RESEARCH ARTICLE

The Widely Used ATB FUNGUS 3 Automated Readings in China and Its Misleading High MICs of Candida spp. to Azoles: Challenges for Developing Countries’ Clinical Microbiology Labs

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Abstract

The rapid development in the clinical microbiology diagnostic assays presents more challenges for developing countries than for the developed world, especially in the area of test validation before the introduction of new tests. Here we report on the misleading high MICs of Candida spp. to azoles using the ATB FUNGUS 3 (bioMérieux, La Balme-les Grottes, France) with automated readings in China to highlight the dangers of introducing a diagnostic assay without validation. ATB FUNGUS 3 is the most commonly used commercial antifungal susceptibility testing method in China. An in-depth analysis of data showed higher levels of resistance to azoles when ATB FUNGUS 3 strips were read automatically than when read visually. Based on this finding, the performance of ATB FUNGUS 3, read both visually and automatically, was evaluated by testing 218 isolates of five clinically important Candida species, using broth microdilution (BMD) following CLSI M27-A3 as the gold-standard. The overall essential agreement (EA) between ATB visual readings and BMD was 99.1%. In contrast, the ATB automated readings showed higher discrepancies with BMD, with overall EA of 86.2%, and specifically lower EA was observed for fluconazole (80.7%), voriconazole (77.5%), and itraconazole (73.4%), which was most likely due to the trailing effect of azoles. The major errors in azole drug susceptibilities by ATB automated readings is a concern in China that can result in misleading clinical antifungal drug selection and pseudo high rates of
antifungal resistance. Therefore, the ATB visual reading is generally recommended. In the meantime, we propose a practical algorithm to be followed for ATB FUNGUS 3 antifungal susceptibility for *Candida* spp. before the improvement in the automated reading system.

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**Introduction**

The rapid development in clinical microbiology laboratory diagnostic assays presents more challenges for developing countries than their developed world counterparts, especially with regard to validation and quality control of such assays. The widely used but problematic ATB FUNGUS 3 with ATB Expression Bacteriology Analyzer automated readings (bioMérieux, La Balme-les Grottes, France) in China and its misleading reported high MICs of *Candida* spp. to azoles, gave a very good show case to highlight the challenges faced by clinical microbiology labs in developing countries.

Invasive candidiasis is now widely recognized as an important public health problem, with considerable morbidity, mortality, and associated health care costs [1–3]. Antifungal surveillance programs like ARTEMIS Global Antifungal Surveillance Program play an important role in the management of fungal infections, guiding clinical treatment as well as tracking the development of drug resistance [4–5].

Unlike in the developed world, multicenter and long term antifungal surveillance data is lacking in China. Furthermore, an analysis of reported data on antifungal susceptibility patterns from different centers in China revealed considerable variability, especially for azole drugs. ATB FUNGUS 3 is the most commonly used commercialized antifungal susceptibility test method in China, and an in-depth analysis of data on *Candida* susceptibility to fluconazole showed higher levels of resistance when ATB FUNGUS 3 strips were read automatically than when read visually, suggesting possible errors by the ATB FUNGUS 3 strips automated reading system [6–10].

Thus, the main aim of the present study was to evaluate the performance of the ATB FUNGUS 3 strips (both read visually and automatically) in relation to the Clinical and Laboratory Standard Institute (CLSI) broth microdilution (BMD) method for the *in vitro* antifungal susceptibility testing of *Candida* isolates from multicenters in China. A secondary aim was to develop a practical and efficient algorithm for *Candida* species susceptibility testing by ATB FUNGUS 3 strips, using a combination of visual and automated readings.
Materials and Methods

1. *Candida* isolates

A total of 218 isolates of five clinically important *Candida* species selected from the 2010 CHIF-NET program from 12 study centers (August 2009–July 2010) were used [11]. All the resistant isolates of the five *Candida* species were included in the study, whilst the susceptible isolates were selected randomly. These isolates included 81 isolates of *Candida albicans*, 42 of *Candida glabrata*, 41 of *Candida tropicalis*, 38 of *Candida parapsilosis* complex (20 of *C. parapsilosis* sensu stricto, 14 of *Candida metapsilosis* and 4 of *Candida orthopsilosis*) and 16 of *Candida krusei*. All the isolates were characterized by sequencing of the internal transcribed spacer (ITS) region.

