GENE EXPRESSION UNIVERSITY

October 06-29

THE ULTIMATE WEBINAR SERIES IN GENE EXPRESSION STUDIES

Register here

applied biosystems
by Thermo Fisher Scientific

WILEY

A Low-Cost Tebuconazole-Based Screening Test for Azole-Resistant *Aspergillus fumigatus*

Amelie P. Brackin,[1] Jennifer M. G. Shelton,[1] Alireza Abdolrasouli,[2] Matthew C. Fisher,[1,3] and Thomas R. Sewell[1]

[1]MRC Centre for Global Infectious Disease Analysis, Department of Infectious Disease Epidemiology, Imperial College London, London, United Kingdom
[2]Diagnostic Mycology Service, Department of Medical Microbiology, North West London Pathology, Imperial College Healthcare National Health Service Trust, London, United Kingdom
[3]Corresponding author: *matthew.fisher@imperial.ac.uk*

The global emergence of azole resistance in *Aspergillus fumigatus* is resulting in health and food security concerns. Rapid diagnostics and environmental surveillance methods are key to understanding the distribution and prevalence of azole resistance. However, such methods are often associated with high costs and are not always applicable to laboratories based in the least-developed countries. Here, we present and validate a low-cost screening protocol that can be used to differentiate between azole-susceptible "wild-type" and azole-resistant *A. fumigatus* isolates. © 2020 The Authors.

Basic Protocol 1: Preparation of Tebucheck multi-well plates
Basic Protocol 2: Inoculation of Tebucheck multi-well plates

Keywords: antifungal • *Aspergillus fumigatus* • azole resistance • fungal diagnostic • tebuconazole

How to cite this article:
Brackin, A. P., Shelton, J. M. G., Abdolrasouli, A., Fisher, M. C., & Sewell, T. R. (2020). A low-cost tebuconazole-based screening test for azole-resistant *aspergillus fumigatus*. *Current Protocols in Microbiology*, *58,* e112. doi: 10.1002/cpmc.112

INTRODUCTION

Aspergillus fumigatus is an opportunistic fungal pathogen with an ecological niche of soil and composting vegetation, where it plays a vital role in ecosystem processes including decomposition and nutrient recycling (Rhodes, 2006; Tekaia & Latgé, 2005; Van De Veerdonk, Gresnigt, Romani, Netea, & Latgé, 2017). The fungus has a vast geographical range, facilitated by the rapid dispersion of airborne conidia that are able to tolerate a broad range of biotic stresses (Pringle et al., 2005; Sewell, Zhu, et al., 2019).

Humans inhale hundreds of conidia daily, which are frequently encountered in respiratory samples (Latgé, 1999; Mortensen et al., 2011). Typically, conidia are eliminated by the host's innate immune response, preventing attachment in the lung and subsequent infection (Balloy & Chignard, 2009). However, in immunocompromised individuals, conidia can become pathogenic resulting in a multitude of disorders including severe invasive aspergillosis (Brown et al., 2012). Current estimates indicate that more than 3 million

CURRENT PROTOCOLS
A Wiley Brand

Current Protocols in Microbiology e112, Volume 58
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpmc.112

people have invasive or chronic *A. fumigatus* infections (Bongomin, Gago, Oladele, & Denning, 2017). With the increase of immunocompromised individuals among the global population and rise in immunosuppressive therapies to treat human illnesses, successful management of globally emerging *Aspergillus*-related infections is largely dependent on early diagnosis and the initiation of effective antifungal treatment plans (Bernal-Martínez, Alastruey-Izquierdo, & Cuenca-Estrella, 2016; Denning et al., 2017; Walsh et al., 2008).

Triazoles are the most widely used class of antifungals owing to their efficacy and broad-spectrum activity (Price, Parker, Warrilow, Kelly, & Kelly, 2015). Their fundamental mode of action prevents fungal cell growth by inhibiting the lanosterol 14-α-demethylase enzyme in the ergosterol pathway, which is encoded by the *cyp*51A gene (Bossche, Koymans, & Moereels, 1995). However, continual exposure to these compounds exerts selective pressures that can facilitate the evolution of azole-resistant *A. fumigatus* (AR*Af*). The recent emergence of two AR*Af*-associated *cyp*51A alleles, TR_{34}/L98H and TR_{46}/Y121F/T289A, has raised concern due to their environmental association and increasing prevalence in azole-naive patients (Abdolrasouli et al., 2018; Chowdhary, Kathuria, Xu, & Meis, 2013; Mellado et al., 2007; Sewell, Zhu, et al., 2019; Snelders, Camps, et al., 2012; Stensvold, Jorgensen, & Arendrup, 2012; Van Der Linden et al., 2013; Zhang et al., 2017).

Several similar azole compounds belonging to the same structural class as the clinical triazoles, with near identical structures, are used extensively in agriculture for crop protection. Substrates with residual azoles, which effectively support the growth and reproduction of *A. fumigatus*, have the potential to become hotspots for azole resistance (Dunne, Hagen, Pomeroy, Meis, & Rogers, 2017; Prigitano et al., 2014; Schoustra et al., 2019). Environmental surveillance and resistance monitoring are needed to fully appreciate the prevalence and distribution of AR*Af* isolates in the environment (Fisher, Hawkins, Sanglard, & Gurr, 2018). Rapid diagnostics and surveillance methods will inevitably aid outbreak recognition, response, and prevention. However, current methods are targeted specifically for clinically derived samples, are associated with high costs, and are not widely available to laboratories based in low-income developing countries (Buil et al., 2017; Kahlmeter et al., 2006; White, Posso, & Barnes, 2017).

