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An invisible threat: mutation-mediated resistance to triazole drugs in *Aspergillus*

Cau D. Pham and **Shawn R. Lockhart***

Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA

Abstract

Aspergillosis has emerged as an important contributor to infection-related morbidity and mortality in susceptible populations. This comes at a time when we are also seeing an increase in the vulnerable populations themselves. At the same time, some parts of the world are reporting an increased incidence of aspergillosis refractory to triazole therapy. Resistance to triazole drugs may have major implications for aspergillosis management since our antifungal armamentarium is limited. This review gives an overview of populations at risk of developing aspergillosis and highlights resistance mechanisms that may contribute to morbidity and mortality in these vulnerable populations.

Introduction

Aspergillus is a large genus of ubiquitous environmental molds that includes the major clinically relevant species *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus* [1•,2]. Conidia of these species are found in both indoor and outdoor environments [3,4]. Consequently, chronic exposure to *Aspergillus* conidia is an unavoidable consequence of breathing. Aspergillosis is a spectrum of disease resulting from the inhalation of airborne conidia by susceptible hosts. Once inhaled, disease development and progression depends on the immune status of the host. Clinical disease ranges from allergic bronchopulmonary aspergillosis (ABPA) to chronic pulmonary aspergillosis (CPA) and finally to invasive aspergillosis (IA).

Invasive aspergillosis is an important contributor to longer hospital stays and increased morbidity and mortality in susceptible populations [5–8]. Advances in the antifungal arsenal and novel tools for early detection of infection may have improved the outcomes for patients with IA. However, favorable prognosis for CPA and IA still remains unacceptably low with the overall mortality rates around 42% and 75%, respectively [9•,10•,11]. Unfavorable outcome is attributed to host factors as well as failure to respond to antifungal treatment. Antifungal treatment failure can be a consequence of bioavailability, which is poor for many antifungal agents, and to some degree, to the development by *Aspergillus* spp. of mechanisms that confer resistance [12,13•,14•,15]. This review gives a description of susceptible populations, a history of emergence of antifungal resistance in Aspergilli

*Send all correspondence to: Dr. Shawn R. Lockhart, Mycotic Diseases Branch, Centers for Disease Control and Prevention, 1600 Clifton Rd., Mailstop G-11, Atlanta, GA 30333, Phone: (404)639-2569, FAX: (404)639-3546,: gyi2@cdc.gov.

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worldwide, and an overview of advances made in understanding the mechanisms underlying resistance to antifungal agents.

Populations at risk

Individuals with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), tuberculosis, asthma, or receiving immunosuppressive therapy for cancer are at increased risk of developing CPA [9•,10•,16–20] and *Aspergillus* spp. are commonly present in their respiratory tracts [21,22]. A 2010 Canadian CF registry report showed that the prevalence of *A. fumigatus* infection/colonization was ≥19% in CF patients and had increased by 6% between 2007 and 2010 [18]. Smith and Denning retrospectively evaluated 126 CPA patients and found that their major underlying conditions were tuberculosis (32.5%), ABPA (11.9%), treated lung cancer (9.5%), COPD and/or emphysema (9.5%), and pneumothorax (9.5%) [16]. Moreover, data from the World Health Organization (WHO) estimate that about 4.8% of pulmonary tuberculosis (PTB) patients around the world were at risk of CPA [19]. The global five-year period prevalence for CPA in PTB patients was calculated to be between 11–15%.

Individuals with hematologic malignancy, those using corticosteroids, recipients of hematopoietic stem cell transplants (HSCT), and to a lesser extent recipients of solid organ transplants (SOT) are at risk for IA [9•,10•,23,24]. These three groups have the highest risk of developing IA, and outcome is particularly poor for those with acute myeloid leukemia (AML) where IA is commonly diagnosed within the first 100 days post-onset immunosuppressive therapy [25]. In one study, between 2004 and 2007 22% of 262 AML patients developed IA and 86% of these IA cases died within two years [26]. A large scale surveillance study in Italian hospitals estimated that more than 2.6% of 3228 HSCT patients had IA as an infectious complication [27]. Data from the Transplant Associated Infections Surveillance Network (TRANSNET), a network of 23 US transplant centers, showed a one-year cumulative incidence of 1.6% among the 875 HSCT patients and 0.7% among the 1063 SOT recipients were diagnosed with IA during the study period [9•,10•]. The one year mortality rate for IA in transplant recipients has been reported to range from 41% to 78% [9•,10•].