2. CLSI BMD method

CLSI BMD susceptibility testing was performed in accordance with the CLSI M27-A3 guidelines [12]. The following drugs and concentrations were tested: 5-flucytosine (0.125–64 μg/mL); amphotericin B, voriconazole, itraconazole (all 0.03–16 μg/mL); and fluconazole (0.125–64 μg/mL). Testing was done at 35°C (air) for 24 h. The MICs of all drugs were read following CLSI M27-A3 guideline [12].

3. ATB FUNGUS 3

An ATB FUNGUS 3 strip consists of 16 pairs of cupules including two growth control wells and five antifungal drugs at different concentrations: 5-flucytosine (4, 16 μg/mL), amphotericin B (0.5–16 μg/mL), fluconazole (1–128 μg/mL), itraconazole (0.125–4 μg/mL) and voriconazole (0.06–8 μg/mL). Testing on the ATB FUNGUS 3 strip was performed simultaneously with CLSI BMD. Following the manufacturer’s instructions, a suspension with a turbidity of 2 McFarland was prepared and 20 μL of this suspension was transferred to an ampule of ATB FUNGUS 3 Medium. After this, 135 μL of the inoculated medium was transferred into each cupule.

After incubation at 35°C for 24 h, the strips were read both visually and automatically on the ATB Expression Bacteriology Analyzer automatic system (bioMérieux, La Balme-les Grottes, France). According to the manufacturer's instructions, the MICs were determined by the growth score for each of the cupules compared with the control cupules (Figure 1). For amphotericin B, the MIC corresponds to the lowest concentration enabling complete growth inhibition (score=“0”). For 5-flucytosine, fluconazole, itraconazole and voriconazole, the MIC corresponds to the lowest concentration of the antifungal agent with which a score of “2”, “1”, “0” is obtained.
4. Quality control

Quality control was ensured by testing the CLSI-recommended quality control strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6528 for both CLSI BMD and ATB FUNGUS 3 [12].

5. Data analysis

The results obtained with the ATB FUNGUS 3 (both visual and automated readings) were compared with those of CLSI BMD after 24 h incubation. The results were considered to be in essential agreement (EA) when the ATB FUNGUS...
3 results were within ±2 dilutions of the BMD reference value (eg. visual reading=1 μg/mL, and automated reading=4 μg/mL, defined as in EA; whilst visual reading=1 μg/mL, automated reading=8 μg/mL, defined as not in EA). The rates of EA (%) were the percentage of the isolates in EA with BMD. The results were considered to be in categorical agreement (CA) when the ATB FUNGUS 3 and CLSI BMD results fell within the same interpretive category (i.e., susceptible, susceptible dose dependent [SDD], intermediate, or resistant, depending on the drugs tested). If established CLSI M27-S4 interpretive breakpoints were available, they were used to determine CA. Epidemiological cutoff values (ECVs) were used for species/drugs without CLSI breakpoints (the susceptibility results are defined as either wide type [WT] or non-WT by ECVs) [13, 14]. Very major errors were identified when BMD indicated a resistant/non-WT result and the ATB FUNGUS 3 indicated a susceptible/WT one. Major errors were identified when BMD showed a susceptible/WT result while the ATB FUNGUS 3 showed the opposite. Minor errors were identified when one method recorded a susceptible or resistant result whilst the other recorded a SDD result.

6. Proposing a Candida species susceptibility testing algorithm
We developed an algorithm based on combination of ATB FUNGUS 3 automated and visual readings, which could be used when the isolates are identified to species level (Figure 2). Using this algorithm, the results are initially read by ATB automated reading system, and the isolates were put into categories [13, 14]. The isolates with non-resistant/WT results for all drugs can be reported as the final results, but those which show resistant results for any drug should be checked by visual reading. If there is still difficulty in the visual reading or the resistance type is atypical, then the susceptibility result should be confirmed by BMD assay.