Here, we present a screening protocol to help identify AR*Af* isolated from the environment at a cost of ∼£0.70 per isolate. Our method is based on the agricultural azole tebuconazole, owing to it its use in the environment, its low cost, and its ability to differentiate between azole-susceptible "wild-type" *A. fumigatus* and AR*Af* (Alvarez-Moreno et al., 2017; Chowdhary, Kathuria, et al., 2012; Sewell, Zhang, et al., 2019; Snelders, Camps, et al., 2012; Verweij, Snelders, Kema, Mellado, & Melchers, 2009).

A. fumigatus is a Hazard Group 2 pathogen; therefore, suitable procedures and regulations for the handling of pathogenic microorganisms should always be followed. All open plate work should be carried out in a class 2 biological safety cabinet (BSC2) while wearing appropriate personal protective equipment. Extra care should be taken when handling due to the highly sporulating nature of the fungus.

BASIC PROTOCOL 1

PREPARATION OF TEBUCHECK MULTI-WELL PLATES

The first step in generating Tebucheck assay plates is to make the tebuconazole-incorporated agar at differing concentrations. Other culture media such as potato dextrose agar or malt extract agar can alternatively be used, although discrepancies in growth characteristics may be observed. Tebuconazole, 1-(4-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol (CAS no. 107534-96-3), is an agricultural triazole fungicide that inhibits sterol biosynthesis (demethylation inhibitor). The primary target of tebuconazole is lanosterol 14-α-demethylase (Kelly, Lamb, Corran, Baldwin, &

Table 1 Volumes Used to Generate the Final Concentrations of Tebuconazole-Incorporated Agar

Tube	Stock[a] tebuconazole (μl)	DMSO (μl)	Final tebuconazole concentration (mg/L)
1	0	12.8	0
2	4.8	8	6
3	6.4	6.4	8
4	12.8	0	16

[a]Stock concentration = 50 mg/ml.

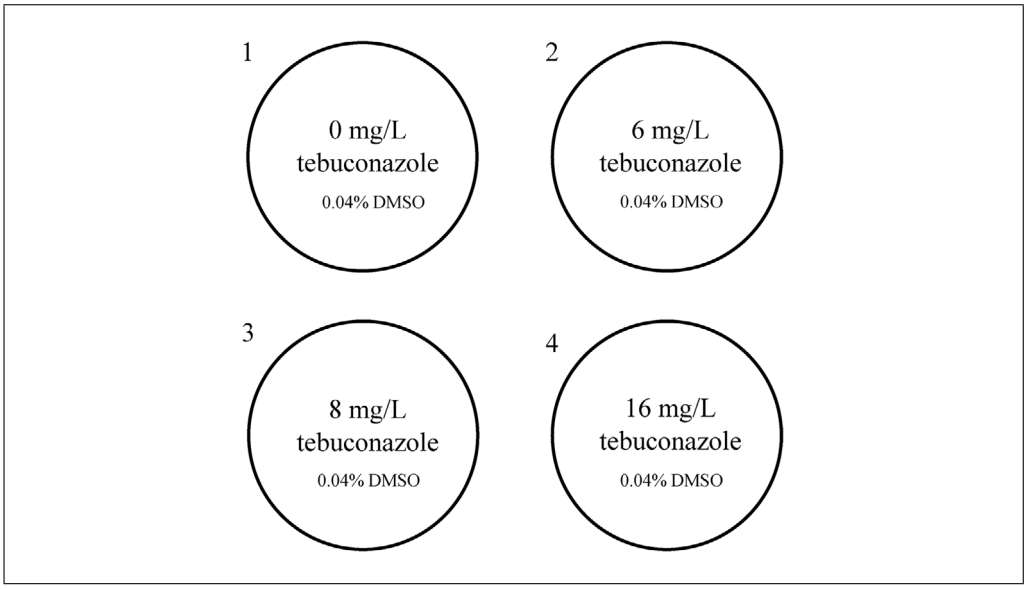

Figure 1 Layout of the Tebucheck assay multi-well plate.

Kelly, 1995), which is a key regulatory enzyme in the ergosterol biosynthetic pathway in fungi. Tebuconazole is classed as moderately toxic to mammals and can be dangerous in high doses (National Center for Biotechnology Information, 2020); consequently, all steps handling tebuconazole should be performed with care and while wearing single-use gloves. Table 1 provides the user with the required volumes/concentrations of tebuconazole and dimethyl sulfoxide (DMSO) for convenience. Tebuconazole stock solutions can be prepared in advance and stored in small-volume vials at −20°C for up to 1 month. To standardize the concentration of DMSO across the four wells of the assay, a supplementary volume must be added to give a final concentration of 0.032% (Table 1). Alternative methods for generating the tebuconazole-incorporated agar are acceptable; however, this may result in differential growth characteristics. The Tebucheck assay was developed using a 4-well multi-dish plate for standardization, reproducibility, and convenience. Other culture plates can be used, although discrepancies in growth characteristics may be observed. The stated volumes produce ∼32 4-well Tebucheck plates. Each well contains 1 ml Sabouraud dextrose agar (SDA) supplemented with three different concentrations of tebuconazole (Fig. 1). The first well is a drug-free control (0 mg/L tebuconazole), and wells 2, 3, and 4 contain SDA supplemented with 6, 8, or 16 mg/L tebuconazole, respectively. Tebucheck plates can be stored at 4°C for up to a month, although inspection of plates prior to use is recommended. The plates should be discarded if there is any obvious damage or contamination.