While we know that vulnerable patient populations continue to increase [28,29], we have very little data measuring the incidence of aspergillosis [13•,18]. There have been no recent surveillance studies assessing the prevalence of aspergillosis outside of specific patient populations. This leaves us with only anecdotal evidence to support the hypothesis that the prevalence of this disease is increasing. Mortality from this disease remains unacceptably high, and the contribution of azole antifungal resistance may be significant. Missing from our understanding of this disease is the overall prevalence of aspergillosis in the total patient population and longitudinal trend analysis. Without these data, appropriate prevention and control resources cannot be distributed.

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Antifungal resistance in *Aspergillus*

One possible contributor to the observed mortality rate in IA is antifungal treatment failure. Thirty-two percent of AML patients from 21 tertiary care centers in Italy who developed IA did not respond to first-line antifungal drugs (amphotericin B (AmB), voriconazole (VOR), caspofungin (CAS), posaconazole (POS)) [5]. Likewise, Denning *et al.* suggest that less than half of ABPA and CPA patients respond to antifungal treatment [19]. Poor bioavailability of many antifungal agents has been well documented. VOR shows the best penetrability at only 50% systemic concentration [30,31]; however, when disease progresses to the central nervous system (CNS), i.e., cerebral abscess, the prognosis is extremely poor despite appropriate antifungal therapy [32].

There are no established MIC breakpoints for *Aspergillus* and any antifungal agent. In order to distinguish non-wild type isolates, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has established epidemiological cut-off values (ECV) for clinically relevant *Aspergillus* spp. against AmB, ITZ (itraconazole), POS, VOR, and CAS [33]. MIC values of isolates from patients who have experienced treatment failure have largely fallen beyond these ECVs, thus supporting the definition of ECVs as the MIC threshold that allows the identification of isolates likely to carry resistance mechanisms. These ECVs have also been used in the literature to define resistance, which is used in this manuscript to describe isolates with MIC values above the ECV.

Triazole drugs generally possess good *in vitro* efficacy against common etiological agents of aspergillosis. For example, more than 95% of *A. fumigatus* clinical isolates have MICs of 1 μg/ml or less for VOR, ITZ, and POS [1•,34–37••]. All three triazoles have similar efficacy against *A. niger*, *A. flavus*, and *A. terreus* [1•,37••]. Furthermore, EUCAST guidelines indicate that most clinically-relevant *Aspergillus* species exhibit low ECVs for ITZ, POS, and VOR. In addition to good efficacy, fewer toxic side effects when compared to amphotericin B contribute to the recommendation of triazole compounds for treatment of aspergillosis [38,39]. Triazoles are used as empirical prophylaxis during HSCT and SOT transplantations as well as in targeted-therapy for CPA and IA of the orbit, sinus, lungs, brain, and central nervous system [40–45]. In addition to their medical applications, triazole compounds are widely employed by the agriculture industry. The extensive usage of triazoles in both medicine and agriculture is implicated in the emergence of triazole resistant *Aspergillus*, as will be discussed below [46••].

In the past decade, antifungal resistant clinical isolates have been associated with treatment failure and mortality in aspergillosis patients. In the Netherlands, more than 4% of the 2,012 *Aspergillus* isolates cultured from patients between 2007 and 2009 showed *in vitro* resistance to ITZ [15]. In addition, 88% of patients with triazole-resistant IA died during the course of this study, including those who received VOR therapy.

