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24 **Abstract**

25 To investigate the efficacy of voriconazole for the treatment of aspergillosis, three groups of six
26 racing pigeons (*Columba livia domestica*) were inoculated in the apical part of the right lung with
27 2×10^7 conidia of an avian derived *Aspergillus fumigatus* strain. The minimal inhibitory
28 concentration of voriconazole for this strain was 0.25 µg/ml. In two groups, voriconazole
29 treatment was started upon appearance of the first clinical signs and continued for fourteen days.
30 The third group was sham treated. The voriconazole treated pigeons received voriconazole orally
31 at a dose of 10 mg/kg body weight (BW) q12h (group 1) or 20 mg/kg BW q24h (group 2).
32 Sixteen days post inoculation all surviving pigeons were euthanized. Weight loss, clinical scores,
33 daily mortality, lesions at necropsy and isolation of *A. fumigatus* were compared between all
34 groups. In both voriconazole treated groups, a significant reduction in clinical signs and lesions
35 was observed. Administering voriconazole at 10 mg/kg BW q12h eliminated *A. fumigatus* and
36 administering voriconazole at 20 mg/kg BW q24h reduced *A. fumigatus* isolation rates. Mild
37 histological liver abnormalities were found in group 1 (10 mg/kg BW q12h), while mild
38 histological as well as macroscopic liver abnormalities were found in group 2 (20 mg/kg BW
39 q24h).
40 In conclusion, voriconazole at 10 mg/kg BW q12h in pigeons reduces clinical signs and
41 eliminates *A. fumigatus* in racing pigeons experimentally infected with *A. fumigatus*.

42

43

44

45

46 **Key words**

47 voriconazole, experimental *Aspergillus fumigatus* infection, antifungal treatment, avian

48 1. Introduction

49 Aspergillosis is the most common fungal disease in birds and is in most cases caused by
50 *Aspergillus fumigatus* although other *Aspergillus* spp. including *A. flavus*, *A. niger*, *A. glaucus*
51 and *A. nidulans* are occasionally involved. These fungi are ubiquitous and can be isolated from
52 soil, air and vegetation (Okoye and Okeke, 1986; Barton et al., 1992; Perelman and Kuttin, 1992;
53 de Wit et al., 1993; Oglesbee, 1997). Treatment of this infectious and noncontagious disease is
54 probably one of the most challenging tasks of avian practitioners (Jenkins, 1991).

55 The novel triazole antifungal agent voriconazole has become the preferred drug of treatment of
56 respiratory and disseminated aspergillosis in humans (Gothard and Rogers, 2004). Voriconazole
57 has a potent *in vitro* activity against many clinically important fungal pathogens, including
58 *Aspergillus* species from birds (Diekema et al., 2003; Silvanose et al., 2006). The *in vivo* efficacy
59 of voriconazole has been demonstrated in several experimental *A. fumigatus* infections in
60 mammals and has been shown to be superior to that of amphotericin B and itraconazole (George
61 et al., 1996; Martin et al., 1997; Murphy et al., 1997; Chandrasekar et al., 2000; Kirkpatrick et al.,
62 2000;). In birds, pharmacokinetics of voriconazole have been determined in chickens, falcons,
63 African grey parrots and racing pigeons (Schmidt et al., 2007; Burhenne et al., 2008; Flammer et
64 al., 2008; Beernaert et al., 2009). However, in avian patients, the use of voriconazole has been
65 implemented on an empirical basis, for example in a cockatiel, African grey parrots and in
66 falcons (Langhover, 2004; Di Somma et al., 2007; Schmidt et al., 2007; Scope et al., 2007).
67 Therefore, the development of an appropriate treatment schedule for avian aspergillosis based on
68 available pharmacokinetic data is needed.

69 Voriconazole shows time-dependent fungistatic activity against *Candida* species and time-
70 dependent slow fungicidal activity against *Aspergillus* species (Theuretzbacher et al., 2006).
71 Because of its time-dependent activity, maximizing the duration of exposure of a fungus to the

72 drug or maintaining the voriconazole blood concentration above the minimal inhibitory
73 concentration (MIC) value would optimize its activity. From a pharmacokinetic study conducted
74 in racing pigeons, it was concluded that the dosage schedules 10 mg/kg BW q12h and 20 mg/kg
75 BW q24h could be appropriate in treating pigeons with aspergillosis (Beernaert et al., 2009).
76 Based on these pharmacokinetic results, the *in vivo* efficacy of the voriconazole treatment
77 schedules 10 mg/kg BW q12h and 20 mg/kg BW q24h was investigated in an experimental *A.*
78 *fumigatus* infection in racing pigeons.

