Mongolia, Xinjiang, and Itenan. Cattle including buffalos are more numerous in 2001 ($129x10^6$ of heads) than in 1965 ($66.6x10^6$ of heads). The number of horses followed the same trend during that period ($8.8x10^6$ *versus* 0.79). On the contrary, camel population decreased slightly (336 000 versus 448 000).

In 2001, China produced also more honey (254 000 tons) and silk (94 000 tons or 75% of the global production) than any other country in the World.

Backyard, part-time animal raising is still the dominant form of livestock production in China. Smallholders made up around 99% of all livestock operations, and produced 73% of hogs, 82% of cattle, over 60% of poultry, 73% of sheep and 65% of dairy cattle (MOA 2003a). China's current policy encourages more scale, standardization, modernization, and integration in hog farming beside numerous small "backyards" farms. Subsequently, recent structural changes have seen a decline in the proportion of rural households (Zhang 2006, Zhang & Somwaru 2004), and the emergence of specialist household producers as well as commercial, large scale enterprises especially in suburban regions of large cities which are more market oriented (Tian 2007). For example the output share of backyard (non-specialist) hog producers fell at the rate of around 2% per year from 1990 to 2001, while that of specialist hog households rose at the annual rate of 6.5% compared with a growth rate of just under 6% for commercial hog enterprises (Rae *et al.* 2006). However the household sector, whether specialized or not, still remains dominant in China's livestock production (Chen & Rozelle, 2003)

In 2009, Guangxi has ranked fourth for meat production in China with $3.7x10^6$ tons. In terms of tonnage, food production in this province is dominated by aquatic products followed by pork, eggs and poultry (table IV).

Table IV. Livestock and aquatic productions in Guangxi in 2009 (China, 2010)

Productions	Volume (in tons)	Percentage share of total production
Pork	2 323 000	35.4%
Beef	133 600	2.0%
Mutton	31 600	0.5%
Poultry	1 183 600	18.0%
Eggs	1 934 00	2.9%
Milk	807 00	1.2%
Aquatic products	2 618 100	39.9%

4.3. Focus on poultry products market in China

The Chinese poultry production has experienced a dramatic expansion and development since the past thirty years. In 2009, total poultry production represented 21% of total meat production and is forecast to account for 23% in 2012. Poultry meat becomes the best alternative meat of pork, which still dominates the meat market. Furthermore, poultry is both less grain intensive and less pollution intensive than beef and pork. The production follows the tremendous increase of broiler meat's consumption, which was multiplied by a factor 8 since 1984 to reach 12kg per capita per year, which is still low (table V).

Poultry production is dominated by chicken husbandry, which accounts for 70% of meat volume (represented by broiler chickens) and 85% of egg production. China is both major importer and exporter in the world broiler market. The country is a net importer of broiler meat in terms of volume because it imports only low-value products such as chicken feet and wings while exporting higher priced products such as de-boned chicken breast. Chicken claws, wings, and legs/feet account for the vast majority of Chinese retail chicken purchases. Claw imports dominate China's total broiler product imports, accounting for more than two-thirds of total volume essentially coming from the United States whose exports are composed of 70% of byproducts (wings and feets), which meet the preferences of Chinese consumers. Exports to Russia, Japan, and Saudi Arabia consist of processed poultry products (Han & Hertel 2003).

Table V. Chronological evolution of annual per capita chicken meat consumption in China

Year	Percentage of chicken meat in poultry meat	Percentages of poultry meat in total meat	Percentages of chicken meat in total meat
1961	**70.3**	**27.2**	**19.1**
1978	**70.4**	**13.8**	**97.0**
1985	**72.1**	**9.6**	**6.9**
1990	**71.2**	**12.3**	**8.8**
1995	**69.8**	**18.0**	**12.6**
2000	**71.4**	**20.4**	**14.6**
2005	**70.9**	**19.7**	**14.0**
2006	**71.2**	**19.7**	**14.0**
2007	**70.6**	**21.4**	**15.1**
2008	**69.9**	**21.2**	**14.8**

Waterfowl gathers two important minor species (15% of poultry Chinese meat production): duck meat ($5.7x10^6$ tons) and goose meat ($2.3x10^6$ tons) represent 69% and 94% of the World output respectively. Down and other byproducts are valuable in the market. Duck meat production grows faster than domestic consumption and may slow down in the future as far as there is no export. Finally, the Chinese have a tradition of eating duck eggs and process them into salted or preserved preparations. Turkey production is insignificant in China. Consequently, almost all turkey products mostly consumed in larger cities are imported.

Poultry is geographically concentrated in southern and eastern provinces of China although fast-growing broiler types are also raised in the North (Shandong, Liaoning, Henan and Jiangsu Province) (figure 20). There is a strong association between duck husbandry and southern lowland tropical areas devoted to rice culture (Prosser *et al*. 2011).

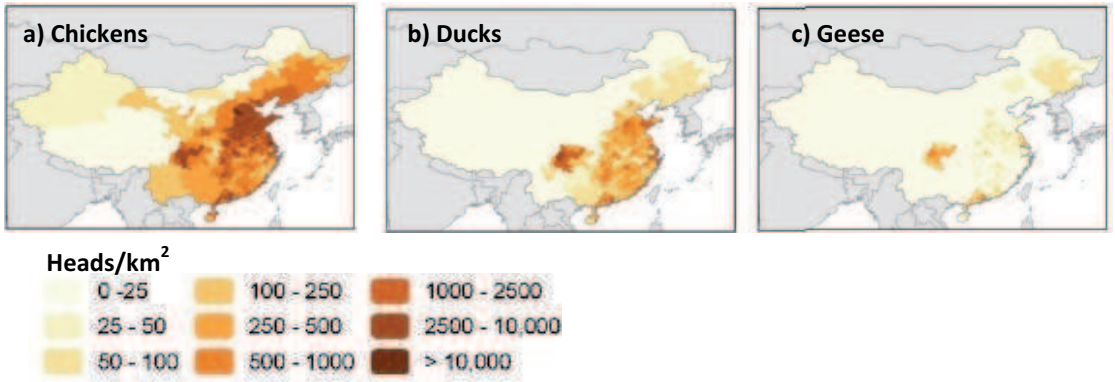


Figure 20. Observed densities for a) chicken, b) ducks and c) geese in China (Prosser *et al.* 2010)

Due to the low price of feedstuffs in the littoral regions, layer production started to shift from the traditional northern region to the southern area, such as Guangdong and Guangxi. The egg sector of China mainly produces table eggs and a small quantity of egg products. Every Chinese eats 285 eggs (= 17kg) a year.

4.4. Poultry genetic resources in China

A great variety of species and breeds

Pigeons, ducks, and geese were bred in China more than 5,000 years ago. Chickens, developed from Asian jungle fowl species, were domesticated probably about the same time (FAO 2010).

China can rely on a diversified genetic reservoir composed of numerous color-feathered local breeds to improve its poultry production. In the last 30 years, modern breeding techniques have been used to develop a lot of specialized lines and new breeds in domestic species including poultry. Economic advantages have been obtained through selection of particular characters of some local breeds, which improves the production performance by a big margin. For instance, the quality Yellow chickens widely used in Chinese production derived from the local chicken breed. They are also fully used to develop package lines of crossbreeding (FAO, 2003).

According to their phenotype combining physical traits such as size, feather and skin color, comb shape, number of toes, pattern of feathering, egg color, and their geographic origin, 81 native chicken breeds, 27 native duck breeds and 26 native geese breeds have been officially recognized in whole China (GuiFang & KuanWei 2003). Some of them have been selected and gathered on a conservation list of key breeds at the State level (table VI).

Table VI. List of key conservation poultry breeds at State level (FAO, 2003)

Species	Breeds
Chicken	Jiujinhuang Chicken, Dagu Chicken, Chinese Game Chicken, Buff Baier Chicken, Xianju Chicken, Beijing You Chicken, Sillkies, Chahua Chicken, Langshan Fowl, Qingyuan Partridge Chicken and Tibetan Chicken
Duck	Beijing Duck, Youxian Partridge Duck, Liancheng White Duck, Jianchang Duck, Jinding Duck, Shaoxing Duck, Putian Black Duck and Gaoyou Duck
Goose	Sichuan White Goose, Yili Goose, Lion Head Goose, Wanxibai Goose, Yan Goose and Huoyan Goose

Broilers and layers breeds (*Gallus gallus domesticus*)

Three types of broilers based on how fast they grow can be distinguished: fast-growing types (raised to 49 days and 1.5kg), medium type (marketed after 80 days and 1.5kg) and slow-growing birds (120 days and 1.1 to 1.5kg). Most of these 4 billions birds are produced in southern China (Guangdong, Guangxi, Anhui, Sichuan and Jiangsu province) and marketed alive at wet market (figure 21) according to dining custom. Most chicken purchased there by the household and those used in the traditional cooking is the slow-growth 'native' breed, so-called 'tu-ji'. One of the best-known Chinese broiler breeds is the three-yellow chicken whose name refers to Huang Yu (yellow beak, yellow feather and yellow shank). The soft skin must be yellow too. This family bird grows slowly, accumulates a quality fat, gives delicious meat but remains quite expensive. Yellow broilers whose products are really appreciated by Chinese consumers are mainly produced in Huanan, Huadong, Guangxi, Sichuan, Hunan and Hubei (figure 22).

Figure 21. Poultry sold in a wet market of Nanning, Guangxi (Pascal Arné, ENVA)

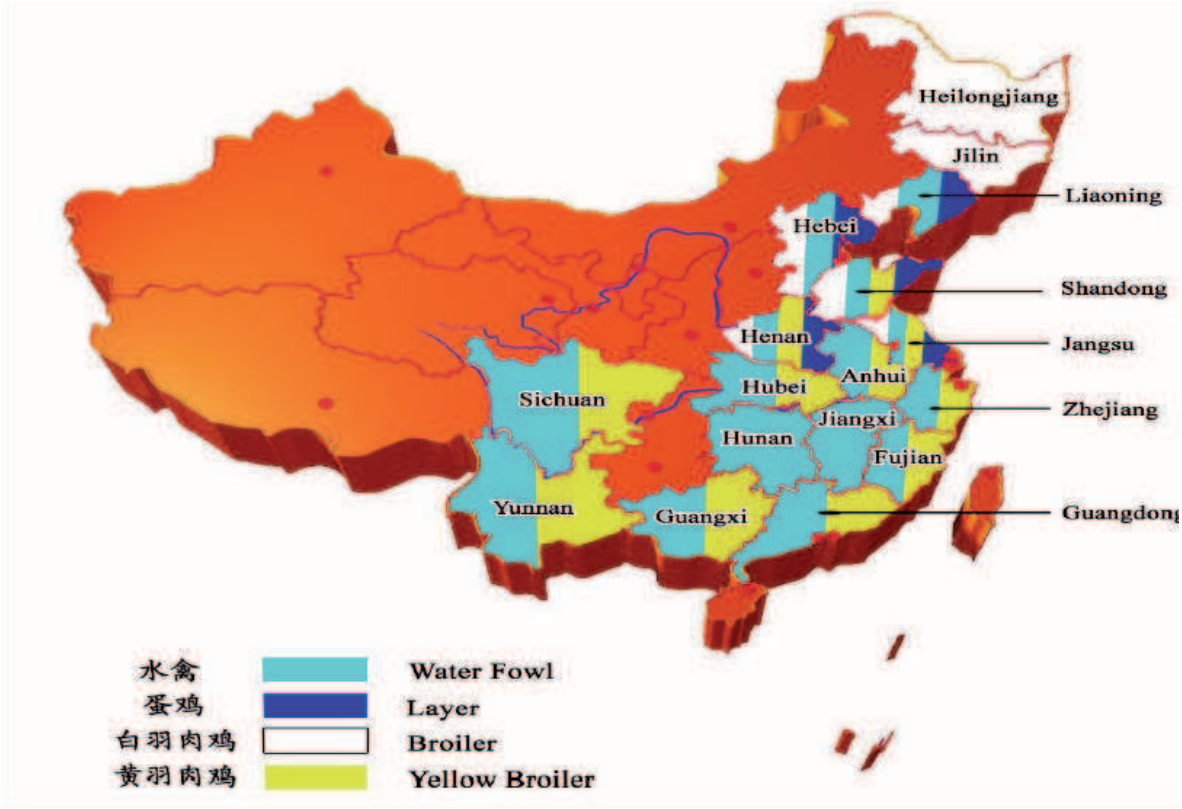


Figure 22. Geographic distribution of poultry production in China
(China Animal Agriculture Association)

Langshan chicken is also a famous native breed presenting three colour varieties (black, yellow or white). Birds are generally high with long legs and tail. They weight between 2.28 kg (female) and 2.85 kg (male). Like most of the native breeds, Langshan belongs to dual-purpose breeds and produces both meat and 135-175 brown-shelled eggs a year. Males can be caponized with profit. Beijing You Chicken and Huiyang Chicken are renowned for the tenderness and the flavor of their meat.

Beside autochthonous breeds, fast-growing white-feathered western type breeds are also reared for meat production. These heavy breeds generally exhibit high feed conversion ratio but a poor quality of the meat. Hence, broiler carcasses are generally processed (nuggets, fried chicken…) and marketed through fast-food chains (chicken burgers), supermarkets, factories restaurants, airline companies or schools. Most common breeds are Arbor Acres, Ross 308 with some Cobb500. Every year since 1980's, grandparent-breeding stock is imported from the United States. Crosses between native and commercial breeds are also used to produce broilers used in Chinese farms.

Layer breeds changed from imports dominated to current equal share with autochthonous breeds. In parallel,

egg production (1.2 billions layers), which started to shift from the traditional northern region to the southern major consumption area is still realised by millions of small and medium size farms. The average productivity of layer is 15.5kg per year. Native breeds, like Xianju Chicken (200 eggs per year weighing 50g) reared in familial facilities are generally less productive, laying small but cheap eggs.

Waterfowl species and breeds

Ducks present several advantages over other poultry species particularly their relative resistance to diseases. They are hardy, excellent foragers and easy to raise, especially in wetlands where they tend to flock together. Jinding, Jingjiang, Sansui, Putian, Youxian, Shanma, Shaoxing breeds are efficient egg-layers. The latter has an annual egg production of 280-300 with egg weight of 68-70g, which is among the highest in the world. Peking duck is a large-size meat duck famous in the world whether Gaoyou Duck, used to make processed or salted duck is particularly known for its double yolk eggs production.

Mainly local breeds of geese are still raised in China but also a few European ones like Toulouse and White Roman breeds, imported for cross-breeding purposes. The great variety in size permits the use of appropriated breeds under various management conditions. Smaller-sized birds (weighing approximately 4kg, such as the Lingxhian or Zi'e breeds in China) are chosen in small farms where they are generally kept under extensive conditions. The broodiness behaviour is generally marked in geese compared with other avian domesticated species. Consequently they lay only 30 to 40 hatching eggs (in three to five laying cycles) per year. However some smaller breeds of geese breeders, which have been counterselected for broodiness expression are very fertile and can lay 70 to 100 eggs annually like the Zi'e breed. The importance of the wide gene pool variety in China is significant for the Asian region in particular and for the world in general. Lion's head goose is the biggest breed used for meat production originated from China. Xupu breeder goose is famous for its large body size, compact body structure and rapid growth. Huoyan geese are one of the more efficient producers of eggs with an average of 129±26.5 eggs per year weighing 120-130g. Sichuan white geese, Kouxi white geese, Eastern Zhejiang white geese are excellent breeders for multiple usages. In duck and goose production, purebreds are generally used but the use of package lines is growing rapidly in order to use heterosis in commercial production (FAO, 2003). Several local breeds of chickens (Sanhuang Chicken, Xiayan, Nandan Yao, spotty plumage chicken, Longshen Feng chicken) ducks (Jingxi Large Partridge, Guangxi Small Partridge) and geese (Youjiang) can be observed in Guangxi (FAO, 2003).

4.5. Poultry systems in China

Two main systems of poultry production still coexist in parallel in China: smallholders on one part and an industrial sector on the other part.

The extensive (or pasture-based) production system

A significant part of poultry production is still realized in small scale diversified farms. These little structure generally lack management skills, stringent biosecurity measures or quality housing. Family poultry is defined as small-scale poultry keeping by households using unpaid family labor and, wherever possible, locally available feed resources. The birds range freely in the farm ("free-range" husbandry) and find much of their own food in their environment, on pastures, getting supplementary amounts from the farmer. Improved birds or local breed, may be raised in either extensive or more intensive farming systems. An original and ancestral model of species association is constituted by the duck-fish farming system found in eastern China. The origin of this common type of integration between fish culture and animal husbandry is greatly attributed to the mutual need of fish and duck for water in addition to other considerations and benefits (FAO, 2010).

The poultry sector is no longer dominated by hundreds of millions of smallholders keeping birds as a sideline activity. Many small farmers have given up production, especially in the economically more developed eastern provinces of the country.

The intensive (or cereal-oilseed protein-based) production system

Chinese poultry industry is quite diverse and scattered. It is not as tightly integrated and efficient as in western countries but more and more big companies emerge nowadays. However integration process progresses rapidly both for egg and meat production with the development of large-scale companies accounting now for more than 50% market share together with animal feed industry and slaughterhouses. This trend leads to a marked spatial and structural concentration of the national production. Specialized farmers invest heavily in large scale automated and environment controlled standard production facilities for better biosecurity, disease prevention and management. China imports massively soybean and more recently corn to feed the poultry. Additional proteins sources such as corn by products, rapeseed meal and cottonseed meal are used to complement the classical soybean-corn meal. Under intensive systems all the nutrients required by the birds must be provided in the feed, usually in the form of a balanced and mixed feed, which is precisely adapted to

physiological needs.

Tasks specialization is the rule in the industrial sector: selection (pedigree lines and crossbreeding), multiplication (parental breeders and hatcheries for egg artificial incubation and hatching) and production operations are carried on by distinct actors. At the production level, batches of thousand or even far more of one-day-old chicks are regularly delivered by the hatchery and subsequently raised indoor in huge buildings, often under confined conditions, respecting an all-in-all-out housing for sanitary and economic purposes. Under intensive conditions of production, it is particularly important to optimize building ambient air renewal (mechanical ventilation), water and feed availability, litter quality and manure management.