Currently the prevalence and trend of azole-resistant *Aspergillus* in clinical isolates is not clear, but several studies shed light on this issue. *Aspergillus* isolates with MICs above the established ECVs for triazoles have been recently reported from several European countries. Both the Netherlands and the United Kingdom have seen an increase in the number of *A.*

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fumigatus isolates with elevated MICs to triazoles [13•,47,48]. A single-center study from the UK found that more than 20% of their *A. fumigatus* clinical isolates were resistant to triazoles in 2009, a 17% increase from 2000 [13•,47]. Likewise, the Netherlands reported a 7.5% increase in that same time span and now have a collection encompassing more than 100 individual isolates [15,34]. The emergence of triazole resistant *Aspergillus*, predominantly *A. fumigatus*, has also been documented in countries outside Europe: the United States (3.6%, n=274), China (4%, n=107), India (2%, n=103), and Japan (11%, n=196) [1•, 2,49•,50]. The ARTEMIS global antifungal surveillance study revealed that more than 5.8% of *Aspergillus fumigatus* isolates collected between 2008 and 2009 had MIC values above the established ECV for the mold-active triazoles [14•]. Furthermore, more than 5% of 76 environmental *A. fumigatus* isolates collected from three different sites around Denmark displayed MICs above the ECV to multiple triazoles [51•].

In vitro resistance to polyenes and echinocandins has also been reported in *Aspergillus*, although at lower frequencies [52,53]. AmB resistance has been observed in two *Aspergillus* species, *A. terreus* and *A. flavus* [54,55]. Among TRANSNET *Aspergillus* isolates, less than 5% of 288 isolates had a minimum effective concentration (MEC) value above the established ECVs for *Aspergillus* spp. against echinocandins [56]. Because very few clinical cases of polyene and echinocandin resistance have been reported in the last 5 years, we will not address these topics in this review.

A. Triazole Resistant Aspergilli

A well-defined target for triazole compounds is the Cyp51 protein family comprised of essential sterol demethylase enzymes that catalyze the conversion of lanosterol to ergosterol, an essential constituent of the fungal cell membrane. Triazole agents compete with lanosterol for the binding site on Cyp51. As a result, these compounds inhibit fungal growth by disrupting ergosterol synthesis [57]. In *A. fumigatus*, there are two known homologues of the Cyp51 protein, Cyp51A and Cyp51B. Although Cyp51A is proposed to be the primary target for triazole compounds, it is Cyp51B that possesses a higher affinity for triazole compounds [58]. Hu *et al.* demonstrated that repression of Cyp51A expression highly sensitizes *A. fumigatus* to fluconazole [59]. Likewise, a *cyp51A*-null *A. lentulus* strain, a sibling of *A. fumigatus*, showed a decrease in MIC to triazoles by up to 100-fold. However, both studies suggested that eliminating Cyp51B expression does not affect triazole sensitivity in *A. fumigatus* [58,59].

A few less frequently encountered *Aspergillus* species are innately less susceptible to triazoles. For example, the MIC_{90} of ITZ, POS, and VOR against *A. calidoustus* are higher than 4μg/ml [1•]. Likewise, *A. versicolor* also displays reduced susceptibility to these three triazole drugs with ECVs of 2.0, 1.0 and 2.0, respectively [37••]. In contrast to the inherent resistance in *A. calidoustus* and *A. versicolor*, other *Aspergillus* species can develop resistance to triazole compounds by Cyp51-dependent and Cyp51-independent mechanisms.

i. Single amino acid substitutions in Cyp51 contribute to triazole resistance in Aspergillus spp—Many triazole-resistant isolates recovered from aspergillosis patients who failed therapy possess single amino acid substitutions within the Cyp51A protein,

