7. SUMMARY AND CONCLUSION
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1. "Study of medically important Candida species with special reference to different typing methods and virulence testing in laboratory animals" was undertaken in the "Mycology division " of Department of Microbiology, Dr.V.M. Medical College, Solapur, from January 1996 to 1998.

2. To undertake this study, 643 clinically suspected patients presenting with detectable signs and symptoms of clinical diseases wherein immunocompromisation was thought to be most likely and species of Candida were thought to be "likely pathogens" were selected from patients seeking treatment in clinical wards of "Shri. Ch. Shivaji Maharaj Sarvopchar Rugnalaya", Solapur and in clinical wards of private hospital like "Shri Siddheshwar Sahakari Cancer Hospital", and "Ashwini Sahakari Rugnalaya", Solapur.

3. Relevant clinical specimen from among these patients with clinically categorised six representative syndromes viz pulmonary tuberculosis, chronically hospitalised and catheterised patients, diabetes mellitus, cancer, clinically suspected cases with HIV infection and patients of burns, were collected for appropriate laboratory procedures. (Table 5.1)

4. The frequency distribution of various types of clinical specimen is as- urine 835, oral gargle 354, sputum 301, wound swabs 125, body fluids and aspirates 42 and stool 23. Wherever feasible, relevant clinical specimens were collected on more than one occasions i.e. second and third time, to demonstrate persistent colonisation of Candida for better clinical correlation. (Table 5.3, 5.4)

5. Fifty age and sex match healthy controls, without any apparent clinical signs and symptoms, but with some personal history that can be correlated as relevant predisposing factor promoting Candida colonisation, namely
tobacco-pan chewing, smoking with or without tobacco-pan chewing and ill fiting denture were selected for control study. Specimen like oral gargle and urine were selected as representative specimen for control study. Similar laboratory procedures as for clinically suspected candidiasis cases were carried out. (Table 5.50.1)

For identification of Candida species if present in the clinical specimen, detailed mycology laboratory procedure were under taken.

6. Direct microscopic demonstration of yeast or yeast like organism was attempted in a total of 1680 clinical specimen collected from patients and 100 specimen collected from healthy controls.

Gram strain smear positivity was found in 16.19% specimen. Higher positivity was observed in specimen like stool (26.08%), sputum (24.58%), body fluid (23.80%) and oral gargle (20.05%).(Table 5.5.6)

a. From among patients of PTB, sputum smear positivity was more in cavitatory type (25.86%) than non-cavitatory type (17.14%). Urine specimen collected on two or three occasions from these cases revealed smear positivity in (9.37%) cases. (Table 5.7.A)

b. In the clinical group of chronically hospitalised and catheterised patients, urine deposit smears revealed presence of Candida in 21.83% specimen. (Table 5.7.C)

c. Among the patients of diabetes mellitus oral gargle specimen collected from 15 patients on two or three occasions revealed the presence of Candida in 30 (15.78%) samples.

Urine specimens from 6 cases collected on two or three occasions with duration of illness for 11 years also revealed smear positivity in 7.22% specimen. (Table 5.5.D)

d. From 104 patients of cancer examined, 41 (25%) oral gargle specimen revealed presence of Candida in direct smear. Whereas from 101 urine specimen examined, Candida was demonstrated in 18(17.81%) samples. (Table 5.7.E)
e. Among clinically suspected HIV infected cases, maximum smear positivity was found in body fluid specimen (29.62%) followed by oral gargle (18.51%), stool (26.08%) and urine deposit (8.64%). Out of 83 cases, 38 (16.74%) cases revealed smear positivity. (Table 5.7.G)

f. Among burn patients higher smear positivity (6.84%) was found in urine deposit than smear prepared from wound swabs (4%). (Table 5.7.1)

7. When the samples were processed for cultivation of Candida in recommended suitable culture media, out of 1701 clinical specimen, 681 (40.03%) specimen revealed Candida isolation. Isolation of Candida was higher in the sputum sample (53.48%) followed by body fluids (50%), stool sample (39.13%), oral gargle (30.79%) and urine (25.98%). (Table 5.8)

8. Isolation of Candida was highest in patients of pulmonary tuberculosis (56.79%), followed by patients of cancer (53.14%), suspected HIV infection (44.50%), uncontrolled diabetes (30.27%), catheterized patients (22.53%) and burns patients (20.29%) in that sequential order. (Table 5.9)

