J M R R

Journal of Microbiology and Related Research

Red Flower Publication Pvt. Ltd.
48/41-42, DSIDC, Pocket-II, Mayur Vihar, Phase-I
Delhi - 110 091 (India)
Tel: 91-11-22754205, 45796900, Fax: 91-11-22754205
E-mail: info@rfppl.co.in
Website: www.rfppl.co.in

Journal of Microbiology and Related Research (pISSN: 2395-6623 , eISSN: 2455-832X) is a leading source of up-to-date information in the field of microbiology. The journal is a peer-reviewed official organ of the Red Flower Publication Pvt. Ltd. It publishes papers based on original research in fundamental and applied biology of bacteria, archaea and lower eukaryotes as well as on all aspects of microbiological research. JMRR cover areas of agricultural, food, environmental, industrial, medical, pharmaceutical, veterinary and molecular microbiology.

Subscription Information

Institutional (1 year): INR8000/USD571

Payment methods

Bank draft / cashier & order / check / cheque / demand draft / money order should be in the name of **Red Flower Publication Pvt. Ltd.** payable at **Delhi**.

International Bank transfer / bank wire / electronic funds transfer / money remittance / money wire / telegraphic transfer / telex

1. **Complete Bank Account No.** 604320110000467
2. **Beneficiary Name (As per Bank Pass Book):** Red Flower Publication Pvt. Ltd.
3. **Address:** 41/48, DSIDC, Pocket-II, Mayur Vihar Phase-I, Delhi – 110 091(India)
4. **Bank & Branch Name:** Bank of India; Mayur Vihar
5. **Bank Address & Phone Number:** 13/14, Sri Balaji Shop,Pocket II, Mayur Vihar Phase- I, New Delhi - 110091 (India); Tel: 22750372, 22753401. **Email:** mayurvihar.newdelhi@bankofindia.co.in
6. **MICR Code:** 110013045
7. **Branch Code:** 6043
8. **IFSC Code:** BKID0006043 (used for RTGS and NEFT transactions)
9. **Swift Code:** BKIDINBBDOS
10. **Beneficiary Contact No. & E-mail ID:** 91-11-22754205, 45796900, E-mail: sales@rfppl.co.in

Online You can now renew online using our RFPPL renewal website. Visit http://rfppl.co.in/subscribe.php?mid=7 and enter the required information and than you will be able to pay online.

Send all Orders to: **Red Flower Publication Pvt. Ltd.,** 48/41-42, DSIDC, Pocket-II, Mayur Vihar Phase-I, Delhi – 110 091(India). Phone: 91-11-22754205, 45796900, Fax: 91-11-22754205, E-mail: sales@rfppl.co.in, Website: www.rfppl.co.in

Journal of Microbiology and Related Research

JMRR

January - June 2017
Volume 3, Number 1

Contents

Original Articles

ICI Journals Master List 2014

Now available! **Annual Report ICI Journals Master List 2014** summarizing the 2014 year with full list of journals and publishers from database of Index Copernicus.

Index Copernicus Search Articles

Log in
to international indexing database ICI Journals Master List

Register journal
in an international indexing database ICI

Home ⇒ Journal passport ⇒

Journal of Microbiology and Related Research [JMRR]

ISSN: 2395-6623, 2455-832X

ICV 2015: 65.86

No historical ratings

Area: Technical science

Print version: yes

Electronic version: yes

The journal is indexed in:

Original Article

Journal of Microbiology and Related Research
Volume 3 Number 1, January - June 2017
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.3117.1

Seroprevalence of Hepatitis B Virus and Hepatitis C Virus as Causes of Acute Viral Hepatitis in North India: A Hospital Based Study

Mohd Suhail Lone*, Junaid Ahmad*, Dalip K Kakru, Lubna Samad*, Shagufta Roohi*, Abiroo Jan*, Akeela Fatima*, Nayeemuddin Wani*, Muneer Bhat*****

Author Affiliation
*Senior Resident **Professor & Head ***Research Scientist, Department of Clinical Microbiology, Sher I Kashmir Institute of Medical Sciences, Soura, J&K.

Reprint Request
Dalip K. Kakru, Professor & Head, Department of Clinical Microbiology Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Soura, J&K - 190011.
E-mail: dkkakru@yahoo.co.in

Received on 14.02.2017,
Accepted on 23.02.2017

Abstract

Context: Hepatitis is an inflammation of the liver, most commonly caused by a viral infection. Acute viral hepatitis (AVH) is a major public health problem and is an important cause of morbidity and mortality. *Aim:* The aim of the present study was to determine the prevalence of parenterally-transmitted hepatitis viruses, hepatitis B virus (HBV) & hepatitis C virus (HCV) as causes of AVH in a tertiary care hospital of North India. *Settings and Design:* Blood samples were collected from patients with clinically suspected acute infectious hepatitis over a 1 year period. *Subjects and Methods:* Samples were tested for hepatitis B surface antigen & anti HCV IgG by the enzyme linked immunosorbent assay. Seroprevalence rate was calculated and stratified by age. *Statistical Analysis Used:* Fisher's exact test was used and percentages were calculated for comparison. *Results:* The study population comprised of 9652 patients presenting with acute hepatitis. Samples were collected from patients with clinically suspected acute infectious hepatitis. Seroprevalence rate was calculated and stratified by age. Of the 9652 cases, 558 (5.8%) had a confirmed viral aetiology. HBV (3.96%) was identified as the most common cause of acute hepatitis compared to HCV (1.8%). Co-infections was present in 2 cases. *Conclusions:* Among the parentally transmitted viral hepatitis, HBV is more common and Health education & vaccination is necessary to control these infections.

Keywords: Seroprevalence; Hepatitis B; Hepatitis C; Viral Hepatitis.

Introduction

Hepatitis is an inflammation of the liver, most commonly caused by viral infections. Acute viral hepatitis (AVH) is a major public health problem in India and other developing nations having inadequate sanitary conditions. Major etiological agent of enterically-transmitted viral hepatitis includes Hepatitis A (HAV) and Hepatitis E virus (HEV). Infection due to Hepatitis A virus is endemic in less developed countries, although its prevalence has decreased recently in areas where sanitary conditions have been improving. Hepatitis B virus (HBV) is transmitted by parenteral, vertical and sexual routes, whereas hepatitis C virus (HCV) is usually transmitted by transfusion [1,2].

Clinical presentation due to these viruses largely overlaps. These range from asymptomatic and inapparent to fulminant and acute fatal infections on one hand, and from subclinical persistent infections to rapidly progressive chronic liver disease with cirrhosis and hepatocellular carcinoma on the other.

[3]. According to the World Health Organisation (WHO), approximately 240 million people are chronically infected with HBV worldwide, while 150 million people are infected with HCV [4].

Since there is paucity of data regarding seroprevalence of viral hepatitis in North India therefore, this study was undertaken to determine the prevalence of parenterally-transmitted hepatotropic viruses HBV & HCV among patients presenting with acute viral hepatitis (AVH) so that appropriate management of cases as well as preventive strategies for this part of the country could be planned..

Materials and Methods

This prospective study was conducted in the Department of Microbiology at Sher i Kashmir Institute of Medical Science J&K, which is a tertiary care hospital in Northern India, from January 2016 to December 2016. Samples were collected from patients with clinically suspected acute infectious hepatitis at Hospital. Serum samples were collected from 9652 patients.

Approximately 5 ml blood sample was collected from all cases; serum was separated and stored at -20°C until tested. Following ELISA Kits were used for testing serum samples for the relevant viral markers.

Hepatitis B virus surface antigen (ErbaLisa Hepatitis B)

IgG Hepatitis C virus. (NANBASEC-96 3 Anti HCV)

The optical density (OD value) value was taken in ELISA reader and cut off value was calculated as per manufacturer's guidelines.

