No increase in frequency of antifungal resistance among yeasts isolated from normally sterile sites in patients at Foothills Hospital from 1993 to 1996

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OBJECTIVE: To determine the prevalence of resistance to antifungal drugs among yeasts isolated from sterile sites of patients in one hospital and the relationship of resistance to antifungal use, and to assess whether resistance was increasing.

METHOD: Susceptibility testing performed by National Committee for Clinical Laboratory Standards (NCCLS) (Villanova, Pennsylvania) microdilution method and by E test. Antifungal use was determined by selected chart review and from pharmacy data.

SPECIMENS AND SETTING: Tertiary care adult hospital with neonatal intensive care.

POPLULATION STUDIED: Distinct yeast isolates from sterile site specimens collected during the years 1993 to 1996.

RESULTS: A total of 132 yeast isolates were studied, of which 78 (59%) were Candida albicans. The proportion of Candida albicans remained steady over the four-year period, and there was no trend to increased resistance among Candida albicans. The number of isolates of all species with fluconazole microdilution minimum inhibitory concentration (MIC) greater than 8 mg/L in each of the four years were one of 32 in 1996, three of 26 in 1994, six of 33 in 1995, and one of 41 in 1996. A single isolate had an itraconazole microdilution MIC greater than 0.5 mg/L in each year. Prior use of antifungal therapy was rare in this patient population.

CONCLUSIONS: The increase in resistance to antifungal drugs reported by some centres did not occur in this institution over the course of the study. This experience may reflect differences in infection control practices and in patterns of use of antifungal agents. The NCCLS method was found to be superior to the E test as a routine method for testing susceptibility of yeasts.

Key Words: Antifungal drugs, Candida, Fluconazole, Resistance
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Aucune augmentation de la fréquence de la résistance aux antifongiques parmi des levures isolées de sites habituellement stériles chez des patients du Foothills Hospital de 1993 à 1996

OBJECTIF: Déterminer la prévalence de la résistance aux médicaments antifongiques parmi des levures isolées de sites habituellement stériles chez des patients d’un hôpital et la relation entre cette prévalence et l’utilisation d’antifongiques, et évaluer si la résistance augmente.

MÉTHODE: Tests de sensibilité par méthode de microdilution du National Committee for Clinical Laboratory Standards (NCCLS) (Villanova, Pennsylvania), et par test E. L’utilisation des antifongiques a été déterminée par une revue sélective des dossiers médicaux et l’examen des données de la pharmacie.

SPÉCIMENS ET CONTEXTE: Hôpital de soins tertiaires pour adultes fournissant aussi des soins intensifs néonatals.

POPULATION ÉTUĐIÉE: Des isolats distincts de levures de spécimens de sites stériles prélevés pendant les années 1993 à 1996.

RÉSULTATS: Cent trente-deux isolats de levures ont été étudiés dont 78 (59 %) étaient Candida albicans. La proportion de C. albicans est restée stable pendant les 4 années, et aucune tendance vers une évolution de la résistance chez C. albicans n’a été notée. Le nombre d’isolats de toutes les espèces avec une concentration minimale inhibitrice (CMI) d’une microdilution de fluconazole supérieure à 8 mg/L au cours de chacune des quatre années était de 1/32 en 1993 ; 3/26 en 1994, 6/33 en 1995 et 1/41 en 1996. Un seul isolat avait une CMI d’une microdilution d’itraconazole supérieure à 0,5 mg/L dans chacune des années. L’administration antérieure d’un traitement antifongique était rare dans cette population de patients.

CONCLUSIONS: L’augmentation de la résistance aux agents antifongiques signalée par certains centres ne s’est pas produite au Foothills Hospital pendant la durée de l’étude. Cette expérience peut refléter des différences dans les mesures de contrôle des infections et dans les schémas d’utilisation des agents antifongiques. La méthode du NCCLS n’est révélée supérieure au test E comme méthode de routine pour tester la sensibilité des levures.

