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ARTICLE

Limited evidence of fungicide-driven triazole-resistant *Aspergillus fumigatus* in Hamilton, Canada

Eta Ebasi Ashu, Ga Young Kim, Patrick Roy-Gayos, Kelly Dong, Adrian Forsythe, Victoria Giglio, Gregory Korfanty, Deborah Yamamura, and Jianping Xu

Abstract: *Aspergillus fumigatus* is a ubiquitous opportunistic fungal pathogen that can cause aspergillosis in humans. Over the last decade there have been increasing global reports of treatment failure due to triazole resistance. An emerging hypothesis states that agricultural triazole fungicide use causes clinical triazole resistance. Here we test this hypothesis in Hamilton, Ontario, Canada, by examining a total of 195 agricultural, urban, and clinical isolates using 9 highly polymorphic microsatellite markers. For each isolate, the in vitro susceptibilities to itraconazole and voriconazole, 2 triazole drugs commonly used in the management of patients, were also determined. Our analyses suggested frequent gene flow among the agricultural, urban environmental, and clinical populations of *A. fumigatus* and found evidence for widespread sexual recombination within and among the different populations. Interestingly, all 195 isolates analyzed in this study were susceptible to both triazoles tested. However, compared with the urban population, agricultural and clinical populations showed significantly reduced susceptibility to itraconazole and voriconazole, consistent with ecological niche-specific selective pressures on *A. fumigatus* populations in Hamilton. Frequent gene flow and genetic recombination among these populations suggest greater attention should be paid to monitor *A. fumigatus* populations in Hamilton and other similar jurisdictions.

Key words: Aspergillus fumigatus, gene flow, antifungal resistance, recombination, molecular markers.

Résumé : *Aspergillus fumigatus* est un pathogène opportuniste ubiquiste qui peut causer l'aspergillose chez l'humain. Au cours de la dernière décennie, un nombre grandissant de rapports à travers le monde ont signalé des échecs thérapeutiques à cause de la résistance aux triazoles. Une nouvelle hypothèse propose que l'utilisation de triazoles comme fongicides agricoles soit responsable de la résistance clinique aux triazoles. Les auteurs testent ici cette hypothèse à Hamilton, Ontario, Canada, en examinant un total de 195 isolats agricoles, urbains et cliniques à l'aide de 9 marqueurs microsatellites hautement polymorphiques. Pour chaque isolat, la sensibilité in vitro au itraconazole et au voriconazole, 2 médicaments de la famille des triazoles utilisés généralement dans la prise en charge des patients, a aussi été déterminée. Leurs analyses suggéraient des flux géniques fréquents parmi les populations agricoles, urbaines et cliniques de *A. fumigatus* et apportaient la preuve d'une recombinaison sexuée répandue à l'intérieur et entre les différentes populations. Fait intéressant, tous les 195 isolats analysés dans cette étude étaient sensibles aux deux triazoles testées. Cependant, comparativement à la population urbaine, les populations agricoles et cliniques montraient une sensibilité significativement réduite au itraconazole et au voriconazole, en cohérence avec les pressions sélectives spécifiques à la niche écologique des populations de *A. fumigatus* à Hamilton. Les flux géniques fréquents et la recombinaison génétique au sein de ces populations suggèrent qu'une attention plus grande soit portée pour suivre les populations de *A. fumigatus* à Hamilton et dans d'autres juridictions similaires. [Traduit par la Rédaction]

Mots-clés : Aspergillus fumigatus, flux génique, résistance antifongique, recombinaison, marqueurs moléculaires.

Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen that can thrive in a broad range of ecological niches. There are 2 distinct features of this saprophyte that enhance its pathogenicity: small size of airborne spores and thermo-tolerance (Dagenais and Keller 2009). Each day, hundreds of *A. fumigatus* spores are inhaled by most individuals but without causing any obvious infec-

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E.E. Ashu, G.Y. Kim, P. Roy-Gayos, K. Dong, A. Forsythe, V. Giglio, G. Korfanty, and J. Xu. Department of Biology and the Institute of Infectious Diseases Research, McMaster University, 1280 Main Street W, Hamilton, ON L8S 4K1, Canada.
D. Yamamura. Department of Pathology and Molecular Medicine, McMaster University, 1280 Main Street W, Hamilton, ON L8S 4K1, Canada.
Corresponding author: Jianping Xu (email: jpxu@mcmaster.ca).

tions. However, in immunocompromised individuals, *A. fumigatus* can cause a group of infections collectively known as aspergillosis, a disease with high morbidity and mortality (Dagenais and Keller 2009).