Results

A total of 9652 patients were enrolled in the study. Out of 9652 cases 6853 were males & 2800 were females. Of the 9652 patients, 361 were children, and 7345 were adults. Out of 9652 patients, a total of 558 (5.8%) cases had a confirmed viral aetiology while in 9094 (94.2%) cases, hepatitis virus could not be detected. Among the two viruses Hepatitis B virus (HBV) infection was more common and was found in the maximum number of cases (3.96%) whereas Hepatitis C virus (HCV) infection was seen in 1.8% of cases (Table 1).

In children the overall seroprevelance of Hepatitis C was less ie 0.3% however none of the cases enrolled in the study tested positive for hepatitis B (Table 2).

The age-specific seroprevalence of HBV infection was highest in the age group 31-40 years (7.50%) and 41-50 years (7.15%). However the age-specific seroprevalence of HCV increased with age, peaking at 41-50 years (3.75%) followed by 51-60 years (1.3%) and 1-10 years (2.58%) respectively. Co-infection of HBV with HCV was present in 2 cases (Table 3).

Table 1: Seroprevelance of HBV & HCV

Virus	Investigation type	No of tests performed	Positive tests
HBV	HBsAg ELISA	9652	382 (3.96%)
HCV	IgG ELISA	9652	176 (1.80%)

Table 2: Seroprevelance of viral hepatitis in children and adults

Viral hepatitis	Children	Adults
HBV	0%	3.96%
HCV	0.03%	1.79%

Table 3: Seroprevalence of Hepatitis B and Hepatitis C in different age groups

Age group	Total no of patients	HBV	HCV
0-10yr	129	0 (0%)	0 (0%)
11-20yr	232	0 (0%)	3 (1.29%)
21-30yr	4693	69 (1.47%)	39 (0.83%)
31-40yr	2810	211 (7.50%)	66 (2.34%)
41-50yr	1146	82 (7.15%)	43 (3.75%)
51-60yr	426	16 (3.75%)	11 (2.58%)
Above 60yr	216	4 (1.85%)	5 (2.34%)
Total	9652	382	176

Discussion

The present study was conducted to evaluate the seroprevalence of Hepatitis B & C viral markers among children and adults. In our study, HBV (3.96%) was found to be common cause of Acute viral hepatitis (AVH) than due to HCV (1.8%) among the parentally transmitted viruses. Similar results were seen by P. Jain et al [5] who in their study found a seroprevelance of HBV (16.10%) and HCV (11.98%) respectively.

In our study both HBV & HCV infections were common etiological agents in adults (3.96% & 1.79%) whereas in children only HCV infection was seen however its seroprevalence was low (0.03%). Similar results were seen by Lakshmia et al., [3] from South India who reported HBV as the commonest cause of viral hepatitis in adults followed by HEV (29.8%). Seroprevalence of hepatitis B virus infection was low in our study (3.96%%) when compared to other studies by P. Jain 26.96%6. However LiviaMelo Villar (Livia Melo et al., 2014) from Brazil reported HBV infection 1.8%, Karatekin et al., from Turkey reported 3.8%. Wang YB reported the overall seroprevalence of HBV 6.1% which was comparable to our study [5,6,7,8]. Agewise incidence showed lowest seroprevalence of Hepatitis B in 0-10 years age group ie 0 % which indirectly indicates the low incidence of vertical transmission of hepatitis B infection. The successful introduction of the HBV vaccine into the National Immunization Program in India has had a great impact on the prevalence of HBV markers among children. The results of the present study showed that universal vaccination of infants has contributed directly to the reduction in the prevalence of HBV.

In our study seroprevalence of HCV was low in children (0.03%) and adults (1.79%). Jain et al., reported a high prevalence of HCV was seen in both children (6.29%) and adults (18.54%)[5]. Mushtaq et al., (2009) from Aligarh reported low seroprevalence of HCV in children (2.03%) [9].

The peak prevalence of Hepatitis B and Hepatitis C was observed in the age groups of 31-40 yrs & 41-50 yrs. Higher prevalence in these age groups may be attributed to iatrogenic factors including vaccinations by the use of unsterilized kits, transfusion of unscreened blood, and and higher indulgence in unprotected sexual activities and illicit drug abuse etc. Further the trend of immunization against hepatitis-B has been introduced in the recent few years. Therefore the older age group population having a higher prevalence of HBsAg in our study is presumptively due to lack of immunization against the disease in their time.

References

1. Acharya, S.K., Kaushal Madan, S., Dattagupta, S.K., Panda. Viral Hepatitis In India. The National Med. J. India, 2006; 19(4).
2. De Paula, V.S., M.E. Arruda, C.L. Vitral, A.M.C. Gaspar. Seroprevalence of Viral Hepatitis in Riverine Communities from the Western Region of the Brazilian Amazon Basin Mem Inst Oswaldo Cruz, Rio de Janeiro, 2001 Nov; 96(8).
3. Mohana Lakshmia, T., A., Vaithilingamb, A., Franklina, E., Prabhakar Reddy. The prevalence of serological markers of viruses causing acute hepatitis in south indian population. Int. J. Biol. Med. Res., 2011; 2(4):925–928.
4. Rachna Behal, Renu Jain, Krishan, K., Behal, Ajay Bhagoliwal, Nakshatra Aggarwal and T.N. Dhole. Seroprevalence And Risk Factors For Hepatitis-B Virus Infection Among General Population In Northern India. Arq. Gastroenterol., 2008; 45(2).
5. Jain, P., S. Prakash, S. Gupta, K.P. Singh, S. Shrivastava, D.D. Singh, J. Singh, A. Jain. Prevalence of hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus and hepatitis E virus as causes of acute viral hepatitis in North India: A hospital based study. Indian J. Med. Microbiol., 2013; 31(3):261-265.
6. LiviaMelo Villar, Luciane Almeida Amado, Adilson José de Almeida,Vanessa Salete de Paula, Lia Laura Lewis-Ximenez, and Elisabeth Lampe. Low Prevalence of Hepatitis B and C Virus Markers among Children and Adolescents. BioMed. Res. Int., Volume 2014, Article ID 324638.
7. Karatekin G, Kilinc M, Gulcan Oksuz B, Igde M. Hepatitis B seroprevalence in children and women and the impact of the hepatitis B vaccination program in the Black Sea Region of Turkey. J Infect Dev Ctries. 2013; 7(12):960-5.
8. Wang YB, Chen EQ, Cui YL, Zeng L, Wang YJ, Tang H. Seroprevalence of hepatitis B virus markers in individuals for physical examination in West China Hospital, China. Eur Rev Med Pharmacol Sci. 2011; 15(6):592-6.
9. Mudasir Mushtaq, Seema Alam, Indu Shukla, Rana Sherwani, S., Manazir Ali Prevalence of Hepatitis C virus infection among children with liver disease, 13(1): 1-12.

Subscription Information

India
Institutional (1 year) (Print+Online): INR8000

Rest of the World
Insitutional (1 year) (Print+Online): $571

Payment instructions
Online payment link:
http://rfppl.co.in/payment.php?mid=15

Cheque/DD:
Please send the US dollar check from outside India and INR check from India made. Payable to 'Red Flower Publication Private Limited'. Drawn on Delhi branch

Wire transfer/NEFT/RTGS:
Complete Bank Account No. 604320110000467
Beneficiary Name: Red Flower Publication Pvt. Ltd.
Bank & Branch Name: Bank of India; Mayur Vihar
MICR Code: 110013045
Branch Code: 6043
IFSC Code: BKID0006043 (used for RTGS and NEFT transactions)
Swift Code: BKIDINBBDOS

Send all Orders to: Subscription and Marketing Manager, Red Flower Publication Pvt. Ltd., 48/41-42, DSIDC, Pocket-II, Mayur Vihar Phase-I, Delhi - 110 091(India), Phone: 91-11-45796900, 22754205, 22756995, E-mail: sales@rfppl.co.in, Website: www.rfppl.co.in

Original Article

Journal of Microbiology and Related Research
Volume 3 Number 1, January - June 2017
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.3117.2

Comparative Evaluation of Phenotypic Method and Hicrome ESBL Agar in Detecting ESBL Producing *Enterobacteriaeceae*

Megha S.*, Vijaya D., Venkatesha D.*****

Author Affiliation
*Assistant Professor **Professor and Head(former) ***Professor and Head, Department of Microbiology, Adichunchanagiri Institute of Medical Sciences, B.G.Nagar- 571448, Mandya. India.

