ABOUT AJMR

The African Journal of Microbiology Research (AJMR) (ISSN 1996-0808) is published Weekly (one volume per year) by Academic Journals.

African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Submission of Manuscript

Please read the Instructions for Authors before submitting your manuscript. The manuscript files should be given the last name of the first author.

Click here to Submit manuscripts online

If you have any difficulty using the online submission system, kindly submit via this email ajmr@academicjournals.org.

With questions or concerns, please contact the Editorial Office at ajmr@academicjournals.org.
Editors

Prof. Dr. Stefan Schmidt,
*Applied and Environmental Microbiology*
School of Biochemistry, Genetics and Microbiology
University of KwaZulu-Natal
Private Bag X01
Scottsville, Pietermaritzburg 3209
South Africa.

Prof. Fukai Bao
*Department of Microbiology and Immunology*
Kunming Medical University
Kunming 650031,
China

Dr. Jianfeng Wu
*Dept. of Environmental Health Sciences, School of Public Health, University of Michigan*
USA

Dr. Ahmet Yilmaz Coban
*OMU Medical School, Department of Medical Microbiology, Samsun, Turkey*

Dr. Seyed Davar Siadat
*Pasteur Institute of Iran, Pasteur Square, Pasteur Avenue, Tehran, Iran.*

Dr. J. Stefan Rokem
*The Hebrew University of Jerusalem, Department of Microbiology and Molecular Genetics, P.O.B. 12272, IL-91120 Jerusalem, Israel*

Prof. Long-Liu Lin
*National Chiayi University, 300 Syuefu Road, Chiayi, Taiwan*

N. John Tonukari, Ph.D
*Department of Biochemistry*
*Delta State University, PMB 1, Abraka, Nigeria*

Dr. Thaddeus Ezeji
*Assistant Professor*
*Fermentation and Biotechnology Unit, Department of Animal Sciences, The Ohio State University, 1680 Madison Avenue, USA.*

Associate Editors

Dr. Mamadou Gueye
*MIRCEN/ Laboratoire commun de microbiologie, IRD-ISRA-UCAD, BP 1386, DAKAR, Senegal.*

Dr. Caroline Mary Knox
*Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, Grahamstown 6140, South Africa.*

Dr. Hesham Elsayed Mostafa
*Genetic Engineering and Biotechnology Research Institute (GEBRI), Mubarak City For Scientific Research, Research Area, New Borg El-Arab City, Post Code 21934, Alexandria, Egypt.*

Dr. Wael Abbas El-Naggar
*Head of Microbiology Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.*

Dr. Abdel Nasser A. El-Moghazy
*Microbiology, Molecular Biology, Genetics Engineering and Biotechnology, Dept of Microbiology and Immunology, Faculty of Pharmacy, Al-Azhar University, Nasr city, Cairo, Egypt.*
Dr. Barakat S.M. Mahmoud
Food Safety/Microbiology
Experimental Seafood Processing Laboratory
Coastal Research and Extension Center
Mississippi State University
3411 Frederic Street
Pascagoula, MS 39567
USA

Prof. Mohamed Mahrous Amer
Poultry Disease (Viral Diseases of poultry)
Faculty of Veterinary Medicine,
Department of Poultry Diseases
Cairo university
Giza, Egypt

Dr. Xiaohui Zhou
Molecular Microbiology, Industrial Microbiology,
Environmental Microbiology, Pathogenesis, Antibiotic
resistance, Microbial Ecology
Washington State University
Bustad Hall 402 Department of Veterinary
Microbiology and Pathology, Pullman,
USA

Dr. R. Balaji Raja
Department of Biotechnology,
School of Bioengineering,
SRM University,
Chennai
India

Dr. Aly E Abo-Amer
Division of Microbiology, Botany Department, Faculty
of Science, Sohag University.
Egypt.

Editorial Board

Dr. Haoyu Mao
Department of Molecular Genetics and Microbiology
College of Medicine
University of Florida
Florida, Gainesville
USA.

Dr. Rachna Chandra
Environmental Impact Assessment Division
Environmental Sciences
Sálim Ali Center for Ornithology and Natural History
(SACON),
Anaikatty (PO), Coimbatore-641108, India

Dr. Yongxu Sun
Department of Medicinal Chemistry and
Biomacromolecules
Qiqihar Medical University, Qiqihar 161006
Heilongjiang Province
P.R. China

Dr. Ramesh Chand Kasana
Institute of Himalayan Bioresource Technology
Palampur, Distt. Kangra (HP),
India

Dr. S. Meena Kumari
Department of Biosciences
Faculty of Science
University of Mauritius
Reduit

Dr. T. Ramesh
Assistant Professor
Marine Microbiology
CAS in Marine Biology
Faculty of Marine Sciences
Annamalai University
Parangipettai - 608 502
Cuddalore Dist. Tamilnadu,
India

Dr. Pagano Marcela Claudia
Post doctoral fellowship at Department of Biology,
Federal University of Ceará - UFC,
Brazil.
Dr. EL-Sayed E. Habib  
Associate Professor, 
Dept. of Microbiology, 
Faculty of Pharmacy, 
Mansoura University, 
Egypt.

Dr. Pongsak Rattanachaikunsopon  
Department of Biological Science, 
Faculty of Science, 
Ubon Ratchathani University, 
Warin Chamrap, Ubon Ratchathani 34190, 
Thailand

Dr. Gokul Shankar Sabesan  
Microbiology Unit, Faculty of Medicine, 
AIMST University 
Jalan Bedong, Semeling 08100, 
Kedah, 
Malaysia

Dr. Kwang Young Song  
Department of Biological Engineering, 
School of Biological and Chemical Engineering, 
Yanbian University of Science and Technology, 
Yanji, 
China.

Dr. Kamel Belhamel  
Faculty of Technology, 
University of Bejaia 
Algeria

Dr. Sladjana Jevremovic  
Institute for Biological Research 
Sinisa Stankovic, 
Belgrade, 
Serbia

Dr. Tamer Edirne  
Dept. of Family Medicine, Univ. of Pamukkale 
Turkey

Dr. R. Balaji Raja M.Tech (Ph.D)  
Assistant Professor, 
Department of Biotechnology, 
School of Bioengineering, 
SRM University, 
Chennai. 
India

Dr. Minglei Wang  
University of Illinois at Urbana-Champaign, USA

Dr. Mohd Fuat ABD Razak  
Institute for Medical Research 
Malaysia

Dr. Davide Pacifico  
Istituto di Virologia Vegetale – CNR 
Italy

Prof. Dr. Akrum Hamdy  
Faculty of Agriculture, Minia University, Egypt 
Egypt

Dr. Ntobeko A. B. Ntusi  
Cardiac Clinic, Department of Medicine, 
University of Cape Town and 
Department of Cardiovascular Medicine, 
University of Oxford 
South Africa and 
United Kingdom

Prof. N. S. Alzoreky  
Food Science & Nutrition Department, 
College of Agricultural Sciences & Food, 
King Faisal University, 
Saudi Arabia

Dr. Chen Ding  
College of Material Science and Engineering, 
Hunan University, 
China

Dr Svetlana Nikolić  
Faculty of Technology and Metallurgy, 
University of Belgrade, 
Serbia

Dr. Sivakumar Swaminathan  
Department of Agronomy, 
College of Agriculture and Life Sciences, 
Iowa State University, 
Ames, Iowa 50011 
USA

Dr. Alfredo J. Anceno  
School of Environment, Resources and Development (SERD), 
Asian Institute of Technology, 
Thailand

Dr. Iqbal Ahmad  
Aligarh Muslim University, 
Aligrah 
India
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Josephine Nketsia-Tabiri</td>
<td>Ghana Atomic Energy Commission, Ghana</td>
</tr>
<tr>
<td>Dr. Juliane Elisa Welke</td>
<td>UFRGS – Universidade Federal do Rio Grande do Sul, Brazil</td>
</tr>
<tr>
<td>Dr. Mohammad Nazrul Islam</td>
<td>NIMR; IPH-Bangalore &amp; NIUM, Bangladesh</td>
</tr>
<tr>
<td>Dr. Okonko, Iheanyi Omezuruike</td>
<td>Department of Virology,</td>
</tr>
<tr>
<td></td>
<td>Faculty of Basic Medical Sciences,</td>
</tr>
<tr>
<td></td>
<td>College of Medicine,</td>
</tr>
<tr>
<td></td>
<td>University of Ibadan,</td>
</tr>
<tr>
<td></td>
<td>University College Hospital,</td>
</tr>
<tr>
<td></td>
<td>Ibadan,</td>
</tr>
<tr>
<td></td>
<td>Nigeria</td>
</tr>
<tr>
<td>Dr. Giuliana Noratto</td>
<td>Texas A&amp;M University,</td>
</tr>
<tr>
<td></td>
<td>USA</td>
</tr>
<tr>
<td>Dr. Phanikanth Venkata Turlapati</td>
<td>Washington State University,</td>
</tr>
<tr>
<td></td>
<td>USA</td>
</tr>
<tr>
<td>Dr. Khaleel I. Z. Jawasre</td>
<td>National Centre for Agricultural Research and</td>
</tr>
<tr>
<td></td>
<td>Extension, NCARE,</td>
</tr>
<tr>
<td></td>
<td>Jordan</td>
</tr>
<tr>
<td>Dr. Babak Mostafazadeh, MD</td>
<td>Shaheed Beheshty University of Medical Sciences,</td>
</tr>
<tr>
<td></td>
<td>Iran</td>
</tr>
<tr>
<td>Dr. S. Meena Kumari</td>
<td>Department of Biosciences,</td>
</tr>
<tr>
<td></td>
<td>Faculty of Science,</td>
</tr>
<tr>
<td></td>
<td>University of Mauritius,</td>
</tr>
<tr>
<td></td>
<td>Reduit,</td>
</tr>
<tr>
<td></td>
<td>Mauritius</td>
</tr>
<tr>
<td>Dr. Anju</td>
<td>Department of Biotechnology,</td>
</tr>
<tr>
<td></td>
<td>SRM University, Chennai-603203,</td>
</tr>
<tr>
<td></td>
<td>India</td>
</tr>
<tr>
<td>Dr. Mustafa Maroufpor</td>
<td>Iran</td>
</tr>
<tr>
<td>Dr. Prof. Dong Zhichun</td>
<td>Professor, Department of Animal Sciences and Veterinary Medicine, Yunnan Agriculture University, China</td>
</tr>
<tr>
<td>Dr. Mehdi Azami</td>
<td>Parasitology &amp; Mycology Dept,</td>
</tr>
<tr>
<td></td>
<td>Baghaeei Lab.,</td>
</tr>
<tr>
<td></td>
<td>Shams Abadi St.,</td>
</tr>
<tr>
<td></td>
<td>Isfahan, Iran</td>
</tr>
<tr>
<td>Dr. Anderson de Souza Sant’Ana</td>
<td>University of São Paulo,</td>
</tr>
<tr>
<td></td>
<td>Brazil</td>
</tr>
<tr>
<td>Dr. Juliane Elisa Welke</td>
<td>UFRGS – Universidade Federal do Rio Grande do Sul, Brazil</td>
</tr>
<tr>
<td>Dr. Paul Shapshak</td>
<td>USF Health, Depts. Medicine (Div. Infect. Disease &amp; Internat Med) and Psychiatry &amp; Beh Med, USA</td>
</tr>
<tr>
<td>Dr. Jorge Reinheimer</td>
<td>Universidad Nacional del Litoral (Santa Fe), Argentina</td>
</tr>
<tr>
<td>Dr. Qin Liu</td>
<td>East China University of Science and Technology, China</td>
</tr>
<tr>
<td>Dr. Xiao-Qing Hu</td>
<td>State Key Lab of Food Science and Technology, Jiangnan University, P. R. China</td>
</tr>
<tr>
<td>Prof. Branislava Kocic</td>
<td>Specialis in Microbiology and Parasitology, University of Nis, School of Medicine Institute for Public Health Nis, Bul. Z. Djindjica 50, 18000 Nis, Serbia</td>
</tr>
<tr>
<td>Dr. Rafel Socias</td>
<td>CITA de Aragón, Spain</td>
</tr>
</tbody>
</table>
Prof. Kamal I. Mohamed
State University of New York at Oswego
USA

Dr. Adriano Cruz
Faculty of Food Engineering-FEA
University of Campinas (UNICAMP)
Brazil

Dr. Mike Agenbag (Michael Hermanus Albertus)
Manager Municipal Health Services,
Joe Gqabi District Municipality
South Africa

Dr. D. V. L. Sarada
Department of Biotechnology,
SRM University, Chennai-603203
India.

Dr. Samuel K Ameyaw
Civista Medical Center
United States of America

Prof. Huaizhi Wang
Institute of Hepatopancreatobiliary Surgery of PLA Southwest Hospital,
Third Military Medical University
Chongqing400038
P. R. China

Prof. Bakhiet AO
College of Veterinary Medicine, Sudan
University of Science and Technology
Sudan

Dr. Saba F. Hussain
Community, Orthodontics and Pediatric Dentistry
Department
Faculty of Dentistry
Universiti Teknologi MARA
40450 Shah Alam, Selangor
Malaysia

Prof. Dr. Zohair I.F.Rahemo
State Key Lab of Food Science and Technology
Jiangnan University
P. R. China

Dr. Afework Kassu
University of Gondar
Ethiopia

Prof. Isidro A. T. Savillo
ISCOP
Philippines

Dr. How-Yee Lai
Taylor’s University College
Malaysia

Dr. Nidheesh Dadheech
MS. University of Baroda, Vadodara, Gujarat, India.
India

Dr. Omitoyin Siyanbola
Bowen University,
Iwo
Nigeria

Dr. Franco Mutinelli
Istituto Zooprofilattico Sperimentale delle Venezie
Italy

Dr. Chanpen Chanchao
Department of Biology,
Faculty of Science,
Chulalongkorn University
Thailand

Dr. Tsuyoshi Kasama
Division of Rheumatology,
Showa University
Japan

Dr. Kuender D. Yang, MD.
Chang Gung Memorial Hospital
Taiwan

Dr. Liane Raluca Stan
University Politehnica of Bucharest,
Department of Organic Chemistry “C.Nenitzescu”
Romania

Dr. Muhammed Osman
Senior Lecturer of Pathology & Consultant Immunopathologist
Department of Pathology,
Faculty of Medicine,
Universiti Teknologi MARA,
40450 Shah Alam, Selangor
Malaysia

Dr. Mohammad Feizabadi
Tehran University of medical Sciences
Iran
Prof. Ahmed H Mitwalli
State Key Lab of Food Science and Technology
Jiangnan University
P. R. China

Dr. Mazyar Yazdani
Department of Biology,
University of Oslo,
Blindern,
Oslo,
Norway

Dr. Ms. Jemimah Gesare Onsare
Ministry of Higher, Education Science and Technology
Kenya

Dr. Babak Khalili Hadad
Department of Biological Sciences,
Roudehen Branch,
Islamic Azad University,
Roudehen
Iran

Dr. Ehsan Sari
Department of Plan Pathology,
Iranian Research Institute of Plant Protection,
Tehran,
Iran.

Dr. Snjezana Zidovec Lepej
University Hospital for Infectious Diseases
Zagreb,
Croatia

Dr. Dilshad Ahmad
King Saud University
Saudi Arabia

Dr. Adriano Gomes da Cruz
University of Campinas (UNICAMP)
Brazil

Dr. Hsin-Mei Ku
Agronomy Dept. NCHU 250 Kuo Kuang Rd, Taichung,
Taiwan

Dr. Fereshteh Naderi
Physical chemist,
Islamic Azad University,
Shahre Ghods Branch
Iran

Dr. Adibe Maxwell Ogochukwu
Department of Clinical Pharmacy and Pharmacy Management,
University of Nigeria,
Nsukka.
Nigeria

Dr. William M. Shafer
Emory University School of Medicine
USA

Dr. Michelle Bull
CSIRO Food and Nutritional Sciences
Australia

Prof. Dr. Márcio Garcia Ribeiro (DVM, PhD)
School of Veterinary Medicine and Animal Science-UNESP,
Dept. Veterinary Hygiene and Public Health,
State of Sao Paulo
Brazil

Prof. Dr. Sheila Nathan
National University of Malaysia (UKM)
Malaysia

Prof. Ebiama Andi Brisibe
University of Calabar,
Calabar,
Nigeria

Dr. Julie Wang
Burnet Institute
Australia

Dr. Jean-Marc Chobert
INRA- BIA, FIPL
France

Dr. Zhilong Yang, PhD
Laboratory of Viral Diseases
National Institute of Allergy and Infectious Diseases,
National Institutes of Health

Dr. Dele Raheem
University of Helsinki
Finland

Dr. Li Sun
PLA Centre for the treatment of infectious diseases,
Tangdu Hospital,
Fourth Military Medical University
China
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Biljana Miljkovic-Selimovic</td>
<td>School of Medicine, University in Nis, Serbia; Referent laboratory for Campylobacter and Helicobacter, Center for Microbiology, Institute for Public Health, Nis Serbia</td>
</tr>
<tr>
<td>Dr. Xinan Jiao</td>
<td>Yangzhou University, China</td>
</tr>
<tr>
<td>Dr. Endang Sri Lestari, MD.</td>
<td>Department of Clinical Microbiology, Medical Faculty, Diponegoro University/Dr. Kariadi Teaching Hospital, Semarang, Indonesia</td>
</tr>
<tr>
<td>Dr. Hojin Shin</td>
<td>Pusan National University Hospital, South Korea</td>
</tr>
<tr>
<td>Dr. Yi Wang</td>
<td>Center for Vector Biology, 180 Jones Avenue, Rutgers University, New Brunswick, NJ 08901-8536 USA</td>
</tr>
<tr>
<td>Dr. Heping Zhang</td>
<td>The Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, China</td>
</tr>
<tr>
<td>Prof. Natasha Potgieter</td>
<td>University of Venda, South Africa</td>
</tr>
<tr>
<td>Dr. Alemzadeh</td>
<td>Sharif University, Iran</td>
</tr>
<tr>
<td>Dr. Sonia Arriaga</td>
<td>Instituto Potosino de Investigación Científica y Tecnológica/División de Ciencias Ambientales, Mexico</td>
</tr>
<tr>
<td>Dr. Armando Gonzalez-Sanchez</td>
<td>Universidad Autonoma Metropolitana Cuajimalpa, Mexico</td>
</tr>
<tr>
<td>Dr. Pradeep Parihar</td>
<td>Lovely Professional University, Phagwara, Punjab, India</td>
</tr>
<tr>
<td>Dr. William H Roldán</td>
<td>Department of Medical Microbiology, Faculty of Medicine, Peru</td>
</tr>
<tr>
<td>Dr. Kanzaki, L I B</td>
<td>Laboratory of Bioprospection. University of Brasilia, Brazil</td>
</tr>
<tr>
<td>Prof. Philippe Dorchies</td>
<td>Laboratory of Bioprospection. University of Brasilia, Brazil</td>
</tr>
<tr>
<td>Dr. C. Ganesh Kumar</td>
<td>Indian Institute of Chemical Technology, Hyderabad, India</td>
</tr>
<tr>
<td>Dr. Farid Che Ghazali</td>
<td>Universiti Sains Malaysia (USM), Malaysia</td>
</tr>
<tr>
<td>Dr. Samira Bouhdid</td>
<td>Abdelmalek Essaadi University, Tetouan, Morocco</td>
</tr>
<tr>
<td>Dr. Zainab Z. Ismail</td>
<td>Department of Environmental Engineering, University of Baghdad, Iraq</td>
</tr>
<tr>
<td>Dr. Ary Fernandes Junior</td>
<td>Universidade Estadual Paulista (UNESP), Brasil</td>
</tr>
<tr>
<td>Dr. Papaevangelou Vassiliki</td>
<td>Athens University Medical School, Greece</td>
</tr>
<tr>
<td>Dr. Fangyou Yu</td>
<td>The first Affiliated Hospital of Wenzhou Medical College, China</td>
</tr>
<tr>
<td>Dr. Galba Maria de Campos Takaki</td>
<td>Catholic University of Pernambuco, Brazil</td>
</tr>
</tbody>
</table>
Dr. Kwabena Ofori-Kwakye  
Department of Pharmaceutics,  
Kwame Nkrumah University of Science & Technology, KUMASI  
Ghana

Prof. Dr. Liesel Brenda Gende  
Arthropods Laboratory, School of Natural and Exact Sciences, National University of Mar del Plata  
Buenos Aires, Argentina.

Dr. Adeshina Gbonjubola  
Ahmadu Bello University,  
Zaria, Nigeria

Prof. Dr. Stylianos Chatzipanagiotou  
University of Athens – Medical School  
Greece

Dr. Dongqing BAI  
Department of Fishery Science,  
Tianjin Agricultural College,  
Tianjin 300384  
P. R. China

Dr. Dingqiang Lu  
Nanjing University of Technology  
P. R. China

Dr. L. B. Sukla  
Scientist –G & Head, Biominerals Department,  
IMMT, Bhubaneswar  
India

Dr. Hakan Parlakpinar  
MD. Inonu University, Medical Faculty, Department of Pharmacology, Malatya  
Turkey

Dr Pak-Lam Yu  
Massey University  
New Zealand

Dr Percey Chimwamurombe  
University of Namibia  
Namibia

Dr. Euclézio Simionatto  
State University of Mato Grosso do Sul-UEMS  
Brazil

Dr. Hans-Jürg Monstein  
Clinical Microbiology, Molecular Biology Laboratory,  
University Hospital, Faculty of Health Sciences, S-581 85 Linköping  
Sweden

Dr. Ajith, T. A  
Associate Professor Biochemistry, Amala Institute of Medical Sciences, Amala Nagar, Thrissur, Kerala-680 555  
India

Dr. Feng-Chia Hsieh  
Biopesticides Division, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council of Agriculture  
Taiwan

Prof. Dra. Suzan Pantaroto de Vasconcellos  
Universidade Federal de São Paulo  
Rua Prof. Artur Riedel, 275 Jd. Eldorado, Diadema, SP  
CEP 09972-270  
Brasil

Dr. Maria Leonor Ribeiro Casimiro Lopes Assad  
Universidade Federal de São Carlos - Centro de Ciências Agrárias - CCA/UFSCar  
Departamento de Recursos Naturais e Proteção Ambiental  
Rodovia Anhanguera, km 174 - SP-330  
Araras - São Paulo  
Brasil

Dr. Pierangeli G. Vital  
Institute of Biology, College of Science, University of the Philippines  
Philippines

Prof. Roland Ndip  
University of Fort Hare, Alice  
South Africa

Dr. Shawn Carraher  
University of Fort Hare, Alice  
South Africa

Dr. José Eduardo Marques Pessanha  
Observatório de Saúde Urbana de Belo Horizonte/Faculdade de Medicina da Universidade Federal de Minas Gerais  
Brasil
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Yuanshu Qian</td>
<td>Department of Pharmacology, Shantou University Medical College, China</td>
</tr>
<tr>
<td>Dr. Helen Treichel</td>
<td>URI-Campus de Erechim, Brazil</td>
</tr>
<tr>
<td>Dr. Xiao-Qing Hu</td>
<td>State Key Lab of Food Science and Technology, Jiangnan University, P. R. China</td>
</tr>
<tr>
<td>Dr. Olli H. Tuovinen</td>
<td>Ohio State University, Columbus, Ohio USA</td>
</tr>
<tr>
<td>Prof. Stoyan Groudev</td>
<td>University of Mining and Geology “Saint Ivan Rilski”, Sofia, Bulgaria</td>
</tr>
<tr>
<td>Dr. G. Thirumurugan</td>
<td>Research lab, GIET School of Pharmacy, NH-5, Chaitanya nagar, Rajahmundry-533294, India</td>
</tr>
<tr>
<td>Dr. Charu Gomber</td>
<td>Thapar University, India</td>
</tr>
<tr>
<td>Dr. Jan Kuever</td>
<td>Bremen Institute for Materials Testing, Department of Microbiology, Paul-Feller-Str. 1, 28199 Bremen, Germany</td>
</tr>
<tr>
<td>Dr. Nicola S. Flanagan</td>
<td>Universidad Javeriana, Cali, Colombia</td>
</tr>
<tr>
<td>Dr. André Luiz C. M. de A. Santiago</td>
<td>Universidade Federal Rural de Pernambuco, Brazil</td>
</tr>
<tr>
<td>Dr. Dhruva Kumar Jha</td>
<td>Microbial Ecology Laboratory, Department of Botany, Gauhati University, Guwahati 781 014, Assam, India</td>
</tr>
<tr>
<td>Dr. N Saleem Basha</td>
<td>M. Pharm (Pharmaceutical Biotechnology), Eritrea (North East Africa)</td>
</tr>
<tr>
<td>Prof. Dr. João Lúcio de Azevedo</td>
<td>Dept. Genetics-University of São Paulo-Faculty of Agriculture- Piracicaba, 13400-970, Brasil</td>
</tr>
<tr>
<td>Dr. Julia Inés Fariña</td>
<td>PROIMI-CONICET, Argentina</td>
</tr>
<tr>
<td>Dr. Yutaka Ito</td>
<td>Kyoto University, Japan</td>
</tr>
<tr>
<td>Dr. Cheruiyot K. Ronald</td>
<td>Biomedical Laboratory Technologist, Kenya</td>
</tr>
<tr>
<td>Prof. Dr. Ata Akcil</td>
<td>S. D. University, Turkey</td>
</tr>
<tr>
<td>Dr. Adhar Manna</td>
<td>The University of South Dakota, USA</td>
</tr>
<tr>
<td>Dr. Cícero Flávio Soares Aragão</td>
<td>Federal University of Rio Grande do Norte, Brazil</td>
</tr>
<tr>
<td>Dr. Gunnar Dahlen</td>
<td>Institute of odontology, Sahlgrenska Academy at University of Gothenburg, Sweden</td>
</tr>
<tr>
<td>Dr. Pankaj Kumar Mishra</td>
<td>Vivekananda Institute of Hill Agriculture, (I.C.A.R.), ALMORA-263601, Uttarakhand, India</td>
</tr>
<tr>
<td>Dr. Benjamas W. Thanomsub</td>
<td>Srinakharinwirot University, Thailand</td>
</tr>
<tr>
<td>Dr. Maria José Borrego</td>
<td>National Institute of Health – Department of Infectious Diseases, Portugal</td>
</tr>
<tr>
<td>Name</td>
<td>Institution</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Dr. Catherine Carrillo</td>
<td>Health Canada, Bureau of Microbial Hazards Canada</td>
</tr>
<tr>
<td>Dr. Marcotty Tanguy</td>
<td>Institute of Tropical Medicine Belgium</td>
</tr>
<tr>
<td>Dr. Han-Bo Zhang</td>
<td>Laboratory of Conservation and Utilization for Bioresources</td>
</tr>
<tr>
<td></td>
<td>Key Laboratory for Microbial Resources of the Ministry of Education,</td>
</tr>
<tr>
<td></td>
<td>Yunnan University, Kunming 650091.</td>
</tr>
<tr>
<td>Dr. Ali Mohammed Somily</td>
<td>King Saud University Saudi Arabia</td>
</tr>
<tr>
<td>Dr. Nicole Wolter</td>
<td>National Institute for Communicable Diseases and University of the Witwatersand, Johannesburg South Africa</td>
</tr>
<tr>
<td>Dr. Marco Antonio Nogueira</td>
<td>Universidade Estadual de Londrina CCB/Dept. De microbiologia</td>
</tr>
<tr>
<td></td>
<td>Laboratório de Microbiologia Ambiental</td>
</tr>
<tr>
<td></td>
<td>Caixa Postal 6001 86051-980 Londrina.</td>
</tr>
<tr>
<td>Dr. Bruno Pavoni</td>
<td>Department of Environmental Sciences University of Venice Italy</td>
</tr>
<tr>
<td>Dr. Shih-Chieh Lee</td>
<td>Da-Yeh University Taiwan</td>
</tr>
<tr>
<td>Dr. Satoru Shimizu</td>
<td>Horonobe Research Institute for the Subsurface Environment,</td>
</tr>
<tr>
<td></td>
<td>Northern Advancement Center for Science &amp; Technology</td>
</tr>
<tr>
<td>Dr. Tang Ming</td>
<td>College of Forestry, Northwest A&amp;F University, Yangling China</td>
</tr>
<tr>
<td>Dr. Olga Gortzi</td>
<td>Department of Food Technology, T.E.I. of Larissa Greece</td>
</tr>
<tr>
<td>Dr. Mark Tarnopolsky</td>
<td>McMaster University Canada</td>
</tr>
<tr>
<td>Dr. Sami A. Zabin</td>
<td>Al Baha University Saudi Arabia</td>
</tr>
<tr>
<td>Dr. Julia W. Pridgeon</td>
<td>Aquatic Animal Health Research Unit, USDA, ARS USA</td>
</tr>
<tr>
<td>Dr. Lim Yau Yan</td>
<td>Monash University Sunway Campus Malaysia</td>
</tr>
<tr>
<td>Prof. Rosemeire C. L. R. Pietro</td>
<td>Faculdade de Ciências Farmacêuticas de Araraquara, Univ Estadual Paulista, UNESP Brazil</td>
</tr>
<tr>
<td>Dr. Nazime Mercan Dogan</td>
<td>PAU Faculty of Arts and Science, Denizli Turkey</td>
</tr>
<tr>
<td>Dr. Ian Edwin Cock</td>
<td>Biomolecular and Physical Sciences Griffith University Australia</td>
</tr>
<tr>
<td>Prof. N K Dubey</td>
<td>Banaras Hindu University India</td>
</tr>
<tr>
<td>Dr. S. Hemalatha</td>
<td>Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi. 221005 India</td>
</tr>
<tr>
<td>Dr. J. Santos Garcia A.</td>
<td>Universidad A. de Nuevo Leon Mexico India</td>
</tr>
</tbody>
</table>
Dr. Somboon Tanasupawat  
Department of Biochemistry and Microbiology,  
Faculty of Pharmaceutical Sciences,  
Chulalongkorn University,  
Bangkok 10330  
Thailand

Dr. Vivekananda Mandal  
Post Graduate Department of Botany,  
Darjeeling Government College,  
Darjeeling – 734101.  
India

Dr. Shihua Wang  
College of Life Sciences,  
Fujian Agriculture and Forestry University  
China

Dr. Victor Manuel Fernandes Galhano  
CITAB-Centre for Research and Technology of Agro-  
Environment and Biological Sciences, Integrative  
Biology and Quality Research Group,  
University of Trás-os-Montes and Alto Douro,  
Apartado 1013, 5001-801 Vila Real  
Portugal

Dr. Maria Cristina Maldonado  
Instituto de Biotecnologia. Universidad Nacional de  
Tucuman  
Argentina

Dr. Alex Soltermann  
Institute for Surgical Pathology,  
University Hospital Zürich  
Switzerland

Dr. Dagmara Sirova  
Department of Ecosystem Biology, Faculty Of Science,  
University of South Bohemia,  
Branisovska 37, Ceske Budejovice, 37001  
Czech Republic

Dr. E. O Igbinosa  
Department of Microbiology,  
Ambrose Ali University,  
Ekpoma, Edo State,  
Nigeria.

Dr. Hodaka Suzuki  
National Institute of Health Sciences  
Japan

Dr. Mick Bosilevac  
US Meat Animal Research Center  
USA

Dr. Nora Lía Padona  
Imunoquímica y Biotecnología- Fac Cs Vet-UNCPBA  
Argentina

Dr. Maria Madalena Vieira-Pinto  
Universidade de Trás-os-Montes e Alto Douro  
Portugal

Dr. Stefano Morandi  
CNR-Istituto di Scienze delle Produzioni Alimentari  
(ISPA), Sez. Milano  
Italy

Dr Line Thorsen  
Copenhagen University, Faculty of Life Sciences  
Denmark

Dr. Ana Lucia Falavigna-Guilherme  
Universidade Estadual de Maringá  
Brazil

Dr. Baoqiang Liao  
Dept. of Chem. Eng., Lakehead University, 955 Oliver  
Road, Thunder Bay, Ontario  
Canada

Dr. Ouyang Jinping  
Patho-Physiology department,  
Faculty of Medicine of Wuhan University  
China

Dr. John Sorensen  
University of Manitoba  
Canada

Dr. Andrew Williams  
University of Oxford  
United Kingdom

Dr. Chi-Chiang Yang  
Chung Shan Medical University  
Taiwan, R.O.C.

Dr. Quanming Zou  
Department of Clinical Microbiology and Immunology,  
College of Medical Laboratory,  
Third Military Medical University  
China
<table>
<thead>
<tr>
<th>Name</th>
<th>Institute/University</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prof. Ashok Kumar</td>
<td>School of Biotechnology, Banaras Hindu University, Varanasi, India</td>
</tr>
<tr>
<td>Dr. Chung-Ming Chen</td>
<td>Department of Pediatrics, Taipei Medical University Hospital, Taipei, Taiwan</td>
</tr>
<tr>
<td>Dr. Jennifer Furin</td>
<td>Harvard Medical School, USA</td>
</tr>
<tr>
<td>Dr. Julia W. Pridgeon</td>
<td>Aquatic Animal Health Research Unit, USDA, ARS, USA</td>
</tr>
<tr>
<td>Dr. Alireza Seidavi</td>
<td>Islamic Azad University, Rasht Branch, Iran</td>
</tr>
<tr>
<td>Dr. Thore Rohwerder</td>
<td>Helmholtz Centre for Environmental Research UFZ, Germany</td>
</tr>
<tr>
<td>Dr. Daniela Billi</td>
<td>University of Rome Tor Vergat, Italy</td>
</tr>
<tr>
<td>Dr. Ivana Karabegovic</td>
<td>Faculty of Technology, Leskovac, University of Nis, Serbia</td>
</tr>
<tr>
<td>Dr. Flaviana Andrade Faria</td>
<td>IBIUCL/UNESP, Brazil</td>
</tr>
<tr>
<td>Prof. Margareth Linde Athayde</td>
<td>Federal University of Santa Maria, Brazil</td>
</tr>
<tr>
<td>Dr. Guadalupe Virginia Nevarez Moorillon</td>
<td>Universidad Autonoma de Chihuahua, Mexico</td>
</tr>
<tr>
<td>Dr. Tatiana de Sousa Fiuza</td>
<td>Federal University of Goias, Brazil</td>
</tr>
<tr>
<td>Dr. Indrani B. Das Sarma</td>
<td>Jhulelal Institute of Technology, Nagpur, India</td>
</tr>
<tr>
<td>Dr. Guanghua Wang</td>
<td>Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, China</td>
</tr>
<tr>
<td>Dr. Renata Vadkertiova</td>
<td>Institute of Chemistry, Slovak Academy of Science, Slovakia</td>
</tr>
<tr>
<td>Dr. Charles Hocart</td>
<td>The Australian National University, Australia</td>
</tr>
<tr>
<td>Dr. Guoqiang Zhu</td>
<td>University of Yangzhou College of Veterinary Medicine, China</td>
</tr>
<tr>
<td>Dr. Guilherme Augusto Marietto Gonçalves</td>
<td>São Paulo State University, Brazil</td>
</tr>
<tr>
<td>Dr. Mohammad Ali Faramarzi</td>
<td>Tehran University of Medical Sciences, Iran</td>
</tr>
<tr>
<td>Dr. Suppasil Maneerat</td>
<td>Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, 90112, Thailand</td>
</tr>
<tr>
<td>Dr. Francisco Javier Las heras Vazquez</td>
<td>Almeria University, Spain</td>
</tr>
<tr>
<td>Dr. Cheng-Hsun Chiu</td>
<td>Chang Gung memorial Hospital, Chang Gung University, Taiwan</td>
</tr>
<tr>
<td>Dr. Ajay Singh</td>
<td>DDU Gorakhpur University, Gorakhpur-273009 (U.P.), India</td>
</tr>
<tr>
<td>Dr. Karabo Shale</td>
<td>Central University of Technology, Free State, South Africa</td>
</tr>
<tr>
<td>Dr. Lourdes Zélia Zanoni</td>
<td>Department of Pediatrics, School of Medicine, Federal University of Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil</td>
</tr>
<tr>
<td>Name</td>
<td>Institution</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Dr. Tulin Askun</td>
<td>Balikesir University, Turkey</td>
</tr>
<tr>
<td>Dr. Marija Stankovic</td>
<td>Institute of Molecular Genetics and Genetic Engineering, Republic of Serbia</td>
</tr>
<tr>
<td>Dr. Scott Weese</td>
<td>University of Guelph, Dept of Pathobiology, Ontario Veterinary College,</td>
</tr>
<tr>
<td></td>
<td>University of Guelph, Guelph, Ontario, N1G2W1, Canada</td>
</tr>
<tr>
<td>Dr. Sabiha Essack</td>
<td>School of Health Sciences, South African Committee of Health Sciences,</td>
</tr>
<tr>
<td></td>
<td>University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa</td>
</tr>
<tr>
<td>Dr. Hare Krishna</td>
<td>Central Institute for Arid Horticulture, Beechwal, Bikaner-334 006, Rajasthan,</td>
</tr>
<tr>
<td></td>
<td>India</td>
</tr>
<tr>
<td>Dr. Anna Mensuali</td>
<td>Dept. of Life Science, Scuola Superiore, Sant’Anna</td>
</tr>
<tr>
<td>Dr. Ghada Sameh Hafez Hassan</td>
<td>Pharmaceutical Chemistry Department, Faculty of Pharmacy, Mansoura University,</td>
</tr>
<tr>
<td></td>
<td>Egypt</td>
</tr>
<tr>
<td>Dr. Kátia Flávia Fernandes</td>
<td>Biochemistry and Molecular Biology, Universidade Federal de Goiás, Brasil</td>
</tr>
<tr>
<td>Dr. Abdel-Hady El-Gilany</td>
<td>Public Health &amp; Community Medicine, Faculty of Medicine, Mansoura University,</td>
</tr>
<tr>
<td></td>
<td>Egypt</td>
</tr>
<tr>
<td>Dr. Hongxiong Guo</td>
<td>STD and HIV/AIDS Control and Prevention, Jiangsu provincial CDC, China</td>
</tr>
<tr>
<td>Dr. Konstantina Tsaousi</td>
<td>Life and Health Sciences, School of Biomedical Sciences, University of Ulster</td>
</tr>
<tr>
<td>Dr. Bhavnaben Gowin Gordhan</td>
<td>DST/NRF Centre of Excellence for Biomedical TB Research, University of the</td>
</tr>
<tr>
<td></td>
<td>Witwatersrand and National Health Laboratory Service, P.O. Box 1038,</td>
</tr>
<tr>
<td></td>
<td>Johannesburg 2000, South Africa</td>
</tr>
<tr>
<td>Dr. Ernest Kuchar</td>
<td>Pediatric Infectious Diseases, Wroclaw Medical University, Wroclaw Teaching</td>
</tr>
<tr>
<td></td>
<td>Hospital, Poland</td>
</tr>
<tr>
<td>Dr. Hongxiong Guo</td>
<td>STD and HIV/AIDS Control and Prevention, Jiangsu provincial CDC, China</td>
</tr>
<tr>
<td>Dr. Mar Rodriguez Jovita</td>
<td>Food Hygiene and Safety, Faculty of Veterinary Science, University of</td>
</tr>
<tr>
<td></td>
<td>Extremadura, Spain</td>
</tr>
<tr>
<td>Dr. Jes Gitz Holler</td>
<td>Hospital Pharmacy, Aalesund. Central Norway Pharmaceutical Trust, Professor</td>
</tr>
<tr>
<td></td>
<td>Brochs gt. 6. 7030 Trondheim, Norway</td>
</tr>
<tr>
<td>Prof. Chengxiang FANG</td>
<td>College of Life Sciences, Wuhan University, Wuhan 430072, P.R.China</td>
</tr>
<tr>
<td>Dr. Anchalee Tungtrongchitr</td>
<td>Siriraj Dust Mite Center for Services and Research, Department of Parasitology,</td>
</tr>
<tr>
<td></td>
<td>Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Road,</td>
</tr>
<tr>
<td></td>
<td>Bangkok Noi, Bangkok, 10700, Thailand</td>
</tr>
</tbody>
</table>
Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The cover letter should include the corresponding author’s full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author’s surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process
All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.

