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CONTENTS

	Page No.
Guest Editorial	
The Need for Control of Viral Illnesses in India: A Call for Action <i>C Lahariya, UK Baveja</i>309
Review Article	
Immunobiology of Human Immunodeficiency Virus Infection <i>P Tripathi, S Agrawal</i>311
Special Articles	
Serum Levels of Bcl-2 and Cellular Oxidative Stress in Patients with Viral Hepatitis <i>HG Osman, OM Gabr, S Lotfy, S Gabr</i>323
Rapid Identification of Non-sporing Anaerobes using Nuclear Magnetic Resonance Spectroscopy and an Identification Strategy <i>S Menon, R Bharadwaj, AS Chowdhary, DV Kaundinya, DA Palande</i>330
Original Articles	
Species Distribution and Physiological Characterization of <i>Acinetobacter</i> Genospecies from Healthy Human Skin of Tribal Population in India <i>SP Yavankar, KR Pardesi, BA Chopade</i>336
Extended-spectrum Beta-lactamases in Ceftazidime-resistant <i>Escherichia coli</i> and <i>Klebsiella pneumoniae</i> Isolates in Turkish Hospitals <i>S Hoşoğlu, S Gündeş, F Kolaylı, A Karadenizli, K Demirdağ, M Günaydın, M Altındis, R Çaylan, H Ucmak</i>346
Typhoid Myopathy or Typhoid Hepatitis: A Matter of Debate <i>M Mirsadraee, A Shirdel, F Roknee</i>351
Correlation Between <i>in Vitro</i> Susceptibility and Treatment Outcome with Azithromycin in Gonorrhoea: A Prospective Study <i>P Khaki, P Bhalla, A Sharma, V Kumar</i>354
Comparison of Radiorespirometric Buddemeyer Assay with ATP Assay and Mouse Foot Pad Test in Detecting Viable <i>Mycobacterium leprae</i> from Clinical Samples <i>VP Agrawal, VP Shetty</i>358
Detection of <i>Mycoplasma</i> Species in Cell Culture by PCR And RFLP Based Method: Effect of BM-cyclin to Cure Infections <i>V Gopalkrishna, H Verma, NS Kumbhar, RS Tomar, PR Patil</i>364

Virulence Factors and Drug Resistance in <i>Escherichia coli</i> Isolated from Extraintestinal Infections	369
<i>S Sharma, GK Bhat, S Shenoy</i>	
Antimicrobial Susceptibility Testing of <i>Helicobacter pylori</i> to Selected Agents by Agar Dilution Method in Shiraz-iran	374
<i>J Kohanteb, A Bazargani, M Saberi-Firoozi, A Mobasser</i>	
Outbreak of Acute Viral Hepatitis due to Hepatitis E virus in Hyderabad	378
<i>P Sarguna, A Rao, KN Sudha Ramana</i>	
A Comparative Study for the Detection of Mycobacteria by BACTEC MGIT 960, Lowenstein Jensen Media and Direct AFB Smear Examination	383
<i>S Rishi, P Sinha, B Malhotra, N Pal</i>	
Cytokine Levels in Patients with Brucellosis and their Relations with the Treatment	387
<i>H Akbulut, I Celik, A Akbulut</i>	
Brief Communications	
Rapid Detection of Non-enterobacteriaceae Directly from Positive Blood Culture using Fluorescent <i>In Situ</i> Hybridization	391
<i>EH Wong, G Subramaniam, P Navaratnam, SD Sekaran</i>	
Latex Particle Agglutination Test as an Adjunct to the Diagnosis of Bacterial Meningitis	395
<i>K Surinder, K Bineeta, M Megha</i>	
Helminthic Infestation in Children of Kupwara District: A Prospective Study	398
<i>SA Wani, F Ahmad, SA Zargar, BA Fomda, Z Ahmad, P Ahmad</i>	
Clinical and Mycological Profile of Cryptococcosis in a Tertiary Care Hospital	401
<i>MR Capoor, D Nair, M Deb, B Gupta, P Aggarwal</i>	
<i>Candida</i> spp. other than <i>Candida albicans</i>: A Major Cause of Fungaemia in a Tertiary Care Centre	405
<i>S Shivaprakasha, K Radhakrishnan, PMS Karim</i>	
Case Reports	
<i>Enterobacter sakazakii</i> in Infants: Novel Phenomenon in India	408
<i>P Ray, A Das, V Gautam, N Jain, A Narang, M Sharma</i>	
Ocular Toxocariasis in a Child: A Case Report from Kashmir, North India	411
<i>BA Fomda, Z Ahmad, NN Khan, S Tanveer, SA Wani</i>	
Cutaneous Actinomycosis: A Rare Case	413
<i>SC Metgud, H Sumati, P Sheetal</i>	
Fatal Haemophagocytic Syndrome and Hepatitis Associated with Visceral Leishmaniasis	416
<i>P Mathur, JC Samantaray, P Samanta</i>	
A Rare Case of Mucormycosis of Median Sternotomy Wound Caused by <i>Rhizopus arrhizus</i>	419
<i>R Chawla, S Sehgal, S Ravindra Kumar, B Mishra</i>	
<i>Mycobacterium fortuitum</i> Keratitis	422
<i>C Sanghvi</i>	
Correspondence	
Prevention of Parent-to-Child Transmission of HIV: An Experience in Rural Population	425
<i>N Nagdeo, VR Thombare</i>	

