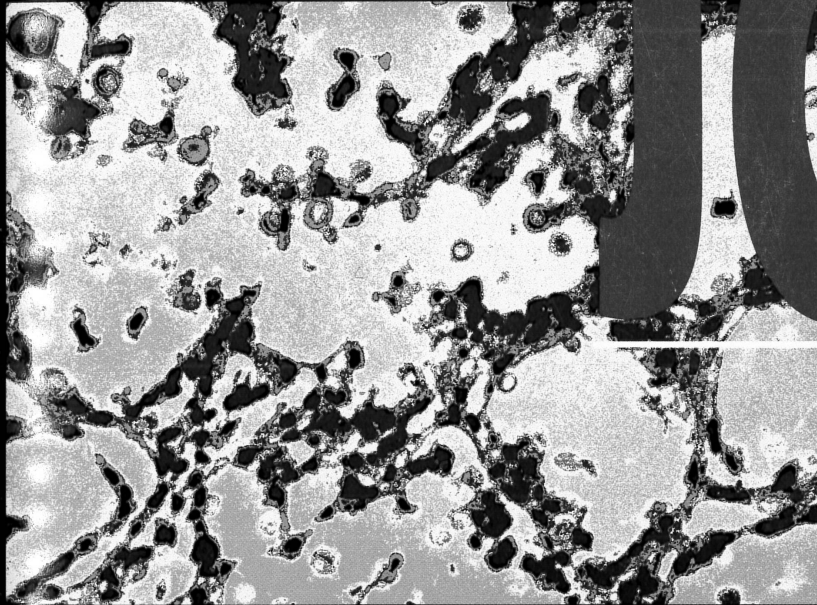


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BACTERIOLOGY

Characterization of <i>Escherichia coli</i> Strains from Cases of Childhood Diarrhea in Provincial Southwestern Nigeria	Iruka N. Okeke, Adebayo Lamikanra, Hartmut Steinrück, and James B. Kaper	7-12
The Efficacy of Laboratory Diagnosis of <i>Helicobacter pylori</i> Infections in Gastric Biopsy Specimens Is Related to Bacterial Density and <i>vacA</i>, <i>cagA</i>, and <i>iceA</i> Genotypes	Leen-Jan van Doorn, Yvonne Henskens, Nathalie Nouhan, Anita Verschuuren, Rolf Vreede, Paul Herbink, Gabriëlle Ponjee, Kees van Krimpen, Ruud Blankenburg, Joost Scherpenisse, and Wim Quint	13-17
Comparative Analysis of PCR versus Culture for Diagnosis of Ulceroglandular Tularemia	Anders Johansson, Lennart Berglund, Ulla Eriksson, Ingela Göransson, Ralf Wollin, Mats Forsman, Arne Tärnvik, and Anders Sjöstedt	22-26
Prevalence of Toxin Types and Colonization Factors in Enterotoxigenic <i>Escherichia coli</i> Isolated during a 2-Year Period from Diarrheal Patients in Bangladesh	Firdausi Qadri, Swadesh Kumar Das, A. S. G. Faruque, George J. Fuchs, M. John Albert, R. Bradley Sack, and Ann-Mari Svennerholm	27-31
Direct Amplification of rRNA Genes in Diagnosis of Bacterial Infections	Kaisu Rantakokko-Jalava, Simo Nikkari, Jari Jalava, Erkki Eerola, Mikael Skurnik, Olli Meurman, Olli Ruuskanen, Anna Alanen, Esa Kotilainen, Paavo Toivanen, and Pirkko Kotilainen	32-39
Convenient Test for Screening Metallo-β-Lactamase-Producing Gram-Negative Bacteria by Using Thiol Compounds	Yoshichika Arakawa, Naohiro Shibata, Keigo Shibayama, Hiroshi Kurokawa, Tetsuya Yagi, Hiroshi Fujiwara, and Masafumi Goto	40-43
Development and Evaluation of a Phage Typing Scheme for <i>Vibrio cholerae</i> O139	A. K. Chakrabarti, A. N. Ghosh, G. Balakrish Nair, S. K. Niyogi, S. K. Bhattacharya, and B. L. Sarkar	44-49
Polymorphism in the Pertussis Toxin Promoter Region Affecting the DNA-Based Diagnosis of <i>Bordetella</i> Infection	Malin Nygren, Elisabet Reizenstein, Mostafa Ronaghi, and Joakim Lundeberg	55-60
CagA Antibodies in Japanese Children with Nodular Gastritis or Peptic Ulcer Disease	Seiichi Kato, Toshiro Sugiyama, Mineo Kudo, Kenji Ohnuma, Kyoko Ozawa, Kazuie Inuma, Masahiro Asaka, and Martin J. Blaser	68-70
Characterization of <i>Streptococcus agalactiae</i> Isolates of Bovine and Human Origin by Randomly Amplified Polymorphic DNA Analysis	Gabriela Martinez, Josee Harel, Robert Higgins, Sonia Lacouture, Danielle Daignault, and Marcelo Gottschalk	71-78
Heterogeneity of <i>Actinobacillus actinomycetemcomitans</i> Strains in Various Human Infections and Relationships between Serotype, Genotype, and Antimicrobial Susceptibility	Susanna Paju, Petteri Carlson, Hannele Jousimies-Somer, and Sirkka Asikainen	79-84
Identification of <i>Mycoplasma fermentans</i> in Synovial Fluid Samples from Arthritis Patients with Inflammatory Disease	Sheena Johnson, David Sidebottom, Felix Bruckner, and David Collins	90-93