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which correlate well with in vitro resistance. A large number of such substitutions have been discovered in *A. fumigatus* over the past decade. Many reside within or adjacent to critical domains of Cyp51A, thus quite often altering its conformation and leading to azole resistance. For example, a frequently encountered hot spot for substitution is the amino acid G54 (Glycine at amino acid position 54), located within the highly conserved region and arguably playing an essential role in substrate-dependent changes to Cyp51 conformation. Substitution of this residue greatly enhances *A. fumigatus* resistance to ITZ and POS [47,60]. Likewise, residues P216, F219, and M220 are situated within the F/G loop, also known as the ligand access channel [15,47,61,62], which has been proposed to facilitate the interaction between triazole agents and Cyp51A. Amino acid substitutions at M220 in *A. fumigatus* confer resistance to all three mold-active triazoles. Specifically, M220R (Arginine substituted for Methionine at amino acid 220), M220I, or M220V conferred resistance to ITZ with a decreased susceptibility to VOR. Similarly, an M220K substitution conferred resistance to ITZ and POS. *A. fumigatus* isolates that harbored a leucine or an isoleucine substitution at residues 216 or 219, respectively, in the Cyp51A protein were resistant to ITZ and POS [15,47,63••]. Camps *et al.* were able to experimentally reproduce this newly discovered resistance mechanism and proposed that these substitutions selectively prevented ITZ and POS, but not VOR, from binding to Cyp51A protein [63••]. Finally, the G (glycine) 448 residue is located within the heme-binding domain of Cyp51A. Substitutions at this residue are predicted to alter the orientation of the heme-binding domain which, in turn, could lead to decreased affinity to triazole compounds. *A. fumigatus* isolates that bear substitutions at amino acid G448 exhibited reduced susceptibility to ITZ and VOR [47,64,65]. In addition to the above substitutions, other substitutions in the Cyp51A amino acid sequence that confer resistance to triazoles are still being discovered and evaluated. Recently, G138C and Y431C substitutions were linked to multiple triazole-resistance (MTR) phenotypes [66]. More importantly, heterologous expression of *A. fumigatus* $Cyp51A^{G138C}$ and $Cyp51A^{Y431C}$ in *Saccharomyces cerevisiae* led to an approximately 10-fold reduction in susceptibility to ITZ, POS, and VOR.

In addition to *A. fumigatus*, single amino acid substitutions conferring triazole resistance have been recently discovered in other *Aspergillus* species. Arendrup *et al.* investigated 20 clinical *A. terreus* isolates and found that 15 isolates had elevated MICs to ITZ, POS, and VOR [67]. DNA sequence analysis of these 15 isolates revealed a mutation in *cyp51A* that corresponded to the single amino acid substitution M217I. In *A. flavus*, Liu *et al.* reported that a missense mutation in the *cyp*-family gene *cyp51C* could confer resistance to VOR [68]. An *A. flavus* isolate cultured from an IA patient following VOR treatment failure harbored a single amino acid substitution, S240A, in its Cyp51C protein [68]. The authors were able to recapitulate the substitution-mediated resistance phenotype using site-directed gene mutagenesis. In addition to the anomaly in *cyp51C*, Krishnan-Natesan *et al.* also reported other genetic aberrations in *cyp51A* and *cyp51B* that may contribute to triazole resistance in *A. flavus* [69]. However, these resistance-associated mutations have not yet been confirmed experimentally.

Single amino acid substitutions in Cyp51A that confer resistance to triazoles have been identified in isolates recovered from patients who have received long-term triazole therapy. There is a high correlation between VOR pre-exposure and *cyp51A* single nucleotide

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polymorphisms [70]. Camps *et al.* reported that substitutions at residues F216, P219, and G54 occurred in a patient who received multiple-triazole therapy for aspergillosis [63••]. The patient was the source of 9 consecutive *A. fumigatus* isolates within a period of 10 months. All isolates were genetically related. However, only the first isolate cultured from the patient was susceptible to triazoles; the eight subsequent isolates were resistant. All 8 resistant isolates acquired additional substitutions including F219I, P215L, and G54E. Similar observations were also reported by other groups [60,64]. Bellete *et al.* reported that *A. fumigatus* harboring a G448S substitution was recovered from a patient one year into VOR therapy [64]. Chen *et al.* detected M220I and G54R substitutions in 4 of 6 *A. fumigatus* isolates cultured serially from a patient who underwent ITZ therapy [60]. In addition to the above clinical observations, triazole-mediated *cyp51A* mutations have also been induced *in vitro*. To demonstrate this point, da Silva Ferreira *et al.* propagated Cyp51A wild type *A. fumigatus* on medium containing 1μg/ml of ITZ [71]. After 10 transfers, G54R and M220I, along with numerous other substitutions that did not confer resistance, were detected in Cyp51A. Snelders *et al.* also demonstrated that G138C and P216L can be induced after 3 ITZ passages [46••]. Taken together, these laboratory and patient studies imply that prolonged exposure to medical triazoles can induce amino acid substitution-mediated resistance in *A. fumigatus*.