Standardized in vitro methods for determining the susceptibility or resistance of yeasts to antifungal drugs have been developed by the National Committee for Clinical Laboratory Standards (NCCLS), Villanova, Pennsylvania (1,2). The NCCLS method provides a standard tool for comparing yeast susceptibility patterns among different institutions and over time. Increasing fluconazole resistance among yeasts from blood cultures has been reported (3) and may compromise the appropriateness of recent guidelines for management of yeast infections (4). We, therefore, set out to determine the susceptibility of yeasts isolated from sterile sites of patients over a four-year period in our hospital, Foothills Hospital, Calgary, Alberta, a major Canadian academic tertiary care institution.

We attempted to relate in vitro resistance to prior antifungal therapy. In addition, we compared the ease of performance and results obtained by E test (AB Biodisc, Solna, Sweden) with the NCCLS method.

SPECIMENS AND METHODS

All yeast isolates were obtained from specimens submitted to the hospital microbiology laboratory for routine diagnostic testing. Blood cultures were performed using the BacT/Alert system (Organon Technika Inc). Other cultures were performed by standard methods. All yeast isolates obtained from blood cultures and other normally sterile clinical specimens types were identified by germ-tube production combined with the Vitek Yeast Biochemical Card (bio-Merieux, Missouri), with further morphological confirmation for non-Candida species. Yeast nomenclature has been changed to the current standard (5) except the name Candida paratropicalis has been retained because this name seemed to identify a cluster of relatively resistant isolates. Where more than one isolate of the same species was obtained from an individual patient within a seven-day period, only the first isolate was selected for susceptibility testing; however, in three instances where both blood and cerebrospinal fluid (CSF) isolates were obtained, both were included.

Both microdilution susceptibility tests were performed by M27-T (1) using antibiotic medium No 3 (Difco) for amphotericin B and Roswell Park Memorial Institute (RPMI) 1640 (Gibco BRL) for the other drugs. Minimum inhibitory concentrations (MICs) were read visually by two technologists after 48 h incubation, following the method in M27-T that defines the MIC for the microdilution assay as the lowest concentration showing a “prominent decrease in turbidity” for fluconazole and azole drugs, and the lowest concentration producing an optically clear well for amphotericin. Plates were also read spectrophotometrically at 492 nm on a Dynatech plate reader (Virginia). In the spectrophotometric method, the plates were shaken to resuspend the yeast cells, and the lowest concentration well with an optical density of less than 50% of the control well was considered to be the MIC (6). Where the visual and spectrophotometric MICs differed, the modal or middle reading was selected for analysis of trends, as described in results.

E test MICs were performed using RPMI agar supplemented with 2% glucose and buffered at pH 7.0 with 0.165 M 3[N-morpholino]propanesulfonic acid. MICs were read, according to the manufacturer’s instructions, at 100% inhibition of growth for amphotericin B and fluconazole, and at an estimated 80% inhibition of growth for itraconazole, ketoconazole and azole drugs. All E test results were read by two technologists after 48 h incubation.

RESULTS

The number of yeast species tested from each year is shown in Table 1. There were 66 isolates from blood cultures, 25 from peritoneal fluids, seven from CSF, and 34 from other tissues and fluids. Nineteen of the isolates came from neonates. The
proportion of infections caused by *Candida albicans* did not change over the study.

**Reproducibility of microdilution fluconazole MIC results:**

The two visual and one spectrophotometric reading of MIC agreed within two doubling dilutions for 127 (96%) of the 132 strains (Table 2). Where there was a discrepancy of more than two dilutions, there was always agreement within one dilution for two of the readings. The MIC result for each isolate was therefore chosen as the result where at least two readings agreed or the middle one where there were three different results. Discrepancies were most commonly seen with those strains where there was a very gradual reduction in growth with increasing drug concentration, as was particularly common with *Candida glabrata*.

**Reproducibility of E test fluconazole MIC results and comparison between E test and microdilution MIC results:**

The E test MIC results for fluconazole and the other imidazoles were felt to be difficult to read, but when results from duplicate readings of the same test were compared, there was agreement within one doubling dilution for 127 (96%) of the 132 strains (Table 2). There was, however, a discrepancy of more than three doubling dilutions between E test and microdilution MICs for 31 strains. The E test tended to give higher MICs for most isolates. Six strains for which the E test gave a result five or six dilutions lower than microdilution were all *Candida tropicalis* or *C paratropicalis*, confirming the findings of Colombo et al (7).