Reprint Request
Megha S., Assistant Professor, Department of Microbiology. Adichunchanagiri Institute of Medical sciences. B.G.Nagar-571448.Mandya. India.
E-mail: meghaptl88@gmail.com

Received on 02.02.2017,
Accepted on 09.02.2017

Abstract

Background and Objective: Beta lactamases are responsible for numerous outbreaks of infection in the world. The occurrence of multiple β-lactamases among bacteria not only limits the therapeutic options but also poses a challenge for microbiology laboratories to identify them. This study has been taken to screen and confirm the production of ESBL by phenotypic method and to compare it with the Crome agar for its efficacy, which is essential for infection control and antimicrobial therapy. *Methodology:* A total of 209 isolates belonging to the family *Enterobacteriaceae*obtained from different clinical samples, received in the Department of Microbiology, Adichunchanagiri Institute of Medical Sciences. B.G. Nagara formed the study group. ESBL screening test was done, followed by phenotypic confirmatory test, for ESBL detection asper CLSI guidelines. This method was compared with thechromogenic media (HiCrome ESBL agar) for detecting ESBL producers. *Results:* On assessing the sensitivity and specificity of HimediaCrome agar with that of the phenotypic method to detect ESBL producers, Crome agar detected 87 isolates as ESBL producers. Sensitivity and specificity of Crome agar considering combination disc method as gold standard was 84.2% and 93.8% respectively. Positive and negative predictive value was 91.95% and 87.7% respectively. *Conclusion:* Cefotaxime/clavulanate disc potentiation test detected maximum number of ESBL compared to Ceftazidime/Clavulunate. HiCrome ESBL agar has high sensitivity and specificity in screening for ESBLproducers and can be used routinely in the laboratory for rapid detection of ESBL producers.

Keywords: ESBL; Combination Disc Method; Hicrome ESBL Agar; *Enterobacteriaeceae*.

Introduction

Enterobacteriaceae may exhibit a reduced susceptibility to the beta lactam antibiotics by a number of mechanisms which includes reduced outer membrane permeability, target site modification and efflux of beta-lactams out of the cell. However by far the most common mechanism of resistance is the enzymatic inactivation of the betalactams by a beta-lactamase [1]. Betalactamases are the enzymes produced by microorganisms which can hydrolyse/open the betalactam ring of beta lactam antibiotics like Penicillins and cephalosporins [2].

Extensive use of third generation cephalosporin has led to the evolution of newer beta-lactamases such as extended spectrum beta-lactamases (ESBL).

Organisms producing ESBLs hydrolysepenicillins, cephalosporins and monobactams [3], and are inhibited by clavulanic acid, tazobactamand sulbactam. They are plasmid coded and are easily transmissible from one organism to the other. They are generally derived from TEM and SHV type [4].

The aimof the present study, was to study thephenotypic method for the detection of ESBL producing *Enterobacteriaceae* and to compare the advantage of chromogenic media with the conventional method for the detection of ESBL producing *Enterobacteriaceae.*

Materials and Methods

The present study was carried out in the department of Microbiology, AIMS, B.G. Nagar, for a period of one year. Ethical committee clearance has been taken from the institution. A total of 209 isolates belonging to family *Enterobacteriaceae* from 200 different clinical samples like urine, pus, blood, sputum, high vaginal swab collected from out-patients and in-patients admitted in the hospital were included in the study. Gram negative bacilli other than *Enterobacteriaceae* were excluded. Cultures yielding no growth or contaminants, and urine samples with insignificant/mixed growth were excluded. Non-repetitive 200 samples of urine, sputum, pus, blood, fluid, stool, high vaginal swab and other samples received in the Microbiology laboratory, AIMS, B.G. Nagar were processed. The specimens were brought to laboratory within 20min of sample collection. Wet films of urine samples were done and examined for pus cells and organisms. Samples were processed as per standard protocols and organisms isolated were identified based on the standard procedures.

Methodology for Detection of ESBL

All 209 *Enterobacteriaceae* isolated from clinical specimens were subjected to screening tests for ESBL. After adjusting the bacterial suspension to 0.5 MacFarland's unit, lawn culture was done on MHA. Amoxicillin- clavulanic acid disc (20µg+10µg) was placed in the centre of the petridish and Cefpodoxime 10µg, Ceftazidime 30µg disc were placed on either side of Amoxicillin- clavulanic acid disc at a distance of 20mm. Extension of zone of inhibition of cefpodoxime or ceftazidime towards Amoxyclav disc was taken as ESBL screening positive.

Chromogenic Medium for ESBL Screening

Bacterial suspension was adjusted to 0.5 Mc Farland's unit and streaked onto HiCrome ESBL agar (Hi media Mumbai) . Plates were incubated at 37^0C for 18-24 hours.

Interpretation [5]

- *Escherichia Coli:* pink to burgundy coloration of β−glucuronidase producing colonies
- *Klebsiella, Enterobacter, Serratia, Citrobacter (KESC):* Green/ blue to browny green coloration of ß-glucosidaseproducing colonies
- *Proteae (Proteus, Providencia, Morganella)*: dark brown /light brown coloration of deaminase expressing strains.

All *Enterobacteriaceae* are subjected to ESBL confirmation.

ESBL confirmatory test- Combination disc method/ Disc diffusion test [6]:

Bacterial suspension was adjusted to 0.5 Mc Farland's unit and lawn culture was done on MHA. Ceftazidime 30µg (CAZ), ceftazidime +clavulanic acid 30µg+10µg (CAZ+CAC) and cefotaxime 30µg (CTX) and cefotaxime+clavulanic acid 30µg+10µg (CTX+CEC) were placed >30mm apart as shown in Figure 1. Plates were incubated at 37^0C for 16-18 hours.

Interpretation

≥ 5 mm increase in the zone of inhibition of Ceftazidime+clavulanicacid and/or cefotaxime+ clavulanic acid discs as compared to Ceftazidime and/or cefotaximediscs alone was taken as ESBL positive.

Results

A total of 209 clinical isolates of *Enterobacteriaceae* from different clinical samples like urine, pus, blood, sputum, high vaginal swab collected from out-patients and in-patients admitted in the hospital were included in the study. Out of 200 clinical samples, majority of the isolates were from urine (55.98%), followed by pus (19.13%), sputum (11.48%), high vaginal swab (6.22%), blood (3.82%), stool (2.87%) and fluid (0.47%). Various Gram negative bacilli belonging to family *Enterobacteriaceae*isolated from different clinical specimens (Table 1) and majority were from female patients. Table 2 shows gender distribution from various clinical specimens.

All the 209 isolates were screened for ESBL production. 84 isolates were detected to be ESBL

screening positive and remaining 125 were negative. Irrespective of screening test results, all the isolates were subjected for phenotypic confirmatory test. 102 isolates were detected to be ESBL positive. Out of 209 isolates inoculated onto Hicrome ESBL agar, 87 were detected to be ESBL producers (Table 3). Table 4 shows various methods that detected ESBL producers of *Enterobacteriaeceae*.

