

Assessment of Aspergillus Fumigatus Burden in Lungs of Intratracheally-Challenged Turkeys (Meleagris Gallopavo) by Quantitative PCR, Galactomannan Enzyme Immunoassay, and Quantitative Culture

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29	Short Title: Aspergillus pulmonary burden in an avian model
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33

34 Abstract – (less than 150 words)

35

Aspergillus fumigatus remains a major respiratory pathogen in birds, but diagnosis is still 36 difficult. We challenged different groups of few-days-old turkeys via intratracheal 37 aerosolisation of increasing concentrations (10^5 up to 10^8) of conidia using a MicroSprayer[®] 38 device. The fungal burden was assessed by real-time PCR, galactomannan dosage, CFU 39 40 counting and histopathological evaluation in order to provide a comparison of these results 41 within each inoculum groups. A significant mortality occurring in the first 96 hours after 42 inoculation was only observed with the highest inoculum dose. Culture counts, GM index 43 and qPCR results on one hand and inoculum size on the other hand appeared to be clearly 44 correlated. The mean fungal burden detected by qPCR was 1.3 log₁₀ units higher than mean values determined by CFU measurement. The new model and the markers will be used for 45 46 the evaluation of the efficacy of antifungal treatments that could be applied in poultry farms. 47 48 49 50

51 Keywords (Up to 10)

Turkey, lung, aspergillosis, *Aspergillus fumigatus*, model, real-time PCR, galactomannan,
culture

54

56 1. Introduction

57 Aspergillosis is the most severe fungal disease in birds involving thermophilic mold species belonging to the genus Aspergillus, among which A. fumigatus is by far the most prevalent 58 59 causative agent. In poultry production and more specifically in turkeys' fast-growing or breeding flocks, this opportunistic infection induces severe economic losses. Generally 60 61 confined to the lower respiratory tract in acute cases affecting young birds, lesions may 62 extend to many other organs via secondary haematogenous dissemination or contiguous 63 infection. The small diameter of conidia allow them, once inhaled, to be deposited directly deep within the respiratory system particularly in posterior air sacs. Therefore, primary 64 65 colonization occurs in an optimal environment for germination and mycelium growth of 66 Aspergillus molds. Considering the physiological specific aspects of their highly efficient 67 respiratory tract and the lack of efficient treatments on declared aspergillosis, the 68 development of avian models is absolutely necessary to acquire a comprehensive knowledge of the physiopathology of a disease which may be responsible for high rates of morbidity 69 70 and mortality, poor performances and subsequent economic losses in poultry farms [1, 2]. 71 Numerous vertebrate models of this disease have been successively developed to study 72 various aspects of the fungal development in the host, the subsequent immune response and 73 to test antifungal drug efficacy. In contrast to rodents or rabbits experimental models, 74 immune modulation is not a necessary prerequisite in avian challenge models [1]. 75 Aspergillosis is readily induced in healthy birds by inhalation of aerosolized conidia in turkeys [3] or chickens [4], intravenous challenge in turkeys [5], intratracheal route in 76 77 starlings [6], Japanese quails [7, 8, 9], rock pigeons and gyr-falcon hybrids [10] or by transcutaneous inoculation into air sacs in turkeys [11] or racing pigeons [12]. Besides 78 79 mortality or clinical signs, the monitoring of host-pathogen interactions following experimental challenge in birds rely on gross lesions and histopathological evidences [13], 80