ii. TR34/L98H has emerged as the predominant mechanism that confers triazole resistance in A. fumigatus—The TR34/L98H triazole-resistant mechanism in *A. fumigatus* was first described by Verweij *et al.* in 2007 [72]. The mutations were discovered through DNA sequence analysis of the *cyp51A* promoter and coding region of *A. fumigatus* isolates from 13 patients, half of whom had already experienced clinical failure of azole therapy. Twelve out of 13 isolates (98%) harbored the TR34/L98H mutation. The TR34 mutation is a 34 nucleotide tandem repeat 288 base-pairs upstream of the start codon, within the promoter region of *cyp51A*. This mutation is coupled with the L98H amino acid substitution. Since the discovery, this resistance mechanism has garnered much attention worldwide because of its highly resistant phenotype. Subsequent studies from Verweij's group using two larger collections of clinical *A. fumigatus* isolates confirmed the high prevalence of TR34/L98H among resistant isolates; it was present in 94% of 41 ITZ-resistant isolates collected between 1994 and 2007 [73]. Similarly, about 90% of 82 ITZ-resistant isolates collected from 2007 to 2009 harbored the same double-mutations [15]. Furthermore, Lockhart *et al.* reported that 26% of 29 *A. fumigatus* isolates from the ARTEMIS collection with triazole MICs above the ECV contained the TR34/L98H mutation [14•].

The TR34 insertion is believed to modulate the expression of *cyp51A*. The mRNA level of *cyp51A* in clinical *A. fumigatus* isolates harboring TR34/L98H is 8-fold higher than in wild type isolates [74,75]. This observation was confirmed experimentally in the laboratory. Strains that harbor only the TR34 mutation exhibit 4- to 8-fold increases in *cyp51A* mRNA expression compared to wild type strains. Overexpression of *cyp51A* is thought to confer resistance to triazoles in *A. fumigatus*. Arendrup *et al.* showed that the mRNA levels of *cyp51A* in two triazole-resistant strains, which displayed no apparent mutation in the *cyp51A* gene or in the promoter region, were 4 to 6-fold higher than in susceptible isolates

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[75]. Interestingly, the transformed TR34 strain without L98H does not confer resistance to ITZ, POS, or VOR. Likewise, *A. fumigatus* strains that harbor the L98H mutation without TR34 are not resistant to triazoles. In contrast, TR34 and L98H double-mutants are resistant to multiple triazoles in both laboratory-generated strains as well as clinical and environmental isolates. Isolates with either the TR34 or L98H single-mutation have yet to be cultured from patients or from the environment. However, Denning *et al.* suggested that such mutations do exist in patients with aspergillosis [76•]. Using real-time polymerase chain reaction (RT-PCR), the authors found both TR34 and L98H single-mutations in the sputum of ABPA and CPA patients at a UK hospital.

A recent molecular epidemiology study of 55 TR34/L98H *A. fumigatus* isolates from the Netherlands suggests that these mutants are genetically less diverse than triazole susceptible isolates and might share the same ancestor [77••]. Furthermore, the authors implied that the few observed genetic variations in these mutants are the result of sexual reproduction. They also showed that strains harboring this mutation had a greater ability to mate, which could increase the propagation of this mutation and enhance the ability of strains carrying this mutation to spread. Since the discovery of the TR34/L98H genotype in the Netherlands, it has been detected in other European nations. TR34/L98H strains have been isolated from Austria, Belgium, Spain, France, UK, Denmark and Norway [34,51•,76•,77••,78–80]. Outside of Europe, the TR34/L98H mutation has also been found in China and India [14•, 49•].

Unlike single amino acid substitutions, the TR34/L98H mutation is not believed to be associated with long-term triazole therapy. This observation is supported by the fact that many TR34/L98H clinical isolates are cultured from triazole-naive patients [15]. Moreover, isolates containing this mutation can be found in indoor and outdoor environments [51•,81]. Snelders *et al.* were able to culture *A. fumigatus* carrying the TR34/L98H mutation from the circulating air and water in a hospital, from flower beds in proximity to the hospital, and in the compost and flower seeds at a nursery where the hospital purchased gardening supplies [81]. Furthermore, these two mutations cannot be induced experimentally by medical triazoles. This supports the theory that this mutation is likely to have originated in the environment. It is hypothesized that triazole fungicides, combined with sexual reproduction, are the facilitators of this mutation. Fungicides such as 14α-demethylase inhibitors (triazoles; DMIs) are widely used in Europe. In the Netherlands, 19 of 33 fungicides commissioned for usage in agriculture from 1979 to 2005 were DMIs [46••]. Of these 19 DMIs, 11 have inhibitory effects similar to ITZ, VOR, and POS. Five of the 11 that inhibited *Aspergillus* growth also shared the same binding configurations as the three medical triazoles. Interestingly, these five fungicides were authorized for agriculture usage in Europe in the early part of the 1990s. By extrapolating the emergence rate of TR34/L98H of 1.37 new genotypes per year, obtained by using 144 *A. fumigatus* isolates harboring TR34/L98H, the author predicted that the first isolate with a TR34/L98H genotype could have emerged around 1997 [46••].