Decisions will be made as rapidly as possible, and the Journal strives to return reviewers’ comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJMR to publish manuscripts within weeks after submission.

Regular articles
All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors’ full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited. Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard Abbreviations should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.
Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author’s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author’s name should be mentioned, followed by ‘et al’. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ‘a’ and ‘b’ after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)

References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:


Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.
Fees and Charges: Authors are required to pay a $550 handling fee. Publication of an article in the African Journal of Microbiology Research is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances.

Copyright: © 2013, Academic Journals.
All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Disclaimer of Warranties
In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the AJMR, whether or not advised of the possibility of damage, and on any theory of liability. This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement. Descriptions of, or references to, products or publications does not imply endorsement of that product or publication. While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.
# African Journal of Microbiology Research

## Table of Contents: Volume 7 Number 41, October 11, 2013

## ARTICLES

### Review

**Biocontrol of *Fusarium* wilt of banana: Key influence factors and strategies**  
Guo Gang, Wang Bizun, Ma Weihong, Li Xiaofen, Yang Xiaolin, Zhu Chaohua, Ming Jianhong and Zeng Huicai

### Research Articles

**Incidence of *Candida albicans* in diabetic patients with a dental prosthesis in Northeast Mexico**  
Garza-Garza Luis Carlos, Gallegos-Canales Anakaren, Elizondo-Garza Nemesio, Martínez-González Gustavo Israel and García-Palencia Patricia

**Antimicrobial activity of *Syzygium aromaticum* extracts against food spoilage bacteria**  
Muhammad Saeed, Muhammad Nadeem, Moazzam Rafiq Khan, Muhammad Asim Shabbir, Aamir Shehzad and Rai Muhammad Amir

**Influence of multi drug resistance Gram negative bacteria in liver transplant recipient**  
Mohamed Abdel Aziz, Iman El-Kholy, Amin Abdo and Samy Selim

**Phosphate solubilization by a few fungal strains belonging to the genera *Aspergillus* and *Penicillium***  
Jyoti Saxena, Paramita Basu, Vanaja Jaligam and Shalini Chandra

**Identification of antagonistic bacteria for *Amorphorallus konjac* soft rot disease and optimization of its fermentation condition**  
Jinping Wu, Zili Ding, Zhenbiao Jiao, Rong Zhou, Xinsun Yang, Zhengming Qiu
Evaluation of the potency of some entomopathogenic bacteria isolated from insect cadavers on Anopheles arabiensis Giles (Order: Diphera; Family: Culicidae) mosquito larvae in Nigeria
Omoya, F.O. and Akinyosoye, F.A.
4877

Typing of Candida species isolated from blood cultures and analysis of their in vitro antifungal susceptibilities
Nurten Baran, Ismail Serkan Salman, Sureyya Gul Yurtseven, Rahim Ozdemir, Serdar Gungor, Senel Yurtseven and Mustafa Demirci
4882

Isolation and identification of microorganism from polyhouse agriculture soil of Rajasthan
Nakuleshwar Dut Jasuja, Richa Saxena, Subhash Chandra and Suresh C. Joshi
4886

A monoclonal antibody-based antigen-capture enzyme-linked immunosorbent assay (ELISA) for the detection of bluetongue virus
Hui-qiong Yin, Shu Yang, Wen-chao Li, Jun Liu, Rui Wang and Jin-gang Zhang
4892

The extraction on polysaccharide of sporocarp and static culture optimization conditions of Morchella esculenta from Qinghai-Tibetan Plateau
Ke-long Chen, Xu Su, Yu-ping Liu, Heng-sheng Wang, Zhao-meng Li, Jian-min Li and Ben La
4896

Molecular characterization of Trichoderma longibrachiatum 21PP isolated from rhizospheric soil based on universal ITS primers
Mohammad Shahid, Mukesh Srivastava, Antima Sharma, Anuradha Singh, Sonika Pandey, Vipul Kumar, Neelam Pathak and Smita Rastogi
4902

Correlation between architect hepatitis C virus (HCV) core antigen and HCV Ribonucleic acid levels in Anti-HCV reactive patients in Turkey
BURUK Kurtulus, BAYRAMOGLU Gulcin, KAKLIKKAYA Nese, AKYUZ Zeynep, KAYA Selcuk, KOKSAL Iftihar, CAN Gamze and AYDIN Faruk
4907
Influence of general anaesthesias on the changes of bacterial flora in bronchial tree
Izabela Duda, Aleksandra Rudnik-Lipińska, Maria Damps, Ewa Musioł and Edyta Gogółka
Review

Biocontrol of *Fusarium* wilt of banana: Key influence factors and strategies

Guo Gang¹²³#, Wang Bizun¹²#, Ma Weihong¹², Li Xiaofen¹², Yang Xiaolin¹⁴, Zhu Chaohua⁴, Ming Jianhong¹²# and Zeng Huical¹²³

¹Haikou Experimental Station/Hainan Key Laboratory of Banana Genetic Improvement, Chinese Academy of Tropical Agricultural Science (CATAS), Haikou 570102, Hainan, China.
²Banana Research and Development Department, National Center of Important Tropical Crops Engineering and Technology Research, Haikou 570102, Hainan, China.
³Institute of Tropical Bioscience and Biotechnology, CATAS, Haikou 571101, Hainan, China.
⁴College of Environment and Plant Protection, Hainan University, Haikou 570228, Hainan, China.

Accepted 8 September, 2013

*Fusarium* wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), is one of the most important and destructive diseases of banana, and is known to be a major biotic limiting factor for the development of the present banana industry. Biocontrol on the destructive disease, the use of antagonist as biocontrol agents (BCAs) against Foc, constitutes an effective option for the management of the disease. The effectiveness of biocontrol agents depends on a range of biological and physico-chemical factors, including the type and properties of the biocontrol agents, the obstacles to the initial colonization of antagonists, as well as the variation factors after initial colonization. Various strategies can be implemented to optimize the biocontrol efficacy, such as the use of endophytes from banana plants as BCAs (favorably *Bacillus* spp.), the development of water and nutrition retaining agent, the application of proper carrier for BCAs, the restoration of soil biodiversity, and combined management of nematodes disease and *Fusarium* wilt. In this review, elements affecting the biocontrol efficacy of *Fusarium* wilt are analyzed in detail, and strategies to promote the biocontrol effects are proposed. Besides, the concept of “post-indigenousness” and “post-indigenous microbes” were firstly suggested.

Key words: *Fusarium* wilt of banana, biocontrol, *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen, *Bacillus* sp., endophytic bacteria.

INTRODUCTION

*Fusarium* wilt of banana, also called Panama disease, is caused by *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen, shortly as Foc (Stover, 1962). *Fusarium* wilt is known to be one of the most important and destructive diseases of banana, which is especially serious in the Central and South Americas, parts of Africa (Viljoen, 2002), Sri Lanka, Burma, Thailand, Indonesia and the Philippines, resulting in heavy enormous economic losses each year (Ploetz, 1994; Stover et al., 1987). In the recent ten years, banana plantation areas in China, influenced by Fusarium wilt, have decreased dramatically, especially in Guandong, Hainan, Fujian provinces.

*Correspondence authors. E-mail: jhming_326@163.com, zhc081@126.com. Tel: 86-898-66754695, 86-898-66707697.

*Authors contributed equally to this work.*
The fungus infects banana plants through the roots and invades the vascular tissue (xylem), causing external symptoms like gradual wilting, progressive yellowing of banana leaves (spreads from leaf margins, and from older leaves to younger leaves), eventual collapse at the petiole, and longitudinal splitting of the outer leaf sheaths in the pseudostem (Yin et al., 2011). The distinguishing internal symptoms of the disease is the typical discoloration of vascular tissues (in roots, corm, pseudostem, fruit stalk) varying from light yellow to dark brown, which appears first in the outer or oldest leaf sheath, then extends up to the pseudostem (Ploetz, 2006). Eventually, the disease leads to the death of banana plants.

The pathogenic isolates of Foc have been traditionally grouped into four physiological races based on pathogenicity to host cultivars under field condition (Fourie et al., 2009). F. oxysporum f. sp. cubense race 1 (Foc R1) attacks ‘Gros Michel’, ‘Lady Finger’ (AAB) and ‘Silk’ (AAB) varieties. Foc R2 infects cultivar ‘Bluggoe’ (ABB), while Foc R3 infects Heliconia spp. (a close relative of banana). And Foc R4 is able to attack cultivar Cavendish and all cultivars that are susceptible to Foc1 and Foc2 (Persley, 1987; Ploetz, 1990).

Banana production was once severely threatened by Fusarium wilt caused by Foc R1. The disease almost devastated the ‘Gros Michel’-based banana industry in the Central American and Caribbean during the mid-1900s (Ploetz, 1994). This adversity persisted until the introduction of Cavendish cultivars, which are unsusceptible to Foc R1 and Foc R2 in tropical banana-growing regions (Fernández-Falcón et al., 2003). However, the prosperity did not last long. The Cavendish cultivars had then been found highly susceptible to a new race of Foc, Foc R4 (Ploetz and Pegg, 2000). Economic losses worldwide are enormous. It brings to the sinister reality that the banana industry is once again threatened by Fusarium wilt.

Fusarium wilt is destructive because it seriously hampers banana production and is difficult to manage. F. oxysporum are able to infect more than 100 plants, and are divided into more than 120 host-specific forms, known as formae specialae (Minerdi et al., 2008). Foc, generally considered to be one of the most destructive formae specialae of F. oxysporum (Ploetz, 1990), is soil-borne and has strong saprophytic ability. The pathogen can survive in the soil for several decades by producing spores (specifically, chlamydomspores), which will re-infect the susceptible banana plants (Stover, 1962). This adds to the difficulty of disease management.

Till date, results from disease management studies have been disappointing. No effective and efficient control strategies available can satisfy the needs for the management of disease worldwide. Although, Huang et al. (2012) reported that Chinese leek-banana rotation is an efficient way for controlling banana Fusarium wilt, the method is not economical because Chinese leek is in low need in the market. Beside, crop rotation tends to have limited effectiveness once a disease outbreak occurs because of its soil-born nature and strong vitality (Fravel et al., 2003). Soil disinfection using chemicals or pesticides are not recommended due to environmental and human health concerns. And fumigated soils with methyl bromide can be re-infected in two or three years in fields with susceptible cultivars (Stover, 1958). Developing resistant banana cultivars may solve this problem but such efforts are progressing slowly (Stover and Buddenhagen, 1986; Hwang and Ko, 2004). Considering the urgency of the disease, acomplementary approach involving Fusarium wilt involves the biological control (or biocontrol), an important component of integrated disease management programs, specifically, in this case, is to use naturally occurring antagonists and active substances (viruses, bacteria, fungi, active substances of natural origin) as biocontrol agents (BCAs) for disease management.

When compared with other approaches, the use of BCAs is proved to be an ecologically safe strategy for disease management. However, the technology is still in its infancy. Enormous efforts should still contributed to the development of effective and safe BCAs for comer-cial application. This review would focus on the elements affecting the biocontrol efficacy of Fusarium wilt, the points needed to be considered in the development of BCAs, and the corresponding strategies to improve the biocontrol efficacy, aiming to provide guidance for the biocontrol of Fusarium wilt and point the way forward for enhanced utilization of BCAs.

ELEMENTS AFFECTING THE BIOCONTROL OF FUSARSIUM WILT AND CORRESPONDING STRATEGIES TO IMPROVE THE BIOCONTROL EFFICACY

It is a systematic work to use biological agents to control banana Fusarium wilt. In order to achieve ideal results, we should at least take into account the following elements: the type and properties of the biocontrol agents, the obstacles to the initial colonization of antagonists, as well as the variation factors after initial colonization.

The type and properties of the biocontrol agents

The first step to achieve effective biocontrol is to find the suitable potential biocontrol agents (BCAs). A primary consideration in the selection of antagonists as BCAs for field application is their type and properties, which directly or indirectly affect the biocontrol efficacy, the production process, the post-processing, as well as the storage and transportation. Production methods for biocontrol agents must be at low cost and yield viable, highly effective propagules of high concentrations. Also, these propagules, such as spores, must be amenable to long-term storage as dry preparations (Jackson, 1997).

Previous studies and experiences implied that Bacillus
species serves as ideal candidates for viable BCAs (Yilmaz et al., 2005; Bertagnolli et al., 1996; Szczech and Shoda, 2004; Farhana et al., 2011; Govindasamy et al., 2011; Tan et al., 2013). *Bacillus* spp. strains are advantageous as BCAs because they are tolerant to adverse environmental stresses by producing endospores, such as heat and desiccation (Schallmey et al., 2004). At present, a variety of strains of *Bacillus* spp. have been extensively applied as BCAs against soil-borne plant diseases (Gurr et al., 2005), including Rhizoctonia (Yu et al., 2002) and *Fusarium* (Schisler et al., 2002; Sun et al., 2011), which have been proven to bring about high biocontrol efficacy. In addition, using *Bacillus* spp. strains as BCAs, the post-processing costs are easy to control, and the storage and transportation conditions needed could be easily fulfilled (Schallmey et al., 2004). On the other hand, the application of other microorganisms as BCAs, such as nonpathogenic *F. oxysporum* (Nel and Steinberg, 2006), *Trichoderma* spp. (Thangavelu et al., 2004), has been demonstrated to result in high cost of production, storage and transportation.

**Obstacles to the initial colonization of antagonists**

The second key point needed to be considered is the colonization of antagonistic microbes. The biocontrol efficacy of the BCAs largely depends on the ability of the antagonistic microbes to colonize the plant root and the rhizosphere, and to produce substances which inhibit pathogens. The colonization of antagonistic microbes is hampered by several natural barriers. Obstacles to the initial colonization refers to the first set of natural barriers encountered by the antagonistic microbes after the application of BCAs, including the predation and phagocytosis from soil protozoa (Ekelund et al., 2001; Ronn et al., 2002), inhibition from the exudates of indigenous microbes (Bolwerk et al., 2003) or plant roots (Chao et al., 1986), the competition with indigenous microbes for ecological sites, nutrients and energy.

Normally, influenced by these barriers, the population of most antagonistic microbes reduced drastically in the first 2 to 3 days after the application of the BCAs (Christoffersen et al., 1995). However, BCAs usually must maintain a certain population of the antagonistic microbes to obtain acceptable levels of disease suppression (Wang et al., 2011). Thus, in order to achieve satisfactory biocontrol efficacy, appropriate measures should be taken to help the antagonistic microbes get through this hard time. One of the alternatives is to increase the original population of the antagonistic microbes in the BCAs, ensuring that a considerable part of the population survive this adverse phase under the regulation of environmental factors. Another recommendation is to apply BCAs repeatedly to maintain certain levels of the antagonistic microbes. Repeated applications of BCAs can be achieved by distributing the BCAs through the irrigation water.

**Predation and phagocytosis from soil protozoa**

Predatory protozoa in the soil to some extent act as the regulator of the population of soil bacteria and fungi (Bird et al., 2003; Ingham et al., 1985). They prey selectively on certain bacteria and fungi, thus exert an effect on the population and diversity of their prey (Burke et al., 2003). The antagonistic microbes in the BCAs, of course, are affected inevitably by this regulation. One proposed solution to counteract this regulation is to inoculate the BCAs directly into the leaves or stems of banana plant through drip injection, but not to apply them into the soil. Specifically, the approach means to inject the BCAs intermittently into the plant vessel from up to down under the law of gravity, and let them spread into the cell eventually. However, one leading problem of this method is the increased costs. Advanced techniques are needed to be developed in order to reduce the costs to an acceptable level for large-scale field applications.

Besides, studies have shown that the spatial heterogeneity of soil may have something to do with the stability of the predator-prey relationship, since certain soil structures are beneficial for the survival of bacteria, for example, in the case that the spaces in the soil are suitable for bacteria to live in but are inaccessible to protozoa (Wardle, 2006). Soil aggregates are clumps of soil particles that are held together by moist clay, organic matter (like roots and fibrous roots), gams (from bacteria and fungi) and by fungal hyphae. Study revealed that well-aggregated soil is thought to be a very desirable habitat for bacteria (Wardle, 2006). Bacteria tend to accumulate inside soil aggregates because this environment provides them with micro-ecological sites that are not accessible to certain protozoa, that is to say, bacteria in this environment are less likely to be eaten by soil animals like protozoa and mites. Such knowledge gives us some clues on the application of BCAs to make the carrier of BCAs into the structure of soil aggregates. This approach can to some extent protect the antagonistic microbes from the prey of indigenous predatory protozoa, thus conserving a substantial number of the microbes as a result. One recommendation currently, is to apply resin-coated controlled-release materials as the carrier of BCAs (Ko et al., 1996; Lunt, 1968).

**Inhibition from the exudates of indigenous microbes or plant roots**

A substantial amount of studies have reported that certain exudates of indigenous microbes and plant roots exert an inhibitory effect on heterologous microorganisms (Quintana et al., 2009; Bais et al., 2006; Hirsch et al., 2003). In addition, plants tend to develop an induced systemic resistance to heterologous microorganisms (Pieterse et al., 1998). These effects exert strong negative influences on the colonization of the heterologous antagonistic microbes in banana plants. To overcome these difficulties,
a proposal is to screen active antagonistic microbes from the endophytic strains of healthy banana plants (Lian et al., 2008). The use of endophytes as antagonist is of great interest because of their ability to colonize plant tissue and produce antibiotics in situ (Sessitsch et al., 2004).

Of course, the endophytes isolated from a certain plant tend to be plant tissue and variety specific. That is to say, the endophytes from one cultivar might not be endophytic to another cultivar due to genetic diversity, which may stand in the way of the colonization process. In fact, the variety of banana cultivars in China is relatively uniform geographically, mostly Brazil and Williams, both belonging to *Musa AAA* group. Normally, it would not be difficult for the endophytes to colonize the varieties from the same group. Our recent studies have preliminarily confirmed the feasibility of endophytes (Wang et al., 2011).

When the BCAs have to be applied into soil, to overcome the inhibition from indigenous microbes and plant roots, or the “induced systemic resistance” mentioned above, a recommended option is to introduce a cover for the antagonistic microbes, which shields the microbes from antimicrobial agents and environmental stress by acting as a physical barrier. For example, to entrap the biocontrol agents in capsule-like materials (Candela and Fouet, 2006) or coated and controlled-release materials mentioned above. The advantages of entrapped BCAs include: a controlled release of agent (controlled by the environment and the properties of the entrapment materials); easy handing and prolonged shelf life; protection against extreme environmental conditions.

**The competition with indigenous microbes for ecological sites, nutrients and energy**

The colonization of the antagonists is also impacted tremendously by indigenous microbes through competition for ecological sites, nutrients and energy (Hyakumachi, 2000). The competition, creating a nutrient-limiting environment, determines how different microbial populations coexist in the same ecosystem. Competition for essential resources is a factor that determines the survival of all organisms (Hyakumachi, 2000). The biocontrol fails if the antagonistic organisms are out-competed by other indigenous microbes in the soil for a limited essential resource. Thus, the antagonists must have the ability to use energy substrates and nutrients at low concentrations. Studies revealed that strains of *Pseudomonas fluorescens* and *Trichoderma harzianum* showed antagonistic effect on Foc. (Rujappan et al., 2002; Saravanan et al., 2003).

**Obstacles to the long-term colonization of antagonists: The variation factors after initial colonization**

The variation factors after initial colonization means the factors that affect the long-term colonization of antagonists. *Fusarium* wilt is a typical soil-borne disease. To achieve ideal biocontrol efficacy, the antagonists must colonize the rhizosphere soil and sustainedly occupy favorable ecological sites in the environment. Previous studies have illustrated that the long-term colonization of antagonists is correlated to soil moisture (or the water content of soil), temperature, soil aeration, pH, soil salinity, as well as the continuous supply of nutrients (Sessitsch et al., 2004; Bashan et al., 1991; Beivinio et al., 2005; Cavaglieri et al., 2005; de-Bashan and Bashan, 2008).

According to latest studies, rhizosphere bacteria exist not in the traditional recognized status (planktonic cells), but in biofilms (Webb et al., 2003). A biofilm is “a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface” (http://en.wikipedia.org, 20090205), formed by an aggregate of microorganisms in which cells adhere to each other and/or to a surface. In nature, biofilms constitute a protected growth modality allowing bacteria to survive in hostile environments (Rinaudi and Giordano, 2010). In this case, we believe biofilm is the best mode for antagonists to occupy heterogeneous habitats, acting as a physical barrier sheltering the antagonists from antimicrobial agents and environmental stress. Thus, favorable conditions should be provided to facilitate the formation of biofilm in practice. As indicated by Chang and Halverson (2003), the formation of rhizosphere bacterial biofilm is mainly affected by water moisture, nutritional status and environmental parameters.

**Soil moisture**

One of the most significant requirements for biofilm formation is sufficient moisture. In banana plantations, soil moisture affects the structure of soil aggregates, and is significantly related to soil aeration, temperature, entropy and pH. Banana plants need plenty of water and fertilizer to grow (Goenaga and Irizarry, 1998), due to their large stems and leaves, shallow roots, rapid growth and high yield. They do best in areas of high humidity (50% or more) and require regular applications of fertilizer. The growing periods and yield of banana plants depend largely on the rational management of fertilizer and water. In China, areas under banana cultivation are mostly dry slopes, platforms, plateaus, or plains in short of water. Irrigation in these areas is normally not convenient (Xia, 2011). The large-scale banana plantations are mostly watered through well irrigation, ditches-guided irrigation, showering irrigation via perforated pipe, or microjet irrigation (Chen, 2001). These irrigation means, although solve the water shortage problem to some extent, are far from enough to satisfy the sustained demand of banana plants for continuous water supply, especially in the vegetative stage, flower bud differentiation stage, flowering and fruit ripening stages. Besides, the irrigation means mentioned above are all based on high costs, which tend to benefit the large-scale plantations owners more and are relatively uneconomical to the smallholders.

Banana plant is also vulnerable to water logging, and the
soil aeration condition directly determines the growth status of its root system (Goenaga and Irizarry, 1998). Waterlogging reduces yield and plant size, restricts root growth, causes shallow root systems, and stops the active uptake of nutrients (Aguilar et al., 2008). In a nutshell, banana plant is vulnerable to both blood (waterlogging) and drought. In this dilemma, most plantations have difficulty in maintaining a balanced water supply throughout the whole growing season, which inevitably results in the intermittent shortage of water. This problem will, undoubtedly, lead to changes in soil microbial communities, mostly, dramatic decrease in the population of soil microbes (which are irreversible for at least one growing season). This is the fundamental reason why most BCAs do not have a sustained effect in practice, according to our continued investigation in the recent 4 years (Guo et al., unpublished data).

Sustained water supply in rhizosphere soil leads to favorable rhizosphere environments for the biofilms formation of antagonists. Through biofilms, the population of antagonists in the rhizosphere can maintain a balance for quite a long time, which in turn prolongs the persistence of the colonization of the antagonists. In conclusion, from our point of view, the population of antagonists is mostly dependent on the stability of sustained water supply in soil. In standard large-scale plantations, this demand can be satisfied through microjet irrigation and drip irrigation facilities. However, in small plantations, where it is not profitable and feasible to set up these facilities, a recommended option is to apply water retaining agents in rhizosphere soil.

There are two types of water retaining agents nowadays, one is chemical and the other is bioactive. Poly-γ-glutamic acid (Zeng et al., 2013), in short γ-PGA, act as a new kind of bioactive water retaining agent in agricultural and environmental applications. It is a biodegradable, water-soluble amino acid polymer with a molecular weight ranging from about 10,000 up to 2 millions, generated by the microbial fermentation in nature (Zeng et al., 2013). It is normally composed of about 5,000 glutamic acids molecules or mono units, with a free carboxyl group on the α-carbon atom of each repeating unit (Do et al., 2011).

Because of the repulsion of the negatively charged carboxyl, the chain space is extended to be particularly large. The interaction between molecules is strong even when γ-PGA is in low concentrations, which makes it an ideal material for super absorbent (Candela and Fouet, 2006). Studies have revealed that γ-PGA can effectively absorb soil water, and can reach a maximum water absorption coefficient of 1108.4 (Tsujimoto et al., 2010). Due to its nature of economical costs, biodegradability and water absorption, γ-PGA, as a water retaining agent, can be used to change soil aggregates structure, maintain soil moisture, and conserve fertilizer in soil. In our study, we have found several γ-PGA-producing strains that are antagonistic against Foc (Guo et al., unpublished data) under the application for a patent. Further studies are still under way to screen more efficient strains. Undoubtedly, the application of these bacteria as BCAs would bring more efficiency.

Nutritional status and environmental parameters

Nutritional status and several environmental parameters are also key factors that influence the formation of biofilm (Pan et al., 2010), which further affect the persistence of the colonization of the antagonists, including carbon source, amount of nitrate, phosphate, calcium and magnesium as well as the effects of osmolarity and pH (Rinaudi et al., 2006).

Availability of nutrition in the form of glucose results in increased biofilm formation of bacteria (Shera et al., 2006; Revdiwala et al., 2012). Increased levels of phosphate, Ca$^{2+}$, Mg$^{2+}$ enhance biofilm formation, whereas osmotic agents, such as NaCl and sorbitol negatively affect biofilm formation through an osmotic effect as their concentrations increase (Rinaudi et al., 2006). Besides, temperatures and pH are also factors that affect biofilm formation (Pettit et al., 2010), and the effects differ from one bacterial species to another. Taken together, the nutritional and environmental requirements for biofilm formation appear to be rather species specific. Thus a specific antagonist should be tested for the optimal nutritional conditions for the biofilm formation, according to which various sugars, osmotic agents or salts should be supplemented to the medium for cultivation to provide feasible conditions for biofilm formation.

Currently, most banana plantations are in the condition of imbalanced soil nutrition (such as redundant nutrition in rhizosphere) and improved salinity (due to years of continuous cultivation and preference in employ of N, P fertilizer) (Zhong et al., 2011), which stand in the way of biofilm formation. Inadaptability to these conditions would definitely result in the rapid decline in the population of the antagonists, thus weakening the biocontrol efficacy.

The development of time release technology, also known as sustained-release (SR), provides an alternative for this problem. The BCAs are recommended to be introduced into a controlled-release system with sustained release of nutrition, for example, the water retaining agent or the slow-release fertilizers. In this system, the nutrients are provided slowly and steadily for an extended duration, which not only protect the antagonists and facilitate the biofilm formation, but also put banana plants in the way they prefer to be fed and helps them grow well. Considering the water shortage problem, it is desirable to develop a water and nutrition retaining agent to maintain both water and nutrition at the same time. In addition, as mentioned above, high salinity exerts negative influence on the formation of biofilms through an osmotic effect. A recommended approach is to use halo-tolerant microbes from mangrove and marine source as antagonists against Foc pathogens (Xu and Dai, 2007).
OTHER FACTORS THAT HELP IMPROVE THE BIOCONTROL EFFICACY AGAINST FUSARIUM WILT OF BANANA

Pay attention to the evolutionary and geographic diversity of Foc

By comparing DNA sequences of nuclear and mitochondrial genes, O’Donnell et al. (1998) concluded that Fusarium wilt of banana is caused by pathogenic strains with independent evolutionary origins, and the Foc races isolated are geographically distinct. A recent field experiment conducted by us supports this conclusion as well, which found that a biocontrol agent proved to have excellent effect in a field in Ledong, Hainan (having a control effect of 99%) exhibited poor performance in the field just 17 km around (having a control effect of 23%). Preliminary analysis revealed that the colony morphology and virulence of Foc R4 in the two fields showed significant difference (Mo et al., 2013). Therefore, the evolutionary and geographic diversity of the pathogen should be taken into consideration in the screening of antagonists against Foc. In the first screening round, it is recommended to use pathogens from various evolutionary origins and geographic areas as targets, in order to improve the biocontrol efficacy.

Take into account the integrated management of banana nematodes

Banana nematodes constitute another major threat to banana production all over the world, which cause yield losses of up to 30 to 60% in many countries (Roderick et al., 2012). The fundamental reason that leads to nematode disease and Fusarium wilt is basically in agreement with previous studies (Zhong et al., 2011; Palomares-Rius et al., 2011), both due to the loss of biodiversity in soil. Banana plants that are infected with nematodes tend to have enhanced susceptibility to Fusarium wilt, even in those lines showing Fusarium tolerance or resistance (Ammar, 2007). Thus, the two diseases could be controlled jointly, and combined management of the two diseases would save us lots of efforts and costs (Pararu et al., 2009). To improve biocontrol efficacy, BCAs against both disease are encouraged to be developed.

Consider addition of non-antagonistic microbes into the biocontrol agents

Microbes that do not have antagonistic effect to pathogens may act as potential biocontrol agents by occupying the ecological sites of the pathogen or competing with the pathogen for nutritious and infection sites (Nel et al., 2006). Therefore, it is recommended to add certain non-antagonistic microbes into the biocontrol agents. Non pathogenic F. oxysporum strains can be developed as biocontrol agents. They are able to compete for nutrients in the soil, which affect the rate of chlamydospore germination of the pathogen (Kidane and Laing, 2010). They can also compete with the pathogens for infection sites on the root, and can trigger plant defense reactions, inducing systemic resistance. To give an example, Fo47, a non-pathogenic F. oxysporum strains, have been successfully applied as biocontrol agent in the field. Studies revealed that the general pattern of colonization in soil was similar for the pathogenic strain and Fo47. However, Fo47 grew faster than the pathogenic strain and, as a consequence, colonized the rhizosphere earlier (Michielse and Rep, 2009).

Considering using secondary metabolites for biocontrol agents

Some antagonistic microbes produce antagonistic substances only under the nutrient-poor conditions (Opelt and Berg, 2004). Based on our experience, this kind of microorganisms cannot act as viable biocontrol agents directly (Mo et al., 2013). A better choice is to take advantage of its secondary metabolites. If they are used as potential viable biocontrol agents, relatively poor culture conditions should be applied in the dual-culture assay during the second screening round, for example, cultured in the water agar. Only the microbes that exhibit antagonistic characteristics in the water agar should be chosen for the third screening round.

Avoid using actinomycetes as viable biocontrol agents

Actinomycetes is not recommended to be applied as viable biocontrol agents for managing Fusarium wilt of banana in Chinese banana plantations, because the favorable conditions needed for actinomycetes to survive is not desirable for the growth of banana roots (Jayasinghe and Parkinson, 2008). However, it is feasible to use the secondary metabolites of Actinomycetes as biocontrol agents.

Introducing limitation factors as many as possible in the screening of antagonists

In the second round of screening for potential BCAs, it is recommended to introduce limitation factors as many as possible, such as extreme pH and temperature. The antagonistic strains that are tolerant to a wide range of environmental conditions have greater market potential as biocontrol agents against Foc. In other words, the advantageous and competitive microbes have greater chance to colonize heterogeneous habitats and become “indigenous inhabitants” in this environment. The process for the microbes to become “indigenous inhabitants” in heterogeneous habitats is defined by us as “post-indigenous-ness”, and these microbes are designated as “post-indi-
genous microbes".

As we all know, the most typical feature of strains belonging to Bacillus family is that they can form spores to get through adverse environmental conditions (Vos et al., 2009), which enable them to survive in a broad range of environmental conditions. The utility of strains of Bacillus sp. as biocontrol agents cannot only obtain sustained colonization in banana plants, but also facilitate the post-processing, storage and transportation. Therefore, antagonistic strains from Bacillus spp. are a recommended choice as biocontrol agents in field application.

**Restoring the soil biodiversity in banana plantations to control Fusarium wilt indirectly**

The most serious problem for banana plantations with years of continuous cultivation is the loss of soil biodiversity (Zhong et al., 2011). Especially, the loss of keystone species in the microbial communities would severely destroy the ecological balance of the environment (Walker, 1992), which offers favorable conditions for the accumulation of Foc pathogens. The toxins secreted by these pathogens first attack the roots of banana plants, and then spread to stem and leaves, eventually resulting in outbreak of Fusarium wilt (Kuo and Scheffer, 1964). The growth of banana plants is closely related to the biodiversity in the rhizosphere. Under constant supply of normal nutrition, the roots of the banana plants are well developed. The exudates from the root hairs or root tip promote the growth of rhizosphere organisms (Bais et al., 2006), which in turn enhance the biodiversity of soil.

**ACKNOWLEDGEMENTS**

This work was supported by the National Natural Science Foundation of China (No. 31271995), the Fundamental Research Funds for Institute of Tropical Biosciences and Biotechnology, CATAS (No. ITBB120203), Agro-Tech System Construction Project on Tropical Crops (No. 13RZNU-39), Department of Agriculture, and Hainan Provincial Key Scientific and Technological Project (ZDZX20130023-1-18).

**REFERENCES**


Incidence of *Candida albicans* in diabetic patients with a dental prosthesis in Northeast Mexico

Garza-Garza Luis Carlos, Gallegos-Canales Anakaren, Elizondo-Garza Nemesio, Martínez-González Gustavo Israel and García-Palencia Patricia*

Department of Oral Microbiology/Molecular Biology, Facultad de Odontología, Universidad Autónoma de Nuevo León, Monterrey, México.

Accepted 20 August, 2013

Diabetes is an important health problem with a high morbidity and mortality. There are infections that are common in patients with diabetes and others that have greater severity and complications. A study was carried out to determine if there is a higher incidence of *Candida albicans* in patients with diabetes who use total and partial dental prostheses in comparison with patients without diabetes. Samples were obtained from 43 patients seven days after receiving a total or partial dental prosthesis. Afterwards, samples from the dentures were cultured in CHROMagar to determine the presence of *Candida*. The frequency of different types of *Candida* was analyzed and it was found that no significant difference exists between patients with diabetes and those without diabetes with regard to the presence of *C. albicans*.

**Key words:** *Candida albicans*, dental prosthesis, diabetes mellitus, CHROMagar.

**INTRODUCTION**

Diabetes is an important health problem with a high morbidity and mortality. It currently affects more than 285 million people worldwide according to the Mexican Federation of Diabetes. Diabetes mellitus is a group of metabolic disorders that are characterized by an increase in blood glucose levels (hyperglycemia). There are infections that are common in patients with diabetes and others which have greater severity and complications (Federación Mexicana de Diabetes, 2012).

A dental prosthesis is an artificial element that is used to restore the anatomy of one or several teeth, also restoring the relationship between the maxillaries, at the same time correcting the vertical dimension and improving both natural dentition and periodontal structures (Rendon, 2007). Prostheses can be classified according to their characteristics, such as the type of support, the material used, and the type of restoration that will be performed; however, they can also be classified as unremovable (fixed prosthesis) and removable (removable partial tooth supported, total mucosal supported and over denture prosthesis). The objective of placing a partial or total prosthesis is to provide function, phonation, swallowing, esthetics, and safety for the patient who uses it.