Table 1: Various *Enterobacteriaceae* isolated from different clinicalspecimens

Organisms	Urine	Sputum	Pus	Blood	Fluid	HVS	Stool	Total
Esch.coli	71(70.29%)	5(4.95%)	9(8.91%)	2(1.98%)	1(0.99%)	7(6.93%)	6(5.94%)	101(48.31%)
Klebsiellaspp	22(38.59%)	18(31.57%)	10(17.54%)	4(7%)	0	3(5.26%)	0	57(27.27%)
Citrobacterspp	9(60%)	0	4(7%)	0	0	2(13.33%)	0	15(7.17%)
Enterobacterspp	5(38.46%)	1(7.69%)	5(38.46%)	2(15.38%)	0	0	0	13(6.22%)
Proteus spp Vulgaris/mirabilis	2(20%)	0	8(80%)	0	0	0	0	10(4.78%)
Providenciaspp	6(66.66%)	0	2(22.22%)	0	0	1(11.11%)	0	9(4.3%)
Morganellaspp	2(50%)	0	2(50%)	0	0	0	0	4(1.91%)
Total	117(55.98%)	24(11.48%)	40(19.13%)	8(3.82%)	1(0.47%)	13(6.22%)	6(2.87%)	209

Table 2: Gender wise distribution of various clinical samples

Sample	Gender		Total
	Male	Female	
Urine	39(34.5%)	74(65.48%)	113(56.5%)
Sputum	16(69.5%)	7(30.4%)	23(11.5%)
Pus	24(64.9%)	13(35.1%)	37(18.5%)
Blood	2(25%)	6(75%)	8(4%)
Fluid	0	1(100%)	1(0.5%)
HVS	0	11(100%)	11(5.5%)
Stool	4(57.1%)	3(42.9%)	7(3.5%)
Total percentage	85(42.5%)	115(57.5%)	200

Table 3: Number of ESBL isolates detected by phenotypic method and HiCrome agar

Result	Phenotypic method	Hi Crome agar
Positive	102	87
Negative	107	122

Table 4: Comparison of various methods in detecting ESBL among *Enterobacteriaceae*

Methods for ESBL detection	Esch. coli	Klebsiellaspp	Enterobacterspp	Citrobacterspp	Proteus spp	Providenciaspp	Morganellaspp
Screening test	48	12	5	4	7	6	3
Combination disc method	55	19	8	6	6	5	3
Chrom agar	47	14	5	4	7	5	3

Table 5: Sensitivity and specificity of HiCrome ESBL agar considering combination disc method as gold standard

Test	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Hi Crome ESBL agar	84.2%	93.8%	91.95%	87.7%

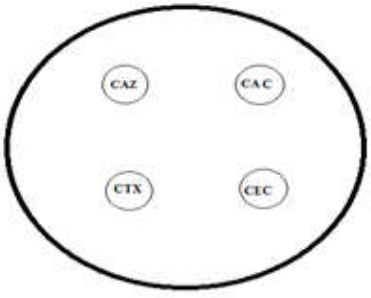

Fig. 1: Template for combination disc test for ESBL detection

Fig. 2: HiCrome ESBL agar
ESBL positive isolate shows growth on Chrom agar. Growth in the tertiary streak line and beyond is considered ESBL positive.

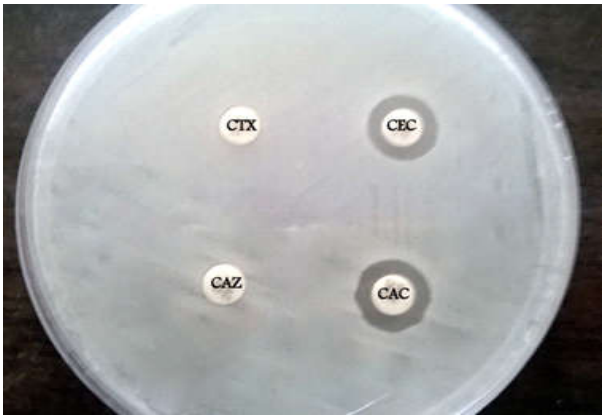

Fig. 3: Combination disc method for ESBL detection.
The isolate is ESBL producer, resistant to CTX and CAZ and the zone of inhibition of CEC and CAC is e" 5mm than CTX and CAZ

Discussion

Members of family *Enterobacteriaceae* accounts for substantial proportion of endemic nosocomial infections. Cephalosporins are the first line drugs used in the treatment of these infections. ESBL detection can be done either by doing confirmatory tests for those isolates which are positive by screening tests or by doing confirmatory tests without prior screening test, as it is notCLSI recommended. Phenotypic ESBL confirmatory test is in routine use [7].

The present study compare the phenotypic method and chromogenic media in detecting the ESBL. In the present study, 209 isolates were screened for the production of ESBL, which showed 84 (40.1%) isolates to be ESBL screening positive and remaining 125 (59.8%) were negative for ESBL production. Irrespective of the screening test all the isolates were put for ESBL confirmatory test. As per CLSI guidelines confirmatory test was put using combination disc method which detected 102 (48.8%) isolates to be ESBL positive, which is comparable with the study of Dalela G in which phenotypic confirmatory disc diffusion test detected 135 (61.6%) out of 219 isolates [8].

In the present study, among the β lactam -inhibitor combination used, CTX and CEC combination detected majority of the ESBL isolates compared to CAZ and CAC. The confirmation of the ESBL production by clavulanic acid inhibition can be difficult in some strains, not only because the activity of the β-lactamase varies with different substrates, but also because the organism may contain additional resistance mechanisms that can mask the presence of the ESBL activity [9]. Shoorashetty RM et al [10] and Sturenberg et al [11] used cefepime/ clavulanate method to detect maximum ESBL.

Apart from phenotypic method, all the isolates were inoculated onto crome agar (HiCrome ESBL agar) for the detection of ESBL. Crome agar detected 87 isolates as ESBL producers.

Sensitivity and specificity of Crome agar considering combination disc method as gold standard was 84.2% and 93.8% respectively. Positive and negative predictive value is 91.95% and 87.7% respectively (Table 5). R.P Helene et al studied the performance of chromID ESBL(bioMerieux) for detection of *Enterobacteriaceae* producing ESBL comparing it with BLSE agar, the sensitivity was 88% for chrom ID ESBL and 85% for BLSE agar [12]. Study by Te-Din Haung et al showed sensitivity of Brilliance ESBL agar (OX; Oxoid, Basingstoke, UK) and Chrom ID ESBL agar (BM; bioMerieux, Marcy l'Etoile, France) 94.9% and specificity was 95.5% and 95.7% respectively [13].

Conclusion

Increased rates of antimicrobial resistance among members of family *Enterobacteriaeceae* which are known to cause clinically significant infections, suggests monitoring mechanisms of antimicrobial resistance as well as simultaneously evaluating the newer antimicrobial agents for their in-vitro activity. Simultaneous production of several β-lactamases in the organisms poses difficulty in detecting each mechanism of resistance as one mechanism can mask the other. Phenotypic methods for detection of these resistant mechanisms are faster, cost effective, easier to perform and less labour intensive.

Combination disc method using cefotaxime/ clavulanic acid detected maximum number of ESBL than ceftazidime/clavulunate but it should be performed with other test using boronic acid for the detection of ESBL in the presence of co-production of AmpC, since the presence of high-level expression of AmpC can mask the recognition of the ESBL's. HiCrome ESBL agar has high sensitivity and specificity. High negative predictive value of this media suggests that this medium though not confirmatory, it can constitute as an excellent screening tool for rapid exclusion of patients for not carrying ESBL producers. Its technically simple, easy to perform, cost effective, can be done routinely in laboratory.