Continued on following page

Isolation, Cultivation, and Characterization of <i>Borrelia burgdorferi</i> from Rodents and Ticks in the Charleston Area of South Carolina	J. H. Oliver, Jr., K. L. Clark, F. W. Chandler, Jr., L. Tao, A. M. James, C. W. Banks, L. O. Huey, A. R. Banks, D. C. Williams, and L. A. Durden	120-124
Clinically Applicable Multiplex PCR for Four Middle Ear Pathogens	Panu H. Hendolin, Lars Paulin, and Jukka Ylikoski	125-132
Specific <i>Taenia crassiceps</i> and <i>Taenia solium</i> Antigenic Peptides for Neurocysticercosis Immunodiagnosis Using Serum Samples	Ednéia Casagrande Bueno, Adelaide José Vaz, Luís Dos Ramos Machado, José Antônio Livramento, and Sílvia Regina Mielle	146-151
Comparison of PCR-Restriction Fragment Length Polymorphism Analysis and PCR-Direct Sequencing Methods for Differentiating <i>Helicobacter pylori ureB</i> Gene Variants	Toshihito Tanahashi, Masakazu Kita, Tadashi Kodama, Naoki Sawai, Yoshio Yamaoka, Shoji Mitsufuji, Fumitaka Katoh, and Jiro Imanishi	165-169
The 1998 Senegal Epidemic of Meningitis Was Due to the Clonal Expansion of A:4:P1.9, Clone III-1, Sequence Type 5 <i>Neisseria meningitidis</i> Strains	Pierre Nicolas, Georges Raphenon, Martine Guibourdenche, Laurent Decousset, Richard Stor, and Abou Beckr Gaye	198-200
<i>Escherichia coli</i> Serotype O15:K52:H1 as a Uropathogenic Clone	Guillem Prats, Ferran Navarro, Beatriz Mirelis, David Dalmau, Nuria Margall, Pere Coll, Adam Stell, and James R. Johnson	201-209
Detection of Clarithromycin-Resistant <i>Helicobacter pylori</i> Strains by a Preferential Homoduplex Formation Assay	Shin Maeda, Haruhiko Yoshida, Hironari Matsunaga, Keiji Ogura, Osamu Kawamata, Yasushi Shiratori, and Masao Omata	210-214
Validation of the INNO-LIA Syphilis Kit as a Confirmatory Assay for <i>Treponema pallidum</i> Antibodies	Anne Ebel, Lies Vanneste, Martine Cardinaels, Erwin Sablon, Isabelle Samson, Katrien De Bosschere, Frank Hulstaert, and Maan Zrein	215-219
Distribution of the Intermedilysin Gene among the Anginosus Group Streptococci and Correlation between Intermedilysin Production and Deep-Seated Infection with <i>Streptococcus intermedius</i>	Hideaki Nagamune, Robert A. Whiley, Takatsugu Goto, Yasuko Inai, Takuya Maeda, Jeremy M. Hardie, and Hiroki Kourai	220-226
Diagnosis of Bubonic Plague by PCR in Madagascar under Field Conditions	L. Rahalison, E. Vololonirina, M. Ratsitorahina, and S. Chanteau	260-263
Etiology of Genital Ulcer Disease in Dakar, Senegal, and Comparison of PCR and Serologic Assays for Detection of <i>Haemophilus ducreyi</i>	Patricia A. Totten, Jane M. Kuypers, Cheng-Yen Chen, Michelle J. Alfa, Linda M. Parsons, Susan M. Dutro, Stephen A. Morse, and Nancy B. Kiviat	268-273
Importance of Inoculum Size and Sampling Effect in Rapid Antigen Detection for Diagnosis of <i>Streptococcus pyogenes</i> Pharyngitis	Bradley Kurtz, Michael Kurtz, Martha Roe, and James Todd	279-281
Species-Specific PCR as a Tool for the Identification of <i>Burkholderia gladioli</i>	Paul W. Whitby, Lauren C. Pope, Karen B. Carter, John J. LiPuma, and Terrence L. Stull	282-285
Prevalence of <i>Campylobacter</i>, <i>Arcobacter</i>, <i>Helicobacter</i>, and <i>Sutterella</i> spp. in Human Fecal Samples as Estimated by a Reevaluation of Isolation Methods for Campylobacters	Jørgen Engberg, Stephen L. W. On, Clare S. Harrington, and Peter Gerner-Smidt	286-291
Molecular Methods for the Epidemiological Typing of <i>Salmonella enterica</i> Serotype Typhi from Hong Kong and Vietnam	J. M. Ling, N. W. S. Lo, Y. M. Ho, K. M. Kam, Nguyen Thi Tuyet Hoa, Le Thi Phi, and A. F. Cheng	292-300

Evaluation of Whole-Cell and OspC Enzyme-Linked Immunosorbent Assays for Discrimination of Early Lyme Borreliosis from OspA Vaccination	Chad A. Wieneke, Steven D. Lovrich, Steven M. Callister, Dean A. Jobe, Jennifer A. Marks, and Ronald F. Schell	313-317
Identification and Characterization of Immunoglobulin G in Blood as a Major Inhibitor of Diagnostic PCR	Waleed Abu Al-Soud, Leif J. Jönsson, and Peter Rådström	345-350
Simplified Protocol for Pulsed-Field Gel Electrophoresis Analysis of <i>Streptococcus pneumoniae</i>	M. Catherine McEllistrem, Janet E. Stout, and Lee H. Harrison	351-353
Temporal Changes in Outer Surface Proteins A and C of the Lyme Disease-Associated Spirochete, <i>Borrelia burgdorferi</i>, during the Chain of Infection in Ticks and Mice	Tom G. Schwan and Joseph Piesman	382-388
Distribution and Antigenicity of Fibronectin Binding Proteins (SfbI and SfbII) of <i>Streptococcus pyogenes</i> Clinical Isolates from the Northern Territory, Australia	Alison M. Goodfellow, Megan Hibble, Susanne R. Talay, Bernd Kreikemeyer, Bart J. Currie, Kadaba S. Sriprakash, and Gursharan S. Chhatwal	389-392
Identification of a Major Cluster of <i>Klebsiella pneumoniae</i> Isolates from Patients with Liver Abscess in Taiwan	Yeu-Jun Lau, Bor-Shen Hu, Wan-Ling Wu, Yu-Hui Lin, Hwan-You Chang, and Zhi-Yuan Shi	412-414
Sequencing the Gene Encoding Manganese-Dependent Superoxide Dismutase for Rapid Species Identification of Enterococci	Claire Poyart, Gilles Quesnes, and Patrick Trieu-Cuot	415-418
Confirmation of <i>psaA</i> in All 90 Serotypes of <i>Streptococcus pneumoniae</i> by PCR and Potential of This Assay for Identification and Diagnosis	Katherine E. Morrison, Derrick Lake, Jennifer Crook, George M. Carlone, Edwin Ades, Richard Facklam, and Jacquelyn S. Sampson	434-437
A Clonal Subpopulation of <i>Leptospira interrogans</i> Sensu Stricto Is the Major Cause of Leptospirosis Outbreaks in Brazil	M. M. Pereira, M. G. S. Matsuo, A. R. Bauab, S. A. Vasconcelos, Z. M. Moraes, G. Baranton, and I. Saint Girons	450-452
CHLAMYDIOLOGY AND RICKETTSIOLOGY		
Molecular Epidemiology of Genital <i>Chlamydia trachomatis</i> Infection in High-Risk Women in Senegal, West Africa	Katharine Sturm-Ramirez, Hunter Brumblay, Khady Diop, Aissatou Guèye-Ndiaye, Jean-Louis Sankalé, Ibou Thior, Ibrahima N'Doye, Chung-Cheng Hsieh, Souleymane Mboup, and Phyllis J. Kanki	138-145
Improved Sensitivity of PCR for Diagnosis of Human Granulocytic Ehrlichiosis Using <i>epank1</i> Genes of <i>Ehrlichia phagocytophila</i>-Group Ehrlichiae	Jennifer J. Walls, Patrizio Caturegli, Johan S. Bakken, Kristin M. Asanovich, and J. Stephen Dumler	354-356
Natural Infection of Domestic Goats with <i>Ehrlichia chaffeensis</i>	Vivien G. Dugan, Susan E. Little, David E. Stalknecht, and Ashley D. Beall	448-449
MYCOBACTERIOLOGY AND AEROBIC ACTINOMYCETES		
Differentiation among Members of the <i>Mycobacterium tuberculosis</i> Complex by Molecular and Biochemical Features: Evidence for Two Pyrazinamide-Susceptible Subtypes of <i>M. bovis</i>	Stefan Niemann, Elvira Richter, and Sabine Rüscher-Gerdes	152-157
Identification of <i>Nocardia</i> Species by Restriction Endonuclease Analysis of an Amplified Portion of the 16S rRNA Gene	Patricia S. Conville, Steven H. Fischer, Charles P. Cartwright, and Frank G. Witebsky	158-164