5. Outline of the thesis

Aspergillus fumigatus and *Aspergillus flavus* are by far predominant causes of fungal diseases in humans and animals. Birds are much more sensitive that mammals and in avian farms, environmental conditions are favorable to the development of a wide variety of fungal species, including *Aspergillus* spp.

The objective of the thesis was to assess the genetic diversity and antifungal susceptibility of *Aspergillus* isolates from avian farms in Guangxi, China.

The first part of the experimental work (chapter II) reports the evolution of fungal contamination in 3 avian farms near the city of Nanning and one farm (including a hatchery) near the city of Guilin.

The second part (chapter III) describes the genetic diversity of *A. fumigatus* and *A. flavus.* For that purpose, the Multiple Locus Variable-number tandem-repeat (VNTR) Analysis was developed and used. For *A. flavus*, VNTR markers were selected and a multiplex reaction was designed.

The third and last part (chapter IV) is about the antifungal susceptibility of *A. fumigatus* isolates collected in avian farms in China and France.

Chapter II

Fungal contamination in avian farms in Guangxi, China

1. Introduction

In animal facilities, environmental conditions are favorable to the development of a wide variety of fungal species (Duchaine *et al.*, 2000). This kind of contamination may cause health problems in workers (Chang *et al.*, 2001; Cormier *et al.*, 2000; Reboux *et al.*, 2006) and in animals, especially avian species (Fulleringer *et al.*, 2006). In order to increase poultry production, domestic birds are usually reared in confined buildings with densely stocked population. Fungi introduced with litter or feed can easily grow and sporulate under appropriate environmental conditions. Subsequently, spores or hyphae fragments can be aerosolized periodically with animal movements or air droughts.

The dynamic impact of airborne fungi on indoor agricultural air quality remains poorly understood. This is partly due to the lack of standardized sampling methodology and the need of appropriate culture media and conditions adapted for surveys in poultry farms.

Feed is a potent source of aflatoxin-producing *Aspergillus*, which may constitute a major threat to the poultry industry. Aflatoxin is expected to be present in eggs under wide range of conditions at almost all over the year because the genus *Aspergillus* is ubiquitous in nature.

The objective of the present study was to characterize the fungal contamination in avian farms in Guangxi, China. We also aimed at collecting a large number of *A. fumigatus* and *A. flavus* isolates in order to develop and test original genotyping methods.

2. Material and methods

2.1. Avian farms

The survey was performed in 4 farms from Guangxi, southwest of China: a farm with broiler chickens, a farm with ducks, a farm with laying hens near Nanning and a farm with reproductive chickens near Guilin.

The first farm (A) corresponded to a single tent, which was located inside a fruit farm. In the building, there were two groups of chickens (group 1, A1, comprised 3000 cocks; group 2, A2, comprised 2500 hens). Animals could get out the building (figure 23).

Figure 23. Chickens in the first farm (A1 and A2) near Nanning

The second farm (B) corresponded to a single pond (about 60×100m) with 3000 ducks (figure 24).

Figure 24. The pond with ducks (farm B) near Nanning

The farm of laying hens (C) comprised 2500 hens raised in cages in a confined building (figure 25).

Figure 25. The farm with laying hens (C) near Nanning

In the farm with reproductive breeders (D), a flock was selected for the investigation. It comprised 3000 hens raised in cages in a confined building (figure 26). Samples were also collected from a hatchery located at the entrance of the farm.

Figure 26. The farm with reproductive breeders (D) near Guilin

Table VII gives an overview of the different farms where the samples were collected in 2009 and 2010.

Table VII. Characteristics of the avian farms in Guangxi

	Location	**Birds**	**Nb of animals**	**Litter**	**Type of breeding**
Avian farm A	near Nanning	Broiler chickens (*Gallus gallus*)	5500	Sawdust, wood shavings	An open tent in a fruit farm
Avian farm B	near Nanning	Ducks (*Anas platyrhynchos*)	3000	none	A large pond
Avian farm C	near Nanning	Laying hens (*Gallus gallus*)	2500	none	A confined but old building with birds in cages
Avian farm D	near Guilin	Reproductive breeders (*Gallus gallus*)	3000	none	A confined and modern building with birds in cages
Hatchery	near Guilin	Chickens (*Gallus gallus*)	-	none	-

2.2. Sampling and fungal isolation

From April to June 2009, pharyngeal swabs were collected from 20 randomly selected birds from farms A and B weekly (for 8 and 6 weeks, respectively). From March to May 2009, pharyngeal swabs were collected from 15 randomly selected birds from farm C every two weeks (5 times). From March to June 2010, pharyngeal swabs were collected from 20 randomly selected birds from farm D, 12 times (a total number of 240 samples were finally collected). For the hatchery of farm D, 3 cycles of hatching were monitored by sampling both egg shells and egg chambers. For each hatching cycle, 120 eggs were randomly selected and sampled (40 eggs three times at day 0, 16 and 21).

In farms A, B, C and D, every week, serial air samples of 100 liters were made with a bio-impactor (Air Strategie Bio-impactor 100-08) loaded with ME (malt-chloramphenicol 0.5%) plates. The air sampler was sampler was placed at 20 cm from the soil (figure 27).

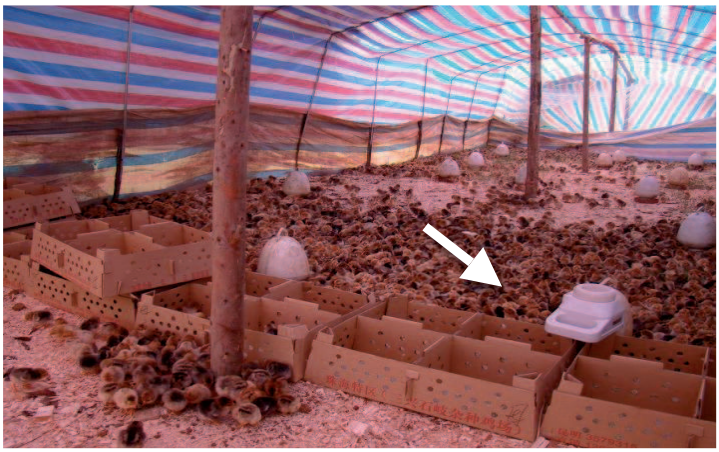


Figure 27. Air sampling with a bio-impactor (arrow) in farm A near Nanning

Pharyngeal swabs were inoculated on malt plates, which were further incubated at 37°C for 7 days. The plates were examined and examined daily. The temperature of 37°C was chosen to select the growth of the thermophilic species which may behave as opportunistic pathogens for birds. Molds were identified by their macroscopic and microscopic appearance after lactophenol cotton blue staining (de Hoog *et al*., 2000).

2.3. Statistical analyses

The results were analyzed statistically by the software Graphpad Prism5.

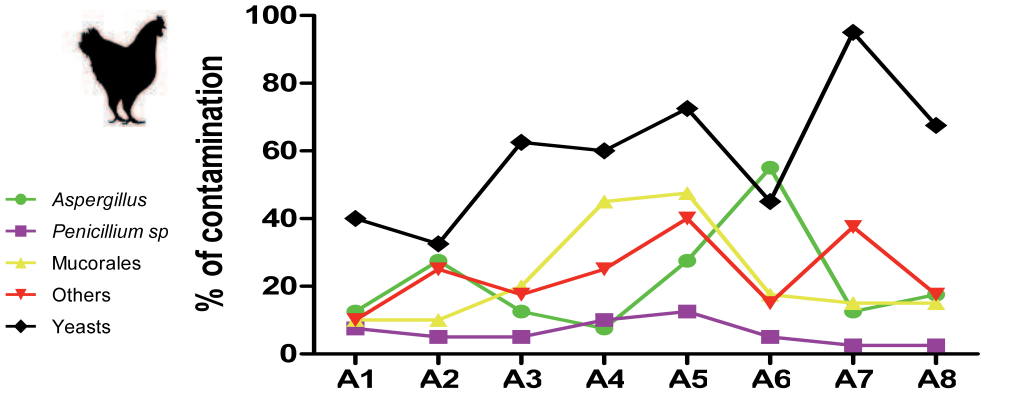
3. Results

The present survey confirmed that a wide variety of fungi, both moulds and yeasts, could be isolated from the pharynx of birds in avian farms from Guangxi. Air samples were also regularly positive for different fungal species or groups. Fungal colonies were recovered from 562 out of 755 (74.4%) pharyngeal or air samples. Figures 28-32 and tables VIII-XII provide the results for pharyngeal swabs and air samples in each farm. Results from swabs are expressed in percentages of positive (at least one colony of the specific fungus) Petri dishes. In the pharynx, the filamentous fungi most frequently isolated were: *Aspergillus* spp. (18.8%), Mucorales (18.0%) and *Penicillium* spp. (8.3%). Yeasts were also very frequently detected (66.6%). Among the genus *Aspergillus, A. fumigatus, A. niger* and *A. flavus* were the most common species (prevalence of isolation 52%, 19% and 13%, respectively). *Aspergillus terreus* was detected sporadically and there were also other *Aspergillus* species, which could not be identified microscopically. In air samples, the most frequently isolated fungi were Mucorales, *Aspergillus* spp., *Penicillium* spp. and yeasts (75.0%, 56.3%, 22.9% and 16.7%, respectively). There were also other filamentous fungi (29.2%). Among *Aspergillus* species, the dominant species isolated were *A. fumigatus*, *A. flavus* and *A. niger* (31.3%, 22.9% and 16.7%, respectively).

In farms A and B, no significant correlation could be detected between the fungal contamination of pharynx and the aerocontamination. At week 6, the contamination level was obviously high with the pharyngeal swabs whereas no *Aspergillus* sp. could be detected from the air sample.

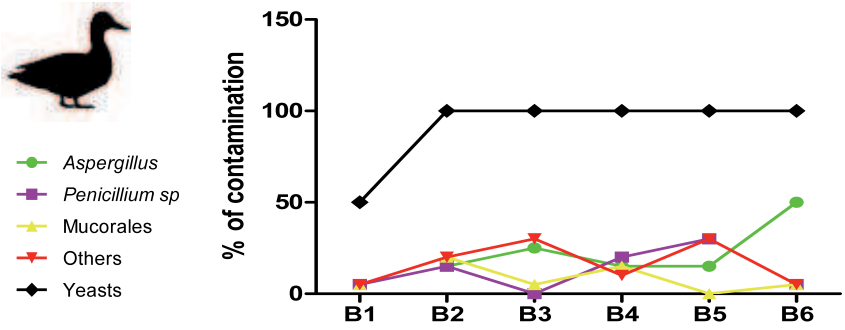
The average contamination level with *Aspergillus* spp. and Mucorales was significantly different according to the farms. In farm B (a pond with ducks), le level of contamination by filamentous fungi was lower than in farms where animals were housed in a tent or a confined building. Furthermore, it seems that the contamination by *Aspergillus* spp. varied according to the climatic conditions. In farm A and B at week 6, elevated fungal contamination was concomitant with rainfall and high humidity.

In the hatchery of farm D, the predominant species were *A. fumigatus* (up to 36.7% of Petri dishes) and *A. flavus* (up to 45.0% of the Petri dishes). *Aspergillus niger* and unidentified *Aspergillus* species were collected sporadically. *Penicillium* spp. and yeasts were isolated occasionally (data not shown). The contamination level seemed to be influenced by rainfall and humidity. During the 2nd and 3rd hatching cycles, there was high rainfall. During the surveys, no case of aspergillosis could be detected in the farms. Finally a total number of 188 *A. fumigatus* and 159 *A. flavus* isolates were collected.



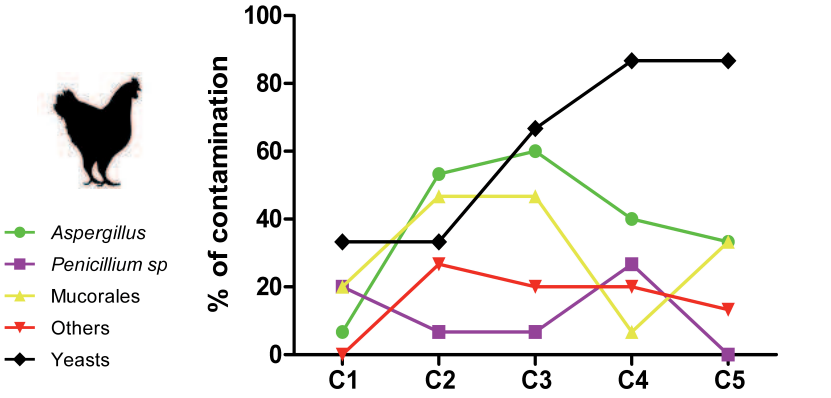
Air samples	1	2	3	4	5	6	7	8
A. fumigatus								
A. flavus								
A. niger								
Mucorale								
Yeasts								
Penicillium								
Others								

Figure 28 and table VIII. Evolution of fungal contamination in **farm A**



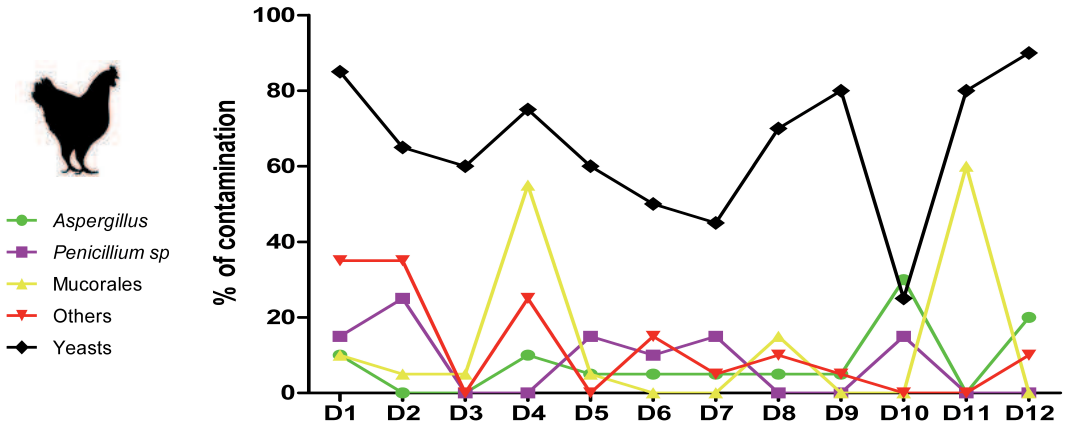
Air samples	1	2	3	4	5	6
A. fumigatus						
A. flavus						
A. niger						
Mucorale						
Yeasts						
Penicillium						
Others						

Figure 29 and table IX. Evolution of fungal contamination in **farm B**



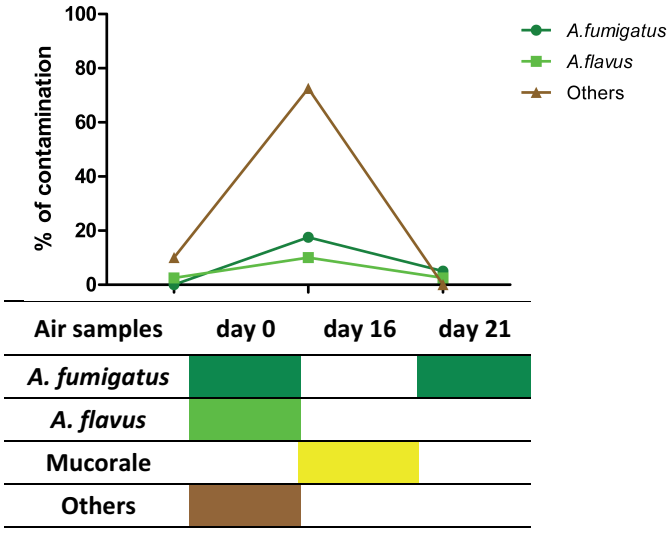
Air samples	1	2	3	4	5
A. fumigatus					
A. flavus					
A. niger					
Mucorale					
Yeasts					
Penicillium					

Figure 30 and table X. Evolution of fungal contamination in **farm C**

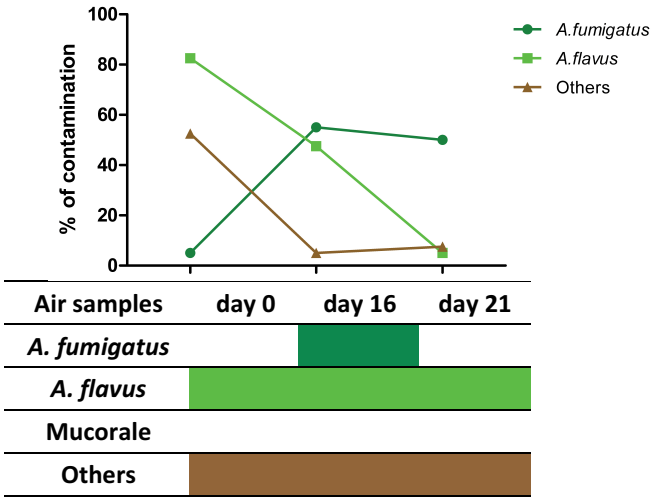


Air samples	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
A. fumigatus												
A. flavus												
A. niger												
Mucorale												
Yeasts												
Penicillium												
Others												

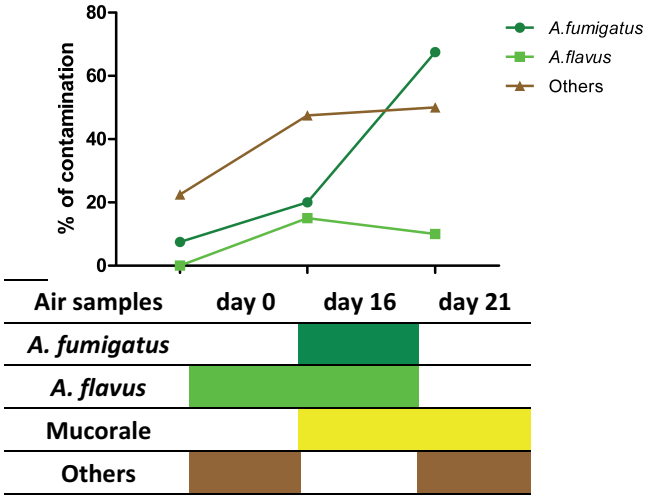
Figure 31 and table XI. Evolution of fungal contamination in **farm D**