81	completed with either the detection of markers of the fungal extension [1] or the
82	quantification of the inflammatory response [7]. Since many years now, non-culture based
83	methods either used alone or in combination have been targeted to Aspergillus DNA by real-
84	time PCR or cell-wall antigens in different tissues or fluids to establish a rapid diagnosis of
85	invasive aspergillosis in humans [14] or to monitor experimental disease in several rodents
86	models [15, 16, 17, 18, 19]. Dosages of galactomannan or 1,3- β -D-glucans have also been
87	tested in different bird species suffering aspergillosis [20], in turkeys following experimental
88	infection [21] and recently on field cases affecting commercial broiler chickens and turkeys
89	[22].
90	Our goal was to validate an accurate model of acute aspergillosis in intratracheally-
91	challenged turkeys. Non-culture-based methods and quantitative culture were compared to
92	assess the fungal burden in lungs. In further investigations this original model may be used
93	to test antifungal treatments in poultry.
94	
95	2. Materials and methods
96	
97	2.1. Aspergillus fumigatus strain, mycological cultures and preparation of the inoculum
98	Aspergillus fumigatus strain CBS 144.89 was used for all experiments. This strain was
99	initially isolated from a human patient with invasive aspergillosis in France and obtained
100	from the Centraalbureau voor Schimmelculture (CBS), Utrecht, The Netherlands. All
101	mycological cultures were done on malt agar plates supplemented with chloramphenicol (50
102	mg/L) and incubated at 37°C till 10 days depending on the fungal growth and species. To
103	prepare the inoculum, the strain was grown for 10 days at 37°C. Conidia were subsequently

104 harvested by flooding the plates with sterile phosphate-buffered saline (PBS) containing

105	0.01% (vol/vol) Tween 20 (PBST). The conidia were concentrated by centrifugation (at
106	3500 g for 30 min) and counted using a Malassez counting chamber.

107

108 2.2. Animals and housing conditions

109 Conventional male turkeys (Le Sayec, Caudan, France) of 1-day-old with an average weight

110 of 65 to 70 g were used. Groups of approximately 20 birds were housed in minimum 2 m²

111 cages on wood chips litter, in the biosafety 3 level sector of the animal facility of the

112 veterinary college of Alfort, France. Animals had permanent access to fresh commercial

113 poultry starter (Versela-Laga) and water. Photoperiod as ambient temperature was regulated

and two infrared lamps provided additional heat during all the experiment.

115

116 2.3. Environmental contamination assessment

117 The fungal contamination of the environment was assessed before and during the

118 experiments by regular sampling of conidia by sedimentation on two opened plates placed in

each cage for 30 min. In parallel, 1 g of litter and feedstuff were sampled and mixed in 45

mL PBST from which $100 \mu l$ was seeded on agar plates.

121

122 2.4. Nebulizer system and intratracheal challenge

123 Two sets of experimental infections were conducted in the same conditions. For the first

experiment, 36 seven-days-old turkeys were inoculated (day 0) with different concentrations

of *A. fumigatus* (12 birds per inoculum): 10^5 , 10^6 or 10^7 conidia per bird (groups named

126 Inoc5, Inoc6 and Inoc7, respectively). Nine turkeys were inoculated with PBST as negative

127 control. In the second experiment, 12 five-days-old turkeys were inoculated with 10^8 conidia

128 (Inoc8) and 7 turkeys were inoculated with PBST. Two birds of each group were

anesthetized immediately after inoculation in order to check the contamination level oflungs.

Turkeys were anesthetized by inhalation of 5 % isoflurane (Aerrane; Baxter) in oxygen

132 and inoculated with 100 µL of A. fumigatus suspension (or PBST) using a stainless steel 19 gauge atomizer (Microsprayer IA-1B®, Penn Century, Philadelphia, PA, USA) inserted into 133 134 the trachea under visual control. No intra-operative deaths or delayed recovery (less than 2 135 min after inoculation) were observed. Birds were monitored at least twice daily and every signs of morbidity or mortalities were recorded. 136 All animal research procedures were approved by the regional ethics committee for 137 138 animal experimentation at the veterinary college of Alfort. 139 140 2.5. Sample collection 141 Targeted organs of the respiratory tract from all dead and euthanized animals were removed 142 aseptically under laminar flow hood. The trachea and left thoracic air sac were sampled with 143 a sterile swab. The left lungs were collected for histology and the right lungs were used for 144 fungal culture, real time PCR and galactomannan dosage.

145

131

146 2.6. Histological evaluation

147 Tissues collected for histology were immediately fixed in 10% neutral buffered formalin and

148 dehydrated. Paraffin wax-embedded specimens were sectioned at 4 µm and stained with

149 hematoxylin-eosin-saffron (HES) to evaluate the inflammatory response. Periodic acid

150 Schiff (PAS) and Grocott-Gomori methenamine-silver stains (MS) were used to visualize

151 Aspergillus in lesions. A blind reading of 1 up to 10 slides per selected turkey of each group

152 was conducted to evaluate the intensity of microscopic lesions.