Candidiasis is a frequent disorder, especially in patients with diabetes. It is caused by excessive growth of *Candida* in the mouth, digestive tract, vagina and other tissues. The risk factors for candidiasis are type 1 diabetes mellitus, characterized by autoimmune destruction...
of Langerhans islet B cells by T cells that cause a lack of production of insulin; type 2 diabetes, in which a family history of the disease as well as other factors such as age >40 years, obesity, the use of drugs (corticosteroids) are present, and gestational diabetes with poor glycemic control in which vaginal candidiasis is more frequent (Nowakowska et al., 2004). Immuno-suppressive states and drugs can also cause candidiasis. A prevalence of denture stomatitis of 15% to over 70% has been reported in denture wearers and can be caused by poor denture hygiene, continual and nighttime wearing of removable dentures, accumulation of denture plaque, and bacterial and yeast contamination of the denture surface. Another cause is mucosal trauma produced by poor-fitting dentures. These factors can increase colonization of the denture and oral mucosal surfaces by Candida albicans, which can produce an opportunistic infection (Gendreau and Loewy, 2011). Candida albicans has been identified as the most frequent etiological agent in oral candidiasis although other studies have isolated Candida dubliniensis, Candida parapsilosis, Candida krusei, Candida tropicalis and Candida glabrata in the pathogenesis of candida-associated denture stomatitis (Liebana Ureña, 2002; Salerno et al., 2011).

The aim of this study was to determine if there is a greater incidence of C. albicans in patients with diabetes in comparison with patients without diabetes when they use a dental prosthesis.

MATERIALS AND METHODS

Study population

We carried out a comparative, open, observational, prospective, cross sectional study in the Total Prosthesis Clinic of the Universidad Autónoma de Nuevo León Dental School. The population consisted of 11250 patients that came for consultation in the prosthesis clinic. To calculate sample size we used the following formula:

\[
n = \frac{Nz^2pq}{e^2(N-1)+z^2pq} = \frac{11250(1.96)^2(0.55)(0.45)}{(0.15)^2(11250-1)+(1.96)^2(0.55)(0.45)} = 42.10 \approx 43 \text{ patients}
\]

with a margin of error of 0.15 and a 95% confidence interval. The population sample consisted of 43 patients (13 men and 30 women) with a mean age of 67 years (range 45 to 85).

The study was approved by the Bioethics Committee of the School of Dentistry of the UANL with registration number SSPI-010613, Page 00008. Patients provided informed consent and were assured of the confidentiality of their personal data and medical history. Men and women with (n = 12) and without diabetes (n = 31) with partial or total dental loss and a dental prosthesis evaluated from August to December 2011 were included. Out of town patients and those less than 44 or greater than 85 years of age were excluded. Patients who did not follow or complete the study protocol or who died during the study were removed.

The presence of diabetes was determined by patient interview. Since C. albicans is part of the normal flora of the oral cavity, we determined its quantitative presence and that of other species of Candida. The independent variables of the study were the presence of diabetes as a nominal qualitative variable. The dependent variables were the quantitative presence of Candida, determined by counting the number of colony forming units (CFU), and the identification of the species of Candida present.

Prostheses were studied at seven days because patients were programmed for revision at that time for examination and necessary adjustments. The opportunity was used to take samples at this time.

Procedures

In the first phase, the prostheses were washed with soap and water, and disinfected with Microdacyn MR for 1 min. They were then rinsed with sterile distilled water to ensure that the prosthesis was clean before giving it to the patient. The device was then placed in the patient’s mouth.

After seven days, the patient returned to the clinic and a sample was obtained using a sterile swab dipped in distilled water. The swab was placed in an Eppendorf tube containing phosphate buffer as a transport medium. Samples were taken to the molecular biology laboratory where 100 µL of the sample were seeded in a Petri dish with previously prepared CHROMagar. Seeding was carried out with a sterile glass pipette.

Samples were incubated at 37°C for 24 to 48 h in CHROMagar™ media and up to 72 h to improve colony pigmentation. Four types of Candida were identified by pigmentation. Candida albicans colonies appear green in CHROMagar, C. tropicalis metallic blue, C. krusei fuzzy pink and other species, white to mauve. A macroscopic count of CFU was performed according to the color of the colony.

Statistical analysis

Descriptive statistics were applied to obtain means, medians, standard deviations and 95% confidence intervals of the variable UFC for Candida; frequencies and percentages for the presence of diabetes in patients were also determined. Cross tabulation was carried out between the presence of diabetes and the Candida values obtained; the mean difference (95% confidence interval) for the presence of Candida in each of the groups was subsequently tested. All statistical procedures were performed using Microsoft Excel 2007 and SPSS, achieving a 95% confidence interval for all cases.

RESULTS

We evaluated 13 (30.2%) men and 30 (69.8%) women with (n = 12; 28%) and without diabetes (n = 31; 72%). Forty two patients had upper dentures and 36 lower dentures. The mean pH among patients with and without diabetes was 7.25 and 7.26, respectively (P = 0.4828). Of the total patients 30 (71.44%) had Candida in the upper denture. In patients with diabetes mellitus, 9 (75%) had some form of Candida in the upper denture. In patients without diabetes mellitus, 21 (70%) had Candida in the upper denture. Of all the patients studied, C. albicans was present in 11 patients (26.2%). In patients with diabetes, C. albicans was present in 3 (25%). Of the
patients without diabetes mellitus only 8 (26.7%) had C. albicans.

In patients with diabetes mellitus, 7 (58.3%) had C. tropicalis. C. krusei was found in 10 (23.8%) patients. This strain of Candida was found in 6 (20%) patients that did not have diabetes mellitus and in 4 (33.3%) patients with diabetes (Figure 1). It was also observed that 3 (7.1%) patients in the study had other species of Candida. In patients with diabetes mellitus, only 1 (8.3%) had other species of Candida. Of the patients who did not have diabetes mellitus, 2 (6.7%) presented other species of Candida.

Regarding those with lower dentures, it was observed that 25 (67.6%) had Candida. In patients with diabetes mellitus, 9 (75%) had some form of Candida. Of the patients who did not have diabetes mellitus, 16 (64%) had some forms of Candida. In patients with lower dentures, 9 (24.3%) had C. albicans. In patients with diabetes mellitus, 3 (25%) had C. albicans. In patients without diabetes mellitus, 6 (24%) had C. albicans (Figure 2).

It was found that 19 (51.4%) patients had C. tropicalis. Of the patients who had diabetes mellitus, 7 (58.3%) had C. tropicalis. Of the patients who did not have diabetes mellitus, 12 (48%) had C. tropicalis.

C. krusei was present in 8 (21.6%) patients. In patients with diabetes mellitus, 4 (33.3%) had C. krusei. Of the patients who did not have diabetes mellitus, 4 (16%) had C. krusei. As for other species of Candida, growth was observed in 3 (8.1%) patients with lower dentures. In
patients with diabetes mellitus, only one (8.3%) had other species of Candida. Of the patients who did not have diabetes mellitus, 2 (8%) had other Candida species.

**DISCUSSION**

We found an incidence of Candida in 71.4% of samples taken from 43 patients. This is in contrast with Zaremba (2006), who isolated Candida in 59.4% of 32 patients with prosthesis and Daniluk et al. (2006), who found an incidence of 66.7% in samples from patients without diabetes, and 43.8% in patients with diabetes. In the present study, 26.2% of the sampled patients had C. albicans and in those with diabetes only 25% had the fungus. Pfaller et al. (1996) evaluated the use of CHROMagar as a differential culture medium that allows the isolation of yeasts and simultaneously identifies colonies of C. albicans, C. tropicalis and C. krusei. They found that more than 95% of the values and clinical isolates of Candida species were correctly identified based on colony morphology and CHROMagar pigmentation. The study by Odds et al. (1994) found that the specificity and sensitivity of the medium in the identification of C. albicans, C. tropicalis and C. krusei exceeded 99% for all three species.

The study by Mata de Henning et al. (2001) showed that 65% of patients had Candida species in 20 patients studied by swabbing the prosthesis in the area in contact with the palatal mucosa. However, their study differs from ours because they observed Candida albicans in 50% of cases and found one case with a species that was not albicans.

In a study by Belazi (2005), of the 128 diabetic and 84 nondiabetic patients, Candida was observed in the oral cavity of 64% of the diabetic patients. However, in contrast with the present research, they found a higher frequency of C. tropicalis. There was also a difference with Daniluk et al. (2006), where it was determined that 43.8% of diabetic patients had C. albicans. In the present study, only 25% had a positive result.

We had a small number of patients with diabetes in our clinic and we believed that there would be a greater incidence of C. albicans, but this was not the case. It would be convenient to perform this study in a greater number of patients.

Our results indicate that colonization by C. albicans is independent of the presence of diabetes mellitus in patients with total and/or partial dentures. Thus, no significant relationship between diabetic and nondiabetic patients with total and/or partial dentures was shown. C. albicans was not as frequent as other types of Candida, since C. tropicalis was found in 52.4% of samples, which was the highest and most significant finding in this study.

**ACKNOWLEDGEMENTS**

We thank the Department of Microbiology and the Molecular Biology Laboratory, School of Dentistry, Universidad Autonoma de Nuevo Leon for their support and the use of their installations for this study. We also thank Sergio Lozano-Rodriguez, M.D. for his help in editing and translating the manuscript.

**REFERENCES**


Full Length Research Paper

Antimicrobial activity of *Syzygium aromaticum* extracts against food spoilage bacteria

Muhammad Saeed¹, Muhammad Nadeem²*, Moazzam Rafiq Khan¹, Muhammad Asim Shabbir¹, Aamir Shehzad¹ and Rai Muhammad Amir¹

¹National Institute of Food Science and Technology, University of Agriculture, Faisalabad-Pakistan. 
²Department of Food Science, University College of Agriculture and Environmental Sciences, The Islamia University Bahawalpur, Bahawalpur-Pakistan.

Accepted 20 September, 2013

In this study, the emphasis was on extraction of aqueous and methanolic extract from whole clove (*Syzygium aromaticum*) that can be efficiently used as an antimicrobial agent with an ultimate objective of developing replacements for the synthetic chemical additives in food products. Antimicrobial activity of extract revealed that the solvent extract of clove has a great potential for the inhibition of microbial load. The value of antimicrobial activities of solvent extract ranged from 12 to 17 mm in the disc diffusion method as compared to aqueous extract i.e ranged from 12 to 16 mm. Minimum Inhibitory Concentrations were found from 17 to 23 mm for solvent extract and 13 to 17 mm for aqueous extract. The sensory characteristics of bread showed that the treatments had a highly significant effect on volume, color of crust, symmetry of form, evenness of bake, character of crust, grain, color of crumb, taste and texture and overall acceptability of bread. Significantly lower total scores for the bread were exhibited by the bread supplemented with 2 and 2.5% clove extract. The results revealed that bread containing up to 1% clove extract is acceptable.

**Key words:** Antimicrobial activity, Clove, *Syzygium aromaticum*, food spoilage bacteria, bread, total plate count.

INTRODUCTION

The distinguished inhibitors of microorganisms are plant essential oils and their extracts. The Spices are reputed to possess several medicinal and pharmacological properties and hence find position in the preparation of a number of medicines. Spices impart aroma, colour and taste to food preparations and sometime mask undesirable odours. Volatile oils give the aroma, and oleoresins impart the taste (Proestos et al., 2008).

Due of their antimicrobial nature, spices are used to improve taste and enhance shelf life. Some of spices are also known to contribute to the self-defence of plants against infectious organisms (Kim et al., 2003). There is considerable potential for utilization of natural antimicrobials in foods, especially in fresh fruits and vegetables. Extract derived from spices and plants have antimicrobial activity against *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenteriae*, *Bacillus subtilis* and *Staphylococcus aureus* at levels between 0.2 and 10 µg ml⁻¹ (Burt, 2004).

Presently, the major problem is that we cannot use chemical preservatives safely now a day due to carcinogenic nature of these chemicals. Residual toxicity is increased due to these chemicals. Due to these reasons, consumers lean to be doubful of chemical additives and thus the requirement has been increased for natural and adequate preservatives (Skandamis et al., 2001). As a
consequence, natural antimicrobials are receiving a good deal of attention for a number of micro-organism-control issues. Reducing the need for antibiotics, controlling microbial contamination, improving shelf-life extension technologies to eliminate undesirable pathogens, decreasing the development of antibiotic resistance by pathogenic micro-organisms or strengthening immune cells in humans are some of the benefits (Fisher and Phillips, 2008).

Clove belongs to a tree Eugenia caryophyllata (Syzygium aromaticum), is used as a spice in almost all the world’s fare. Bud Oil of Clove has natural behavior and the main properties include antioxidant, insecticidal, antifungal and antibacterial properties. By tradition, it has been used in food preservation as flavoring and antimicrobial substance (Velluti et al., 2003). It has a very major role in spice trade and is highly appreciated for their therapeutic properties. Cloves are an excellent source of manganese. They are also a very good source of dietary fiber, vitamin C, vitamin K, and Ω-3 fatty acids and a good source of magnesium and calcium. Cloves consist of a significant amount of proteins, iron, carbohydrates, calcium, phosphorus, potassium, sodium and hydrochloric acid. They are also rich in vitamins A and C, manganese, and dietary fiber (Kim et al., 1998).

The most important constituent of clove is the phenylpropene eugenol due to which it has strong characteristic aroma. Major parts of clove consist of eugenol comprises 70 to 90 % and remaining 15% consist of dry weight (Shobana and Naidu, 2000). Molds, yeast and bacterial growth could be inhibited by the application of clove essential oil (Burt, 2004). Micro-organisms like Alternaria sp., Aspergillus sp., Canninghamella sp., Lactobacillus sp. Fusarium sp., Clostridium sp; Mucor sp., Salmonella sp. Penicillium sp. Bacillus sp. could be repressed by using clove essential oil (Soliman and Badeea, 2002). The cloves are antimutagenic, anti-inflammatory, antioxidant, antiulcerogenic, antithrombotic and antiparasitic. The essential oil extracted from the dried flower buds of clove are used for acne, warts, scars and parasites (Miyazawa and Hisama, 2003; Srivastava and Malhotra, 1991; Chaieb et al., 2007b).

B. subtilis is not a human pathogen but is responsible for causing Ropiness, a sticky and stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough (Priest et al., 1988). B. subtilis has been associated with outbreaks of food poisoning but the exact nature of its involvement has not been established. B. subtilis, like other closely related species in the genus, B. licheniformis, B. pumulis, and B. megaterium, have been shown to be capable of producing lecitinase, an enzyme which disrupts membranes of mammalian cells. However, there has not been any correlation between lecitinase production and human disease in B. subtilis (Collins et al., 1991). Considering the importance clove, the present project has been designed to to analyze the physico-chemical properties of clove extract in order to study the inhibitory effect of clove extract against food spoilage bacteria.

**MATERIALS AND METHODS**

**Sample preparation and extraction**

Fresh whole clove samples were purchased from the local grain market of Faisalabad, Punjab, Pakistan. The samples were obtained from retail spice-sellers in the amount of 1 kg. Grinding was done with grinder MJ-176P in the laboratory grinder. The samples were kept in closed containers after being chopped into small pieces (1 mm). Two types of clove extract were prepared for aqueous and methanolic extracts.

For the preparation of aqueous extract, 150 ml of distilled water was added into 25 g of chopped clove and the mixture was left for agitation in shaker incubator for 8 h at 300 rpm at 39°C. Afterwards, it was filtered. The dark colored extract obtained at the end of this process was used for further analysis. The sample extract was kept in the refrigerator (4°C) for accomplishment of further analysis (Wilson, 1995).

For the preparation of solvent extract, 150 ml of methanol was added into 25 g of chopped clove and the mixture was left for agitation in shaker incubator for 8 h at 300 rpm and 39°C. Afterwards, it was filtered and the methanol was vaporized in rotary evaporator (60°C). The dark colored oily extract obtained at the end of this process was used form for the analysis. The sample extract was kept in the refrigerator (4°C) until the analysis was accomplished (Wilson, 1995).

**Physicochemical analysis of extract**

Electronic digital type pH meter of Wellium model: Inolab pH 720, WTW 82362 was used for pH determination. Total Acidity, Referactive index, Specific gravity and Brix value was determined by following the methods described in AOAC (2007).

**Isolation of bacteria**

Glassware should be autoclave before the start of experiment. Spoiled bread samples were collected from different bakeries located in vicinity of Faisalabad city. The samples were drawn in sterilized screw capped bottles and preserved for further studies. Six sterilized test tubes were taken and labeled as 10⁻¹, 10⁻², 10⁻³... 10⁻⁶. Serial dilutions for each sample were made by the method as recommended by Cappuccino and Sherman (1996). Nutrient agar, Mueller Hinton agar (MH agar) and Potato dextrose agar (PDA) were prepared according to the methods of NCCLS (2000). In each Petri plate 10ml of medium and 1ml of respective dilution were added carefully. Six sterilized test tubes were taken and labeled as 10⁻¹, 10⁻², 10⁻³... 10⁻⁶. Medium and inoculum were mixed immediately by given to and for shaking and circular movement lasting 5 to 7 s. Medium was allowed to solidify. After solidifying inverted Petri plates were placed in incubator at 37°C for 24 to 48 h for bacterial isolation by following the method given by Cappuccino and Sherman (1996).

**Morphological examination of bacteria**

The representative colonies, showing catalase negative and Gram positive, were randomly picked from higher dilution (10⁻²) of nutrient agar plates. The culture isolates were morphologically observed under microscope by following the method described by Cappuccino and Sherman (1996). Gram’s Staining was done according to the method described by Becker et al. (2003).

**Purification of isolates**

The colonies which appeared after 48 h incubation on nutrient agar
plates were subjected to morphological examination. Pure growth of *B. subtilis* were then transferred to potato dextrose agar and incubated at 37°C and preserved in refrigerator at 4°C for further use. The results of colony characteristics were recorded by following the method as described by Harrigan and McCance (1990).

**Determination of antibacterial activity**

Microbiological methods used for the determination of antimicrobial activity of clove extract were disc diffusion method and agar well diffusion method. Treatments T1, T2, T3, T4 and T5 for aqueous extract and methanolic extract were used at concentration 0.5, 1, 1.5, 2 and 2.5% respectively. Antimicrobial activity was determined by standard disc diffusion method as described by saeda et al. (2007). The inoculum suspension of each bacterial strain was swabbed on the entire surface of Mueller-Hinton agar (MHA, pH 7.3 ± 0.1, Difco). Sterile 6 mm filter paper discs (Schleicher & Schuell) immersed with clove extract was aseptically placed on MHA surfaces. The plates were left at ambient temperature for 15 min to allow excess prediffusion of extracts prior to incubation at 37°C for 24 h. Diameter of inhibition zones was measured. Each experiment was done in triplicate.

**Minimum inhibitory concentration (MIC)**

Minimum inhibitory concentration was determined by agar well diffusion method as described by Ogata et al. (2000). The plates containing Mueller Hinton agar medium were spread with either 0.1 ml of the bacterial inoculum. Wells (8 mm in diameter) were cut from agar plates using a sterilized stainless steel borer and were filled with 0.1 ml of clove extract. The plates were incubated at either 37°C for 24 h and the diameter of resultant zone of inhibition was measured. Each combination antimicrobial agent was repeated three times. Microorganisms showing a clear zone of more than 6 mm were considered to be inhibited.

**Preparation of bread**

The breads were prepared from straight grade flour containing clove methanolic extract from 0.5 to 2.5% by method according to the procedure as described in AACC (2000).

**Physical analysis of bread**

The color of bread was determined with the help of Color meter as described by Rocha and Morais (2003). The textual study of bread was conducted by using Texture analyzer (Model TA-XT2, Stable Microsystems, Surrey, UK) as described by Piga et al. (2005). The water activity of bread was determined by using Hygropalm water activity meter (Rotronic Hygropalm water activity meter, series number 601089738) as described by Piga et al. (2005). The loaf volume of the bread was determined using a rapeseed displacement method by the method of Hussain (2009).

**Total plate count of bread**

Six sterilized test tubes were taken and labeled as $10^1$, $10^2$, $10^3$, ..., $10^6$. Nine milliliters of normal saline was poured into each test tube. One grams of crushed spoiled bread sample was shifted into the first test tube and contents were mixed well by gentle shaking. 1m shifted contents from each dilution on to the surface of separate plate count agar plates. Serial dilutions for each sample were made by the method as recommended by Cappuccino and Sherman (1996). One milliliters shifted contents from each dilution on to the surface of separate plate count agar plates and spread well and incubated at 37°C for 24 h. Average number of colonies was counted from those dilutions that showed the colonies size ranging from 30 to 300 with the help of colony counter. The total plate count was calculated using the following formula:

$$\text{Total Plate Count} = \text{Average number of colonies} \times \text{dilution factor/volume factor}$$

**Sensory evaluation**

The prepared bread loaves were evaluated by a panel of judges for external characteristics such as volume, crust color, symmetry of form, evenness of bake and internal characteristics like grain, crumb color, aroma, taste and texture by following the method of Land and Shepherd (1988).

**Statistical analysis**

The data obtained from each parameter was subjected to a statistical analysis using analysis of variance techniques to determine the level of significance in different parameters according to the method described by Steel et al. (1997).

### RESULTS AND DISCUSSION

**Physicochemical analysis of extracts**

The results indicated that acidity of clove extracts differed significantly. The results given in the Table 1 indicated that total acidity was found to be 0.45 and 0.25 for methanolic extract and aqueous extract respectively. With increase in solvent concentration, acidity was increased. The results of present study are in agreement with the findings of Burt (2004) who reported that acidity ranged from 0.20 to 0.55%. The results were also in agreement with the findings of Hammer et al. (1999). They studied 13 different spices solvent extract and reported that titratable acidity ranged from 0.35 to 0.55.

The results for pH of clove extract (Table 1) indicated significant variations for pH of clove extract. The results indicated that pH was ranged from 5.20 to 6.70 for methanolic extract and aqueous extract respectively. The results of present study are in concordance with the findings of Koiche and Bouras (2010) who reported that pH of the clove extract was ranged from 5.0 to 5.5.
et al. (2007) described their results for pH of clove aqueous extract was ranged from 6.50 to 6.90. The results for specific gravity of clove extracts (Table 1) indicated the significant differences for specific gravity.

The results showed that specific gravity was ranged from 1.025 to 1.015 for methanolic extract and aqueous extract respectively. The results of present study are in agreement with the findings of Kumar et al. (2007a) who described that the specific gravity of methanolic extract was ranged from 1.02 to 1.028. Kim et al. (1995) described that the specific gravity of clove extract was ranged from 1.01 to 1.029.

The results pertaining to refractive index of clove extracts (Table 1) indicated significant variations of refractive index. The results showed that refractive index ranged from 1.533 to 1.523 for methanolic extract and aqueous extract respectively. The results of present study are in agreement to the findings of Dorman et al., 2000 who reported that refractive index of clove extract was ranged from 1.520 to 1.535. These results were also in agreement with the findings of Gutierrez et al. (2009) who reported the refractive index of clove extract was ranged from 1.518 to 1.536.

The results for brix of clove extract showed that brix of clove extracts had non significant differences. The results (Table 1) indicated that brix of methanolic and aqueous extracts was 9.80 and 9.80 respectively. The results of present study are in contradiction to the findings of Cowan, 1999 who reported that brix of spices extract are in the range of 8.50 to 9.0. The difference in the results might be due to the chemical reaction of solvents with sugars during the mixing operations.

Microbiological analysis of extract

**Determination of antibacterial activity**

The results for antibacterial activity of aqueous clove extract have been presented in Table 2. The results indicated that aqueous extract of clove extract showed highly significant variations as compared to methanolic extract for antibacterial activity. The highest zone of inhibition of antibacterial activity for clove aqueous extract was showed by $T_5$ (16.66 mm) followed by $T_4$ (15.00 mm).

Minimum antibacterial activity was showed by $T_1$ (12.33 mm)

The results for antibacterial activity of methanolic clove extract (Table 2) indicated that clove methanolic extract showed significant results as compared to aqueous extract for antibacterial activity. Methanolic extract have highest antibacterial activity at $T_5$ (17.66 mm) and minimum zone of inhibition was showed by $T_1$ (14.00 mm). The present results showed that methanolic extract exhibited the strongest inhibitory activity (14 to 17.66 mm) as compared to results of aqueous extract (12 to 16 mm). It is evident from the results that the diameter of inhibition increased significantly as concentration of extract increased. Diameter is showed in millimeter (mm).

The results of present study are in agreement with the findings of Meena and Sethi (1994) who reported that different extracts of clove showed strong antibacterial activity against *P. vulgaris* and *B. subtilis*. They reported the inhibition diameter in the range of 12 to 25 mm. Their results also in concordance with the findings of (Kumar, 2007a) who stated that *C. cumin* showed good activity against *S. aureus* and *B. subtilis*. Both aqueous and methanol extracts of *S. lineare* and *T. asiatica* showed a strong antimicrobial activity against food spoilage bacteria.

Similar results were found in the previous study of Mytle and others (2006) that determined the inhibitory effect of clove extract against *B. subtilis* on chicken frankfurters. *B. Subtilis* was inoculated at low (102 to 103 CFU/g) or high cell numbers (104 to 106 CFU/g), and stored at 5°C for 2 wk or at 15°C for 1 wk. All strains of *B. subtilis* and grew on control frankfurters at 5 and 15°C, but growth was inhibited under both storage conditions in the presence of either 1 or 2% clove oil.

*S. aromaticum*, *P. granatum*, *C. cumin* and *T. asiatica* produced the largest zones of inhibition against *B. subtilis*, *S. aureus* and *S. epidermidis*. (Bevilacqua et al., 2010) reported good antibacterial activity in clove methanolic extract against *E. coli* using aqueous and methanol extracts. The ethanolic extracts of clove, cumin, and kaffir lime peels showed the broadest antibacterial activity by inhibiting growth of all bacterial strains tested (the diameter of inhibition zone, 8 to 22 mm), while the extracts of cardamom, cinnamon, and kaffir lime leaves inhibited the growth of almost all strains (7 to 12 mm), except for *S.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aqueous extract</th>
<th>Methanolic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_0$= control without methanolic extract</td>
<td>12.33 ± 0.22</td>
<td>14.00 ± 0.23</td>
</tr>
<tr>
<td>$T_1$= 0.5% methanolic extract</td>
<td>14.66 ± 0.24</td>
<td>14.66 ± 0.25</td>
</tr>
<tr>
<td>$T_2$= 1.0% methanolic extract</td>
<td>14.85 ± 0.25</td>
<td>15.33 ± 0.28</td>
</tr>
<tr>
<td>$T_3$= 1.5% methanolic extract</td>
<td>15.00 ± 0.25</td>
<td>16.00 ± 0.26</td>
</tr>
<tr>
<td>$T_4$= 2.0% methanolic extract</td>
<td>15.84 ± 0.25</td>
<td>16.66 ± 0.29</td>
</tr>
<tr>
<td>$T_5$= 2.5% methanolic extract</td>
<td>15.84 ± 0.25</td>
<td>16.66 ± 0.28</td>
</tr>
</tbody>
</table>

Values are given as Mean ± Standard Deviation, $T_0$= Control (without methanolic extract).
Table 3. Effect of clove aqueous and methanolic extracts on MIC.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aqueous extract</th>
<th>Methanolic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T₁</td>
<td>14.00 ± 0.22</td>
<td>14.66 ± 0.28</td>
</tr>
<tr>
<td>T₂</td>
<td>16.00 ± 0.25</td>
<td>15.66 ± 0.27</td>
</tr>
<tr>
<td>T₃</td>
<td>17.00 ± 0.23</td>
<td>17.00 ± 0.25</td>
</tr>
<tr>
<td>T₄</td>
<td>19.00 ± 0.24</td>
<td>20.00 ± 0.26</td>
</tr>
<tr>
<td>T₅</td>
<td>21.00 ± 0.27</td>
<td>23.00 ± 0.27</td>
</tr>
</tbody>
</table>

Values are given as Mean ± Standard Deviation, T₀= Control (without methanolic extract), T₁= 0.5% methanolic extract, T₂= 1.0% methanolic extract, T₃= 1.5% methanolic extract, T₄= 2% methanolic extract, T₅= 2.5% methanolic extract.

Typhimurium, S. London, and Serratia marcescens. Plant-derived antimicrobial compounds have been recognized as means of inhibiting undesirable bacteria and numerous research articles have described the antimicrobial properties of plant extracts. Clove extract has shown inhibiting activity against bacteria (Kildeaa, 2004).

Minimum inhibitory concentration (MIC)

The results pertaining to MIC of aqueous extract have been given in the Table 3 showed highly significant differences between aqueous extract and methanolic extract for minimum inhibitory concentration. The results indicated that highest value of MIC with aqueous extract was shown by T₅ (21 mm) and minimum value was shown by T₁ (17 mm).

The results for MIC of methanolic extract (Table 3) showed highly significant variations among aqueous extract and methanolic of MIC. Highest value of MIC methanolic extract was shown by T₅ (23 mm) and minimum value 17 mm was shown by T₁. Results showed that MIC value of methanolic extract is greater as compared to aqueous extract because methanol also acts as a natural antimicrobial agent.

The results of present study are in concordance with the findings of Kim et al. (1994) who showed that the methanolic extract of clove showed inhibitory activity against all the six food associated bacteria in which the diameter of zone of growth inhibition varied between 15 to 25 mm (in clove) and 15 to 20 mm (in garlic). The clove ethanolic extract showed highest diameter of zone of inhibition 32 mm against E. coli followed by S. aureus (21 mm) and B. subtilis (23 mm) (Burt, 2004).

The clove ethanolic extract showed similar zone of inhibition of 20 mm diameter against B. megaterium and B. sphaericus. The minimum inhibitory activity was recorded against B. polymyxa. Our results substantiate the findings of Soliman and Badeaa (2002) that demonstrated the antibacterial activity of clove ethanolic extract against E. coli, S. aureus and B. subtilis and found that the highest antibacterial activity was against B. subtilis.

The MIC values of the clove methanolic extract tested against L. monocytogenes ranged in between 20 to 25 mm for selected ethanol extracts ranged from 0.25 to 11.75 mg/mL. It is apparent from the results that the MIC values are high for lavender and verbena, explaining the extent of resistance offered by L. monocytogenes, against these ethanol extracts. This study revealed that clove extract showed maximum activity against L. monocytogenes with MIC value 0.25 mg/mL followed by mint timija extract with MIC value of 0.315 mg/mL, indicating that clove and mint timija showed excellent antimicrobial activity against L. monocytogenes. L. monocytogenes is fairly sensitive to all ethanol extracts except lavender and verbena and was showing moderate MIC values against rosemary, geranium and camomile. The MIC values were used as guide for the treatment and battle against undesirable microorganisms. The results obtained showed that the MIC values varied according to the extracts and indicated that clove exhibited the strongest antibacterial activity, followed by mint timija. Similar result has been reported by Meena and Sethi (1994).

Analysis of bread

Based on the results obtained during study, it is stated that methanolic extracts had great antibacterial activity and zone of inhibition than aqueous extract. So bread was prepared from methanolic extracts.

Physical analysis of bread

The results pertaining to color of bread have been given in the Table 4. The results indicated that the color value of bread differed significantly due to increasing concentrations of extract. The results illustrated that color of breads prepared from T₀ (control) had the minimum color value that is, 173.0 and its value increased gradually as concentrations of extract increased. The darker color was due to dark oily color of extract. The bread prepared with T₁ was found as best value of color near to control. The breads prepared from T₅ concentration got the maximum color value that is, 186.67. The results of present study are in concordance to the finding of Holley and Patel (2005) who reported that color value of cereal products increase with increase in the concentration of spices extract.

The results regarding the water activity results indicated highly significant variations for water activity of bread. The results indicated that water activity of breads ranged from 0.77 to 0.83. The maximum water activity was found in the bread prepared with T₅ while the minimum water activity was found in the bread prepared with T₀. The water activity was increased as the percentage of extract increased (Dragland, 2003). The results pertaining to the texture of bread (Table 4) showed highly significant results for texture. The results indicated that the texture (firmness) of bread ranged from 1721 to 2043. Maximum
Table 4. Effect of clove methanolic extract on physical characteristics of bread.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Color</th>
<th>Water activity</th>
<th>Texture</th>
<th>Loaf volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>173.00 ± 0.25</td>
<td>0.77 ± 0.25</td>
<td>1721.3 ± 0.28</td>
<td>620.0 ± 0.25</td>
</tr>
<tr>
<td>T₁</td>
<td>175.67 ± 0.26</td>
<td>0.79 ± 0.24</td>
<td>1814.7 ± 0.21</td>
<td>580.3 ± 0.22</td>
</tr>
<tr>
<td>T₂</td>
<td>176.67 ± 1.23</td>
<td>0.80 ± 0.25</td>
<td>1920.7 ± 0.23</td>
<td>550.30 ± 0.24</td>
</tr>
<tr>
<td>T₃</td>
<td>180.00+2.09</td>
<td>0.80 ± 0.26</td>
<td>2016.0 ± 0.25</td>
<td>520.30 ± 0.21</td>
</tr>
<tr>
<td>T₄</td>
<td>183.00+0.76</td>
<td>0.81 ± 0.27</td>
<td>2029.7 ± 0.26</td>
<td>490.30 ± 0.26</td>
</tr>
<tr>
<td>T₅</td>
<td>186.67+0.91</td>
<td>0.83 ± 0.26</td>
<td>2043.0 ± 0.27</td>
<td>440.00 ± 0.28</td>
</tr>
</tbody>
</table>

Values are given as Mean ± Standard Deviation, T₀= Control (without methanolic extract), T₁= 0.5% methanolic extract, T₂= 1.0% methanolic extract, T₃= 1.5% methanolic extract, T₄= 2% methanolic extract, T₅= 2.5% methanolic extract.

Table 5. Effect of clove methanolic extract on external characteristics of breads.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume</th>
<th>Crust color</th>
<th>Symmetry of form</th>
<th>Evenness of bake</th>
<th>Crust character</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>8.80 ± 0.21</td>
<td>8.20 ± 0.26</td>
<td>4.80 ± 0.21</td>
<td>2.60 ± 0.25</td>
<td>2.90 ± 0.28</td>
</tr>
<tr>
<td>T₁</td>
<td>7.95 ± 0.25</td>
<td>7.60 ± 0.23</td>
<td>4.05 ± 0.22</td>
<td>2.60 ± 0.23</td>
<td>2.40 ± 0.21</td>
</tr>
<tr>
<td>T₂</td>
<td>7.60 ± 0.22</td>
<td>7.10 ± 0.28</td>
<td>3.80 ± 0.23</td>
<td>2.30 ± 0.26</td>
<td>2.30 ± 0.27</td>
</tr>
<tr>
<td>T₃</td>
<td>6.30 ±0.23</td>
<td>5.70 ± 0.25</td>
<td>3.60 ± 0.24</td>
<td>2.20 ± 0.24</td>
<td>2.20 ± 0.22</td>
</tr>
<tr>
<td>T₄</td>
<td>5.30 ±0.24</td>
<td>4.30 ±0.24</td>
<td>3.30 ±0.25</td>
<td>2.20 ± 0.22</td>
<td>1.90 ± 0.23</td>
</tr>
<tr>
<td>T₅</td>
<td>4.30 ±0.26</td>
<td>3.40 ±0.21</td>
<td>3.30 ±0.26</td>
<td>2.20 ± 0.27</td>
<td>1.80 ± 0.25</td>
</tr>
</tbody>
</table>

Values are given as Mean ± Standard Deviation, T₀= Control (without methanolic extract), T₁= 0.5% methanolic extract, T₂= 1.0% methanolic extract, T₃= 1.5% methanolic extract, T₄= 2% methanolic extract, T₅= 2.5% methanolic extract.

The firmness value of bread was 2043 prepared with T₅ (2.5%) methanolic extract. Minimum firmness value of bread prepared with T₀ (control) extract concentration was 1721. The results of present study are in agreement with the findings of Piga et al. (2005) who reported that firmness value is increased as the percentage of spices essential oil increased.

The results for loaf volume of breads containing methanolic extract (Table 4) indicated highly significant results for loaf volume of bread. The results revealed that the maximum loaf volume (620 ml) was found by the bread produced from T₅ (control) followed by the T₁ extract (580 ml) while the minimum loaf volume (440 ml) was found in the T₃ extract bread. The results indicated that loaf volume of bread was affected significantly by different concentrations level of extract. It is obvious from results that loaf volume of bread containing methanolic extract was higher than prepared from the bread having no extract.

The decrease in loaf volume of the bread may be attributed due to the reduction in wheat structure forming proteins and low ability of dough to trap air. The protein quantity, alpha amylase activity, damaged starch and genetic factors might have significant effect on bread volume and baking quality for different composite flours (Burt et al., 2007).

Sensory evaluation of bread

The sensory evaluation of bread for various attributes such as volume, colour, symmetry of form, evenness of bake, character of crust, grain, and colour of crumb, aroma, taste and texture was carried out. The product was evaluated by a panel of judges and the results are described below.