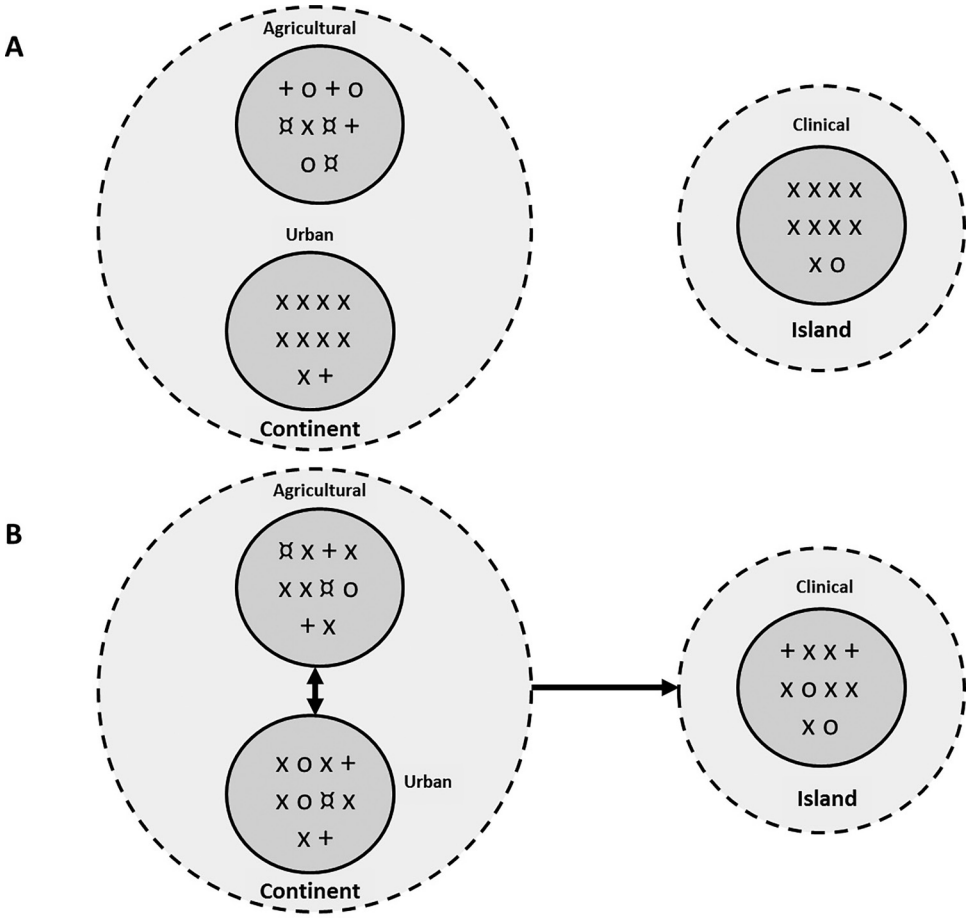
The antifungal triazole drugs, such as itraconazole and voriconazole, have emerged as the first-line drugs in the treatment and prevention of aspergillosis. Similarly, triazole fungicides such as propiconazole, prochloraz, pebuconazole, and priadimenol are commonly used to manage plant fungal infections in agriculture (Bowyer and Denning 2014). Triazoles impede proliferation of *A. fumigatus* by inhibiting lanosterol-14-α-demethylase (CYP51A), a protein that is essential for the synthesis of ergosterol in fungal cell membranes. Over the last decade, there have been increasing reports of triazole resistance in clinical and environmental isolates from countries across Eurasia, and North and South America, including those from the United Kingdom (UK), the Netherlands, Demark, Germany, India, United States, and Colombia (Pfaller et al. 2011; Verweij et al. 2016). However, there is limited surveillance data in Canada. Two hypotheses have been proposed to explain recent increases in triazole resistance: (*i*) the use of triazoles for prophylaxis in highly immunocompromised patients, and (*ii*) the extensive use of triazole fungicides in agriculture (Enserink 2009). In favor of the latter hypothesis, there have been several reported cases of triazole-resistant aspergillosis in triazole-naïve patients in the Netherlands, India, and the UK (Chowdhary et al. 2012*a*; Enserink 2009; Howard et al. 2009; van der Linden et al. 2013). Furthermore, it has been shown that clinically resistant strains of *A. fumigatus* exhibit cross-resistance to agriculture triazole fungicides (Chowdhary et al. 2012*b*). This cross-resistance is largely due to the fact that both agricultural and clinical triazoles have similar molecular structures and adopt similar conformations while docking to their target protein, CYP51A (Chowdhary et al. 2012*b*; Snelders et al. 2012).

Understanding the frequency of triazole resistance in clinical settings and its relation to agricultural fungicide use is of significant importance to agronomists, mycologists, physicians, public health practitioners, and policymakers worldwide. Thus far, 3 major field studies have been conducted to test if agricultural fungicide use can lead to triazole-resistant *A. fumigatus*. One of these field studies was done in the UK and showed evidence supporting the agricultural fungicide-driven resistance hypothesis (Bromley et al. 2014). However, the 2 other studies done in Japan showed little evidence to support the said hypothesis (Kano et al. 2014; Toyotome et al. 2016). These studies suggest that there might be some geographic specificity in the relationship between agricultural triazole fungicide use and drug resistance in *A. fumigatus*. At present, the agricultural fungicide-driven resistance hypothesis has yet to be investigated using a molecular epidemiological approach that tracks resistant strains from farms, through urban areas to carriage in patients within a community.

Located at the west end of Lake Ontario, Hamilton is home to ~530 000 people. Hamilton hosts 12 specialized healthcare facilities and hospitals. Some of these hospitals and healthcare facilities serve as referral centers for patients in southern Ontario, thus there is a frequent influx of patients, including those that are immunocompromised, into Hamilton. Although there is a frequent influx of high-risk patients and use of triazole fungicides on farms around Hamilton, very little is known about the prevalence of triazole-resistant *A. fumigatus* or the link between agricultural triazole fungicide use and the acquisition of clinically important resistant strains.

To understand the prevalence of triazole-resistant *A. fumigatus* and the link between triazole fungicide use in agriculture and the acquisition of clinically important resistant strains, we examined agricultural, urban environmental, and clinical *A. fumigatus* populations in and around Hamilton. We hypothesized that there should be triazole-resistant strains in agricultural fields with long-term triazole fungicide use history. In addition, given the prevalence of asexual reproduction in this species and the high dispersal ability of its asexual conidial spores, we postulated that there would be shared resistant genotypes between the environmental and clinical populations. Our postulate was based on a continent–island gene flow model wherein the environmental sample acts as the continental source population and the clinical sample as the island population. Our proposed continental population in Hamilton consists of 2 main demes (urban and agricultural), which we assumed to have frequent gene flow (Fig. 1). Supposing the agricultural population was under strong triazole selective pressure (Fig. 1A), our proposed model would suggest an eventual convergence of resistant phenotypes and genotypes between agricultural and urban populations due to frequent gene flow between the 2 populations (Fig. 1B). In this model, change in allelic frequency (Δq) due to gene flow from the continental to the island population can be calculated using the formula $\Delta q = -m(qo - Q)$ (Macdonald 2004), where m is the proportion migrants in the island population, Q the frequency of resistant strains in the continental population, and qo the frequency of resistant strains in the island population. At equilibrium ($qo = Q$), after several cycles of gene flow, resistant allele frequency in continental and island populations are expected to converge as well. Prior to equilibrium, qo is expected to be less than Q. Being a potential source population for triazole resistance, the environmental population was expected to show a higher allelic and genotype diversities of resistant strains. In addition, environmental and clinical populations were expected to share identical triazole resistant genotypes.