154 2.7. Fungal culture of respiratory organs

155 The right lungs were weighed and grinded with a homogenizer of tissue (Bio-Gen PRO200

156 Homogenizer, Pro Scientific, Oxford CT, USA) in 6 mL of PBS containing 0.01% (vol/vol)

157 Tween 20. 100 μ L of the primary lung homogenates were immediately used for fungal

158 culture, the rest was frozen at -20° C until use.

159 Dilutions of primary lung homogenates and swabs of trachea and air sac were seeded

160 on malt chloramphenicol agar plates and incubated at 37°C. The count of *A. fumigatus*

161 colonies (CFU) were assessed 1 and 2 days after plate inoculation.

162

163 2.8. Assessment of conidial equivalent by real time PCR

164 The primary lung homogenates were subjected to a secondary homogenization step. $500 \,\mu L$

165 of lung homogenate was transferred to a sterile 2 mL screw-cap centrifuge tube containing

166 0.5 mm-diameter glass beads (Biospec, Bartlesville, USA) and placed in a MagNa Lyser

167 homogenizer (Roche, Meylan, France) for 3 bursts of 30 s at 3500 rpm with incubation on

168 ice between bursts. The secondary lung homogenate was collected by centrifugation at 800 g

169 for 5 min at 4° C and 100 μ L of this homogenate was directly used for DNA extraction with

the QIA amp DNA Mini Kit (Qiagen, Courtabeouf, France) according to the manufacturer's

171 directions. Fungal DNA and secondary homogenate were stored at -20°C until use. Real-

time TaqMan PCR quantified the amount of A. fumigatus cell nuclei by targeting the single-

173 copy *FKS* gene and the multi-copy gene encoding the 28S rRNA. The sequences of the *FKS*

primers were previously reported [23]: sense amplification primer, AFKS1 (5'-GCC TGG

175 TAG TGA AGC TGA GCG T-3'), antisens amplification primer, AFKS2 (5'-CGG TGA

176 ATG TAG GCA TGT TGT CC-3') and the hybridization *FKS* probe (5'-6-FAM-TCA CTC

177 TCT ACC CCC ATG CCC GAG CC-BHQ1-3'). The 28S primers were reported by Challier

178 et al. [24]: 28S-466 (5'-CTC GGA ATG TAT CAC CTC TCG G-3') and 28S-533 (5'-TCC

TCG GTC CAG GCA GG-3') and the hybridization 28S probe (5'-6-FAM-TGT CTT ATA
GCC GAG GGT GCA ATG CG- TAMRA-3').

181 A standard curve was established with DNA samples extracted from uninfected lungs of turkeys inoculated with different doses of A. *fumigatus* conidia (from 10^2 to 10^8 conidia) and 182 183 allowed us to quantify the Aspergillus DNA in the organs by calculating conidial equivalents 184 (CE) [15]. DNA samples were analysed in duplicate by using the LightCycler® 480 185 detection system (Roche, Meylan, France). The reaction mixture consisted in 5 µL of DNA, 186 900 or 500 nM sense and antisens primers from FKS or 28S targets, 200 nM FKS probe or 250 nM 28S probe and LightCycler® 480 Probes Master 2x (Roche, Meylan, France). All 187 assays were run under the following conditions: 50°C for 2 min, 95°C for 10 min, then 50 188 189 cycles of 15 s at 95°C and 1 min at 65°C or 60°C respectively for FKS or 28S primers. 190 191 2.9. Galactomannan (GM) quantity assessment

supernatant was placed into a fresh tube. Lung supernatants (300 μ l) were used for GM

500 µL of primary lung homogenates were centrifuged at 2,300 g for 5 min and the

determination with the Platelia GM-EIA kits (Bio-Rad Laboratories) according to the

195 manufacturer's directions. The minimum and maximum thresholds of DO detected by the

196 ELISA reader (Biorad PR1300) ranged between 0 and 3 Abs. A 1:100 dilution of lung

197 homogenates was realized for all groups and a 1:1000 dilution for the Inoc8 group in order

to avoid the saturation of the signal detected in some crude lung homogenates.