External characteristics of breads

The results pertaining regarding volume of breads have been given in Table 5 revealed that methanolic extract showed highly significant effect on the scores assigned to loaf volume of the breads. The results revealed that the scores assigned to volume of breads were affected significantly by the level of extracts concentrations. The results that the scores assigned to loaf volume of breads ranged from 4.30 to 8.80. The judges assigned maximum scores to the volume of control breads (8.80) followed by the breads prepared from the T₁ (0.5% extract). However, the breads prepared from T₅ (2.5%) got minimum scores with respect to volume. Decrease in volume was observed after baking of breads. In the present study the breads prepared from methanolic extract showed variable trends as breads from T₁ and control got statistically closest scores for volume where as breads from other conc. got significantly lower scores for volume of breads.

Crust color is an important sensory parameter concerning the consumer’s acceptability of bread. The results (Table 5) indicated that the clove methanolic extract showed significant effect on the scores given by judges to crust color of the breads. The results indicated that there was a significant decrease in scores assigned to crust
color of breads prepared from different extract concentrations as compared to control bread. The breads prepared from T_0 got significantly the highest scores for crust color followed by the breads produced from T_1. The results also indicated that bread prepared with T_5 has minimum crust color value. Crust colour of the bread was light brown which darkened progressively with the increasing level of extract concentration. The darkened colour of crust may be due to the Maillard reaction taking place during baking of loaves, due to high lysine contents. The results of the present study are comparable with those of Lee and Shibamoto (2000) who studied the behavior of different spices extract during bread making process and found that the addition of extract has a positive effect for the crust color of sourdough breads.

Symmetry of form is an important bread parameter in deciding the characteristics like uneven top, low ends and shrunken sides of the bread. The results pertaining to the effect of different clove methanolic extract concentration indicated that scores assigned to evenness of bake differ significantly due to variation in extract concentration (Table 5). It is evident from the results that breads prepared from T_0 clove extract got significantly the highest scores. The scores for symmetry of form were assigned to be the lowest (3.3) to the breads prepared form T_5. It was also evident from the data that the score for symmetry of form decreased proportionally with increase in the concentration of clove extract. The results of present study are in agreement with the findings of Chavan et al. (1991) who reported that the score assigning to symmetry of form of bread decreased as the concentration level of methanolic extract increased. Burt (2007) also found that spices extracts significantly reduce the score for symmetry of form of bread.

The evenness of bake reflects that all sides including top and the bottom are uniformly baked and it also reflects the intensity of baking whether the sides having lighter or darker shade. The results pertaining regarding the effect of clove methanolic extract indicated that scores assigned to evenness of bake showed non significant variations (Table 5). The results for the evenness of bake of breads indicated that evenness of bake among breads varied from 2.0 to 2.6 prepared from different concentration of methanolic extract. The scores assigned to evenness of bake decrease significantly. The loaf should be evenly baked on all sides, including the bottom. Pan breads should be evenly colored with no light or burned spots. The shade of the sides and bottom should conform to that of the crust.

The results regarding scores given by judges to crust character of sourdough breads made from clove methanolic extract at different concentration have been presented in Table 5. The results showed that methanolic extract exhibited highly significant effect on scores given to crust character of the breads. The mean scores assigned by the panelists to the crust character of breads prepared from methanolic extract with the difference in concentration showed that the breads prepared from T_0 and T_1 got the highest scores 2.90 and 1.80 respectively for crust character.

### Internal characteristics of bread

The results (Table 6) showed that methanolic exhibited highly significant effect on scores given to grain of the breads. The scores assigned by the panelists to the grain of breads prepared from clove extract with the difference in concentration ranged from 7.20 to 14.00 among the breads. The breads from T_5 (7.20) got significantly lower scores while maximum scores were given to the control breads (14.0). The breads prepared from T_1 and T_2 got almost similar scores for grain.

A soft creamy white crumb color is preferred in white breads. However, in some geographic areas, a bright white color is also preferred for the bread crumb. The results for the effect of clove methanolic extract on crumb color of breads have been given in Table 6 indicated that clove extract showed significant effect on crumb color of the breads. The crumb color got significantly the highest scores for breads prepared from the T_0 (8.40) followed by the T_1 bread (7.50). The scores given to the breads decreased with the increase in extract concentrations. The results for crumb color of bread prepared from different concentration levels of clove extract indicate that clove extract significantly affected the crumb color of breads. The scores assigned by the panelists to the crumb color of breads prepared from different concentration of extract. Results indicated that breads prepared with T_0 got

---

**Table 6. Effect of Clove methanolic extract on internal characteristics of bread.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Grain</th>
<th>Crumb color</th>
<th>Aroma</th>
<th>Taste</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_0</td>
<td>14.00 ± 0.23</td>
<td>8.40 ± 0.25</td>
<td>8.70 ± 0.27</td>
<td>15.30 ± 0.21</td>
<td>13.80 ± 0.24</td>
</tr>
<tr>
<td>T_1</td>
<td>12.20 ± 0.22</td>
<td>7.50 ± 0.28</td>
<td>7.10 ± 0.26</td>
<td>14.80 ± 0.22</td>
<td>12.80 ± 0.25</td>
</tr>
<tr>
<td>T_2</td>
<td>11.30 ± 0.21</td>
<td>6.50 ± 0.27</td>
<td>6.20 ± 0.23</td>
<td>13.30 ± 0.24</td>
<td>11.10 ± 0.26</td>
</tr>
<tr>
<td>T_3</td>
<td>9.80 ± 0.25</td>
<td>5.20 ± 0.26</td>
<td>5.20 ± 0.24</td>
<td>12.84 ± 0.25</td>
<td>9.10 ± 0.28</td>
</tr>
<tr>
<td>T_4</td>
<td>8.30 ± 0.26</td>
<td>4.20 ± 0.29</td>
<td>4.40 ± 0.25</td>
<td>11.30 ± 0.26</td>
<td>7.80 ± 0.26</td>
</tr>
<tr>
<td>T_5</td>
<td>7.20 ± 0.27</td>
<td>3.30 ± 0.22</td>
<td>3.40 ± 0.21</td>
<td>10.20 ± 0.27</td>
<td>6.50 ± 0.23</td>
</tr>
</tbody>
</table>

Values are given as Mean ± Standard Deviation, T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2=1.0% methanolic extract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.
Table 7. Effect of clove methanolic extract on Total Plate Count of bread.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>6.5 × 10^2</td>
</tr>
<tr>
<td>T₁</td>
<td>5.6 × 10^2</td>
</tr>
<tr>
<td>T₂</td>
<td>5.0 × 10^2</td>
</tr>
<tr>
<td>T₃</td>
<td>4.4 × 10^2</td>
</tr>
<tr>
<td>T₄</td>
<td>3.4 × 10^2</td>
</tr>
<tr>
<td>T₅</td>
<td>3.1 × 10^2</td>
</tr>
</tbody>
</table>

T₀= Control (without methanolic extract), T₁= 0.5% methanolic extract, T₂= 1.0% methanolic extract, T₃= 1.5% methanolic extract, T₄= 2% methanolic extract, T₅= 2.5% methanolic extract.

significantly the highest scores (8.40) for crumb color while the breads prepared with T₅ were ranked at the bottom (3.30) by the judges. It is also evident from the results that breads prepared from T₁ is close to control value followed by T₂ extract concentration.

The results of aroma (Table 6) showed that aroma of breads prepared from different clove extract concentrations significantly affected the scores given to aroma of breads. The scores assigned to the aroma of different breads indicated that breads prepared from T₀ extract got statistically the highest scores (8.7) for aroma followed by breads from T₁ (7.10) whereas the minimum aroma scores were assigned to the breads prepared from T₅. The results are in concordance to the findings of Meena (1994) who reported that aroma of the food products decreased as the concentration of extract increased.

The results of scores allocated to taste of the bread samples prepared from different clove extract concentrations (Table 6) indicated significant effect of clove extract. The scores assigned to taste of breads prepared from clove extract showed that the breads prepared from control i.e. T₀ extract got the highest scores (15.30) for taste by the panelists. It is obvious from the results that breads prepared from control were graded at the bottom with respect to taste scores. The results in Table 6 further exposed that breads prepared from T₁ (14.80) and T₂ (13.30). There was a decline in assigning the scores to breads by increasing the level of extract concentration.

Texture and appearance are two major sensory characteristics of the cereal products. The textural properties of a food has been described as that group of physical characteristics that are sensed by the feeling of touch, disintegration and flow of the food under the application of a force, time and distance. The results for texture of breads prepared from different extract concentrations (Table 6) indicated that scores given to texture of breads differed significantly due to differences in extract concentration level. The scores given to the texture of breads prepared with extract indicated that the breads from T₀ extract got the maximum scores for texture (13.80) and the scores decreased progressively as the level of extract concentration increased. The breads prepared from T₅ got the minimum scores by the judges for texture.

Microbial analysis

Total plate count

The results pertaining to total plate count of bread have been presented in the Table 7. The results indicated the highly significant values for Total plate count of bread prepared with clove methanolic extract.

The results indicated that the total plate count of all the bread samples decreased significantly. Decrease in the total plate count occurred from 6.5 × 10^2 to 3.1 × 10^2 for all breads. Bread with clove methanolic extract T₁ (0.5%) had TPC value 5.6 × 10^2. TPC for bread with 1% extract decreased from 6.5 × 10^2 to 5.6 × 10^2 and 5.6 × 10^2 to 3.1 × 10^2 from the rest. The best results of TPC were obtained from the bread made with 0.25% methanolic extract. Additions of clove extract which is used as antimicrobial substance, showed decrease in the growth rate of microorganisms.

It is evident from the results that there was decrease in the total plate count occurred as the extract level increased. All the results were in the agreement with the Lewis and ausubel (2006) and Lane et al. (1991) who showed there was significant effects on spices extract addition on the TPC of bread.

Conclusion

The preliminary study of this project revealed that spices can be used as natural antimicrobial in food products due to their less lethal effects as compared to synthetic chemical additives. Consumers are very conscious about food safety nowadays. The Present study reveals that there is a great potential of using spices extract as natural antimicrobials for controlling food spoilage and pathogenic bacteria. Our results signify the fact that natural products like spices can be seen as alternatives to chemical preservatives used in various food industries so as to minimize their side effects and simultaneously improving the shelf life of the food products. The inhibitory factor responsible for the antimicrobial activity can further be identified and used as an alternative to currently used drugs against the pathogenic microbes. Nowadays microbes are increasingly developing resistance against the drugs in use. To combat against these drug resistant microbes, a large library of novel compounds is required. Natural products from plants may give us a solution to this alarming problem.

REFERENCES


Full Length Research Paper

Influence of multi drug resistance Gram negative bacteria in liver transplant recipient

Mohamed Abdel Aziz¹, Iman El-Kholy², Amin Abdo³ and Samy Selim¹,4*

¹Microbiology and Botany Department, Faculty of Science, Suez Canal University, Ismailia, P.O. 41522, Egypt.  
²Ain Shams University Specialized Hospital, Cairo, Egypt.  
³Gomohory Hospital, Ministry of Health, Yemen.  
⁴Department of Medical Laboratory Sciences, College of Applied Medical Science, Al Jouf University, Sakaka, P.O 2014, Saudi Arabia.

Accepted 20 September, 2013

Infection is a common cause of morbidity and mortality after liver transplantation, which are often caused by Gram negative bacteria and the most frequently occurring infectious complications after liver transplantation (LT). The aim of this study was to investigate, incidence, pathogenic spectrum, and risk factors for bacteria due to multidrug resistant (MDR) Gram-negative bacteria, and its impact on mortality after LT. In total, 150 consecutive patients who underwent liver transplantation between January 2012 and March 2013, 115 isolates of bacteria and 10 isolates fungal from 80 patients. MicroScan® microbiology and API20 were used to identify the all isolates. Bacterial infections represented the most frequent event (92.0%) and fungal infections (8.0%). The presumed sources of infection were the Urine (41 events, 51%), followed by the bile drain (14 events, 17.5%). Infections of the sputum, ascetic fluid, and blood site accounted for 13.8, 5.0 and 6.3%, respectively. Antibiotics the most susceptible antibiotic against Pseudomonas aeruginosa was colistin (94.1%). Patients with Gram-negative bacilli after liver transplantation show a significantly worse prognosis. Gram-negative bacteria after liver transplantation have been a major problem in our center.

Key words: Gram-negative bacteria, multi drug resistance, liver transplantation, mortality.

INTRODUCTION

Infection is a common cause of morbidity and mortality after liver transplantation (LT). The effect of infections in LT is higher compared with recipients of other organs. Bacterial infections are among the most relevant causes of morbidity and mortality after organ transplantation. The high prevalence of multidrug-resistance among bacterial pathogens causing infections in solid organ transplant recipients is an additional concern. In a recent report, infections with multidrug-resistant Gram negative bacteria were associated with higher mortality among liver transplant recipients (Shi et al., 2009). Emergence of nosocomial infections, especially Acinetobacter baumannii, Pseudomonas aeruginosa with multidrug resistance have become a major problem among transplant recipients in hospital settings (Diab et al., 2002, 2004; Patel et al., 2010). These infections can result in a wide range of complications, including bacteremia, pneumonia, urinary tract infection and peritonitis. The aim of this study was to investigate the clinical characteristics, mortality, and outcomes among liver transplant recipients with Gram-negative infection. Over the past 30 years, colistin use has been limited due to concerns regarding its toxicity along with the development of newer antibiotics with better safety profiles (Jain et al., 2004). However, the increasing
incidence of multidrug-resistant *A. baumannii* in addition to a lack of new antimicrobial agents has reawakened interest in the utilization of colistin due to its good activity against this organism.

**MATERIALS AND METHODS**

In total, 150 consecutive patients (50 female and 100 male) who underwent liver transplantation between January 2012 and March 2013 at the Ain Shams University Specialized Hospital, Cairo, Egypt, were enrolled in this study. Patients range age from 18 to 70 years. Specimens included blood, pus, tissues and body fluids, urine, sputum and peritoneal dialysate.

The strains had been isolated on blood agar, desoxycholate citrate agar, thiosulphate citrate bile salts sucrose agar tests for growth at 30, 37°C were performed in brain heart infusion broth in a water bath. Sheep blood (5%) agar plates were used to detected hemolysis, gelatin stab method was used to test for gelatin liquefaction. The susceptibility tests were read after overnight incubation, and the tests for hemolysis and growth at 30, 37°C were read on the first and second day. For gelatin liquefaction the incubation temperature was 22°C. Antimicrobial susceptibility was determined by the Kirby-Bauer disk diffusion method (Selim, 2011; Selim et al., 2012, 2013). The interpretive criteria followed the latest National Committee for Clinical Laboratory Standards (NCCLS) recommendations (NCCLS, 2002). Identified at least to genus level by MicroScan® microbiology, biochemical tests and API 20E system (bioMe’rieux, France), in a previous study and cryopreserved in 20% glycerol at -80°C. Working cultures were maintained in Luria Bertani (LB) agar and broth. We collected data from hospital and operative records of each liver transplantation recipient, including perioperative demographic and clinical characteristics (that is, age, gender, Child-Pugh), operative variables (that is, operation time, blood loss) and clinical events with 6 months post transplant (that is, duration of initial intubation, intensive care unit (ICU) stay, reoperation, dialysis, and rejection).

**Statistical analysis**

The data was analyzed using Chi-square test, Z-test and two way classification of ANOVA.

**RESULTS AND DISCUSSION**

In total, 150 patients (median age 40 years) were included in this study and contributed a total of 64.4, 80 patients positive infection, events 70 cases are negative. 125 isolated, bacterial infections represented the most frequent (115 events, 92%), followed by fungal infections (10 events, 8.0%).

**Bacterial infections**

Among the bacterial infections, the most frequent pathogen was *Pseudomonas aeruginosa* with 34 isolates (29.6%). The second most common isolated organism was *Klebsiella pneumonia* (25 events, 21.7%), followed by *E. coli* (24 events, 20.9%), *Acinetobacter baumannii* (13 events, 11.3%), *Staphylococcus* spp (12 events, 10.4%), *Enterobacter* spp (5 events, 4.3%), *Aeromonas* spp (2 events, 1.7%). The majority of the bacterial infections occurred during the first 6 months, with the highest incidence of infection during the first 30 days (30 events/month), Table 1.

**Fungal infections**

In total, 10 events of fungal infections were documented. In 7 cases (70.0%), *Candida* species were isolated (5 events of *Candida albicans* and 2 cases with *Candida krusei*). Infections with *Aspergillus* accounted for 30% of fungal infections (3 cases of *Aspergillus fumigatus*). One case with pulmonary involvement was documented during the first 30 days.

**Manifestation of infection**

The most common site of pathogen isolation overall was the urine (41 events, 51%), followed by the bail drain
Table 2. Postoperative Gram negative bacteria isolates from various sites in 80 patients after LT.

<table>
<thead>
<tr>
<th>Site</th>
<th>Pseudomonas aeruginosa 34 in (25)</th>
<th>Klebsiella pneumonia 25 in (20)</th>
<th>E. coli 24 in (20)</th>
<th>Acinetobacter spp. 13 in (9)</th>
<th>Aeromonas hydrophila 2 in (2)</th>
<th>Enterobacter Spp. 5 in (4)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>16 (15)</td>
<td>13(11)</td>
<td>12(11)</td>
<td>2(2)</td>
<td>1(1)</td>
<td>2(1)</td>
<td>46(41)</td>
</tr>
<tr>
<td>Bile drain</td>
<td>9 (5)</td>
<td>3(2)</td>
<td>4(3)</td>
<td>3(2)</td>
<td>1(1)</td>
<td>1(1)</td>
<td>21(14)</td>
</tr>
<tr>
<td>Sputum</td>
<td>4(2)</td>
<td>5(4)</td>
<td>3(3)</td>
<td>2(1)</td>
<td>None</td>
<td>1(1)</td>
<td>15(11)</td>
</tr>
<tr>
<td>Fluid</td>
<td>None</td>
<td>2(1)</td>
<td>2(1)</td>
<td>3(2)</td>
<td>None</td>
<td>None</td>
<td>7(4)</td>
</tr>
<tr>
<td>Blood</td>
<td>3(2)</td>
<td>1(1)</td>
<td>None</td>
<td>1(1)</td>
<td>None</td>
<td>1(1)</td>
<td>6(5)</td>
</tr>
<tr>
<td>Abdominal</td>
<td>2(1)</td>
<td>1(1)</td>
<td>3(2)</td>
<td>2(1)</td>
<td>None</td>
<td>None</td>
<td>8(5)</td>
</tr>
<tr>
<td>Total</td>
<td>34(25)</td>
<td>25(20)</td>
<td>24(20)</td>
<td>13(9)</td>
<td>2(2)</td>
<td>5(4)</td>
<td>103(80)</td>
</tr>
</tbody>
</table>

Total = isolates of bacteria/number of patients.

Table 3. Antibiotic resistance profiles of predominant Gram-negative bacilli in 80 recipients after LT.

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Pseudomonas aeruginosa (n=34)</th>
<th>Klebsiella pneumonia (n=25)</th>
<th>E. coli (n=24)</th>
<th>Acinetobacter spp. (n=13)</th>
<th>Aeromonas hydrophila (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin-clavulanate</td>
<td>17.6%(6/34)</td>
<td>20%(5/25)</td>
<td>16.7%(4/24)</td>
<td>92.3%(12/13)</td>
<td>40%(2/5)</td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>23.5%(8/34)</td>
<td>20%(5/25)</td>
<td>20.8%(5/24)</td>
<td>100%(13/13)</td>
<td>20%(1/5)</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>11.8%(4/34)</td>
<td>16%(4/25)</td>
<td>20.8%(5/24)</td>
<td>100%(13/13)</td>
<td>60%(3/5)</td>
</tr>
<tr>
<td>Ceftiraxone</td>
<td>17.6%(6/34)</td>
<td>28%(7/25)</td>
<td>25%(6/24)</td>
<td>92.3%(12/13)</td>
<td>20%(1/5)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>20.6%(7/34)</td>
<td>16%(4/25)</td>
<td>16.7%(4/24)</td>
<td>84.6%(11/13)</td>
<td>20%(1/5)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>17.6%(6/34)</td>
<td>16%(4/25)</td>
<td>16.7%(4/24)</td>
<td>100%(13/13)</td>
<td>20%(1/5)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>94.1%(32/34)</td>
<td>80%(20/25)</td>
<td>83.3%(20/24)</td>
<td>100%(13/13)</td>
<td>100%(5/5)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>88.2%(30/34)</td>
<td>88%(22/25)</td>
<td>83.3%(20/24)</td>
<td>100%(13/13)</td>
<td>100%(5/5)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>44.1%(15/34)</td>
<td>52%(13/25)</td>
<td>62.5%(15/24)</td>
<td>100%(13/13)</td>
<td>100%(5/5)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>11.8%(4/34)</td>
<td>8%(2/25)</td>
<td>8.3%(2/24)</td>
<td>100%(13/13)</td>
<td>20%(1/5)</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>14.7%(5/34)</td>
<td>20%(5/25)</td>
<td>8.3%(2/24)</td>
<td>92.3%(12/13)</td>
<td>40%(2/5)</td>
</tr>
<tr>
<td>Colistin</td>
<td>5.9%(2/34)</td>
<td>72%(18/25)</td>
<td>29.2%(7/24)</td>
<td>7.7%(1/13)</td>
<td>20%(1/5)</td>
</tr>
</tbody>
</table>

Values in the parentheses are number (n) of antibiotic resistance isolates/number of total isolates tested, respectively.

(14 events, 17.5%). Infections of the sputum, blood and ascitic fluid site accounted for 13.8, 6.3 and 5.0%, respectively. Other common sites of infection included the abdominal (6.3%) (Table 2).

Antimicrobial resistance of main Gram-negative bacilli

The antibiotic resistance patterns of predominant Gram negative bacilli were listed in Table 3. The most susceptible antibiotic against *P. aeruginosa* was colistin (94.1%). Imipenem was the most resistance antibiotic against all isolates (80 to 100%).

All antimicrobial agents showed relative low susceptibility against *Acinetobacter* spp except colistin. Piperacillin/tazobactam (84%) high antibiotic susceptibility to *K. pneumonia*.

Phenotypic identification

All isolates were subjected to simplified phenotypic tests as described in Table 4. Briefly, tests for growth at 30, 37°C were performed in brain heart infusion broth in a water bath. Sheep blood (5%) agar plates were used to detected hemolysis, gelatin stab method was used to test for gelatin liquefaction. The susceptibility tests were read after overnight incubation, and the tests for haemolysis and growth at 30, 37°C were read on the first and second day. For gelatin liquefaction the incubation temperature was 22°C.

DISCUSSION

Studies of infectious complications after liver transplantation exist in the literature (Piselli et al., 2007).
However, most of these data are older than 10 years and all of the studies were performed in the United States and Europe. Therefore, we analyzed the incidence, presentation, and risk factors of infections in liver transplant recipients in Egypt population treated between 2012 and 2013, and compared our findings with data worldwide.

The 1-year survival in our population was 89%, similar to that of other transplant groups. The incidence of infection during the period of 12 months was comparable to data by an Italian study (Garbino et al., 2005), who reported infections in 56% of patients during the first year, and somewhat lower than data surveyed by Kusne et al. (1998) from the University of Pittsburgh Medical Center between 1984 and 1985, who found infections in 83% of the patients, or results from a Swiss single-center study, who reported that 80% of patients developed infections after liver transplantation (Garbino et al., 2005).

Bacterial infections were the most frequent event in our population, as found by other groups (9, 4). In our study, the proportion of bacterial events was higher (92.0%) in comparison with 47% observed in the Swiss study (Garbino et al., 2005), 48% reported by Torbenson et al. (1998), about 54% at the Pittsburgh Medical Center, or 57% in the Italian study (Piselli et al., 2007). Infection by Gram-negative enteric organisms was the main cause of bacterial infection in our population, which shows that the epidemiology of infections at our hospital is similar to those of Western Europe and the United States. In accordance with other studies (Piselli et al., 2007, Garbino et al., 2005), most bacterial infections occurred within the first month, and the bloodstream was the leading infection site. Other significant locations were urinary tract infections, and abdominal and surgical site infections.

Fungal infections accounted for 8.0% in our patients which compared with other studies that showed infection rates between 12 and 29% (Piselli et al., 2007). Pappas et al. (2006) reported a rate of 4% of invasive fungal infections during the first 100 days post transplantation. Most of the infections in our study were attributed to Candida species and peaked during the first month. In summary, liver transplant recipients within the first month are most susceptible to nosocomial infections similar to those seen in non-immunosuppressed surgical patients.

Positive cultures by routine investigations, obtained according to the protocols of harvesting systematic samples after the transplant, or the identification of a microorganism of the normal human flora were not taken into account, unlike some studies that included such events (Dawwas et al., 2007). Multiple screening cultures likely result in an over diagnosis of infection; however, screening can be the only early guidance for early treatment of infection.

The literature lacks randomized controlled studies for bacterial prophylaxis comparing the efficacy of different antibiotic regimens in transplant recipients. Most centers used amoxicillin-clavulanate or a second- or third-generation cephalosporin against bacterial infections. We used amoxicillin-clavulanate in combination with gentamicin, a standard prophylaxis, and reported an infection rate that was comparable with data worldwide. As described previously, most infections were caused by Gram-negative aerobic bacteria such as E. coli, which are inhabitants of the digestive tract.

However, the incidence of multiple-antibiotic-resistant bacteria is increasing, so the choice of prophylaxis also has to take into consideration the bacterial isolates that are commonly present in the center performing the transplantation. Therefore, it is difficult to compare our results with other studies reports regarding prophylaxis and infection rate, because many other variables affect infection rates. In one of the largest studies with more than 1200 patients, more than 8 types of antibiotics or antibiotic combinations were used for surgical prophylaxis (Asensio et al., 2008); however, the most important risk factor for surgical site infection was the choledochojejunual reconstruction (Asensio et al., 2008).

The recent study could confirm the data by other groups and supports the impact of post-transplant infections on morbidity and mortality of liver recipients. Infections were mainly of bacterial origin and occurred more frequently during the early post-transplant period. The bloodstream, abdomen, urinary tract, and drain were the most common sites of infection. Parenteral nutrition and a prolonged stay in the ICU were associated significantly with an increased infection rate. Early detection and treatment of infections is essential to obtaining a better outcome in

### Table 4. Phenotypic characteristics of Gram negative bacteria isolates from patients after LT.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Lipase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteolytic</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Congo red</td>
<td>30</td>
<td>23</td>
<td>20</td>
<td>11</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

N= number of isolates.
liver transplant patients. The choice of prophylactic regimen has to take into account the bacterial isolates that are commonly present in the center performing the transplantation. Our results provide additional insight into the risk of infection after liver transplantation and could motivate performance of new studies concerning the understanding and improved prophylaxis of post-transplant infections.

Conclusion

Infection due to MDR Gram-negative bacilli are common after LT, and associated with allograft acute rejection, post-transplant reoperation, and abdominal infection. Patients with MDR Gram-negative bacillus have significantly worse prognosis, and are associated with pre LT bacterial infections, renal dysfunction, post-LT abdominal infection, and prolonged endotracheal intubation. The increasing isolates of MDR Gram-negative bacilli pose a great challenge for clinical treatment.

REFERENCES


Phosphate solubilization by a few fungal strains belonging to the genera Aspergillus and Penicillium

Jyoti Saxena¹, Paramita Basu²*, Vanaja Jaligam³ and Shalini Chandra⁴

¹Biochemical Engineering Department, BT Kumaon Institute of Technology, Dwarahat, Uttarakhand 263653, India.
²Department of Pharmaceutical and Biomedical Sciences, Touro College of Pharmacy, New York, NY 10027, USA.
³Charles River Laboratories, 20401 Seneca Meadows Pkwy Germantown, MD 20876, USA.
⁴Department of Statistics, Banasthali University, Distt. Tonk, Rajasthan 304022, India.

Accepted 20 September, 2013

Many phosphate solubilizing fungi (PSF) are found in soil and their introduction in the rhizosphere of crops not only increases the availability of phosphorus from insoluble sources of phosphate but also increases the efficiency of phosphate fertilizers such as superphosphate and rock phosphate. Studies on 2 strains belonging to Aspergillus niger, and 1 strain each of Aspergillus flavus, Penicillium aurantiogriseum and Penicillium claviformis with special reference to phosphate solubilization were performed in this communication. All the fungal strains showed halo zone around them on Pikovskaya plates. Quantitative estimation of 3 indigenous strains along with the other 2 non-indigenous strains taken as reference strains was done in two nutrient broths namely: Czapek’s Dox and Pikovskaya containing dicalcium phosphate (DCP) as insoluble source of phosphorus. Out of the two media selected for the study, Pikovskaya broth supported better solubilization. The insoluble phosphates DCP, TCP (tricalcium phosphate) and hypt (hydroxyapatite) were tested, DCP was least solubilized by all strains but there was no significant difference in solubilization of TCP and hydroxyapatite. There was significant negative correlation between pH and phosphate solubilized in all forms of insoluble phosphates. Types of nitrogen sources and metal ions were also screened during the study. (Na₄)₂SO₄ supported more solubilization than NaNO₃, whereas phosphate solubilizing activity decreased (except in A. niger ATCC 282) in presence of Mn⁺² and Fe⁺³ but presence of Al⁺³ did not have statistically significant effect on solubilization. Though various species of Penicillium have been reported for phosphate solubilization in literature but to the best of our knowledge this is first report of phosphate solubilization by P. claviformis.

Key words: Aspergillus, Penicillium, phosphate solubilisation, dicalcium phosphate, tricalcium phosphate, hydroxyapatite.

INTRODUCTION

Phosphorus (P) is the second most important macro-nutrient required for plant growth next to nitrogen. In particular, Indian soils are poor to medium in available phosphorus (Hasan, 1994). Besides, there is also the problem of its fixation in nature. As soon as it is applied to soil in the form of fertilizer, a large amount of it quickly combines with other chemicals forming compounds which do not release the phosphorus for plants. In acidic soil it is fixed by free oxides and hydroxides of aluminium (Al) and iron (Fe), while in alkaline soil by calcium (Ca). In order to reduce P deficiencies and ensure plant productivity, large quantities of expensive chemical phosphate

*Corresponding author. E-mail: paramita.basu@touro.edu.
fertilizers are applied worldwide every year. Microorganisms play critical role in natural P cycle, and the use of phosphate-solubilizing microorganisms (PSMs) has been proposed as a low-cost input to increase the agronomic effectiveness of insoluble phosphates. Several scientific reports showed that microorganisms such as bacteria, fungi, and actinomycetes were indeed able to promote the P solubilization and increase crop yields (Whitelaw, 2000; Oberson et al., 2001; Hamdali et al., 2008; Minaxi et al., 2010). These PSMs render insoluble phosphate into soluble form through the process of acidification, chelation, and exchange reactions (Assialed et al., 2003).

Out of all microbes, fungi are superior to their bacterial counterpart for P solubilization both on precipitated agar and in liquid (Kucey, 1983a; Banik and Dey, 1982; Singhal et al., 1994; Whitelaw et al., 1997; Sheshadri and Ignacimuthu, 2004). Fungal hyphae are able to reach greater distances in soil more easily than bacteria. Furthermore, it has been observed that PSB upon repeated sub-culturing lose the phosphate solubilizing activity (Halder et al., 1990a; Illmer and Schinner, 1992) but such losses have not been observed in PSF (Kucey, 1983b).

Among the fungal genera with the phosphate solubilization ability are Aspergillus, Penicillium, Trichoderma, Mucor, Candida, Yeast, Discosia, Eupenicillium and Gliocladium (Xiao et al., 2008; Rahi et al., 2009). The strains from the genera Aspergillus and Penicillium are among the most powerful phosphate solubilizers. Solubilization of PSF depends on the insoluble inorganic phosphate source, type of carbon, nitrogen and metal ions in soil, as well as on culture conditions (Kucey, 1983b; Nahas, 2007; Jain et al., 2012).

Thus, in view of above facts, there is an increasing realization to explore the possibilities of utilizing a viable alternative for chemical fertilizers for sustainable agriculture. The present study is an effort to see the solubilization pattern of a few fungi belonging to Aspergilli and Penicillia on insoluble forms of phosphate salts of calcium and the effect of different nitrogen sources and metal ions on P solubilization.

MATERIALS AND METHODS

Organisms

Two known organic acid producing fungal strains namely: Aspergillus niger ATCC 282 and Penicillium aurantiogriseum MTCC 2285 were obtained from IMTECH, Chandigarh, India in lyophilized state and maintained in laboratory on Czapek’s Dox Agar slants at 4°C in a refrigerator. The other 3 strains namely; A. niger, A. flavus and P. claviformis were isolated from the rhizosphere region of wheat fields of Banasthali, India.

The soil was collected from rhizosphere region of 5 wheat plants of 3 agricultural fields in sterilized polythene bags and composite mixtures of soil were prepared from each field. Serial dilution and enrichment methods were used for isolation of fungi. The phosphate solubilizing species were identified based on morphological characteristics by manuals and expertise available in the department. These were designated as A. niger bv, A. flavus bv and P. claviformis bv. Due to lowest solubilization capacity of different phosphate sources, A. flavus was not selected for further studies.

Media used

The following media have been used:

i) Pikovskaya agar medium obtained from Hi Media Labs was used to determine the halo formation.

ii) To see the effect of 3 different forms of insoluble phosphates namely: DCP, TCP and Hyp, Pikovskaya broth having following composition Glucose 10.0, Tricalcium phosphate 2.0, (NH₄)₂SO₄ 0.5, KCl 0.2, MgSO₄ 7H₂O, MnSO₄ Trace, FeSO₄ Traces, Yeast extract 0.5, Distilled water 1000 ml (pH 7.0) was used. All these salts being insoluble in water, 0.5 g of each form of phosphate was added in 100 ml of broth in separate flasks.

iii) For experiments on effect of different nitrogen sources and metal ions modified Czapek’s Dox broth was used (composition: Sucrose 30.000, Sodium nitrate 2.000, Dipotassium phosphate 1.000, Magnesium sulphate 0.500, Potassium chloride 0.500, Ferrous sulphate 0.010, Final pH (at 25°C) 7.3 ± 0.2 in which soluble KH₂PO₄ was replaced by insoluble CaHPO₄,2H₂O as it is dominant in alkaline soils of study area). Czapek’s being a synthetic medium, it is easy to replace or add different ions. To see the effect of nitrogen source, NaN₃ was replaced by equal amount of (NH₄)₂SO₄. For metal ions viz. Fe⁺², Al⁺³ and Mn⁺², 0.1 g of FeCl₃, Al₂(SO₄)₃ and MnSO₄ respectively were added separately to modified Czapek’s Dox broth.

Solubilization of phosphate on solid medium

The strains were subjected to screening test for their phosphate solubilization potential. Pikovskaya’s medium containing TCP was prepared and poured into sterilized Petri plates. The isolates were spotted on these plates and incubated at 28°C for 4 to 7 days. Those showing halo zones around the colonies were supposed to be phosphate solubilizing ones. Solubilizing efficiency (S.E) was calculated according to Nguyen et al. (1992) by following formula:

\[
\% \text{ Solubilization efficiency (SE)} = \frac{\text{Diameter of solubilization zone (S)}}{\text{Diameter of the colony}} \times 100
\]

Microdetermination of phosphorus:

Two ml of 10⁶ spores/ml of sterilized water was used for inoculation of flasks. Control flasks were also prepared with 2 ml of sterilized water instead of spore suspension. The flasks were incubated at 28 ± 2°C and 120 rpm in an incubator shaker for 2, 4, 6, 8, 10 and 12 days. After completion of respective incubation periods, cultures in triplicate were harvested and filtered with Whatman filter paper no. 42 into separate 250 ml beakers to separate the broth containing phosphate from fungal mat. Chen’s method (Chen et al., 1956) was used for quantitative estimation of solubilized phosphate. Absorbance at 820 nm was read by spectrophotometer. Standard curve was plotted to determine the values. The pH of fungal filtrate was also taken using a glass electrode pH meter.

All the glassware used for biochemical studies were soaked in chromic acid overnight. Thereafter they were washed with distilled water, rinsed with dilute HCl, washed again with distilled water and then dried in glassware drying oven to remove organic matter from the glasswares, especially phosphorus, if present.

Statistical analysis

A statistical analysis for the comparison of phosphate solubilization
process by different strains has been done using two-way and three-way incomplete ANOVA. For the multiple pair wise comparisons Tukey test and l.s.d. were used.

**RESULTS**

**Phosphate solubilization by different strains**

The screening of phosphate solubilizing fungi was done on Pikovskaya agar plates. All the fungi selected for the study showed a clear halo zone around them (Figure 1 A and B). *A. flavus* solubilized 46.5 µg/ml DCP and nearly half the amount of TCP and hydroxyapatite, hence discarded for further studies. The percent phosphate solubilization efficiency of this strain was lowest i.e. 90% in comparison to other strains which was in between 300 to 380%, which shows that percent efficiency was directly related to P solubilized in medium.