Materials

SDA (see recipe *or* purchase from a commercial vendor; e.g., Oxoid, cat. no. CM0041)

Tebuconazole, molecular biology grade, >98% purity (e.g., abcam, ab143703)
DMSO, molecular biology grade (e.g., Sigma-Aldrich, cat. no. D4540)

500-ml glass bottle (e.g., Duran, cat. no. 21 801 36 5)
Magnetic stir plate and stir bar
Benchtop autoclave
50°C water bath
50-ml conical tubes (e.g., Sarstedt, cat. no. 62.547.254)
Tube rack suitable for 50-ml tubes
50-ml serological pipette (e.g., Corning cat. no. CLS4490)
20-µl pipette and tips
4-well multi-dish plate (e.g., Thermo Fisher Scientific, cat. no. 176740)
1000-µl pipette and tips

Agar preparation

1. Add 19.5 g SDA to a 500-ml glass bottle.
2. Add 300 ml distilled water.
3. Mix gently on a magnetic stirrer for 5 min using a suitable-sized magnetic stir bar.
4. Autoclave in an automated benchtop autoclave to sterilize.
5. Allow molten medium to cool to ∼60°C inside a BSC2.
6. Move a suitable water bath inside the BSC2, and set to 50°C.
7. Label four 50-ml conical tubes 1 through 4, which correspond with the 4-well microplates used later.
8. Place tubes into the 50°C water bath using a 50-ml tube rack.
9. Using a serological pipette, transfer 40 ml molten SDA into the four conical tubes.
10. Allow SDA to equilibrate in the tubes for 5 min.

Incorporating tebuconazole

11. While the agar is equilibrating to temperature, prepare a 50 mg/ml stock solution of tebuconazole by dissolving 100 mg tebuconazole in 2 ml molecular biology–grade DMSO.

    The 50 mg/ml stock solution can be stored at −20°C for up to 1 month.

12. Using a 20-µl pipette, remove 12.8 µl SDA from each tube.
13. Using a 20-µl pipette, transfer the correct volume of tebuconazole stock for each corresponding tube (Table 1).
14. Gently mix each tube.
15. Using a 20-µl pipette, transfer the correct volume of DMSO into each corresponding tube (Table 1).
16. Gently mix each tube.
17. Leave tubes to infuse before immediately moving to Basic Protocol 2.
18. Using a 1000-µl pipette, transfer 1000 µl tebuconazole-incorporated agar from each tube into the corresponding well of the 4-well microplate (Fig. 1) starting with the control (0 µg/ml tebuconazole).

    Regularly change pipette tips to reduce the chance of volume discrepancies.

19. Once complete, allow plates to dry for 10 min in a BSC2.

INOCULATION OF TEBUCHECK MULTI-WELL PLATES

Inoculation of Tebucheck plates includes two incubation steps: (1) the initial culturing of *A. fumigatus* isolates to be tested and (2) incubation of the inoculated Tebucheck plates. The user must therefore allow 72 to 120 hr to complete this protocol. This time frame includes culture and preparation of pure single colonies plus the Tebucheck incubation period. Known wild-type *A. fumigatus* isolates can be purchased from CBS-KNAW Collections (*http://www.wi.knaw.nl/Collections/)* and used as a negative control. For positive controls, *A. fumigatus* isolates harboring the resistance alleles TR_{34}/L98H and TR_{46}/Y121F/T289A can be obtained by contacting the Fisher laboratory (*https://www.fisherlab.co.uk*). After incubation of Tebucheck plates, the presence or absence of growth on the surface of the four wells can be classified using the following scale: 0, no growth (0% to <10% growth coverage); 0.5, partial/visible growth (10% to <50% growth coverage); and 1, growth (50% to 100% growth coverage).

The end point of susceptibility should be recorded where visual growth was 0% to 10% coverage. For example, an isolate that grows vigorously in well 1, grows partially in well 2, and does not grow in wells 3 and 4 would receive a final Tebucheck score of 1.5. A population of Tebucheck *A. fumigatus* isolates can be analyzed together using a custom R script downloadable at *https://github.com/abrackin/tebucheck*.

Materials

SDA (see recipe *or* purchase from a commercial vendor; e.g., Oxoid, cat. no. CM0041)
A. fumigatus negative and positive controls
0.05% (v/v) Tween 80, sterile
Tebucheck plates (see Basic Protocol 1)

500-ml glass bottle (e.g., Duran, cat. no. 21 801 36 5)
Magnetic stir plate and stir bar
Benchtop autoclave
25-cm^2 cell culture flask (e.g., Thermo Fisher Scientific, cat. no. 156340) *or* appropriate alternative
37°C cell culture incubator
Custom R script, available at *https://github.com/abrackin/tebucheck*

A. fumigatus culture

1. Add 26 g SDA to a 500-ml glass bottle.
2. Add 400 ml distilled water.
3. Mix gently on a magnetic stir plate for 5 min using a suitable-sized magnetic stir bar.
4. Autoclave in an automated benchtop autoclave to sterilize.
5. Allow molten medium to cool to ∼60°C inside a BSC2.
6. Carefully pour ∼15 ml molten SDA into a 25-cm^2 cell culture flask or an appropriate alternative.
7. Place flasks horizontally (base down), and allow agar to solidify for 20 min.
8. Inoculate *A. fumigatus* sample (glycerol stock or agar punch) in the center of each cell culture flask.
9. Incubate at 37°C for 24 to 72 hr or when the colony has occupied ∼50% of the cell culture flask.

Tebucheck screening

10. Once fully grown, add 10 ml of 0.05% (v/v) Tween 80 to each cell culture flask.

    Use a 0.45-μm filter to sterilize the 0.05% (v/v) Tween 80 before use. Sterilized solution can be stored at room temperature for up to 3 months.

11. Gently shake each flask to disrupt the spores.
12. Leave spore suspension for 5 min to allow any aerosolized spores to settle.
13. Inoculate each well of the Tebucheck plates with 5 μl spore suspension, taking care to introduce the suspension into the center of the well.
14. Incubate inoculated Tebucheck plate for 48 hr at 37°C.
15. Score Tebucheck end point by visually assessing presence or absence of growth on each well.
16. Analyze multiple isolates using the custom R script.