In order to prevent the spread of these multidrug resistant organisms, it is necessary to identify and

detect them routinely in the laboratories using simple phenotypic methods as this helps the clinician to provide appropriate antimicrobial therapy. It is advisable for all the health care settings to have hospital infection control committee with hospital antibiotic policy, with regular updates.

Abbreviations

PCT - Phenotypic confirmatory test, CLSI - Clinical and Laboratory Standard, CAZ- Ceftazidime, CAC- Ceftazidime + Clavulanic acid, CTX- Cefotaxime, CEC- Cefotaxime + Clavulanic acid.

References

1. Laghawe A, Jaitly N. Thombare V. The simultaneous detection of ESBL and the AmpC β-lacatamases in Gram negative bacilli. J ClinDiagReas. 2012; 6(4): 660-663.
2. Bush Karen. β-lactamase inhibitors from Laboratory to clinic. Clinical Microbiological Reviews. 1988; 1(1):109-23.
3. Thomson KS. Controversies about Extended spectrum betalactamases. Emerginf dis. 2001; 7(2): 333-36.
4. Rupp ME, Fey PD. Extended Spectrum β lactamase (ESBL) –Producing *Enterobacteriaceae*- Consideration for Diagnosis, Prevention and Drug Treatment. 2003; 63(4):353-65.
5. Himedia HiCrome agar. Technical data M1829. 2013.
6. Clinical and Laboratory standard institute. Performance standards for antimicrobial susceptibility testing; 23rd informational supplement. 2013; 31(1):44-61.
7. CLSI 2005 M 100-S15 Clinical and Laboratory standard institute. Performance standards for antimicrobial susceptibility testing; 15th informational supplement. 2005; M100-S15.
8. Dalela G. Prevalence of Extended spectrum â lactamase (ESBL) producers among Gram negative bacillifrom various clinical isolates in a tertiary care hospital at Jhalawar, Rajasthan, India. J ClinDiag Res. 2012; 6(2): 182-87.
9. Steward CD ,Rasheed JK, Hubert SK et al. characterization of the clinical isolates of *Klebsiellapneumoniae* from 19 laboratories by using the National Committee for Clinical Laboratory Standards extended- spectrum β-lactamase detection methods. J.ClinMicrobiol 2001; 39:2864-72.
10. Shoorashetty RM, Nagarathnamma T, Prathibha J. Comparison of the boronic acid disc potention test and cefepime-clavulunic acid method for the detection of ESBL among among Amp C producing *Enterobacteriaceae*. Indian J Med Microbiol 2011; 29 (3):297-301.
11. Sturenberg E, Mack D. Extended Spectrum â lactamases: Implications for the clinical microbiology laboratory, therapy and infections control. J. Inf 2003; 47(4):273-95.
12. Poupet H R, Naas T, Carrer A, Cady A et al. Performance of ChromID ESBL, a chromogenic medium for detection of ESBL producing *Enterobacteriaceae*. J Med Microbiol 2008; 57:310-15.
13. Huang T, Bogaerts P, Berhin C,Guisset A, and Glupczynsk Y. Evaluation of Brilliance ESBL agar, a Novel Chromogenic Medium for the detection of Extended-spectrum-beta-lactamase-producing *Enterobacteriaceae*. J ClinMicrobiol 2010; 48(6):2091-6.

Journal of Microbiology and Related Research

Library Recommendation Form

If you would like to recommend this journal to your library, simply complete the form below and return it to us. Please type or print the information clearly. We will forward a sample copy to your library, along with this recommendation card.

Please send a sample copy to:

Name of Librarian

Name of Library

Address of Library

Recommended by:

Your Name/ Title

Department

Address

Dear Librarian,

I would like to recommend that your library subscribe to the **Journal of Microbiology and Related Research**. I believe the major future uses of the journal for your library would provide:

1. useful information for members of my specialty.
2. an excellent research aid.
3. an invaluable student resource.

I have a personal subscription and understand and appreciate the value an institutional subscription would mean to our staff.

Should the journal you're reading right now be a part of your University or institution's library? To have a free sample sent to your librarian, simply fill out and mail this today!

Stock Manager

Red Flower Publication Pvt. Ltd.

48/41-42, DSIDC, Pocket-II

Mayur Vihar Phase-I

Delhi - 110 091(India)

Phone: Phone: 91-11-45796900, 22754205, 22756995, Fax: 91-11-22754205

E-mail: sales@rfppl.co.in

Original Article

Journal of Microbiology and Related Research
Volume 3 Number 1, January - June 2017
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.3117.3

A Study of Biofilm Producers and Its Correlation to Antimicrobial Resistance among Orthopaedic Implant Associated Infections in a Tertiary Care Centre

Shobha Medegar K.R.*, Pooja Mansabdar, Asha B. Patil***, Harshika Y.K.*, Ganesh Perumal**, Neeta P.N.******

Author Affiliation
*Tutor **Post Graduate Student ***Professor and HOD, Department of Microbiology, KIMS, Hubballi. ****Assistant Professor, Department of Community Medicine, VIMS, Ballari.

Reprint Request
Neeta P.N., Assistant Professor, Dept. of Community Medicine, Vijayanagar Institute of Medical Sciences, Hospet Rd, Cantonment, Ballari, Karnataka 583104.
E-mail: drnita10@gmail.com

Received on 28.03.2017,
Accepted on 07.04.2017

Abstract

Background: Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. These biofilm forming organisms are frequently associated with implant associated infections and they are intrinsically more resistant to antimicrobial agents than planktonic cells. *Objectives*: 1. This study aims to detect the biofilm formation in the orthopaedic implant associated infections 2. to correlate its antibiotic susceptibility pattern with emphasis on multi drug resistance pattern. *Methodology*: A prospective study was done on a total of 150 cases,of aspirated pus sample, of all orthopaedic implant associated infections over a period of one year and sent to the department of microbiology, KIMS, Hubballi, whereinthey were processed according to the standard laboratory protocol. The isolates were identified and subjected to biofilm detection by three methods (Tube method, Tissue culture plate method and Congo red Agar method) and subsequently antibiotic susceptibility testing was performed on Muller Hinton Agar (MHA) by Kirby Bauer's Disc Diffusion method according to Clinical and Laboratory Standard Institute(CLSI) guidelines 2016. *Results*: Among 150 samples which were processed for biofilm detection 48% were detected as positive. Majority were Gram Positive Cocci (GPC) accounting to 63.88%. Methicillin Resistant Staphylococcus Aureus (MRSA) accounted to 60%, multi-drug resistance (MDR) was noted in 69.56% in case of GPC and 100% MDR Gram negative bacteria (GNB). *Conclusion*: Biofilm detection methods and its antimicrobial susceptibility testing should be routinely employed especially in case of implant associated infections, so that we can formulate antibiotic regimen for the multi-drug resistant isolates, by appropriate screening of MRSA, Extended Spectrum Beta Lactamase (ESBL), AMP-C and MBL and thus prevent treatment failures.

Keywords: AMP-C Co-producers; Biofilm; ESBL; Implant; Multi-Drug Resistance and MRSA.

Introduction

Bone and joint degenerative and inflammatory problems affect millions of people worldwide [1]. The introduction of an implant in the body is always associated with the risk of microbial infection, particularly for the fixation of open-fractured bones and joint-revision surgeries [2]. Infection is a major

problem in orthopedics leading to implant failure due to formation of biofilm, making it challenging to treat [3]. Biofilms are a group of microbes along with their exopolysaccharide matrix which adhere on biotic and abiotic surfaces conferring antibiotic resistance especially in indwelling medical devices [4].

Biofilm formation is dependent on adhesion properties such as adsorption, extracellular polymeric substances, attachment to hydrophobic (Teflon)or hydrophilic (glass) substratum and presence of fimbriae, flagella, pilli or glycocalyx, oxygen concentration, nutrient composition of medium and antimicrobial drug concentration [5,6,7]. Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents, as high as 1000 times, when compared to planktonic cells, hence effective antimicrobial agents are needed to inactivate them [8].