Restriction Fragment Length Polymorphism Analysis of <i>Mycobacterium tuberculosis</i> Isolated from Countries in the Western Pacific Region	Young-Kil Park, Gill-Han Bai, and Sang-Jae Kim	191-197
Sequence-Based Identification of <i>Mycobacterium</i> Species Using the MicroSeq 500 16S rDNA Bacterial Identification System	Jean Baldus Patel, Debra G. B. Leonard, Xai Pan, James M. Musser, Richard E. Berman, and Irving Nachamkin	246-251
Differentiation of Phylogenetically Related Slowly Growing Mycobacteria by Their <i>gyrB</i> Sequences	Hiroaki Kasai, Takayuki Ezaki, and Shigeaki Harayama	301-308
Evaluation of the BACTEC MGIT 960 and the MB/BacT Systems for Recovery of Mycobacteria from Clinical Specimens and for Species Identification by DNA AccuProbe	Fernando Alcaide, Miguel Angel Benítez, Josep M. Escribà, and Rogelio Martín	398-401
Differentiation of <i>Mycobacterium tuberculosis</i> Complex and Nontuberculous Mycobacterial Liquid Cultures by Using Peptide Nucleic Acid-Fluorescence In Situ Hybridization Probes	F. A. Drobniewski, P. G. More, and G. S. Harris	444-447

MYCOLOGY

New Monoclonal Antibody Specific for <i>Candida albicans</i> Germ Tube	Agnes Marot-Leblond, Linda Grimaud, Sandrine Nail, Sandrine Bouterige, Veronique Apaire-Marchais, Derek J. Sullivan, and Raymond Robert	61-67
Recovery of <i>Candida dubliniensis</i> from Non-Human Immunodeficiency Virus-Infected Patients in Israel	Itzhack Polacheck, Jacob Strahilevitz, Derek Sullivan, Samantha Donnelly, Ira F. Salkin, and David C. Coleman	170-174
Typing of <i>Histoplasma capsulatum</i> Isolates Based on Nucleotide Sequence Variation in the Internal Transcribed Spacer Regions of rRNA Genes	Bingdong Jiang, Marilyn S. Bartlett, Stephen D. Allen, James W. Smith, L. Joseph Wheat, Patricia A. Connolly, and Chao-Hung Lee	241-245
Determination of Antifungal MICs by a Rapid Susceptibility Assay	Marcia H. Riesselman, Kevin C. Hazen, and Jim E. Cutler	333-340
Antifungal Susceptibility Testing of Dermatophytes: Establishing a Medium for Inducing Conidial Growth and Evaluation of Susceptibility of Clinical Isolates	C. J. Jessup, J. Warner, N. Isham, I. Hasan, and M. A. Ghannoum	341-344
Pulmonary Infection Caused by <i>Gymnascella hyalinospora</i> in a Patient with Acute Myelogenous Leukemia	Peter C. Iwen, Lynne Sigler, Stefano Tarantolo, Deanna A. Sutton, Michael G. Rinaldi, Rudy P. Lackner, Dora I. McCarthy, and Steven H. Hinrichs	375-381
<i>Microascus cinereus</i> (Anamorph <i>Scopulariopsis</i>) Brain Abscess in a Bone Marrow Transplant Recipient	John W. Baddley, Stephen A. Moser, Deanna A. Sutton, and Peter G. Pappas	395-397
Fungemia Due to <i>Fusarium sacchari</i> in an Immunosuppressed Patient	Josep Guarro, Marcio Nucci, Tiyomi Akiti, Josepa Gené, M. Da Gloria C. Barreiro, and Renato T. Gonçalves	419-421
<i>Aspergillus fumigatus</i> Antigen Detection in Sera from Patients at Risk for Invasive Aspergillosis	Bernabé F. F. Chumpitazi, Claudine Pinel, Bernadette Lebeau, Pierre Ambroise-Thomas, and Renee Grillot	438-443