Table 2 *Aspergillus fumigatus* Isolates Used for Tebucheck Validation

Isolate no.	Isolate name	Mutation	Country	Date of isolation
1	AFU-379/E12/2	Wild-type	India	2012
2	47-154	Wild-type	UK	2012
3	TF4-19	Wild-type	Japan	2013
4	47-181	Wild-type	Hawaii	1997
5	47-137	Wild-type	South Africa	2002
6	47-191	Wild-type	Sri Lanka	1991
7	47-151	Wild-type	Australia	1988
8	47-120	Wild-type	Brazil	1991
9	47-105	Wild-type	Hungary	1983
10	AF293 (reference)	Wild-type	UK	1993
11	HYDE42	TR_{34}/L98H	UK	2017
12	HL102-1	TR_{34}/L98H	Taiwan	2015
13	D007	TR_{34}/L98H	Taiwan	2014
14	CDC2014730721	TR_{34}/L98H	USA	2014
15	BUU10	TR_{34}/L98H	Thailand	2016[a]
16	BUU06	TR_{34}/L98H	Thailand	2016[a]
17	NAN121	TR_{34}/L98H	Colombia	2017[a]
18	ComPos-1	TR_{34}/L98H	UK	2017
19	NAN077	TR_{46}/Y121F/T289A	Colombia	2017[a]
20	302Wg	TR_{46}/Y121F/T289A	Wales	2015
21	NAN087	TR_{46}/Y121F/T289A	Colombia	2017[a]
22	DUB_48	TR_{46}/Y121F/T289A	Ireland	2015
23	DUB_54	TR_{46}/Y121F/T289A	Ireland	2015
24	DUB_50	TR_{46}/Y121F/T289A	Ireland	2015
25	E276	TR_{46}/Y121F/T289A	Germany	2012
26	E224	TR_{46}/Y121F/T289A	Germany	2012

[a]Denotes publication date rather than isolation date.

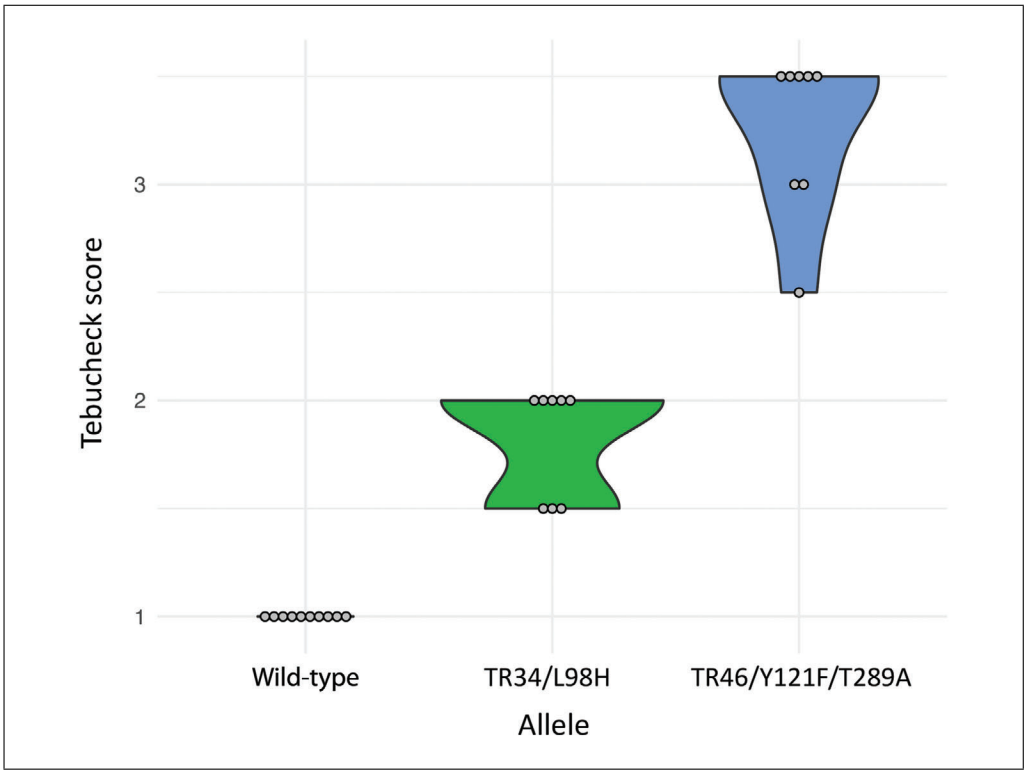

Figure 2 Violin plot representing the distribution of Tebucheck scores given to a panel of *A. fumigatus* isolates (n = 26) with known *cyp*51A alleles.

Sample data

Validation of Tebucheck was performed using a panel of 26 environmental *A. fumigatus* isolates (Table 2) selected from the Imperial College whole genome sequencing *A. fumigatus* culture collection (total collection >500 isolates). The panel represents the genetic population structure of the fungus, its vast geographical distribution, two of the most common environmentally associated azole antifungal-resistant genotypes (TR_{34}/L98H [n = 8] and TR_{46}/Y121F/T289A [n = 8]), and wild-type representatives (n = 10).

All wild-type isolates were susceptible to tebuconazole at 6 mg/L. Preliminary testing of the Tebucheck protocol used 4 mg/L, although this did not fully distinguish between wild-type and azole-resistant isolates. Isolates with known resistance alleles grew on the control well and exhibited various levels of growth in wells 2, 3, and 4 (Fig. 2). Of the isolates harboring the TR_{34}/L98H resistance allele, 100% had growth in well 2, and 62.5% showed partial growth in well 3 (up to 8 mg/L tebuconazole susceptibility). All TR_{34}/L98H isolates were scored 0 (0 to <10% growth coverage) in well 4 (16 mg/L), suggesting an end point of tebuconazole susceptibility at 8 mg/L. Of the isolates harboring the TR_{46}/Y121F/T289A allele, 100% showed growth in well 2 (6 mg/L), 100% grew in well 3, and 62.5% grew in well 4 (16 mg/L), suggesting an end point of tebuconazole susceptibility ≥16 mg/L (Fig. 3).