199

192

200 2.10. Genotyping of *Aspergillus fumigatus* isolates

201 A subset of strains recovered either from the environment or from internal organs was

202 collected and genotyped by Multi Locus VNTR Analysis [25].

204 2.11. Statistical allaryses	204	2.11.	Statistical	analyses
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205	Statistical analyses were performed with the JMP 10.0 software. A log rank t test has been
206	used to analyse the survival of birds and to evaluate the effect of the inoculum dose on
207	mortality. A nonparametric one-way analysis of variance, Wilcoxon test, linear regression
208	models and Spearman's rank correlation were performed for multiple comparisons and to
209	evaluate the relationship between the different biomarkers (\log_{10} CFU/g of lung, \log_{10} CE/g
210	of lung and the GM index) and the inoculum doses.
211	
212	3. Results
213	
214	3.1. Body weight, clinical signs and mortality
215	A dose dependent response was observed concerning weight gain evolution when inoculated
216	turkeys were compared to non-challenged birds in both sets. If birds from Inoc5 and Inoc6
217	were not affected by Aspergillus challenge, growth average of birds exposed to higher
218	concentrations (Inoc7 and Inoc8) decreased significantly as soon as 2 days post inoculation
219	(figure 1).
220	No clinical signs or mortality were observed in non-challenged turkeys and Inoc5.
221	The clinical signs observed in turkeys of Inoc6, Inoc7 and Inoc8 included lethargy, ruffled
222	feathers, progressive weight loss and respiratory distress such as open-mouthed breathing
223	leading to lateral recumbency in severe cases. Two birds out of 9 in Inoc6 exhibited clinical
224	signs 6 days after inoculation, but did not die before the end of the experiment. Eight turkeys
225	out of 10 in Inoc7 presented clinical signs, which appeared between 3 and 6 days after
226	inoculation. Among them, only 3 animals died at day 5. In set B, an acute respiratory
227	distress was registered between 1 and 3 days post inoculation of A. fumigatus in all turkeys

of Inoc8 (n=10), which died 24 to 48h later (figure 2).

229 3.2. Macroscopic and microscopic lesions

230 No macroscopic lesions were observed on non-challenged and Inoc5 birds. An increasing proportion of turkeys from Inoc6 (5/10), Inoc7 (8/10) and Inoc8 (10/10) exhibited few 231 232 granulomas on thoracic air sacs and a densification of variable proportions of the pulmonary 233 parenchyma. Histological lesions associated with Aspergillus development, mostly 234 represented by multifocal either isolated or coalescing granulomas with necrotic centers, and 235 occurrence of multinucleated giant cells (figure 3) were confirmed in lungs of Inoc6, Inoc7 236 and Inoc8 turkeys but were absent in Inoc5 or non-challenged birds. Percentages of altered parenchyma varying from 30 to 80% have been estimated on lungs preparation coming from 237 238 different birds belonging to Inoc7 and Inoc8. However, the hyphae burden seemed to be 239 more important in the latter group.

240

241 3.3. Fungal cultures from trachea, air sacs and lungs

242 The lower limit of the assay is approximately 300 CFU/g of lung (10 CFU/plate). All non-243 challenged turkeys exhibited negative cultures. Semi-quantitative numerations from trachea 244 and air sacs showed variable levels of contamination immediately after inoculation in all challenged groups. However no Aspergillus could be recovered from both organs of all birds 245 246 of Inoc5, Inoc6 and Inoc7 that were euthanized between 5 and 8 days post challenge. For 247 Inoc8, a high level of contamination (>100 CFU/plate) was still present in air sacs till day 4, 248 but not in trachea even in turkeys which died spontaneously 2 days after inoculation. Positive counts ranging from 3.28 up to 9.75 \log_{10} CFU/g have been registered on lungs of 249 250 all turkeys sacrificed immediately after the inoculation. In the remaining birds, the 251 proportion of positive samples and the mean of log CFU/g of lung established on those 252 samples increased with the inoculum size (table 1). The mean of \log_{10} CFU/g of lung for the

13 turkeys (Inoc7 and Inoc8) that died spontaneously between 2 and 5 days pi was 5.47 ±
0,41.