PARASITOLOGY

- Differentiating *Taenia solium* and *Taenia saginata* Infections by Simple Hematoxylin-Eosin Staining and PCR-Restriction Enzyme Analysis** H. Mayta, A. Talley, R. H. Gilman, J. Jimenez, M. Verastegui, M. Ruiz, H. H. Garcia, and A. E. Gonzalez 133-137
- Performance of Immunoblotting in Diagnosis of Visceral Leishmaniasis in Human Immunodeficiency Virus-*Leishmania* sp.-Coinfected Patients** G. Santos-Gomes, S. Gomes-Pereira, L. Campino, M. De Almeida Araújo, and P. Abranches 175-178
- Serodiagnosis of Recently Acquired *Toxoplasma gondii* Infection with a Recombinant Antigen** Shuli Li, Greg Maine, Yasuhiro Suzuki, Fausto G. Araujo, Gina Galvan, Jack S. Remington, and Stephen Parmley 179-184
- Optimized PCR Using Patient Blood Samples for Diagnosis and Follow-Up of Visceral Leishmaniasis, with Special Reference to AIDS Patients** Laurence Lachaud, Jacques Dereure, Elisabeth Chabbert, Jacques Reynes, Jean-Marc Mauboussin, Eric Oziol, Jean-Pierre Dedet, and Patrick Bastien 236-240
- A Polymorphic Multigene Family Encoding an Immunodominant Protein from *Babesia microti*** M. J. Homer, E. S. Bruinsma, M. J. Lodes, M. H. Moro, S. Telford III, P. J. Krause, L. D. Reynolds, R. Mohamath, D. R. Benson, R. L. Houghton, S. G. Reed, and D. H. Persing 362-368
- Evaluation of a Combination Rapid Immunoassay for Detection of *Giardia* and *Cryptosporidium* Antigens** Raymond Chan, Jing Chen, Mary K. York, Norman Setijono, Raymond L. Kaplan, Fitzroy Graham, and Herbert B. Tanowitz 393-394
- Serological Detection of *Capillaria hepatica* by Indirect Immunofluorescence Assay** Martina Juncker-Voss, Heinrich Prosl, Helga Lussy, Ulrike Enzenberg, Herbert Auer, and Norbert Nowotny 431-433

VIROLOGY

- Development and Application of a PCR-Based Method Including an Internal Control for Diagnosis of Congenital Cytomegalovirus Infection** Rachel N. Jones, M. Lynne Neale, Brian Beattie, Diana Westmoreland, and Julie D. Fox 1-6
- Automated Specific Capture of Hepatitis C Virus RNA with Probes and Paramagnetic Particle Separation** Hayato Miyachi, Atsuko Masukawa, Toshio Ohshima, Toru Hirose, Chaka Impraim, and Yasuhiko Ando 18-21
- Neutralization Assay for Human Group C Rotaviruses Using a Reverse Passive Hemagglutination Test for Endpoint Determination** Ritsushi Fujii, Mitsutaka Kuzuya, Masako Hamano, Hajime Ogura, Masao Yamada, and Tadashige Mori 50-54
- Relationship of Incremental Specimen Volumes and Enhanced Detection of Human Immunodeficiency Virus Type 1 RNA with Nucleic Acid Amplification Technology** Donald J. Witt, M. Kemper, Andrew Stead, Christine C. Ginocchio, and Angela M. Caliendo 85-89
- Development of a TT Virus DNA Quantification System Using Real-Time Detection PCR** Takanobu Kato, Masashi Mizokami, Motokazu Mukaide, Etsuro Orito, Tomoyoshi Ohno, Tatsunori Nakano, Yasuhito Tanaka, Hideaki Kato, Fuminaka Sugauchi, Ryuzo Ueda, Noboru Hirashima, Kazuhide Shimamatsu, Masayoshi Kage, and Masamichi Kojiro 94-98

Continued on following page

Performance of Indirect Immunoglobulin M (IgM) Serology Tests and IgM Capture Assays for Laboratory Diagnosis of Measles	Samuel Ratnam, Graham Tipples, Carol Head, Micheline Fauvel, Margaret Fearon, and Brian J. Ward	99-104
Comparison of Antibody Titers Determined by Hemagglutination Inhibition and Enzyme Immunoassay for JC Virus and BK Virus	R. S. Hamilton, M. Gravel, and E. O. Major	105-109
In Vitro and In Vivo Evaluations of Sodium Lauryl Sulfate and Dextran Sulfate as Microbicides against Herpes Simplex and Human Immunodeficiency Viruses	Jocelyne Piret, Julie Lamontagne, Julie Bestman-Smith, Sylvie Roy, Pierrette Gourde, André Désormeaux, Rabea F. Omar, Julianna Juhász, and Michel G. Bergeron	110-119
VP7 and VP4 Genotyping of Human Group A Rotavirus in Buenos Aires, Argentina	M. H. Argüelles, G. A. Villegas, A. Castello, A. Abrami, P. D. Ghiringhelli, L. Semorile, and G. Glikmann	252-259
Nested PCR for Rapid Detection of Mumps Virus in Cerebrospinal Fluid from Patients with Neurological Diseases	Gustavo Palacios Poggio, Claudia Rodriguez, Daniel Cisterna, María Cecilia Freire, and Jerónimo Cello	274-278
Recovery and Analysis of Human Immunodeficiency Virus Type 1 (HIV) RNA Sequences from Plasma Samples with Low HIV RNA Levels	Jordi Niubò, Wuyi Li, Keith Henry, and Alejo Erice	309-312
Stability of Plasma Human Immunodeficiency Virus Load in VACUTAINER PPT Plasma Preparation Tubes during Overnight Shipment	Mark Holodniy, Lynne Rainen, Steve Herman, and Belinda Yen-Lieberman	323-326
Improved Amplification of Genital Human Papillomaviruses	P. E. Gravitt, C. L. Peyton, T. Q. Alessi, C. M. Wheeler, F. Coutlée, A. Hildesheim, M. H. Schiffman, D. R. Scott, and R. J. Apple	357-361
Genotype Profiles of Rotavirus Strains from Children in a Suburban Community in Guinea-Bissau, Western Africa	Thea Kølsten Fischer, Hans Steinsland, Kåre Mølbak, Rui Ca, Jon R. Gentsch, Palle Valentiner-Branth, Peter Aaby, and Halvor Sommerfelt	264-267
Quantitation of Human Immunodeficiency Virus Type 1 Group O Load in Plasma by Measuring Reverse Transcriptase Activity	J. Gerardo García Lerma, Vincent Soriano, Antonio Mas, Miguel E. Quiñones-Mateu, Eric J. Arts, and Walid Heneine	402-405
Polyomaviruria in Renal Transplant Patients Is Not Correlated to the Cold Ischemia Period or to Rejection Episodes	P. Priftakis, G. Bogdanovic, G. Tyden, and T. Dalianis	406-407
Additional Human Papillomavirus Types Detected by the Hybrid Capture Tube Test among Samples from Women with Cytological and Colposcopic Atypia	József Kónya, György Veress, Attila Juhász, Krisztina Szarka, Tamás Sápy, Zoltán Hernádi, and Lajos Gergely	408-411
Mink Lung Cells and Mixed Mink Lung and A549 Cells for Rapid Detection of Influenza Virus and Other Respiratory Viruses	Yung T. Huang and Brian M. Turchek	422-423
2-Hour Cytomegalovirus pp65 Antigenemia Assay for Rapid Quantitation of Cytomegalovirus in Blood Samples	Marie L. Landry and David Ferguson	427-428
Impact of Sample Type on Rapid Detection of Influenza Virus A by Cytospin-Enhanced Immunofluorescence and Membrane Enzyme-Linked Immunosorbent Assay	Marie L. Landry, Sandra Cohen, and David Ferguson	429-430