Materials and Methods

This isa prospective study, carried out in the department of microbiology, KIMS, Hubballi, on all orthopaedic implant associated infections from September 2015 to September 2016. A total of 150 non repetitive clinical specimens of pus, collected from implanted area or swabs from discharging sinuses were taken for culture, out of which 101 were culture positive and subjected to biofilm detection. All the bacterial isolates were identified by standard biochemical tests. Antibiotic susceptibility test of bacterial isolates was performed by Kirby Bauer disc diffusion method according to Clinical and Laboratory Standard Institute (CLSI) guidelines. A reference strain of Staphylococcus epidermidis ATCC 35984 (positive biofilm producer) and Staphylococcus epidermidis ATCC 12228 (non biofilm producer) were used as positive and negative controls respectively. Biofilm detection was done by the following methods:

1. *Tube Adherence Method:* Described by Christensen et al [9] this is a qualitative method for biofilm detection.
2. *The Congo Red Agar (CRA) method:* According to the Freeman et al [10], it is a simple qualitative method to detect biofilm production.
3. *The Tissue Culture Plate (TCP) method:* This is a quantitative test considered as the gold standard method for biofilm detection. The interpretation of biofilm was done according to the criteria of Stepanovicet al [11].

Results

Among the total 150 samples which were processed, 101 samples were culture positive. Out of these, biofilm producing organisms were 72 isolates (which were positive by any one of the method)

Table 1 shows that highest number of isolates were of Staphylococcus aureus 54.45%(55 isolates), followed by Klebsiella species 14.8%(15 isolates), CONS 11.8%(12 isolates), Pseudomonas species 4.9% (5 isolates), NFGNB 4.9% (5 isolates), Citrobacter freundi 3.9%(4 isolates), Escherichia coli 2.9%(3 isolates) and Providencia species 1.9% (2 isolates). For the Staphylococcal isolates, screening for Methicillin Resistant Staphylococcus aureus (MRSA) was done by using 30µg of cefoxitin disk on Muller Hinton Agar (MHA). The isolate with zone of inhibition <=21 mm was considered to be methicillin resistant.

Table 1: The distribution of isolates and biofilm production

Organisms Isolated	Biofilm Producers	Non Bio Film Producers
Escherichia coli	02(66.66%)	01
CONS (Coagulase negative staphylococcus)	06(50%)	06
Klebsiella species	12(80%)	03
Pseudomonas species	05(100%)	00
NFGNB (Non fermenting gram negative bacteria)	03(60%)	02
Citrobacter species	03(75%)	01
Providencia species	01(50%)	01

The high percentage of MRSA (60%) in biofilm producers was a contributing factor for high rate of drug resistance among them. Table 2 shows that majority of the isolates were highly resistant to Beta lactam antibiotics and macrolides. The isolates were sensitive to aminoglycosides, flouroquinolones, linezolid and 100% sensitive to teicoplanin and Vancomycin. The resistance of ampicillin and amoxyclav was statistically significant among biofilm producers and non biofilm producers. The statistical significance was calculated by using Chi Square test with p value <0.05 considered as significant.

Table 2: Drug resistance pattern among grampositive cocci isolates(n=67)

Antibiotics (mcg)	Biofilm producer	Non biofilm producer
Ampicillin(10)	89%(41)	57%(12)
Amoxicillinclavulanic acid(30)	78.2%(36)	42.86%(9)
Erythromycin(15)	65.2%(30)	57.14%(12)
Clindamycin(2)	41.3%(19)	38.09%(8)
Cefoxitin(30)	58.67%(27)	47%(10)
Linezolid(30)	13%(6)	0%
Vancomycin(30)	0%	0%
Teicoplanin(30)	0%	0%
Gentamycin(10)	19.5%(9)	4.76%(1)
Amikacin(30)	10.86%(5)	0%
Ciprofloxacin(30)	32.6%(15)	19.04%(4)
Levofloxacin(5)	4.34%(2)	0%
Cefepime(30)	30.4%(14)	23.8%(7)

Table 3: Multi drug resistance pattern in biofilm producing gram positive cocci(GPC) isolates:

Antibiotics	Number of isolates
A, AX, E, Cl,	10
A, AX,E,Cl,Cip,Cfm	8
A,AX, E, Cl,Gen,Ami,	4
A,AX,Cip,CX	8
A,AX,E,Cl,LN,CX	2

Table 3 shows that among the 46 biofilm producing GPC, 32 isolates were multidrug resistant accounting to 69.56%. Ampicillin (A), Amoxycillin clavulanic acid (AX), Erythromycin (E), Clindamycin (Cl), Ciprofloxacin (Cip), Cefepime (Cfm), Gentamycin (Gen), Cefoxitin (CX), Amikacin (Ami), Linezolid (LN)

Table 4 shows that there is high resistance among biofilm producers for Beta-lactam group of antibiotics. Resistance amongst biofilm and non biofilm producers for ampicillin, amoxy clavulanic acid, co-trimoxazole, ceftazidime and cefoxitinwas statistically significant and less resistance to Quinolones, Piperacillin tazobactam and aminoglycoside group of antibiotics was seen. All the isolates were sensitive to imipenem. The statistical significance was done by Fischer's test with p value <0.05 were considered significant.

Table 4: Theantibiotic resistance pattern among biofilm and non biofilm isolates of gram negative bacteria(n=34)

Antibiotics (mcg)	Biofilm producers(26)	Non-biofilm producers(8)	Chi square value	p- value
Ampicillin (10)	24(92%)	03(37.3%)	11.18	0.004
Amoxicillin clavulanic acid(30)	22(84.6%)	02(25%)	10.44	0.002
Ciprofloxacin(30)	11(42.3%)	02(25%)	1.329	p>0.05
Levofloxacin (5)	02(25%)	00(0%)	0.765	p>0.05
Co-trimoxazole(25)	22(84.7%)	02(25%)	10.3	0.001
Ceftriaxone(30)	21(80.7%)	05(62.5%)	0.126	p>0.05
Gentamycin(10)	18(69.2%)	02(25%)	4.936	0.03
Amikacin(30)	08(30.7%)	02(25%)	0.3	p>0.05
Ceftazidime(30)	21(80%)	03(37.8%)	5.5	0.01
Cefoxitin(30)	22(84.6%)	03(37.8%)	6.97	0.008
Pipercillin/tazobactam (100/10)	07(26.92%)	00(00%)	3.12	p>0.05
Imepenem(10)	00	00	00	0

Table 5: Multi drug resistance in biofilm producing Gram negative bacteria isolates(n=26)

Antibiotics	Number of isolates
A, AX, Cip,Ctr, Caz	10
A,AX, Cip, Gen, Ami,Caz, Ctr	06
A,AX,Ctr, Caz, Cx, Cot	05
A,AX, PTZ, Caz, Cx, Ctr	05

Table 5 shows that among the 26 biofilm producers all were multidrug resistance accounting to 100% (A:Ampicillin, AX:Amoxyclavulinic acid, Cip: Ciprofloxacin, Ctr: Ceftriaxone, Gen: Gentamycin, Ami:Amikacin, Caz: Ceftazidime, Cx: Cefoxitin, PTZ: Piperacillin/tazobactam, Cot:Cotrimoxazole). Among the total GNB organisms, (ESBL) producing isolates were 20%, AMP-C producers were 06%, ESBL and AMP-C co-producers were 44%. All the Pseudomonas isolates were biofilm positive conferring 100% biofilm producing isolates andall were non Metallo Beta Lactamase (MBL) producers.