79

80 **2. Materials and methods**

81 **Animals.**

82 Eighteen clinically healthy 4-5-week-old pigeon squabs were divided into three groups of six
83 pigeons. The animals were free from *Salmonella* spp. and endoparasites. During the experiment
84 each bird was housed individually. All rooms were maintained at 20-22°C with a 12-hour
85 photoperiod. The birds received a commercial pigeon diet *ad libitum* and had free access to fresh
86 drinking water.

87 The experiment was performed with the permission of the Ethical Committee of the Faculty of
88 Veterinary Medicine, Merelbeke, Ghent University, Belgium (EC 2006/099).

89 **Inoculum.**

90 The *Aspergillus fumigatus* strain K24-1 used in this study was isolated from a racing pigeon with
91 aspergillosis. The isolate was identified based on the macro- and micromorphology of the fungus.
92 Determination of partial DNA sequences of the Beta-tubulin and rodlet A genes (Alcazar-Fuoli et
93 al., 2008) and the ability to grow at 48°C was used to confirm species identity. Five day old
94 cultures of this strain on Sabouraud dextrose agar were washed with 5 ml 0.01% Tween 80 in
95 Hank's balanced salt solution (HBSS) to harvest *A. fumigatus* conidia. The conidia were washed

96 three times in 0.01% Tween 80 in HBSS (3200 g, ten minutes at 4°C) and the suspension was
97 adjusted to a concentration of 10^8 *A. fumigatus* conidia/ml suspension in HBSS by
98 hemacytometer count. Numbers of viable conidia were determined by plating serial 10-fold
99 dilutions in 0.01% Tween 80 in HBSS on Sabouraud dextrose agar plates. The number of colony
100 forming units (CFU)/ml was calculated after incubation at 37°C for 20 hours. The final conidial
101 suspension had a viable count of 1.75×10^7 CFU/ml.

102 **Susceptibility testing.**

103 The minimal inhibitory concentration (MIC) of voriconazole for the *A. fumigatus* strain was
104 determined as described in the Clinical and Laboratory Standards Institute (CLSI) document
105 M38-A2. *Candida parapsilosis* IHEM 3270 (ATCC 22019) and *C. krusei* IHEM 9560 (ATCC
106 6258) were included as quality control strains for this procedure and their MICs were within the
107 established reference MIC ranges described by Barry et al. (2000).

108 **Experimental design.**

109 Three groups of six pigeons were inoculated in the apical part of the right lung with 0.2 ml of a
110 10^8 *A. fumigatus* conidia/ml suspension in HBSS (= a total of 3.5×10^6 viable conidia per pigeon)
111 as described previously (Beernaert et al., 2008). In two groups, voriconazole treatment was
112 started upon appearance of the first clinical signs (forty-two hours post inoculation (p.i.)) and
113 continued for fourteen days. Voriconazole tablets (Vfend® 200 mg; Pfizer Limited, Sandwich,
114 Kent, UK) were crushed into a fine powder, suspended in water by shaking thoroughly and given
115 by crop gavage using a 2.5 ml syringe and a 14 G 2" catheter. The powder residue in the syringe
116 was suspended twice in water to maximize the precision of the dose. The total application volume
117 was approximately 1.5 ml. Group 1 received voriconazole orally at a dose of 10 mg/kg BW q12h,
118 group 2 at a dose of 20 mg/kg BW q24h, the sham-treated pigeons received water. The animals
119 were observed at least twice daily. The presence of a fluffed posture and dyspnea (abdominal

120 breathing and tail hopping) were scored as described below. Other clinical symptoms were also
121 recorded, if present. Pigeons with severe dyspnea (open beak breathing) or extreme weakness
122 were euthanized. After a treatment of fourteen days all survived pigeons were euthanized by an
123 intravenous injection of 0.2 ml T61 (Intervet, Mechelen, Belgium) in the wing vein. All pigeons
124 were weighed prior to inoculation and at necropsy.

125 **Clinical score.**

126 A clinical score was given to each pigeon daily. This clinical score is the sum of a score for the
127 pigeon's posture and a score for dyspnea. The day a pigeon died, a maximum clinical score of 3
128 was given. The score for a fluffed posture, as a general sign of sickness ranged from 0-1
129 (0=absent, 0.33=mild, 0.67=pronounced, 1=highly pronounced). The score for dyspnea consists
130 of the average of the scores for tail hopping and abdominal breathing for a given pigeon on a
131 given day. These scores ranged from 0-1 (0=absent, 0.2=barely visible, 0.4=mild, 0.6=moderate,
132 0.8=pronounced, 1=highly pronounced).