Cycle 1



Cycle 2



Cycle 3

Figure 32 and table XII. Evolution of fungal contamination in **the hatchery of farm D**

4. Discussion

Avian farms in Guangxi were contaminated by a wide variety of fungi, including moulds and yeasts. The fungal species or groups detected in the present study were the same as those detected in avian farms in France (Fulleringer *et al.* 2006, Nieguitsila *et al.* 2011). Initial contamination of poultry farms may occur through use of a mouldy litter or introduction of one-day-old birds whose down has retained conidia in hatchery facilities. Further contamination may involve inappropriate bedding management (Dyar *et al.* 1984; Perelman & Kuttin 1992; Zafra *et al.* 2008), poor quality feedstuffs or admission of outside air loaded in conidia (Martin *et al.* 2007). Organic substrates like litter, feed and even feathers (Santos *et al.* 1996) can easily fullfil nutrient requirements of many fungal species including *A. fumigatus* or *A. flavus* (Kunkle 2003; Latgé 1999, 2001). Humidity and temperature conditions encountered in poultry farms promote the rapid growth of hyphae and efficient asexual multiplication resulting in a high production of easily airborne hydrophobic conidia, which are subsequently dispersed and inhaled by the birds (Kunkle, 2003; Pinello *et al.*, 1977). Transfers of conidia between the putative bedding reservoir (Dyar *et al.* 1984; Pinello *et al.* 1977) and indoor atmosphere are still poorly understood (Debey *et al.* 1995). Constant animal movements under high stocking densities, litter refreshing (Bacon & Burdick 1977; Dyar *et al.* 1984; Huton 1966) or deficient ventilation (Debey *et al.*, 1995; Pinello *et al.* 1977) may contribute to generate a conidial aerosol. A short-time exposure to heavily contaminated wood shavings induced an experimental pulmonary aspergillosis in chickens (Ghori & Edgar 1979; Julian & Goryo 1990) and turkeys (Fate *et al.* 1987). Birds inhale the air and contact litter with continual exposure to the conidia. Therefore, a comprehensive understanding of contributory factors leading to productive infection requires precise information on instantaneous conidia concentration on one hand and fluctuations in aerosol composition on the other hand prevailing in production facilities. Numerous longitudinal surveys have been conducted in different production systems including layers (Fate *et al.* 1987; Lovett *et al.* 1971; Lugauskas *et al.* 2004) and broilers (Bacon & Burdick 1977; Fulleringer *et al.* 2006; Gigli *et al.* 2005; Nieguitsila *et al.* 2011; Pinello *et al.* 1977; Sauter *et al.* 1981) in order to characterize the mycoflora of litter, feedstuffs and air inside the buildings. Several investigations were associated with current (Dyar *et al.* 1984; Huton 1966) or previous (Fulleringer *et al.* 2006) outbreaks of aspergillosis.

In previous investigations, air samples were collected either by sedimentation (Lugauskas *et al.* 2004; Nichita *et al.* 2010), filtration (Nieguitsila *et al.*, 2011; Pinello *et al.*, 1977; Sauter *et al.*, 1981) or impaction (Fulleringer *et al.* 2006; Lugauskas *et al.* 2004; Nieguitsila *et al.* 2011) with appropriate biocollectors (like the Air Strategie

Bio-impactor 100-08). Viable and cultivable fungi were generally counted on standard agar media designed for mycological identification (Sabouraud or malt agars most frequently). Culture-independent techniques, like PCR-TTGE or PCR-D-HPLC have been developed to monitor fungal aerosol communities in broiler farms and proved complementary with classical methods (Nieguitsila *et al.* 2007; Nieguitsila *et al.* 2010).

In farms, which were free from aspergillosis (like those in Guangxi), the concentration of *Aspergillus* spp. in the air varied from 10 to 10^4 CFU/m^3 either in chicken (Gigli *et al.* 2005; Nichita *et al.* 2010; Nieguitsila *et al.* 2011; Nieguitsila *et al.* 2007; Sauter *et al.* 1981) or turkey houses (Debey *et al.* 1995; Fulleringer *et al.* 2006). Although up to more than sixty different species have been identified in a turkey confinement brooder house (Pinello *et al.* 1977), a few genera (*Aspergillus, Penicillium*, *Cladosporium, Fusarium* and *Scopulariopis*) constitute the majority of fungal isolation (Debey *et al.* 1995; Gigli *et al.* 2005; Lugauskas *et al.* 2004; Nichita *et al.* 2010; Nieguitsila *et al.* 2011; Nieguitsila *et al.* 2007; Sauter *et al.* 1981). Prevalence and relative importance of *Aspergillus* species can vary significantly (Debey *et al.*, 1995; Fulleringer *et al.*, 2006; Lugauskas *et al.* 2004; Nichita *et al.* 2010). In fact, air contamination is characterized by cyclic variations as evidenced by weekly sampling (Fulleringer *et al.* 2006; Sauter *et al.* 1981). In several healthy turkey flocks, air concentration of *Aspergillus* spp. measured in the winter was fifteen times higher than in summer (Debey *et al.* 1995). Concentration of *Aspergillus* spp. which predominated in air and litter in a turkey farm decreased drastically when the windows were opened (Pinello *et al.* 1977) whereas no significant quantitative differences were attributable to house ventilation design in other surveys (Debey *et al.* 1995; Gigli *et al.* 2005). The negative correlation between relative humidity and the number of *Aspergillus* conidia in air may indicate that xerophilic *Aspergillus* conidia more readily discharge in dry conditions than in humid atmosphere. Interestingly, high counts of *A. fumigatus* conidia in air coincided with high levels of respirable dust particles suggesting a possible physical association or a similar response to indoor conditions (Debey *et al.*, 1995). Sawdust generated by both litter and feed, harboured numerous fungi as dormant propagules due to low moisture that could serve as an inoculum for fresh litter (Bacon & Burdick 1977; Dennis & Gee 1973; Hamet 1990).

The biodiversity of litter mycoflora depends on material choice, litter aging and handling techniques (Dennis & Gee 1973). More than thirty different taxa were identified in wood chips of turkey facilities (Pinello *et al.* 1977) and shavings in broiler houses with a predominance of *Aspergillus, Scopulariopis* and *Penicillium* (Bacon & Burdick 1977; Dennis & Gee 1973; Lovett *et al.* 1971; Pinello *et al.* 1977; So *et al.* 1978). Using an immersion

technique with strip baits, Bacon and Burdick (1977) isolated 18 fungal species from poultry litter. The same fungal species were isolated from both litter and air (Pinello *et al.* 1977). The mean total fungal counts in shavings or wood chips from broiler and layer houses ranged between 10^2 and 10^8/g with many samples exhibited variable counts for most of the species defining contrasted growth patterns (Bacon & Burdick 1977; Dennis & Gee 1973; Lovett *et al.* 1971). Global densities were slightly lower than that of the final samples of litter (Dennis & Gee 1973). Conidial populations in five broiler litters showed significant differences varying from $1.4x10^5$ to $7.8x10^5$/g (So *et al.* 1978). The role of variations in pH and moisture content on fungal population densities remained controversial although localized damp and soiled areas under troughs or feeders might promote fungal development (Bacon & Burdick 1977; Huton 1966; Lovett *et al.* 1971; So *et al.* 1978). In turkey farms of central Iowa, increased numbers of *Aspergillus* conidia correlated with higher counts of moulds in bedding suggesting that the species arose from the litter or that environmental conditions were favourable to growth and sporulation (Debey *et al.* 1995). Lair-Fulleringer *et al.* (2006) reported very low and constant densities of *A. fumigatus* in fresh straw litter in a turkey house. Lovett (Lovett, 1972) isolated a toxinogenic *A. fumigatus* from a poultry litter, which induced chick embryo death at 9 days.

Cases of acute aspergillosis have been attributed to changes in litter management. Dyar *et al.* (1984) incriminated the addition of a dry hardwood mixture, that had been used for the treatment of moist places, to explain the increased mortality of 3.5-week-old turkeys due to *A. fumigatus* infection. The added litter was highly contaminated with $2.5x10^6$ cfu/g and contained at least 25 times more fungal organisms per gram than the original litter. Subsequent treatment of the bed with a fungistatic compound reduced both mould counts and mortality. In two broiler houses, the replacement of rice-hull beddings by *A. fumigatus*-contaminated sunflowers shell was associated with a severe aspergillosis. Removal of mouldy litter resulted in health improvement (Zafra *et al.* 2008). The direct application of feed on the litter and an average concentration of $1.3x10^4$ *Aspergillus* cfu/g of wood shavings were incriminated in an outbreak of aspergillosis affecting a broiler breeder flock (Akan *et al.* 2002). The sporadic use of fresh sugarcane bagasse instead of traditional storage stacked bagasse was associated with up to 90% mortality in six flocks of young chickens on the Island of Barbados. The very high moisture of the fresh material seemed to be highly favourable for *A. fumigatus* growth and sporulation. No clinical case occurred after reintroduction of stored bagasse as litter (Huton 1966).

In a recent survey, the contamination of eggs by *Aspergillus* was up to 14% (Neamatallah *et al.* 2009). It is known that in avian aspergillosis outbreaks, the role of incubator is essential where the high humidity and optimal temperature create prerequisites for development of molds, introduced with contaminated eggs (Enev

et al. 2005). Filamentous fungi can penetrate through the egg shell during storage in storerooms, thus deteriorating their quality and making them inappropriate for consumption (Pavlov *et al.* 2006).

Mucormycoses have been reported in several avian species, including chickens (Steinlage *et al.* 2003), ducks (McCaskey & Langheinrich 1984), parakeets (Barros & Londero 1972) and penguins (Bigland *et al.* 1961).

Chapter III

Development and use of VNTR-based methods for typing *Aspergillus flavus* and *A. fumigatus* isolates from avian farms in Guangxi, China

1. Introduction

In Europe and North America, the majority of human and animal aspergillosis are caused by *Aspergillus fumigatus* (chapter I). The second most frequent pathogenic species is *Aspergillus flavus* (Krishnan *et al.* 2009, Pasqualotto 2008). In many countries from the Middle East, Africa and Southeast Asia however, *A. flavus* is the principal *Aspergillus* species causing human invasive forms of aspergillosis but also sinusitis, keratitis, endophthalmitis and cutaneous infections (Hedayati *et al.*, 2007; Khairallah *et al.*, 1992; Taj-Aldeen *et al.*, 2003; Wong *et al.*, 1997). During the epidemiological survey in avian farms in Guangxi, we demonstrated that *A. flavus* was frequently isolated from air, eggshells and the respiratory tract of birds (chapter II).

Tracking the sources of contamination is essential to prevent *A. flavus* infection in humans or animals. For that purpose, molecular tools have been proposed (Hadrich *et al.*, 2011) but the number of techniques validated for *A. flavus* is much lower than that currently used for *A. fumigatus* in hospitals or animal facilities (chapter I).

The Multiple Locus Variable-number tandem-repeat Analysis (MLVA) is based on polymorphism of tandemly repeated genomic sequences called VNTR (Variable-Number Tandem-Repeats). VNTRs are classically separated into microsatellites (up to 8 bp) and minisatellites (9 bp and more) (Chapter I). The MLVA technique has been used for the genotyping of many bacterial pathogens. It allows resolving closely related isolates for the investigation of disease outbreaks and provides information on the phylogenetic patterns among isolates (Top *et al.*, 2004; Wang *et al.*, 2009). The usefulness of MLVA was demonstrated for *A. fumigatus* by Thierry *et al*. in 2010.

The objective of the present study was to develop a new typing method based on the detection of VNTRs in the filamentous fungus, *A. flavus*. This part of the study was made with the help of Leila Hadj-Henni (master 2 in 2011).

We also described the genetic diversity of *A. fumigatus* isolates collected in avian farms in Guangxi.

2. Materials and methods

2.1. Origin of fungal isolates

In order to develop a scheme for MLVA and select discriminant VNTR markers, a total number of 36 *A. flavus* geographically or temporally unrelated isolates were selected. Thirty isolates were collected from the environment, from the pharynx or the respiratory tract of birds or from human cases of aspergillosis. Six reference strains (UMIP20.65, UMIP954.67, UMIP597.69, UMIP855.64, UMIP1145.76 and NRRL3357) were also included (table XIII).

Table XIII. Origin and period of collection for 30 unrelated isolates and 6 reference strains of *Aspergillus flavus* used for the selection of VNTRs markers

Isolates and strains no	Origin	Period of collection
Ge12, Ge20, Ge51, Ge78, Ge106	Eggshell (chicken), Guangxi, China	2009
Np124, Np121, Np130, Np127, Np134	Chicken (*Gallus gallus*), asymptomatic Carriage in pharynx*, Guangxi, China	
Nl136, Nl133, Nl153, Nl143	Chicken (*Gallus gallus*), lung samples**, Guangxi, China	
1137-1, 1137-2, V8, V11	Dogs and cats, asymptomatic carriage in hair, Paris, France	2010, 2011
Ss588, Sn1069, Ss1268, Ss19, Sn944, Ss1058***	Humans with aspergillosis, Sfax, Tunisia	2005-2007
AFL2, AFL3, AFL5, AFL6, AFL8, NLPT34	Mouldy peanuts, Benin	2009
Strain UMIP20.65	Human nasopharynx polyp, Beyrouth, Lebanon	np
Strain UMIP954.67	Human endocarditis, Montreal, Canada	np
Strain UMIP597.69	Human endocarditis, Montreal, Canada	np
Strain UMIP855.64	Plant (*Zea mays*)	np
Strain UMIP1145.76	Environment, Caen, France	np
Strain NRRL3357	Moldy peanut	np

* pharyngeal swabs were made in living animals, which were handled for regular sanitary control in the farm

** lung samples were collected from pre-deceased animals (birds that spontaneously died in the farm)

*** isolates came from the already-existing collection of the Sfax hospital in Tunisia (Pr Ali Ayadi)

np: not precised

To test the MLVA technique, a second group of 55 *A. flavus* isolates was examined. These isolates represented distinct epidemiological situations: (i) 41 isolates from avian farms in Southern China and (ii) 14 isolates from human cases of invasive aspergillosis in Tunisia (table XIV).

Table XIV. Origin and period of collection for 55 epidemiologically related isolates of *Aspergillus flavus* from China and Tunisia

Isolates no	Samples	Period of collection	Geographic origin
Ga27	Air samples	2010	**Avian farm D** in Guilin, China (Chapter II)
Ge11, Ge13, Ge15, Ge25, Ge32, Ge35, Ge40, Ge45, Ge46, Ge47, Ge50, Ge54, Ge60, Ge65, Ge98, Ge81, Ge75, Ge80, Ge81, Ge85, Ge90, Ge100, Ge181	Samples from egg surface and air chamber (chicken)		
Gp115, Gp111, Gp114	Pharyngeal swabs in chickens* (*Gallus gallus*)		
Np122, Np125，Np128, Np129 Np140, Np141, Np145, Np157	Pharyngeal swabs in chickens* (*Gallus gallus*)	2009	**Avian farms A, B, C** in Nanning, China (Chapter II)
Nl131, Nl132, Nl138, Nl164	Lung samples in chickens** (*Gallus gallus*)		
Nl167, Nl170	Lung samples in ducks** (*Anas platyrhynchos*)		
Sb16, Sb33, Sb31***	Broncho-alveolar lavages from patients with aspergillosis	2005-2007	A single hospital in Sfax,Tunisia
Ss1403, Sn855, Sn1076, Ss1368, Sn897, Ss938, Ss948, Sn887***	Sputum or nasal from patients with aspergillosis		
Se870, Se811, Se890***	Environmental sample		

* pharyngeal swabs were made in living animals, which were handled for regular sanitary control in the farm

** lung samples were collected from pre-deceased animals (birds that spontaneously died in the farm)

*** isolates came from the already-existing collection of the Sfax hospital in Tunisia (Pr Ali Ayadi)

In the present study, we also examined a large number (n=188) of *A. fumigatus* isolates collected in the four Chinese farms described in chapter II (table XV).

Table XV. Origin and period of collection for 188 epidemiologically related isolates of *Aspergillus fumigatus* from avian farms in China

Isolates no	Samples	Period of collection	Geographic origin
Gul15-23, Gul78-85	Air samples	2010	**Avian farm D** in Guilin, China (Chapter II)
Gue24-77, Gue 86-109	Samples from egg surface and air chamber (chicken)		
Gup1-14	Pharyngeal swabs in chickens* (*Gallus gallus*)		
NCP114-117，Ncp119-134, Ncp139, Ncp140, Ncp142-145, Ncl147-154, Ncp177-187, Ncp189-192	Pharyngeal swabs in chickens* (*Gallus gallus*)	2009	**Avian farms A, B, C** in Nanning, China (Chapter II)
Ncl110-113, Ncl118, Ncl135-138, Ncl141, Ncl146	Lung samples in chickens** (*Gallus gallus*)		
Ncp155, Ncp156, Ncp158-161, Ncp165-172, Ncp188,	Pharyngeal swabs in ducks** (*Anas platyrhynchos*)		
Ncl157, Ncl162-164	Lung samples in ducks** (*Anas platyrhynchos*)		

* pharyngeal swabs were made in living animals, which were handled for regular sanitary control in the farm

** lung samples were collected from pre-deceased animals (birds that spontaneously died in the farm)

All isolates from avian farms were collected specifically for this study (see chapter II). Pharyngeal samples were obtained from living animals, which were handled for regular sanitary control and lung samples were collected from pre-deceased animals (birds that spontaneously died in the farm). The animal protocol followed the *Guide for the Care and Use of Agricultural Animals* in Agricultural Research and Teaching (Federation of Animal Science Societies, third Edition, January 2010).