7. Review of previously published antifungal susceptibility data
We reviewed a series of previous reports on ATB FUNGUS 3 by searching in China “Wanfang Med Online” (http://eng.med.wanfangdata.com.cn/) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed) up to August 2014. The susceptibilities of C. albicans and C. tropicalis to fluconazole using ATB FUNGUS 3 (ATB Expression Bacteriology Analyzer automated read) in single centers, as well as ATB FUNGUS 3 (visual read) and the antifungal susceptibility data from surveillance programs were summarized in Table 1 for references [4, 6–11, 15].

Results
1. Data reviewed
As shown in Table 1, using the ATB automated reading system, the reported level of resistance to fluconazole reached as high as 42.3% for C. albicans and 52.6% for C. tropicalis, which is in sharp contrast to the results from visual readings (highest: 6.6% for C. albicans and 10.2% for C. tropicalis). Furthermore, published
surveillance data for 12 institutions in China (CHIF-NET) showed fluconazole resistance rates of 0.4% (*C. albicans*) and 5.7% (*C. tropicalis*). The ARTEMIS global antifungal surveillance program also showed fluconazole resistance rates of only 1.4% for *C. albicans* and 4.1% for *C. tropicalis* (*Table 1*).

2. Essential agreement (EA) between ATB FUNGUS 3 and CLSI BMD

The overall EA between ATB visual readings and BMD was 99.1%. The highest concordance between the two methods for all the *Candida* species tested was observed for amphotericin B (100%) and 5-flucytosine (100%) (*Table 2*). Of all the *Candida* species tested, *C. glabrata* showed the lowest concordance between the two methods (*Table 2* and *3*). The overall EA between ATB automated readings and BMD was only 86.2%, which was significantly lower (P<0.05) than those of visual readings (99.1%). The highest agreement was observed for
amphotericin B (99.5%) and 5-flucytosine (99.5%), but much lower EA for fluconazole (80.7%), voriconazole (77.5%), and itraconazole (73.4%). Most isolates with discrepant results had MICs of more than 2 dilutions higher than that of BMD for azole drugs.

C. tropicalis isolates showed the lowest EA, with only 31.7% for fluconazole, 24.4% for voriconazole and 22.0% for itraconazole. In contrast, C. parapsilosis and C. krusei showed very high EA levels with BMD (Tables 2 and 3).

3. Categorical agreement (CA) between ATB FUNGUS 3 and CLSI BMD

The overall CA for the comparisons of the ATB visual reading results with the BMD results was 97.5%, and 95.4% and 95.0% for fluconazole and voriconazole, respectively (Table 2). In contrast, the overall CA between the ATB automated readings and the BMD results was only 84.0%, and 78.4% and 72.9% for fluconazole and voriconazole, respectively. The highest major error rate was observed for susceptibility of C. tropicalis to either fluconazole (23 of 41 isolates studied, 56.1%) or voriconazole (24 of 41, 58.5%), followed by C. albicans (8 of 81 [9.9%] isolates for fluconazole and 12 of 81 [14.8%] to voriconazole (Table 2).

4. Validation of the proposed algorithm

Based on this algorithm, all C. tropicalis isolates can be directly read visually because of poor accuracy by the ATB automated reading system. However, the local prevalence of C. tropicalis should be considered when choosing this proposal. When we applied the proposed algorithm to the 218 isolates in the present study

Table 1. Reported susceptibilities of C. albicans and C. tropicalis to fluconazole using ATB visual and automated readings in single centers of China and susceptibilities by disc diffusion in global and national antifungal surveillance programs.

| Method             | Study site                      | Susceptibility to fluconazole (% resistant) | Reference   |
|--------------------|---------------------------------|--------------------------------------------|-------------|
|                    |                                 | C. albicans | C. tropicalis |
| **Single center**  |                                 |              |                |
| ATB automated      | a 3B-grade hospital in Shandong, China | 60/270 (22.2) | 15/60 (25.0)   | Yu et al. 2007 (6) |
| ATB automated      | a 2A-grade hospital in Xinjiang, China | 19/143 (13.3) | 10/19 (52.6)   | Mi et al. 2008 (7) |
| ATB automated      | a 3A-grade hospital in Liaoning, China | 50/118 (42.3) | 7/25 (26.9)    | Wang et al. 2012 (8) |
| ATB visual         | a 3A-grade hospital in Hainan, China | 44/825 (5.3) | 20/206 (9.7)   | Huang et al. 2012 (9) |
| ATB visual         | a 3A-grade hospital in Beijing, China | 9/136 (6.6) | 6/59 (10.2)    | Li et al. 2013 (10) |
| ATB visual         | an institution in Taiwan         | 13/388 (3.3) | 10/171 (5.8)   | Chen et al. 2009 (15) |
| **Surveillance program** |                                 |              |                |
| Disc diffusion     | 133 institutions in the world (ARTEMIS) | 1800/128625 (1.4) | 637/15546 (4.1) | Pfaller et al. 2010 (4) |
| Disc diffusion     | 24 institutions in Asia-Pacific region (ARTEMIS) | 259/28781 (0.9) | 336/5178 (6.5) | Pfaller et al. 2010 (4) |
| Disc diffusion     | 12 institutions in China (CHIF-NET) | 1/282 (0.4) | 7/123 (5.7)    | Wang et al. 2012 (11) |