133 **Post mortem analyses.**

134 At necropsy macroscopic lesions were described. Samples of trachea, lungs, air sacs, heart,
135 pericardium, liver, kidney, spleen and brain were inoculated on Sabouraud dextrose agar plates
136 and incubated for fourteen days at 37°C under aerobic conditions to isolate *A. fumigatus*. Samples
137 from the liver were fixed in phosphate buffered formaldehyde solution, sectioned and stained
138 with Haematoxylin & Eosin (H&E).

139 **Statistical analyses.**

140 Differences in the average total weight loss and in the average clinical daily scores between the
141 groups were statistically analyzed using Paired Two-Sample T test For Means and Two-Sample
142 T test Assuming Unequal Variances, respectively (Microsoft Office Excel).

143

144 **3. Results**

145 **Susceptibility testing.**

146 The MIC of voriconazole for the *A. fumigatus* strain used in this study was 0.25 µg/ml.

147 **Clinical signs.**

148 The pigeons of both voriconazole treated groups did not show any significant weight loss (on
149 average -0.7% and 0.5% of the body weight prior infection for 10 mg/kg BW q12h and 20 mg/kg
150 BW q24h, respectively). On the other hand, the sham treated pigeons showed significant weight
151 loss ($p < 0.05$) (on average 15% of the body weight prior infection). One pigeon of group 2
152 showed excessive weight loss (6.6%), while another pigeon of this group showed excessive
153 weight gain (9.5%). Voriconazole treated pigeons of both treatment regimens showed
154 significantly less severe clinical signs compared to the sham treated animals ($p = 0.0099$ and
155 $p = 0.029$ for 10 mg/kg BW q12h and 20 mg/kg BW q24h, respectively). The pigeons treated with
156 voriconazole at 10 mg/kg BW q12h showed also significantly less severe clinical signs compared
157 to the pigeons treated with voriconazole at 20 mg/kg BW q24h ($p = 0.045$).

158 **Mortality.**

159 None of the six pigeons died in both voriconazole treated groups, opposed to five out of six
160 pigeons in the sham treated group, in which one pigeon has been euthanized due to open beak
161 breathing, severe weakness and fluffed posture two days (fifty-three hours) p.i. and one, two and
162 one pigeon(s) died at three, five and fourteen days p.i., respectively.

163 **Post mortem findings.**

164 The number of pigeons with lesions with a granulomatous aspect in different organs is presented
165 in Table 1. In only a minority of voriconazole-treated pigeons, lesions with a granulomatous
166 aspect were present and these were only observed in the respiratory tract. On the other hand, five
167 of six sham-treated pigeons showed lesions with a granulomatous aspect in both the respiratory

168 tract and visceral organs. Livers appeared to be normal in group 1 (received voriconazole at 10
169 mg/kg BW q12h) except for one pigeon (slightly blunt edges), while the livers of the pigeons in
170 group 2 (voriconazole at 20 mg/kg BW q24h) showed a zonal pattern (5/6), had a pale aspect
171 (3/6), were swollen in general (1/6) or in one lobe (1/6), showed slightly blunt edges (3/6) or
172 were normal (1/6). Histopathology (H&E staining) revealed moderate oval cell proliferation of
173 the limiting plate (3/6), moderate bile duct proliferation (3/6), moderate hepatocellular
174 vacuolization (3/6), portal heterophilic and lymphocytic infiltration (6/6), congestion of the
175 sinusoids (1/6) in group 1 (received voriconazole at 10 mg/kg BW q12h); and moderate
176 hepatocellular vacuolization (1/6), portal heterophilic and lymphocytic infiltration (6/6) and
177 diffuse fatty hepatocellular degeneration (2/6) in group 2 (received voriconazole at 20 mg/kg BW
178 q24h). In group 3 (sham treated) histopathology revealed limited (2/6) to moderate (4/6)
179 heterophilic and lymphocytic infiltration, multifocal hepatocellular necrosis with hyphae (2/6),
180 multiple foci of colliquative necrosis with giant cells (1/6), pyogranulomatous hepatitis with
181 intralesional hyphae (3/6), diffuse fatty hepatocellular degeneration (2/6), oval cell proliferation
182 of the limiting plate (1/6), bile duct proliferation (1/6), cholestase (1/6) and hemosiderin-loaded
183 macrophages (1/6).

184 **Mycological findings.**

185 The mycological findings are summarized in Table 1. *A. fumigatus* was not isolated from the
186 organs of the pigeons that received voriconazole at a dose of 10 mg/kg BW q12h. In the group of
187 pigeons that received voriconazole at a dose of 20 mg/kg BW q24h *A. fumigatus* was only
188 isolated from the inoculation site (right lung) in two out of six pigeons. In the sham-treated
189 pigeons *A. fumigatus* was isolated from the respiratory tract (5/6) as well as from the visceral
190 organs (5/6).