9. On the basis of germ tube positivity, 419 (61.52%) Candida isolates were presumptively identified as *Candida albicans* and 262 (38.47%) as Candida species other than *C. albicans*. (Table 5.10)

10. The distribution of isolated Candida species was studied according to the clinical syndromes.

a. The isolation of Candida was high among PTB cases (56.79%), compromising of 107 *C. albicans* and 123 non-albicans Candida species. Culture positivity was distinctly more in chronic cavitatory type of cases (36.15%) than fresh cavitatory type (23.72%). In these cases sputum and urine collected on two consecutive occasions revealed Candida culture positivity. (Table 5.10.A)

Among the non-cavitatory PTB cases, the culture positivity was marginally more in chronic cases (20.90%) than in fresh cases (19.20%), and *C. albicans* strains were more frequently isolated than non-albicans Candida.

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b. In chronically hospitalised and catheterised patients, isolation of Candida was (22.53%), comprising of 32 Candida isolates from urine samples. The isolation of *C. albicans* (28 strains, 87.5%) was the dominant feature as against four strains of non-albicans Candida species. The isolation of Candida was more frequent when catheterisation lasted for two weeks or more. (Table 5.10.B,5.17)

c. Among diabetes mellitus patients, isolation of Candida was (30.27%), where *C. albicans* (n=103,91.96%) was the predominant Candida species isolated. The Candida culture positivity was found to increase with increase in the duration of illness. (Table 5.10.C, 5.17)

d. Next frequent isolates of Candida were found in patients of cancer (53.14%), representing 101 *C. albicans* and 51 non-albicans species of Candida. When compared to malignancies at other sites, the oral cancer was found to be frequent type of cancer. The isolation of *C. albicans* (66.44%) was the dominant feature in these cases. (Table 5.10.D1, 5.17)

e. Among suspected HIV patients, isolation of Candida was (n=100 strains, 44.05%), comprising 43 % *C. albicans* and 57% non-albicans species of Candida. (Table 5.10.E1,5.10. E2 & 5.17)

f. In patients with burns, isolation of Candida was (20.29%), patients with thermal burn injury and electric burn injury revealed higher Candida isolation. Thirty *C. albicans* strains (54.54%) were recovered, as against 25 (45.45%) non-albicans Candida. Higher culture positivity (28.46%) was seen in urine specimen collected from these patients. (Table 5.10F, 5.17)

11. Isolation of 681 Candida species comprising of 412 (60.49%) *C. albicans* and 269 (39.50%) non albicans Candida is a significant finding.

12. Identification of Candida species on basis of carbohydrate fermentation and assimilation patterns revealed 6 different species of Candida. The distribution of these species is as, *Candida albicans* 412 (60.49%), *C. tropicalis* 101(14.83%), *C.parapsilosis* 61(8.91%), *C.krusei* 43(6.31%), *C.kefyr* 33(04.84%) and *C.guilliermondii* 31 (4.55%).(Table 5.11)
13. Of the 681 clinical isolates of Candida species 412 were \textit{C. albicans}, which were more frequently isolated from oral gargle (41.52%) and sputum (40.19%) samples than other clinical specimen. Whereas 101 clinical isolates of \textit{C. tropicalis} showed sputum (18.93%) and blood (14.28%) as the common clinical source. \textit{Candida parapsilosis} (n=61), was the third most frequent Candida species isolated in sputum (6.97%) and body fluid (9.52%) including urine (4.19%). (Table 5.18)

14. All Candida isolates were also identified by observing morphological patterns developed on 0.1% glucose agar with tween 80 & corn meal agar. On glucose agar better morphological discrimination among the six species of Candida was observed. Out of 681 Candida strains tested, as many as 407 strains were identified within 48 hrs. as against only 44 strains on corn meal agar. (Table 5.15)

15. The 681 Candida isolates obtained in the present study were also identified on the basis of chemical & dye sensitivity using disc diffusion test. The six species of Candida were subgrouped into 12 different codes. \textit{Candida albicans} into 3 codes 120406, 000406 and 100406, the code 120406 represent the most predominant code. \textit{C. tropicalis} into two codes 123456 and 103456. \textit{C. krusei} into 2 codes 123456 and 023456. The \textit{Candida kefyr} & \textit{C. guilliermondii} could not be subgrouped & were identified on basis of code 123456 and 120456. (Table 5.16)