Combining Vital Staining with Fast Plaque: TB Assay426
<i>D Rawat, MR Capoor, A Hasan, D Nair, M Deb, P Aggarwal</i>	
Disseminated Histoplasmosis427
<i>PK Maiti, MS Mathews</i>	
Authors' Reply428
<i>RS Bharadwaj</i>	
Microwave Disinfection of Gauze Contaminated with Bacteria and Fungi428
<i>VH Cardoso, DL Gonçalves, E Angioletto, F Dal-Pizzol, EL Streck</i>	
Endoscope Reprocessing: Stand up and Take Notice!429
<i>A Das, P Ray, M Sharma</i>	
Prevalence of <i>Toxoplasma gondii</i> Infection amongst Pregnant Women in Assam, India431
<i>BJ Borkakoty, AK Borthakur, M Gohain</i>	
Evaluation of Glucose-Methylene-Blue-Mueller-Hinton Agar for E-Test Minimum Inhibitory Concentration Determination in <i>Candida</i> spp.432
<i>MR Capoor, D Rawat, D Nair, M Deb, P Aggarwal</i>	
Resurgence of Diphtheria in the Vaccination Era434
<i>N Khan, J Shastri, U Aigal, B Doctor</i>	
A Report of <i>Pseudomonas aeruginosa</i> Antibiotic Resistance from a Multicenter Study in Iran435
<i>MA Boroumand, P Esfahanifard, S Saadat, M Sheihkvatan, S Hekmatyazdi, M Saremi, L Nazemi</i>	
Trends of Antibiotic Resistance in <i>Salmonella enterica</i> Serovar Typhi Isolated from Hospitalized Patients from 1997 to 2004 in Lagos, Nigeria436
<i>KO Akinyemi, AO Coker</i>	
Book Review	
Hospital-Acquired Infections: Power Strategies for Clinical Practice438
<i>Reba Kanungo</i>	
Title Index, 2007440
Author Index, 2007442
Scientific Reviewers, 2007446

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Evaluation of Glucose-Methylene-Blue-Mueller-Hinton Agar for E-Test Minimum Inhibitory Concentration Determination in *Candida* spp.

Dear Editor,

A rise in invasive fungal infections and their emerging resistance have necessitated the need for antifungal susceptibility testing (AFT) for clinical work-up.¹ The standardized broth micro-dilution (BMD) method is expensive, laborious and cumbersome for routine use in clinical microbiology laboratory.² Recently, a disc-diffusion method has been approved by CLSI using glucose-methylene-blue (GMB) Mueller-Hinton agar (MHA). Despite being easy and practical, this needs to be confirmed by BMD to exclude false resistance.¹

Recent reports have documented comparable results between BMD (NCCLS-27-A)³ and agar-based E-test.^{4,5} The manufacturer-recommended media for E-test is glucose-supplemented RPMI agar (RPMI-G). The end point for azoles is poorly defined on this medium. Therefore, we undertook this study to determine whether GMB-MHA could be used in the E-test method.

A total of 31 blood stream isolates from candidaemia cases were selected. These were speciated using germ tube test, CHROM agar, cornmeal agar and tetrazolium reduction test (Himedia, Mumbai). Antifungal susceptibility of these isolates was performed by E-test and BMD for amphotericin-B and fluconazole. The E-strip (AB-Biodisk, Solna) minimum inhibitory concentration (MIC) was determined on RPMI-G (RPMI + 1.5% agar + 2% glucose) media and GMB-MHA (MHA + 2% glucose + 0.5 µg of methylene blue). For agar diffusion E-test, 0.5 McFarland standard inocula were applied to GMB-MHA and RPMI-G media with a cotton swab. The plates were allowed to dry for at least 15 min before the E-strip was applied to the