Three forms of most commonly found insoluble phosphates namely: DCP, TCP and hydroxyapatite were chosen to see the effect of solubilization by different fungal strains in Pikovskaya broth. The results are depicted in Table 1. In general, the amount of soluble phosphate increased with the increase of incubation period in all types of insoluble phosphates solubilized by all the strains,
Table 1. Comparison of solubilization of different forms of insoluble phosphates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Days</th>
<th>Dicalcium phosphate</th>
<th>Hydroxyapatite</th>
<th>Tricalcium phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amount of phosphate</td>
<td>pH</td>
<td>Amount of phosphate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>solubilized (μg/ml)</td>
<td></td>
<td>solubilized (μg/ml)</td>
</tr>
<tr>
<td>A. niger ATCC 282</td>
<td>0</td>
<td>8.00</td>
<td>7.00</td>
<td>8.50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26.50</td>
<td>5.11</td>
<td>19.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>45.91</td>
<td>3.63</td>
<td>29.12</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>54.25</td>
<td>3.10</td>
<td>52.14</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>56.50</td>
<td>2.87</td>
<td>72.23</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>57.12</td>
<td>2.75</td>
<td>77.40</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>58.40</td>
<td>2.73</td>
<td>78.11</td>
</tr>
<tr>
<td>A. niger bv</td>
<td>0</td>
<td>8.00</td>
<td>7.00</td>
<td>8.52</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24.22</td>
<td>5.80</td>
<td>17.30</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>40.12</td>
<td>3.72</td>
<td>25.51</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>49.71</td>
<td>3.31</td>
<td>47.00</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>55.73</td>
<td>3.25</td>
<td>62.33</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>56.81</td>
<td>3.04</td>
<td>68.89</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>57.41</td>
<td>2.96</td>
<td>72.00</td>
</tr>
<tr>
<td>P. aurantiogriseum MTCC 2285</td>
<td>0</td>
<td>8.00</td>
<td>7.00</td>
<td>8.51</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26.05</td>
<td>5.80</td>
<td>19.50</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>31.63</td>
<td>4.61</td>
<td>30.50</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>36.14</td>
<td>4.25</td>
<td>25.82</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>39.74</td>
<td>3.80</td>
<td>56.90</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>36.72</td>
<td>3.62</td>
<td>66.48</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>40.21</td>
<td>4.32</td>
<td>72.33</td>
</tr>
<tr>
<td>P. claviformis bv</td>
<td>0</td>
<td>8.00</td>
<td>7.00</td>
<td>8.42</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24.07</td>
<td>5.78</td>
<td>21.42</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>38.50</td>
<td>4.58</td>
<td>32.20</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>35.11</td>
<td>3.99</td>
<td>30.05</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>32.72</td>
<td>3.91</td>
<td>63.20</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>46.01</td>
<td>4.25</td>
<td>83.51</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>30.52</td>
<td>3.95</td>
<td>69.49</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>8.00</td>
<td>7.00</td>
<td>8.52</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.50</td>
<td>6.98</td>
<td>9.25</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10.00</td>
<td>6.86</td>
<td>10.81</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>11.41</td>
<td>6.74</td>
<td>9.75</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.60</td>
<td>6.72</td>
<td>11.50</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>10.50</td>
<td>6.63</td>
<td>11.65</td>
</tr>
</tbody>
</table>

although the amount varied with the phosphate type and the fungal strain. The pattern of solubilization of different forms of insoluble phosphates was significantly different in all four strains with $F= 2.579$ and $p$-value < 0.1. The solubilization of TCP was highest, closely followed by hydroxyapatite, while DCP was most difficult to solubilize. However, there was no significant difference in solubilization of TCP and hydroxyapatite at 5% level of significance. $A. niger$ strains solubilized phosphates efficiently for first 6 days, then the rate of solubilization finally attained an almost constant values. However, the values increased in DCP whereas; there were more fluctuations in case of TCP and hydroxyapatite. But the $Penicillium$ spp. did not give constantly increasing values at all, as shown by more evident fluctuations in solubilized phosphate concentrations. Also, after 10 to 12 days of incubation...
there was a slight increase in pH.
Table 1 also showed that the rise in the available phosphate concentration was accompanied by concomitant decrease in pH of the medium. It decreased from 7 at day zero to 2.20 at 12th day in case of A. niger bv solubilizing hydroxyapatite. In other strains also the decline in pH from 7 to 2 to 4 was observed. In all, statistical analysis revealed a significant negative correlation (-0.879 with p value<0.01) between amount of phosphate solubilized and pH values.

Microdetermination of phosphorus in liquid medium

Phosphate solubilizing activity of four strains was observed on Czapek’s Dox and Pikovskaya broth containing DCP as the insoluble phosphate source (Figure 2 A-D). There was a significant difference between amounts of phosphate solubilized in vitro by all the four strains in Czapek’s Dox and Pikovskaya broth with t = 3.370 and p value<0.01. It was found that Pikovskaya broth overall supported better solubilization than Czapek’s Dox broth.
The results of effect of NaNO3 and (NH4)2SO4 as nitrogen sources and Mn3+, Al3+ and Fe3+ as metal ions on phosphate solubilization by all four strains is depicted in Figures 3 to 4. There was a significant difference between amounts of phosphate solubilized by all the four strains in case of nitrate and ammonium ions as nitrogen source with t=-2.471 and and p value<0.05. NaNO3 proved inferior nitrogen source to (NH4)2SO4 in supporting phosphate solubilization activity.

DISCUSSION
In general, TCP supported highest phosphate solubilizing activity, closely followed by TCP and hydroxyapatite while DCP was least solubilized. Calcium phosphates are the dominant insoluble inorganic phosphates present in neutral to alkaline soils. The soil of study area is also alkaline (Anamika et al., 2007), therefore, locally isolated strains are expected to solubilize these forms of phosphates well. Out of three insoluble inorganic phosphate sources,
TCP was solubilized the most which is in agreement with Narsian et al. (1995). This could be due to the difference in chemical structure and charge of three forms of phosphates. CaHPO$_4$ gives HPO$_4^{2-}$ after release of phosphate while Ca$_3$(PO$_4$)$_2$ gives PO$_4^{3-}$ which bears more negative charge. This would react more readily with or have higher affinity for H$^+$ present in water in dissociated form, thus go into solution more easily. This was further supported from the data obtained from control samples which did not have inocula. For all three forms of insoluble phosphates, the control showed a very small and gradual increase in available phosphorus concentration with increase in number of days of incubation. This may be explained by the phenomenon of leaching, an abiotic process, caused due to formation of traces of organic acids due to chemical interaction between the components of media (Johnston, 1954).

The rise in the available P concentration was accompanied by decrease in pH of the medium. This drop in pH indicates the production of organic acid (Pradhan and Sukla, 2005; Jain et al., 2011). A. niger is known to produce citric, oxalic and gluconic acids (Fomina et al., 2004), thus lowering the pH of medium and facilitating the solubilization. The rate of fall in pH was sharp and uniform during the first 8 days and then it gradually lowered to attain a more or less stable value. These results coincide with Halder and his team mates (Halder et al., 1990b). The lower values of pH in case of Aspergillus spp. in comparison to Penicillus spp. may be due to less organic acid produced by Penicillia. After 10 to 12 days of incubation there was a slight increase in pH in all the cases which can be explained by the start of autolysis phase (Halder et al., 1990a; b).

In the present study, NH$_4^+$ was found to be a good nitrogen source in comparison to NO$_3^-$ Our results corroborate with Jain et al. (2011) who also reported NO$_3^-$ as inferior nitrogen source for phosphate solubilization. The possible reason could be the acid production in form of H$^+$ release in response to the assimilation of cations such as NH$_4^+$, which has been reported as a well known phenomenon in fungi (Kucey, 1983a; b; Roos and Luckner, 1984). Asea et al. (1998) attributed higher phosphate concentration in plant amended with NH$_4^+$ to better development of roots and acidification of rhizosphere. Roos and Luckner (1984) have reported that the presence of NH$_4^+$ in growth medium of P. cyclopium resulted in the development of inorganic acid following an operation of NH$_4^+$/H$^+$ exchange mechanisms.

The phosphate solubilizing activity decreased (except in A. niger ATCC 282) in presence of Mn$^{2+}$ and Fe$^{3+}$ as...
compared to control but presence of Al\(^{3+}\) did not have statistically significant effect on these strains. Gaur and Sachar (1980) found that Al\(^{3+}\), Fe\(^{3+}\) and Ca\(^{2+}\) were inhibitory to growth and activity of fungi or caused a change in pH of the medium, which in turn affected phosphate solubilization. In the present study Al\(^{3+}\) did not make any remarkable difference but the other two ions had inhibitory effect. Sayer et al. (1995) observed the activity of *P. simplicissimum* and *A. niger* in presence of 8 insoluble metal compounds. Cobalt phosphate was the most toxic whereas, zinc phosphate was the least toxic and most resistant to solubilisation, while zinc oxide was the most readily solubilized compound.

The type of insoluble phosphate present in the soil of a particular locality seems to affect the efficiency of phosphate solubilization by microbes present locally or applied as biofertilizer. Also, NH\(_4^+\) which was proved to be a better source of nitrogen, could be considered while choosing nitrogen fertilizer if these microbes were simultaneously applied in the same soil as a part of integrated management of nutrients. More studies are in offing with regard to metal ions which could play important role in selecting the biofertilizer, if any of the salts containing these ions were present in the soil.

The advantage of using fungi as bioinoculant includes their tolerance to high concentrations of potentially toxic metals (Sayer et al., 1995), and better acid and alkali tolerance than bacteria (Chuang et al., 2007), although fungi might be inferior to bacteria in their ability to colonize plant root. Overall, fungi may have a much better potential to serve as an agent to convert insoluble inorganic P into a soluble form (for example, HPO\(_4^{2-}\), H\(_2\)PO\(_4^-\)) usable by plants in low or high soil pH.

The present study has generated useful information about the phosphate-solubilizing fungi under control and stressful conditions *in vitro*. It is expected that these fungal strains could serve as suitable candidates for solubilizing P in rigorous environments. However, since the conditions in soil are much more complex than those in vitro, further study of environmental factors affecting phosphate solubilization by these strains in soil should be of practical importance for crops.

REFERENCES


Identification of antagonistic bacteria for *Amorphorallus konjac* soft rot disease and optimization of its fermentation condition

Jinping Wu, Zili Ding, Zhenbiao Jiao, Rong Zhou, Xinsun Yang* and Zhengming Qiu

Hubei Academy of Agricultural Sciences, Wuhan 430064, China.

Accepted 26 August, 2013

Soft rot disease of *Amorphorallus konjac*, an important and potentially destructive corms disease, is caused by *Pectobacterium* species. Now, the conventional methods of controlling the disease include the breeding variety and the chemical control, but the effects are uncomfortable. The aim of this study was to screen antagonistic bacteria for soft rot disease and optimize its fermentation conditions. The antagonistic bacterium (strain C12) confirmed *Bacillus subtilis* by the identification of Biolog system and analysis of 16S rDNA gene sequence. Antimicrobial spectrum of the strain C12 was determined by growth rate method, which could restrain the growth of 12 pathogens. The fermentation conditions of the strain C12 were studied by using the single-factor method. The optimal fermentation conditions for antagonistic bacteria were as follow: Medium initial pH 7.0, the fermentation temperature 31°C, the quantity of medium 50 mL in a 250 mL flask, the inoculation volume 2.5%, the incubation time 22 h and the rotation speed 180 rpm, the glucose as carbon source and yeast as nitrogen source. The fermentation liquor of the strain C12 was twice than the streptomycin in control effect of pot experiment. The research provides reference for controlling soft rot disease of *A. konjac*. The findings suggested that the strain C12 could be exploited as a biocontrol agent for soft rot pathogens.

Key words: Soft rot disease, antagonistic bacteria, characterization, fermentation condition, optimization.

INTRODUCTION

Soft rot disease is one of the destructive diseases of vegetables. It causes a greater total loss of produce than any other bacterial disease. The disease can be found on crops in the field, in transit, in storage and during marketing, and results in great economic losses (Bhat et al., 2010). The disease is conventionally controlled by cultivation measures (such as crop rotation, intercropping) and chemical control (Ronald et al., 2004). Chemical control is usually inappropriate because pathogens can develop resistance and also pesticides can pollute the environment. Therefore, people pay much attention to the biological control. It was reported that *Pseudomonas fluorescens* (Hendawy et al., 1998), *Lysobacter enzymogenes* (Folman et al., 2003), *Erwinia carotovora* subsp. *Betavasculorum* Ecb168 (Costa and Joyce, 1994), *E. carotovora* subsp. *carotovora* Ecc 32 (Seo et al., 2004) and *Streptomyces* (Zamanian et al., 2005) and so on could restrain the *Pectobacterium carotovorasub* sp. *carotovora*. *Amorphophallus konjac* K. Koch ex N.E.Br. (Araceae) originates in South East Asia (Hetterscheid

*Corresponding author. E-mail: wjp9188@aliyun.com. Tel: 86 27 87280817; fax: 86 27 87280817.

Abbreviations: *P.c.c.* Pectobacterium carotovorasub sp. Carotovora; PCR, polymerase chain reaction; BLAST, Basic Alignment Search Tool; DO, dissolved oxygen.
and Ittenbach, 1996) and is now mainly distributed throughout Southern and South Eastern China and Vietnam (Brown and Aroids, 2000). China is the main producer of konjac with an area under cultivation of ~200 million acres (Xu et al., 2001) and has approximately 400 factories devoted to the production of konjac flour and related goods (En, 2008). Due to the increasing demand for konjac flour, konjac is now regarded by the Chinese government as an agronomically important crop which has great potential in both domestic and international markets (WFS, 2003). Projects involving planting konjac in mountainous regions of Southern China have been implemented by provincial governments to help combat rural poverty (WFS, 2003). But the soft rot disease of konjac could cause losses between 30 and 50% in the total production, some as high as 80% and even the complete destruction (Xiu et al., 2006). This disease is becoming the bottleneck of konjac industry. No konjac cultivars resistant to the soft rot disease have been reported so far. Recent researches have focused on biological control. Zhou et al. (2004) found that the extracts from Orostachys fimbriatus had the antibacterial activities against Pectobacterium spp. Sheng et al. (2007) used the antibiotic extracted from microbial product to prevent and treat soft rot in konjac. Bacillus subtilis BS5 from calli tissue of konjac and Serratia marcescens strain 21-2 from rotten corms of konjac showed antibacterial activity against Pectobacterium carotovora sub sp. Carotovora (P.c.c) (Zhou et al., 2007; Wu et al., 2012). But until now effective ways to control this pathogen in the field have not been available. The primary objective of this study is to screen and identify the antagonistic bacteria against from P.c.c the soil of the rhizosphere of konjac, and grope the optimal fermentation conditions, so that the antagonistic bacteria could be quickly developed as a biological control agent of soft rot disease of konjac.

MATERIALS AND METHODS

Strains and culture medium

The strain of P.c.c (Registry number: FJ463871) and other pathogens were provided by the laboratory. PSA medium (200 g·L⁻¹ potato, 18 g·L⁻¹ sucrose) and KMB medium (20 g·L⁻¹ peptone, 15 mL glycerol, 2.5 g·L⁻¹ KH₂PO₄, 0.73 g·L⁻¹ MgSO₄) were reference of the microbiology experiment (Fang, 1998).

Isolation of bacterial antagonist

One gram of soil sample, collected from rhizosphere soil of konjac, was suspended in 10 mL of sterile water and vortexed for 45 s. The sample was serially diluted and 100 μL of each dilution was added to molten PSA agar maintained at 55°C along with 10⁶ cfu·mL⁻¹ of P.c.c and poured in sterile petri dishes. After incubation at 28°C for 2 days, bacterial colonies showing zones of inhibition were selected. Further, the antagonistic bacteria were inoculated onto PSA at 180 rpm, 28°C for 24 h. The cell-free supernatants were obtained by centrifugation at 5000×g for 15 min and filtered on a 0.45 μm Millipore filter (Millipore Co., USA). Culture supernatants were added in the wells, made in PSA using sterile metal cylinders, and the plates were incubated at 28°C for 24 h. Bacterial colonies showing zones of inhibition were reselected (Fang, 1998). The streptomycin (0.24 mg·mL⁻¹) (ZHONGNONG, Co., China) was controlled to compare the effect of the antagonistic bacteria. The experiment was repeated three times.

Inhibition of the different fungi

Inhibition of the different fungi for antagonistic bacteria was determined by growth rate method. The bacteriostasis rate was calculated by the antibacterial circle diameter.

The bacteriostasis rate (%) = (Control bacteria colony diameter - Treatment bacteria colony diameter) / (Control bacteria colony diameter - The colony diameter)

Identification of the strain based on 16S rRNA and Biolog plates

The sequence of the 16S rDNA was obtained from the total DNA of the antagonistic bacteria by polymerase chain reaction (PCR) amplification with sense primer 5'-AQAGTTTGTACGCTTCA G-3' and antisense primer 5'-CGCCTACCTTTGATCAGCTTT-3' (Weisburg et al., 1991). The PCR amplification conditions were as follows: one denaturation step (3 min at 95°C), 35 cycles of amplification (30 s at 95°C, 30 s at 50°C, 1.5 min at 72°C), and a final elongation step of 10 min at 72°C. PCR products were inserted into the pGEM T-easy vector for sequencing. The Biolog system was used to support the 16S rDNA genus identification. All protocols for preparation and identification of microorganisms are outlined in the Biolog™.

Fermentation conditions of the antagonistic bacteria

The antagonistic bacteria grew in KMB medium for these experiments. The fermentation conditions of the antagonistic bacteria were studied by using the single-factor method. Different factors were regulated by the requirement of experiment. The antagonistic bacteria was cultured at 31°C, pH 7.0, broth content 50 mL/250 mL, inoculum concentration 2.5%, the fermentation cycle 22 h and the shaker revolution 180 rpm. Different factors: pH (2-11), fermentation temperature (22, 25, 28, 31, 34, 37 and 40°C), liquid volume (30, 50, 70, 100, 130 and 150 mL medium in 250 mL triangular flask), inoculation amount (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) (volume fraction), culture time (12, 18, 24, 30, 36, 42 and 48 h), rotation speed (150, 180, 210, 240 and 270 rpm), carbon source (glucose, fructose, lactose, mannitol, maltose, sucrose and glycerin) and nitrogen source (NaNO₃, C₆H₅NO₂, Yeast, (NH₄)₂SO₄, KNO₃, Tryptone, (NH₄)₂H₂PO₄, NH₄NO₃, L-Glu). The optical density (OD) was surveyed by spectrophotometer (INESA, Co., China). Each treatment was repeated for three times.

Pot experiments

A. konjac plants were grown in a greenhouse until the 3-month-old, where the temperature ranged between 25 and 30°C, and the humidity was more than 80%. The healthy plants were drenched with the 24-h-old-grown culture (approximately 1×10⁸ cfu·mL⁻¹, 50 ml/plant) of the antagonistic bacteria. At the same time, the sterile distilled water and streptomycin (0.24 mg·mL⁻¹) were as the negative control. After one week, the plants were challenge-inoculated with 50 mL of P.c.c strain (approximately 1×10⁷ cfu·mL⁻¹) by pouring the bacterial suspension around the root zone. The incidence of soft rot was recorded periodically over a period of time up to 30 days.
Figure 1. The antimicrobial activity in fermentation supernatants of strain C12 to *P. cc* on PDA medium in 9-cm-diameter Petri dish. The antimicrobial activity was determined by measuring the translucent inhibition zones after incubating the dish 24 h at 30°C. A. streptomycin; B, fermentation liquor of strain C12.

Each treatment was repeated three times. The data were analysed statistically.

RESULTS

Screening of antagonistic bacteria

Twenty-four antagonistic bacteria from the soil of the rhizosphere of konjac against *P. cc* were obtained by zones of inhibition. The antagonistic effect was further studied by using sterile metal cylinders. The strain C12 had the largest inhibition zone diameter (about 2.6 cm) among the twenty-four antagonistic bacteria. The inhibition zone diameter of the streptomycin was about 2.1 cm. The antibacterial circle diameter of the strain C12 was about 0.5 cm wider than the streptomycin (Figure 1).

Determination of the antifungal spectrum

The antifungal spectrum of the antagonistic bacteria strains C12 was determined by growth rate method (Table 1). The strains C12 could inhibit the 12 kinds of pathogens, and the inhibition rate of the 11 pathogens was above 50%. The inhibition rate was greater than 90% of the strawberry root rot (*Rhizoctonia solani*) and Fusarium Wilt of watermelon (*Fusarium oxysporum* f.sp. *niveum*). The results showed that strain C12 had a wide antifungal spectrum.

Identification the antagonistic bacteria

Similar degree of strain C12 with *B. subtilis* was 0.562 by the BIOLOG system (Gin III 5.2 Microstation). The whole 1,420 bp nucleotides 16S rDNA of strain C12 (Registry number: JX960647) was aligned with all related sequences in the NCBI database by the Basic Alignment Search Tool (BLAST) program. The Max ident was 99% with *B. subtilis* in Genbank. So the strain C12 was proved to be *B. subtilis*.

Fermentation conditions of the antagonistic bacteria

The effect of pH value on cell density indicated that the strain C12 could grow under 5 < pH < 10 (Figure 2a). The strain C12 could grow in a wide pH range. The fermentation temperature has little influence on the growth of strain C12. The strain C12 grew best at 31°C (Figure 2b). Soft rot disease of konjac is easily occurred at 25-30°C. Therefore, the optimum growth temperature of the strain C12 was consistent with the outbreak temperature of soft rot disease.

Liquid volume had great influence in the growth of strain C12. The strains C12 grew best when liquid volume was 50 mL, and then liquid volume was 30 and 70 mL. So the ventilation volume was too large or small, which are not beneficial to the growth of strain C12 (Figure 2c). The strain C12 grew best under the inoculation amount 2.5%, then the 2.0 and 3.0% (Figure 2d). Antagonistic bacteria strain C12 reached a maximum growth value when culture time in 22 h, then the strain C12 started aging period (Figure 2e). The antagonistic bacteria strains grew best at 180 rpm. When the shaking speed was more than 180 rpm, the growth of the strain C12 was gradually reduced (Figure 2f).

We could see that the carbon source utilization rate was glucose > sucrose > fructose > maltose > mannitol > lactose > starch > glycerin for antagonistic bacteria (Figure 2g). The highest utilization rate of antagonistic bacteria was yeast, then tryptone, and the other nitrogen source utilization rate was very low (Figure 2h).

Effects of biological bacteria against soft rot disease in pot experiments

30 trees konjac were experimented in each treatment. The plants were all deaths after 30 d by the water treatment, the survival rate of the plant was 16.7% by streptomycin, and the survival rate of the plant was 33.3% by the fermentation liquor of strain C12 (Table 2). The results of pot experiments illuminated that the strain C12 could effectively control soft rot disease of konjac.

DISCUSSION

The strain C12 was identified to be *B. subtilis* by the BIOLOG system and 16S rDNA. Because the bacterium *B. subtilis* produces a variety of antibacterial and antifungal antibiotics such as Zwittermicin-A, kanosamine and lipopeptides from iturin, surfactin and fenzycin fami-
Table 1. The antibacterial spectrum of strain C12.

<table>
<thead>
<tr>
<th>Pathogenic bacteria</th>
<th>The inhibition rate (%)</th>
<th>Pathogenic bacteria</th>
<th>The inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria alternata</td>
<td>86.7</td>
<td>Fusarium graminearum</td>
<td>72.9</td>
</tr>
<tr>
<td>Colletotrichum acutatum</td>
<td>55.0</td>
<td>Rhizoctonia bataticola</td>
<td>80.0</td>
</tr>
<tr>
<td>Colletotrichum gloeosporioides</td>
<td>78.0</td>
<td>Rhizoctonia solani</td>
<td>98.7</td>
</tr>
<tr>
<td>Myrothecium inundatum</td>
<td>45.8</td>
<td>Sclerotinia sclerotiorum</td>
<td>86.7</td>
</tr>
<tr>
<td>Fusarium oxysporum f.sp. batatas</td>
<td>68.3</td>
<td>Sphaeronaemella fragariae</td>
<td>51.2</td>
</tr>
<tr>
<td>Fusarium oxysporum f.sp. niveum</td>
<td>94.9</td>
<td>Sclerotium rolfsii Sacc</td>
<td>71.8</td>
</tr>
</tbody>
</table>

Figure 2. Effect of different factor on cell density for the strain C12. a, Effect of pH value on cell density; B, effect of temperature on cell density; C, effect of the volume of liquid on cell density; D, effect of inoculation volume on cell density; E, effect of incubation time on cell density; F, effect of rotation speed on cell density; G, effect of different C-source on cell density; H, effect of different N-source on cell density.
families, so the strain C12 not only inhibited the soft rot pathogen of konjac but also exhibit strong inhibitory activity against various plant pathogenic fungi, such as *R. solani*, *Alternaria alternate*, *Fusarium graminearum* among others. Many bacteria could be used as biological control agents. Among them, *B. subtilis* is now widely recognized as a powerful tool in bio-control as a prevalent soil inhabitant. As a soil-dwelling rhizobacterium, naturally present in the immediate vicinity of plant roots, *B. subtilis* is able to maintain stable contact with higher plants and promote their growth. In addition, *B. subtilis* had broad host range, formed endospores and produced different antibiotics with a broad spectrum activity. Soft rot disease of konjac was a soil-borne disease, and almost no chemical measure was effective in the control of this disease.

Figure 2. Contd.
(Krzysztofa et al., 2007). Therefore, the strain C12 was expected to be a bio-control bacterium for soft rot disease of konjac.

The optimal medium volume in the flask and the rotary speed were around 50 mL and 180 rpm, respectively. Our study revealed that rotary speeds, medium volume in the flask are related to the dissolved oxygen (DO) in shaken flasks (Yan et al. 2012). A proper DO level was beneficial to the growth of antagonistic bacteria. Lai et al. (2005) found that the shear effect on cell morphology such as mycelium growth or pellet formation was closely influenced by the DO level.

Carbon sources and nitrogen sources played an important role in the growth of the strain C12, not only because of limiting the supply of an essential nutrient is an effective means of restricting growth but also because of the choice of limiting nutrients can have specific metabolic and regulatory effects (Doull and Vining, 1990; Elibol, 2004). The initial pH, the fermentation temperature, the inoculation volume, the incubation time and so on were disadvantage to the growth of the strain C12 when they were too large or small. The information obtained is considered fundamental and useful for developing a cultivation process for efficient production of antibiotics on a large scale for the strain C12.

**ACKNOWLEDGMENTS**

This work was financially supported by National Science and Technology Supporting Program [Grant No. 2011BAD33B03], Hubei Public Science and technology Program [Grant No. 2012DBA09 and Grant No. 2012DBA43] and the Morning Program of the Youth Science and technology in Wuhan, Hubei [Grant No. 201271031408].

Table 2. Control efficiency of antagonistic bacteria against soft rot of konjac in the greenhouse.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Inoculate time (d)</th>
<th>The number of inoculated plants</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12</td>
<td>$1 \times 10^7$cfu·mL$^{-1}$</td>
<td>30</td>
<td>30</td>
<td>33.3</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.24 mg·mL$^{-1}$</td>
<td>30</td>
<td>30</td>
<td>16.7</td>
</tr>
<tr>
<td>CK</td>
<td>—</td>
<td>30</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>
REFERENCES


Hetterscheid WL, Ittenbach S (1996). Everything you always wanted to know about Amorphophallus but were afraid to stick your nose into. Aroideana 19: 7-129.


Xu L, Xu HH, Huang TS, Du TF, He XR, Shen LH (2001). New Techniques of Cultivation for Good Agricultural Practice (GAP) and Industrializing Develop-ment on the Chinese Rare-Medicinal Herbs. Xie He Medical University, Beijing (in Chinese).


Evaluation of the potency of some entomopathogenic bacteria isolated from insect cadavers on Anopheles arabiensis Giles (Order: Diptera; Family: Culicidae) mosquito larvae in Nigeria

Omoya, F.O.* and Akinyosoye, F.A.
Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria.

Accepted 10 May, 2013

The laboratory evaluation of larvicidal activities of some bacteria namely; Pseudomonas aeruginosa, Bacillus polymyxa and Bacillus subtilis was assessed against the second and fourth instar of Anopheles arabiensis collected from South West Nigeria. Concentrations ranging from $1.3 \times 10^7$ cfu/mL to $6.5 \times 10^7$ cfu/mL were tested on the larvae for a period of 48 h. The disparity in the activities was monitored with B. subtilis displaying the highest activity in both the second and fourth instar with $LC_{50}$ of 0.865 and 2.361 mg/mL respectively. P. aeruginosa showed $LC_{50}$ of 1.931 and 4.205 mg/mL while the least activity was recorded in B. polymyxa with $LC_{50}$ of 5.776 and 7.403 mg/mL. There were significant differences in the $LC_{50}$ value of the bacteria on the tested instars. Values obtained from the fourth instar group were significantly different from those obtained from the second instar group in all the treatments. Finding from this study show that B. subtilis may be a potential biocontrol agent of A. arabiensis, the main malaria vector in Nigeria.

Key words: Biocontrol, larvicidal activities, Anopheles mosquito larvae, malaria.

INTRODUCTION

Mosquito (Diptera) is one of the social insects that has posed serious problems to the health of man by serving as a vector to the etiologic agents of diseases such as malaria, dengue fever, filariasis and Japanese encephalitis which remain endemic in the sub-sahara region of developing countries (Omoya et al., 2009). Malaria is most challenging among the afore mentioned diseases considering the morbidity-mortality ratio. According to Breman et al. (2004), globally, 300 to 500 million cases of infection and over one million deaths are reported annually; 90% of these occur in tropical Africa. Nigeria is known for its high prevalence of malaria and available records show that about 50% of the population suffer from at least one episode of malaria each year. Malaria accounts for over 45% of all out-patient cases in Nigeria (Federal Ministry of Health, 2001). The global increase of malaria toward the end of the 1970s coupled with the inadequate results of malaria eradication strategies in many countries especially the tropical countries have led to concerted search for new methods of control. This was due largely to the emergence of strains of Plasmodium falciparum resistant to chloroquine and sulphadoxine-pyrimethamine (SP), the mainstays of treatment (Trape et al., 1998). The use of chemical insecticides as prophylaxis measure in applications such as coils, sprays, insecticide-treated nets (ITNs) have posed threat to human health and the ecosystem (Philip, 2001).

An important alternative measure to chemical insecticides is biological control measure which involves the regulation of pest population using natural control agents.
such as predators, nematodes and microbial insecticides (Merritt et al., 2005). It is the use of one biological organism to control another; releasing beneficial bacteria, fungi or arthropods to limit pest infestation. Weinzierl et al. (2005) noted that the organisms used in microbial insecticides are essentially non-toxic and non-pathogenic to non-target organisms. The safety offered by microbial insecticides is their greatest strength. Bacteria and fungi have been shown to kill mosquitoes to varying degrees (Orduz and Axtel, 1991; Su et al., 2002). Bacillus thuringiensis var israelensis (BTI) and B. sphaericus are being used in worldwide field test designed to control mosquitoes’ population (Philip, 2001). These microorganisms have their own limitations which include low persistence of the bacterial larvicidal crystal protein in warm environment as a result of sunlight inactivation. The direct presence of the bacterial larvicidal crystal protein in warm environment is toxic and non-specific. Therefore, the isolation of bacterial strains with larvicidal activity having a broad host range specifically, stable habitation and non-pathogenic properties is desired. In order to achieve this, we investigate the larvicidal activities of Pseudomonas aeruginosa, Bacillus polymyxa and Bacillus subtilis on different instars of Anopheles mosquito larvae at varying concentrations under laboratory condition.

MATERIALS AND METHODS

Insect rearing

The mosquito larvae were collected from stagnant waters. They were selected and differentiated using both physical and molecular characterisations. The A. arabiensis larvae were reared in a meshed cage at 25°C and 70% relative humidity under 14L:10D photoperiod with slight modifications according to Zhong et al. (2006). They were fed daily with Tetramin® fish food. This allowed them to reach maturity stage and where offered blood meal. Eggs laid on wet filter papers were transferred to water trays. Larvae were fed and sorted for bioassays.

Entomopathogenic bacteria

This study was conducted at the Federal University of Technology Akure, Nigeria. Cockroaches and housefly were collected into sterile containers from their natural breeding habitats (cupboards for cockroach and housefly around the refuse dumps) in Akure, Nigeria. In the laboratory, adult cockroaches were placed inside a sterile Petri dish containing 10 mL of sterile water each (in triplicate). The Petri dish was properly shaken to ensure good washing away of particles that were on the cockroaches. 1 ml was taken from the wash water, serially diluted to 10⁻² and 0.1 ml of the 10⁻⁴ serial dilution was poured plated using molten nutrient agar and potato dextrose agar. Incubation was done at 37°C for 24 h and the plates were observed for growth. Identification of the bacterial isolates was done using cultural, morphological and biochemical characteristics according to the methods of Holt et al. (1994). The identification of fungi was by comparison of the observed morphological characteristics with those described by Onions et al. (1995), after examination under the microscope. The same procedure was repeated using housefly. B. subtilis, P. aeruginosan and B. polymyxa were selected for bioassay due to high larvicidal activity recorded on preliminary assay conducted in our laboratory.

Cultivation of bacteria

A basal medium containing K₂HPO₄ (17.4 g), NH₄SO₄ (1.98 g), MgSO₄ (0.48 g), FeSO₄·7H₂O (0.0025 g) and glucose (2.0 g) in 100 mL of sterile distilled water was used. Each isolate was inoculated into 10 mL of sterile basal medium, incubated at 37°C for 24 h. The cells were centrifuged at 12,168 × 10³ g for 15 min (Centrifuge MSE Minor 35) and re-suspended into 2 mL sterile water. The cells were counted and diluted. At inoculation onto mosquito, the diluted cells were poured plated into nutrient agar, incubated and counted using colony counter.

Susceptibility of A. arabiensis larvae to P. aeruginosa, B. polymyxa and B. subtilis

One hundred (100) A. arabiensis larvae were used for each concentration in this experiment. The mosquito larvae were surface sterilised in separate Petri dishes using 75% alcohol and rinsing with sterile water. There were four replicates and control per treatments with 25 mosquito larvae in each container. The mosquito larvae were starved for 24 h prior to inoculation. Each mosquito larva was inoculated with the cells of bacterial isolates at varying cell loads. Incubation was carried out for 48 h. The cadavers were removed daily and counted.

Statistical analysis

All data were analysed using analysis of variance (ANOVA). Duncan Multiple Range Test was used to separate mean while Probit analysis (Finney, 1971) was used to determine the LC₅₀ that will kill 50% of test population and resistance ratio.

RESULTS AND DISCUSSION

Based on our previous findings (Omoya et al., 2010) and by the new outcomes, we selected the tested microorganisms for evaluation in the successive bioassay. Cell population and incubation time were seen to affect the degradation and subsequently lead to increase in the percentage mortality (Figures 1 to 3). This implies that there is need to increase the contact time to effectively eradicate mosquito larvae particularly when low cell number is used. The degradation rate of mosquito larvae was rapid when sufficient high cell number was used. B. subtilis exhibited higher larvicidal activity than both P. aeruginosan and B. polymyxa. This observation was noticed after 24 h of infesting the larvae with the organisms. In all the treatments, the percentage mortality recorded was seen to increase with increase in concentration with B. Subtilis treatment showing the highest percentage mortality. Although, there was no significant
difference in percentage mortality of *P. aeruginosa* and *B. polymyxa* at 24 h of exposure (Figure 1), at 48 h of exposure to all the treatments, gradual increase in percentage mortality was recorded and results from each treatment were significantly different from each other (Figure 2). This might be as a result of increase in the feeding rate of the larvae which is in agreement with the results of Gunasekaran et al. (2004). In Figure 3, decrease in percentage mortality of the inoculated mosquito larvae was observed. This could be as a result of decrease in the ingestion rate due to the age of the larvae. During this present study, the mosquito larvae showed greatest susceptibility to *B. Subtilis* when compared to the other tested bacterial isolates. Toxin concentration of 4 to 5 folds of *B. polymyxa* was necessary to induce the same effect of 50% mortality.
Figure 3. Effect of different concentration of bacterial isolates on the mortality of 4th instar Anopheles mosquito larvae.

Table 1. Relative potency of Bacillus subtilis, Pseudomonas aeruginosa and Bacillus polymyxa on second instar.

<table>
<thead>
<tr>
<th>Line name</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Lower limit</th>
<th>Upper limit</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Index</th>
<th>RR</th>
<th>Slope</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>0.862</td>
<td>0.433</td>
<td>1.219</td>
<td>*</td>
<td></td>
<td></td>
<td>100</td>
<td>1</td>
<td>1.249</td>
<td>0.862</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1.931</td>
<td>1.23</td>
<td>2.501</td>
<td>*</td>
<td></td>
<td></td>
<td>44.64</td>
<td>2.24</td>
<td>0.926</td>
<td>1.931</td>
</tr>
</tbody>
</table>

LC<sub>50</sub>, lethal concentration at which the extract kill 50% of the organisms at a given time. *1, 2 and 3 showed a significant difference in the test organisms; RR, resistance ratio: resistance ratio (RR) is calculated by dividing the LC<sub>50</sub> of the most potent microorganisms with other susceptible strains.

Table 2. Relative potency of Bacillus subtilis, Pseudomonas aeruginosa and Bacillus polymyxa on fourth instar.