Fig. 1. Continent–island model of genetic relationships between environmental and clinical populations of *Aspergillus fumigatus*. Crosses (+) represent susceptible strains while other signs represent resistant strains with a diversity of resistance mechanisms. Double arrow thick line is indicative of equal gene flow in either direction, while the thick single arrow line represents a predominantly one-way gene flow. (A) A model where a triazole selective pressure is solely exerted on the agricultural population. (B) Prior model after many cycles of gene flow.



Materials and methods

Environmental and clinical isolates

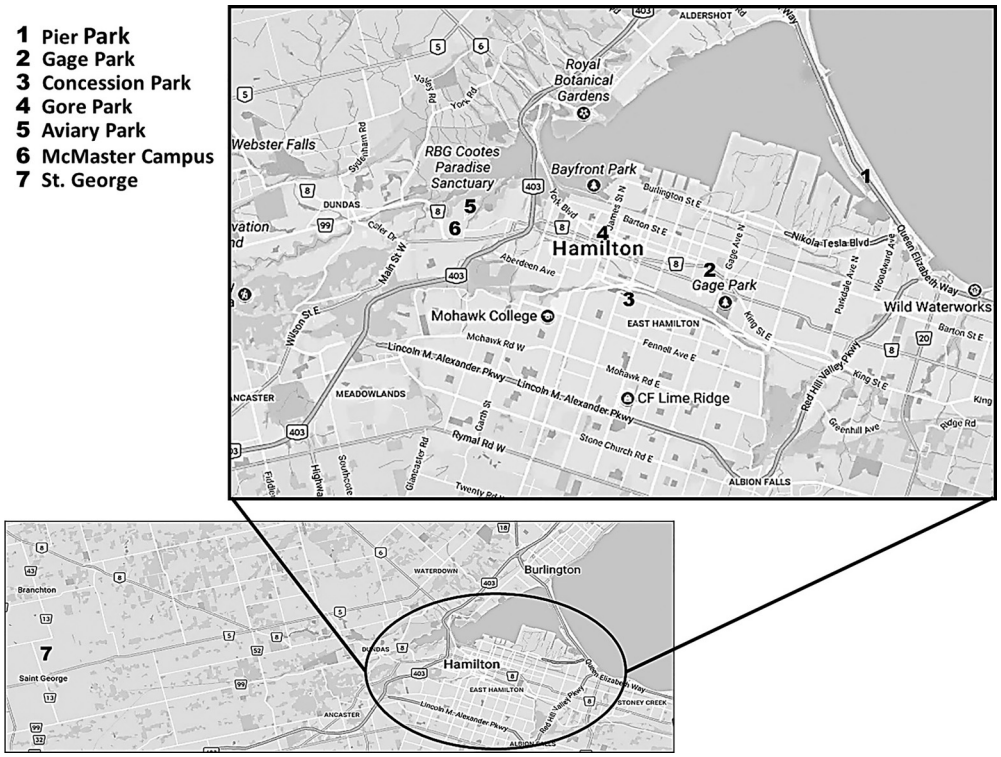
A total of 781 soil samples were obtained from 3 agricultural and 6 urban sites between September 2014 and January 2015 (Fig. 2). Agricultural samples were obtained from 3 farms around St. George, a township that is ~35 km west of Hamilton, Ontario. Wheat, soybeans, or barley were grown on these farms. Triazole fungicides were used on these farms every other year for at least the previous 10 years. Triazole fungicides with trade names Stratego (propiconazole and tebuconazole), Prosaro (prothioconazole and tebuconazole), and Headline (pyraclostrobin and tebuconazole) were used. Urban sampling was done throughout the city of Hamilton: at McMaster University, Aviary Park, Gore Park, Concession Park, Gage Park, and Pier Park. These urban sites have no known fungicide usage. For each collection site, soil samples were obtained ~5 m apart from each other in all 4 major cardinal directions. Environmental sampling was done by adding about 1 g of soil to microcentrifuge tubes each containing 1 mL of Sabouraud dextrose (SD) broth supplemented with chloramphenicol (50 mg/L) (Sigma-Aldrich, Mississauga).

Clinical *A. fumigatus* isolates were obtained from the Microbiology Laboratory of Hamilton Health Sciences, located at the Hamilton General Hospital site. This laboratory serves as the regional mycology laboratory for Hamilton Health Sciences and St. Joseph's Healthcare. Between January and October 2015, clinically significant *A. fumigatus* isolates from respiratory, wound, tissue, and sterile fluid were obtained and evaluated. A retrospective chart review (approved by the Hamilton Integrated Research Ethics Board, project No. 3328-C) to obtain patient demographics and prior antifungal use was performed. All isolates of *A. fumigatus* were archived by pipetting 750 μL of spore solutions (~10^8 spores/mL of sterile 0.9% NaCl) into cryotubes containing 250 μL of glycerol and storing them at –80 °C.

A. fumigatus identification

To differentiate *A. fumigatus* from other species of *Aspergillus* (e.g., *A. lentulus*, *A. udagawae*, *A. fumigatiaffinis*,

Fig. 2. Map of Hamilton showing *Aspergillus fumigatus* sampling sites. The agricultural site (No. 7) consisted of 3 different farms (not shown in this figure). Map was retrieved Google and modified.



A. novofumigatus, *A. fumisynnematus*, *A. viridinutans*, *A. fischeri*, and *A. thermomutatus* (section Fumigati)), both environmental and clinical isolates were incubated on SD agar supplemented with chloramphenicol at 50 °C for 48 h (Samson et al. 2007; Lamoth 2016). *Aspergillus fumigatus* was then identified by macroscopic and microscopic features such as colony color, texture, conidial heads, and seriation. *Aspergillus fumigatus* was further confirmed and analyzed by using a set of 9 species-specific microsatellite markers developed previously (Valk et al. 2005).