16. All Candida species isolated in the present study showed variable degree of mono microbial and poly-microbial pattern of isolation. Combined isolation of Candida species on 116 occasion is a significant finding.

a. On 372 instances \textit{C. albicans} were isolated in the mono-microbial form, whereas 40 \textit{C. albicans} strains were isolated with other common Candida species viz. \textit{C. tropicalis} (n=12 strains), \textit{C. parapsilosis} (n=10 strains), \textit{C. krusei} (n=12 strains), 2 and 4 strains of \textit{C. kefyr} and \textit{C. guilliermondii}, respectively, more frequently from sputum and oral gargle specimen than other clinical specimens. (Table 5.20)
b. Out of 101 *C. tropicalis* isolated 81 (70.20%) isolates were isolated as pure isolates, where as 20 (19.80%) were in combination with other species viz. *C. albicans* (n=12 strains), *C. parapsilosis* (n=4 strains) and *C. guilliermondii* (n=2 strains). (Table 5.21)

c. A total of 43 *C. krusei* isolates were obtained of which as many as 19 (44.18%) were associated with other Candida species. *C. albicans* (n=12 strains) are the common Candida isolates in combination. (Table 5.22)

d. Out of 61 *C. parapsilosis* 44 (72.13%) were pure isolates and 17 (27.86%) were combined isolations. *C. albicans* (n=10 strains), *C. tropicalis* (n = 4 strains) and *C. kefyr* (n=4 strains) were the other Candida species isolated more commonly. (Table 5.23)

e. 33 *C. kefyr* strains were isolated of which only eight (24.24%) revealed combined isolation. (Table 5.24)

f. Out of 31 *C. guilliermondii* 12 (38.70%) were isolated in combination with other Candida species, *C. kefyr* is more commonly found species. (n=4 strains) in association with *C. guilliermondii*. (Table 5.25)

17. Germ tube formation, since is an important criteria for differentiating Candida species, was evaluated for certain relevant features such as use of different support media, effect of starvation period and effect of growth phase. Highest germ tube formation (98%) was seen when support medium contains pooled normal human serum and when Candida was starved for a period of 45 minutes. Germ tube positivity was found to be highest (98%) when inocula were prepared from 72 hrs. old cultures of Candida and when pooled normal human serum was used. (Table 5.12,5.13,5.14)

18. All the 412 *C. albicans* isolates were subjected for further typing procedure viz. serotyping, morphotyping, resistotyping and biotyping. In situations where *C. albicans* was isolated on more than one occasions, the isolation was correlated with serotyping, morphotyping, resistotyping and biotyping and the clinical syndrome associated.

a. 81.06% strains of *C. albicans* were serotype as serotype A, and 18.93% as serotype B. Higher occurrence of serotype A in patients of PTB
(81.04%), diabetes mellitus (85.43%) and cancer patients (93.06%) was noted whereas higher occurrence of serotype B was noted in patients of burns (40%) and suspected HIV infected cases (48.83%). (Table 5.28)

b. All the 412 C. albicans isolates were typed into different morphotypes on the basis of culture streak morphology viz presence or absence of fringe, surface topography, texture and width of fringe. 18 different morphotypes were defined from among 412 C. albicans isolates. Bulk of isolates i.e. 388 were grouped under nine common morphotypes, of which fringless variant "000/0" was the most common morphotype found among 254 strains, the second common was morphotype "324/0" showing discontinuous fringe followed by morphotype "323/0" in 37 strains. Discontinuous fringe morphotype "324/0" was seen common in sputum of PTB cases and oral gargle of cancer patients. Whereas, morphotype "323/0" was more commonly found in oral gargle of diabetes mellitus patients. Same morphotype was also found in sputum, body fluid of suspected HIV patients and in the wounds of burn patient. Different varieties of morphotypes were recovered from sputum of PTB patients than the sputum specimen collected from HIV patients. Similarly oral gargle from cancer patients, urine from burn patients revealed variety of morphotypes.