REAGENTS AND SOLUTIONS

SDA

10 g/L mycological peptone
40 g/L glucose
15 g/L agar
Adjust pH to 5.6 ± 0.2 when at 25°C
Autoclave at 121°C for 15 min
Store at room temperature for up to 3 months

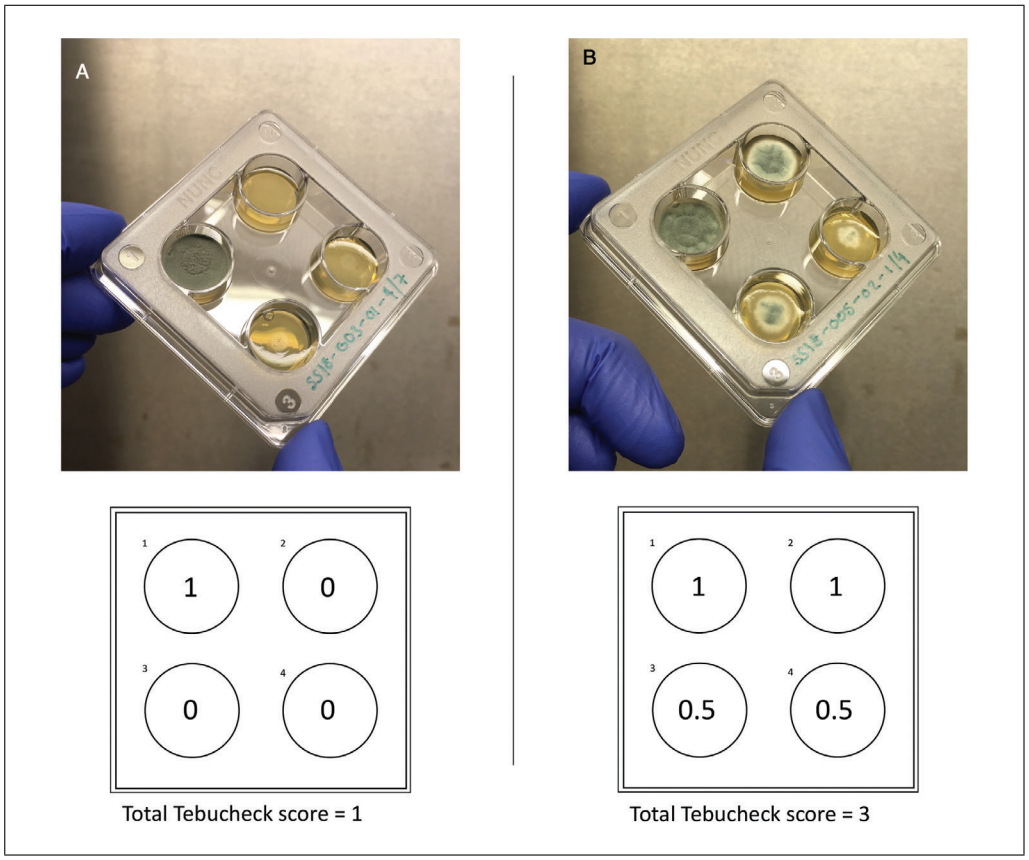

Figure 3 An example of the Tebucheck assay displaying growth characteristics and representative Tebucheck scores of (**A**) an isolate with a wild-type *cyp*51A allele and (**B**) an isolate harboring the AR*Af*-associated cyp51A allele TR_{46}/Y121F/T289A. Growth on the surface of each of the four wells can be scored using the following scale: 0, no growth (0% to <10% growth coverage); 0.5, partial/visible growth (10% to <50% growth coverage); 1, growth (50% to 100% growth coverage).

COMMENTARY

Background Information

The emergence of environmental AR*Af* and its associated pathological risks to immunocompromised individuals is of global concern (Fisher et al., 2018). Currently, the majority of azole resistance monitoring studies are constrained to European countries, motivated primarily by the clinical emergence of AR*Af* in these regions (Snelders, van der Lee, et al., 2008; Van Der Linden et al., 2013; Verweij et al., 2009). However, there has been an increasing number of reports of AR*Af* in the least-developed countries (Meis, Chowdhary, Rhodes, Fisher, & Verweij, 2016) where, typically, financial assets, specialized equipment, trained staff, and testing capacity are limited. Precise estimates of prevalence are largely unknown in developing countries where knowledge is limited by a lack of surveillance and reporting. Without environmental screening it is difficult to determine whether variations in prevalence may be due to climatic conditions, fungal distributions, varying use of agrichemical products, or incomplete investigations. While advances in technology have resulted in a variety of diagnostic tools to save time and cost, they are not always widely available to some laboratories.

Molecular tools such as quantitative PCR, loop-mediated isothermal amplification (LAMP), and Sanger sequencing are effective diagnostic tools used in many research laboratories and are regularly used to characterize AR*Af*-associated resistance alleles (Sewell, Zhang, et al., 2019; White et al., 2017; Yu et al., 2019). Biological growth assays such as VIP-check™ (Buil et al., 2017) and EUCAST (Kahlmeter et al., 2006) are used to determine the organism's resistance phenotype. Many of these assays are targeted specifically at clinical AR*Af* isolates despite the environmental source of *A. fumigatus*. While these techniques are extremely effective and broadly applicable in many pathology and genomic research facilities, they can become costly when applied to large screening projects,

particularly for those included in large-scale, longitudinal studies.

For many mycology laboratories, cost-effective culture-based diagnostics are paramount to research. Surveys conducted by the Asia Fungal Working Group comprising 241 mycology laboratories within seven Asian countries revealed that 89% had culture facilities, whereas only 17% performed DNA sequencing, and just 22% had molecular diagnostic facilities (Chindamporn et al., 2018). With the rise of antifungal resistance, routine susceptibility testing is essential, particularly in countries where the burden of fungal disease is high.