Aspergillus flavus isolates from infected humans were received from the already-existing collection of the Sfax hospital in Tunisia (Pr Ali Ayadi). All the isolates from humans were anonymized.

Aspergillus isolates were microscopically identified after cultivation on Malt Agar plates at 37°C until conidia formation. For all isolates, the species identification was confirmed by amplification and sequencing of partial ß-tubulin gene using primer set ßtub1 and ßtub2 (Balajee *et al.*, 2005; Balajee *et al.*, 2006).

2.2. DNA isolation

For each isolate, conidia were collected from the pure culture and transferred into a microtube for extraction. DNA extraction was performed with QIAamp DNA Kit (Qiagen) according to the manufacturor's instructions.

2.3. Selection of VNTR markers for *A. flavus*

The availability of sequences of *A. flavus* (strain NRRL3357) (http://www.aspergillusflavus.org/) allowed us to identify exhaustively tandem-repeat sequences using the Tandem Repeat Finder on-line software (http://tandem.bu.edu/trf/trf.html) (Benson, 1999). Loci with tandem repeats consisting of more than 20 nucleotides and more than 3 repeats were selected. Primers were designed using Primer Express® 2.0 software. PCR were performed in a total volume of 15 µl containing 1-5 ng of DNA, 1x mixed buffer and 0.5 µM of each primer. The initial denaturation step at 95°C for 10 min was followed by 35 cycles consisting of denaturation at 95°C for 40 s, primer annealing at 61°C for 40 s, and elongation at 72°C for 40 s. The final extension step was at 72°C for 10 min. Six microliters of amplification product were loaded onto a 2% standard agarose gel. Gels stained with ethidium bromide were visualized under UV light, and photographed. The size marker used was a Quick-load 100-bp ladder (New England BioLabs, Ipswich UK).

The alleles observed on each VNTR were sequenced to confirm the observations made on electrophoresis gel. The number of repeats was estimated from the amplicon size. The sequencing of one example of allele allowed to check whether microdeletions occurred and to evaluate the internal variation of the repeats. A total number of 50 amplicons were sequenced by Qiagen (Courtaboeuf, France) and then aligned and compared, in order to confirm the exact number of repeats.

Taking into account the maximum and minimum size of the repeats, combinations of VNTR markers were finally tested to obtain a multiplex technique with a clear and unambiguous separation of amplicons on agarose gels. All the combinations were tested by double, triple and fourfold PCR. The primer annealing temperature was the same (61°C) for all multiplex PCRs.

2.4. VNTR markers for *A. fumigatus*

For the molecular typing of *A. fumigatus* isolates, we used the markers previously selected by Thierry *et al*. (2010). These markers are located on 4 different chromosomes (1, 5, 6 and 8). Five VNTRs are on chromosome 1 (Asp_167, Asp_202, Asp_330, Asp_443 and Asp_446). VNTRs Asp_165, Asp_252 and Asp_345 are on

chromosome 5. VNTRs Asp_204 and Asp_20 are on chromosome 6 and 8, respectively. PCR were performed in a total volume of 15 µl containing 1-5 ng of DNA, 1X PCR reaction buffer, 0.5 U of Taq polymerase (Takara Bio Inc, Shiga Japan), 250 µM of each deoxynucleotide triphosphate, and 0.5 µM of each flanking primer. Primers were designed using Primer Express® 2.0 software. The initial denaturation step at 95°C for 10 min was followed by 35 cycles consisting of denaturation at 95°C for 30 s, primer annealing at 58°C for 40 s, and elongation at 72°C for 30 s. The final extension step was at 72°C for 10 min.

Ten microliters of amplification product were loaded onto a 3% standard agarose gel. Gels stained with ethidium bromide were visualized under UV light, and photographed. The size marker used was a Quick-load 100-bp ladder (New England BioLabs, Ipswich UK).

2.5. Specificity

To test the specificity of the MLVA technique for *A. flavus*, isolates from other *Aspergillus* species were also included: 4 reference strains of the *Flavi* section (*A. parasiticus* UMIP1142.76, *A. tamarii* UMIP1017.70 and *A. oryzae* UMIP1042.72, UMIP1141.76), 3 strains of other sections of the genus *Aspergillus*: *A. fumigatus* (CBS 14489), *A. niger* (CBS 733.88) and *A. nidulans* (CBS 589.65).

2.6. Stability and reproducibility

The stability of the VNTR markers was estimated by analysis of 5 distinct isolates of *A. flavus* subcultured 12 times in 2 months. The reproducibility of the method was assessed by the analysis of 25 isolates in 2 different units situated in two different buildings of the Animal Health Laboratory of ANSES (Agence Nationale de Sécurité Sanitaire) at Maisons-Alfort, France, and by 2 different technicians.

2.7. Discriminatory power

The discriminatory power was calculated by using the Simpson index of diversity (D):

$$D=1-\frac{1}{N(N-1)}\sum_{j=1}^{s} n_j(n_j-1)$$

where N is the total number of isolates in the test population (30 unrelated isolates and 6 reference strains), s is the total number of types described, and n_j is the number of isolates belonging to the j^{th} type (Hunter & Gaston 1988); a D value of 1.0 indicates that the typing method is able to discriminate between all isolates; a D value of 0.0 indicates that all isolates exhibit exactly the same genotype.

2.8. Clustering analysis for *A. flavus* and *A. fumigatus* isolates

Amplicon size was determined with Quantity One software package version 4.6.9 (Bio-Rad Laboratories, USA). The number of repeats in each allele was derived from the amplicon size. The size of flanking sequences was subtracted from the band size and the number was divided by the repeats size. The result of this calculation corresponded to the number of repeats. Data were analyzed with Bionumerics software package version 6.5 (Applied-Maths, Saint-Martens-Latem, Belgium) as a character dataset. Two different techniques were used to represent the relationships between isolates (Wang *et al.* 2009): a phenogram using phenetic UPGMA method and a graphing algorithm termed Minimum Spanning Tree (MST). The priority rule for constructing MST was set in order that the type that had the highest number of single-locus variants (SLVs) would be linked first. A cutoff value of maximum differences of one/two VNTRs out of 8/10 was applied to define cluster in the MST method.

3. Results

3.1. Development of the MLVA method for *A. flavus*

The use of the Tandem Repeat Finder software allowed the detection of 24 tandem-repeat loci with a repeat unit larger than 20 bp. These putative VNTR markers were found in the 8 chromosomes of *A. flavus*. Out of 24 tandem-repeat loci, 14 had a homology of more than 90% between the different repeats and a number of repeats higher than 3. Only 8 loci were finally deemed suitable for genotyping because they displayed variation (more than 3 alleles among the 36 tested isolates) and were present in all the isolates. Final markers were located on 6 different chromosomes (1, 2, 3, 5 7 and 8). Two markers were located on chromosome 5 (AFL5-66 and AFL5-81), 2 on chromosome 7 (AFL7-57 and AFL 7-78) and one on chromosomes 1, 2, 3 and 8 (AFL1-75, AFL2-66, AFL3-236 and AFL8-135, respectively). Characteristics of final VNTRs and respective primer sets are listed in table XVI.

Table XVI. Characteristics of VNTR markers for fingerprinting of *Aspergillus flavus*

VNTR markers	Primer sequences (5' to 3')	Unit repeat size (bp)	Range of repeat number	Simpson diversity index*	Marker location (non coding region or name of the gene if coding)
AFL1-75	GTCAGAGTGCTGTTGGGCG CGTCTCCCAGGCCGTTAGT	75	0-8	0.494	Chromosome 1, non coding
AFL2-66	CGCGAATGTCGATGATCACT AACAGGTAGGGCTGGGTTCC	66	3-6	0.618	Chromosome 2, GPI anchored protein, putative
AFL3-236	CAGAATTTTCAGTTAGCAAAGTGCTC TAAGACTTGGAGATATGTGACAAGGCTATA	236	1-3	0.541	Chromosome 3, non coding
AFL5-66	TCCACAGGCTGTATCGTTATCCT CAGTGACCCTTTCGGTGAAGAC	66	0-5	0.740	Chromosome 5, conserved hypothetical protein
AFL5-81	GGTTGCATCACAGTTATAGCGCT CCGGCACGACTGTGGAC	81	2-9	0.802	Chromosome 5, conserved hypothetical protein
AFL7-57	CACCGCAATGGAGCACAAG TGGTCGAGCTGTTCCTGGA	57	6-17	0.818	Chromosome 7, hypothetical protein
AFL7-78	GCTTCGTCATTGGCCCAT CTCGATCAATGTGTACTCATAAATGCT	78	2-6	0.655	Chromosome 7, conserved hypothetical protein
AFL8-135	GGTTTGCAGTGAGGATCTGCT GATGTGAGCCAGGCCATTG	135	0-3	0.398	Chromosome 8, conserved hypothetical protein

* Each index was calculated with the results from the 36 unrelated *Aspergillus* isolates

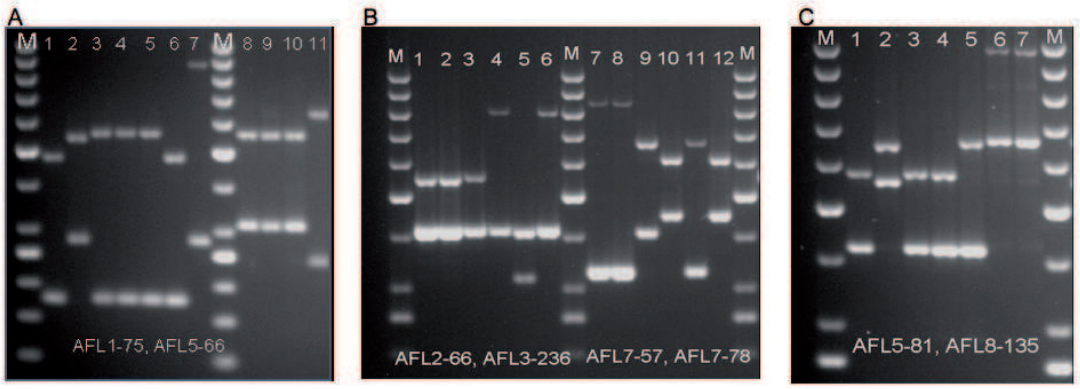


Figure 33. Electrophoretic gels showing VNTR profiles after multiplex amplifications

Gel A: co-amplification of AFL1-75 and AFL5-66 for 11 isolates.

Gel B: co-amplification of AFL2-66 and AFL3-236 for 6 isolates and co-amplification of AFL7-57 and AFL7-78 for 6 isolates.

Gel C: co-amplification of AFL5-81 and AFL8-135 for 7 isolates.

Line M represents the DNA Ladder.

For multiple MLVA analysis, 4 pairs of markers were finally chosen: the combinations of AFL2-66 and AFL3-236, AFL1-75 and AFL5-66, AFL5-81 and AFL8-135, AFL7-57 and AFL7-78. These combinations allowed a clear and unambiguous separation of amplicons on agarose gels (figure 33). Multiple (more than 2)-bands patterns were never detected.

When VNTR primer sets were tested with DNA from *A. fumigatus*, *A. niger* and *A. nidulans* no amplification was observed. On the contrary with DNA from *Aspergillus* species from the Flavi section, amplification was obtained with 4 to 5 out of 8 markers (AFL1-75, AFL2-66, AFL5-66 and AFL8-135 for *A. parasiticus* and *A. oryzae*; AFL1-75, AFL2-66, AFL3-236, AFL7-78 and AFL8-135 for *A. tamarii*). As a consequence, the observation of 8 amplicons following the combination of 8 VNTRs should be considered as specific of *A. flavus* and 3 markers (AFL1-75, AFL2-66 and AFL8-135) may be specific of the section *Flavi*.

The 35 samples (5 isolates subcultured 7 times in 2 months) used for the evaluation of stability were typed by MLVA and yielded exactly the same MLVA pattern for each isolate.

The 50 samples used for the evaluation of reproducibility (25 isolates tested by 2 different technicians in 2 different laboratories) yielded exactly the same MLVA pattern.

Simpson diversity index was first calculated for each VNTR and for the panel of 8 markers tested on the 30 unrelated isolates and 6 reference strains. The index for individual markers ranged from 0.398 to 0.818. A combined loci index calculated with all of 8 markers yielded an index of 0.998.

3.2. Genetic diversity of *A. flavus* isolates

A total number of 91 *A. flavus* isolates, including 6 reference strains were typed with the panel of 8 VNTRs. This analysis yielded 78 different genotypes, which corresponds to a combined loci index of 0.993. Among all genotypes, 71 were only found once. Five genotypes were shared by two isolates, one genotype was shared by three isolates and one genotype was shared by 7 isolates. Analysis of the details of those isolates in a single genotype revealed that 7 of them were isolated from eggshells in May and June 2010 in the same avian farm in China (farm D). Three isolates collected from eggshells and pharyngeal swabs (in laying hens) from the same farm shared a single but distinct genotype. Two isolates from another avian farm in China (farm A) shared a single genotype. The same situation was detected for two pairs of isolates collected in a hospital in Tunisia: one genotype corresponded to two isolates collected in bronchoalveolar lavages (from two different patients) and

another one corresponded to two isolates collected from nasal swabs (from two distinct patients).

UPGMA analysis did not allow a clear clustering of the isolates (figure 34) whereas the graphing algorithm termed Minimum Spanning Tree (MST) demonstrated one major cluster of isolates (figure 35). This cluster comprised 19 out of 32 isolates collected in the avian farm D in Guilin, China. Additional but smaller clusters could be defined for isolates collected in the hospital in Sfax or from avian farm A near Nanning, China. These clusters comprised from 2 to 5 closely related genotypes. Five out of these 6 small clusters correspond to a group of isolates sharing the same geographic origin (Paris, Nanning or Sfax). Apparently, there was no correlation between the genotype of the isolates and their pathogenicity.

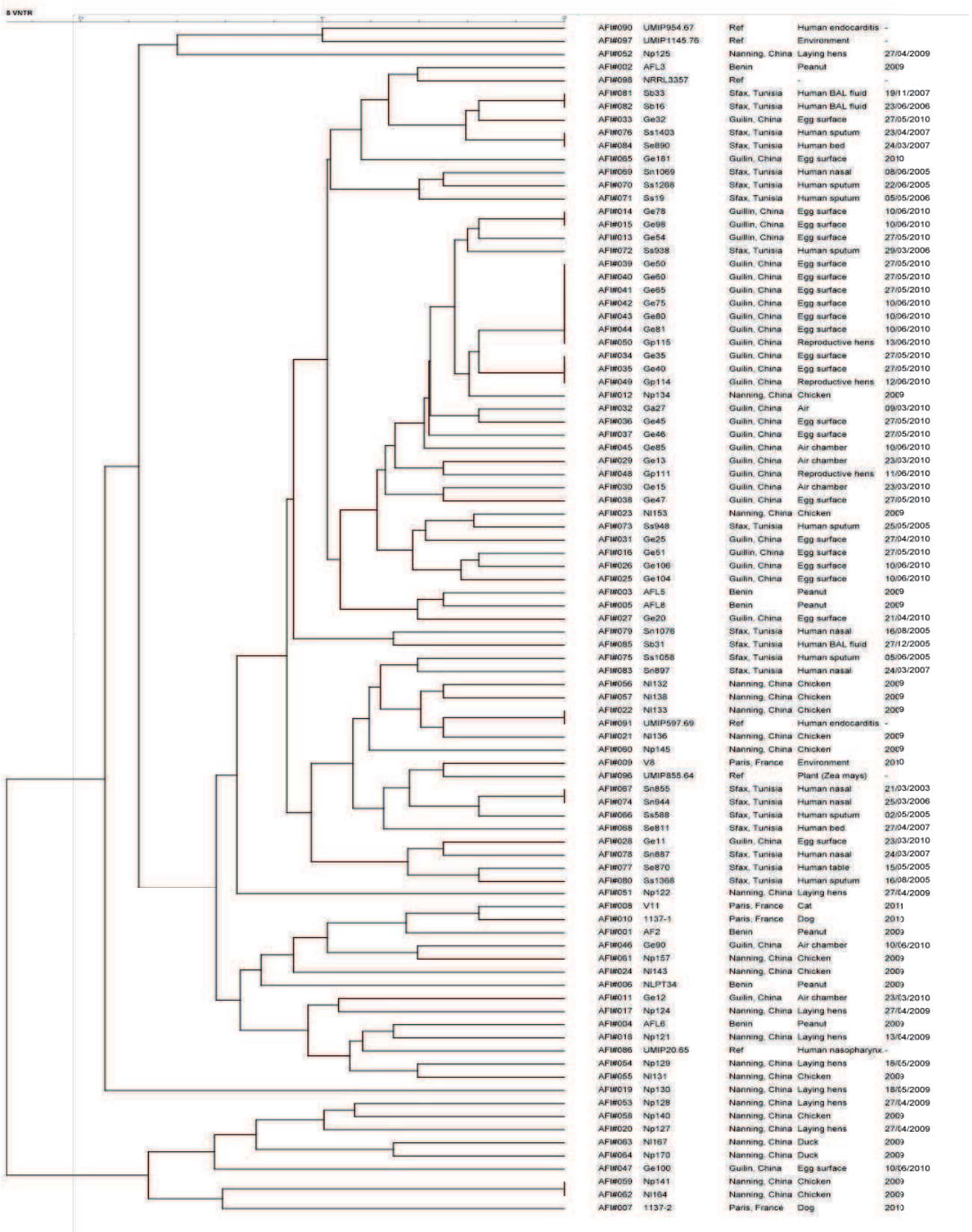


Figure 34. Dendrogram generated from genotyping 91 *A. flavus* isolates. The scale bar above the dendrogram indicates the percentage identity between the genotypes.