*aFive institutions from China were included. 
All 12 institutions were tertiary hospitals.

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Table 2. Essential agreement (EA) and categorical agreement (CA) between ATB FUNGUS 3 (visual and automated readings) and CLSI BMD.

| Species (no. of isolates tested) | Antifungal agent | Test method | EA no. (%) | CA no. (%) | Errors |
|----------------------------------|-----------------|-------------|------------|------------|--------|
|                                  |                 |             | Visual     | Automated  |        |
|                                  |                 |             | Percentage | Percentage | Errors |
|                                  |                 |             | Very major error | Major error | Minor error |
| C. albicans (81)                 | fluconazole<sup>a</sup> | ATB visual | 81 (100) | 79 (97.5) | 0 (0) | 0 (0) | 2 (2.5) |
|                                  |                 | ATB automated | 71 (87.7) | 61 (80.2) | 0 (0) | 8 (9.9) | 0 (0) |
|                                  | voriconazole<sup>a</sup> | ATB visual | 81 (100) | 81 (100) | 0 (0) | 0 (0) | 0 (0) |
|                                  |                 | ATB automated | 67 (82.7) | 65 (80.5) | 0 (0) | 12 (14.8) | 1 (1.2) |
|                                  | itraconazole | ATB visual | 81 (100) | 80 (98.8) | 1 (1.2) | 0 (0) | 0 (0) |
|                                  |                 | ATB automated | 59 (72.6) | 58 (71.3) | 0 (0) | 19 (23.5) | - |
|                                  | amphotericin B  | ATB visual | 81 (100) | 81 (100) | 0 (0) | 0 (0) | 0 (0) |
|                                  |                 | ATB automated | 81 (100) | 81 (100) | 0 (0) | 0 (0) | 0 (0) |
| C. glabrata (42)                 | fluconazole<sup>a</sup> | ATB visual | 38 (90.5) | 37 (88.1) | - | - | 5 (11.9) |
|                                  |                 | ATB automated | 38 (90.5) | 36 (85.7) | - | - | 6 (14.3) |
|                                  | voriconazole | ATB visual | 40 (95.2) | 39 (92.9) | 0 (0) | 3 (7.1) | - |
|                                  |                 | ATB automated | 38 (90.5) | 30 (71.4) | 0 (0) | 12 (28.6) | - |
|                                  | itraconazole | ATB visual | 38 (90.5) | 37 (88.1) | 2 (4.8) | 3 (7.1) | - |
|                                  |                 | ATB automated | 38 (90.5) | 27 (64.3) | 1 (2.4) | 14 (33.3) | - |
|                                  | amphotericin B  | ATB visual | 42 (100) | 42 (100) | 0 (0) | 0 (0) | - |
|                                  |                 | ATB automated | 41 (97.6) | 41 (97.6) | 0 (0) | 1 (2.4) | - |
|                                  | 5-flucytosine  | ATB visual | 42 (100) | 42 (100) | 0 (0) | 0 (0) | - |
|                                  |                 | ATB automated | 42 (100) | 42 (100) | 0 (0) | 0 (0) | - |
| C. tropicalis (41)               | fluconazole<sup>a</sup> | ATB visual | 41 (100) | 40 (97.6) | 0 (0) | 0 (0) | 1 (2.4) |
|                                  |                 | ATB automated | 13 (31.7) | 17 (41.5) | 0 (0) | 23 (56.1) | 1 (2.4) |
|                                  | voriconazole<sup>a</sup> | ATB visual | 41 (100) | 38 (92.7) | 0 (0) | 0 (0) | 3 (7.3) |
|                                  |                 | ATB automated | 10 (24.4) | 14 (34.2) | 0 (0) | 24 (58.5) | 3 (7.3) |
|                                  | itraconazole | ATB visual | 41 (100) | 41 (100) | 0 (0) | 0 (0) | - |
|                                  |                 | ATB automated | 9 (22.0) | 9 (22.0) | 0 (0) | 32 (78.0) | - |
|                                  | amphotericin B  | ATB visual | 41 (100) | 41 (100) | 0 (0) | 0 (0) | - |
|                                  |                 | ATB automated | 41 (100) | 41 (100) | 0 (0) | 0 (0) | - |
|                                  | 5-flucytosine  | ATB visual | 41 (100) | 41 (100) | 0 (0) | 0 (0) | - |
|                                  |                 | ATB automated | 41 (100) | 41 (100) | 0 (0) | 0 (0) | - |
| C. parapsilosis (38)             | fluconazole<sup>a</sup> | ATB visual | 38 (100) | 37 (94.7) | 0 (0) | 0 (0) | 1 (5.3) |
|                                  |                 | ATB automated | 38 (100) | 30 (79.0) | 0 (0) | 1 (2.6) | 7 (18.4) |
|                                  | voriconazole<sup>a</sup> | ATB visual | 38 (100) | 38 (100) | 0 (0) | 0 (0) | 0 (0) |
|                                  |                 | ATB automated | 38 (100) | 36 (94.7) | 0 (0) | 0 (0) | 2 (5.3) |
|                                  | itraconazole | ATB visual | 38 (100) | 38 (100) | 0 (0) | 0 (0) | - |
|                                  |                 | ATB automated | 38 (100) | 38 (100) | 0 (0) | 0 (0) | - |
|                                  | amphotericin B  | ATB visual | 38 (100) | 38 (100) | 0 (0) | 0 (0) | - |
|                                  |                 | ATB automated | 38 (100) | 38 (100) | 0 (0) | 0 (0) | - |
|                                  | 5-flucytosine  | ATB visual | 38 (100) | 38 (100) | 0 (0) | 0 (0) | - |
|                                  |                 | ATB automated | 38 (100) | 38 (100) | 0 (0) | 0 (0) | - |
| C. krusei (16)                   | fluconazole     | ATB visual | 16 (100) | 15 (93.7) | 1 (6.3) | 0 (0) | - |