In order to address the issues outlined above, we present Tebucheck, a simple cost-effective method for the detection of AR*Af* using the agricultural antifungal tebuconazole. The method is intended for the use of determining the level of azole resistance in environmental samples and to aid in the forward planning of in-depth AR*Af* investigations. Affordable, easy-to-use identification tools such as Tebucheck can be used to identify AR*Af* hotspots and their implications on public health. It allows for screening and the subsequent selection of putative AR*Af* to be subjected to further downstream characterization and analysis, thus reducing the number (and cost) of high-precision testing.

Critical Parameters

Tebucheck has been designed to be a basic and easy-to-use assay, meaning it has very few parameters and a low chance of user error. However, a few critical conditions should be considered. Cell density is an important consideration when handling Tebucheck. If a very turbid spore suspension is used to inoculate the Tebucheck wells, uncharacteristic growth may occur. Users should aim to have a turbidity of ∼0.5 McFarland or a cell density reading at 600 nm between 0.08 and 0.13 using a standard spectrophotometer. However, if the guidelines set out in this protocol for the generation of *A. fumigatus* spore suspension are followed closely (Basic Protocol 2, steps 1 to 12), then there should not be an issue at this step, and cell density readings will not need to be verified. When creating the tebuconazole-incorporated medium, it is vitally important the user allows the tebuconazole stock solution to properly mix with the molten SDA. Gently mixing SDA and allowing it to equilibrate in the water bath for 5 min is enough to ensure a consistent concentration of tebuconazole is maintained across all wells of the Tebucheck assay plates. Growth rates between *A. fumigatus* isolates can vary and may affect the assessment and scoring after the mandatory 48-hr incubation period. If growth in the control well (0 μg/ml tebuconazole) is underdeveloped after 48 hr, then Tebucheck plates can be left to incubate to the point at which the control well exhibits ∼50% growth coverage (maximum 5-day incubation due to drug exhaustion).

Troubleshooting

Our findings suggest that Tebucheck could be a useful addition to the current range of AR*Af* diagnostics available. Here, we show that it can distinguish between resistant and susceptible isolates and putatively predicted, environmentally associated resistance genotypes. Despite this finding, other resistance mechanisms can also explain AR*Af* phenotypes, and there may be others yet undiscovered. It is important, therefore, that Tebucheck is predominantly used to distinguish between resistant and non-resistant environmentally acquired isolates and that any putative genotype calls be properly assessed with quantitative PCR, LAMP assay, or direct sequencing of the *cyp*51A allele (Sewell, Zhang, et al., 2019; White et al., 2017; Yu et al., 2019).

Understanding Results

Tebucheck was validated using a panel of *A. fumigatus* isolates that were subjected to three concentrations of tebuconazole plus a control. Growth of all non-resistant isolates harboring the wild-type *cyp*51A allele was restricted to the control well only, clearly defining azole-susceptible wild-type isolates from resistant isolates. All resistant isolates were able to grow on at least one concentration of tebuconazole-incorporated growth medium. By using the scoring system outlined in Basic Protocol 2, it is possible for users to analyze a population of *A. fumigatus* isolates and determine its level of resistance.

The four different concentrations of tebuconazole in the Tebucheck assay can also provide insight into the level of resistance the isolates exhibit, providing the user with a more detailed characterization of their AR*Af* samples. During validation, it became clear that isolates with different resistance genotypes had different growth characteristics on Tebucheck, with TR_{46}/Y121F/T289A genotypes more likely to grow across all four wells of the assay and TR_{34}/L98H restricted to two wells (Figs. 2 and 3). It is possible, therefore, to not only gauge the isolates level of

resistance but also putatively suggest its *cyp*51A genotype.

Time Considerations

Time considerations for this protocol depend on the number of *A. fumigatus* isolates the user wishes to process using Tebucheck. Considering a single set of Tebucheck plates (32 plates), Basic Protocol 1 should take ∼30 min, while Basic Protocol 2 can take between 3 and 5 days due to the incubation steps involved in prior culturing of *A. fumigatus* isolates and the mandatory 48-hr Tebucheck incubation period. Generally, the total time to perform the whole protocol, including incubation steps, is between 2 and 5 days.

Acknowledgments

The authors acknowledge joint Centre funding from the UK Medical Research Council and Department for International Development. T.R.S., A.P.B., J.M.G., and M.C.F. were supported by the Natural Environmental Research Council (NERC; NE/P001165/1), and all authors were supported by the Medical Research Council (MRC; MR/R015600/1). M.C.F. is a CIFAR Fellow in the Fungal Kingdom program.

Author Contributions

A.P.B., J.M.G.S., A.A., M.C.F., and T.R.S. conceived and designed the study. A.P.B., J.M.G.S., and T.R.S. tested the protocol and collected the data. A.P.B. and T.R.S. analyzed the data. A.P.B. and T.R.S. wrote the manuscript. A.P.B., J.M.G.S., A.A., M.C.F., and T.R.S. commented on the manuscript.

Literature Cited

Abdolrasouli, A., Scourfield, A., Rhodes, J., Shah, A., Elborn, J. S., Fisher, M. C., … Armstrong-James, D. (2018). High prevalence of triazole resistance in clinical *Aspergillus fumigatus* isolates in a specialist cardiothoracic centre. *International Journal of Antimicrobial Agents*, *52*, 637–642. doi: 10.1016/j.ijantimicag.2018.08.004.

Alvarez-Moreno, C., Lavergne, R.-A., Hagen, F., Morio, F., Meis, J. F., & Le Pape, P. (2017). Azole-resistant *Aspergillus fumigatus* harboring TR 34 /L98H, TR 46 /Y121F/T289A and TR 53 mutations related to flower fields in Colombia. *Scientific Reports*, *7*, 45631. doi: 10.1038/srep45631.