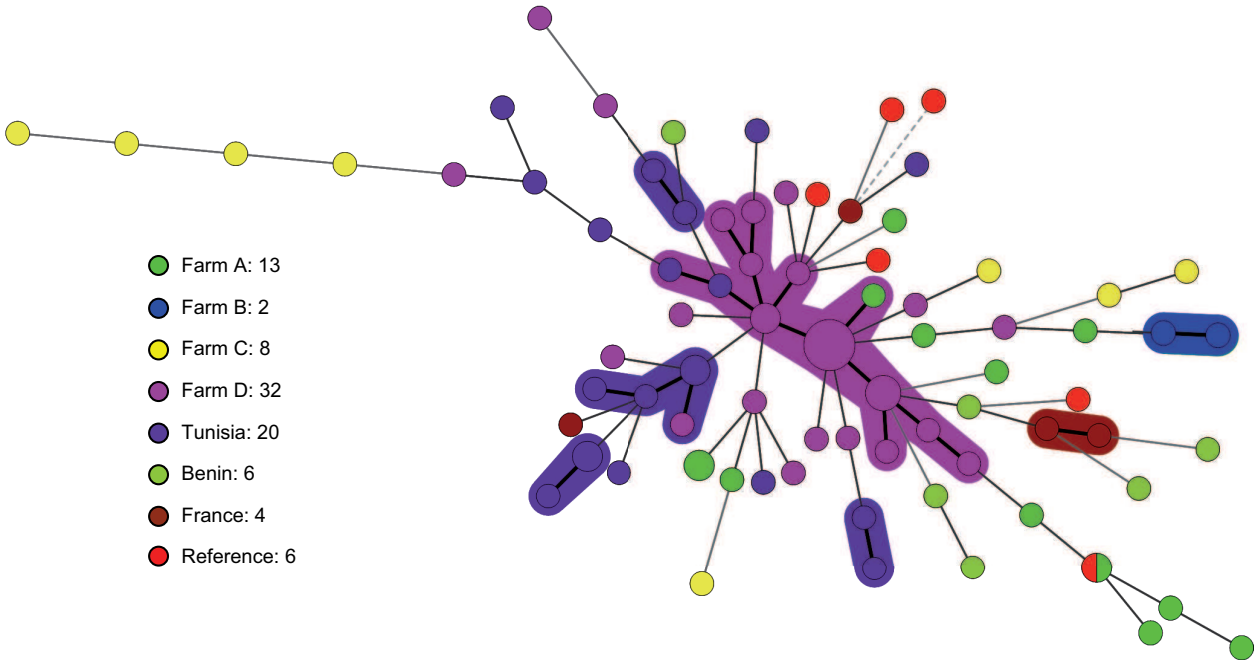


Figure 35. Minimum spanning tree of 91 *A. flavus* isolates based on categorical analysis of 8 VNTRs
Each circle represents a unique genotype. The diameter of each circle corresponds to the number of isolates with the same genotype. Genotypes connected by a shaded background differ by a maximum of one VNTR marker and could be considered a "clonal complex". Thick connecting lines represent one marker difference; regular connecting lines represent two or three marker differences; thin interrupted lines represent four or more differences. The length of each branch is also proportional to the number of differences. Each epidemiological situation is represented by a specific colour: green for 13 isolates collected in avian farm A (broiler chickens) in Guilin, China; blue for 2 isolates collected avian farm B (ducks) in Nanning, China; yellow for 8 isolates collected avian farm C (laying hens) in Nanning, China; pink for 32 isolates collected avian farm D (reproduction hens) in Guilin, China; purple for 20 isolates collected in patients and in the hospital of Sfax, Tunisia; olive for 6 isolates collected from a stock of mouldy peanuts in Benin; brown for 4 isolates collected from the fur of domestic carnivores in France and red for 6 reference strains.

The VNTR patterns obtained in the present study were incorporated in a specific database (http://minisatellites.u-psud.fr/MLVAnet/). On this website, it is now possible to compare *A. flavus* VNTR patterns using complete panel of 8 markers or just a selection of them. This database also allows building dendrograms with the query. Grissa *et al.* detailed all the possibilities provided by the website and database (Grissa *et al.*, 2008).

3.3. Genetic diversity of *A. fumigatus* isolates

The analysis of 188 *A. fumigatus* isolates using 10 VNTR markers led to the resolution of 142 distinct genotypes. Clusters of isolates could be defined by using the graphing algorithm MST. The genetic structuration of *A. fumigatus* isolates seems to depend on the type of poultry production. Large clusters were defined for isolates collected in avian farm D where birds were kept indoor. On the contrary, most isolates collected from open breeding systems (broiler chickens in farm A or ducks in farm B) were not genetically related (figure 36).

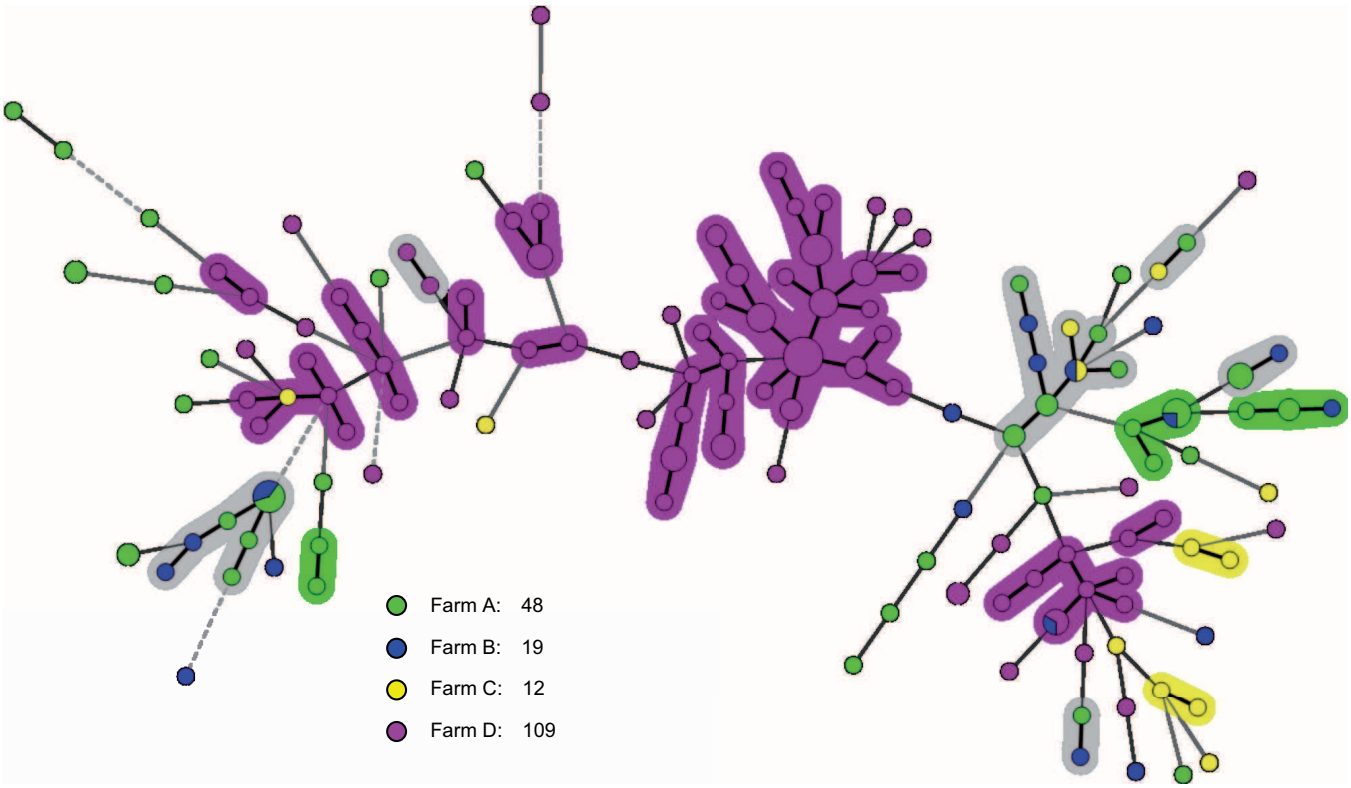


Figure 36. Minimum spanning tree of 188 *A. fumigatus* isolates based on categorical analysis of 10 VNTRs

Each circle represents a unique genotype. The diameter of each circle corresponds to the number of isolates with the same genotype. Genotypes connected by a shaded background differ by a maximum of one VNTR marker and could be considered a "clonal complex". Thick connecting lines represent one marker difference; regular connecting lines represent two or three marker differences; thin interrupted lines represent four or more differences. The length of each branch is also proportional to the number of differences. Each epidemiological situation is represented by a specific colour: green for 48 isolates collected in avian farm A (broiler chickens) in Guilin, China; blue for 19 isolates collected avian farm B (ducks) in Nanning, China; yellow for 12 isolates collected avian farm C (laying hens) in Nanning, China; pink for 109 isolates collected avian farm D (reproduction hens) in Guilin, China.

4. Discussion

Aspergillus species are not equally distributed all over the globe. The species *A. flavus* is particularly prevalent in the air of some tropical countries (Adhikari *et al.*, 2004) while in countries like Saudi Arabia, Sudan, and Tunisia with semi-arid and arid dry weather conditions, *A. flavus* is frequently described as the cause of invasive aspergillosis in immunocompromised patients (Hadrich *et al.*, 2011; Khairallah *et al.*, 1992; Krishnan *et al.*, 2009). The species *A. flavus* is also prevalent in India, Pakistan, Qatar and Iran where it could be responsible for localised infections in immunocompetent humans (Hedayati *et al.*, 2007; Taj-Aldeen *et al.*, 2003). In these countries, the development of a simple and cost-effective typing method is required in order to study the molecular epidemiology of this emerging fungal pathogen.

In a recent review, Hadrich *et al*. (2011) listed the different molecular techniques, which have been tested so far for the typing of *A. flavus* or for the distinction between the clades or species within the *Flavi* section. Various DNA fingerprinting systems have been described such as restriction fragment length polymorphism (RFLP) (Bagyalakshmi *et al.*, 2007; James *et al.*, 2000; Moody and Tyler, 1990), random amplified polymorphic DNA (RAPD) (Heinemann *et al.*, 2004; Midorikawa *et al.*, 2008), amplified fragment length polymorphism (AFLP) (Montiel *et al.*, 2003) and, very recently, microsatellite length polymorphism (MLP) (Grubisha & Cotty, 2010; Hadrich *et al.*, 2010; Rudramurthy *et al.*, 2011). Most of these typing techniques were developed in order to resolve closely related isolates for the purposes of outbreak investigation in hospitals and disease surveillance in humans. In only one study (Grubisha & Cotty, 2010), the genetic markers (24 microsatellite loci and the mating type locus) were used to assess population structure and potential gene flow among *A. flavus* vegetative compatibility groups in sympatric populations in Arizona and Texas. The RAPD method was used for *A. flavus* probably because it requires simple equipment and no genomic sequence information, but it suffered from limited discriminatory power and reproducibility. MLP typing methods were proved to be highly discriminant and reproducible. Hadrich *et al*. (2010) selected 12 microsatellite markers for the typing of 63 *A. flavus* isolates (15 from Marseille, France and 48 from Sfax, Tunisia, including some of the isolates we examined in the present study. The use of all the markers yielded 35 different genotypes with a diversity index of 0.970. A 5 markers combination yielded 27 different genotypes with a diversity index of 0.952. Isolates from Sfax and Marseille displayed distinct genotypes suggesting a geographical structuring in *A. flavus*. This type of structuring has already been observed for *A. fumigatus* using repeat sequence analysis with the CSP method (Balajee *et al.*, 2007) and MLVA (Thierry *et al.*, 2010). In 2011, Rudramurthy *et al*. developed a multicolor microsatellite panel for genotyping of *A. flavus*. Nine microsatellite markers were finally used for the typing of

162 clinical isolates from India. The diversity index for the individual markers ranged from 0.657 to 0.954. The diversity index of the panel of nine markers combined was 0.997.

In the present study, a new molecular typing method for *A. flavus* based on the study of 8 VNTR markers with repeat size larger than 9 bp was developed and further applied to 55 environmental or clinical isolates from China and Tunisia. Size differences between alleles of the 8 selected VNTRs were large enough to allow a multiplex amplification and further efficient differentiating on agarose gel. This makes the present MLVA scheme easy to implement in laboratories with basic molecular biology equipment. The method showed a good reproducibility, which could be increased by the production of an internal ladder (including an example of each allele amplicon size). The MLVA was shown to be rapid and very discriminant. With the panel of isolates of the present study, we obtained a diversity index of 0.993. If we exclude the 7 isolates collected in the same avian farm in China and which shared the same genotype, the value of the diversity index increases to 0.998. With the MLVA method, 20 isolates from Sfax yielded 18 genotypes whereas they were clustered into 12 genotypes when the polymorphism of microsatellite markers was examined by Hadrich *et al*. (2010).

For the clustering analysis of VNTR profiles, we used a graphing algorithm termed minimum spanning tree (MST). This method was introduced to improve analysis of VNTR profiles (Balajee *et al.*, 2006). Similar to maximum-parsimony phylogenetic tree reconstruction methods, MST constructs a tree that connects all the genetic profiles in such a way that the summed genetic distance of all branches is minimized. The differences in mathematical approach between MST and UPGMA methods may account for the changes in isolates clustering. In the present study, MST allowed to group *A. flavus* isolates, which were not so clearly clustered with UPGMA. A large cluster included most of the isolates from farm D in China whereas small clusters included a few isolates from Tunisia (figure 35). As a consequence, MST results are in accordance with the geographical structuring already suggested by Hadrich *et al*. (2011) for *A. flavus*. Additional isolates from other countries should be examined to confirm this result.

To date, it is still a matter of controversy whether certain isolates are more virulent and genetically distinct from other isolates, or whether infection by *A. flavus,* like that by *A. fumigatus,* is simply a matter of contracting infection from any environmental source. In the present study, only a few clinical isolates (from patients in Tunisia) were examined but these isolates yielded genotypes that could not be distinguished from those corresponding to environmental isolates. A larger number of clinical isolates (from humans but also from birds) should be examined to confirm this preliminary result.

In conclusion, we developed a multiplex VNTR panel for *A. flavus* genotyping. The markers in this VNTR assay

were highly discriminating and stable over time. The typing method could be used for molecular epidemiological studies of *A. flavus* in many countries without the need for sophisticated equipment. Furthermore, data obtained by the present method could be easily shared in a web database. The clustering analysis was in accordance with a geographical structuring of *A. flavus* isolates similar to that detected in *A. fumigatus*.

Chapter IV

Antifungal susceptibility of *A. fumigatus* isolates from avian farms in France and China

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1. Introduction

Itraconazole resistance in *Aspergillus* spp. was first reported in 1997 in 3 clinical isolates obtained from California in the late 1980s (Denning *et al*. 1997). Since then, an increasing number of clinical cases have been published. In 2007, Verweij *et al*. observed the emergence of multiple-triazole-resistance in *A. fumigatus* isolates in the Netherlands (Verweij *et al*. 2007). Patients with azole-resistant *A. fumigatus* presented with primary invasive aspergillosis or with breakthrough infection while receiving itraconazole or voriconazole. The patients were from six different hospitals in The Netherlands, and there was no evidence for spread of a single clone. The isolates were highly resistant to itraconazole and showed elevated minimum inhibitory concentrations (MICs) of voriconazole, posaconazole and ravuconazole. In 2009, Howard *et al*. investigated the frequency of *A. fumigatus* itraconazole resistance in a referral laboratory collection in UK, defined the azole cross-resistance pattern and identified mutations in the *Cyp51A* gene (table XVII). In the clinical collection of *A. fumigatus* isolates, the frequency of itraconazole resistance was 5% with a significant increase since 2004. In 2008, 92 *A. fumigatus* isolates were tested for triazole susceptibility, of which 21 (23%) were resistant to at least one azole. In 2009, 43 of 138 (31%) isolates tested were azole resistant (Bueid *et al*. 2010) (Figure 37).

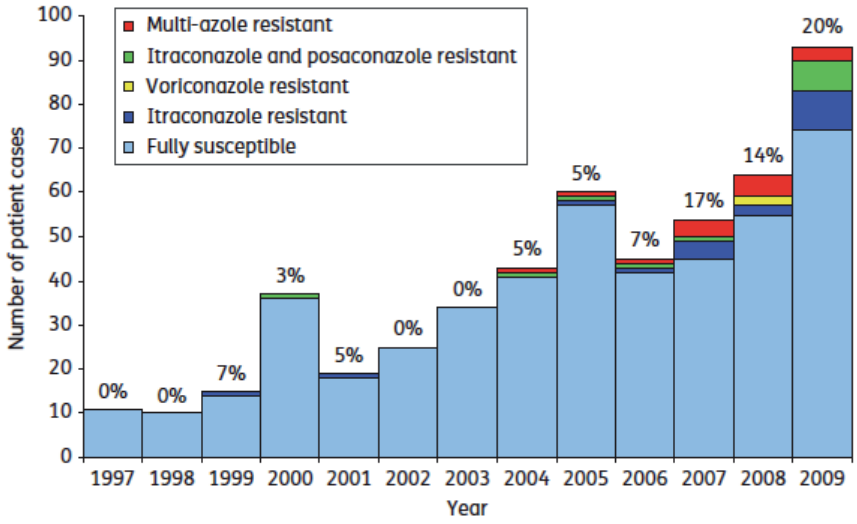

Figure 37. Azole resistance frequency in *A. fumigatus* by patient 1997–2009 in UK. Overall azole resistance for each year is shown above each column as a percentage (Bueid *et al*. 2010)

Prominent amino acid substitutions in the target site, lanosterol demethylase encoded by the gene *Cyp51A*, account for more than 80% of acquired resistance in *A. fumigatus*. Howard *et al*. (2009) demonstrated that the

position and type of amino acid substitution within the Cyp51A protein determines the pattern of azole cross-resistance (table XVII), which is consistent with predicted structural properties of the demethylase enzyme.