<table>
<thead>
<tr>
<th>Line name</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Lower limit</th>
<th>Upper limit</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Index</th>
<th>RR</th>
<th>Slope</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>2.36</td>
<td>0.433</td>
<td>1.219</td>
<td>*</td>
<td></td>
<td></td>
<td>100</td>
<td>1</td>
<td>1.021</td>
<td>2.36</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>4.205</td>
<td>3.805</td>
<td>5.586</td>
<td>*</td>
<td></td>
<td></td>
<td>33.25</td>
<td>1.78</td>
<td>1.623</td>
<td>4.205</td>
</tr>
<tr>
<td>Bacillus polymyxa</td>
<td>7.403</td>
<td>4.442</td>
<td>9.415</td>
<td>*</td>
<td></td>
<td></td>
<td>16.78</td>
<td>3.34</td>
<td>1.862</td>
<td>7.403</td>
</tr>
</tbody>
</table>

LC<sub>50</sub>, lethal concentration at which the extract kill 50% of the organisms at a given time. *1, 2 and 3 showed a significant difference in the test organisms; RR, resistance ratio: resistance ratio (RR) is calculated by dividing the LC<sub>50</sub> of the most potent microorganisms with other susceptible strains.

(LC<sub>50</sub>) on the larvae when compared to B. Subtilis while 2 folds of P. aeruginosa concentration will cause the same effect and resistance ratio (RR) showed that the three tested organisms in second instar varied. P. aeruginosa showed that the resistance ratio (RR) values was 2.24 folds above that of B. substilis while B. Polymyxa was 6.70 folds above B. substilis (Table 1). Variation in the LC<sub>50</sub> and RR values was recorded in Table 2. Toxin concentration of above 3 folds of B. polymyxa was necessary to induce the same effect of 50% mortality (LC<sub>50</sub>) on the larvae when compared with B. substilis while 2 folds of P. aeruginosa concentration will cause the same effect and RR values of P. aeruginosa was 1.78 folds above that of B. substilis while B. polymyxa was 3.34 folds above Bacillus substilis in fourth instar. The observed difference in the susceptibility might be due to their ingestion rate (Sun et al., 1980). Hence more mortality of the mosquito larva was recorded...
in the second instar treatments. Therefore, from the data obtained in this study, we conclude that the application of *B. Subtilis* to kill malaria vector larvae could significantly reduce parasite transmission, be economical and therefore lead to reduction of malaria risk. Hence this method of biological control has potential as a new strategy for malaria control in Nigeria.

REFERENCES


Typing of *Candida* species isolated from blood cultures and analysis of their *in vitro* antifungal susceptibilities

Nurten Baran¹, Ismail Serkan Salman¹, Sureyya Gul Yurtsever¹*, Rahim Ozdemir¹, Serdar Gungor¹, Senel Yurtsever² and Mustafa Demirci¹

¹Department of Medical Microbiology, Ataturk Training and Research Hospital, Izmir Katip Celebi University, Izmir, Turkey.
²Department of Dermatology, Tepecik Training and Research Hospital, Izmir, Turkey.

Accepted 24 September, 2013

The aim of the present study was to investigate antifungal susceptibility of Candida strains isolated from blood cultures in our tertiary hospital. Patients hospitalized between December 2008 and April 2011 whose more than one blood cultures revealed growth of *Candida* species and Candida strains isolated in these cultures were included in the study. In order to identify isolated yeast species, appearance and configuration of the colonies, germ tube test results and their morphological appearance in corn flour Tween 80 agar were evaluated. Within the study period, among 65 Candida strains isolated, 36 (55.4%) *Candida parapsilosis*, 17 (26.2%) *Candida albicans*, 6 (9.2%) *Candida glabrata*, 4 (6.2%) *Candida tropicalis*, and 2 (3.1%) *Candida lusitaniae* isolates were identified. According to Fungifast susceptibility panel, antifungal susceptibility rates were as follows: amphotericin B and flucytosine (100%) fluconazole (93.8%), itraconazole (87.6%) and voriconazole (96.9%). Antifungal susceptibility rates of Candida isolates based on E-test method were as follows: amphotericin B (100%), voriconazole (92.3 %) itraconazole (53.8%) and fluconazole (89.2%). In consideration of higher morbidity, mortality and economic burden of the cases with fungemia, measures against emergence of these infections convey crucial importance. Typing of fungi isolated from intensive care units in particular, and their antifungal susceptibility tests should be done regularly to reveal resistance patterns of pathogens, and any increase in resistance (if any) over time, must be determined with scientific methods. We think that similar studies will guide the clinician in planning treatment of Candida infections especially in patients at risk.

Key words: Candidemia, candida, *in vitro*, microbial sensitivity tests.

INTRODUCTION

Hematologic infections due to *Candida* species in the world are becoming increasingly important. In the United States, *Candida* spp. yeasts ranked fourth among the most frequently isolated microorganisms from blood cultures (Martin et al., 2005). In recent years, the incidence of nosocomial Candida infections increased due to increasing number of patients receiving chemotherapy and other immunosuppressive therapies, innovations in transplantation surgery, use of broad-spectrum antibio-

*Corresponding author. E-mail: sgul71@yahoo.com. Tel: 00905052363081. Fax: 00902322431530.
Candida spp. differ between countries and hospitals (Pfaller et al., 2003; Dimopoulos et al., 2008). In species other than Candida albicans, especially in Candida krusei and Candida glabrata resistance againstazole group antifungal agents develops which further complicates choice of treatment in candidemias. Antifungal susceptibility tests guide the antifungal therapy during episodes of fungemia.

In this study, our aim was to investigate antifungal susceptibility of Candida strains isolated from blood cultures in our tertiary hospital.

MATERIALS AND METHODS

Our study included patients hospitalized in various services of our tertiary hospital between December 2008 and April 2011 whose more than one blood cultures sent to Medical Microbiology Laboratory revealed growth of Candida spp. and also Candida strains isolated in these cultures. Among the same results obtained from the cultures of the same patient, only one blood culture results were included in the analysis.

In order to identify isolated yeast spp., appearance and configuration of the colonies, germ tube test results and their morphological appearance in corn flour Tween 80 agar were evaluated. In addition to these conventional methods, commercial Funfast (ELITech France SAS) kit which provided quick identification and antifungal susceptibility results were used in accordance with the manufacturer’s instruction.

Antifungal susceptibility tests were performed using E-test method. For this purpose, CLSI M27-A M44-A guidelines published by CLSI were followed. As a culture medium, Mueller-Hinton agar (Oxoid CM0337) (GM-MH) containing 2% glucose and 5 µg/ml methylene blue (RM 956-Hmedra) was used. In the study, for E-test application, yeasts were suspended in 0.85% NaCl so as to achieve 0.5 McFarland turbidity standard. Surface of GM-MH Agar were scratched in zigzags with sterile cotton swab to provide homogenous growth of Candida. E-test strips (bioMerieux AB, Sweden) containing fluconazole, itraconazole, amphotericin B and voriconazole were applied on agar surface using sterile forceps. E-test strips containing culture medium were incubated at 35°C. Incubation was maintained up to 24 or 48 till distinct appearance of inhibition ellipse which indicated growth of Candida. The first intersection point between the inhibition ellipse and the scale on the strip where a significant inhibition was observed was evaluated as minimal inhibitory concentration (MIC).

RESULTS

Within the study period, among 65 Candida strains isolated, 36 (55.4%) Candida parapsilosis, 17 (26.2%) Candida albicans, 6 (9.2%) Candida glabrata, 4 (6.2%) Candida tropicalis, and 2 (3.1%) Candida lusitaniae isolates were identified. In addition, two standard Candida strains (C. krusei ATCC 6258 and C. albicans ATCC 90 029) were included in the study. Distribution of the patients among services is shown in Table 1, and of isolates of Candida spp. in Table 2.

In the study, according to Fungifast susceptibility panel, antifungal susceptibility rates were as follows: amphotericin B and flucytosine (100%) fluconazole (93.8%), itraconazole (87.6%), and voriconazole (96.9%).

Antifungal susceptibility rates of Candida isolates based on E-test method were as follows: amphotericin B (100%), voriconazole (92.3%) itraconazole (53.8%) and fluconazole (89.2%).

Using E-test in the study, MIC range of amphotericin B for Candida albicans was 0004-0125 µg/ml, and for non-albicans Candida pp. it was 0.003-0.38 µg/ml, respectively. In our study, amphotericin B resistance was not observed in none of the 65 isolates. MIC50, and MIC90 values of amphotericin B against C. albicans were 0.047 and 0.125 µg/ml, while for non-albicans Candida spp. MIC50, MIC90 values were 0.064 and 0.19 µg/ml, respectively.

MIC range of fluconazole for Candida albicans and non-albicans Candida spp. were 0.38-64 and 0.19-256 µg/ml, respectively. Seven of the sixty five isolates in our study (10.7%) were resistant to fluconazole. Four Candida, one C. tropicalis and two C. glabrata strains were resistant strains to fluconazole. In our study, a dose-dependent susceptible strain for fluconazole was not detected. Fluconazole MIC 50 and MIC 90 values for Candida albicans, and non-albicans Candida spp. were detected as 0.75 vs. 64 µg/ml, and 0.50 vs. 4 µg/ml, respectively.

MIC range of itraconazole was 0023-32 µg/ml for Candida albicans, and 016-32 µg/ml for non-albicans Candida spp. In our study, 17 of 65 isolates (26.1%) were resistant to itraconazole. However, 13 (20%) of these strains were susceptible to itraconazole in a dose-dependent manner. Five strains of Candida albicans (29.4%) twelve (25%) of non-

Table 1. Distribution of the patients among hospital departments.

<table>
<thead>
<tr>
<th>Department</th>
<th>Number of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaesthesia Intensive Care Unit</td>
<td>37 (56.9)</td>
</tr>
<tr>
<td>Internal Medicine</td>
<td>8 (12.3)</td>
</tr>
<tr>
<td>Cardiovascular Surgery</td>
<td>7 (10.8)</td>
</tr>
<tr>
<td>Neurosurgery</td>
<td>1 (6.2)</td>
</tr>
<tr>
<td>General Surgery</td>
<td>4 (6.2)</td>
</tr>
<tr>
<td>Haematology</td>
<td>2 (3.1)</td>
</tr>
<tr>
<td>Urology</td>
<td>2 (3.1)</td>
</tr>
<tr>
<td>Emergency Medicine</td>
<td>1 (1.5)</td>
</tr>
<tr>
<td>Infectious diseases</td>
<td>1 (1.5)</td>
</tr>
<tr>
<td>Nephrology</td>
<td>1 (1.5)</td>
</tr>
<tr>
<td>Neurology Intensive Care Unit</td>
<td>1 (1.5)</td>
</tr>
</tbody>
</table>

Table 2. Candida spp. isolated from blood cultures and their distribution.

<table>
<thead>
<tr>
<th>Candida spp.</th>
<th>Number of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parapsilosis</td>
<td>36 (55.4)</td>
</tr>
<tr>
<td>C. albicans</td>
<td>17 (26.2)</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>6 (9.2)</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>4 (6.2)</td>
</tr>
<tr>
<td>C. lusitaniae</td>
<td>2 (3.1)</td>
</tr>
</tbody>
</table>
albicans Candida spp. were resistant. Among all non-albicans Candida spp., all C. glabrata (100%), three C. tropicalis (75%), and three C. parapsilosis (8.3%) strains were resistant to itraconazole. One C. albicans, two C. lusitaniae, and ten C. parapsilosis strains were susceptible to itraconazole in a dose-dependent manner. MIC50 and MIC 90 values of itraconazole against C. albicans were 0.0125 and 0.032 µg/ml, and for non-albicans Candida spp. MIC 50 and MIC 90 values of the drug were 0.0125 and 0.032 µg/ml, respectively.

MIC range, MIC50 and MIC 90 of voriconazole against C. albicans, and non-albicans Candida spp. were 0.0012-0.032, 0.0094 and 0.032 vs. 0.0012-0.032, 0.032 and 0.1225 µg/ml, respectively. A total of five (7.6%) strains were found to be resistant among 65 strains investigated for their susceptibilities to voriconazole. Four of these resistant strains belonged to the C. albicans, and one to the C. tropicalis spp. None of the strains of C. parapsilosis, C. glabrata and C. lusitaniae spp. were resistant to voriconazole.

According to Fungifast susceptibility panel, all (100%) strains of C. albicans and non-albicans Candida spp. were susceptible to amphotericin B, while the corresponding susceptibility rates for fluconazole (88.2 vs. 95.8%), itraconazole (82.3 vs. 89.6%) and voriconazole (94.1 vs. 97.9%) were also estimated as indicated in parentheses.

We used E-test in our study, and detected rates of susceptibility of C. albicans and non-albicans Candida spp. to amphotericin B (100 vs. 100%), fluconazole (76.5 vs. 93.7%), itraconazole (64.7 vs. 50%) and voriconazole (76.5 vs. 97.9%) as indicated in parentheses.

**DISCUSSION**

Since fungemias are often severe, rapidly progressive, and treatment-resistant diseases which are difficult to diagnose, they cause serious morbidity and mortality. Literature studies have been emphasizing potential differences among regions, and patient groups served by hospitals (Martin et al., 2005; Cheng et al., 2004; Ostrosky-Zeichner et al., 2003).

In many studies performed, various causative agents of candidemia vary from country to country, annually in the same country, and also among the hospitals were reported. According to some population based studies in the United States and Europe four strains of Candida, namely C. albicans, C. glabrata, C. parapsilosis and C. tropicalis are responsible for about 95% of the cases of candidemia (Tan et al., 2008). In a study performed by the International Fungal Surveillance Group which included 32 nations, and related data recorded between years 1992 and 2001, observed that C. parapsilosis candidemia took the first place among cases of candidemia (Pfaller et al., 2004). In our study in 36 (55.4%) of 65 strains of Candida, C. parapsilosis was identified and ranked first with more than twice the number of C. albicans isolates. C. albicans was identified in 17 (26.2%) isolates and took the second place. Subsequently 6 (9.2%) C. glabrata, 4 (6.2%) C. tropicalis, 2 (3.1%) C. lusitaniae strains were identified in order of decreasing frequency.

The International Fungal Surveillance Group isolated 6082 strains of Candida spp. during a study lasting for 12 years, and detected susceptibility of Candida spp. to fluconazole as 90%, while rates of dose-dependent susceptibility, and resistance to this antifungal medication were 7 and 3%, respectively. In the same study, C. glabrata was determined as the least fluconazole susceptible strain (Pfaller et al., 2004). Matta et al. (2007) used microdilution antifungal susceptibility test in their investigation in Brazil conducted between 1995 and 2003 with 1000 Candida isolates obtained from blood cultures, and reported susceptibility rates for amphotericin B, fluconazole, itraconazole, and voriconazole as 100, 97, 93, and 99.7%, respectively. In our country, resistance to amphotericin B seems to have changed among geographic regions. Yucesoy et al. (2000) investigated in vitro susceptibilities of blood culture isolates of Candida spp. to antifungal agents, and all strains of Candida spp. were susceptible to amphotericin B. However, according to another study performed by Dograman et al. (2000) resistance against amphotericin B was reported for ten C. tropicalis spp, two C. albicans spp, and one C. parapsilosis spp. Strains. In studies performed in our country, regions with higher resistance against azole antifungals were detected. In an investigation, significant in vitro resistance against azole antifungals was not detected in isolates identified (Arikan et al., 2001). Koc et al. (1999) retrospectively evaluated yeast growth in blood cultures for one year, and found MIC 50 vs. MIC 90 values of fluconazole for C. glabrata and C. krusei, which are mostly encountered Candida spp. after C. albicans as 64 vs.64 and 64 vs. 128 µg/ml, respectively.

In our study, we evaluated antifungal susceptibilities using E-test method, and commercial Fungifast susceptibility panel. Based on standardization techniques, and experiences gathered, correlation between E-test, and referenced methods as for Candida spp and azole antifungals is at an acceptable level. Besides, E-test is a relatively valuable method in the determination of MIC value for amphotericin B, and it is one of the reliable methods for the identification of resistant isolates. In a multicenter study, Pfaller et al. (2000) detected a 86-100% concordance between results of E-test, and macrodilution test methods with respect to amphotericine B, fluconazole, fluucytosine, and itraconazole. In our study, we performed E-test method using GM-MH agar instead of RPMI 1640 culture medium. In a study, the authors reported that reference macrodilution method, and E-test method used with RPMI 1640 medium are far from being practical methods that can be used routinely by every laboratory (Lee et al., 2009). In this study, for 182 isolates, GM-MH of fluconazole was used, and in comparisions with E-test and reference macrodilution methods, MIC values of these tests were concordant at a level of 82.9%. In our study, antifungal susceptibility was evaluated
by E-test method and 100, 92.3 53.8, and 89.2% of Candida isolates were found to be susceptible to amphotericin B, voriconazole, itraconazole, and fluconazole, respectively. This 100% susceptibility to amphotericin was in agreement with other studies performed in this country and abroad. Although higher rates of resistance against fluconazole were detected in our study when compared with rates in developed countries, these rates are closer to those reported in research centers localized in different regions of our country.

Susceptibility rate of all Candida spp. to itraconazole was 53.8%. Concordance of this susceptibility rate detected for itraconazole in literature results is controversial. In our study, itraconazole-resistance detected in all strains of C. glabrata spp. when compared with other drugs, resistance of all C. glabrata strains to itraconazole and higher rates of itraconazole-resistance among strains of non-albicans Candida spp. to itraconazole is a striking phenomenon. Besides, detection of 10 (27.7%) dose-dependent susceptible and three (8.3%) resistant strains of C. parapsilosis spp., higher incidence of nosocomial infection in our hospital caused by this agent, and also the fact that it was the most commonly isolated strain in this study increase the importance of this etiologic factor. Susceptibility rates for voriconazole appear to be in concordance with the literature findings. In our study, all strains were susceptible to both itraconazole and fluconazole at the same time. All five strains resistant to voriconazole were also resistant to fluconazole, and itraconazole.

Fungifast susceptibility panel, and E-test results were compared, and a p value of 0.001 was determined for susceptibility estimations of non-C. albicans to itraconazole, and p <0.05 was accepted as the level of significance. Therefore, when compared with the E-test method, Fungifast susceptibility rating method was found to be insufficient for the determination of susceptibility to itraconazole. A statistical difference was not found between two tests with respect to amphotericin B, fluconazole and voriconazole.

When susceptibilities of all isolates identified in our study were evaluated, susceptibility of 89.2% of Candida isolates to fluconazole demonstrates drug’s suitability for initial antifungal therapy for patients with candidemia in our hospital in consideration of its relative lack of toxicity, ease of use, affordable cost and availability. All strains of Candida spp. responsible for candidemia were found to be susceptible to amphotericin B. In this regard, it is a suitable drug for patients with higher risk of mortality.

In conclusion, in consideration of higher morbidity, mortality, and economic burden of the cases with fungemia, measures against emergence of these infections convey crucial importance. Typing of fungi isolated from intensive care units in particular, and their antifungal susceptibility tests should be done regularly to reveal resistance patterns of pathogens, and any increase in resistance (if any) over time, must be determined with scientific methods.

We think that similar studies will guide the clinician in planning treatment of Candida infections especially in patients at risk.

REFERENCES


Isolation and identification of microorganisms from polyhouse agriculture soil of Rajasthan

Nakuleshwar Dut Jasuja¹*, Richa Saxena¹, Subhash Chandra¹ and Suresh C. Joshi²

¹Department of Biotechnology and Allied Sciences, Jayoti Vidyapeeth Women's University, Jaipur-303007, Rajasthan, India.
²Department of Zoology, University of Rajasthan, Jaipur-302004, India.

Accepted 21 September, 2013

The present work deals with the isolation and characterization of microorganisms from polyhouse agriculture soil of Jharna village (Rajasthan). Several bacteria and fungi were isolated from polyhouse soil, using serial dilution method. These bacterial isolates were Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes, Shigella sp., Proteus mirabilis, Bacillus anthracis, Bacillus subtilis, Staphylococcus aureus and Staphylococcus epidermidis species which were further identified on the basis of colony morphology, Gram staining, biochemical tests and using selective and differential media. Identification of fungi such as Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Trichoderma sp. and Rhizopus sp. was carried out by cultivating on potato dextrose and sabouraud’s dextrose agar media and microscopic method. Microorganisms play an important function in biodegradation of solid agriculture waste and also help in the crop production.

Key words: Bacteria, fungi, agriculture, polyhouse.

INTRODUCTION

Microorganism are frequently present in soil, manure and decaying plant tissues which are able to degrade wastes that are correlated with the substrate organic matter (Alexander, 1977). Agriculture soil is a dynamic medium in which a large number of pathogenic and non-pathogenic bacterial and fungal flora live in close association. Microbes in the soil are the key to carbon and nitrogen recycling. Microorganisms produce some useful compounds that are beneficial to soil health, plant growth and play an important role in nutritional chains that are important part of the biological balance in the life in our planet (Paul and Clerk, 1966; Kummerer, 2004). Polyhouses are basically naturally ventilated climate controlled. Polyhouse cultivation has been evolved to create favorable micro-climates, which favours the crop production and could be possible all through the year or part of the year as required. Wherein, off season crops are also grown under a favorable controlled environment and other conditions viz. temperature, humidity, light intensity, ventilation, soil media, irrigation, fertigation and other agronomical practices throughout the season irrespective of the natural conditions outside. Therefore, the soil of polyhouse may have different conditions for growth of microorganism than natural environment. The present study aimed to isolate effective microorganisms that are present in polyhouse soil and find out the optimize culture.
conditions.

MATERIALS AND METHODS

Collection of soil samples

Agricultural soil sample were collected from polyhouse of Jayoti Vidyapeeth Women’s University campus, located at East Longitude-75° 27’ 38”, North Latitude-26° 49’ 34”, 450-500 m above sea level, weather: Temperature - 28-34°C in summer, 12°- 18° C in winter, Jaipur, Rajasthan (Figure 1). Soil samples were taken with the help of sterile spatula, in sterile plastic bags. The samples were brought to the microbiology laboratory.

Determination of physiochemical properties of soil

Freshly collected soil samples were taken for determination of physiochemical properties. The moisture content of the sample was measured in a hot air incubator at 105°C to constant weight. The pH, temperature, humidity, and air pressure was determined using digital pH meter, thermometer, hygrometer and barometer, respectively (Pramer and Schmidt, 1964; Iyengar and Bhave, 2005).

Isolation of microorganism

The microorganisms were isolated by serial dilution technique on Potato Dextrose Agar (PDA) and Nutrient Agar Media (NAM). In this technique, a sample suspension was prepared by adding 1.0 g sample to 10 ml distilled water and mixed well for 15 min and vortexed. Each suspension was serially diluted 10⁻¹ to 10⁻⁶. 0.1 ml was pipetted onto plates with PDA and NAM media, spread with a glass spreader and incubated at 28°C for fungal and 37°C for bacterial observation. Each colony that appeared on the plate was considered as one colony forming unit (cfu) (Waksman, 1927; Nazir, 2007).

Identification of fungi

The fungal isolates were identified by morphological examination and its characteristics. Morphological characteristics were examined under microscope (Onion et al., 1981).

Optimization of culture condition for fungi

For the determination of optimum condition of isolated fungi, three media were used (Potato dextrose agar media, Sabouraud’s dextrose agar, Czapek Dox agar). The media were adjusted to pH 4 to 7. For optimization of the incubation period, the culture plates were incubated at 28°C for 4 to 7 days (Bhattacharyya and Jha, 2011; Azzaz et al., 2012).

Identification and characterization of Bacteria

Gram staining was performed to check the morphology of the cells and spore chain morphology was identified by spore staining technique. The pure culture was grown on nutrient agar medium and transferred to Mac-conkey agar medium, EMB agar medium, Endo-Agar medium and mannitol salt agar medium for differentiate and identified bacteria. The plates were incubated at 37°C in the incubator and readings were taken 24 h after inoculation. The bacterial isolates were biochemically characterized by catalase test, oxidase test, urease test, motility test, TSI test, nitrate reduction test and IMVIC test (Collins and Lyne, 1989; Harold, 2002; Zaved et al., 2008).

RESULTS AND DISCUSSION

This study revealed that polyhouse soil samples were analyzed with respect to different types of bacteria and fungi. The common bacteria, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes, Shigella sp., Proteus mirabilis, Pseudomonas aeruginosa, Bacillus anthracis, Bacillus subtilis, and S. epidermidis are dominating species in the soil samples. Similarly, when the soil samples were tested for different types of fungi, Aspergillus niger, Aspergillus flavus, Trichoderma sp., Fusarium oxysporum, Rhizopus sp. were dominating species in soil samples. The physiochemical properties of soil play an important role in the growth of microorganism. The polyhouse soil was slightly acidic. The humidity, moisture content, and temperature were 35, 45.5 and 32°C, respectively (Table 1).

In the present study, the isolated fungi were identified on the basis of cultural, microscopic and morphological characteristics (Figure 2). Earlier work reported that for maximum growth of fungi, potato dextrose agar was most favorable (Maheshwari, 2000). The isolated fungi were
Table 1. Physiochemical properties of soil samples.

<table>
<thead>
<tr>
<th>Physiochemical properties of soil</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.5 ±1.0</td>
</tr>
<tr>
<td>Temperature</td>
<td>32°C</td>
</tr>
<tr>
<td>Humidity</td>
<td>35% Fair</td>
</tr>
<tr>
<td>Moisture content</td>
<td>45.5%</td>
</tr>
<tr>
<td>Air pressure</td>
<td>743 mb</td>
</tr>
</tbody>
</table>

cultured in PDA, SDA and Czapec Dox agar media. It was observed that the PDA and SDA media were most suitable for good growth of *A. niger*, *A. flavus* and *Trichoderma* sp., *Rhizopus* sp. and *Fusarium oxysporum* at optimum range of pH 6 to 8 and a suitable incubation period was 4 to 7 days, respectively. However, czapec dox agar medium was also suitable for *A. niger*, *A. flavus*, *Trichoderma* sp., *Rhizopus* sp. and *F. oxysporum*. Most of the fungi show favorable growth in alkaline side of neutral pH but they can also tolerate the pH range from 6 to 8 (acidic to basic) (Table 2). The enzymatic activities and use of bacteria, actinomycetes and fungi in the decomposition of organic matter provides beneficial metabolic products to the soil (Tiquia et al., 2002; Singh et al., 2003). Similarly, Karthik et al. (2011) also reported the isolation, identification of microorganisms such as *Bacillus* species from agricultural waste dump soil for screening of pectinase producing activity. The isolation of various fungal, bacterial species showed that the agricultural soil is quite rich in microbial flora. In agriculture process, soil microorganisms such as bacteria and fungi may play important roles in soil fertility and in the form of loss and gain in the production of grains, fruits, vegetables. Moreover, it also helps to maintain or enhance the environment quality and conserve natural resources. Most of the bacteria such as *Bacillus anthracis*, *B. subtilis*, and *S. aureus* and *S. epidermidis* isolated in this study have been reported by other researchers (Amir and Pineau, 1998; Okoh et al., 1999). Several bacteria were isolated from agriculture soil samples. Identification and characterization of isolated bacteria were performed by morphological, microscopically (Figure 3), biochemical tests such as shape, arrangement, colonies, temperature, growth, indole production test, ethyl red and Voges-proskauer test, citrate utilization test, urease test, catalase test, Mac-conkey’s test, TSI test, growth at 37°C (Table 3). Further, selective and differentiate media were prepared and the isolated bacteria were inoculated under sterilized conditions incubated at 37°C for 18 to 24 h and the results were recorded (Table 4). In different fermentation media, isolates showed different level of growth. The growth of *S. aureus* was observed on mannitol salt agar medium. In the presence of high salt concentration, *S. aureus* fermented mannitol producing acid which changes the pH turning phenol red to yellow (Figure 4). The present study, concluded on isolation and identification of bacterial and fungal floras with optimum pH, temperature and other cultural condition. These isolated polyhouse soil bacteria and fungi are also used in the seasonal and off season crop production with different environment condition. These microorganisms are to supply nutrients to crop, to encourage plant growth; for example, through the production of plant hormones and to control or inhibit the activity of plant pathogen. This study provides knowledge on microorganisms of Rajasthan semi arid soil which is used in polyhouse and produce seasonal and off season foods and vegetables which are not able to grow in normal environment condition.

Conclusion

The environment where we live is the habitat for various microorganisms; mostly bacteria and fungi which are used for various industrial applications like enzymes production, fabric manufacturing, bioremediation, pharmaceutical production, etc. Micro-organisms play an important role in composting of organic waste and can be an important contributor to optimal agricultural waste. This study revealed the isolation and identification of diversity of microorganisms which are present in agricultural soil habitat.

ACKNOWLEDGEMENT

The authors are thankfull to Jayoti Vidyapeeth Women’s University for providing the laboratory and technical facilities.
Table 2. Effect of pH and incubation period on the growth of isolated fungi on PDA, SDA, Czapek - Dox agar media at 28°C temperature.

<table>
<thead>
<tr>
<th>pH of the Medium</th>
<th>Isolated fungi</th>
<th>PDA Media</th>
<th>SDA Media</th>
<th>Czapek Dox agar Media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incubation period (days)</td>
<td>Incubation period (days)</td>
<td>Incubation period (days)</td>
</tr>
<tr>
<td>6</td>
<td>Aspergillus niger</td>
<td>+1 2 3 4</td>
<td>+2 2 3 4</td>
<td>+2 3 4 4</td>
</tr>
<tr>
<td></td>
<td>Aspergillus flavus</td>
<td>+1 2 3 4</td>
<td>+1 2 3 4</td>
<td>+2 2 3 4</td>
</tr>
<tr>
<td></td>
<td>Trichoderma sp.</td>
<td>+2 3 4 4</td>
<td>+2 3 4 4</td>
<td>+2 2 3 4</td>
</tr>
<tr>
<td></td>
<td>Fusarium oxysporum</td>
<td>+1 1 2 3</td>
<td>+1 2 3 4</td>
<td>+2 3 3 4</td>
</tr>
<tr>
<td></td>
<td>Rhizopus sp.</td>
<td>+1 2 3 4</td>
<td>+1 2 3 4</td>
<td>+1 2 3 4</td>
</tr>
<tr>
<td>7</td>
<td>Aspergillus niger</td>
<td>+2 3 4 4</td>
<td>+2 3 4 4</td>
<td>+2 3 4 4</td>
</tr>
<tr>
<td></td>
<td>Aspergillus flavus</td>
<td>+2 2 3 4</td>
<td>+2 3 4 4</td>
<td>+1 2 3 4</td>
</tr>
<tr>
<td></td>
<td>Trichoderma sp.</td>
<td>+3 3 4 4</td>
<td>+2 3 4 4</td>
<td>+1 2 3 4</td>
</tr>
<tr>
<td></td>
<td>Fusarium oxysporum</td>
<td>+1 2 3 4</td>
<td>+1 2 3 4</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>Rhizopus sp.</td>
<td>+1 2 3 4</td>
<td>+1 2 3 4</td>
<td>+1 2 3 4</td>
</tr>
<tr>
<td>8</td>
<td>Aspergillus niger</td>
<td>+1 2 3 4</td>
<td>+2 2 3 4</td>
<td>- 2 3 4</td>
</tr>
<tr>
<td></td>
<td>Aspergillus flavus</td>
<td>+2 2 3 4</td>
<td>+1 2 3 4</td>
<td>- 1 2 3</td>
</tr>
<tr>
<td></td>
<td>Trichoderma sp.</td>
<td>+3 3 4 4</td>
<td>+2 3 4 4</td>
<td>- 2 3 4</td>
</tr>
<tr>
<td></td>
<td>Fusarium oxysporum</td>
<td>- - - -</td>
<td>- - - -</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>Rhizopus sp.</td>
<td>+1 1 2 3</td>
<td>+1 1 2 3</td>
<td>- 2 3 4</td>
</tr>
</tbody>
</table>

- No growth, +1 poor growth, +2 moderate growth, +3 good growth, +4 massive growth.

Figure 3. Microscopic images showing A. *Bacillus anthracis*, B. *Bacillus subtilis* C. *Pseudomonas aeruginosa*, D. *Klebsiella pneumoniae*. 
Table 3. Biochemical characterization of bacterial isolates.

<table>
<thead>
<tr>
<th>Identified bacteria</th>
<th>Gram stain</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Nitrate reductase</th>
<th>VP</th>
<th>MR</th>
<th>Ind</th>
<th>Citrate</th>
<th>Motility</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>-ve bacilli</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve Motile</td>
<td>-ve</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>-ve bacilli</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve Non motile</td>
<td>+ve</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>-ve, bacilli</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve Motile</td>
<td>-ve</td>
</tr>
<tr>
<td>Shigella sp.</td>
<td>-ve rods</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve Non motile</td>
<td>-ve</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>-ve, bacilli</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve Motile</td>
<td>-ve</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>-ve, bacilli</td>
<td>+ ve</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve Motile</td>
<td>-ve</td>
</tr>
<tr>
<td>B. anthracis</td>
<td>+ve, bacilli</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve Non motile</td>
<td>+ve</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>+ve, bacilli</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve Motile</td>
<td>-ve</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>+ve , cocci in clusters</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve Non motile</td>
<td>+ve</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>+ve , cocci</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve Non motile</td>
<td>+ve</td>
</tr>
</tbody>
</table>


Table 4. Growth of bacterial isolates on different media.

<table>
<thead>
<tr>
<th>Identified bacteria</th>
<th>Mac- Conkey agar media</th>
<th>EMB agar media</th>
<th>TSI agar media</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>LF</td>
<td>Metallic sheen</td>
<td>A/A, Gas (+), H2S (-)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>LF</td>
<td>Dark</td>
<td>A/A, Gas (+), H2S (-)</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>LF</td>
<td>Dark</td>
<td>A/A, Gas (-ve), H2S (-)</td>
</tr>
<tr>
<td>Shigella sp.</td>
<td>NLF</td>
<td>Colorless</td>
<td>A/ALK Gas (-), H2S (-)</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>NLF</td>
<td>Colorless</td>
<td>A/Alk, Gas (+), H2S (+)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>NLF</td>
<td>Light</td>
<td>Alk /no change, Gas (-), H2S (-)</td>
</tr>
</tbody>
</table>

LF: Lactose fermentation, NLF: lactose non fermentation, A: yellow slant or butt, Alk: slant or butt red.

Figure 4. (A). Staphylococcus aureus and (B). Staphylococcus epidermidis on mannitol salt Agar media.

REFERENCES

A monoclonal antibody-based antigen-capture enzyme-linked immunosorbent assay (ELISA) for the detection of bluetongue virus

Hui-qiong Yin#, Shu Yang#, Wen-chao Li, Jun Liu, Rui Wang and Jin-gang Zhang*

Viral Detection Laboratory of National Center of Biomedical Analysis, Institute of Transfusion Medicine, the Academy of Military Medical Sciences, Beijing 100850, China.

Accepted 24 September, 2013

A monoclonal antibody-based antigen-capture enzyme-linked immunosorbent assay (ELISA) was developed for the detection of bluetongue virus (BTV) in cell culture lysates and blood samples from sheep. The monoclonal antibody 3E2 and 1C11 specific to BTV VP7 were used as capture antibody and detection antibody, respectively. The assay has detected BTV 1-22 specifically, and had no cross-reactivity with the closely related epizootic hemorrhagic disease virus (EHDV) serotypes 5. The limit of sensitivity of the assay was 9 ng/ml for purified recombinant BTV VP7 and 10^0.5 TCID_{50}/ml for BTV-5. The coefficient of variation (CV) of intra-assay and inter-assay range from 3.45 to 6.10%. The developed antigen-capture ELISA showed good coincident rate (100%) with INGEZIM BTV DAS in 5 serotypes BTV and 8 blood samples from sheep. Therefore, the antigen-capture ELISA may be useful for testing large number of samples in a convenient and short time.

Key words: Bluetongue virus, antigen-capture enzyme-linked immunosorbent assay (ELISA), serotypes, monoclonal antibody.

INTRODUCTION

Bluetongue (BT) is an insect-borne viral disease of ruminants. Among domestic animals, clinical disease occurs most often in sheep, and can result in significant morbidity. The economic consequences of the outbreak were dramatic. For instance, in the Netherlands, the estimated total net costs of the 2006 and 2007 outbreak were 200 million Euros (Velthuis et al., 2010). It has been included in the World Organization for Animal Health (OIE) list of notifiable diseases (formerly List A) (OIE, 2011). The distribution of BT is determined by the geographic distribution of the arthropod vector and extends globally between latitudes 35°S and 53°N (Martin et al., 2008; Orru et al., 2004). Outbreaks have occurred in many countries in northern and western Europe since 2006 (Saegerman et al., 2008; Carpentera et al., 2009; Kampen and Ortega et al., 2010).

Bluetongue virus (BTV) is the prototype member of the genus Orbivirus within the family Reoviridae. Thus far 26 serotypes are recognized. BTV is non-enveloped with a double shelled structure and a double-stranded (ds) 10 segment RNA genome. The virus contains 7 structural proteins and its genome encode also for 5 non structural proteins: NS1, NS2, NS3, NS3a and NS4. The outer capsid proteins, VP2 and VP5, are the serotype determinants and are responsible for generation of serotype-specific neutralizing antibody. The antibodies against VP7, the major core protein, will specifically detect the whole BTV serogroup. And the genomic segment 5, encoding NS1, is the most highly conserved of the 10 segments (Roy, 1989).