Discussion

Implant related infection is a major concern, to the patients and in the orthopaedic community. The use of prosthetic implants in orthopaedics provides an ideal environment for biofilm formation as they are highly susceptible to infection. This is due to preoperative/post-operative infection, local host immune response or device rejection leading to device failure [12]. This necessitates further studies to determine the causative organisms and their susceptibility pattern to treat the patient. The diagnosis and treatment of these infections are complicated by the formation of a bacterial biofilm and an increase in the number of multidrug resistant bacteria. This stresses the value of an early diagnosis, leading to appropriate therapy of these patients.

In the present studya total of 150 samples were processed from all implant associated infections by collecting pus/swabs from wound discharge. Out of them 101 were cultures positive. Majority of the isolates were gram positive cocci accounting for 67 in number (66.33%) in comparison to gram negative organisms which were 34 in number (33.66%). Among the Gram positive cocci (GPC) Staphylococcus aureus isolates were 55 and Coagulase negative Staphylococcus species (CoNS) were 12 in number. Among Gram negative bacteria (GNB), majority were Klebsiella species 15 in number, followed by Pseudomonas species. A total of 72 biofilm producers were isolated which almost matches to a study conducted by Carla Renata Arciola et al [13] which shows 66% biofilm producers in orthopaedic implantsout of 80 isolates. Among the Staphylococcus aureus isolates 60% biofilm producers were Methicillin Resistant Staphylococcus aureus (MRSA) and 37.5% were MRSA in non biofilm producers. The present study results were in accordance to Khosravi et al [14] and Anisha F et al [15] which also reported Staphylococcus aureus as the most frequent isolatein orthopaedic implant associated infections.

The antimicrobial susceptibility testing revealed high rate of antimicrobial resistance in Staphylococcus aureus isolates to most of the routinely used antibiotics. All the Gram Positive Cocci, showed sensitivity to Vancomycin and Teicoplanin as seen in Afreenish Hassan et al [16] and Nixon M et al where Vancomycin was the most effective antibiotic [17]. The percentage of Methicillin Resistant Coagulase negative Staphylococcus was 50% in biofilm producing isolates and in gram negative biofilm producing organisms high prevalence of non MBL and high ESBL and Amp C co-producer isolates accounting to 44%, contributing to implant failures. Probably the prolonged hospitalization in these patients contributes to hospital acquired infection leading to such high drug resistance [18]. Also the lowered immune status, extremes of age, patient with steroid therapy and other conditions like diabetes mellitus and open wound fractures could have contributed to high rate of infections. Studies also suggest use of titanium implants rather than stainless steel implants to minimize the risk of infections. But affordability is also a major concern in a government setup unless the government subsidizes it.

We found the following antibiotics-vancomycin, teicoplanin, amikacin, levofloxacin to be more effective for biofilm producing Gram Positive Cocci and amikacin, levofloxacin,Imipenem and piperacillin/tazobactam effective for biofilm producing Gram Negative Bacilli.

Conclusion

The result of this test shows that there is high prevalence of biofilm producing organisms in orthopaedic implants, showing multi drug resistance, hence routine screening tests for MRSA, ESBL and AMP-C producing isolates should be emphasized to prevent treatment failure.

Recommendation

However we would recommend that similar studies need to be done with larger sample size to identify biofilm producing isolates and their antibiotic susceptibility pattern in prosthetic implants.

Conflicts of Interest: None

Source of Support: Nil

References

1. Ribeiro M, Monteiro FJ, Ferraz MP. Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions. Biomatter. 2012; 2(4):176-194.
2. Duan K, Wang R. Surface modifications of bone implants through wet chemistry. J Mater Chem. 2006; 16:2309–21.
3. Ercan B, Kummer KM, Tarquinio KM, Webster TJDecreasedStaphylococcus aureus biofilm growth on anodized nanotubular titanium and the effect of electrical stimulation.ActaBiomater. 2011 Jul; 7(7):3003-12.
4. Donlan RM, Biofilm formation: a clinically relevant microbiological process, Clinical Infectiuos Diseases, 2001; 33(8):1387-1392.
5. Arciola CR et al Biofilm formation in Staphylococcus implant infections.a review of molecular mechanisms and implications for biofilm resistant materials, Biomaterials, 2012; 33:5967-5982.
6. Romano CL et al Antibiofilm agents and implant related infections in orthopaedics:where are we?, Journal of chemotherapy, 2013; 25(2):67-80.
7. RM Donlan, Biofilms and device associated infections, Emerging Infectious Diseases, 2001; 7 (2):277-281.
8. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet 2001.p.135-8.
9. Christensen GD, Simpson WA, Bisno AL, Beachey EH et al. Adherence of slime producing strains of Staphylococciepidermidis to smooth surfaces. Infect Immun 1982; 37:318-26.
10. Freeman J, FalkinerFR,Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol 1989; 42:872-4.
11. Stepanovic S, Vukovi D, Hola V et al. Quantification of biofilm in microtitre plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. APMIS. 2007; 115:891-9.
12. Zimmerli W. Clinical presentation and treatment of orthopaedic implant associated infection. J intern Med. 2014; 276:111-119.
13. Arciola, C R, Campoccia D, Baldassarri L, Donati M E, Pirini V, Gamberini S, Montanaro L. Biofilm formation in Staphylococcus epidermidis from implant infections. Comparison of a PCR-method that recognizes the presence of ica genes with two classic phenotypic methods.J.Biomed. Mater. Res, 76-A:425-430.
14. Khosravi AD, Ahmadi F, Salmanzadeh S, Dashtbozorg A, Montazei EA. Study of bacteria isolated from orthoctions and their antimicrobial susceptibility pattern. Microbial Research J. 2009; 4: 158-163.
15. Anisha F, Meena D. The microbiological profiles of infected prosthetic implants with emphasis on the organisms which form biofilms. Journal of clinical and diagnostic research. 2013; 7(2):219-223.
16. Afreenish Hassan, JavaidUsman, Fatima Kaleem, Maria Omair, Ali Khalid, Muhammad Iqbal. Detection and antibiotic susceptibility pattern of biofilm producing Gram positive and Gram negative bacteria isolated from a tertiary care hospital of Pakistan. Malaysian Journal of Microbiology, 2011; 7(1);57-60.
17. Nixon M, Jackson B, Varghese P, Jenkins D, Taylor G. Methicillin resistant Staphylococcus on orthopaedic wards. J Bone Joint surgery(br) 2006; 88-B:812-817.
18. Sinha P, Sharma R, Rishi S, Sood S, Pathak.D. Prevalence of extended spectrum beta lactamase producers among Escherichia coli isolates in a larefe tertiary care hospital in Jaipur. Indian J Pathol Microbiol. 2008; 51(3):367-369.

Original Article

Journal of Microbiology and Related Research
Volume 3 Number 1, January - June 2017
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.3117.4

Prevalance of Enteric Parasites among Hiv Seropositive Patients and Evaluation of Different Concentration Techniques

Chincholkar V.V.*, Abdagire N.V., Nilekar S.L.***, More S.R.******

Author Affiliation
*Associate Professor, Dept. of Microbiology, GMC Latur. **Assistant Professor ***Associate Professor ****Professor and Head, Dept. of Microbiology, S.R.T.R, Govt. Medical College Ambajogai.