Table XVII. Reported Cyp51A amino acid substitutions and associated cross-resistance patterns in azole-resistant *Aspergillus fumigatus* isolates from the Regional Mycology Laboratory of Manchester 1992-2007 (Howard *et al*. 2009)

Mutations	Number of isolates	Amino acid substitutions	MIC mg/L		
			ITZ	VOR	POSA
F46	4	Y	>8	2–4	0.125–0.5
G54	5	E, R, V	>8	0.125–1	1–>8
L98+TR	2	H	>8	8	1–2
G138	10	C	>8	8–>8	2–>8
H147	1	Y	>8	>8	0.5
M172	4	V	>8	2–4	0.125–0.5
P216	1	L	>8	1	1
M220	4	K, T	>8	1-4	0.5–>8
N248	1	T	>8	2	0.25
D255	1	E	>8	2	0.25
E427	5	G, K	>8	2–4	0.125–0.5
Y431	1	C	>8	4	1
G434	1	C	>8	4	1
G448	2	S	>8	>8	0.5–1
No substitution	3	-	>8	2–8	0.25–1

To date, 17 mutations have been identified in azole-resistant isolates. The dominant genetic changes in itraconazole-resistant isolates in the *Cyp51A* gene were a point mutation leading to the substitution of leucine 98 for histidine, together with the presence of two copies of a 34-base pair sequence in tandem in the promoter of cthe gene. Eight additional mutations have been detected in both azole-resistant or susceptible isolates (table XVIII).

Recently van der Linden *et al*. (2011) demonstrated that multiazole resistance in *A. fumigatus* is widespread in the Netherlands and is associated with a high death rate for patients with invasive aspergillosis. From June 2007 through January 2009, all clinical *Aspergillus* isolates were screened for itraconazole resistance. In total, 2,062 isolates from 1,385 patients were screened; the prevalence of itraconazole resistance in *A. fumigatus* in the patient cohort was 5.3% (range 0.8%-9.5%). Patients with a hematologic or oncologic disease were more

likely to harbor an azole-resistant isolate than were other patient groups ($p<0.05$). Most patients (64.0%) from whom a resistant isolate was identified were azole naive, and the case-fatality rate of patients with azole-resistant invasive aspergillosis was 88.0%.

The ARTEMIS global antifungal susceptibility program was designed to detect trends in antifungal resistance through sentinel surveillance of more than 100 medical centers worldwide. In 2008 to 2009, an increase in *A. fumigatus* isolates with elevated MIC values to triazole compounds was detected (Pfaller *et al*. 2011).

In fungi, the evolution of drug resistance is more likely to proceed by the sequential accumulation of adaptive mutations. However, fungi are able to rapidly adapt in response to environmental challenges by antifungal drugs. In the setting of infection in individuals coinfected with HIV, *Candida albicans* has evolved various résistance mechanisms in response to short periods of azole exposure. Acquired resistance was also seen in *A. fumigatus* isolates cultured from patients with aspergilloma during treatment with azoles (Howard *et al.* 2009). Another route of resistance development is the exposure of *A. fumigatus* to azole compounds in the environment. Exposure of saprophytic fungi to azole compounds could take place in agriculture, where such chemical compounds are commonly used for plant protection. The fungicides are applied repeatedly over a long period of time and could thereby create a persistent pressure of azole compounds on saprophytic fungi, including *A. fumigatus*. The researchers from the University of Nijmegen suggested that an environmental route of resistance development in *A. fumigatus* should be considered because a single mechanism of azole resistance (TR/L98H substitution) was found in 94% of clinical isolates from different hospitals in the Netherlands (Verweij *et al.* 2009, Snelders *et al*. 2011). To date, clinical *A. fumigatus* isolates with the TR/L98H substitution have been reported in patients from the Netherlands, Spain, France, Norway, United Kingdom, Belgium and China (Howard *et al*. 2009, Lokhart *et al*. 2011, Mellado *et al*. 2007, Mortensen *et al*. 2010, Snelders *et al*. 2008). Environmental *A. fumigatus* isolates with the TR/L98H substitution been recovered in the Netherlands and Denmark from agricultural samples of soil (Mortensen *et al*. 2010, Snelders *et al*. 2008).

The hypothesis that the use of antifungal drugs in avian farms may also be responsible for the emergence of azole resistance has never been investigated. That's why we decided to assess the susceptibility of a large number of *A. fumigatus* isolates collected from birds or from the environment of avian farms in France and China. The isolates were characterized using phenotypic and molecular methods.

Table XVIII. Mutations in Cyp51A identified in *A. fumigatus* isolates (adapted from Howard *et al*. 2011)

Mutations	Amino acid substitutions	Phenotype	References
Hot spot mutations commonly found in azole-resistant isolates			
G54	E, K, R, V, W	Itraconazole and posaconazole resistant, voriconazole susceptible	Howard *et al*. 2009, Rodriguez-Tudela *et al.* 2008
L98+TR	H	Pan-azole resistant	Howard *et al*. 2009, Snelders *et al*. 2008
M220	K, I, T, V	Itraconazole and posaconazole resistant, voriconazole variable	Howard *et al*. 2009, Rodriguez-Tudela *et al.* 2008
Mutations found less frequently in azole-resistant isolates			
N22	D	Itraconazole resistant, other azole susceptibilities not reported	da Silva Ferreira *et al*. 2004
S52	T	Pan-azole resistant	Snelders *et al*. 2009
G138	C, R	Pan-azole resistant	Howard *et al*. 2009
Q141	H	Pan-azole resistant	Snelders *et al*. 2009
P216	L	Itraconazole and posaconazole resistant, voriconazole susceptible	Howard *et al*. 2009
M236	K, T, V	Itraconazole resistant	Ferreira *et al*. 2005
S297	T	Itraconazole and posaconazole resistant, voriconazole susceptible	Snelders *et al*. 2008, Mellado *et al.* 2007
P394	L	Itraconazole resistant, other azole susceptibilities not reported	da Silva Ferreira *et al*. 2004
Y431	C	Pan-azole resistant	Howard *et al*. 2009
G434	C	Pan-azole resistant	Howard *et al*. 2009
T440	A	Itraconazole resistant, other azole susceptibilities not reported	da Silva Ferreira *et al*. 2004
G448	S	Pan-azole resistant	Howard *et al*. 2009
Y491	H	Itraconazole resistant, other azole susceptibilities not reported	da Silva Ferreira *et al*. 2004
F495	I	Itraconazole and posaconazole resistant, voriconazole susceptible	Snelders *et al*. 2008, Mellado *et al.* 2007
Mutations found in azole susceptible and resistant isolates			
F46	Y	Azole susceptible and resistant	Howard *et al*. 2009, Rodriguez-Tudela *et al.* 2008
F165	L	Azole susceptible and resistant	Escribano *et al*. 2011
M172	V	Azole susceptible and resistant	Howard *et al*. 2009, Rodriguez-Tudela *et al.* 2008
N248	T	Azole susceptible and resistant	Howard *et al*. 2009, Rodriguez-Tudela *et al.* 2008
D255	E	Azole susceptible and resistant	Howard *et al*. 2009, Rodriguez-Tudela *et al.* 2008
D262	Y	Azole susceptible and resistant	Escribano *et al*. 2011
E427	G, K	Azole susceptible and resistant	Howard *et al*. 2009, Rodriguez-Tudela *et al.* 2008
N479	D	Azole susceptible and resistant	Escribano *et al*. 2011

2. Materials and methods

2.1. Origin of fungal isolates

We examined a total number of 177 *A. fumigatus* isolates. A first set of 51 isolates came from 4 avian farms in Guangxi, China (chapter II); 57 isolates were collected by Marine Gricourt (Master 2) in two avian farms in France in 2010; 69 additional isolates were collected from birds in France during the PhD thesis of Simon Thierry (2008-2011). Chemoprophylaxis with parconazole was applied in the two avian farms examined by Marine Gricourt in 2010. All the isolates were microscopically identified after cultivation on Malt Agar plates at 37°C until conidia formation. For 55 randomly selected isolates, the species identification was confirmed by amplification and sequencing partial sequencing of *ß-tubulin* gene (Balajee *et al.* 2005).

2.2. Antifungal susceptibility testing

The Etest was performed in accordance with the manufacturer's instructions. *Aspergillus fumigatus* isolates were inoculated on Malt at 37°C for 7 days. Conidia suspensions were prepared in sterile saline and adjusted to a concentration of 10^6 conidia/mL. Petri dishes were filled with RPMI 1640 medium, supplemented with glucose (2%) with final pH 7.0 (AES, Combourg, France). Plates were inoculated 200µL of the appropriate cell suspension, dried at room temperature for 15 min before the Etest gradient strips of itraconazole (concentration range, 0.002 to 32.0 µg/mL, BioMérieux, France) were applied. The plates were incubated at 37°C and read at 48 h or 72h. The Etest minimum inhibitory concentrations (MICs) were estimated as the drug concentrations at which the border of the elliptical inhibition zone intersected the scale on the antifungal test strip (figure 38).

Isolates were considered resistant to itraconazole when the MIC was 2 µg/mL or higher.

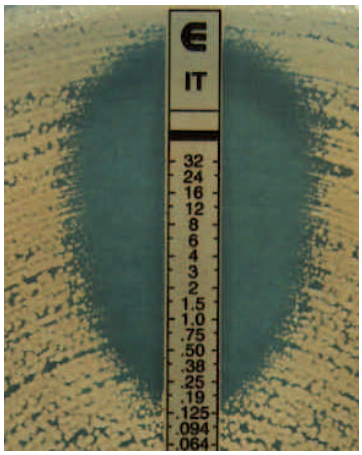

Figure 38. An example of Etest result. In the present case, the MIC is 0.125 µg/mL

2.3. Sequencing of *Cyp51A*

For the sequencing of *Cyp 51A* (the coding gene of the enzyme 14-α-sterol demethylase), 32 out of 57 isolates from avians farms in France and 29 out of 51 isolates from avian farms in China were selected. For each isolate, conidia were collected from the pure cultures and transferred into a microtube for extraction. DNA extraction was performed with QIAamp DNA miniKit (Qiagen). For amplification we used two primer sets: P450A1 (5'-ATG GTG CCG ATG CTA TGG-3') - P450A2 (5'-CTG TCT CAC TTG GAT GTG-3') (Diaz-Guerra *et al*. 2003) and A7 (5'-TCA TAT GTT GCT CAG CGG-3') - A2 (GGG GTC GTC AAT GGA CTA -3') (Mellado *et al*. 2001). The two sequences were assembled and edited with SeqMan II and EditSeq software packages (Lasergene; DNAStar, Inc., Madison, WI). Sequences were compared with that of the reference strain CM-237 (GenBank accession number AF338659) (Mellado *et al*. 2001).

2.4. MLVA typing

The Multiple Locus Variable-number tandem-repeat (VNTR) Analysis (MLVA) was performed according to Thierry *et al.* (2010). A total number of 81 *A. fumigatus* isolates were analyzed using 10 markers (chapter III). VNTR profiles were analyzed with Bionumerics software package version 4.6 (Applied-Maths, Belgium) as a character dataset. Two different techniques were used to represent the relationships between isolates: a phenogram using UPGMA method and a graphing algorithm termed Minimum Spanning Tree (MST). The priority rule for constructing MST was set in order that the type that had the highest number of single-locus variants (SLVs) would be linked first. A cutoff value of maximum differences of 2 VNTRs out of 8 or 10 was applied to define cluster in the MST method.

3. Results

3.1. Susceptibility to itraconazole

Susceptibility testings demonstrated that 4 isolates should be considered as resistant to itraconazole: 2 isolates from avian farms in Guangxi, China, C3 (from farm A) (MIC = 3 µg/mL) and C15 (from farm C) (MIC = 3 µg/mL) and 2 isolates from avian farms in France, F78 (MIC = 2µg/mL) and F82 (MIC = 3 µg/mL).

Most of the isolates from China were susceptible to itraconazole with a MIC comprised between 0.38 and 0.75 µg/mL. Six isolates had a MIC of 1 µg/ml (figure 39).

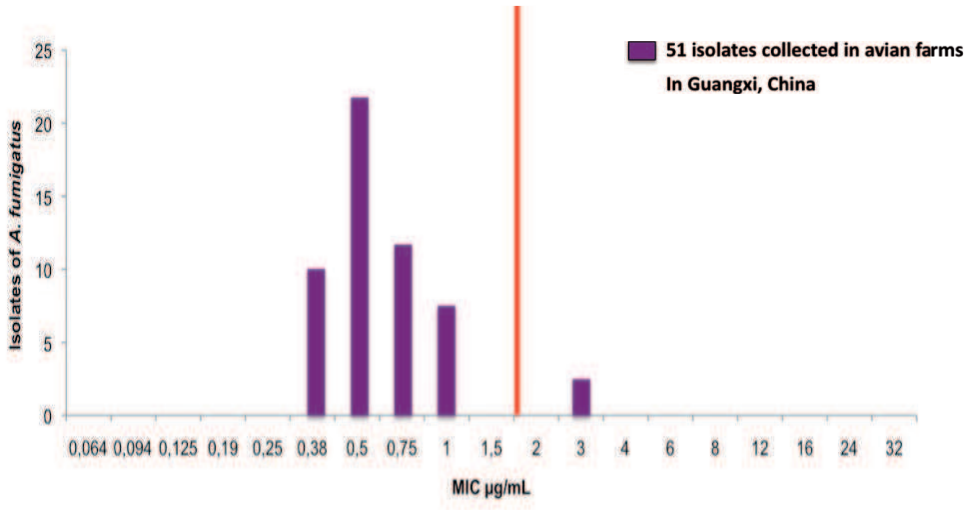

Figure 39. Distribution of MIC values for *A. fumigatus* isolates collected from avian farms in Guangxi, China. The red line indicates the cut-off value (2 µg/mL) for the definition of itraconazole resistance.

Most of the isolates from birds and avian farms in France were susceptible to itraconazole with a MIC comprised between 0.19 and 1µg/mL. Three isolates had a MIC of 1.5µg/mL (figure 40). MIC values of isolates collected in farms with chemoprophylaxis were not higher than those of isolates collected from birds (that never received antifungal drugs before the sampling).

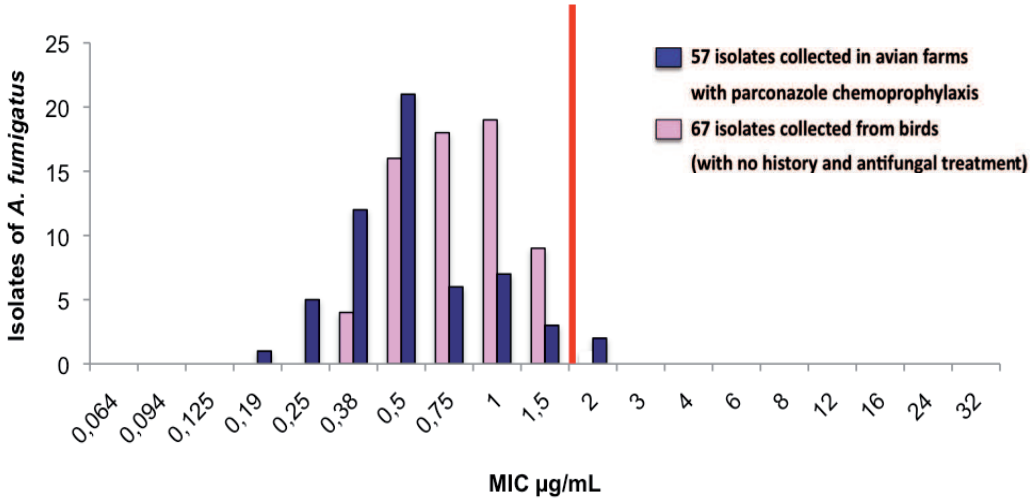

Figure 40. Distribution of MIC values for *A. fumigatus* isolates collected from birds or avian farms in France. The red line indicates the cut-off value (2 µg/mL) for the definition of itraconazole resistance.

3.2. Mutations of *Cyp51A* and corresponding protein

Direct sequencing of *Cyp51A* was performed in 61 *A. fumigatus* isolates.

For 50 isolates, we obtained the same sequence as that reported for the reference strain CM-237 (GenBank accession number AF338659). This first group of isolates included the itraconazole-resistant isolate F78, which was collected from an avian farm in France.

A modification of the *Cyp51A* sequence was identified in 11 isolates (3 azole-resistant and 8 azole-susceptible isolates). We detected 21 nucleotidic mutations. Eleven of these mutations were silent and 10 yielded to amino acid substitutions in the protein. Seven of these substitutions had already been described (table XVIII) whereas mutations A116R, E130D and Q131H were original ones.

Mutations were detected in both resistant and susceptible isolates (table XIX) but the number of nucleotidic mutations seemed to be higher in itraconazole-resistant isolates (figure 41).

Three amino acid substitutions were specifically detected in itraconazole-resistant isolates: phenylalanine to tyrosine (F46Y), methionine to valine (M172V), and glutamic acid to lysine (E427K). Two isolates from China had two additional amino acids changes: asparagine to threonine (N248T) and aspartic acid to glutamic acid (D255E).

The hot spot mutations (G54, L98+TR and M220) commonly found in azole-resistant isolates (collected in hospitals) were never detected in the present study.