**Trends in fluconazole resistance over time and by species:**

There was no trend to increased fluconazole resistance over the four-year period (Table 3); in fact, the last year studied had the highest proportion of strains that could be categorized as fully susceptible to fluconazole on the basis of a MIC 8 mg/L or less (2). The high frequency of fluconazole resistance (MIC 64 mg/L or greater) in 1995 was accounted for by a cluster of five fluconazole-resistant *C paratropicalis* and one fluconazole-resistant *C tropicalis* in that year. *C albicans* isolates were consistently susceptible to fluconazole, though two strains with MIC of 4 mg/L were noted (Table 4).

**Itraconazole and ketoconazole:**

Reproducibility of itraconazole and ketoconazole MICs on triplicate readings of microdilution plates or duplicate readings of E test plates were similar to the reproducibility obtained for fluconazole MICs. There were again major discrepancies between the microdilution MICs and the E test MICs for itraconazole and ketoconazole (not shown). There were no increases in itraconazole or ketoconazole MICs over the four years.

**Correlation between itraconazole, ketoconazole and fluconazole MICs:**

Several patterns of relationship between the MICs of these drugs were observed, of which two were the most

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### Table 1

**Numbers of yeast species isolated from normally sterile sites from Foothills Hospital patients, 1993 to 1996**

| Species                  | 1993 | 1994 | 1995 | 1996 | Total |
|--------------------------|------|------|------|------|-------|
| *Candida albicans*       | 20 (62) | 15 (58) | 16 (48) | 27 (66) | 78    |
| *Candida glabrata*       | 2    | 8    | 4    | 4    | 18    |
| *Candida kefyr*          | 1    | 1    | 1    | 2    |
| *Candida krusei*         | 2    | 2    |      |      |
| *Candida lusitaniae*     | 5    | 5    | 5    |      |
| *Candida parapsilosis*   | 1    | 1    | 1    | 2    |
| *Candida paratropicalis* | 1    | 1    | 2    | 8    |
| *Candida tropicalis*     | 11   | 12   | 2    |      |
| *Saccharomyces cerevisiae* | 1  | 1    | 2    | 4    |
| All species              | 32   | 26   | 33   | 41   | 132   |

*Count excludes duplicate isolates from individual patients within seven days, as described in Specimens and Methods section*

### Table 2

**Comparison between fluconazole minimum inhibitory concentration determined by first reader and second reader and by spectrophotometric interpretation**

| Difference to spectrophotometric result | −3 | −2 | −1 | 0 | +1 | +2 | +3 | +4 | +8 |
|----------------------------------------|----|----|----|---|----|----|----|----|----|
| Difference to first reader result      |    |    |    |   |    |    |    |    |    |
| −3                                     | 1  |    |    |   |    |    |    |    |    |
| −2                                     |    | 1  | 2  | 1 |    |    |    |    |    |
| −1                                     |    |    | 8  | 32|    |    |    |    |    |
| 0                                      |    |    |    | 1 | 4  | 59 | 5  | 2  |    |
| +1                                     |    |    |    |   | 2  | 4  | 2  |    |    |
| +2                                     |    |    |    |   | 1  | 3  |    |    |    |
| +3                                     |    |    |    |   | 1  |    |    |    |    |
| +4                                     |    |    |    |   |    |    | 1  |    |    |
TABLE 3
Distribution of fluconazole minimum inhibitory concentrations (MICs) by year for all yeast species tested

| Year | 0.125 or less | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 or greater | Total |
|------|---------------|------|-----|---|---|---|---|----|----|--------------|-------|
| 1993 | 7             | 13   | 8   | 2 | 1 |    |    |     |    |              | 32    |
| 1994 | 6             | 7    | 2   | 1 | 6 | 1  |    | 1   | 2  |              | 26    |
| 1995 | 3             | 15   | 2   | 2 | 1 | 3  | 1  |     |    |              | 33    |
| 1996 | 6             | 22   | 6   | 1 | 3 | 2  | 1  |     |    |              | 41    |
| All years | 22 | 57   | 18  | 5 | 3 | 12 | 4  | 1  | 1 |              | 132   |