c. Resistotyping of 412 C. albicans strains was done by using method of Warnock. et. al. (1979)¹⁸³ and McCreight et.al (1985)²⁹⁷. The seven chemicals / dyes / antimicrobial used for resistotyping were sodium selenite, orthoboric acid, cetrimide, malachite green, cupric sulfate, mercurochrome and sodium chloride, abbreviated from A to G. Eleven different resistogram patterns were obtained. 30 strains represented A- - D - - G pattern, while 79 strains revealed A - CD – FG pattern. 412 C. albicans isolated from different clinical syndromes and clinical specimen were well distributed without significant clumping into eleven-resistogram pattern. (Table 5.34)
d. The three-digit nine-test scheme of biotyping proposed by Odds et al. (1980) was used to delineate 412 C. albicans isolates. Total 24 biotypes were recognised of which biotype 356 (n=52), 322 (n=51), 355 (n=43), 336 (n=29), 545 (n=8), 252 (n=27) and 325 (n=24) were the predominant biotypes. Certain biotype for example 322 was seen in specimen like sputum, oral gargle, urine, as well as body fluid. Similarly, biotype 356 and 355 was seen in sputum, oral gargle and urine, while biotype 532 in sputum, urine and body fluid. Biotype 336 was seen in sputum as well as oral gargle and stool. (Table 5.36)

19. Combination of all four phenotypic methods were used to designate each C. albicans strain a specific combined phenotype code. The C. albicans strains were therefore designated by its serotype, morphotype, resistotype and biotype for example A/324/0/A - - D - - /315. In this manner all 412 cases isolates could be designated into 214 combined phenotypes. In some instances when C. albicans were isolated on more than one occasions, strain with same serotype, morphotype, resistotype but for the difference in biotype was found, where as in other instance strains with different morphotype or resistotype or serotype were found, inferring the importance of each typing system in strain discrimination. Clustering of 412 C. albicans isolates into 2 serotypes (serotype A and B) 18 morphotypes, 11 resistotypes, and 24 biotypes was noted. However, when combination of these 4 typing methods was used to designate each C. albicans strain a "combined phenotype", better degree of strain delineation was achieved resulting into 214 designate combine phenotype of C. albicans. Thus emphasising the importance of use of combination phenotyping method, for achieving better discrimination of cluster of homogenous C. albicans strains.

20. Analysis of frequency of combined phenotype in each clinical group and clinical specimen revealed that some combine phenotype are more frequently associated with clinical condition and the specimen, for example in PTB cases A/324/0/A - - D - - /315 type was recovered on 8
occasions from sputum specimen. Whereas, B/324/0/A - - D - - -/356 type was recovered on 3 occasions from urine of the PTB patients.

21. In some clinical situations when \textit{C. albicans} was isolated on more than one/two occasions, the combined phenotype of \textit{C. albicans} was correlated. Analysis of such correlation revealed that in majority of patients when \textit{C. albicans} was isolated on multiple occasions same combination phenotype was obtained in some clinical specimen, indicating that a particular \textit{C. albicans} strain had etiopathogenic relatedness. Whereas, in some patients different combined phenotype was obtained from same patient, when clinical specimen was collected on 2 occasions, indicating that the need of independent clinical interpretation of such cases.

22. Out of 107 \textit{C. albicans} strains isolated from PTB cases 31 combined phenotype were obtained from sputum and 8 from urine. Different combined phenotypes were found in cavitatory and non-cavitatory PTB cases. In most cases, when sputum specimen was collected on 2/3 occasions, same combined phenotypes e.g. A / 000 /0 / A- -D - - -/ 322 were obtained, but the combined phenotypes of \textit{C. albicans} obtained from one patient is not identical to the type of \textit{C. albicans} obtained from other patient. Interestingly, in some instances, the combined phenotype of the \textit{C. albicans} isolated on first occasion is different from that obtained on second occasion e.g. A / 524 /0/- -CDE - - / 325 and A / 254 /0/ ABC - -G/ 356. Indicating those different strains of \textit{C. albicans} colonises the respiratory tract of PTB patients. (Table 5.37A, 5.37B & 5.43.A)

13 \textit{C. albicans} isolates obtained from urine of PTB cases revealed 8 combined phenotypes. The combined phenotypes of \textit{C. albicans} isolated on second or third occasion are different from the type of \textit{C. albicans} isolated on first occasion. e.g. A / 000 /0/ - - CDE- - / 555, B / 254/0 / A-- D- - -/ 555 and B / 754/0 / - - CDE- - / 356 combined phenotype were obtained on first, second and third occasions respectively. (Table 5.43.B)
Whereas, same combined phenotype was seen in sputum and urine specimen of some patient providing scope for correlation between source and site of colonisation.