Balloy, V., & Chignard, M. (2009). The innate immune response to *Aspergillus fumigatus*. *Microbes and Infection*, *11*, 919–927. doi: 10.1016/j.micinf.2009.07.002.

Bernal-Martínez, L., Alastruey-Izquierdo, A., & Cuenca-Estrella, M. (2016). Diagnostics and susceptibility testing in *Aspergillus*. *Future Microbiology*, *11*, 315–328. doi: 10.2217/fmb.15.140.

Bongomin, F., Gago, S., Oladele, R. O., & Denning, D. W. (2017). Global and multi-national prevalence of fungal diseases—estimate precision. *Journal of Fungi*, *3*, 57. doi: 10.3390/jof3040057.

Bossche, H. V., Koymans, L., & Moereels, H. (1995). P450 inhibitors of use in medical treatment: Focus on mechanisms of action. *Pharmacology and Therapeutics*, *67*, 79–100. doi: 10.1016/0163-7258(95)00011-5.

Brown, G. D., Denning, D. W., Gow, N. A. R., Levitz, S. M., Netea, M. G., & White, T. C. (2012). Hidden killers: Human fungal infections. *Science Translational Medicine*, *4*, 165rv13. doi: 10.1126/scitranslmed.3004404.

Buil, J. B., van der Lee, H. A. L., Rijs, A. J. M. M., Zoll, J., Hovestadt, J. A. M. F., Melchers, W. J. G., & Verweij, P. E. (2017). Single-center evaluation of an agar-based screening for azole resistance in *Aspergillus fumigatus* by using VIPcheck. *Antimicrobial Agents and Chemotherapy*, *61*, e01250–17. doi: 10.1128/AAC.01250-17.

Chindamporn, A., Chakrabarti, A., Li, R., Sun, P. L., Tan, B. H., Chua, M., … Chayakulkeeree, M. (2018). Survey of laboratory practices for diagnosis of fungal infection in seven Asian countries: An Asia Fungal Working Group (AFWG) Initiative. *Medical Mycology*, *56*, 416–425. doi: 10.1093/mmy/myx066.

Chowdhary, A., Kathuria, S., Xu, J., & Meis, J. F. (2013). Emergence of azole-resistant *Aspergillus fumigatus* strains sue to agricultural azole use creates an increasing threat to human health. *PLoS Pathogens*, *9*, e1003633. doi: 10.1371/journal.ppat.1003633.

Chowdhary, A., Kathuria, S., Xu, J., Sharma, C., Sundar, G., Singh, P. K., … Meis, J. F. (2012). Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR_{34}/L98H mutations in the *cyp*51A gene in India. *PLoS ONE*, *7*, e52871. doi: 10.1371/journal.pone.0052871.

Denning, D. W., Perlin, D. S., Muldoon, E. G., Colombo, A. L., Chakrabarti, A., Richardson, M. D., & Sorrell, T. C. (2017). Delivering on antimicrobial resistance agenda not possible without improving fungal diagnostic capabilities. *Emerging Infectious Diseases*, *23*, 177–183. doi: 10.3201/eid2302.152042.

Dunne, K., Hagen, F., Pomeroy, N., Meis, J. F., & Rogers, T. R. (2017). Intercountry transfer of triazole-resistant *Aspergillus fumigatus* on plant bulbs. *Clinical Infectious Diseases*, *65*, 147–149. doi: 10.1093/cid/cix257.

Fisher, M. C., Hawkins, N. J., Sanglard, D., & Gurr, S. J. (2018). Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science*, *360*, 739–742. doi: 10.1126/science.aap7999.

Kahlmeter, G., Brown, D. F. J., Goldstein, F. W., MacGowan, A. P., Mouton, J. W., Odenholt, I., & Stetsiouk, O. (2006). European Committee

on Antimicrobial Susceptibility Testing (EUCAST) technical notes on antimicrobial susceptibility testing. *Clinical Microbiology and Infection*, *12*, 501–503. doi: 10.1111/j.1469-0691.2006.01454.x.

Kelly, S. L., Lamb, D. C., Corran, A. J., Baldwin, B. C., & Kelly, D. E. (1995). Mode of action and resistance to azole antifungals associated with the formation of 14α-methylergosta-8,24(28)-dien-3β,6α-diol. *Biochemical and Biophysical Research Communications*, *207*, 910–915. doi: 10.1006/bbrc.1995.1272.

Latgé, J.-P. (1999). *Aspergillus fumigatus* and aspergillosis. *Clinical Microbiology Reviews*, *12*, 310–350. doi: 10.1128/CMR.12.2.310.

Meis, J. F., Chowdhary, A., Rhodes, J. L., Fisher, M. C., & Verweij, P. E. (2016). Clinical implications of globally emerging azole resistance in *Aspergillus fumigatus*. *Philosophical Transactions of the Royal Society of London*, *371*, 20150460. doi: 10.1098/rstb.2015.0460.

Mellado, E., Garcia-Effron, G., Alcázar-Fuoli, L., Melchers, W. J. G., Verweij, P. E., Cuenca-Estrella, M., & Rodríguez-Tudela, J. L. (2007). A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. *Antimicrobial Agents and Chemotherapy*, *51*, 1897–1904. doi: 10.1128/AAC.01092-06.

Mortensen, K. L., Jensen, R. H., Johansen, H. K., Skov, M., Pressler, T., Howard, S. J., & Arendrup, M. C. (2011). *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: A laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *Journal of Clinical Microbiology*, *49*, 2243–2251. doi: 10.1128/JCM.00213-11.

National Center for Biotechnology Information. (2020). PubChem compound summary for CID 86102, tebuconazole. Retrieved June 16, 2020 from https://pubchem.ncbi.nlm.nih.gov/compound/Tebuconazole.