TABLE 4
Distribution of fluconazole minimum inhibitory concentrations (MICs) by species for all years tested

| Species               | 0.125 or less | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 or greater | Total |
|-----------------------|---------------|------|-----|---|---|---|---|----|----|--------------|-------|
| Candida albicans       | 21            | 51   | 3   | 1 | 2 |    |    |     |    |              | 78    |
| Candida glabrata       | 1             |      |     |   |   | 2  | 9  | 4  |    |              | 18    |
| Candida kefyr          | 1             |      |     |   |   | 1  |    |    |    |              | 2     |
| Candida krusei         |               |      |     |   |   |    |    |    |    |              | 2     |
| Candida lusitaniae     | 2             |      |     |   |   |    |    |    |    |              | 2     |
| Candida parapsilosis   | 1             | 8    | 3   |   |   |    |    |    |    |              | 12    |
| Candida paratropicalis | 1             |      |     |   |   | 6  | 7  |    |    |              | 7      |
| Candida tropicalis     | 1             | 3    | 3   |   |   |    |    |    |    |              | 8     |
| Saccharomyces cerevisiae|            |      |     |   |   | 2  |    | 1  | 1 |              | 4     |
| Total                 | 22            | 57   | 18  | 5 | 3 | 12 | 4  | 1  | 1 |              | 132   |

TABLE 5
Use of antifungal drugs at Foothills Hospital, Calgary, Alberta, 1992 to 1996

| Drug, route and dose | 1992-93 | 1993-94 | 1994-95 | 1995-96 |
|---------------------|---------|---------|---------|---------|
| Amphoterin 50 mg     | 933     | 912     | 761     | 746     |
| Fluconazole          | 160     | 138     | 320     | 336     |
| Oral fluconazole 100 mg | 2131   | 2366    | 4793    | 4229    |
| Oral ketoconazole 200 mg | 588    | 252     | 149     | 208     |
| Oral itraconazole 200 mg | 165   | 208     |         |         |

common. Six strains of *C. tropicalis* or *C. paratropicalis* had fluconazole MICs of 64 mg/L but were apparently sensitive to itraconazole and ketoconazole with MICs of 0.03 to 0.06 mg/L. Thirteen strains of *Candida glabrata* had fluconazole MICs of less than 0.5 mg/L, and seven of these had ketoconazole MICs of 0.5 mg/L or greater.

Flucytosine: The flucytosine microdilution MICs gave very consistent results. E test replicate readings were less consistent (not shown). There were nine isolates with flucytosine MICs of 32 mg/L or greater, all of which were fully susceptible to fluconazole. Seven of the nine flucytosine-resistant strains were *C. albicans*.

Amphotericin: The modified microdilution assay for amphotericin B, using antibiotic medium number 3, gave much sharper end-points than did the RPMI 1640 broth assay for azole drugs, and was, therefore, highly reproducible between observers. The E test was also highly reproducible between observers, but tended to give MICs one or two dilutions higher than the microdilution method. There was no trend to increased resistance over the four years. All strains had MICs of 0.25 mg/L or less by the NCCLS method, except for six strains of *Candida parapsilosis* that had MICs of 0.5 to 1.0 mg/L. The highest MIC detected by E test was 2.0 mg/L for two strains, which had MICs of 0.25 and 1.0 mg/L by the NCCLS method.

**Use of antifungal agents in Foothills Hospital:** Considerable amounts of antifungal agents are used in Foothills Hospital (Table 5). There has been increased use of fluconazole, but a fall in use of amphotericin and ketoconazole over the four years from 1992 to 1996. There is no defined treatment of choice for candidemia in Foothills Hospital, but the amounts of drug used appear to reflect a change in the consensus treatment of choice from amphotericin to fluconazole. Prophylactic fluconazole is not generally used in the hospital, not even in bone marrow transplant recipients, because the rate of yeast infection has been very low without prophylaxis. However, patients who are enrolled in multicentre treatment trials may receive fluconazole prophylaxis. Charts were reviewed for all patients where fluconazole MIC was 8/g/mL or greater, and none had documented prior antifungal therapy.