Problematic Automatic Readings of ATB FUNGUS 3
as shown in Table 4, the algorithm offered a very good balance between accuracy and automation. As can be seen, the proposed testing algorithm is practical, labor and time saving for Candida susceptibility testing in large surveillance programs (Table 4).

Discussion

ATB FUNGUS 3 is the most commonly used commercialized antifungal susceptibility test method in China, and is also widely used in parts of Africa, South America and Europe [16–19]. A former version (ATB FUNGUS 2) of this current system has been evaluated by Torres-Rodriguez et al. [17]. In agreement to our findings, lower agreement with BMD was observed for fluconazole and itraconazole, particularly with C. tropicalis and C. albicans. [17].

Table 3. The number of strains for ATB visual and automated readings which were not in essential agreement (EA) with CLSI BMD.

| Species         | ATB visual readings a | ATB automated readings a | P value |
|-----------------|-----------------------|--------------------------|---------|
|                 | One drug | Two drugs | Three drugs | Agreement (%) b | One drug | Two drugs | Three drugs | Agreement b | visual vs. automated |
| C. albicans (81)| 0        | 0         | 0           | 81 (100)     | 7        | 6         | 9           | 59 (72.8)    | P<0.5               |
| C. glabrata (42)| 1        | 3         | 1           | 37 (88)      | 9        | 2         | 0           | 31 (85.6)    | P>0.5              |
| C. tropicalis (41)| 0    | 0         | 0           | 41 (100)     | 3        | 2         | 28          | 8 (19.5)     | P<0.5              |
| C. parapsilosis (38)| 0    | 0         | 0           | 38 (100)     | 0        | 0         | 0           | 38 (100)     | P>0.5              |
| C. krusei (16)   | 0        | 0         | 0           | 16 (100)     | 1        | 0         | 0           | 15 (93.8)    | P<0.5              |

aThe strains with susceptibility errors were divided into errors in one drug, two drugs and three drugs.

bThe number of strains of which all the five drugs were in EA with CLSI BMD.