Genotyping

Genotyping of all isolates was done at 9 highly polymorphic microsatellite markers following protocols previously described by Valk et al. (2005). Briefly, each reaction contained a total volume of 13.5 μL, which was made up of 0.25 μL of each primer (0.16 μmol/L), 7 μL of 2× GoTaq Master Mix (Promega, Markham, Ontario, Canada), and 5 μL of nuclease-free water. The amplification conditions were as follows: an initial denaturation step of 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and by a final elongation step at 72 °C for 5 min. Capillary electrophoresis was carried out at the MoBix Laboratory (Hamilton, Ontario, Canada). Peak Scanner software version 1.0 (ThermoFisher Scientific) was used for fragment analysis. To further categorize the genetic makeup of our set of isolates, a mating type allele-specific PCR was carried out as previously described (Chang et al. 2016). The *MAT1-1* and *MAT1-2* mating types were identified by their corresponding band sizes of 834 and 438 bp, respectively.

Antifungal susceptibility testing

The in vitro susceptibilities of our set of isolates to itraconazole and voriconazole (Selleckchem, San Francisco) were tested following the M38-A2 guidelines of the Clinical and Laboratory Standard Institute (Clinical and Laboratory Standards Institute 2008). On the basis of previously described epidemiological cutoff values (Pfaller et al. 2011, 2009; Rodriguez-Tudela et al. 2008), isolates with voriconazole and itraconazole minimal inhibitory concentrations (MICs) of ≥2 mg/L were considered resistant. To prepare the isolates for testing, all isolates were first plated on SD agar at 37 °C for 48 h. Spores were harvested by washing culture plates with 1 mL of 0.9% sterile saline and adjusted to a range of approximately 4×10^5 to 5×10^6 CFU/mL. These suspensions were then diluted 50 times in standard medium. In-house strains with known MICs were used as controls (accession Nos. KU575343 and KU575805). Strains KU575343 and KU575805 are 2 *A. fumigatus* strains that have been previously confirmed by means of β-tubulin gene sequencing and microscopic and macroscopic morphology. These strains were used because their MICs fall on the 20th and 80th percentile of the range of itraconazole and voriconazole concentrations tested. After MICs were visually determined, growth in the microtiter plates was also assessed by exposing microtiter plates to light at a

Table 1. Patients from whom 2 or more isolates were obtained and analyzed.

Patient No.	Isolate source*	2A	2B	2C	3A	3B	3C	4A	4B	4C
15-4a	BAL	11	30	13	3	7	18	13	6	7
15-5a	BAL	11	29	13	3	10	18	14	4	7
15-6b	BAL RLL	13	31	15	27	10	33	8	9	10
15-7b	BAL RLL	12	31	14	4	11	19	12	8	9
15-19c	Sputum	11	30	13	10	9	25	12	7	8
15-58c	Sputum	11	30	13	10	9	25	12	7	8
15-21d	ETT	10	29	12	20	12	32	11	8	9
15-22d	ETT	16	34	18	24	30	16	7	7	8
15-32e	BAL LUL	28	46	30	36	9	5	16	7	8
15-36e	BAL RUL	4	23	6	12	9	25	8	6	6
15-42f	BR wash	23	41	25	6	2	25	14	6	10
15-52f	BR wash	22	40	25	28	9	13	8	6	7
15-43g	BAL	21	39	23	11	3	27	8	8	6
15-57g	Sputum	21	39	23	11	3	27	8	6	9
15-46h	Sputum	17	35	19	9	9	24	8	9	10
15-48h	Sputum	14	32	16	2	9	15	15	8	15
15-49i	BAL RLL	8	27	10	30	10	12	14	6	7
15-50i	BAL RLL	11	30	13	34	15	12	15	8	7
15-62i	BAL LLL	10	29	12	9	8	31	13	6	7
15-65i	BAL LLL	14	33	16	28	9	13	12	3	8
15-68i	BAL RLL	14	33	16	28	9	13	12	7	8
15-66j	Sputum	9	28	11	11	17	11	7	4	5
15-67j	Sputum	9	28	11	11	17	11	7	4	5
15-61k	BR wash	6	25	8	41	16	12	7	10	11
15-63k	Tissue	9	28	11	2	31	17	7	7	8
15-69k	Tissue	4	23	6	11	8	24	8	6	7

Note: The same letter after a number indicates the isolates are from the same patient. Adjacent highlighted isolates are from the same patient and share the same genotypes at all 9 microsatellite loci.

*BR wash, bronchial wash; BAL, bronchoalveolar lavage; RLL, right lower lung; LLL, left lower lung; RUL, right upper lung; LUL, left upper lung; ETT, endotracheal tube.

wavelength of 590 nm and recording the optical density (OD) in individual wells. ODs for all 195 agricultural, urban, and clinical isolates were obtained. The above procedure was repeated 3 times for a representative set of 15 isolates from each population and their optical densities were also obtained. As an independent confirmation of our MIC results, 5 representative isolates from our collection were further tested at the Public Health of Ontario Laboratory in Toronto.

Population and statistical analysis

Simpson's unbiased diversity index (uh) and analysis of molecular variance (AMOVA) as implemented by GENALEX (version 6.5) were used to determine levels of ecological niche differentiation and genetic diversity (Peakall and Smouse 2012). To score levels of linkage disequilibrium, MULTILOCUS (version 1.3b) was used to determine the index of association (Smith et al. 1993). Significance was determined by 1000 permutations. Analysis of variance (ANOVA) was used to elucidate differences in triazole susceptibility between ecological niche groups at different drug concentrations. The Wilcoxon rank sum test was used to elucidate differences in triazole susceptibility between ecological niche populations.

Results

Environmental and clinical *A. fumigatus* isolates in and around Hamilton

Aspergillus fumigatus isolates were distinguished from other thermophilic fungi by their distinct greyish-green suede-like colonies. Furthermore, microscopic observation confirmed the presence of uniseriate and club-shaped conidial heads characteristic of *A. fumigatus*. Isolates not amplified by the aforementioned set of highly discriminatory microsatellite markers were not included in our analysis. A total of 124 environmental and 71 clinical *A. fumigatus* isolates were identified.