255

256 3.4. Assessment of conidial equivalent by real time PCR

257 The sensitivity of real time PCR on 28S target was higher than on FKS gene with limits of detection varying from 5 CE (corresponding to 10^3 CE/g of lung) to 250 CE per reaction (5 x 258 10^4 CE/g of lung) respectively. Immediately after experimental infection, qPCR signals 259 260 remained undetectable for the 2 birds of Inoc5, one turkey of Inoc6 but highly positive for the others (Inoc7 and 8). At day 8, no amplification at all was detected with both targets for 261 262 Inoc5 and only 3/9 samples from Inoc6 were detected with the 28S target. The numbers of 263 CE/g established of lung detected with FKS target and 28S target were positively correlated $(\log_{10} \text{CE}_{\text{FKS}}/\text{g of lung} = -2.92 + 1.34*\log_{10} \text{CE}_{28S}/\text{g of lung})$ when assessed on the 17 DNA 264 265 samples (7/10 Inoc7 and 10/10 Inoc8) exhibiting amplification with both DNA targets (data not shown). Similarly to fungal numerations both proportions of positive amplifications and 266 267 means of log CE_{28S} /g of lung in positive results appeared to be correlated to the inoculum 268 size (table 1).

269

270 3.5. Galactomannan (GM) quantity assessment

GM index obtained on crude lung homogenates presented huge variations from 0.5 to 6 (signal saturation) in all groups including non-challenged birds. GM index repartition after 1:100 dilution (figure 4) was quite similar in control, Inoc5 and Inoc6 birds (p=0.0084). The highest variation of GM index values was observed in Inoc7 (mean GM index = $3.99 \pm$ 0.90). All turkeys that died spontaneously (including all Inoc8) exhibited index higher than 5. Interestingly, GM index of all birds immediately euthanized after inoculation were lower than 0.35. 278 3.6. Correlation between the different markers

279	The number of \log_{10} CE28S/g of lung was strongly correlated to the number of \log_{10} CFU/g
280	of lung (p<0.0001) when the data of all groups were included (figure 5) but not in each
281	groups considered separately. Both values increased significantly with inoculum size
282	(p<0.0001). As featured by the regression line, the sensitivity threshold of CE values was
283	almost 10 fold higher than CFU counts in lung samples ($\log_{10} \text{CE28S/g of lung} = 1.30*\log_{10}$
284	CFU/g of lung). Birds, which died quickly, have constantly more CFU and CE in lungs than
285	the other birds. GM index applied to diluted lung homogenates (1:1000) of Inoc8 was
286	significantly correlated to log_{10} CE/g (p=0.0172) but not with log_{10} CFU/g (p=0.526) (figure
287	6).
288	
289	3.7 Environmental contamination
200	Naglicible levels of contamination by molds (Aspensillus app. and Musereles) have been
290	Regrigible revers of containination by molds (<i>Aspergulus</i> spp. and Mucorales) have been
291	detected in the litter or in the room's atmosphere prior to the installation of the birds or after
292	inoculation (controls at day 1 and day 8). Feedstuffs remained constantly culture-negative.
293	
294	3.8. Molecular typing of A. fumigatus
295	Six A. fumigatus isolates collected from the animals and the environment presented the
296	same MLVA genotype as that of the reference strain (CBS 144.89) used for the
297	experimental infection.
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299	
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301	