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In the present evaluation, ATB visual readings showed good concordance with BMD, whilst a high pseudo resistant rate (defined as major errors) to azole drugs was observed for ATB automated readings. The potential explanation for the high major errors with the ATB automated readings is the trailing effect of azoles. We found that *C. tropicalis* and *C. albicans* showed obvious trailing growth in testing with azole drugs. The activation of calcineurin and altered regulation of genes mediating resistance could partially explain the trailing phenomenon [20, 21].

The major errors in azole drug susceptibility testing by ATB automated readings are a cause for concern for several reasons. Firstly, the very high pseudo-resistance may result in misleading clinical antifungal drug selection and increased costs for antifungal drugs. Secondly, national and international surveillance studies and epidemiological comparison studies may potentially get misleading results and conclusions if they simply used the ATB automated reading data - generally will over-estimate the China azoles antifungal resistance rates. Thirdly, the unwarranted use of alternative antifungal agents may promote further resistance in these agents, making future drug selection difficult [22]. Therefore, the ATB visual reading is generally recommended.

The present study highlights the flaws encountered in commercial automated identification and susceptibility testing systems especially in developing countries. Very recently, similar problems were reported by Chowdhary *et al.* in Asia, where VITEK 2 (bioMérieux, Marcy l’Etoile, France) misidentified 10 *Candida auris* isolates as *Candida haemulonii* and 2 as *Candida famata* [23, 24]. The problems highlighted the need for an improvement and validation of the automated systems as well as alerting laboratory staff of the potential errors. Before the improvement of the automated reading system, we developed an algorithm to make good use of the ATB reading system, which is especially practical for surveillance programs.

Generally, the ATB FUNGUS 3 (visual reading) is a simple and accurate method for the determination of MICs for *Candida* spp., which is suitable for clinical microbiology use in developing countries like in Asia, Africa and South America. The present study served as an important experience in ATB FUNGUS 3 practical use for other countries where it is routinely used.

### Table 4. Comparison of four strategies for susceptibility testing of 218 isolates of *Candida* using ATB FUNGUS 3.

| Strategy                                   | No. isolates read automatically | No. isolates read visually | Final agreement<sup>a</sup> (%) |
|--------------------------------------------|---------------------------------|---------------------------|---------------------------------|
| All read automatically                     | 218                             | 0                         | 151/218 (69.3)                  |
| All read visually                          | 0                               | 218                       | 216/218 (99.0)                  |
| Proposed algorithm                         | 218                             | 88 “resistant/non-WT”     | 213/218 (97.7)                  |
| Proposed algorithm (C. tropicalis read visually directly) | 177                             | 93 (41 C. tropicalis+52 “resistant/non-WT”) | 213/218 (97.7) |

<sup>a</sup>The number of re-checked strains in proposed algorithm is a little high because all the resistant isolates of the five *Candida* species in CHIF-NET 10 were included in the present study.

<sup>b</sup>The number of the strains of which the susceptibilities to the five drugs were all in EA with BMD.

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Problematic Automatic Readings of ATB FUNGUS 3

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The major errors in azole drug susceptibility testing by ATB automated readings are a cause for concern for several reasons. Firstly, the very high pseudo-resistance may result in misleading clinical antifungal drug selection and increased costs for antifungal drugs. Secondly, national and international surveillance studies and epidemiological comparison studies may potentially get misleading results and conclusions if they simply used the ATB automated reading data - generally will over-estimate the China azoles antifungal resistance rates. Thirdly, the unwarranted use of alternative antifungal agents may promote further resistance in these agents, making future drug selection difficult [22]. Therefore, the ATB visual reading is generally recommended.

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Author Contributions
Conceived and designed the experiments: HW LZ MX YCX. Performed the experiments: HW LZ LLM HRZ MX. Analyzed the data: LZ MX HW. Wrote the paper: LZ HW MX TK FK.

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