Table XIX. Mutations in the protein Cyp51A identified in *A. fumigatus* isolates collected in avian farms in China and France

Mutations	Nucleotidic changes	Amino acid substitutions	*A. fumigatus* isolates (MIC values)
Mutations detected in itraconazole-resistant isolates			
F46	TTT→TAT	**Y**	C3 (3 µg/mL), C15 (3 µg/mL), F82 (2 µg/mL)
M172	ATG→GTG	**V**	C3 (3 µg/mL), C15 (3 µg/mL), F82 (2 µg/mL)
N248	AAT→ACT	**T**	C3 (3 µg/mL), C15 (3 µg/mL)
D255	GAC→GAG	**E**	C3 (3 µg/mL), C15 (3 µg/mL)
E427	GAG→AAG	**K**	C3 (3 µg/mL), C15 (3 µg/mL), F82 (2 µg/mL)
Mutations detected in itraconazole-resistant or susceptible isolates			
M172	ATG→GTG	**V**	C3 (3 µg/mL), C15 (3 µg/mL), F82 (2 µg/mL), C2 (0.5 µg/mL), C8 (1 µg/mL), C10 (0.38 µg/mL), C23 (0.38 µg/mL), C24 (0.5 µg/mL), C26 (0.5 µg/mL)
Mutations detected in itraconazole-susceptible isolates			
A116*	GGA→AGA	**R**	C2 (0.5 µg/mL)
E130*	GAG→GAC	**D**	C19 (0.5 µg/mL)
Q131*	CAG→CAT	**H**	C19 (0.5 µg/mL)
N248	AAT→AAA	**K**	C28 (0.38 µg/mL)

* mutations which had never been reported in previous investigations

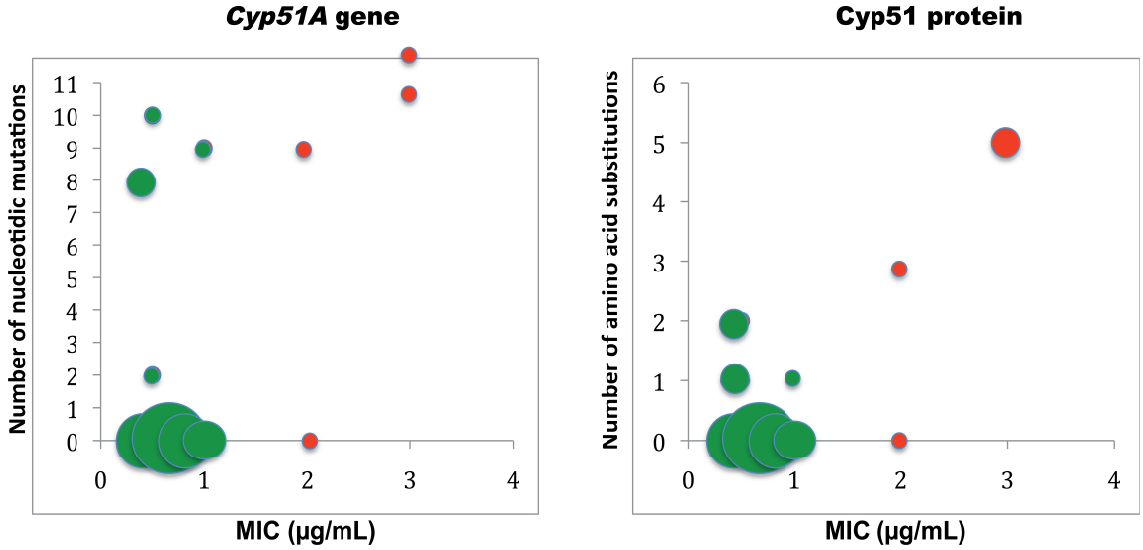

Figure 41. Distribution of nucleotidic or proteic mutations according to the MIC values (itraconazole) of the isolates. The diameter of each circle corresponds to the number of susceptible (green) or resistant (red) isolates.

3.3. MLVA typing

A total number of 81 *A. fumigatus* isolates were typed with the panel of 10 VNTRs. This analysis yielded 74 different genotypes. Among all genotypes, 70 were only found once. Two genotypes were shared by 2 isolates and one genotype was shared by 3 or 4 isolates.

UPGMA analysis did not allow a clear clustering of the isolates (data not shown) whereas the graphing algorithm termed Minimum Spanning Tree (MST) demonstrated small clusters of isolates (figure 42). These clusters comprised a small number of isolates collected in the same avian farms in China or in France.

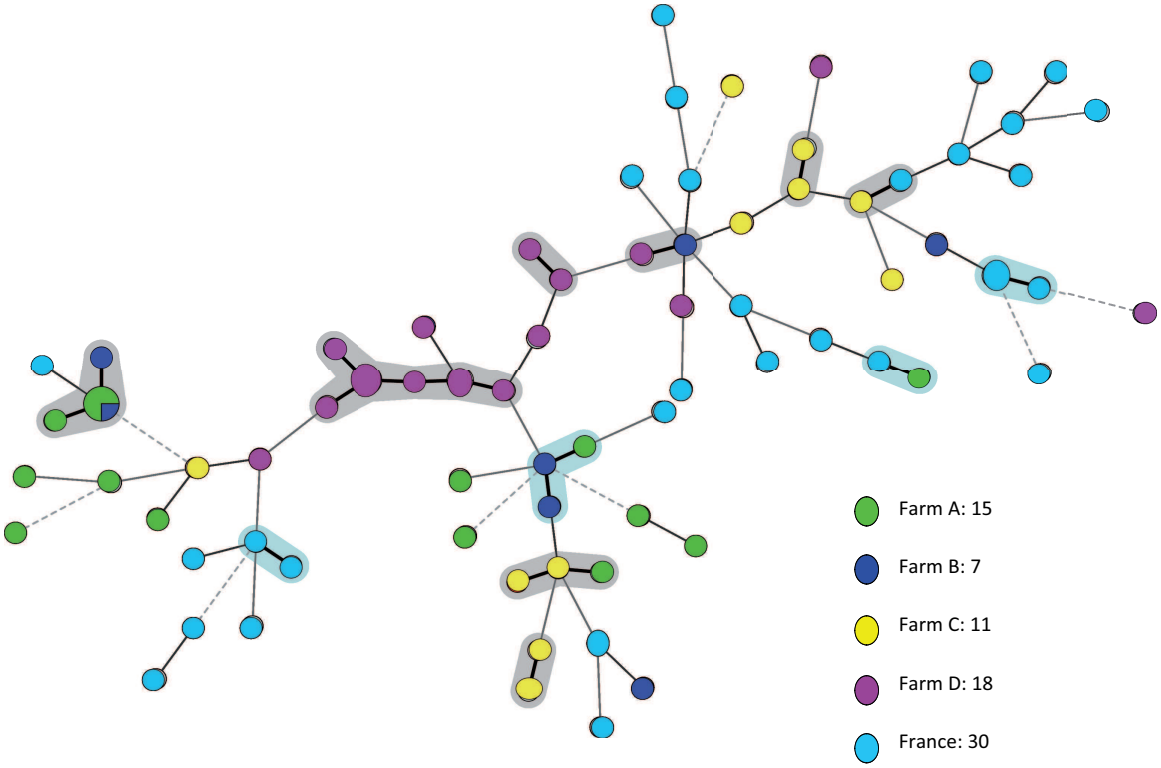

Figure 42. Minimum spanning tree of 81 *A. fumigatus* isolates based on categorical analysis of 10 VNTRs (Thierry *et al*. 2010). Colours correspond to the origin of the isolates.

Each circle represents a unique genotype. The diameter of each circle corresponds to the number of isolates with the same genotype. Genotypes connected by a shaded background differ by a maximum of one VNTR marker and could be considered a “clonal complex”. Thick connecting lines represent one marker difference; regular connecting lines represent two or three marker differences; thin interrupted lines represent four or more differences. The length of each branch is also proportional to the number of differences. Each epidemiological situation is represented by a specific colour: green for 15 isolates collected in avian farm A (broiler chickens) in Guilin, China; blue for 7 isolates collected avian farm B (ducks) in Nanning, China; yellow for 11 isolates collected avian farm C (laying hens) in Nanning, China; pink for 18 isolates collected avian farm D (reproduction hens) in Guilin, China; brown for 30 isolates collected in avian farms in France.

When azole susceptibility was considered as the discriminant parameter, MST analysis revealed that itraconazole-resistant isolates (F78, F82, C3 and C15) were genetically distinct (figure 43). However small clusters of *Cyp51A*-mutated isolates could be detected. A first cluster comprised isolate F82 and 6 susceptible isolates (C2, C8, C10, C24 and C26). The point mutation M172V was detected in all these isolates harbored (table XIX). Another small cluster comprised isolates C28 and C15. These isolates were both mutated at position N248 (but the nucleotidic mutation was not the same and the amino acid substitution was different). In the MST representation, isolate C19, the only one with mutations E130D and Q131H, was genetically distinct from all other isolates. The same situation was observed for isolate F78, an azole-resistant isolate without any mutations of *Cyp51A*.

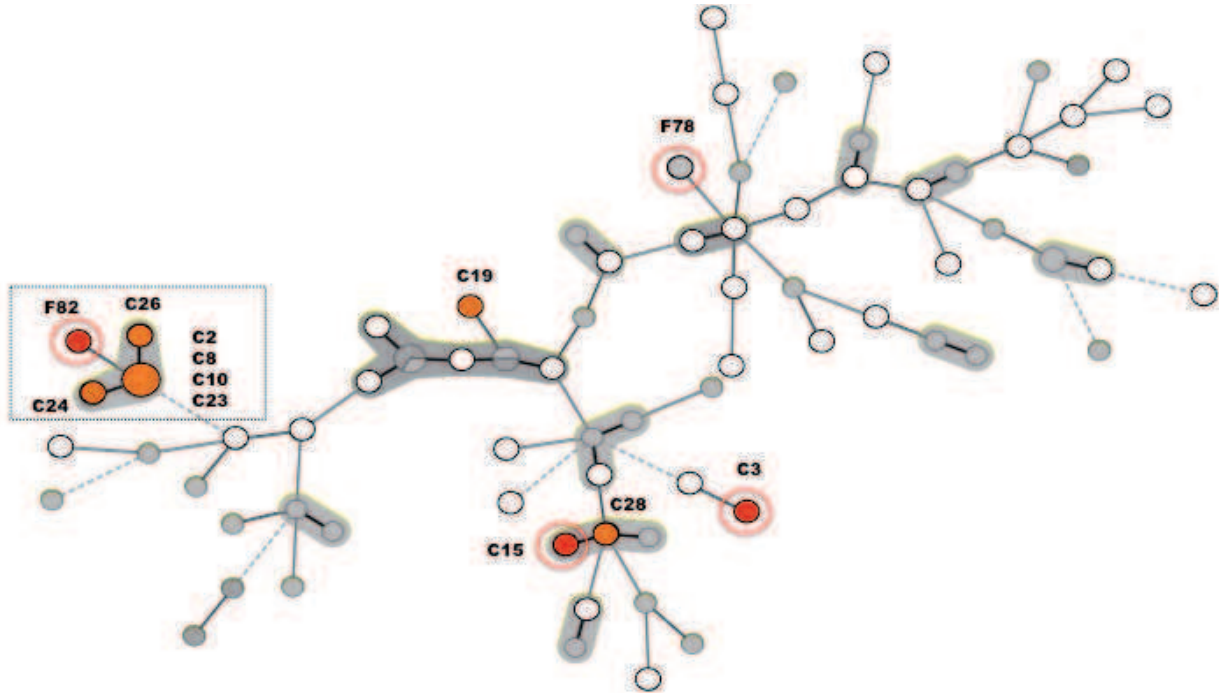

Figure 43. Minimum spanning tree of 81 *A. fumigatus* isolates based on categorical analysis of 10 VNTRs (Thierry *et al*. 2010). Colours correspond to the azole susceptility of the isolates.

Each circle represents a unique genotype, The diameter of each circle corresponds to the number of isolates with the same genotype. Genotypes connected by a shaded background differ by a maximum of 1 of the 10 VNTR markers and could be considered a "clonal complex". Thick connecting lines represent one marker difference; regular connecting lines represent two or three marker differences; thin interrupted lines represent four or more differences. The length of each branch is also proportional to the number of differences. Each situation is represented by a specific colour: red for 3 resistant isolates (MIC≥2µg/mL) with mutations of Cyp51A, orange for 8 susceptible isolates (MIC<2µg/mL) with mutations of Cyp51A; the red ring for the 4 isolates with a MIC≥3 µg/ml; grey for sensitive isolates without mutation and white for isolates whose *Cyp51A* was not sequenced.

4. Discussion

Multiple mechanisms of resistance are now known to be responsible for azole resistance in fungi. In yeasts of the genus *Candida*, most of the reports on azole resistance are related to the increased efflux of azole drugs due to the overexpression of efflux pumps (Cowen *et al*. 2002, Prasad and Kapoor 2005). In filamentous fungi, polymorphisms in *Cyp51A* (and subsequent modifications in Cyp51A, the target of azole drugs) is now recognized as the major mechanism of resistance. This has been demonstrated in crop plant pathogens (like *Mycosphaerella graminicola* or *Blumeria graminis*) exposed to different demethylase inhibitors (DMIs) used for agricultural purposes (Leroux and Walker 2011, Wyand and Brown 2005). In *A. fumigatus*, azole drug resistance has been described for both laboratory mutants and clinical isolates and has mainly been attributed to alterations in *Cyp51A* (Howard *et al*. 2011). Mutations of *Cyp51A*, reducing the binding of azoles to the enzymatic site, have been associated with different antifungal susceptibility profiles, depending on the azole structure and concomitant binding affinities. Even though analysis of the *A. fumigatus* genome has shown the existence of more than 40 ATP binding cassette transporter (ABC) homologs and more than 100 major facilitator transporter genes (five times more than the number in yeasts) (Tekaia and Latgé, 2005), isolates that overexpress efflux pumps as an azole resistance mechanism have rarely been described.

In the present study, we demonstrated that azole-resistant isolates may circulate in avian farms in France and in Guangxi, China. However the proportion of resistant isolates was limited and MIC values were low (ie close to the cut-off value of 2 µg/mL). Furthermore, the proportion of azole-resistant isolates was not different in avian farms with or without parconazole prophylaxis. Hot spot mutations already found in isolates from hospitals were not detected.

One isolate (F78) with elevated itraconazole MIC value had no *Cyp51A* mutation. This is not unusual. In a 2008 to 2009 survey in England, Bueid *et al*. (2010) found that 43% of their itraconazole-resistant isolates had a wild-type *Cyp51A* sequence and Snelders *et al*. (2008) found itraconazole-resistant isolates with wild-type *Cyp51A* genes during environmental sampling. As others have found, there is probably more than one mechanism working in these isolates to overcome the pressure of triazole exposure.

Only a very few studies aimed at determining MICs for fungi isolated from birds or from their environment. Silvanose *et al*. (2006) collected fungal isolates from the air sacs of falcons before and during antifungal treatment with amphotericin B nebulisation and oral itraconazole or voriconazole. Before treatment, 95% of

the isolates, including *A. fumigatus* were susceptible to voriconazole (at MICs up to 0.38µg/mL). Before treatment, 21% of the isolates, including *A. fumigatus* (27.6%), were less susceptible or resistant (MIC≥1µg/mL) to itraconazole; 51% of the isolates, including *A. fumigatus* (31%), had MICs over 1 µg/mL to amphotericin B, and after treatment their MICs increased significantly. In contrast, there were no significant differences between the MICs of voriconazole and itraconazole for the different *Aspergillus* species before and during treatment with these antifungal agents.

In 2009, Beernaert *et al*. examined 59 *A. fumigatus* isolates from domestic and wild birds. The isolates were obtained from a wide range of avian groups (including Falconiformes, Galliformes and Psittaciformes) from Belgium and The Netherlands. Two of the 4 resistant isolates were collected from birds that received itraconazole. The possible effect of antifungal treatment in birds is unknown, and because isolates were not available before treatment, this could not be further investigated. Determination of resistant isolates in birds can also be considered an indication of the presence of acquired resistance in the surrounding environment.

The genetic distances between azole-resistant and susceptible *A. fumigatus* isolates were estimated in two investigations. Using microsatellite markers, Snelders *et al*. (2008) demonstrated that 32 itraconazole-resistant isolates had distinct genotypes but clustered (figure 44). These observations suggested that the TR/L98H isolate was not transmitted from person to person, but might be present in the environment in the Netherlands.

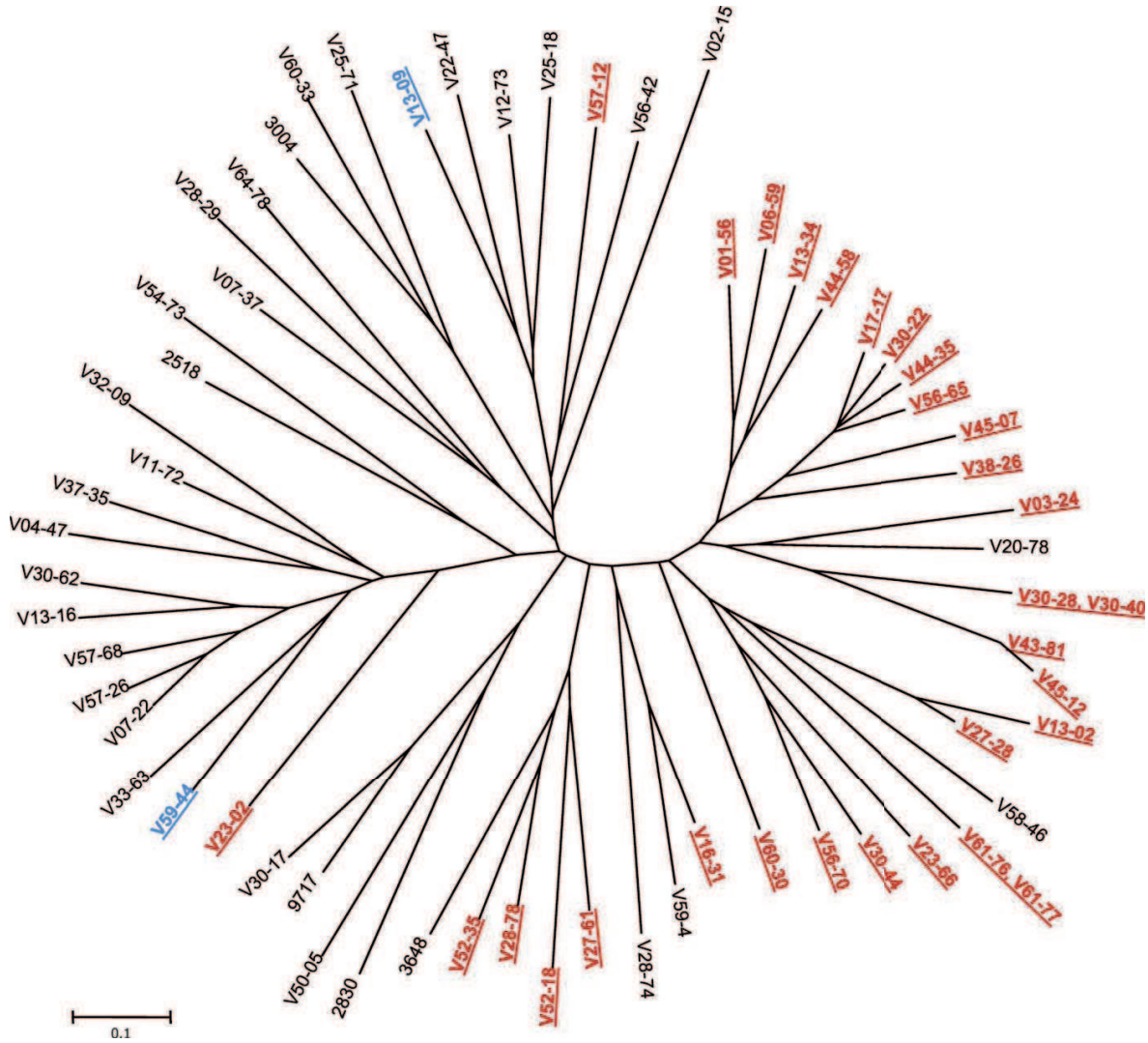

Figure 44. Genotypic Relatedness of 32 itraconazole-resistant *A. fumigatus* Isolates and 32 itraconazole-susceptible controls (Snelders *et al*. 2008).