23. 12 combined phenotype patterns of 28 *C. albicans* strains were obtained from urine of chronic hospitalised and catheterised patients. Same combined phenotype viz. A/000/0/A-CD-FG/154, A/000/0/A - - D - - - /545, A/000/0/A B C - - - /555 and A/254/0/A B - - E - - /545, was obtained when urine was collected on two occasions from 4 cases with duration of catheterisation from 13-15 days. (Table 5.38,5.44)

No single predominant combined phenotype was found among chronically hospitalised and catheterised patients urine specimen.

24. From 106 strains of *C. albicans* isolated from oral gargle (n=75 strains) and urine (n=28 strains) specimen total 28 combined phenotypes were found. (Table 5.39A,& 5.39B.)

Six strains of *C. albicans* recovered from patients with first time presentation of diabetes revealed same combined phenotype. A/000/0/A - C D - F G/322.

In 2 patients with duration of diabetes from 6 years upto 10 years, same combined phenotype i.e. A/000/0/A - - D - - G/336 was found when oral gargle was collected on 3 occasions.

12 strains of *C. albicans* recovered from diabetes mellitus patients with duration of illness from 11 to 15 years same combined phenotype were found except for the difference in biotypes for example A/000/0/A - C D E F -/356 and A/000/0/A - C D E F/256.

While in same group of patients when oral gargle was collected on 3 occasions from 2 patients same combined phenotype A/000/0/A B C - - - G/336, but for difference in biotype was found.

The urine specimen collected on 2 occasions, from 2 patients with duration of diabetes 6 to 10 years, yielding 4 strains of *C. albicans* revealed same combined phenotype A/000/0/A - C D - F G/152.
Similarly in a group of patients with duration of illness from 11 to 15 years, 8 C. albicans strains from urine of 4 patients collected on 2 occasions revealed same combined phenotype i.e. A/000/0/A B C - - -/545. (Table 5.45.A,B)

25. From the 72 strains of C. albicans isolated from the oral gargle specimen of cancer patients 21 combined phenotype were obtained. Whereas from urine specimen 14 combined phenotypes were found. One strain of C. albicans isolated from blood specimen revealed A/323/0/A - - D - - -/315 combined phenotype.

From oral gargle collected on 3 occasions from 2 oral cancer cases, the same combined phenotype but for the difference in biotype i.e. A/000/0/A - C D - F G/355 and A/000/0/A - C D - F G/252 was found. In 2 patients of ovarian cancer, the oral gargle collected on 3 occasions same combined phenotype A/000/0/A - - E - - /355 was found. While in 2 patients of lung cancer when oral gargle was collected on 3 occasions same combined phenotype A/000/0/A - C D - F G/323 was found.

In the oral gargle collected on 3 occasions from 2 CML patients the 6 strains of C. albicans were isolated which revealed the same combined phenotype viz. A/000/0/A B C - - - G/322.

Three patients of oral cancer were found to be colonized with C. albicans same combined phenotype pattern but for the differences in biotype in two cases and difference in morphotype in one case, highlighting the importance of morphotyping and biotyping. (Table5.40A&B, 5.46.A&B)

26. Of the 43 C. albicans strains isolated from suspected HIV patients 26 combined phenotypes were found.

From among the 5 different types of clinical specimen used for isolation of Candida, no single, "the predominant" combined phenotype was found in these different clinical specimen. (Table 5.41A&B, 5.47.A&B)

27. From burn patients, the C. albicans isolated from wound swabs revealed different combined phenotypes and total 14 combined phenotypes were found.
Ten *C. albicans* strains isolated on 2 occasions from urine specimen of 5 patients, same combined phenotype were found in these patients.

While 6 *C. albicans* strain isolated from urine of 3 burn patients on 2 occasions revealed same combined phenotype A/000/0/A B C - - - G/545. (Table 5.42, 5.48)

In the present study out of 681 number of Candida isolates, 200 strains comprising of 70 *C. albicans*, 40 *C. tropicalis*, 30 *C. kefyr*, 20 each of *C. parapsilosis*, *C. krusei* and *C. guilliermondii* were subjected for antifungal susceptibility testing against AmB, Mcz and Fcz by using agar disc diffusion method.