**DISCUSSION**

There are still many questions to be answered about the methodology, interpretation and utility of susceptibility testing for yeasts, despite the major effort involved in the development of NCCLS guidelines (2,8). One methodological problem is the gradual reduction in growth with increasing drug concentrations.
Antifungal resistance among yeasts isolated from normally sterile sites

centration frequently seen with the azoles, which makes it difficult to determine an exact MIC. This problem has been addressed by defining the MIC in the broth macrodilution method as the lowest concentration of drug showing an 80% reduction in growth, and in the microdilution method as the lowest concentration of drug giving a “prominent decrease in turbidity” (1). To make the microdilution method more objective, one of the authors of M27-T has suggested using a microplate reader, and has shown that an end-point of 50% reduction in optical density correlates well with M27-T results for control strains (6). Because of the subjective nature of the M27-T microdilution method, we chose to read all MICs twice, and also to read them by the more objective but less well tested method suggested by Pfaller et al (6). We found that the results agreed closely most of the time, and chose to use the modal or middle result where there were discrepancies.

There has been no detectable increase in the frequency of nonalbicans Candida species, and no increase in fluconazole MICs among C. albicans or other yeast species, over a four-year period despite the increasing use of the drug. The sample size is small and the time-frame is short, so that this finding has to be interpreted with caution. A longer time-frame in particular may be needed to observe a trend to increasing resistance. However, our results are consistent with other Canadian data that showed no reduction in the proportion of C. albicans and no change in antifungal susceptibility patterns among blood culture isolates over an 11-year period (9).

The rise in fluconazole resistance reported by others in invasive yeast isolates is largely explained by a rise in the proportion of species other than C. albicans. The proportion of C. albicans in our population was 78 of 132 (59%) overall, or 39 of 66 (59%) for blood culture isolates, and remained fairly constant over the four-year period. This is similar to the proportion reported by Karlowsky et al (9) and in the middle of the range observed by Price et al (3) who reported that the frequency of C. albicans among yeasts isolated from blood cultures fell from 87% to 31% between 1987 and 1992.

The rise in the proportion of yeast isolates that are fluconazole resistant has been linked to increasing use of fluconazole (3), and particularly to use of prophylactic fluconazole (10), but has not been observed in all centres (11) and has not been shown in prospective controlled trials (12). It may be, therefore, that the absence of increasing resistance in our population is explained by the virtual absence of prophylactic fluconazole, or it may be due to other factors, such as good infection control practices.

Our findings support the recent Canadian guideline for the management of Candida infections in intensive care patients (4), in which fluconazole is preferred over amphotericin B as empirical treatment for hemodynamically stable patients. A trend to increasing fluconazole resistance would be a concern for the implementation of these guidelines. Our data support the view that identification of a yeast isolate as C. albicans is highly predictive of fluconazole susceptibility, and would be reasonable grounds on which to switch from amphotericin B to fluconazole in patients who were initially treated with amphotericin B because of hemodynamic instability. Identification of an isolate as Candida krusei clearly implies fluconazole resistance. According to our findings, the identification of an isolate by the Vitek system as C. paratropicalis also indicates a high probability of fluconazole resistance, but this association may be due to temporary local prevalence of an unusual clone. Up-to-date local data are required to draw conclusions about the susceptibility of yeast species other than C. krusei.

Useful technical lessons were learned concerning the most appropriate way to test yeast susceptibility. The NCCLS microdilution method was relatively easy to perform. Microdilution plates or strips can be stored frozen for six months so that this method could be used to test occasional clinical isolates on demand. However, it is still fairly slow, requiring subculture of the initial isolate and controls, and 48 h incubation of the plates. Maintaining consistent quality when testing small numbers of strains on demand is also a problem, although the spectrophotometric method provides a degree of objectivity for the final reading of results. The E test, on the other hand, was found unsatisfactory. The results were felt to be difficult to read, and did not correlate well with the NCCLS method in our hands, despite what has been reported by others (13,14). The E test is also less convenient for occasional clinical use because of the need to keep special agar plates available.

Therefore, our conclusion regarding the choice of methodology is that the NCCLS broth microdilution method is preferable to the E test for our diagnostic laboratory. Antifungal susceptibility testing remains slow and labour intensive, and requires a high level of skill. It is, therefore, best adapted to periodic batch testing to guide empirical therapy, rather than rapid testing of individual isolates. Commercially prepared frozen or dried (15) microdilution panels would reduce the burden of preparation and quality control required.

Other institutions should monitor their own susceptibility data, and we should continue to monitor ours to provide relevant data on which to base treatment of yeast infections.

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