191

192 **4. Discussion**

193 Despite the pharmacokinetic limitations (e.g. liver enzyme induction during multiple dose
194 experiments) of voriconazole in racing pigeons (Beernaert et al., 2009), the present study
195 demonstrated good *in vivo* activity of voriconazole in these animals. This phenomenon is
196 consistent with other studies describing good responses in treating aspergillosis in falcons and
197 psittacines (field studies) and in an experimental model of invasive aspergillosis in rabbits
198 (George et al., 1996; Di Somma et al., 2007; Scope et al., 2007). As in racing pigeons, plasma
199 levels of voriconazole above the MIC value are also difficult to maintain in these animal species
200 (George et al., 1996; Schmidt et al., 2007; Flammer et al., 2008; Beernaert et al., 2009).

201 Both treatment regimens used in this study (10 mg/kg BW q12h and 20 mg/kg BW q24h)
202 resulted in a significant reduction of aspergillosis associated clinical signs and lesions compared
203 to the sham-treated animals. The treatment schedule of 10 mg/kg BW q12h was shown to be
204 superior to that of 20 mg/kg BW q24h as it eliminated *A. fumigatus* in the near absence of
205 macroscopic liver pathology. On liver histology, however, moderate oval cell proliferation of the
206 limiting plate and moderate bile duct proliferation in three out of six pigeons was seen, which is
207 in accordance with a previous study (Beernaert et al., 2009). These features might be a sign of
208 regeneration as a result of hepatocellular degeneration. A hepatic injury due to voriconazole
209 could be the cause, but further toxicological investigations with control pigeons are necessary to
210 reveal the true impact of voriconazole on the liver of pigeons. Hypothetically, the absence of
211 these features in group 2 (20 mg/kg BW q24h) could be due to the longer dose interval which
212 allows the hepatocytes to recuperate in between doses.

213 The reason for the macroscopic liver abnormalities in group 2 (20 mg/kg BW q24h) remains
214 uncertain. Diffuse fatty liver degeneration in two out of six pigeons resulted in pale and swollen
215 livers at necropsy. The cause of the fatty hepatocellular degeneration might be associated with

216 excessive weight loss (6.6% of the initial weight) in one pigeon and excessive weight gain (9.5%
217 of the initial weight) in another pigeon during the experiment. The reason for the pronounced
218 weight fluctuation in these pigeons in comparison with the others is unknown.

219

220 **5. Conclusion**

221 Voriconazole at 10 mg/kg BW q12h in pigeons reduces clinical signs and eliminates *A. fumigatus*
222 in racing pigeons experimentally infected with *A. fumigatus*. The safety of long-term usage of
223 this dosage schedule, however, remains to be confirmed by toxicological studies with control
224 pigeons.

225

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228 Technology in Flanders (IWT Vlaanderen), Brussels, Belgium. The skillful technical assistance
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230

231 **Conflict of interest**

232 None

233

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333

334 Table 1. Pathological and mycological findings in pigeons inoculated with *A. fumigatus* conidia
 335 in the right lung and treated with voriconazole at 10 mg/kg BW q12h (group 1), 20 mg/kg BW
 336 q24h (group 2) and sham treated (group 3).

Organs	Number of pigeons with granulomatous lesions (L) and number of pigeons from which <i>A. fumigatus</i> was isolated (I) from the respiratory tract or visceral organs in the different groups ^a .					
	Group 1		Group 2		Group 3	
	L	I	L	I	L	I
Trachea	0	0	0	0	0	1
Left abdominal air sac	0	0	0	0	4	1
Right abdominal air sac	0	0	1	0	4	1
Left thoracic air sac	0	0	0	0	5	3
Right thoracic air sac	0	0	0	0	5	3
Left lung	1	0	0	0	1	2
Right lung	1	0	0	2	5	3
Pericard	0	0	0	0	5	4
Kidney	0	0	0	0	0	1
Liver	0	0	0	0	5	5
Spleen	0	0	0	0	1	1
Brain	0	0	0	0	0	0

337 ^a Each group consisted of six animals.

338 Figure 1. The average clinical score (\pm stdev) per group of pigeons inoculated with *A. fumigatus*
339 conidia in the right lung and treated with voriconazole at 10 mg/kg BW q12h (group 1), 20 mg/kg
340 BW q24h (group 2) and sham treated (group 3).

341

342

343 ^a, ^b, ^c: Similar superscripts refer to a statistically significant difference.

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