CLINICAL VETERINARY MICROBIOLOGY

- Prevalence of *Escherichia coli* O157:H7 from Cull Dairy Cows in New York State and Comparison of Culture Methods Used during Preharvest Food Safety Investigations Patrick L. McDonough, Christine A. Rossiter, Robert B. Rebhun, Susan M. Stehman, Donald H. Lein, and Sang J. Shin 318-322
- Comparison of *Pasteurella* spp. Simultaneously Isolated from Nasal and Transtracheal Swabs from Cattle with Clinical Signs of Bovine Respiratory Disease D. C. DeRosa, G. D. Mechor, J. J. Staats, M. M. Chengappa, and T. R. Shryock 327-332
- Molecular Cloning and Characterization of the 120-Kilodalton Protein Gene of *Ehrlichia canis* and Application of the Recombinant 120-Kilodalton Protein for Serodiagnosis of Canine Ehrlichiosis Xue-jie Yu, Jere W. McBride, C. Marcela Diaz, and David H. Walker 369-374
- Quality Control Guidelines for Disk Diffusion and Broth Microdilution Antimicrobial Susceptibility Tests with Seven Drugs for Veterinary Applications Brant A. Odland, Meredith E. Erwin, and Ronald N. Jones 453-455

EPIDEMIOLOGY

- Phenotypic and Molecular Typing of Nosocomial Methicillin-Resistant *Staphylococcus aureus* Strains Susceptible to Gentamicin Isolated in France from 1995 to 1997 Jacques-Olivier Galdbart, Anne Morvan, and Nevine El Solh 185-190
- Typing of *Candida glabrata* in Clinical Isolates by Comparative Sequence Analysis of the Cytochrome *c* Oxidase Subunit 2 Gene Distinguishes Two Clusters of Strains Associated with Geographical Sequence Polymorphisms Gerdine F. O. Sanson and Marcelo R. S. Briones 227-235
- Pulsed-Field Gel Electrophoresis-Based Molecular Comparison of *Vibrio cholerae* O1 Isolates from Domestic and Imported Cases of Cholera in Japan Eiji Arakawa, Toshiyuki Murase, Shigeru Matsushita, Toshio Shimada, Shiro Yamai, Takeshi Ito, and Haruo Watanabe 424-426

CASE REPORTS

- Peritonitis Due to *Roseomonas fauriae* in a Patient Undergoing Continuous Ambulatory Peritoneal Dialysis Evangelia Bibashi, Danai Sofianou, Konstantina Kontopoulou, Efsthios Mitsopoulos, and Elisabeth Kokolina 456-457
- Bacteremic Pneumonia Caused by a Single Clone of *Streptococcus pneumoniae* with Different Optochin Susceptibilities Hsiu-Yuan Tsai, Po-Ren Hsueh, Lee-Jene Teng, Ping-Ing Lee, Li-Min Huang, Chin-Yun Lee, and Kwen-Tay Luh 458-459
- Isolation of *Pantoea agglomerans* in Two Cases of Septic Monoarthritis after Plant Thorn and Wood Sliver Injuries C. De Champs, S. Le Seaux, J. J. Dubost, S. Boisgard, B. Sauvezie, and J. Sirot 460-461
- Streptococcus bovis* Meningitis in an Infant Russell J. Grant, Terence R. Whitehead, and James E. Orr 462-463

FAST-TRACK COMMUNICATION

- Achieving 100% Typeability of *Pseudomonas aeruginosa* by Pulsed-Field Gel Electrophoresis Ute Römling and Burkhard Tümmler 464-465

LETTERS TO THE EDITOR

- Is a Large Number of Sputum Specimens Necessary for the Bacteriological Diagnosis of Tuberculosis? Alessandro Cascina, Anna Fietta, and Lucio Casali 466

Continued on following page

Detection of *Bordetella holmesii* Using *Bordetella pertussis* IS481 PCR Assay

Mike J. Loeffelholz, Curt J. Thompson, Karla S. Long, and Mary J. R. Gilchrist 467

Two *Actinobacillus pleuropneumoniae* Serotype 8 Reference Strains in Circulation

Trine Gram, Peter Ahrens, and Øystein Angen 468

Acute Renal Failure in an Infant Associated with Cytotoxic *Aeromonas sobria* Isolated from Patient's Stool and from Aquarium Water as Suspected Source of Infection

Guido Filler, Jochen H. H. Ehrich, Eckhard Strauch, and Lothar Beutin 469-470

Rapid Mini-Preparation of Fungal DNA for PCR

Don Liu, Sue Coloe, Rob Baird, and John Pedersen 471

Obtaining Unacceptable Results in Assays for Quantitation of Human Immunodeficiency Virus Type 1 RNA in Plasma Samples

A. Aguilera, A. Vela, M. Treviño, E. Varela, R. Seoane, and B. J. Regueiro 472-473

ERRATA

Differentiation of *Campylobacter jejuni* Serotype O19 Strains from Non-O19 Strains by PCR

Naoaki Misawa, Ban Mishu Allos, and Martin J. Blaser 474

Case of *Staphylococcus schleiferi* Endocarditis and a Simple Scheme To Identify Clumping Factor-Positive Staphylococci

Michael J. Leung, Nicholas Nuttall, Margaret Mazur, Tania L. Taddei, Michael McComish, and John W. Pearman 474

AUTHOR'S CORRECTION

Characterization of *Bacteroides forsythus* Strains from Cat and Dog Bite Wounds in Humans and Comparison with Monkey and Human Oral Strains

M. K. Hudspeth, S. Hunt Gerardo, M. F. J. Maiden, D. M. Citron, and E. J. C. Goldstein 475

Antifungal Susceptibility Testing of Dermatophytes: Establishing a Medium for Inducing Conidial Growth and Evaluation of Susceptibility of Clinical Isolates