Clinical isolates were obtained from a total of 56 patients, 30 female and 26 male. Of all 56 patients, 27 lived in Hamilton; 4 in St. Catharines; 3 each in Grimsby, Burlington, and Niagara Falls; and 2 each in Haldimand, Simcoe, Brantford, and Welland. One patient each lived in Oakville, Guelph, Waterloo, Milton, and Glencairn. Three patients' postal codes were unknown. Patients' ages ranged from 8 to 97 years, with the median and modal ages being 64 and 65 years, respectively. More than 1 *A. fumigatus* isolate was obtained from a total of 11 patients: 2 isolates each were obtained from 9 patients, 3 isolates from 1 patient, and 5 isolates from 1 patient. When 2 or more *A. fumigatus* isolates were obtained from

Table 2. Proportions of *Aspergillus fumigatus* isolates obtained from 6 urban sites and 3 agricultural fields.

Sample region	No. of soil samples	No. (%) of thermophilic fungal isolates	No. (%) of *A. fumigatus* isolates
Urban			
West			
McMaster University	38	26 (68)	10 (26)
Aviary Park	94	62 (66)	17 (18)
Centre			
Gore Park	36	25 (69)	4 (11)
Concession Park	36	24 (67)	6 (17)
East			
Gage Park	36	22 (61)	0 (0)
Pier Park	91	43 (47)	26 (29)
Agricultural			
Field A	34	21 (62)	0 (0)
Field B	33	11 (33)	0 (0)
Field C	383	205 (54)	65 (17)
Total	781	439 (56)	128 (16)

a single patient, they often (9/11) did not share the same microsatellite genotype (Table 1), suggesting the existence of multiple carriages among Hamiltonian patients. Ten of the 56 patients surveyed in this study had unknown triazole histories while 4 patients had previously used either fluconazole or voriconazole within 3 months prior to their sampling dates. A total of 6 *A. fumigatus* isolates were obtained from all 4 patients who had previously used triazoles.

Sixty-two *A. fumigatus* isolates were each obtained from urban and agricultural soil samples (Table 2). At the first attempt, *A. fumigatus* was not isolated from Fields A and B, likely due to limited decay matter available in and around these fields. Given that Field C was surrounded by a notable amount of vegetation and had a higher isolation rate at the first attempt, it was extensively further sampled. This was where all of our agricultural isolates were eventually obtained. Overall, there was no statistically significant difference in isolation rates between urban and agricultural ecological niches. Similarly, the difference in isolation rates among west, center, and east Hamilton urban environments was statistically insignificant. The distribution of *A. fumigatus* isolated by location is shown in Table 2.

Evidence of gene flow

AMOVA showed that only 0.4% of total genetic variation in the whole Hamiltonian *A. fumigatus* population was contributed by grouping our set of isolates by ecological niche, i.e., urban, agricultural, and clinical. Although very small, differences between the clinical population and the other 2 populations were statistically significant, consistent with limited differentiations [clinical and urban PhiPT = 0.006 (P = 0.03), clinical and agricultural PhiPT = 0.006 (P = 0.05); PhiPT refers to pairwise population heterogeneity index of the proportion of total genetic variance]. However, there was no significant differentiation between the urban and agricultural populations (PhiPT = 0.001, P = 0.33).

Susceptibility patterns

All 195 isolates obtained and analyzed here were susceptible to itraconazole and voriconazole. Our MICs were concordant with those done at Public Health of Ontario laboratory. Similarly, the OD from technical repeats corroborated with each other. Compared with the urban population, both agricultural and clinical populations showed a significantly reduced susceptibility to itraconazole (in both cases, P value of <0.001) (Table 3). Similarly, spectrophotometry readings revealed that agricultural and clinical populations grew significantly better than the urban population at itraconazole concentrations higher than 0.25 mg/L (Fig. 3). Comparably, the clinical population also grew significantly better than the urban and agricultural populations at voriconazole concentrations higher than 0.125 mg/L (Fig. 3). These results suggest that even though there is currently no evidence for triazole resistance in *A. fumigatus* from Hamilton, there is significant evidence of reduced triazole susceptibility in both clinical and agricultural populations.

Patterns of diversity and evidence of recombination

We found high levels of allelic and genotypic diversities within ecological niche populations of *A. fumigatus* from Hamilton. Out of 62 urban isolates, we identified 60 genotypes. Similar to the urban population, only 2 genotypes were shared by 4 clinical isolates; the remaining 67 isolates belonged to 67 different genotypes. Within the agricultural population, only 1 genotype was shared by 3 isolates; the remaining 59 isolates belonged to 59 different genotypes. Simpson's unbiased allelic diversity indices for urban, agricultural, and clinical populations were 0.893, 0.891, and 0.890, respectively. Locus 3C showed the most allelic polymorphism in urban (uh = 0.951) and clinical (uh = 0.946) populations, while locus 3A had the highest diversity in the agricultural population (uh = 0.943). Locus 4C showed the least allelic polymorphism in agricultural (uh = 0.788) and clinical (uh = 0.805) populations. Locus 4A in the urban population had an unbiased diversity index of 0.772. Consistent with high levels allelic polymorphism, no genotype was shared between the ecological populations.

All 3 ecological niche populations showed significant evidence of phylogenetically incompatible pairs of loci. Specifically, phylogenetic incompatibility indices for urban, agricultural, and clinical populations were 0.97, 0.94, and 0.92, respectively. These results are consistent with widespread recombination in natural populations of this organism in Hamilton. However, there is also evidence for some clonality within each population. Though the index of association (I_A) values were low (0.81 for the urban, 0.88 for the clinical, and 1.14 for the agri-

Table 3. In vitro antifungal susceptibility profiles of the Hamiltonian *Aspergillus fumigatus* isolates against 2 triazoles: itraconazole and voriconazole.