302 4. Discussion

303 4.1. Experimental model

Many experimental aspergillosis have already been published using different avian models 304 305 and testing various bird species, age at inoculation, challenge routes and even the 306 Aspergillus species [1]. Both field data [2] and experimental results [26] clearly demonstrate a higher susceptibility of turkeys and quails to A. fumigatus infection when compared to 307 308 chickens, but also intraspecific differences in host responses [4, 27] highlighting the 309 necessity to establish models on relevant species or lineages reared for meat or egg production. Different routes of Aspergillus inoculation have been tested in turkeys including 310 311 intrapulmonary [28], intra-air-sac administration [11, 21] and finally aerosolization in 312 confined plastic chambers [3]. With the exception of the latter method, which is difficult to 313 standardize [4], the intratracheal inoculation, which has not been yet tested in turkeys, is the 314 closest to the natural way of infection and only bypasses the upper airways and their putative 315 defenses [1]. We propose a relevant model of acute aspergillosis in one-week-old turkeys 316 following intratracheal nebulization of Aspergillus fumigatus conidia with a MicroSprayer® 317 aerosolizer. The use of this device has already been validated in immunosuppressed 6-8 weeks-old Sprague-Dawley rats [16] and immunocompetent Japanese quail [7] to 318 319 administrate precisely calibrated inoculum of *Aspergillus fumigatus* in the respiratory tract. The evaluation of the impact of four inoculum concentrations (10^5 to 10^8 conidia per turkey) 320 allowed us to record mortality following a dose response with values of 0, 0, 30 and 100% 321 322 respectively. Global parameters such as the evolution of body-weight, the occurrence and 323 severity of clinical signs and the chronology of deaths appeared strictly in concordance with 324 the concentration of conidia administered. Lower mortality rates (respectively 16, 55, 56 and 20%) have been observed in turkeys treated with 10^8 conidia in the air sac [21] or submitted 325 to dry aerosol delivering doses of 5.10^9 spores per animal [3] or in quails receiving $1.5 \ 10^6$ 326

[9] conidia intratracheally. A similar protocol $(2.10^7 \text{ conidia administered intratracheally})$ 327 applied to the same species resulted in 55% [29] and 100% mortality [8]. As mentioned by 328 Goetting et al. [7] who compared two modalities of inoculation, the nebulization by 329 330 Microsprayer® should not be considered to be strictly equivalent to a "classical" 331 intratracheal inoculation using an intravenous catheter after removing the stylet. Higher mortality (88% versus 33%) has indeed been observed after nebulization compared to the 332 "classical" method after the same inoculum size (4.7 10^7 spores). This could be attributed to 333 differences in dispersion parameters (velocity, dropplet size) through the respiratory tract. 334 Similarly to [7], the macroscopic lesions registered in our model were strictly limited to the 335 336 respiratory apparatus and particularly marked in lungs but much more rarely on caudal air 337 sacs. Extensive parenchyma necrotic damage consecutive with the presence of multifocal 338 typical granulomas [11, 13] were seen in the present experiment in all birds of Inoc8 but also 339 in some animals belonging to Inoc7, which have been euthanized at the end of our 340 experiment. We cannot therefore exclude the possibility of delayed mortality (up to 50%) in 341 the latter group if the experiment took place over a longer post-inoculation period [3].

342

343 4.2. Biomarker evaluation

The relevance of three putative biomarkers (CFU, galactomannan and real-time PCR) has been concomitantly assessed on lung homogenates for the first time in turkeys. In contrast with already published models in the immunosuppressed rat [15] or Guinea pig [17, 18], our primary purpose was to study the kinetics of fungal development in several target organs but to compare the effect of variable inoculum sizes on these parameters and, in addition, we tried to establish potential correlations to characterize fungal burden states in the lung since it was the sole organ to present extensive macroscopic lesions in our model. 351 Semi-quantitative culture-based methods used to evaluate the Aspergillus load in organs are 352 easy to perform and common but only detect viable organisms. Furthermore, temporal studies in rodents suggest that these tools may not be accurate when monitoring the 353 354 development of multicellular filamentous fungi particularly in parenchyma submitted to 355 mechanical homogenization [30] leading to possible underestimation [31, 32]. Except for 356 some air sacs samples obtained from turkeys, which died in the first four days following 357 inoculation, no fungus could be detected on the trachea or air sac swabs. This may be 358 explained by fungal clearance, or low sensitivity of the method used on these organs. Although not strictly comparable because the interval post-inoculation differed between 359 360 dead and euthanized birds, the mean lung numerations, the proportion and the homogeneity 361 of positive results seemed clearly to increase with the inoculum dose. All dead animals presented at least 5.10^5 spores/g of lung tissue which was estimated by Richard *et al.* [3] as 362 363 the dose necessary to kill about 50% of 3-week-old turkeys exposed to an aerosol of Aspergillus conidia which appeared to be a clear threshold in younger birds unable to mount 364 365 an effective innate response [4, 11, 13] in the critical phase of the first days following 366 experimental infection. The detection of viable Aspergillus was possible in a high proportion of apparently healthy turkeys, which were euthanized at the end of the experiment, but may 367 368 persist to least 8 weeks post-exposure [3]. However, fungal load in surviving birds as 369 assessed by culture seemed to decrease post-inoculation as in other avian [4, 9] or mammal 370 models [18, 19].