surface. The MIC for the E-test was measured after 24 h, at transition point where growth abruptly decreased (reduction in colony, size, number and density: approximately 80% growth inhibition standards). BMD-MIC was performed using RPMI and 0.165 M morpholine propanesulphonic acid (Himedia, Mumbai). The interpretation was done spectrophotometrically after 24 and 48 h of incubation, as per NCCLS guidelines.³ The optical density (OD) of the medium control well was subtracted from the ODs of all other wells and MIC concentration was computed mathematically. Briefly, the BMD-MIC of amphotericin B was determined as the lowest concentration with an OD corresponding to greater than or equal to 90% decrease in turbidity compared to that of growth control and the MICs of fluconazole, corresponding to a 50% decrease in turbidity.³ The quality control was performed by testing *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019) with each batch of clinical isolates. All the MIC experiments were repeated twice and mean was taken.

The isolates included in the study comprised of *C. tropicalis* (12), *C. parapsilosis* (8), *C. albicans* (8), *C. krusei* (2) and *C. glabrata* (1). Twenty-four (77.4%) of the isolates that were found to be susceptible by BMD were identified as susceptible by RPMI-G agar to amphotericin B and fluconazole. The similar figures for GMB-MHA was 24 (77.4%) and 25 (80.6%) for amphotericin B and fluconazole, respectively. Higher MIC levels (1-2 dilutions) were noted by E-strip method as compared to BMD method. Table shows the comparison of susceptibility by the E-test method on GMB-MHA and RPMI-G media.

Table: Comparison of susceptibility by the E-test method on RPMI-G and GMB-MHA

<i>Candida</i> spp.** (n)	Total no. of susceptible isolates			
	RPMI-G		GMB-MHA	
	Amphotericin B ($\mu\text{g/mL}$)*	Fluconazole ($\mu\text{g/mL}$)*	Amphotericin B ($\mu\text{g/mL}$)*	Fluconazole ($\mu\text{g/mL}$)*
<i>C. tropicalis</i> (12)	10	10	10	11
<i>C. parapsilosis</i> (8)	7	6	7	6
<i>C. albicans</i> (8)	7	8	7	8

*Interpretive criteria: amphotericin - $\leq 1 \mu\text{g/mL}$ (sensitive), $> 2 \mu\text{g/mL}$ (resistant); fluconazole - $\leq 8 \mu\text{g/mL}$ (sensitive), $\geq 64 \mu\text{g/mL}$ (resistant);

***C. krusei* (2) and *C. glabrata* (1) were resistant to amphotericin B and fluconazole on RPMI-G and GMB-MHA

Prior studies have shown that agar-based susceptibility testing (E-test and DD method) are more practical as compared to BMD due to their simplicity and reproducibility. Requirement of additional equipment (spectrophotometer) is also eliminated.⁴ The results obtained by using both media were in acceptable concordance ($>93\%$) with those obtained by MBD method. Similar findings were corroborated by previous studies.⁴ Disparate readings were attributable to 'trailing growth phenomenon' in an isolate of *C. tropicalis*. This discrepancy was also observed in prior studies.^{2,6}

A large number of recent studies have compared E-test or DD on different media.^{1,2,6} But studies comparing E-test MIC on RPMIG and GMB-MHA could not be seen in the pertinent literature. The overall levels of agreement between E-strip MICs obtained by RPMIG and GMB-MHA at 24 h were comparable (97% agreement) for both amphotericin-B and fluconazole for resistant and susceptible isolates. However, a single disparity in an isolate of *C. tropicalis* in fluconazole susceptibility was observed, where RPMI-G and BMD gave a resistant and GMB-MHA depicted a susceptible MIC. This disparity could not be explained as repeat testing yielded the same result. Higher MIC levels (1-2 dilutions) were noted by E-strip method as compared to BMD method. These findings have been reported previously.⁵

It was observed in the current and prior studies that GMB-MHA has enhanced growth, simplified reading and minimal microcolonies around zone of inhibition.^{1,4} Thus GMB-MHA appears to be a useful medium for E-test, as it compared well with BMD and RPMI-G (except a single *C. tropicalis* outlier despite repeat testing). The stock-solution of GMB can be refrigerated and added to the molten and cooled MHA which is made routinely. In a resource constraint and busy clinical laboratory, AFT by MIC method on GMB-MHA followed by confirmation by BMD of resistant strains is recommended to exclude false resistance. Further, meaningful large-scale studies and continued refinement of all susceptibility techniques are required for routine AFT.

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