Drug and niche	No. of isolates tested	Drug concn. (mg/L) 0.03	0.06	0.125	0.25	0.5	1	Modal MIC
Itraconazole								
Urban	62	0	0	6	56	0	0	0.25
Agricultural	62	0	0	0	17	45	0	0.5
Clinical	71	1	0	0	13	57	0	0.5
Total	195	1	0	6	86	102	0	0.5
Voriconazole								
Urban	62	1	0	4	14	43	0	0.5
Agricultural	62	0	0	0	25	37	0	0.5
Clinical	71	1	0	1	56	13	0	0.25
Total	195	2	0	5	95	93	0	0.25

cultural populations), all 3 populations strongly rejected the null hypothesis of random recombination ($P < 0.01$).

Although slightly skewed in urban and agricultural populations, mating type ratios were also consistent with sexual recombination. Thirty-eight of the urban isolates belonged to the MAT1-1 mating type, while 24 were categorized as MAT1-2. Similarly, 40 and 22 agricultural isolates were categorized as MAT1-1 and MAT1-2 mating types. The clinical population's mating type ratio was however a lot closer to 1:1. However, the mating type loci for 4 clinical isolates could not be amplified using previously described AFM1, AFM2, and AFM3 primers, likely due to polymorphisms at the primer sites (Paoletti et al. 2005).

Discussion

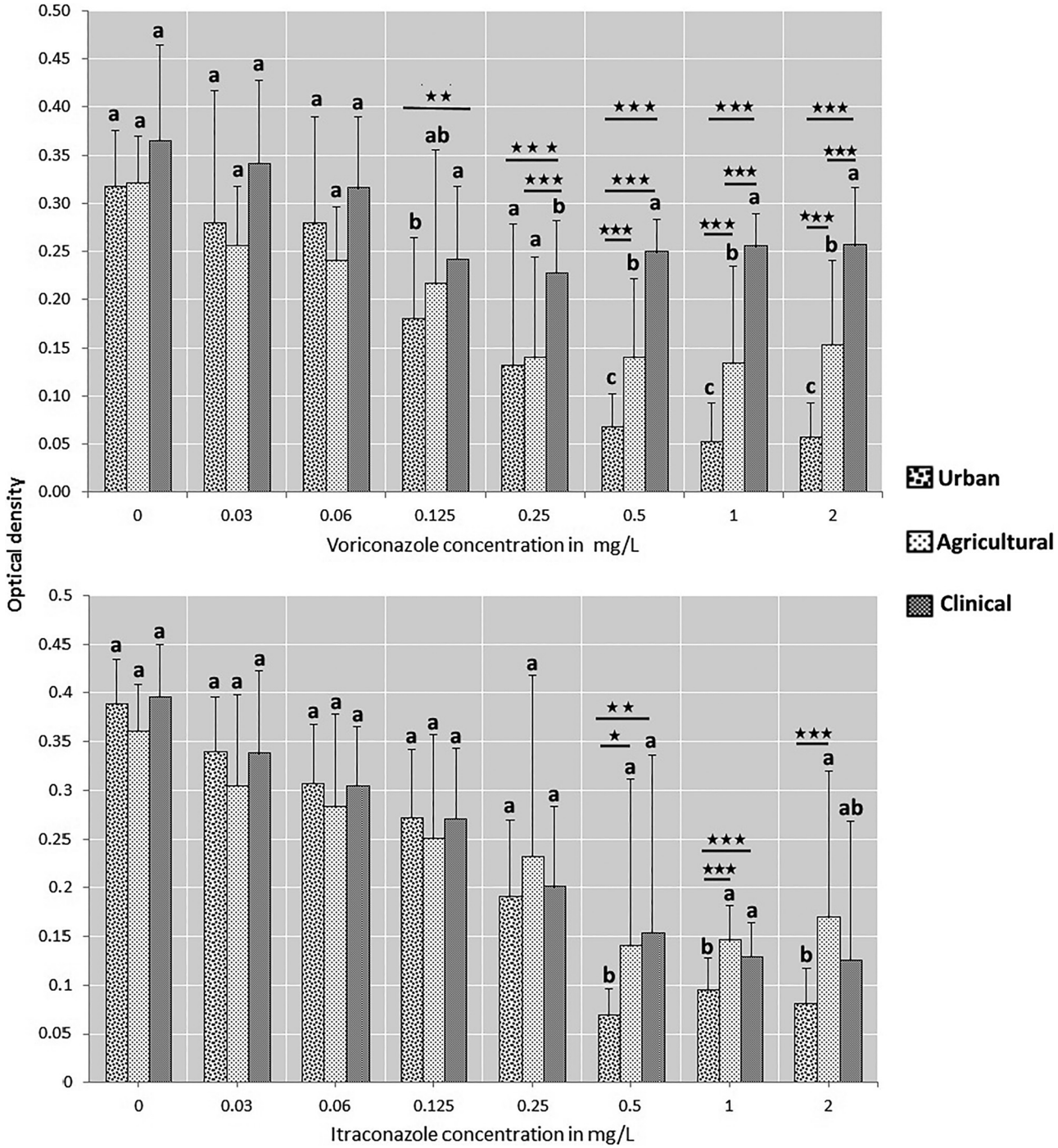
In several geographic regions, the effectiveness of triazoles in the management of patients with aspergillosis has been compromised because of increasing resistance to the azole class of antifungals. It has been found that the use of triazole fungicides in agriculture can cause resistance in environmental *A. fumigatus* strains of clinical importance (Chowdhary et al. 2012*b*). However, there has been no report of such a link in Canada. Here, we investigated the potential link between triazole fungicide use and the acquisition of clinically important resistant strains in Hamilton, Ontario. While all 195 isolates examined in this study were susceptible to both itraconazole and voriconazole, there was evidence of increased MICs in both clinical and agricultural *A. fumigatus* isolates over the urban isolates. Our results suggest that continued triazole selection pressure could drive some of these isolates to become resistant. Furthermore, we found evidence of widespread recombination and gene flow among the local ecological niche samples. Such gene flows and sexual recombination could have a significant implication in the initiation and spread of resistant genes throughout the Hamiltonian population. Below, we discuss the implications for our results to *A. fumigatus* epidemiology and to public health practitioners and farmers in Canada.