*Corresponding author. E-mail: zhangjg@nic.bmi.ac.cn.

# Authors contributed equally and are co-first authors for this work.
Traditionally, laboratory confirmation of BTV is done by intravenous egg inoculation followed by passages in mammalian cells (World Organization for Animal Health, 2008). Virus isolation is tedious and may take up to 5 weeks for completion. Consequently, alternative methods for virus detection have been sought, which include enzyme-linked immunosorbent assay (ELISA), immunoelectron microscopy, reverse transcription polymerase chain reaction (RT-PCR) (Yin et al., 2008), real-time RT-PCR (Yin et al., 2010), bio-barcode assay (Yin et al., 2011) and so on. ELISA techniques have a number of advantages including being economical, specific and rapid. Additionally, large number of clinical or laboratory samples could be screened by the assay in a very short time during sero-epidemiological studies. In this study, a monoclonal antibody-based antigen-capture ELISA was developed for detecting of VP7 protein.

MATERIALS AND METHODS

Monoclonal antibody to VP7 of bluetongue virus

The gene of serogroup-specific antigen VP7 was amplified from BTV-5 (China Animal Health and Epidemiology Center, China) and cloned into pET-Dsba (Promega, Madison, U.S.A). Then, the recombinant VP7 was expressed in *Esherichia coli* BL21 (DE3) pLysS and purified using a His-tag affinity chromatography column on Ni²⁺-nitrotriacetate (NTA) resin (Qiagen, Hilden, Germany) (Li et al., 2007).

BTV-5 propagated in confluent monolayers of baby hamster kidney (BHK)-21 cells was purified through gradient centrifugation. BALB/c mice were immunized with the purified BTV-5 antigens. Spleenocytes from the immunized mice were fused with SP2/0 myeloma cells, and positive hybridoma clones were screened through the expressed recombinant VP7. Monoclonal antibodies (Mab) 3E2 and 1C11 specific to BTV VP7 were prepared and determined to IgG2b (κ) (Yang et al., 2008).

The antigen-capture ELISA

In the antigen-capture ELISA, flat bottom, 96-well plates were incubated with 3E2 Mab and incubated overnight at 4°C. After three washes with PBST, 1% bovine serum albumin (BSA) blocking solution was added to the wells and incubated at 37°C for 60 min and washed three times with PBST. VP7 or BTV samples were added to the wells and incubated at 37°C for 60 min. After three washings, horseradish peroxidase-conjugated 1C11 Mab was added to the wells and incubated at 37°C for 30 min. The solutions were developed by adding 3,3',5,5'-tetramethylbenzidine (TMB) at room temperature. Reactions were stopped after 15 min by the addition of 2 M H₂SO₄. The absorbance in each well was read at 450 nm wavelength on an ELISA reader. A value twice (or more) the mean OD value of the negative antigen control was considered as the positive/negative cut-off value (positive to negative (P/N) ratio=2).

The working dilutions of the capture and detection antibodies were selected by checker board titration, and the best and most satisfactory result was obtained at a 1:2000 dilution for the capture antibody and a 1:1000 dilution for the detection antibody, while the dilution of the antigen (recombinant VP7) and negative control was a 1:10 dilution. At this dilution, the protein content of the controls was between 10 and 15 μg per well, and the P/N ratio was 4.0. These dilutions of reagents were followed throughout the study.

Evaluation of the antigen-capture ELISA

The specificity of the antigen-capture ELISA was confirmed by performing it to detect BTV serotypes 1-22 and the closely related orbivirus epizootic hemorrhagic disease virus (EHDV), using reference strains of serotype 5 (China Animal Health and Epidemiology Center, China).

The sensitivity of the assay was evaluated by conducting the antigen-capture ELISA to detect the recombinant VP7 protein at different concentrations and viral 10-fold serial dilutions of a BTV-5 strain cultured in BHK-21 cell, respectively.

The antigen-capture ELISA was carried out to detect strong-positive sample (0.25 μg/ml VP7), weak-positive sample (0.010 μg/ml VP7) and negative sample (distilled water) to determine the reproducibility of the assay. The samples were tested on 5 separate occasions, with 5 identical samples each time. The coefficients of variation (CV) were analyzed according to sample to negative (S/N) ratio (OD₅₄₀ value).

Five serotypes of BTV cultured in BHK-21 cell were 10-fold diluted respectively, and these BTV samples and eight sheep blood samples (a gift from China Animal Health and Epidemiology Center) were processed using the antigen-capture ELISA and INGEZIM BTV DAS (INGENASA, Madrid, Spain) to investigate the coincidence rate.

RESULTS

The specificity

Different serotypes BTV and EHDV-5 were detected using the antigen-capture ELISA, and the results indicated that the assay could detect VP7 at concentrations as low as 9 ng/ml protein. Then, 10-fold serial diluted BTV-5 was tested using the assay. Results showed that titers as low as 100.5 TCID50/ml BTV-5 were detected positively (Table 2).

The sensitivity

VP7 of BTV at different concentrations was detected using the antigen-capture ELISA, and the results indicated that the assay could detect VP7 at concentrations as low as 9 ng/ml protein. Then, 10-fold serial diluted BTV-5 was tested using the assay. Results showed that titers as low as 100.5 TCID50/ml BTV-5 were detected positively (Table 2).

The reproducibility

The reproducibility of the antigen-capture ELISA was evaluated by detecting the VP7 protein. And the results showed that the CVs (%) of the strong-positive sample, weak-positive sample and negative sample were 3.79, 4.55 and 3.45 intra-test respectively, and 5.38, 6.10 and 4.88 inter-test, respectively (Table 3).

Coincidence analysis

Five serotypes of 10-fold diluted BTV and eight sheep blood samples were detected using the antigen-capture ELISA and INGEZIM BTV DAS. And the results indicated
Table 1. Detection of BTV and EHDV strains cultured in BHK-21 cell with the antigen-capture ELISA.

<table>
<thead>
<tr>
<th>Viral sample</th>
<th>BTV serotype</th>
<th>EHDV serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 5</td>
<td></td>
</tr>
</tbody>
</table>

*P: positive; N: negative.

Table 2. Detection of BTV VP7 and BTV at different concentrations with the antigen-capture ELISA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>The recombinant VP7 of BTV (μg/ml)</th>
<th>BTV-5 (TCID&lt;sub&gt;50&lt;/sub&gt;/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 0.5 0.05 0.01 0.009 0.008 10&lt;sup&gt;3&lt;/sup&gt; 10&lt;sup&gt;2&lt;/sup&gt; 10&lt;sup&gt;1&lt;/sup&gt; 10&lt;sup&gt;0.5&lt;/sup&gt; 10&lt;sup&gt;0&lt;/sup&gt;</td>
<td>P P P P P P P P P P N</td>
</tr>
<tr>
<td>Results</td>
<td>P P P P P N N N N N N N N N N N N N N</td>
<td>P P P P P P P P P P P P</td>
</tr>
</tbody>
</table>

*P: positive; N: negative.

Table 3. Reproducibility of the antigen-capture ELISA for positive and negative samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CV&lt;sup&gt;a&lt;/sup&gt; (%) of S/N&lt;sup&gt;b&lt;/sup&gt; of OD&lt;sub&gt;450&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-test</td>
</tr>
<tr>
<td>Strong-positive sample</td>
<td>3.79</td>
</tr>
<tr>
<td>(0.25μg/ml VP7)</td>
<td></td>
</tr>
<tr>
<td>Weak-positive sample</td>
<td>4.55</td>
</tr>
<tr>
<td>(0.01μg/ml VP7)</td>
<td></td>
</tr>
<tr>
<td>Negative sample</td>
<td>3.45</td>
</tr>
<tr>
<td>(distilled water)</td>
<td></td>
</tr>
</tbody>
</table>

*CV: Coefficient of variation; S/N: sample to negative ratio.

that the coincidence rate of the antigen-capture ELISA and INGEZIM BTV DAS was 100% in detecting BTV-5, 14, 16, 21, 22 (10<sup>1.57</sup>, 10<sup>1.81</sup>, 10<sup>2.67</sup>, 10<sup>2.93</sup>, 10<sup>3.50</sup> TCID<sub>50</sub>/ml) and these blood samples (Table 4).

**DISCUSSION**

Monitoring and controlling of BTV infection in cattle, sheep and goats remains a top priority in BTV-endemic and epidemic countries interested in exporting livestock free of this disease or in restricting the introduction of new serotypes into existing endemic populations. The recent outbreaks of BT in Europe, 2006 to 2010, emphasize the need for surveillance and monitoring of BT, and the clinical and laboratory diagnosis of BT.

Serological assays provide evidence of earlier animal exposure to BTV. VP7 has a highly conserved sequence, displays antigenicity across all serotypes, and is the major group-specific antigen (Mertens et al., 2005). Not surprisingly, VP7 is frequently used in immunoassays designed to detect BTV (Nagesha et al., 2001; Reddington et al., 1991). Many countries use ELISAs that use Abs raised against BTV to detect the virus, even though ELISAs that utilize MAbs that specifically recognize VP7 have greater specificity (Afshar et al., 1992; Reddington et al., 1991).

A recent study demonstrated that the sensitivity of the polyclonal antibody-based sandwich ELISA was estimated to be between 10<sup>2.4</sup> and 10<sup>2.6</sup> TCID<sub>50</sub>/ml with different serotypes of BTV (Chank et al., 2009). In the present study, the analytical detection limit of the antigen-capture ELISA for VP7 protein and BTV-5 was 9 ng/ml VP7 and 10<sup>0.5</sup> TCID<sub>50</sub>/ml BTV-5, respectively. The sensitivity of the assay was compared with the real-time RT-PCR...
(Yin et al., 2010). As expected, real-time RT-PCR (10⁻¹ TCID₅₀/ml BTV-1) was found to be at least 10 times more sensitive than the antigen-capture ELISA for detection of BTV in cell culture.

The analytical specificity of the assay has been studied with EHDV, since the virus is closely related to BTV. The results indicate that the 22 serotypes of BTV reference strains in our laboratory tested were detected specifically, with no cross-reactivity against the closely related orbivirus EHDV.

The antigen-capture ELISA developed in this study is based on MAbs specific to BTV VP7, which further improves the specificity of the assay of antibody-based ELISA. The assay is a simple and rapid test for high-throughput detecting BTV in cell culture and blood samples, and will be helpful at least for national purpose at least in China.

ACKNOWLEDGEMENTS

We are grateful to China Animal Health and Epidemiology Center for providing the viruses and the whole blood samples of sheep. This work was partially supported by the National Hi-Tech Research and Development Program (2006AA10Z446) and the National Supporting Program of Science and Technology (2008BAI54B06-21) of China.

REFERENCES


**Table 4. Detection of BTV strains cultured in BHK-21 cell and sheep blood samples with the antigen-capture ELISA and INGEZIM BTV DAS.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>BTV serotype</th>
<th>Sheep blood sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>The antigen-capture ELISA</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>INGEZIM BTV DAS</td>
<td>P</td>
<td>P</td>
</tr>
</tbody>
</table>

* INGEZIM BTV DAS: double antibody sandwich ELISA for detection of BTV VP7 protein purchased from INGENASA (Spain); \(^{a}\) P: positive; \(^{b}\) N: negative.
In this paper, adopting orthogonal design method, the *Morchella esculenta* mycelia from Qinghai-Tibetan plateau was preliminary studied in different growing conditions such as pH, temperature, carbon sources, nitrogen sources and growth factors. The results showed that the *M. esculenta* mycelium liquid fermentation pH was between 6.0 to 7.0, cultivating optimum temperature 24°C, training required for 1.2% of potassium nitrate nitrogen (KNO₃), carbon source was 4.5% soluble starch, growth factor biotin was 0.1%. At the same time, we also took the plateau *M. esculenta* polysaccharide fermentation liquid extraction, the extraction time was optimal combination 2 h, precipitated with ethanol concentration of 90% extraction ratio 1:20, the highest yield of polysaccharides.

**Key words:** Qinghai-Tibetan Plateau, *Morchella esculenta*, sporocarp, mycelium, static culture.

**INTRODUCTION**

Polysaccharide is the material basis of life, it has a comparable information function of nucleic acid or protein, involved in cell-cell recognition, regulation of immune function and intercellular substance during transport, cell transformation, apoptosis, and most non-toxic. The research found that polysaccharides had anti-tumor and immunomodulatory (Zhang et al., 2007), anti-bacterial, anti-viral (Li et al., 2003), antioxidant and anti-aging, and many other pharmacological activities, it had become the ideal drug and natural sources of antioxidants. Polysaccharides exist in the mycelium, sporocarp and fermentation broth, which consists of more than 10 monosaccharide glycoside linkage to a polymer made of polymers with complex biological activity and function. Among them, the most important one is immunomodulatory activity. Recently, fungal polysaccharides have been widely used into immunodeficiency diseases, autoimmune diseases, cancer and other diseases and clinical treatment and medical field for other purposes such as preparation of pharmaceutical materials, drug delivery agents, blood plasma substitutes. In recent years, the study of fungal polysaccharide and its compound has attracted more and more attention. So it has become one of the hot research areas such as molecular biology, medicine, food science, etc.

*Morchella esculenta* is famous for edible fungus with rich...
nutritious and delicious taste, which is welcomed by the international market. The content of amino acid lies on the first of all kinds of edible fungi. *M. esculenta* is internationally recognized as valuable medicinal fungus, its medicinal value of Chinese "broad spectrum of bacteria" and "compendium of materia medica" and other classics have been reported. Traditional medicine thought *M. esculenta* was natural, sweet and benefited the stomach and phlegm and lung airflow at ease. Modern medical research showed that *M. esculenta* polysaccharide is effective medicinal ingredients; it has enhanced immunity, anti-fatigue, anti-viral, tumor suppression and many other effects (Ren and Zhang, 1999).

In this article, on the basis of single factor experiment, static culture conditions were optimized and extraction technology of polysaccharide was selected adopting the orthogonal experiment. This research will provide the theoretical foundation for the antioxidant of natural polysaccharide development.

### MATERIALS AND METHODS

Static culture conditions optimized of *M. esculenta* mycelium.

#### Test materials

**Tested strains**: the optimal use of screening strain Y2 (North mountain forestry farm, Huzhu, Qinghai, gathered on May 3, 2005, by isolating stalk of *Morchella vulgaris* to obtain the pure strain).

**Culture medium**

Mother Culture Media: PDA integrated medium (potato 200 g, glucose 20 g, KH₂PO₄ 1 g, MgSO₄·7H₂O 1 g, VB₃ 50 μg, agar 15 g, chloramphenicol 0.5 g), add distilled water to 1000 ml, pH 6.5, 121°C, sterilize 20 min.

**Liquid seed medium**: PDA medium without agar.

**Test method**

Let the above medium become into plate medium, then take a different number of punch block *M. esculenta* mycelium, and take them to plant in the fresh medium (five repeats of each group). Finally place them in incubator.

<table>
<thead>
<tr>
<th>Level</th>
<th>Factor</th>
<th>A (%)</th>
<th>B (%)</th>
<th>C (%)</th>
<th>D (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3 KNO₃</td>
<td>0.25 Biotin</td>
<td>10</td>
<td>2.5 C₆H₁₂O₆</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5 Peptone</td>
<td>0.5 VB₃</td>
<td>20</td>
<td>3 Corn flour</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.3 Soy flour</td>
<td>0.75 IAA</td>
<td>30</td>
<td>3.5 Soluble starch</td>
<td></td>
</tr>
</tbody>
</table>

Note: The content of other components is respectively 0.5% KH₂PO₄, 0.1% MgSO₄ and 100mL H₂O.

**Table 1. The level table of L₉ (3⁴) on nutritional conditions**

**Determination on dry weight of colonies**

Training 120 h, place the colonies and medium from the plates in a beaker with distilled water, each plate processing corresponds to a beaker, then the beaker is heated in the dry box. When the medium melted, mycelium will singled out with a sterile glass rod and the excess water was absorbed by the filter paper. Then place them in a weighing bottle and dry at 60°C until constant weight. At the end, they will be weighed (Wang et al., 2010). Based on the preliminary experiments of single factor, the orthogonal experiment was made by selecting the more important factor, including nitrogen (A), the type and concentration of growth factors (B), inoculum (C), carbon (D), pH (E), incubation time (F), incubation temperature (G), ventilation (H), impact on dry weight of *M. esculenta* mycelium. As there were more experimental factors and there are in order, the orthogonal experiment carried out twice (Wang, 2006). In the vicinity of the optimum value of each factor, three levels were taken and four factors and three levels orthogonal experiments were done with indicators for dry weight of mycelium. According to the L₉(3⁴) cross-table, the technology of polysaccharide extraction was optimized (Tables 1 and 2).

**Polysaccharide extraction of *M. esculenta* fermentation liquor**

**Tested strains**

The optimal screening strain Y2.

**Media and culture conditions**

Make media by applying the above, the best media and culture conditions obtained by the experiments.

**Determination of polysaccharide content**

*M. esculenta* fermentation broth was filtered after 21 days of culture. Then the filtrate was concentrated in a water bath at 90°C to the original volume of 1/3, slowly add 3 volumes of 80% ethanol solution, stand and overnight. The next day, the precipitation is extracted by centrifugation, dissolve in a small amount of hot water and wash three times with ethanol, centrifuge for 15 min with 3000 rpm. The precipitate was dried naturally in order to obtain polysaccharide fermentation broth of *M. esculenta*.

**Extraction technology of polysaccharide of *M. esculenta* sporocarp**

**Tested materials**

*M. esculenta* sporocarp: collected from North mountain forestry farm of Huzhu in Qinghai Province.
Test Method

Best extraction selection: select three main factors impact on polysaccharide extraction of *M. esculenta* sporocarp including extraction ratio, extraction time and ethanol concentration. The orthogonal experiment was made by using L$_2$(3$^4$) and regarding the ratio of *M. esculenta* polysaccharide as investigated indicators (Table 3).

Extraction method: dry *M. esculenta* sporocarp in 60°C and grind them for used. Take 9 parts of *M. esculenta* sporocarp with the weight of 10 g, then place them into 1000 ml beaker, which was added 20 times with distilled water (200 ml), 30 times (300 ml), 40 times (400 ml), and finally take them into thermostatic water bath with the water temperature controlled at about 95°C. They were extracted with water volume and extraction time under orthogonal test. Centrifuge for 10 min with 4000 r/min speed, take the supernatant liquid centrifugal, derived from sediments into the Petri dish, dried at 60°C in the roasting oven, and get morel fruting polysaccharides (Meng et al., 2013).

RESULTS AND ANALYSIS

Static culture conditions optimized of *M. esculenta* mycelium

Through the intuitive analysis of data (Tables 4 and 6), the static culture conditions of *M. esculenta* mycelium was selected out by orthogonal test. Namely, when use water as solvent and take dry weight as evaluation indicators, the best mix programme was A$_1$B$_2$C$_2$D$_3$ and E$_2$F$_3$G$_2$H$_2$. What is more, 0.3% KNO$_3$, 0.5% VB$_2$, 10% vaccination volume, 3.5% soluble starch as carbon source, pH 6.5, 120 min training time, the most suitable temperature for 24°C and ventilation volume for 250 ml. Analysis of variance table (Tables 5 and 7) showed that the impact extent order of various factors on dry weight of mycelium is: carbon source > growth factor > nitrogen > inoculation, incubation temperature > pH > incubation time > ventilation. The carbon source, growth factors, nitrogen, temperature, pH and incubation time have significantly effect on the dry weight of *M. esculenta*.

Study on extraction technology of polysaccharide from *M. esculenta* sporocarp

The results were shown in Table 8. When the ratio of extraction was 1:30, extraction time was 2 h and concentration of ethanol was 90%, the production of polysaccharides yield was the highest and reached for 13.1%, which was basically consistent with the result reported by Wu and An (Wu and An, 2005), but it was lower than Li and Qiu (Li and Qiu, 2005) who reported. Thus, we thought that it was a certain relationship with the extraction method of polysaccharide fermentation liquid. Li and Qiu (Li and Qiu, 2005) used a method of enzyme extraction, so extraction yield of polysaccharide could be increased significantly through this method. They determined the optimum conditions of pectinase action were 15% enzyme dosage, 50°C and 3 h and the optimal conditions of cellulase enzyme were 15% enzyme dosage, 45°C and 3 h. But it could be seen from the tables, the polysaccharides yield is little difference when extraction ratio were 1:20, 1:30 and 1:40. If considering the practicality of technology, the choice of 1:20 was the best extraction ratio. Extraction time had the greater impact on the extraction of polysaccharide. When extraction time was 2 h, the average yield of polysaccharide was the highest, so 2 h should be used for the best extraction time. At the same
M. esculenta sporocarp growing on the ecological environment requirements was extremely strict and has obvious season. Because mycelium and sporocarp of M. esculenta were different, they were not the same with demand for nutrients, temperature, pH, moisture, light, oxygen and so on. In different nutritional conditions, the shape, color and other cultural characteristics of mycelium, which were growing in the same strain of M. esculenta, are not identical. These differences reflected the diversity of physiological characteristics about M. esculenta. This diversity caused the volatility of domesticated conditions and increased the difficulty of domestication. At present, the artificial cultivation could still not achieve commercialized cultivation and wild resources of M. esculenta was very limited, but the demand for M. esculenta became more and more, so we must find another way in order to do the sustainable development and utilization of M. esculenta resources. Through the experiment, we found that the nutrients contained in mycelium produced by liquid fermentation were basically consistent with those from sporocarp, so mycelium and mycelium polysaccharide should be obtained by the massively artificial liquid fermentation and those products were developed and utilized. Because Qinghai province had the unique geographical environment and location, and M. esculenta resources were rich and various, so the wild resources of M. esculenta were realized sustainable development. On
Table 6. The orthogonal test \(L_9(3^4)\) design scheme and results of *Morchella esculenta* culture conditions.

<table>
<thead>
<tr>
<th>Number</th>
<th>Factor</th>
<th>Dry weight of mycelium (mg/100ml)</th>
<th>Sum</th>
<th>Average value mg/100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E (h)</td>
<td>F (℃)</td>
<td>G (ml)</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1(6.0)</td>
<td>1(20)</td>
<td>1(150)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2(96)</td>
<td>2 (24)</td>
<td>2(250)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>3(120)</td>
<td>3 (28)</td>
<td>3(350)</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

**T1** 1546 1556.3 1621.7 1593.7
**T2** 1646.6 1602.2 1725 1607.1
**T3** 1610.7 1644.8 1516.6 1602.5
**X3** 179.0 182.8 168.5 178.0
**R** 11.2 9.8 16.5 1.5

Optimization parameters: \(E_2\) \(F_3\) \(G_2\) \(H_2\)

Table 7. Analysis of variance table of orthogonal experiment on *Morchella esculenta* culture conditions.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block</td>
<td>2317.43</td>
<td>8</td>
<td>289.68</td>
<td>12.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E</td>
<td>577.60</td>
<td>2</td>
<td>288.80</td>
<td>12.77</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>F</td>
<td>435.33</td>
<td>2</td>
<td>217.66</td>
<td>9.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G</td>
<td>1294.20</td>
<td>2</td>
<td>647.10</td>
<td>28.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>H</td>
<td>10.30</td>
<td>2</td>
<td>5.15</td>
<td>0.23</td>
<td>0.7985</td>
</tr>
<tr>
<td>Experimental error</td>
<td>406.97</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total variation</td>
<td>2724.40</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 8. The experimental results and intuitive analysis on polysaccharide extraction of *Morchella esculenta* sporocarp.

<table>
<thead>
<tr>
<th>Number</th>
<th>Extraction ratio A</th>
<th>Extraction time (h) B</th>
<th>Concentration of ethanol precipitation (%) C</th>
<th>Polysaccharide production (g)</th>
<th>Polysaccharide yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.21</td>
<td>12.1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1.20</td>
<td>12.0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1.24</td>
<td>12.5</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1.32</td>
<td>13.1</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>0.70</td>
<td>7.0</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1.10</td>
<td>11.1</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>1.20</td>
<td>12.0</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1.05</td>
<td>10.5</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1.09</td>
<td>12.9</td>
</tr>
<tr>
<td>K_1</td>
<td>1.217</td>
<td>1.243</td>
<td>1.120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K_2</td>
<td>1.040</td>
<td>0.983</td>
<td>1.203</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K_3</td>
<td>1.113</td>
<td>1.143</td>
<td>1.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.177</td>
<td>0.260</td>
<td>0.156</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Optimization parameters: \(A_1\) \(B_1\) \(C_2\)
Table 9. Analysis of variance table of orthogonal experiment on polysaccharide extraction.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block</td>
<td>0.19</td>
<td>6</td>
<td>0.03</td>
<td>0.89</td>
<td>0.62</td>
</tr>
<tr>
<td>A</td>
<td>0.05</td>
<td>2</td>
<td>0.02</td>
<td>0.67</td>
<td>0.60</td>
</tr>
<tr>
<td>B</td>
<td>0.10</td>
<td>2</td>
<td>0.05</td>
<td>1.46</td>
<td>0.41</td>
</tr>
<tr>
<td>C</td>
<td>0.04</td>
<td>2</td>
<td>0.02</td>
<td>0.52</td>
<td>0.66</td>
</tr>
<tr>
<td>The experimental error</td>
<td>0.07</td>
<td>2</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total variance</td>
<td>0.26</td>
<td>8</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

On the one hand, liquid fermentation of *M. esculenta* were studied and related products were developed in order to research and develop mycelium polysaccharides and related products; On the other hand, the relevant departments should formulate corresponding policies to manage and control the collection, purchase, sales, and actively establish production and experimental base for the implementation of artificial and artificial cultivation so as to achieve the purpose of effective protection and rational utilization of *M. esculenta* resources and realize sustainable development.

ACKNOWLEDGEMENTS

We gratefully acknowledge the comments and suggestions made by the reviewers and the associate editor. This research was supported by the Scientific Research Fund of Ministry of Education "Chunhui Plan" (Z20100078), State Key Laboratory Breeding Base-Key Laboratory of Qinghai Province for Plateau Crop Germplasm Innovation and Utilization (2013-01), Natural Science Fund of Qinghai Province (2011-Z-745) and the National Science Foundation of China (31260052).

REFERENCES


Molecular characterization of *Trichoderma longibrachiatum* 21PP isolated from rhizospheric soil based on universal ITS primers

Mohammad Shahid¹*, Mukesh Srivastava¹, Antima Sharma¹, Anuradha Singh¹, Sonika Pandey¹, Vipul Kumar¹, Neelam Pathak² and Smita Rastogi²

¹Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur-208002, Uttar Pradesh, India.
²Department of Biosciences, Integral University, Lucknow 226026, Uttar Pradesh, India.

Accepted 20 September, 2013

*Corresponding author. Email: shahid.biotech@rediffmail.com.

**African Journal of Microbiology Research**

Full Length Research Paper

Molecular characterization of *Trichoderma longibrachiatum* 21PP isolated from rhizospheric soil based on universal ITS primers

Mohammad Shahid¹*, Mukesh Srivastava¹, Antima Sharma¹, Anuradha Singh¹, Sonika Pandey¹, Vipul Kumar¹, Neelam Pathak² and Smita Rastogi²

¹Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur-208002, Uttar Pradesh, India.
²Department of Biosciences, Integral University, Lucknow 226026, Uttar Pradesh, India.

Accepted 20 September, 2013

*Corresponding author. Email: shahid.biotech@rediffmail.com.

**African Journal of Microbiology Research**

Full Length Research Paper

Molecular characterization of *Trichoderma longibrachiatum* 21PP isolated from rhizospheric soil based on universal ITS primers

Mohammad Shahid¹*, Mukesh Srivastava¹, Antima Sharma¹, Anuradha Singh¹, Sonika Pandey¹, Vipul Kumar¹, Neelam Pathak² and Smita Rastogi²

¹Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur-208002, Uttar Pradesh, India.
²Department of Biosciences, Integral University, Lucknow 226026, Uttar Pradesh, India.

Accepted 20 September, 2013

*Corresponding author. Email: shahid.biotech@rediffmail.com.

**African Journal of Microbiology Research**

Full Length Research Paper

Molecular characterization of *Trichoderma longibrachiatum* 21PP isolated from rhizospheric soil based on universal ITS primers

Mohammad Shahid¹*, Mukesh Srivastava¹, Antima Sharma¹, Anuradha Singh¹, Sonika Pandey¹, Vipul Kumar¹, Neelam Pathak² and Smita Rastogi²

¹Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur-208002, Uttar Pradesh, India.
²Department of Biosciences, Integral University, Lucknow 226026, Uttar Pradesh, India.

Accepted 20 September, 2013

*Corresponding author. Email: shahid.biotech@rediffmail.com.

**African Journal of Microbiology Research**

Full Length Research Paper

Molecular characterization of *Trichoderma longibrachiatum* 21PP isolated from rhizospheric soil based on universal ITS primers

Mohammad Shahid¹*, Mukesh Srivastava¹, Antima Sharma¹, Anuradha Singh¹, Sonika Pandey¹, Vipul Kumar¹, Neelam Pathak² and Smita Rastogi²

¹Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur-208002, Uttar Pradesh, India.
²Department of Biosciences, Integral University, Lucknow 226026, Uttar Pradesh, India.

Accepted 20 September, 2013

*Corresponding author. Email: shahid.biotech@rediffmail.com.

**African Journal of Microbiology Research**

Full Length Research Paper

Molecular characterization of *Trichoderma longibrachiatum* 21PP isolated from rhizospheric soil based on universal ITS primers

Mohammad Shahid¹*, Mukesh Srivastava¹, Antima Sharma¹, Anuradha Singh¹, Sonika Pandey¹, Vipul Kumar¹, Neelam Pathak² and Smita Rastogi²

¹Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur-208002, Uttar Pradesh, India.
²Department of Biosciences, Integral University, Lucknow 226026, Uttar Pradesh, India.

Accepted 20 September, 2013

*Corresponding author. Email: shahid.biotech@rediffmail.com.
MATERIALS AND METHODS

Isolation of Trichoderma from soil sample

Rhizospheric soil samples were collected from various experimental fields of an Indian district Kaushambi (25° 31' 50.68" N, 81° 22' 38.25" E). The strain was isolated and identified in Potato Dextrose Agar (PDA) with low sugar medium by serial dilution method (Johnson and Crul, 1972; Nirenberg, 1976).

DNA isolation of Trichoderma

Pure culture of the target fungal was grown overnight in liquid PDB for the isolation of genomic DNA using a method described by Hiney et al. (1992).

Identification of Trichoderma

The isolated strain was then identified at molecular level using PCR amplification of the specific gene sequence with universal ITS primers ITS-1 (forward) and ITS-4 (reverse) primers. It is then deposited to the Indian Type Culture Collection (ITCC), IARI (New Delhi, India).

Molecular characterization

The total genomic DNA was extracted from isolate of Trichoderma based on Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) mini extraction method as was described by Crowhurst et al. (1995) in their study.

Agarose gel electrophoresis

Ten microlitre of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0% agarose with ethidium bromide at 8 V/cm and the reaction product was visualized under a UV transilluminator.

Amplification of isolated DNA using ITS

The internal transcribed spacer (ITS) regions of the rDNA repeat from the 3' end of the 28S and the 5' end of the 28S gene were amplified using the two primers, ITS-1 and ITS-4 which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene (White et al., 1990). The PCR-amplification reactions were performed in a 50 µl mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.0 mM MgCl2, 200 µM of each of the four deoxynucleotide triphosphates (dNTPs), 0.2 µM of each primer, 40 ng/µl of template and 2.5 U of Taq polymerase (Fermentas). The cycle parameters included an initial denaturation for 5 min at 94°C, followed by 40 cycles of denaturation for 1 min at 94°C, primer annealing for 2 min at 55°C, primer extension for 3 min at 72°C, and a final extension for 10 min at 72°C. Amplified products were separated on 1.2% agarose gel in TAE buffer, pre-stained with ethidium bromide (1 µg/ml) and electrophoresis was carried out at 60 V for 3 h in TAE buffer. One Kb ladder (MBI, Fementas) was used as a marker. The gel was observed in a transilluminator over ultraviolet light. The desired bands were cut from the gel with minimum quantity of gel portion using QIAGEN gel extraction kit.

Purification of PCR product

The PCR product was purified by QIAGEN gel extraction kit using the protocol described in the manufacturer’s manual.

DNA sequencing of the 28S rDNA fragment

A pair of universal ITS primers ITS-1 (forward) and ITS-4 (reverse) was used for sequencing of the amplified product and this step was carried out at the Merck Laboratory (Bangalore, India). The details of the primers are mentioned in Table 1.

Sequence analysis and phylogeny

A comparison of 28S rRNA gene sequence of the test strain against nucleotide collection (nr/nt) database was done using BLAST program (Zhang et al., 2000). Sequences that shared about 90% similarity with the test sequence were selected for a multiple sequence alignment that was carried out using ClustalW (Thompson et al., 1994). Subsequently, an evolutionary distance matrix was generated from these nucleotide sequences in the dataset. A phylogenetic tree was then constructed using the Neighbor Joining method (Saitou and Nei, 1987). Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 (Tamura et al., 2007). The 16S rRNA gene sequence of test strain was compared with a different set of sequence database such as small subunit ribosomal RNA (SSU rRNA) and large subunit ribosomal RNA (LSU rRNA)

Table 1. Gene sequence with universal primers.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
<th>Length</th>
<th>Tm (°C)</th>
<th>GC content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS-1</td>
<td>TCTGATTGTAAGCTGCGT</td>
<td>19</td>
<td>53</td>
<td>58</td>
</tr>
<tr>
<td>ITS-4</td>
<td>TCCTCCTATTGATATGC</td>
<td>20</td>
<td>50</td>
<td>45</td>
</tr>
</tbody>
</table>
using Ribosomal RNA BLAST (Altschul et al., 1997). 28S rRNA gene sequence of the test strain was also compared against those sequences, in Ribosomal Database Project (Cole et al., 2009) by using the RDP Classifier check program (Wang et al., 2007). The annotated information for the sequence in the database to which 28S rRNA aligns is used for fungal identification.

RESULTS

Rapid identification of microorganisms is necessary in the pathological laboratory in an attempt to proceed for the preparation of a bioformulation that can be useful for the farmers. The rRNA based analysis is a central method in pathology used not only to explore microbial diversity but also to identify new strains. The genomic DNA was extracted from isolated fungal strain T. longibrachiatum 21PP and universal ITS-1 (19F) and ITS-4 (20R) primers were used for the amplification and sequencing of the 28S rRNA gene fragment. A total of 664 bp of the 28S rRNA gene was sequenced (Figure 1).

The sequenced gene fragment of the specific strain was then searched for other closely related and similar templates using BLAST program. Sequences that shared 90% similarity to the test sequence were selected for a multiple sequence alignment. Figure 2 shows the phylogenetic relationship of the test strain (T. longibrachiatum 21PP) with others strains of Trichoderma. Comparison of test strain against known sequences of SSU and LSU rRNA databases showed that the gene sequence of the isolate 21PP has 90% sequence similarity (Score=664 bits, Expect=0.0) with 28S rRNA gene sequence of Trichoderma (GenBank Acc. No.: JX978542). Thus, it can be concluded from the above data that the isolate 21PP is a member of the genus T. longibrachiatum.

Conclusion

The study is focused on the identification of bioagent T. longibrachiatum 21PP at species level. Biocontrol activity of fungal strains in laboratory is generally determined by biological assays. To check the presence or absence of these biocontrol agents is a need to develop some efficient method. In past, serial dilution method had some disadvantages as it cannot distinguish between indigenous and artificially introduced strains that is why the first step of utilizing the full potential of fungal strains is identified at molecular level.

ACKNOWLEDGEMENT

The authors are grateful for the financial support granted.
Figure 2. Shows the phylogenetic relationship of the test strain with other strains of *Trichoderma*.

by the ICAR under the Niche Area of Excellence on “Exploration and Exploitation of *Trichoderma* as an antagonist against soil borne pathogens” running in the Biocontrol Laboratory, Department of Plant Pathology, C.S.A University of Agriculture and Technology, Kanpur, India.

REFERENCES


Wang Q, Garrity GM, Tiedje JM, Cole JR (2007). Naive Bayesian...
Classifier for Rapid Assignment of rRNA Sequences into the New
sequencing of fungal ribosomal DNA for phylogenetics. In: Innes MA,
Gelfand DH, Sninsky JJ, White TJ (Eds.), PCR protocols: a guide to
315-322.
DNA polymorphisms amplified by arbitrary primers are useful as

domain: the primary kingdoms. Proc. Natl. Acad. Sci. USA, 74: 5088-
5090.
Yadav V, Prakash S, Srivastava S, Verrna PC, Gupta V, Basu V,
Rawat, AK (2009). Identification of Comamonas species using 16S
rRNA gene sequence. Bioinformation 3(9): 381-383.
Correlation between architect hepatitis C virus (HCV) core antigen and HCV Ribonucleic acid levels in Anti-HCV reactive patients in Turkey

BURUK Kurtulus¹, BAYRAMOGLU Gulcin¹, KAKLIKKAYA Nese¹*, AKYUZ Zeynep¹, KAYA Selcuk², KOKSAL Ifthihar², CAN Gamze³ and AYDIN Faruk¹

¹Karadeniz Technical University Medical Faculty, Department of Medical Microbiology, Trabzon, Turkey.
²Karadeniz Technical University Medical Faculty, Department of Infectious Diseases, Trabzon, Turkey.
³Karadeniz Technical University Medical Faculty, Department of Public Health, Trabzon, Turkey.