iii. Cyp51-independent triazole resistance mechanisms in A. fumigatus—Many triazole-resistant *A. fumigatus* isolates cultured from patients have a wild type *cyp51A* gene. In one study, 5 of 19 (26%) clinical *A. fumigatus* isolates with an MTR phenotype did not

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have any mutations in the *cyp51A* gene or the promoter region and in another study 7 of 22 (32%) triazole resistant isolates had a wild type copy of *cyp51A* [50,72]. This implies that *A. fumigatus* may possess other triazole resistance mechanism(s).

Overexpression of two classes of efflux pumps, ATP-binding cassette (ABC) and major facilitator superfamily (MFS) are shown to contribute to antifungal resistance in fungi. These efflux pumps are responsible for transportation of substances, such as antifungals, out of the cytoplasm. Overexpression of ABC efflux pumps has been observed in *A. nidulans* and *A. fumigatus* isolates resistant to triazoles. Slaven *et al.* demonstrated that increased expression of *atrF*, an *A. fumigatus* ABC transporter gene, conferred resistance to itraconazole [82]. Moreover, da Silva Ferriera *et al.* demonstrated that mRNA levels of *A. fumigatus* ABC genes *Afumdr1 Afumdr2*, *Afumdr4*, and *AtrF* as well as the MFS gene *Afumdr3* increased from 0.2 to 23-fold, depending on the gene, when ITZ was included in the growth medium [83]. Using restriction-mediated plasmid integration (REMI) Bowyer *et al.* demonstrated that disruption of an ABC transporter gene led to the hyper-sensitization to triazoles in the affected *A. fumigatus* strain [84]. Progress in this area of research is slow due to the large number of ABC (n=37) and MFS (n=251) genes in *A. fumigatus*.

Conclusion

In the last five years some regions of the world have witnessed the emergence of triazole resistance in *Aspergillus* species, especially *A. fumigatus*. Clinical isolates with high *in vitro* MICs have been associated with antifungal treatment failure and high mortality. These high MICs can be linked in most cases to mutations in the genetic loci responsible for expression of drug targets. Although epidemiological cutoff values for *A. fumigatus* exist and can be accurately used to predict which isolates will likely be less responsive to therapy, clinical interpretive breakpoints are not available. Therefore, routine susceptibility testing of *Aspergillus* is not recommended, but testing of isolates from patients failing therapy in conjunction with therapeutic drug level monitoring may have a role in treatment of aspergillosis.

The appearance of antifungal drug resistant Aspergilli has come at a time when we are also seeing an increase in vulnerable patient populations. A desirable outcome for aspergillosis patients is dependent on timely diagnosis and effective therapeutic intervention. Earlier diagnosis is available with new non-culture-based diagnostics and advances in radiology, so that culture and susceptibility testing of *Aspergillus* is not now routinely performed in many critically ill IA patients. While we know that antifungal resistant isolates of *Aspergillus* exist in some parts of the world, we do not know the prevalence in many countries because surveillance studies have not yet been initiated. An evidence base is urgently needed to determine the magnitude of the threat posed by triazole resistant Aspergilli to patient outcome, and to assess whether the prevalence of these isolates is increasing and spreading throughout the world. Sentinel surveillance screening by reference laboratories is needed to help develop a baseline data set. With the diagnostic landscape shifting away from procurement of isolates for identification of causative agents, more assays need to be developed for the detection of resistant Aspergilli directly from clinical samples. Without robust surveillance, we will never know the impact of this invisible threat.

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The findings and conclusions of this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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