Link between triazole fungicide use and the acquisition of clinically important resistant strains

Using the epidemiological resistance cutoffs of 2 mg/L, all isolates examined in this study were susceptible to both itraconazole and voriconazole. However, the agricultural and clinical *A. fumigatus* populations were significantly less susceptible to itraconazole and voriconazole than the urban population (Fig. 3). Likewise, the clinical population showed an overall reduced susceptibility to voriconazole than both the agricultural population and even more so the urban population (Fig. 3). We hypothesized that in the advent of a significant link between triazole fungicide use and the acquisition of resistance in strains of clinical importance, agricultural and urban populations would share similar triazole susceptibility profiles and be more resistant than the clinical population (Fig. 1). However, we found that the urban population was significantly more susceptible to both itraconazole and voriconazole than the other 2 populations were. Given that we found significant evidence of gene flow, more so between agricultural and urban populations, these results are consistent with independent selective pressures acting on ecological niche populations of *A. fumigatus* in Hamilton.

Two agricultural fungicides used on the sampled farms in this study, propiconazole and tebuconazole, are structurally similar to medical triazoles and they dock similarly to the CYP51A protein (Snelders et al. 2012). The continuous use of these fungicides could have contributed to the decreased itraconazole and voriconazole susceptibility in the agricultural population of *A. fumigatus* around Hamilton. Acquired triazole resistance in *A. fumigatus* due to repeated exposure to triazoles has been previously demonstrated both in vivo and in vitro (Dannaoui et al. 2001; Escribano et al. 2011).

Fig. 3. Graphs showing growth responses of urban, agricultural, and clinical populations of *Aspergillus fumigatus* to voriconazole (top) and itraconazole (bottom). The *x* axis refers to triazole drug concentration. The *y* axis refers to optical density reading at 590 nm. Different letters at a given concentration indicate a statistically significant difference in the mean optical density reading. The number of stars depicts the level of significance. Adjustments were made to *P* values using the Bonferroni correction.



Similar to what has been reported elsewhere (Toyotome et al. 2016; Meletiadis et al. 2012; Pfaller et al. 2011, 2009), our voriconazole modal MICs in environmental and clinical populations were 0.5 and 0.25 mg/L, respectively. However, a more in-depth analysis of voriconazole susceptibility using spectrophotometry readings revealed that clinical isolates grew significantly better at higher concentrations than agricultural and urban populations did (Fig. 3). Escribano and colleagues previously reported that after progressive and direct exposure to itraconazole, there was a statistically significant increase in the geometric mean MICs of 2 other triazoles (Escribano et al. 2011). It is worth noting that although prior itraconazole exposure led to a significant

Table 4. Percentage of farmland with crops to which fungicides (including triazoles) were applied in Canada from 1996 to 2006.

Province	1996	2001	2006	% Change from 1996 to 2006
Manitoba	8.3	15.7	16.5	98.70
Saskatchewan	3.7	5.9	6.6	77.57
Ontario	5.2	5.3	7.2	40.09
Alberta	5.7	5.6	6.8	19.26
New Brunswick	17.6	17.8	19.9	13.16
Newfoundland and Labrador	2.2	3.7	2.5	10.74
Quebec	3.8	4.0	4.0	5.21
British Columbia	4.4	4.0	4.3	−3.56
Nova Scotia	9.7	10.1	9.2	−5.17
Prince Edward Island	26.0	25.4	24.4	−6.22

Note: Source: Statistics Canada.

increase in the geometric mean MICs of voriconazole and posaconazole, itraconazole MICs were the most affected among the 3 triazoles. In this study, although the clinical population showed reduced susceptibility to both itraconazole and voriconazole when compared with the urban population, voriconazole MICs were more affected than itraconazole MICs (Fig. 3). However, given that only 2 patients surveyed in this study had been previously exposed to voriconazole, selection by voriconazole was unlikely the reason for the overall reduced voriconazole susceptibility in the whole clinical sample. Instead, adaptation to host microenvironments by the secretion of SrbA (sterol regulatory element-binding protein) or similar proteins might have been responsible. A previous study showed that adaptation to hypoxic environments due to altered expression of SrbA could lead to resistance to voriconazole (Willger et al. 2008).

At present, the dominant mutations associated with triazoles resistance in *A. fumigatus* are found in the CYP51A gene. However, Escribano and colleagues also noted that increases in geometric mean MICs of *A. fumigatus* strains can be independent of the presence of *CYP51A* mutations (Escribano et al. 2011). Indeed, sequencing 1 kb of the *CYP51A* gene of 95 randomly chosen isolates from our sample set revealed no mutations known to be associated with decreased triazole susceptibility (data not shown). Although mechanisms leading to decreased triazole susceptibility in clinical and agricultural populations are not fully understood, several mechanisms could have contributed, including target overexpression, upregulation of multidrug transporter, secretion of sterol regulatory element-binding proteins, and (or) other cellular stress responses (Pfaller et al. 2011; Willger et al. 2008).

Abundant evidence of gene flow between ecological niche populations was found in Hamilton (PhiPT = 0.006). Surprisingly, on a population scale, these exchanges do not seem to cause a convergence of triazole susceptibility phenotypes between these populations. The results are consistent with our inference of independent selective triazole pressures on ecological niche populations of *A. fumigatus* in Hamilton. A possible explanation for the observed difference in triazole susceptibility despite abundant evidence of gene flow is that decreased itraconazole and voriconazole susceptibility in agricultural and clinical populations is likely caused by non-*CYP51A* mechanisms, which may have a fitness cost on these populations. A previous study showed that a *CYP51A* wild-type isolate with an acquired non-*CYP51A* itraconazole resistance mechanism (>16 mg/L) experienced a significant increase in susceptibility (1 mg/L) after 5 weeks of proliferation in triazole-free medium (Escribano et al. 2011). It is plausible that a majority of strains migrating from the farm to the urban environment adapt to their new environment by losing triazole tolerance, which may help restore their fitness in the urban, triazole-free environment.