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A standardized reference method for dermatophyte in vitro susceptibility testing is lacking. In a previous study, Norris et al. (H. A. Norris, B. E. Elewski, and M. A. Ghannoum, *J. Am. Acad. Dermatol.* 40(6, part 2):S9–S13) established the optimal medium and other growth variables. However, the earlier study did not address two issues: (i) selection of an optimal medium for conidial formation by dermatophytes and (ii) validation of the method with a large number of dermatophytes. The present study addresses these two points. To select which agar medium best supported conidial growth, representative isolates of dermatophytes were grown on different agars. Preliminary experiments showed that only oatmeal cereal agar supported the production of conidia by *Trichophyton rubrum*. We tested the abilities of 251 *T. rubrum* isolates to form conidia using three different cereal agars and potato dextrose agar. Overall, oatmeal cereal and rice agar media were comparable in their abilities to support *T. rubrum* conidial growth. Next, we used the oatmeal cereal agar for conidial formation along with the optimal conditions for dermatophyte susceptibility testing proposed by Norris et al. and determined the antifungal susceptibilities of 217 dermatophytes to fluconazole, griseofulvin, itraconazole, and terbinafine. Relative to the other agents tested, terbinafine possessed the highest antifungal activity against all of the dermatophytes. The mean \pm standard error of the mean MICs of fluconazole, itraconazole, terbinafine, and griseofulvin were 2.07 ± 0.29 , 0.13 ± 0.01 , 0.002 ± 0.0003 , and 0.71 ± 0.05 $\mu\text{g/ml}$, respectively. This study is the first step in the identification of optimal conditions that could be used for the standardization of the antifungal susceptibility testing method for dermatophytes. Inter- and intralaboratory agreement as well as clinical correlations need to be established.

In the last two decades the incidence of infections caused by dermatophytes and other fungi has increased considerably (1, 7, 11). With an increasing variety of drugs available for the treatment of dermatophytoses, the need for a reference method for the testing of the antifungal susceptibilities of dermatophytes has become apparent (3, 7, 9, 11). Establishment of a reference susceptibility testing method may allow the clinician to select the appropriate therapy for the treatment of infections caused by dermatophytic fungi. Recently, a standard method for antifungal susceptibility testing of yeasts has been established by the National Committee for Clinical Laboratory Standards (NCCLS; M27-A document) (6). This reference method for yeast is the first step in the establishment of a reliable, standardized, and clinically useful technique for the susceptibility testing of filamentous and dermatophytic fungi. Efforts to develop a reference method for broth dilution antifungal susceptibility testing of filamentous fungi are being pursued by NCCLS (1a, 8). This paper represents the first attempt at standardizing the antifungal susceptibility testing of dermatophytes.

In developing this method for antifungal susceptibility testing of dermatophytic fungi, many variables need to be considered. An earlier investigation by Norris et al. (7) evaluated inoculum size, temperature and duration of incubation, medium, and endpoint determination. Although the earlier study established many important variables concerning those condi-

tions necessary for optimization of the susceptibility testing method for dermatophytes, it did not address which medium is appropriate for conidial formation. Identification of such a medium is critical since dermatophytes (particularly *Trichophyton rubrum*) are known to be poor producers of conidia (11). An isolate's inability to produce conidia will hamper our ability to determine the susceptibility or resistance of that particular isolate.

In this study, we established oatmeal cereal agar and rice agar as the optimal agar media for support of conidial growth. We also expanded the antifungal susceptibility findings of Norris et al. (7) by determining the antifungal susceptibilities of a larger number of dermatophytes isolates to four clinically used antifungal agents. The results from this study serve as a foundation for the development of a standardized susceptibility testing method for dermatophytes.

MATERIALS AND METHODS

Identification of an appropriate medium for production of conidia by various dermatophytes. (i) Agar. Three types of agar media were initially used to evaluate the conidial growth of the selected dermatophyte isolates (see below): Mycosel agar with 1% yeast extract, potato dextrose agar (both from Becton Dickinson and Company, Cockeysville, Md.), and Heinz oatmeal cereal (H. J. Heinz Co., Pittsburgh, Pa.). On the basis of preliminary data, a continuation study evaluating a larger number of isolates for their conidial growth was performed by using two different cereal agars (mixed grains [H. J. Heinz Co.] and Beech-Nut Rice [Beech-Nut Nutrition Corp., St. Louis, Mo.]), along with oatmeal cereal agar and potato dextrose agar. To prepare the cereal agars, 100 g of the dried ingredients was mixed with 15 g of Bacto agar (Difco Laboratories, Detroit, Mich.). These components were then stirred into 1 liter of distilled water and were mixed thoroughly. The mixture was then autoclaved for 20 min and was then allowed to cool by placing it into a 10°C water bath. Once it was cooled, the mixture was autoclaved again for an additional 20 min. Preliminary experiments

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TABLE 1. Percentages of conidium-forming *T. rubrum* isolates on three types of agar media at 4 and 7 days of incubation^a

Medium	% of <i>T. rubrum</i> isolates with the following conidial growth at the indicated times:							
	No conidia		1 to 15 conidia/field		16 to 25 conidia/field		≥26 conidia/field	
	4 days	7 days	4 days	7 days	4 days	7 days	4 days	7 days
Heinz oatmeal cereal	0	0	0	0	0	0	100	100
Potato dextrose	40	30	20	10	10	10	30	50
Mycosel (1% yeast extract)	40	40	60	30	0	10	0	20

^a A total of 10 isolates were tested.

revealed that the hold time and the second autoclaving are critical to kill any *Bacillus* spores present in the mixture.

(ii) **Organisms.** We initially tested 43 isolates of dermatophytes including 10 strains each of *T. rubrum*, *Trichophyton mentagrophytes*, and *Trichophyton tonsurans*, 7 strains of *Epidermophyton floccosum*, and 6 strains of *Microsporium canis*. The continuation study for conidial growth involved 251 strains of *T. rubrum*. All strains were clinical isolates obtained from nail or hair specimens received from clinicians by the Center for Medical Mycology, University Hospitals of Cleveland. Dermatophytes were identified to the species level by conventional methods (5). Isolates were stored at -80°C on potato dextrose agar slants until the time of use.