Out of 200 strains tested, 180 (90%) showed sensitivity to AmB, 173 (86.5%) each to Mcz and Fcz, 17 (8.5%) strains were intermediate to AmB and Fcz, whereas, 18 (9%) were intermediate to Mcz. Only three (1.5%) were resistant to AmB, whereas 10 (5%) and 9 (4.5%) were resistant to Mcz and Fcz respectively.

Most of the clinical Candida isolates are susceptible to the commonly used anti-fungal agent and only a few non-albicans Candida are showing resistance, the resistance to azoles is more common than to polyenes.

All *C. albicans* strains are sensitive to drugs used in the present study.

The study has revealed vast scope for analysis of clinical infections noted in hospital, with a definite prospect of clinicoepidemiological correlation.

It is further observed that, not all the clinical candidal infections are due to *C. albicans* and importantly not all the non-albicans Candida are insignificant isolates. (Table 5.49)

Out of 100 specimen obtained from 50 healthy controls, 19 oral gargle and nine urine samples demonstrated smear positivity. Culture positivity was seen in 24 oral gargle and 13 urine samples. (Table 5.50.3)

Out of 37 isolates from healthy controls, the predominant isolate was *C. albicans*, with higher isolation from oral gargles (n=13) than from urine(n=7). Candida isolation has relationship with tobacco chewing and smoking. (Table 5.50.5)
31. The 20 strains of *C. albicans* isolated from healthy controls 20 number of combined phenotype were obtained. The combined phenotype of *C. albicans* isolated from oral gargle and urine of healthy controls is not same. The combined phenotypes of *C. albicans* isolated from oral gargle and urine of healthy controls are not same to those found in clinical specimen collected from patients with clinical categorised 6 representative syndromes. (Table 5.51.10)

32. The mice inoculated with strains of *C. albicans* and *C. tropicalis* revealed abscess formation at the site of inoculation, the size of abscess was larger in animal inoculated with *C. albicans* than *C. tropicalis*. (Table 5.51.1)

33. White patches of abscesses in parenchymal organs of peritoneal cavity were seen in 2/3 animals in *C. albicans* and 1/3 animals in *C. tropicalis*. Abscess at the site of inoculation and white patches of micro abscesses in the peritoneal cavity are not seen in the animal inoculated with _C. psuedotropicalis_, *C. parapsilosis, C. krusei, and C. guilliermondii_. (Table 5.51.2)

34. *Candida albicans* was demonstrated in smear and culture of the specimen obtained from site of inoculation in all 3/3 animals, from peritoneal fluid of 2/3 animals, lung and liver homogenate of 1/3 animals. (Table 5.51.2)

35. *Candida tropicalis* was demonstrated in smear and culture of the specimen obtained from 1/3 animals, peritoneal fluid of 2/3 animals, lung and liver homogenate of 1/3 animals. (Table 5.51.2)

36. *Candida psuedotropicalis, C. parapsilosis, C. krusei, and C. guilliermondii* were not recovered in smear/culture of specimen obtained from animals inoculated with these Candida species. (Table 5.51.2)

37. Histopathological changes were observed in animals inoculated with *C. albicans* in form of disseminated lesions, and abscesses in lungs, liver, kidney, heart, spleen and pancreas. In animals inoculated with *C. tropicalis* disseminated lesions in form of abscess were seen in lungs and spleen only. (Table 5.51.3)

inoculated intra-peritoneally into mice, only *C. albicans* and *C. tropicalis* revealed pathogenicity potential in form of disseminated lesions in different visceral organs (Table 5.51.3)

39. The present study has been highly rewarding, in our effort of isolating Candida strains from clinical patients with immunocompromise, identification and speciation of all the Candida isolates.

40. Isolation of 269 non albicans Candida has been very interesting aspect of the study.

41. Typing of *Candida albicans* by four methods of established importance has been very fascinating facet of the study.

42. Combined phenotyping has provided great scope for differentiating the clustered type of *C. albicans*.

43. The present study emphasizes need for setting up of elaborate mycological investigation of clinical infections among immunocompromised patients.