The enzyme immune assay of galactomannan, a specific cell-wall component of fungi is relatively easy to perform and is likely to reflect the progressive increase in mycelium load by tip extension of hyphae [31]. The availability of commercial tests has allowed this polysaccharide component to be measured as a valuable biomarker in the sera of different bird species suffering from aspergillosis [20]. It has been used in field studies on 376 commercial broiler chickens and turkeys [22], but also in mammals [16, 32] and turkeys 377 [21] experimental models. As a potential diagnosis tool, contrasting results have been obtained due to variable sensitivity or specificity depending on the species tested. Additional 378 379 difficulties exist in the definition of an appropriate cut-off value and the stage of the disease 380 [20] but also to the ubiquitous presence of galactomannan in the poultry environment leading to false positives [1]. This last point may partially explain the positive GM values 381 382 obtained from crude lung homogenates of control birds housed in a clean but not sterile 383 environment. This necessitates work on dilutions to evaluate this parameter. The GM index on 1:100 dilutions were very low in turkeys submitted to the two lowest doses as for non-384 385 exposed birds but were much more variable in Inoc7. This may reflect individual differences 386 in host-response efficiency. The high GM index systematically seen in turkeys, which died 387 spontaneously in contrast to almost undetectable values in birds euthanized immediately 388 after receiving variable doses of non-germinated conidia, is consistent with the relationship 389 between the index value and the progression of the fungus [17, 31]. Furthermore, GM index correlated with the extent of infection as demonstrated by histopathological examination 390 391 [18].

A real-time quantitative PCR assay has been developed recently in rodent models to 392 393 measure the fungal load and its progression in different organs in order to evaluate the 394 efficacy of antifungal therapy [15, 17, 18, 19]. This molecular tool allows the detection of 395 both conidia and hyphae and can be very sensitive and specific but like the GM enzyme 396 immunoassay, it does not indicate viability [31]. Gene targets included a monocopy gene 397 like FKS1 [18] or multicopy genes, in particular the ribosomal DNA genes [15] and internal 398 transcribed spacer. The choice of multicopy genes alone or in the frame of a nested PCR 399 [17] to improve sensitivity may not be ideal for standardization of the method [32]. To our knowledge, qPCR in avian lungs was evaluated for the first time in this study. The real-time 400

401 PCR signal from a sample of infected tissue was compared with a standard curve composed 402 of conidia added to naïve pulmonary parenchyma and we chose to express the fungal 403 biomass in terms of CE [15]. The performances of the monocopy FKS gene and the 404 multicopy 28SrDNA amplification in turkey lungs proved to be equivalent although the 405 sensitivity of the latter PCR was logically about $1 \log_{10}$ CE/g lower. Mathematical extrapolation predicted that the detection limit of this assay was approximately 10^3 CE/g of 406 lung, which is quite similar to 2.10^3 CE/g (18S) estimated in an haematogenous mouse 407 408 model of aspergillosis [15], but could further be improved with a nested qPCR [17]. As demonstrated by results on euthanized birds just after inoculation, the qPCR seemed to be 409 410 less sensitive than CFU numerations, which may be explained by a low efficiency of 411 extraction of conidia from tissue samples [19, 30]. Although no signal could be amplified in 412 Inoc5 and there was no sign of illness, both the proportion of positive samples and mean 413 $log_{10}CE/g$ tended to increase with the dose used. Finally, the onset of induced mortality 414 coincided clearly with a peak in fungal burden in Inoc8 as observed in a mouse model [15]. 415 A strong correlation between qPCR results and culture numerations was seen when positive 416 data from all groups were included and the molecular method demonstrated a higher sensitivity. This relationship appears to be stronger when tissues contain hyphae rather than 417 418 conidia [30]. When data from Inoc8 were compared with similar mammal models [15, 18], 419 GM index and qPCR appeared to be more closely correlated with the rise of infection in 420 comparison with CFU values which may partially be explained by the fact that the culture-421 numerations stagnates or decreases with time [19]. 422