Accepted 20 September, 2013

Quantitative tests for the detection of hepatitis C virus ribonucleic acid (HCV-RNA) levels and HCV core antigen have been mainly used for the management of patients with HCV infection. The aim of this study was to evaluate the correlation between HCV core antigen and HCV RNA levels in patients reactive for anti-HCV antibodies. Three hundred and twenty-five anti-HCV reactive sera samples were included in the study. HCV core antigen and HCV RNA levels were determined using the Architect HCV Ag test and Abbott RealTime™ HCV RNA test (RT-PCR), respectively. The correlation coefficient between the levels of HCV core antigen and HCV RNA test results was calculated using Spearman’s rank test, and linear regression analysis was applied. One hundred and sixteen of the 325 samples were detected positive by both methods. Three additional samples by RT-PCR, and 4 samples by Architect HCV Ag, the negative samples were found positive by the other method. All of these contradictory results were obtained from the low level HCV RNA or HCV core antigen including samples. A correlation coefficient (r) was determined as 0.899 between the levels of HCV core antigen and HCV RNA (p<0.0001). The sensitivity, specificity, positive predictive value and negative predictive value of the HCV core antigen test were 97.48, 98.06, 96.67 and 98.54%, respectively, using the HCV RNA test as a reference. The Architect HCV core antigen test exhibits a good correlation with the HCV RNA test. It can be used as an alternative method, especially when the HCV RNA test is unavailable.

Key words: Anti-hepatitis C virus, Architect hepatitis C virus Ag test, hepatitis C virus RNA.

INTRODUCTION

The most frequently used screening method in the diagnosis of hepatitis C virus (HCV) infections is the detection of anti-HCV antibodies in serum or plasma. Nevertheless, anti-HCV antibodies reached detectable levels in the serum after a long window period of HCV infection. Additionally, even if the virus is eliminated from the blood its presence may persist for many years, for which reason this method cannot be used for the differentiation of active or past infection or for monitoring antiviral therapy. At the same time, tests used for determining anti-HCV antibodies can give false positive results for various reasons (Chevaliez, 2011; Richter 2002; Alter et al., 2003).
The recombinant immunoblot test is used in order to determine true positive results. It has disadvantages, such as giving a large number of indeterminate results and being a time-consuming procedure (Makuria et al., 2012). For these reasons, HCV RNA tests are the most commonly used technique in the confirmation of HCV infections and in the monitoring of antiviral therapy. Tests used for the detection of HCV RNA have high sensitivity and specificity, and low detection limits such as 15 to 30 IU/ml and can give quantitative results. However, these tests also require special technical and laboratory facilities, carry a risk of contamination and also impose high costs on users (Chevaliez, 2011; Richter 2002; Alter et al., 2003).

A large number of immunologically-based tests have been developed over the last 20 years for the detection of HCV core antigens in plasma or serum as potential alternatives to HCV RNA tests. In first-generation HCV core antigen tests, there is no preliminary procedure intended to differentiate between antigen and antibody. Therefore, HCV core antigens can only be detected in the period up to the emergence of anti-HCV antibodies using first-generation tests (Takahashi et al., 1992; Aoyagi et al., 1999; Tanaka et al., 1995; Kashiwakuma et al., 1996; Icardi et al. 2003). With second-generation tests, preliminary procedures aimed at distinguishing HCV core antibodies and anti-HCV antibody complexes are performed beforehand. HCV core antigens can thus be detected during seroconversion. Preliminary procedures are performed manually in these tests carried out on microplates, and the lowest detectable limit is about 1.5 pg/ml (Tobler et al., 2005; Tanaka et al., 2006; Fabrizi et al., 2005).

A fully automated system has recently been developed by Abbott Diagnostics (Abbott Park, IL, USA) for the detection of HCV core antigen in serum or plasma including preliminary procedures for differentiation of antigen-antibody complexes. This test also provides quantitative results. The lowest detectable limit in the test is 3 fmol/l (0.06 pg/ml). This value is approximately 25 times lower than that for previous tests (Mederake et al., 2009).

The purpose of this study was to compare the results of the architect HCV core antigen test, which has newly been introduced in Turkey with the HCV RNA results obtained from the Abbott RealTime™ HCV RNA test (Abbott Molecular Inc., Des Plaines, IL, USA) and to determine the correlation between the two.

### MATERIALS AND METHODS

In this study, HCV RNA test and HCV core antigen test were consecutively and simultaneously administered on three hundred and twenty-five sera samples between June 2010 and July 2012. All the samples were anti-HCV reactive.

Anti-HCV levels in the serum samples reaching our laboratory were determined using the chemiluminescent micro particle immunoassay (CMIA) technique with Architect Anti-HCV kits (Abbott Diagnostics, Wiesbaden, Germany) on an Architect i2000SR (Abbott Diagnostics, Abbott Park, IL, USA) device. Sample/cutoff values (S/CO) ≥1 were regarded as reactive.

HCV core antigen levels in serum were investigated using the Architect HCV Ag test (Abbott Diagnostics, Wiesbaden, Germany) with CMIA technology following the manufacturer’s recommendations. Since the HCV core antigen test and Anti-HCV antibody test were investigated on the same device, it was subjected to daily maintenance using a 0.5% sodium chloride solution in order to prevent cross-contamination. The lowest level detectable by the test, 3 fmol/l (0.06 pg/ml), was adopted as the cut-off value.

HCV RNA isolation was performed using the Sample Preparation System (Promega Corporation Madison, WI, USA) on an Abbott m2000sp platform (Abbott Molecular Inc., Des Plaines, IL, USA). HCV RNA levels were determined using the Abbott m2000rt Instrument System (Abbott Molecular Inc., Des Plaines, IL, USA) and Abbott RealTime™ HCV test (Abbott Molecular Inc., Des Plaines, IL, USA). This test, based on the quantitative determination of the HCV viral load in plasma using the real time polymerase chain reaction method (RT-PCR) after reverse transcription, was performed with a 0.2 ml plasma sample, in line with the manufacturer’s instructions. The test’s lowest detection limit, 30 IU/ml, and values above that were regarded as positive.

Statistical analysis was performed using SPSS 13.0 (Series no: 9069728). Descriptive data were expressed as number and percentage. The Spearman rank test and linear regression analysis were used to determine the correlation between HCV RNA and HCV core antigen levels.

### RESULTS

One hundred and sixteen of the 325 samples were detected positive by the two tests. HCV RNA levels were greater than 30 IU/ml in 119 of the 325 samples (35.69%). HCV core antigen levels were greater than 3 fmol/l in 120 samples (36.92%) (Table 1). When the HCV RNA test was regarded as a reference test, HCV core antigen test sensitivity, specificity, PPV and NPV rates were calculated as described by Akobeng AK (2007), and found as 97.48, 98.06, 96.67 and 98.54%, respectively. The Spearman’s correlation coefficient (r) was determined as 0.899 (p<0.0001) at comparison of HCV RNA and HCV core antibodies (Figure 1). HCV core antigen was deter-

---

**Table 1. Comparison of HCV RNA and HCV core antigen tests’ results.**

<table>
<thead>
<tr>
<th>HCV Core Ag</th>
<th>Positive (%)</th>
<th>Negative (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>116 (35.69)</td>
<td>4 (1.23)</td>
<td>120 (36.92)</td>
</tr>
<tr>
<td>Negative</td>
<td>3 (0.92)</td>
<td>202 (62.16)</td>
<td>205 (63.08)</td>
</tr>
<tr>
<td>Total</td>
<td>119 (36.61)</td>
<td>206 (63.39)</td>
<td>325 (100)</td>
</tr>
</tbody>
</table>
mined as negative in four HCV RNA positive samples, and positive in three HCV RNA negative tests. Detailed results from the seven samples in which the HCV RNA and HCV core antigen tests were inconsistent are shown in Table 2.

**DISCUSSION**

This study compared HCV RNA test and Architect HCV core antigen test results in specimens with anti-HCV antibodies identified as reactive. The ability of Architect HCV core antigen test's to provide quantitative results is being fully automated, and having a low detection limit of 3 fmol/l represent superior features compared to previous HCV core antigen tests. The device's total working time, that is, maintenance once daily has been performed and is approximately 40 min; some five times shorter than that of previously manufactured tests (around 3.5 h) (Mederake et al., 2009; Ergunay K et al., 2011; Morota et al., 2009; Ross et al., 2010; Kesli et al., 2011; Park et al., 2010; Medici MC et al., 2011).

Positivity rates of HCV RNA, and HCV core antigen tests were found to be quite similar, 36.61 and 36.92%, respectively. When the HCV RNA test was taken as refe-
rence, the HCV core antigen test exhibited rather high sensitivity, specificity, PPV and NPV (97.48, 98.06, 96.67 and 98.54%, respectively). These values are slightly higher than those obtained in previously manufactured tests. Fabrizi et al. (2005) used the second-generation Ortho Trak-C test (Ortho-Clinical Diagnostics, Raritan, N.J., USA) and determined sensitivity, specificity, PPV and NPV of 92.7, 97.4, 94.7 and 96.5%, respectively, in hemodialysis patients.

HCV RNA and HCV core antigen test results were inconsistent in seven specimens in our study. The HCV RNA test was lower than 30 IU/ml or negative in four of these specimens in those HCV core antigen levels were positive with amount lower than 10 fmol/l. The manufacturers recommend that if HCV core antigens are measured at 3 to 10 fmol/l, the test should be repeated two times, and that if positivity above 3 fmol/l is seen in at least one of these tests, the test should be interpreted as positive. Samples with a level of 3-10 fmol/l were not retested in our study for economic reasons. This may be regarded as a limitation of the study. The possibility of there being insufficient RNA for quantification in specimens identified as HCV core antigen positive and with HCV RNA ≤30 IU/ml must also be borne in mind.

HCV RNA was positive in three samples although the HCV core antigen test results of them were below 3 fmol/l. HCV RNA levels in these three samples were 10^3 IU/ml or less. We therefore think that care is needed when interpreting HCV core antigen results if HCV RNA levels are ≤10^3 IU/ml.

When the correlation between HCV RNA and HCV core antigen levels was analyzed, the correlation coefficient between the two tests was quite high (r=0.899). Kesli et al. (2011) determined HCV RNA levels using the Qiagen HCV RNA test (Qiagen, Hilden, Germany) and identified a correlation with the Architect HCV core antigen test as 0.864. Ergünay et al. (2011) determined HCV RNA levels with COBAS Ampliprep/COBAS Taqman HCV Real-time PCR (Roche Diagnostics, Germany) and calculated a correlation coefficient as 0.915. Mederacke et al. (2009) determined HCV RNA levels using Cobas Taqman or Amplicor HCV Monitor (Roche Diagnostics, Germany) and reported a correlation coefficient as 0.75. Medici et al. (2011) determined a correlation coefficient ranging from 0.713 to 0.870 for the HCV core antigen test and different HCV RNA kits. The values determined in our study and the results from other studies show that HCV RNA tests and the HCV core antigen test give quite comparable results.

In conclusion, the Architect HCV core antigen test and HCV RNA test produced highly compatible findings in our study. The HCV RNA test is still regarded as standard in the detection of active infection in individuals in whom anti-HCV antibodies are detected, in the confirmation of anti-HCV antibody tests and in the commencement and monitoring of treatment (Alter et al., 2003). However, we conclude that the HCV core antigen test can be used as an alternative to HCV RNA tests, particularly when the HCV RNA test is unavailable.

REFERENCES


Medici MC, Furlini G, Rodella A, Micheli MC, Furlini G, Rodella A, Ansaldi F (2009). Determination of HCV RNA levels using Cobas Taqman or Amplicor HCV Monitor (Roche Diagnostics, Germany) and reported a correlation coefficient as 0.75. Medici et al. (2011) determined a correlation coefficient ranging from 0.713 to 0.870 for the HCV core antigen test and different HCV RNA kits. The values determined in our study and the results from other studies show that HCV RNA tests and the HCV core antigen test give quite comparable results.

In conclusion, the Architect HCV core antigen test and HCV RNA test produced highly compatible findings in our study. The HCV RNA test is still regarded as standard in the detection of active infection in individuals in whom anti-HCV antibodies are detected, in the confirmation of anti-HCV antibody tests and in the commencement and monitoring of treatment (Alter et al., 2003). However, we conclude that the HCV core antigen test can be used as an alternative to HCV RNA tests, particularly when the HCV RNA test is unavailable.


Influence of general anaesthesias on the changes of bacterial flora in bronchial tree

Izabela Duda, Aleksandra Rudnik-Lipińska, Maria Damps, Ewa Musioł and Edyta Gogółka

Department of Anesthesiology and Intensive Care, Medical University of Silesia Katowice, Poland.

Accepted 20 September, 2013

The aim of the study was to assess the influence of general anesthesia on bacterial growth in bronchial tree, depending upon the applied method of anesthesia. The artificial airway is the additional gate for respiratory tract infections and mechanical ventilation which can have an influence on postoperative complications such as pneumonia and atelectasis. Patients undergoing 4h surgeries were sampled. Due to selection of anesthetics, volatile or intravenous, patients were divided into groups VGA (volatile general anesthesia) and TIVA (total intravenous anesthesia). Material collected with mini-bronchoalveolar lavage method directly after intubation and just before extubation. In 40% of all patients no bacteria growth was noted in both time points. In VGA group, from the bacteria cultured in 61.9% of patients in first sample, 62.5% of colonies diminished or eradicated, only 6.25% multiplied. In TIVA group 42.9% patients presented bacteria in first sample. All bacteria got reduced. Length of hospitalization preceding surgery (p=0.036) and number of smoked cigarettes (p=0.028) significantly correlated with colonization of bacteria. General anesthesia has no influence on the respiratory tract microorganism contamination and can even favour the eradication of the colonizing bacterial flora.

Key words: Respiratory tract, general anesthesia, contamination.

INTRODUCTION

Natural defensive mechanisms, such as mucociliary transport, lysozyme activity, lactoferrins, macrophages, natural killer (NK) cells, as well as, specific humoral and cell response play significant roles in prevention and control of respiratory system infections (Ficker, 2008). After general anaesthesia of long duration, due to incorrect ventilation and retention of secretion in bronchial tree, the risk of pulmonary infections and atelectasis increases (Sachdev and Napolitano, 2012). Endotracheal intubation can cause mucosal injury, loss of cilia and promotes metaplasia of respiratory epithelium. That artificial respiratory tract is an additional entry of infection. Bacteria reach the lower respiratory tract mainly via aspiration from the upper part of pharynx, as well as leakage of secretion containing bacteria around the sealing collar, which slows down the tracheal flow of mucus. Inhalation of colonized bacteria from oropharynx, sinus cavities, nares, dental plaque can be a reason of postoperative lungs infections (Brusselaers et al., 2013).

Mechanical ventilation disturbs the correct cough reflex, sneezing, and efficient mucociliary transport. The mucociliary clearance rate in bronchial tree is optimal in the temperature of 34 to 40°C. Greater sensitivity of lower respiratory tract to decreased temperature brings about the threat that substitutive ventilation with dry and cold gases may lead to impairment of ciliated epithelium functions. That may promote the development of microorganisms in respiratory tract environment. Anaesthetics, inhalants and intravenous drugs, also affect the movement and function of cilia. The mechanism of impaired cleaning
of respiratory tract during anaesthesia has been suggested to exist, in the form of inhibition of Cl− ions secretion and increased stickiness of mucus, caused by inhaled anaesthetics. Propofol, on the other hand, would enhance the activity of cilia by increasing the intracellular concentration of Ca2+ ions in cells of respiratory epithelium, stimulation of nitrogen oxide, and production of cGMP (Shirakami et al., 2000).

Some authors report inhibition of inflammatory reaction by anaesthetics being inhalants, or even their bactericidal properties noted in experiments in vitro (Mollie et al., 1998; Karabijik et al., 2007). Among the risk factors for infections of lower respiratory tract in hospital conditions, apart from intubation, mechanical ventilation, surgery performed, one can also list prolonged hospitalization (Niedermann et al., 2005). Dependence has been proven to exist between the aetiology of hospital acquired pneumonia (HAP) and length of hospitalization. In early hospital infections, usually the dominating micro-organisms are those which are sensitive to antibiotics, in late ones the strains that dominate are resistant to treatment (Trouillet, 2012). The responsibility for infections of lower respiratory tract rests with pathogenic microorganisms, such as: Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae, Moraxella catarrhalis, Pseudomonas aeruginosa, and in patients with hypoimmunity, also bacteria which colonize upper respiratory tract, such as: Streptococcus viridans, Neisseria species, Corynebacterium species (Ionas et al., 2002). The material for microbiological examinations concerning patients with suspected pulmonary pathology may be obtained by means of BAL (bronchoalveolar lavage) bronchoscopy or other, less invasive, techniques, such as BPSB (blinded protected specimen brush) or mini-BAL (mini bronchoalveolar lavage).

The aim of the study was to assess the influence of mechanical ventilation used during general anaesthesia upon bacterial growth in bronchial tree, depending upon the applied method of anaesthesia: inhalation (VGA, volatile general anaesthesia), or purely intravenous administration (TIVA, total intravenous anaesthesia) and their influence on post-operative pneumonia development.

**MATERIAL AND METHODS**

Having obtained the consent of the Bioethics Committee of Medical University of Silesia in Katowice, Poland, the study comprised a group of 38 patients, operated in the Central University Hospital in Katowice, Poland, between May and November, 2011. Patients who have been qualified for the study met the physical status criteria for classes I and II according to ASA scale, also those patients had the predicted time of general anaesthesia exceeding four hours. Not included in the study have been those patients, whose general physical status was assessed according to ASA as II class III, patients with history of chronic diseases of the respiratory system, namely asthma, COPD (Chronic Obstructive Pulmonary Disease), restrictive lesions, confirmed in spirometry, inflammatory and neoplastic tumours of the lungs. Patients who were currently treated with antibiotics as well as those who received recently anti-biotic therapies have been excluded from the study. On the eve of the surgery, patients were consulted by an anaesthesiologist. Patients were informed about the study, potential threats, and gave informed consent for taking part in research study. Due to the selection of anaesthetics, patients have been divided into two groups.

Randomization was performed according to the number of patient's case history. In the VGA group, where patients were anaesthetized before the procedure by means of inhalants, 23 patients were studied, age range 41 to 78 years. In the TIVA group, 15 patients were studied, age range 29 to 66 years. Surgical procedures in that group were performed under general, total intravenous anaesthesia. All patients, 30 minutes before the scheduled procedure, were premedicated with midazolam in doses of 0.1 to 0.15 mg. kg−1 orally. After two-minute pre-oxygenation with 100% oxygen, anaesthesia was induced by means of two methods. In the VGA group, the following were used for inducing anaesthesia: midazolam in doses of 0.02 to 0.05 mg.kg−1 intravenously (i.v.), fentanyl 1 to 2 μg.kg−1 i.v., propofol and their mixture 0.8 mg. kg−1 i.v. Anaesthesia was sustained by a mixture of 50% oxygen with air and inhaled anaesthetic - sevoflurane (0.8 to 1.0 MAC), and repeated doses of fentanyl 0.1 to 0.2 mg i.v. and rocuronium 0.3 mg. kg−1 i.v. In the TIVA group anaesthesia was induced by propofol by means of infusion pump intravenous drop 1.5 to 2 mg. kg−1 h−1, fentanyl in the dose of 0.1 mg i.v. After the cilia reflex subsided, rocuronium was administered in dose 0.8 mg. kg−1 i.v. After intubation anaesthesia was maintained by means of administration of propofol via pump in intravenous drop, at the rate of 6 mg. kg−1 h−1, and of remifentanil at the rate of 0.05 to 0.25 μg. kg−1 min−1, the mixture of oxygen and air was used in proportion of 50/50%. Rocuronium was used for relaxation, in maintenance doses of 0.3 mg. kg−1 i.v.

Patients, in the supine position, after intubation were ventilated mechanically in the IPPV (intermittent positive pressure ventilation) mode, applying the volume of 8 ml.kg−1 and frequency of 8-12 breaths per minute, depending upon end tidal CO2 which was maintained at the range of 33-38 mmHg.

In order to provide qualitative and quantitative assessment of bacteria, the material from respiratory tracts has been collected twice: directly after induction of anaesthesia and again before the end of anaesthesia that is before extubation. All the procedures concerning tracheal intubation were performed by anaesthesiologist using surgical facemasks and sterile gloves after proper hands hygiene with sterile equipment (face mask, laryngoscope, single-use tracheal tube, single-use filter placed between the patient and the breathing circuit (a new filter for each patient) and standardised staff technique in order to minimise the contamination of tracheal tree. The mini-BAL technique has been used for collecting the material and was performed by the same single operator every time, carrying out a sterile procedure under full aseptic conditions.

The mini-BAL technique applied consisted of “blind” collection of washings from lower respiratory tract. After injecting, via endotracheal tube, of 20 ml of sterile solution of saline, heated to body temperature, the patient was ventilated manually five times, by means of self-reinflating bag. After re-installation of the intrabronchial catheter, through endotracheal tube, the bronchoalveolar washings in the amount of 2 to 5 ml have been collected by means of aspirator directly to sterile test tubes. The material for the first test tube was obtained after intubation and stabilization of ventilation and circulation parameters, whereas the washings for the second test tube were collected after minimum four hours of anaesthesia, directly before extubation. Each test tube was immediately sent to microbiological laboratory for qualitative and quantitative assessment of bacterial flora. The time elapsed between collection of material and beginning of culture did not exceed 15 min. In laboratory of Central University Hospital, Katowice, Poland, the samples were processed in line with the generally accepted procedures mandatory in a microbiological laboratory. The material
obtained was cultured with use inoculating loop onto blood agar, chocolate agar, Chapman and MacConkey agar plates. Plates were incubated in the temperature of 35 to 37°C, in aerobic conditions. Then read after 48 h. Plates were visualized for growth by two different observers. CFU – colony forming unit was based on the laboratory formula: CFU = n × v where n stands for number of colony on space of plate, v means inverse of inoculating loop volume in 1 ml. Results more than 10^5 CFU implied infection of lower respiratory tract, results less than 10^4 CFU – bacterial colonization. 

The statistical analysis has been prepared using the Statistica v8.0 package, by StatSoft. The graphic illustration of results has been prepared mainly by means of Statistica software, partly also by means of the graphics editor contained in the MS Office 2007 package. Verification of hypotheses concerning normal distribution of variables has been carried out using Kolmogorov-Smirnov test, and Shapiro-Wilk test. For variables having distribution consistent with normal distribution, the assessment of significance of differences between groups has been made by means of t-Student test. The use of that test required that the condition of variance homogeneity was met, which has been attained using Snedecor test. The significance of differences for variables, the distribution of which did not meet the condition of normality has been assessed by means of rank sum test by U Mann-Whitney. For analysis of significance of differences between groups resulting from division of the investigated sample, in accordance with a determined criterion for one variable in relation to another variable, the independence chi-squared test has been used. The condition required for the application of that test is that the number in each class established as a result of sample division must be greater than 8. For those subgroups, where the size of the group was smaller, the Yates’s chi-squared independence test was used. In selected cases, for 2×2 tables, the Fisher’s exact test has been used, while in case of tables of larger size that is, the test of highest likelihood. P value < 0.05 was considered statistically significant. Power analysis calculation was conduct using G Power software. The sample size calculation was based on a 95% confidence level with a power of 80%.

**RESULTS**

From the total of 38 patients, three patients have been excluded from the study. Two of them due to too short operation time, not exceeding two hours, one because of difficult intubation and early death caused by circulatory insufficiency, within 24 h after the procedure. 35 patients who have been subject to final analysis are characterized in Table 1.

Of the 35 patients studied, 14 (40%) no micro-organisms have been isolated in both points in time assumed for material collection. In the VGA group that amounted 38.1% of patients (n=8), in the TIVA group to 57.1% of cases (n=8).

In the VGA group, the average time of general anesthesia amounted to 239.90 min. The most frequently isolated flora was alpha-hemolytic Streptococcus. The list of micro-organisms isolated in washings obtained is provided in Table 2.

In 13 patients (61.9%), on material collected before the operation micro-organisms have been cultured. The respiratory tracts of nine patients were colonized by one species of bacteria, in four patients by two or more species of micro-organisms. Of the bacteria cultured on pre-operative material 62.5% of colonies diminished or

---

**Table 1.** Data characterizing studied patients. Values are number (proportion) or mean (SD); n, number of patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total number of patients</th>
<th>VGA group</th>
<th>TIVA group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=35</td>
<td>n=21</td>
<td>n=14</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>n=14 (60%)</td>
<td>n=9 (64.3%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>n=14 (40%)</td>
<td>n=5 (35.7%)</td>
<td></td>
</tr>
<tr>
<td>Average age</td>
<td>57.0 (11.31)</td>
<td>59.81 (10.38)</td>
<td>52.71 (11.67)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(range 29-78)</td>
<td>(range 41-78)</td>
<td>(range 29-66)</td>
<td>p=0.21</td>
</tr>
<tr>
<td>Average BMI</td>
<td>24.99 (3.18)</td>
<td>24.83 (3.15)</td>
<td>25.22 (3.33)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=21</td>
<td>n=12</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=11</td>
<td>n=7</td>
<td>n=4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=3</td>
<td>n=2</td>
<td>n=1</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>n=12 (34.2%)</td>
<td>n=7 (33.3%)</td>
<td>n=5 (35.7%)</td>
<td></td>
</tr>
<tr>
<td>Number of smoked cigarettes</td>
<td>4.43 (7.25)</td>
<td>4.52 (7.40)</td>
<td>4.29 (7.30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(range 7-8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average number of days of hospitalization before the operation</td>
<td>9.31 (7.56)</td>
<td>6.90 (6.46)</td>
<td>12.93 (7.77)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(range 1-32)</td>
<td>(range 1-31)</td>
<td>(range 2-32)</td>
<td></td>
</tr>
<tr>
<td><strong>Type of operation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Craniotomy</td>
<td>n=20</td>
<td>n=10</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>Laparotomy</td>
<td>n=15</td>
<td>n=11</td>
<td>n=4</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Bacteria isolated from lower respiratory tract in the VGA group.

<table>
<thead>
<tr>
<th>Case</th>
<th>Bacteria isolated</th>
<th>Number of colonies ml(^{-1}) in pre-surgery material, directly after intubation</th>
<th>Number of colonies ml(^{-1}) in post-surgery material directly before extubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Streptococcus alpha-hemolytic</em></td>
<td>(10^4)</td>
<td>(10^3)</td>
</tr>
<tr>
<td>2.</td>
<td><em>Streptococcus alpha-hemolytic</em></td>
<td>(10^4)</td>
<td>none</td>
</tr>
<tr>
<td>3.</td>
<td><em>Streptococcus alpha-hemolytic</em></td>
<td>(10^3)</td>
<td>(10^4)</td>
</tr>
<tr>
<td>4.</td>
<td><em>Streptococcus alpha-hemolytic</em></td>
<td>(10^3)</td>
<td>(10^3)</td>
</tr>
<tr>
<td>5.</td>
<td><em>Neisseria species</em></td>
<td>(10^3)</td>
<td>none</td>
</tr>
<tr>
<td>6.</td>
<td><em>Streptococcus alpha-hemolytic</em></td>
<td>(10^3)</td>
<td>none</td>
</tr>
<tr>
<td>7.</td>
<td><em>Streptococcus alpha-hemolytic</em></td>
<td>(10^3)</td>
<td>none</td>
</tr>
<tr>
<td>8.</td>
<td><em>Streptococcus alpha-hemolytic</em></td>
<td>(10^3)</td>
<td>none</td>
</tr>
<tr>
<td>9.</td>
<td><em>Klebsiella oxytoca</em></td>
<td>none</td>
<td>(10^3)</td>
</tr>
<tr>
<td>10.</td>
<td><em>Haemophilus influenzae</em></td>
<td>none</td>
<td>(10^3)</td>
</tr>
<tr>
<td>11.</td>
<td><em>Streptococcus pneumonia</em></td>
<td>(10^3)</td>
<td>(10^3)</td>
</tr>
<tr>
<td>12.</td>
<td><em>Acinetobacter calcoaceticus baumani</em></td>
<td>(10^4)</td>
<td>(10^3)</td>
</tr>
<tr>
<td>13.</td>
<td><em>Moraxella species</em></td>
<td>(10^3)</td>
<td>none</td>
</tr>
<tr>
<td>14.</td>
<td><em>Serratia marcescens</em></td>
<td>(10^3)</td>
<td>none</td>
</tr>
<tr>
<td>15.</td>
<td><em>Haemophilus influenzae</em></td>
<td>(10^3)</td>
<td>none</td>
</tr>
</tbody>
</table>

Table 3. Bacteria isolated from lower respiratory tract in the TIVA group.

<table>
<thead>
<tr>
<th>Case</th>
<th>Bacteria isolated</th>
<th>Number of colonies ml(^{-1}) in pre-surgery material, directly after intubation</th>
<th>Number of colonies ml(^{-1}) in post-surgery material directly before extubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Streptococcus pneumonia</em></td>
<td>(10^5)</td>
<td>(10^2)</td>
</tr>
<tr>
<td>2.</td>
<td><em>Streptococcus pneumonia</em></td>
<td>none</td>
<td>(10^4)</td>
</tr>
<tr>
<td>3.</td>
<td><em>Streptococcus alpha-hemolytic</em></td>
<td>(10^4)</td>
<td>(10^3)</td>
</tr>
<tr>
<td>4.</td>
<td><em>Streptococcus alpha-hemolytic</em></td>
<td>(10^4)</td>
<td>none</td>
</tr>
<tr>
<td>5.</td>
<td><em>Moraxella species</em></td>
<td>(10^3)</td>
<td>none</td>
</tr>
<tr>
<td>6.</td>
<td><em>Haemophilus influenzae</em></td>
<td>(10^3)</td>
<td>none</td>
</tr>
</tbody>
</table>

eradicated, 31.25% of colonies remained unchanged, and 6.25% multiplied. In one patient bacteria culture increased to bacterial index level which may signify infection. In two patients, sample two revealed additional bacterial strains, which were not present in the first sample, having a bacterial index of \(10^3\) CFU.

In the TIVA group the average time of general anaesthesia amounted to 247.14 min. The bacteria most often isolated in the material analysed are presented in Table 3. In six cases (42.9%), bacteria were cultured from bronchoalveolar washings obtained just after intubation. In four patients the respiratory tracts were colonised by one species of bacteria, in two cases by two strains of bacteria. In two patients the titre of sample one indicated infection, however, bacterial colonies were reduced after the anaesthesia ended. The bacterial index of bacteria isolated in samples collected after intubation got reduced in all cases. 57.1% of bacterial colonies underwent total eradication. In one patient in sample two additional bacterial colonies were detected, in the amount of \(10^4\) CFU, which were not present in sample one.

In the analysis, to determine the predictors of bronchial bacterial colonization we have assessed variables: age, sex, BMI, smoking habits, hospitalization time preceding surgery. Three of these variables proved to be statistically significant in analysed VGA group: BMI (p=0.048).
As discussed, hospitalization time preceding surgery (p = 0.036) and number of smoking cigarettes (p=0.028). Bacteria were cultured in smokers as well as non-smokers in both samples. Of the patients who had no bacteria cultured in samples more often were non-smoking patients (Figure 2 and 3). There was significant difference between patients smoking more cigarettes in comparison to patients smoking less in VGA group (Figure 4).

The complication in the form of bronchial spasm, which appeared immediately after administration of physiological saline and insertion of catheter to bronchial tree, occurred in nine patients (25.7%). Higher peak and plateau pressures were noted in respiratory tract, as well as changes on auscultation, in the form of wheezing, dry rales and absence of vesicular murmur. Bronchial spasm usually subsided after five to ten minutes after administration of 100 to 300 mg hydrocortison i.v. In two cases there was a significant drop of blood saturation to 84 and 90%, in case of ventilation with 100% oxygen. The patients required intravenous administration of aminophillin, repeated removal by sucking from intubation tube, and temporary change of ventilation mode to PCV (pressure controlled ventilation) mode.

**DISCUSSION**

Bacterial colonization of lower respiratory tract has been noted in 54.3% of patients in our study. Micro-organisms that have been most often isolated were Streptococcus alpha-hemolytic (37% of colonies cultured) and Streptococcus pneumoniae (18.5% of colonies cultured). As opposed to strains of bacteria, which dominate in VAP (ventilator associated-pneumonia) - the Gram negative bacteria, Gram positive bacteria dominated in our material. Probably time factor might play an important role there, especially the length of hospitalization preceding the operation. It is well known that prior hospitalization is a risk factor of bacterial colonization. Complications, in the form of post-operative pneumonia, depend also on the underlying disease and type of operation performed (Ficker, 2008). In our study, patients had the procedures of laparotomy and craniotomy. In such cases, suppression of the cough reflex, for example to prevent the increase of intracranial pressure or to restrain pain level might have promoted the retention of secretion in bronchial tree, and pulmonary infection. Decreased BMI<25 was also an independent risk factor for bronchial colonization. That conclusion differs from those noted in literature (Ionas et al., 2002). Despite the existing pneumonia risk factors in VGA and TIVA groups, no infections of lower respiratory tract have been recorded.

The amount of bacteria isolated in both groups, in the majority of cases decreased in relation to the initial bacteriological index. In literature the influence of inhalational anaesthetics: halothane, enflurane, isoflurane, as well as intravenous anaesthetics upon the movement of cilia, and transport of mucus were presented. Inhalational anaesthetics would inhibit ciliary movement and increase the volume of retained mucus. Propofol, due to the properties stimulating ciliary movement and influencing the mechanisms of secretion liquefaction would appear a safer drug in case of patients with history of pulmonary pathology.
Figure 2. Comparison of bacteria presence in sample 1 according to smoking. No, non-smokers; Yes, smokers.

Figure 3. Comparison of bacteria presence in sample 2 according to smoking. No, non-smokers; Yes, smokers.

(Ledowski et al., 2006). In his study, Molliex in *in vitro* conditions demonstrated a direct influence of inhalational general anesthesia drugs upon bacterial cells of the *Pseudomonas aeruginosa* strain, a pathogen participating in hospital-acquired pneumonia. The author has proven a maximum inhibition of bacterial cultures after four hours of exposure to isoflurane, enflurane, halothane, greater in the concentration of anesthetic equal to
2.0 MAC, in comparison with 1.0 MAC (Molliex et al., 1998).

Only in one patient, in the material examined was there an increase of bacteria to the value of $10^6$ CFU and cultures of additional species from the family Klebsiella and Haemophilus influenzae. Suspicion of pneumonia, in accordance with the latest recommendations of American Thoracic Society, may be had on the basis of the following typical lesions detected in lung radiography, and minimum two of the following three criteria: body temperature, bronchial secretion, and leucocytosis. (American Thoracic Society, 2005). On the fifth day after operation, each patient participating in the project has been examined for parameters of inflammation - physical examination, leukocyte count, and taking body temperature. The woman patient whose bacteriological index could have indicated lung infection has been referred to her original ward and put under detailed observation. In the end, without signs of infection (no changes detected above lung fields on auscultation, leucocytosis, or elevated temperature). The woman has been discharged home.

Most studies concerning bacterial colonization of respiratory tract are devoted to patients with chronic obstructive pulmonary disease (COPD) (Domenech et al., 2012; King et al., 2013; Marin et al., 2012). A positive result of culture in one of our woman-patients, a compulsive smoker (20 cigarettes a day) may be considered colonization of respiratory tract.

In order to avoid the drawbacks of bronchoscopy, such as steep cost, side effects, experience required because of the performance technique, a cheaper and quicker mini-BAL method has been used. The method labelled as mini-BAL is an alternative for traditional bronchoscopy. Examination sensitivity is assessed at about 82% (63 to 100%), whereas the specificity compared with that of a bronchoscopy is assessed at 66 to 96% (Campbell, 2000).

**Conclusion**

To sum up, none of the patients participating in the study manifested marks of pneumonia in the postoperative period. It appears that general anesthesia does not have influence upon the increase of bacterial contamination of bronchial tree, and may even promote eradication of the colonizing bacterial flora. In the existing literature, there is no explicit standpoint concerning the influence of general anesthesia with the use of inhalants or intravenous anesthetics upon changes of bacterial flora in bronchial tree, especially in clinical aspect. However, it is too early to draw ultimate conclusions. The study reported here should be treated as preliminary communication.
REFERENCES


UPCOMING CONFERENCES

International Conference on Pharmaceutical and Biological Sciences, Abu Dhabi, UAE, 18 Nov 2013

Conferences and Advert

**November 2013**
International Conference on Pharmaceutical and Biological Sciences, Abu Dhabi, UAE, 18 Nov 2013

**December 2013**
International Conference on Molecular Virology and Microbiology, Bangkok, Thailand, 24 Dec 2013

International Conference on Cellular and Molecular Biology, Phuket, Thailand, 25 Dec 2014

**January 2014**
African Journal of Microbiology Research

Related Journals Published by Academic Journals

- African Journal of Biotechnology
- African Journal of Biochemistry Research
- Journal of Bacteriology Research
- Journal of Evolutionary Biology Research
- Journal of Yeast and Fungal Research
- Journal of Brewing and Distilling