(iii) **Determination of conidial growth.** Dermatophytes were grown on different agar media at 30°C for 4 to 7 days. At the end of the incubation period, cellophane tape preparations were used to quantitate the conidial formation microscopically. For each isolate the numbers of conidia were determined in five viewing fields. The average numbers of conidia were calculated for each strain. The ability of various media to support conidial formation was scored on a scale of from 0 to 3, where 0 implies no conidiation and 3 implies proliferative conidiation. The data were expressed as conidial formation as a percentage of that for *T. rubrum* isolates forming conidia on different agar media.

In vitro susceptibility testing. (i) Organisms. We tested 217 isolates of dermatophytes including *T. rubrum* ($n = 132$), *T. mentagrophytes* ($n = 32$), *T. tonsurans* ($n = 42$), *E. floccosum* ($n = 3$), and *M. canis* ($n = 8$). Two American Type Culture Collection (ATCC; Rockville, Md.) quality control organisms were used: *Candida parapsilosis* ATCC 22019 and *Paecilomyces variotti* ATCC 22319. All strains were clinical isolates obtained from nail or hair specimens received from clinicians by the Center for Medical Mycology, University Hospitals of Cleveland. Dermatophytes were identified and were stored as described above.

(ii) **Antifungal agents.** Four antifungal drugs, supplied by the manufacturers as powders, were used: fluconazole (Pfizer Pharmaceuticals Group, New York, N.Y.), terbinafine (Novartis, E. Hanover, N.J.), itraconazole (Janssen Research Foundation, Beerse, Belgium), and griseofulvin (Sigma Chemical Company, St. Louis, Mo.). Fluconazole was dissolved in sterile water, itraconazole and griseofulvin were dissolved in 100% dimethyl sulfoxide (Curtin Matheson Scientific Inc., Houston, Tex.), and terbinafine was dissolved in dimethyl sulfoxide with 5% Tween 80 (Curtin Matheson Scientific Inc., Houston, Tex.). All drugs were prepared as stock solutions of 1 mg/ml.

(iii) **Medium.** RPMI 1640 (American Bionanics Inc., Niagara Falls, N.Y.) with L-glutamine but without sodium bicarbonate and buffered at pH 7.0 with 3-(*N*-morpholino)propanesulfonic acid, monosodium salt (MOPS), was the medium used for broth microdilution susceptibility testing.

(iv) **Drug dilutions.** Serial twofold dilutions were prepared according to the NCCLS M27-A (6) proposed standard. Fluconazole and griseofulvin had MIC ranges of 0.13 to 64.0 µg/ml. Itraconazole and terbinafine had MIC ranges of 0.06 to 32.0 µg/ml.

(v) **Inoculum preparation.** We prepared a standardized inoculum by counting the microconidia microscopically. Cultures were grown on oatmeal cereal agar slants for 7 days at 30°C. Sterile normal saline (85%) was added to the slant, and the culture was gently swabbed with a cotton tip applicator to dislodge the conidia from the hyphal mat. The suspension was transferred to a sterile centrifuge tube, and the volume was adjusted to 5 ml with sterile normal saline. The resulting suspension was counted on a hemocytometer and was diluted in RPMI 1640 to the desired concentration.

(vi) **Broth microdilution testing.** Microdilution plates were set up in accordance with the NCCLS M27-A (6) reference method; the exception was the inoculum preparation, which was set up as described above. Column 1 was filled with 200 µl of medium to serve as a sterility control. Columns 2 through 11 were filled with 100 µl of the inoculum and 100 µl of the serially diluted antifungal agent. Column 12 was filled with 200 µl of the inoculum and served as a growth control.

(vii) **Incubation time and temperature.** The microdilution plates were incubated at 35°C and were read visually after 4 days of incubation.

(viii) **Endpoint criteria.** The MIC was defined as the point at which the organism was inhibited 80% compared with the growth in the control well. All isolates were run in duplicate and the results were read visually. For the two isolates tested with fluconazole as quality controls, MICs were within the ex-

pected range (for *C. parapsilosis*, 4.0 µg/ml; for *P. variotti*, 64.0 µg/ml) specified in document M-27A (6).

RESULTS

Comparison of conidial growth of common dermatophytes on different agar media. Three types of agar media (potato dextrose, Mycosel with 1% yeast extract, and Heinz oatmeal cereal) were compared for their abilities to induce conidiation. The representative dermatophyte isolates tested were *T. rubrum* ($n = 10$), *T. mentagrophytes* ($n = 10$), *T. tonsurans* ($n = 10$), *E. floccosum* ($n = 7$), and *M. canis* ($n = 6$). All isolates of the last four species produced abundant conidia (≥26 conidia/field), irrespective of the medium used. The ability of *T. rubrum* to form conidia was medium dependent. Potato dextrose agar and Mycosel supported only limited growth of *T. rubrum* conidia at both 4 and 7 days of incubation; i.e., between 10 and 60% of the isolates were able to produce a small number of conidia (1 to 15 conidia/field). In contrast, by 4 days, oatmeal cereal agar promoted the production of a large number of conidia (≥26 conidia/field) from all *T. rubrum* isolates examined in this preliminary screen. Comparison of the abilities of various isolates to produce conidia at 4 and 7 days showed that 4 days was sufficient for growth of abundant conidia from all isolates of *T. mentagrophytes*, *T. tonsurans*, *E. floccosum*, and *M. canis*. *T. rubrum*, on the other hand, produced abundant conidia following growth for 4 and 7 days when it was cultured on oatmeal cereal but not potato dextrose or Mycosel (Table 1). On the last two media, conidial production by *T. rubrum* was enhanced when the organisms were incubated for 7 days. However, there was no conidial growth for 30 to 40% of the isolates even after 7 days of incubation.

Having identified oatmeal cereal agar as a potentially useful medium for conidial formation for all dermatophytic species tested, we wanted to test the ability of a large number of *T. rubrum* isolates ($n = 251$) to form conidia with three different cereal agars (oatmeal cereal, rice, and mixed grains) and potato dextrose agar. Overall, even though the oatmeal cereal

TABLE 2. Percentages of conidium-forming *T. rubrum* isolates on three types of cereal media agar and potato dextrose agar at 7 days of incubation^a

Medium	% of <i>T. rubrum</i> isolates with the following conidial growth:			
	No conidia	1 to 15 conidia/field	16 to 25 conidia/field	≥26 conidia/field
Heinz oatmeal cereal	15.7	15.7	24.1	44.6
Beech-Nut rice	14.7	14.4	31.6	38.6
Heinz mixed grains	26.1	41.1	18.4	14.5
Potato dextrose	28.8	21.6	16.5	33.0

^a A total of 251 isolates were tested.