Contrary to our results, Bromley et al. (2014) showed evidence of fungicide-driven resistance in Manchester, UK. Their study, however, did not include a clinical population that could be useful in tracking resistant strains from farms through urban settings to carriage in patients. Nonetheless, they sampled for a period of over 3 years, which was longer than what was done in this study. Given that we sampled within a period of only 4 months, we could not completely exclude the possibility of a link between triazole fungicide use and the acquisition of clinically important resistant strains. However, even if there were a link, our results suggested that agricultural triazole fungicide use in Hamilton has not been a dominant mechanism for the spread of triazole resistance.

The importance of crop rotation

At present, the detailed triazole fungicide usage pattern in Hamilton or elsewhere in Canada is not known. However, the combined fungicide usage pattern suggests that the increase in fungicide usage in Ontario is among the highest in Canada (Table 4). Generally speaking, a tendency towards specialized and intensive crop production is thought to have played a major role in

increasing fungicide use. Specialized and intensive farming is also thought to have resulted in little to no crop rotation in provinces like Ontario (Gossen et al. 2014). Crop rotation has been shown to reduce infection severity of blackleg on western Canadian canola plants (Kutcher et al. 2013). Similarly, Bailey and colleagues showed that populations of fungal pathogens *Septoria tritici*, *Bipolaris sorokiniana*, and *Stagonospora nodorum* on wheat decreased after increasing rotating crop diversity (Bailey et al. 2001). Frequent crop rotation often results in less fungal plant diseases and consequently less fungicide use. Although the increase in fungicide usage in Ontario is among the highest in Canada, the field from which we sampled rotated wheat, soybeans, and barley. Furthermore, triazole fungicides were only used every other year. Our analyses thus suggest that crop rotation and farmer's fungicide use patterns could have contributed to the observed overall triazole susceptibility pattern (MICs < 2 mg/L) in the agricultural *A. fumigatus* population in this study.

The need for continuous surveillance in Hamilton and similar jurisdictions

Given that wild-type CYP51A isolates seldom have itraconazole MICs of ≥1 mg/L (Ingen et al. 2015; Meletiadis et al. 2012), we compared our results with those of wild-type isolates obtained from around the world. Compared with the modal itraconazole MICs in wild-type isolates reported elsewhere (Toyotome et al. 2016; Meletiadis et al. 2012; Pfaller et al. 2011, 2009; Rodriguez-Tudela et al. 2008), the modal MIC reported in this study is slightly higher. For example, Pfaller et al. (2011) previously determined the modal itraconazole MIC for a set of 1221 wild-type isolates obtained from over 60 medical centers worldwide to be 0.25 mg/L. Interestingly our modal itraconazole MIC comprises up to 76.7% of all agricultural and clinical isolates, which is noticeably higher than was reported by Rodriguez-Tudela et al. (2008), 54.8%; Pfaller et al. (2009), 41.3%; and Toyotome et al. (2016), 65%. In light of the overall observed reduced itraconazole susceptibility, there is a need for close monitoring in hospitals and on farms that use azole fungicides in Hamilton, as a strong selection pressure could drive some of these isolates to become resistant to clinical triazoles drugs. Despite following CLSI guidelines and using multiple controls, authors acknowledge that lab-to-lab variability in susceptibility testing is a potential limitation to the above inference.

Different from most previous studies where abundant evidence for clonality was often found, the Hamiltonian samples showed notable evidence of recombination. For instance, significant numbers of both mating types were found in all 3 ecological populations. Recombination is known to allow for faster adaptation to stressful environments. Coupled with abundant gene flow, the frequent recombination observed in Hamilton can significantly facilitate the dissemination of resistance genes among the ecological niche populations. We thus call on necessary public health stakeholders such as Public Health Ontario and clinical microbiology and academic research labs to monitor *A. fumigatus* populations in Hamilton and similar jurisdictions in Ontario.

Epidemiology

According to Statistic Canada, only 23.7% of Hamilton's population is 60 years and over. However, this age group was disproportionately represented among patients surveyed in this study (59%), suggesting old age and (or) old-age-associated risk factors as potential contributors to the high prevalence of aspergillosis in this age group around Hamilton, Ontario. Further epidemiological surveys are needed to ascertain whether age as well as other factors such residence, occupation, and health status are related to aspergillosis in Hamilton. Evidence of multiple carriages was found in 82% (9/11) of patients from who 2 or more isolates were obtained. This result is not unique to this study; 75% (9/12) of studied patients from 3 European hospitals were also shown to carry at least 2 genotypes (Bertout et al. 2001). Multiple carriages are of public health and clinical significance for 2 main reasons. First, multiple carriages can complicate antifungal therapy, leading to treatment failure or disease persistence, as both triazole susceptible and resistant *A. fumigatus* genotypes can be present in the same patient (Mortensen et al. 2011). The cohabitation of both triazole susceptible and resistant genotypes could also lead to the spread of resistance alleles from resistant to susceptible strains within and even between host microenvironments. Second, in the event of aspergillosis outbreaks, tracking the sources of infections can be complicated in these cases.

Conclusion

This study, which aimed to understand the relationship between agricultural triazole fungicide use and the acquisition of clinically important resistant strains, is the first to be conducted in Canada. Although our results suggest that agricultural triazole fungicide use has not caused triazole resistance among clinical samples of *A. fumigatus* in Hamilton, we cannot exclude the possibility that other regions in Canada could have such a link. For example, increased usage of fungicides in Canada is the highest in Manitoba and Saskatchewan (Table 4). Such a greater selective pressure could have facilitated the emergence and spread of triazole-resistant strains. Investigating the fungicide-driven hypothesis in those provinces, with a focus on the local areas with the highest triazole fungicide use, could provide vital information to public health scientists and policy makers in Canada. During the course of this study, we were unable to obtain data on the specific types and amounts of agricultural fungicides used by individual provinces. With the growing global triazole resistance, it's essential that all public health stakeholders including Statistics Canada collect and curate data on the types and amounts of azole

fungicide used by different provinces in Canada to have a better understanding of the link between agricultural fungicide use and clinical antifungal resistance.

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