Price, C. L., Parker, J. E., Warrilow, A. G. S., Kelly, D. E., & Kelly, S. L. (2015). Azole fungicides - Understanding resistance mechanisms in agricultural fungal pathogens. *Pest Management Science*, *71*, 1054–1058. doi: 10.1002/ps.4029.

Prigitano, A., Venier, V., Cogliati, M., De Lorenzis, G., Esposto, M. C., & Tortorano, A. M. (2014). Azole-resistant *Aspergillus fumigatus* in the environment of northern Italy, May 2011 to June 2012. *Eurosurveillance*, *19*, 20747. doi: 10.2807/1560-7917.es2014.19.12.20747.

Pringle, A., Baker, D. M., Platt, J. L., Wares, J. P., Latgé, J.-P., & Taylor, J. W. (2005). Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigatus*. *Evolution*, *59*, 1886–1899. doi: 10.1111/j.0014-3820.2005.tb01059.x.

Rhodes, J. C. (2006). *Aspergillus fumigatus*: Growth and virulence. *Medical Mycology*, *44*, 77–81. doi: 10.1080/13693780600779419.

Schoustra, S. E., Debets, A. J. M., Rijs, A. J. M. M., Zhang, J., Snelders, E., Leendertse, P., & Verweij, P. E. (2019). Environmental hotspots for azole resistance selection of *Aspergillus fumigatus*, the Netherlands. *Emerging Infectious Diseases*, *25*, 1347–1353. doi: 10.3201/eid2507.181625.

Sewell, T. R., Zhang, Y., Brackin, A. P., Shelton, J. M. G., Rhodes, J., & Fisher, M. C. (2019). Elevated prevalence of azole-resistant *Aspergillus fumigatus* in urban versus rural environments in the United Kingdom. *Antimicrobial Agents and Chemotherapy*, *63*, e00548–19. doi: 10.1128/AAC.00548-19.

Sewell, T. R., Zhu, J., Rhodes, J., Hagen, F., Meis, J. F., Fisher, M. C., & Jombart, T. (2019). Nonrandom distribution of azole resistance across the global population of *Aspergillus fumigatus*. *mBio*, *10*, e00392–19. doi: 10.1128/mBio.00392-19.

Snelders, E., Camps, S. M. T., Karawajczyk, A., Schaftenaar, G., Kema, G. H. J., van der Lee, H. A., & Verweij, P. E. (2012). Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS ONE*, *7*, e31801. doi: 10.1371/journal.pone.0031801.

Snelders, E., van der Lee, H. A. L., Kuijpers, J., Rijs, A. J. M. M., Varga, J., Samson, R. A., & Verweij, P. E. (2008). Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Medicine*, *5*, 1629–1637. doi: 10.1371/journal.pmed.0050219.

Stensvold, C. R., Jorgensen, L. N., & Arendrup, M. C. (2012). Azole-resistant invasive *Aspergillosis*: Relationship to agriculture. *Current Fungal Infection Reports*, *6*, 178–191. doi: 10.1007/s12281-012-0097-7.

Tekaia, F., & Latgé, J.-P. (2005). *Aspergillus fumigatus*: Saprophyte or pathogen? *Current Opinion in Microbiology*, *8*, 385–392. doi: 10.1016/j.mib.2005.06.017.

Van De Veerdonk, F. L., Gresnigt, M. S., Romani, L., Netea, M. G., & Latgé, J.-P. (2017). *Aspergillus fumigatus* morphology and dynamic host interactions. *Nature Reviews Microbiology*, *15*, 661–674. doi: 10.1038/nrmicro.2017.90.

Van Der Linden, J. W. M., Camps, S. M. T., Kampinga, G. A., Arends, J. P. A., Debets-Ossenkopp, Y. J., Haas, P. J. A., … Verweij, P. E. (2013). Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clinical Infectious Diseases*, *57*, 513–520. doi: 10.1093/cid/cit320.

Verweij, P. E., Snelders, E., Kema, G. H. J., Mellado, E., & Melchers, W. J. G. (2009). Azole resistance in *Aspergillus fumigatus*: A side-effect of environmental fungicide use? *The Lancet Infectious Diseases*, *9*, 789–795. doi: 10.1016/S1473-3099(09)70265-8.

Walsh, T. J., Anaissie, E. J., Denning, D. W., Herbrecht, R., Kontoyiannis, D. P., & Marr, K. A., & Infectious Diseases Society of America (2008).

Treatment of aspergillosis: Clinical practice guidelines of the Infectious Diseases Society of America. *Clinical Infectious Diseases*, *46*, 327–360. doi: 10.1086/525258.

White, P. L., Posso, R. B., & Barnes, R. A. (2017). Analytical and clinical evaluation of the PathoNostics AsperGenius assay for detection of invasive aspergillosis and resistance to azole antifungal drugs directly from plasma samples. *Journal of Clinical Microbiology*, *55*, 2356–2366. doi: 10.1128/JCM.00411-17.

Yu, L.-S., Rodriguez-Manzano, J., Malpartida-Cardenas, K., Sewell, T., Bader, O., & Armstrong-James, D. (2019). Rapid and sensitive detection of azole-resistant *Aspergillus fumigatus* by tandem repeat loop-mediated isothermal amplification. *The Journal of Molecular Diagnostics*, *21*, 286–295. doi: 10.1016/j.jmoldx.2018.10.004.

Zhang, J., van den Heuvel, J., Debets, A. J. M., Verweij, P. E., Melchers, W. J. G., Zwaan, B. J., & Schoustra, S. E. (2017). Evolution of cross-resistance to medical triazoles in *Aspergillus fumigatus* through selection pressure of environmental fungicides. *Proceedings of the Royal Society B: Biological Sciences*, *284*, 20170635. doi: 10.1098/rspb.2017.0635.