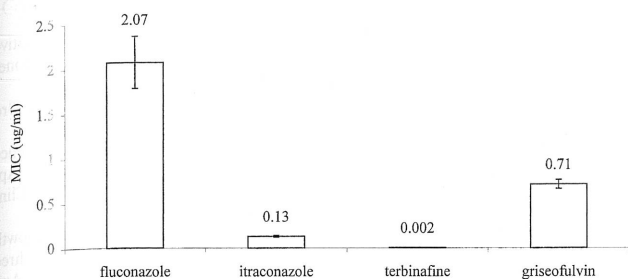


FIG. 1. Mean MICs of fluconazole, itraconazole, terbinafine, and griseofulvin for 217 dermatophytes by the proposed method for in vitro susceptibility testing.

agar was slightly more effective than rice in its ability to support production of conidia by approximately 84% of the *T. rubrum* isolates tested, these two media were comparable in their abilities to support *T. rubrum* conidial growth, as seen in Table 2. About 15% of *T. rubrum* isolates failed to produce conidia in any of the media tested.

Determination of antifungal susceptibilities of 217 dermatophytes by using optimized conditions. By using the optimized conditions the MICs of griseofulvin, itraconazole, terbinafine, and fluconazole for 217 dermatophyte isolates including *T. rubrum* ($n = 132$), *T. mentagrophytes* ($n = 32$), *T. tonsurans* ($n = 42$), *E. floccosum* ($n = 3$), and *M. canis* ($n = 8$) were determined. Our data show that terbinafine was the most active antifungal agent tested against dermatophytes. As shown in Fig. 1, the mean \pm standard error of the mean MICs were 2.07 ± 0.29 , 0.13 ± 0.01 , 0.002 ± 0.0003 , and 0.71 ± 0.05 $\mu\text{g/ml}$ for fluconazole, itraconazole, terbinafine, and griseofulvin, respectively. Additionally, the MIC at which 50% of isolates are inhibited (MIC_{50}) and the MIC_{90} of terbinafine (MIC_{50} , 0.001 $\mu\text{g/ml}$; MIC_{90} , 0.004 $\mu\text{g/ml}$) were 130- and 250-fold lower, respectively, than those of the second most active antifungal agent (itraconazole), as seen in Table 3.

DISCUSSION

A standardized dermatophyte susceptibility testing technique should encompass the following: an ideal growth medium, a specific protocol with reference to the initial inoculum size, a specific incubation time, a specific incubation temperature, and an MIC endpoint determination which is applicable to all dermatophytes. Another important factor for the determination of antifungal susceptibility which is particularly important for the testing of dermatophytes is the selection of the most appropriate medium that will support conidial growth. Norris et al. (7) were the first to identify the optimal param-

eters to be used in performing antifungal susceptibility testing of dermatophytes. In their study, variables such as growth medium, inoculum size, and length and temperature of incubation were addressed.

In this study, we showed that, of those media tested, oatmeal cereal agar and rice agar preparations are the most appropriate for the production of conidia from various dermatophyte species, specifically, *T. rubrum*. Identification of an appropriate medium for conidial production is of critical importance since an inability to produce spores will limit our ability to prepare the necessary inoculum for the initiation of testing. Although the majority of dermatophytes are capable of producing conidia in different media, the induction of conidiation by *T. rubrum* has proven to be difficult (7). Since *T. rubrum* is an important pathogen responsible for a significant number of dermatophyte infections (e.g., over 90% of nail infections are caused by this organism) (1), we decided to investigate the medium appropriate for induction of conidiation by this species. Our data suggest that both oatmeal cereal agar and rice agar could be adopted as media for the induction of conidiation for the standard in vitro dermatophyte susceptibility testing method. Unfortunately, approximately 15% of the isolates tested failed to produce conidia even in the most efficient media (oatmeal cereal and rice). Determination of the antifungal susceptibilities of these organisms will not be possible by currently accepted methods. Other conditions or media that may enhance the sporulation ability of these *T. rubrum* strains for which induction of sporulation is difficult should be investigated.

In this study we extended the findings of Norris et al. (7) and provided a more comprehensive investigation by testing a larger number of isolates. Using the proposed conditions, we were able to determine the susceptibility profiles of the antifungal agents used to treat dermatophyte infections. Importantly, this profile agrees with earlier reports (10) comparing the in vitro activities of various agents. For example, our data confirm earlier reports showing that terbinafine has the highest level of activity against dermatophytes (10). Furthermore, there was very little difference in the activity of terbinafine against different species, illustrating its uniformly high level of activity.

Our study shows that various parameters, RPMI 1640 medium, an incubation temperature of 35°C, an incubation time of 4 days, and an inoculum of 10^3 conidia/ml, are optimal for determination of the antifungal susceptibilities of dermatophytes. These conditions, along with our suggested agar medium (oatmeal cereal or rice), combine to provide optimal conditions by which one can obtain the MICs of antifungal agents for dermatophytes. Therefore, these proposed parameters form the foundation for a standardized antifungal sus-

TABLE 3. MIC_{50} s and MIC_{90} s of fluconazole, itraconazole, terbinafine, and griseofulvin for 217 dermatophytes

Organism	Total no. of isolates	MIC ($\mu\text{g/ml}$)							
		Fluconazole		Itraconazole		Terbinafine		Griseofulvin	
		50%	90%	50%	90%	50%	90%	50%	90%
<i>T. rubrum</i>	132	1.0	2.0	0.13	0.5	0.001	0.002	0.5	2.0
<i>T. mentagrophytes</i>	32	2.0	16.0	<0.06	0.5	0.001	0.001	0.13	0.25
<i>T. tonsurans</i>	42	1.0	8.0	<0.06	0.06	0.002	0.008	0.5	4.0
<i>M. canis</i>	8	0.25	2.0	<0.06	0.06	0.008	0.03	0.25	1.0
<i>E. floccosum</i>	3	4.0	4.0	<0.06	<0.06	0.015	0.015	1.0	2.0
All dermatophytes	217	1.0	4.0	0.06	0.25	0.001	0.004	0.5	2.0

ceptibility testing method for dermatophytes. A number of future studies that use these conditions are proposed. A larger sample of dermatophytes needs to be tested to determine the inter- and intralaboratory agreements of such a method. Additionally, MICs need to be correlated with clinical outcome to develop interpretive breakpoints for dermatophyte susceptibility testing.

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