423 4.3. Conclusion

424 We have developed a repeatable and standardized model of acute and fatal primary

425 pulmonary pneumonia in the young, immunocompetent turkey using an atomizer, which

426	may be applicable to chemotherapy trials. Housing birds in groups in a controlled
427	environment enables field conditions of production to be mimicked and to evaluate the
428	treatment of aspergillosis by nebulized molecules such as azole compounds [2]. Besides
429	survival, still considered the most reliable measure to assess antifungal therapy, the
430	combination of additional markers of fungal development may contribute to an earlier and
431	more precise detection of treatment efficiency in birds submitted to lethal or sub-lethal
432	challenges but also to the refinement and the reduction objectives of in vivo experimental
433	research [31, 32].
434	
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439 **Table 1.** Fungal burdens in lungs of inoculated turkeys measured by CFU and real time PCR

440 (28S) assays.

Groups	Mean log ₁₀ CFU/g of lung	Mean $\log_{10} CE_{_{28S}}$ /g of lung
Inoc5	BLD ^a	BLD
Inoc6	2.84 ± 0.30 (5/9) ^b	5.32 ± 0.13 (3/9)
Inoc7	4.34 ± 0.29 (7/10)	5.73 ± 0.14 (7/10)
Inoc8	5.60 ± 0.12 (10/10)	7.17 ± 0.11 (10/10)

^a BLD : below level of detection

^b Mean ± standard error (no. of positive values/total no. of birds tested)

443 Figure 1. Mean weight variations of inoculated birds (black line) compared to non-

444 challenged turkeys (grey line) from set A (solid line) or B (dashed line). Inoculum of 10^5

445 (\triangleright), 10⁶ (\triangle), 10⁷ (\diamondsuit) or 10⁸ (O) *Aspergillus fumigatus* conidia.

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Figure 2. Survival time of non-challenged (grey line) and inoculated (black line) turkeys from set A (solid line) and B (dashed line) Inoculum of 10^5 (\triangleright), 10^6 (\triangle), 10^7 (\diamondsuit) or 10^8 (O) *Aspergillus fumigatus* conidia.



- Figure 3. Histological sections of lungs from 2 turkeys (Inoc8). 3a (HES stain): Voluminous
 granuloma with a necrotic centre. 3b, 3c (HES stain) and 3d (MS stain): Detailed of a
 granuloma. A: necrosis; B: granuloma; C: red blood cells in capillary; D: giant cells with
- 464 multiple nuclei; E: *Aspergillus* hyphae.



465

467 Figure 4. Comparison of mean Galactomannan index (1:100 dilution) in lung samples from468 non challenged and inoculated groups.



Figure 5. Relationship between CFU and CE (\log_{10} CE28S/g of $\log = 1.30*\log_{10}$ CFU/g of lung) as shown by linear regression. Turkeys inoculated with 10^5 (\triangleright), 10^6 (\triangle), 10^7 (\diamondsuit) or 10^8 (\bigcirc) conidia. *Significant correlation (p < 0.0001) between results by the Spearman rank correlation, n = 21.



Figure 6. Relationship between GM index and CFU (A) or CE (B) per g of lung from turkeys inoculated with 10^8 conidia. *Significant correlation (p = 0.0038) between galactomannan index and CE by the Spearman rank correlation test, n = 10.



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