The numbers are the identification numbers of the individual isolates. The itraconazole-resistant *A. fumigatus* isolates with the L98H substitution and a tandem repeat in the promoter region of *Cyp51A* are underlined and printed in red. The itraconazole-resistant isolates with other or unknown resistance mechanisms are underlined and printed in blue. The itraconazole-susceptible isolates are printed in black.

Recently, Lockhart *et al.* (2011) surveyed 497 *A. fumigatus* isolates collected from 2008 to 2009 as part of the ARTEMIS global surveillance study for elevated MIC values to itraconazole, voriconazole, and posaconazole. Sequencing of the *cyp51A* gene revealed that 8/29 isolates with elevated MIC values to one or more triazoles, all originating from China, contained the TR/L98H mutation associated with resistant European isolates of *A. fumigatus*. Using microsatellite markers, Lockhart *et al.* (2011) demonstrated that all of the isolates with elevated triazole MIC values had distinct genotypes. The eight isolates with the TR/L98H mutation were distributed throughout the dendrogram, with only two sets of two isolates clustering as nearest neighbors (figure 45), ruling out clonal spread of a single isolate between patients within the institutions from which they were cultured.

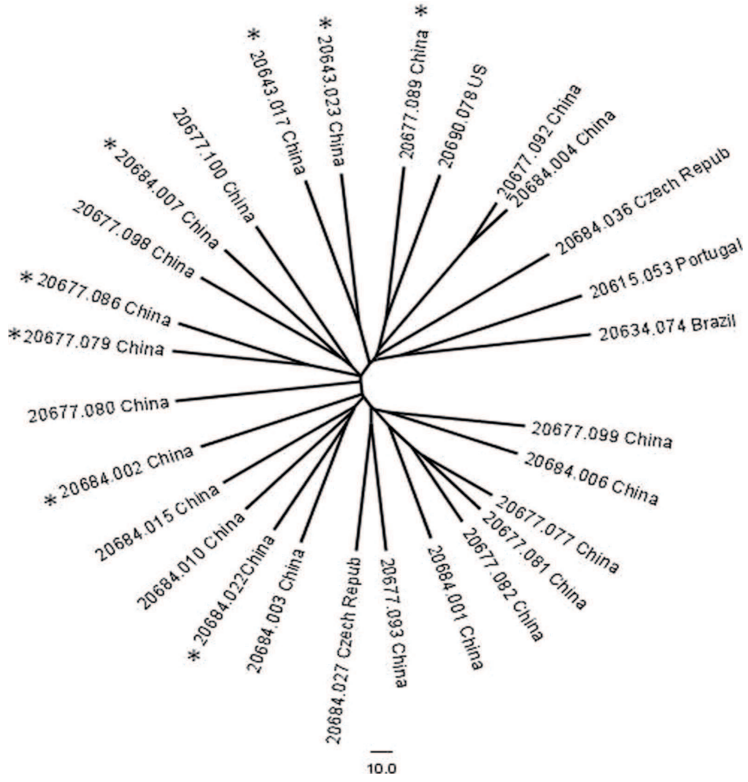

Figure 45. Dendrogram of 28 isolates with elevated triazole MIC values. Isolates with the TR/L98H mutation are marked with an asterisk (Lokhart *et al*. 2011)

In conclusion, continued surveillance of azole resistance should be maintained to determine the resistance rates and any possible trend of increase in the isolation frequency of resistant strains. Based on the association with agricultural azole use, environmental sampling remains significant as well.

General discussion and perspectives

One third of global meat production consists of poultry products. From this single sector emerge some of the most diverse and affordable sources of protein in many countries including China. With a rapidly changing agricultural landscape and growing population, feeding flocks for optimum performance and feeding the world become greater challenges for the future. The Chinese meat market has experienced a vigorous growth in the past two decades due to consumers' demand continuous development, which is driven by demography, regular improved incomes, urbanisation and changes in diet habits. In such circumstances, reducing the impact of Infectious and parasitic diseases in poultry may represent a major objective. Coccidiosis remains the parasitic disease with the greatest economic impact on poultry industries worldwide due to production losses and costs for treatment or prevention. Furthermore, the continuous use and misuse of anticoccidial drugs have led to the emergence of drug-resistant strains. Respiratory diseases including aspergillosis may also have a significant economic impact. However, there are only a very few studies which tried to evaluate the impact of aspergillosis in poultry. None of them was performed in China. In order to increase poultry production, domestic birds are usually reared in confined buildings with densely stocked population. Fungi introduced with litter or feed can easily grow and sporulate under appropriate environmental conditions.

The objective of the present study was to collect information about fungi of the genus *Aspergillus* in avian farms from southern China. We first decided to assess the evolution of fungal contamination in 3 poultry farms near the city of Nanning and in one farm (including a hatchery) near the city of Guilin. Pharyngeal swabs and air samples were collected during several weeks and 3 cycles of hatching were monitored. In the pharynx, the filamentous fungi most frequently isolated were *Aspergillus* spp.*,* Mucorales and *Penicillium* spp. Yeasts were also very frequently detected. The average contamination level with *Aspergillus* spp. and Mucorales was significantly different according to the farms. These results look similar to those reported from avian farms in other countries. However, the lack of standardized sampling methodology and the use of different culture media and conditions make comparative analyses hardly possible. Results from swabs were expressed in percentages of positive (at least one colony of the specific fungus) Petri dishes. Unfortunately, the number of colonies was not recorded and for that reason the dynamic evolution of the fungal contamination could not be precisely analysed. In further studies, we plan to test different air sampling techniques and different culture

conditions to track the fungal contamination in farms from southern China. The performance of two samplers (CIP 10-M and Airport MD8) was recently evaluated in avian farms in France (Nieguitsila *et al*. 2011). The CIP 10-M (*Capteur Individuel de Poussières Microbiologiques*, Arelco Company, Fontenay-sous-Bois, France) is an inhalable aerosol sampler (Courbon *et al*. 1988; Görner *et al.* 2006). It was originally validated for the detection of non-culturable fungi of the genus *Pneumocystis* (Guillot *et al*. 1999). More recently, CIP 10-M has been used for the characterization of fungal aerocontamination by measurement of ergosterol in air (Robine *et al.* 2005) and by TTGE fingerprinting (Nieguitsila *et al*., 2007). The configuration of the CIP 10-M air flow is supposed to cause minimal stress to the microorganisms. There is neither mechanical impaction nor pressure drop shock. The CIP 10-M can be fit to three different particle selectors making possible the sampling of health-related aerosol fractions (inhalable, thoracic and respirable). The second air sampler (Airport MD8, Sartorius, Goettingen, Germany) is a filtration-based bioaerosol collector (Engelhart *et al.* 2007). The filter head contains a gelatine membrane (Sartorius AG, Biotechnology Division, Germany), which can be dissolved in distilled water at 37°C (Parks *et al*. 1996). The Airport MD8 sampler was used for the characterization of fungal aerocontamination in avian farms by D-HPLC fingerprinting (Nieguitsila *et al*. 2010). Nieguitsila *et al*. (2011) also assessed the relative efficiencies of culture conditions for the quantification of airborne culturable fungi in a poultry farmhouse in France. For *Aspergillus fumigatus*, higher colony forming units (cfu) values were obtained with and dichloran glycerol-18 (DG18) and malt extract agar (ME) at 25°C (30.28 and 23.06 cfu/m^3, respectively) than with ME at 37°C (3.20 cfu/m^3). This kind of result should be confirmed in avian farms in southern China. The presence of a large number of *A. flavus* conidia may modify the performance of the air samplers and culture conditions. Once the best procedure is defined for the different types of avian farms in China, a comparative analysis will be possible (according to the different climatic regions but also according to the different poultry systems). The relationship between the environmental fungal contamination and the risk of aspergillosis should be evaluated in a second step. For that purpose, a surveillance program should be defined in a limited number of farms. The reports of carcass condemnation should be collected from the slaughterhouses. The access to slaughter inspection results will probably be very difficult to obtain. However this would be the best way to evaluate the impact of aspergillosis in different regions in China and also this would constitute a simple and efficient tool to select avian farms where the relation between fungal contamination and aspergillosis could be assessed. The relative importance of *A. fumigatus* and *A. flavus* as pathogenic agents for poultry should be determined.

The survey in avian farms from Guangxi allowed us to collect a total number of 188 *A. fumigatus* and 159 *A. flavus* isolates. This series constituted an interesting complement to the already existing collection of isolates in BIPAR Unit. Most of these isolates were genetically characterized by Multiple Locus Variable-number tandem-repeat (VNTR) Analysis (MLVA). For *A. fumigatus,* the MLVA technique had already been developed by Simon Thierry during his PhD thesis (2009-2011). For *A. flavus*, a specific procedure was proposed during the present study. Loci with tandem repeats consisting of more than 20 nucleotides and more than 3 repeats were selected. Eight original VNTR markers were finally selected and a multiplex reaction was designed. Size differences between alleles of the 8 selected VNTRs were large enough to allow a multiplex amplification and further efficient differentiating on agarose gel. This makes the present MLVA scheme easy to implement in laboratories with basic molecular biology equipment. The method showed a good reproducibility, which could be increased by the production of an internal ladder (including an example of each allele amplicon size). The MLVA was shown to be rapid and very discriminant. With the panel of isolates of the present study, we obtained a diversity index of 0.993. If we exclude the 7 isolates collected in the same avian farm in China and which shared the same genotype, the value of the diversity index increases to 0.998. With the MLVA method, 20 isolates from Sfax yielded 18 genotypes whereas they were clustered into 12 genotypes when the polymorphism of microsatellite markers was examined by Hadrich *et al*. (2010). These encouraging results should be confirmed by examining a larger number of isolates. Both techniques (MLVA and microsatellites) should be used and compared. For *A. flavus*, most of the typing techniques have been developed in order to resolve closely related isolates for the purposes of outbreak investigation in hospitals and disease surveillance in humans. In only one study (Grubisha & Cotty 2010), the genetic markers (24 microsatellite loci and the mating type locus) were used to assess population structure and potential gene flow among *A. flavus* vegetative compatibility groups in sympatric populations in Arizona and Texas. In our study, the graphing algorithm termed Minimum Spanning Tree (MST) demonstrated one major cluster of *A. flavus* isolates. This cluster comprised 19 out of 32 isolates collected in the avian farm D in Guilin. Additional but smaller clusters could be defined for isolates collected in the hospital in Sfax or from avian farm A near Nanning. These clusters comprised from 2 to 5 closely related genotypes. Five out of these 6 small clusters correspond to a group of isolates sharing the same geographic origin (Paris, Nanning or Sfax). These preliminary results should be confirmed by examining a larger number of isolates from France, China and Tunisia. We especially need to collect isolates from hospital in China and from avian farms in Tunisia. We also need to collect *A. flavus* isolates

from avian cases of aspergillosis in order to confirm that there is no correlation between the genotype of the isolates and their pathogenicity. We should also examine isolates collected from crops (maize, peanut).

Some isolates from the *Aspergillus* section *Flavi* such as *A. flavus*, *A. parasiticus*, *A. nomius* and *A. caelatus* can produce aflatoxins, which are a serious problem as they have adverse effects on animal and human health (Bennett & Klich 2003). There has been considerable fundamental work on the aflatoxins, especially on their biosynthesis and molecular biology. The first stable step in the biosynthetic pathway is the production of norsolorinic acid, an anthraquinone precursor, by a type II polyketide synthase. An elaborate series of at least 15 postpolyketide synthase steps follows, yielding a series of increasingly toxigenic metabolites. Sterigmatocystin is a late metabolite in the aflatoxin pathway and is also produced as a final biosynthetic product by a number of species such as *Aspergillus versicolor* and *Aspergillus nidulans*. For *A. flavus,* it has been demonstrated that the resting bodies called sclerotia can germinate and have an important role in overwintering and production of mycotoxins (Geiser *et al.* 2000, Nesci *et al.* 2007). It would be interesting to compare the VNTR patterns of *A. flavus* according to the size of the sclerotia (S and L types) and to the level of aflatoxin production. In previous investigations, PCR has been employed to identify the presence of *Aspergillus* fungi in the soil by Accinelli *et al.* (2008), while RT-PCR (reverse transcription PCR) has also been employed to distinguish toxigenic and nontoxigenic *A. flavus* (Degola *et al.* 2006, Scherm *et al.* 2005), as well as to track the presence of aflatoxin biosynthetic genes (Accinelli *et al*. 2008).

The past decade has seen remarkable progress through the development of novel classes of anti-fungal drugs active against *Aspergillus* and other mould infections. It remains evident, however, that despite new drugs and strategies of combination therapy in an attempt to improve response rates, the available drugs can only achieve a limited level of efficacy in a host that is unable to mount an adequate immune response. Furthermore, strains of *A. fumigatus* with resistance to several triazoles have been isolated from some patients in several countries. If multi-azole resistant strains of *A. fumigatus* become common, they could have a serious impact on the management of invasive aspergillosis in humans and probably in animals also. Exposure of saprophytic fungi to azole compounds could take place in agriculture, where such chemical compounds are commonly used for plant protection. The fungicides are applied repeatedly over a long period of time and could thereby create a persistent pressure of azole compounds on saprophytic fungi, including *A. fumigatus*. The researchers from the University of Nijmegen (The Netherlands) suggested that an environmental route of resistance development in *A. fumigatus* should be considered because a single mechanism of azole resistance

(TR/L98H substitution) was found in most of clinical isolates from different hospitals in the Netherlands (Verweij *et al.* 2009, Snelders *et al*. 2011). To date, clinical *A. fumigatus* isolates with the TR/L98H substitution have been reported in patients from the Netherlands, Spain, France, Norway, United Kingdom, Belgium and China (Howard *et al*. 2009, Lokhart *et al*. 2011, Mellado *et al*. 2007, Mortensen *et al*. 2010, Snelders *et al*. 2008). Environmental *A. fumigatus* isolates with the TR/L98H substitution have been recovered in the Netherlands and Denmark from agricultural samples of soil (Mortensen *et al*. 2010, Snelders *et al*. 2008). As azole derivatives are frequently used in poultry farms (for the control of avian mycoses), we decided to assess the antifungal susceptibility of *A. fumigatus* isolates from farms in France and China. Most of the isolates from China were susceptible to itraconazole with a Minimum Inhibitory Concentration (MIC) comprised between 0.38 and 0.75 µg/mL. Most of the isolates from birds and avian farms in France were susceptible to itraconazole with a MIC comprised between 0.19 and 1 µg/mL. MIC values of isolates collected in farms with antifungal chemoprophylaxis were not higher than those of isolates collected from birds that never received antifungal drugs before the sampling. This preliminary result should be confirmed by the examination of a larger number of isolates from farms in which antifungal chemotherapy is frequently performed. Two molecules can be used in poultry farms: enilconazole (by nebulization) and parconazole (with the feed). The two molecules and the two modes of administration may have a different impact in the potential emergence of isolates with a decreased antifungal susceptibility. In the present study, Etest results demonstrated that 4 isolates should be considered as resistant to itraconazole: 2 isolates from avian farms in Guangxi, China and 2 isolates from avian farms in France. This kind of result should be confirmed by the use of other susceptibility tests. In recent years, there have been advances with the development of Clinical Laboratory Standards Institute (CLSI; formerly NCCLS) M38-A (NCCLS, 2002), and European Committee for Antibiotic Susceptibility Testing (EUCAST) methods for filamentous fungi, including *Aspergillus* spp. Both methods include a microtitre format, 48-h incubation at 35–37°C and a no growth visual endpoint. The main differences between the two standards are the inoculum preparation and size of the inocula. We should also include different azole derivatives (other than itraconazole).

A modification of the *Cyp51A* sequence was identified in 11 isolates (3 azole-resistant and 8 azole-susceptible isolates). Twenty-one nucleotidic mutations were detected. Eleven of these mutations were silent and 10 yielded to amino acid substitutions in the protein. Seven of these substitutions had already been described whereas mutations A116R, E130D and Q131H were original ones. One itraconazole-resistant isolate (F78) presented a wild type *Cyp51A* sequence. The absence of mutation may indicate that another mechanism of

resistance is implicated for this isolate. The potential increased efflux of azole drugs due to the overexpression of efflux pumps should be assessed by specific PCR reactions.

One of the most interesting results of the present study was the combination of azole susceptibility results and genotyping by MLVA. When azole susceptibility was considered as the discriminant parameter, clustering analysis revealed that resistant *A. fumigatus* isolates were genetically distinct. However small clusters of *Cyp51A*-mutated isolates could be detected. A first cluster comprised isolate F82 and 6 susceptible isolates. The point mutation M172V was detected in all these isolates. Another small cluster comprised isolates C28 and C15. These isolates were both mutated at position N248. In the MST representation, isolate C19, the only one with mutations E130D and Q131H, was genetically distinct from all other isolates. The same situation was observed for isolate F78 (the azole-resistant isolate without any mutations of *Cyp51A*). In further investigations, we should genotype *A. fumigatus* isolates with the TR/L98H substitution and verify whether they could form clusters in MST representations. The azole susceptibility of *A. flavus* isolates from southern China should also been assessed.

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