Reprint Request
Chincholkar Vijaykumar V., Associate Professor, Dept. of Microbiology, Government Medical College & Hospital, Latur. Maharashtra-413512.
E-mail: dr_vchincholkar@rediffmail.com

Received on 23.02.2017,
Accepted on 04.03.2017

Abstract

Introduction: Human immunodeficiency virus (HIV) infection is a global pandemic, with cases reported virtually from every country. The gastrointestinal involvement in HIV/AIDS is almost universal, and intestinal parasitic infection remains as an important cause of morbidity and mortality in developing countries. If the number of parasites in the stool specimens are low, examination of a direct wet mount may not detect parasites, hence the stool should be concentrated either by sedimentation or floatation techniques. Therefore the present study was undertaken to determine the prevalence of enteric parasites among HIV seropositive patients with and without diarrhea and to evaluate different concentration techniques for their detection. *Material and Method:* A total of 510 HIV seropositive patients were included in the study. Stool specimens were collected in wide mouthed, clean, dry, plastic containers with tight fittings lids. Microscopic examination was done by direct saline and iodine mount. All the specimens were subjected to formol ether centrifugal sedimentation technique and zinc sulphate centrifugal floatation technique for concentration of parasitic ova and cysts. Sheather's sugar floatation technique was performed for detection of coccidian parasites. The data obtained was analyzed by applying appropriate statistics wherever needed. *Result and Observations:* Out of 510 HIV seropositive patients, intestinal parasites were detected in 132 (25.88%) cases. A total of 149 intestinal parasites were isolated from 132 patients of which 116 patients showed single parasite and 16 showed mixed infections. Detection rate of intestinal parasites by saline and iodine mount was 17.06%. The rate of detection of intestinal parasite by formol ether sedimentation technique and zinc sulphate centrifugal floatation technique was 26.67 % and 10.20 % respectively. A total of 78 (15.29%) coccidian parasites were detected by Sheather's sucrose floatation technique. *Conclusion*: Routine screening of the stool samples of all HIV seropositive patients with diarrhoea and without diarrhoea should be done for prompt patient care, to prevent the fulminant form of the disease. Along with saline and iodine mount, formol ether centrifugal sedimentation technique and Sheather's Sucrose floatation technique can be used to increase diagnostic sensitivity.

Keywords: HIV; Diarrhoea; Concentration Technique; Coccidian Parasites.

Introduction

Human immunodeficiency virus (HIV) infection is a global pandemic, with cases reported virtually from every country [1]. HIV has posed a major challenge to public health in the present time. Since 1981, when it first began to spread widely, HIV has caused the deaths of 25 million people worldwide. Globally an estimated 35.3 (32.2–38.8) million people were living with HIV in 2012 with 2.3 (1.9-2.7) million new HIV infections [2].

The gastrointestinal involvement in HIV/AIDS is almost universal, and a significant disease occurs in 50-90% of the patients while diarrhoea can be a presenting manifestation or life threatening complication in HIV patients [3]. The aetiological spectrum of the enteric pathogens includes bacteria, parasites, fungi and viruses [4]. Intestinal parasitic infection remains as an important cause of morbidity and mortality in developing countries [5]. The broad spectrum of the diseases which are caused by intestinal parasites in HIV patients range from asymptomatic infestations to severe life threatening diarrhoea, dehydration and mal-absorption [6].

If the number of parasites in the stool specimens are low, examination of a direct wet mount may not detect parasites, hence the stool should be concentrated either by sedimentation or floatation technique [7]. Various concentration techniques are used for detection of parasites such as formol ether centrifugal sedimentation technique, zinc sulfate centrifugal floatation technique. Therefore the present study was undertaken to determine the prevalence of enteric parasites among HIV seropositive patients with and without diarrhoea. An attempt was also made to evaluate different concentration techniques for their detection.

Aim and Objectives

1. To study the prevalence of intestinal parasitic infections among HIV seropositive patients.
2. To compare different concentration techniques for detection of intestinal parasites.

Material and Method

The present study was conducted in the Department of Microbiology at S.R.T.R. Govt. Medical College Ambajogai for period of two years from Oct. 2012 to Sep. 2014. Ethical clearance from institutional ethical committee was obtained.

A total of 510 HIV seropositive patients were included in the study. HIV seropositive patients, who had received antiparasitic treatment for diarrhoea in past 3 weeks, were excluded from study. Stool specimens were collected in wide mouthed, clean, dry, plastic containers with tight fittings lids. The patients were asked to collect their stool sample preferably in the morning. No attempt was made to isolate bacteria and viruses.

The specimens were examined by naked eye for colour, consistency, presence of blood, mucus and adult or segments of worms. Microscopic examination was done by direct saline and iodine mount. All the specimens were subjected to formol ether centrifugal sedimentation technique and zinc sulphate centrifugal floatation technique for concentration of parasitic ova and cysts. From formol ether centrifugal sedimentation technique, smears were prepared to identify the coccidian parasites by using modified Ziehl-Neelsen stain and examined with oil immersion objective. Sheather's sucrose floatation technique was performed for detection of coccidian parasites. The data obtained was analyzed by applying appropriate statistics wherever needed.

Result and Observations

In the present study, 510 HIV seropositive patients were screened for intestinal parasites amongst which, males (54.12 %) were more as compared to females (45.88%). Maximum numbers of the patients were seen in the age group of 31-40 years followed by 21-30 years. Out of 510 HIV seropositive patients, intestinal parasites were detected in 132 (25.88%) cases. A total of 149 intestinal parasites were isolated from 132 patients of which 116 patients showed single parasite and 16 showed mixed infections. In our study, most commonly observed intestinal parasite was *Cryptosporidium parvum* followed by *Isospora belli* and least commonly observed parasites were *Hymenolepis nana* and *Taenia spp*.

In our study, detection rate of intestinal parasites by saline and iodine mount was 17.06%. Among 87 isolates, *Cryptosporidium parvum* was most commonly observed parasite followed by *Entamoeba histolytica*.

A total of 136 intestinal parasites were detected either alone or in mixed infection among 119 patients by formol ether centrifugal sedimentation technique. 16 (3.14%) patients showed mixed parasitic infections and the combination of *Cryptosporidium parvum* and *Isospora belli* was most frequently observed. Overall *Cryptosporidium parvum* was most frequently

observed parasite in mixed combination.

In our study, 52 (10.20%) intestinal parasites were detected among 51 patients by zinc sulphate centrifugal floatation technique. In only one patient mixed parasitic infection was seen with a combination of *Giardia lamblia* and *Ascaris lumbricoides*. The rate of detection of intestinal parasite by formol ether sedimentation technique and zinc sulphate centrifugal floatation technique was 26.67 % and 10.20 % respectively and difference was found to be statistically significant.

A total of 78 (15.29%) coccidian parasites were detected by Sheather's sucrose floatation technique, among which 56 were *Cryptosporidium parvum* and 22 were *Isospora belli*. Detection rate of *Cryptosporidium parvum* and *Isospora belli* was more by Sheather's sucrose floatation technique as compared to formol ether centrifugal sedimentation technique but difference was statistically non significant.

Table 1: Distribution of intestinal parasites among HIV seropositive patients (n=510)

Intestinal Parasites	Single	%	Mixed	%	Total	%
Cryptosporidium parvum	46	9.02	10	1.96	56	10.98
Isospora belli	15	2.94	07	1.37	22	4.31
Entamoeba histolytica	18	3.53	02	0.39	20	3.92
Giardia lamblia	09	1.76	04	0.78	13	2.55
Ancylostoma duodenale	10	1.96	01	0.20	11	2.16
Ascaris lumbricoides	07	1.37	04	0.78	11	2.16
Strongyloides stercoralis	05	0.98	05	0.98	10	1.96
Hymenolepis nana	03	0.59	00	0.0	03	0.59
Taenia spp.	03	0.59	00	0.0	03	0.59
Total	116	22.75	33	6.47	149	29.22

Table 2: Various intestinal parasites detected by saline and iodine wet mount (n=510)

Intestinal parasites	Isolates	
	Number	Percentage (%)
Intracellular Protozoans		
Cryptosporidium parvum	26	5.10
Isospora belli	09	1.76
Extracellular Protozoans		
Entamoeba histolytica	16	3.14
Giardia lamblia	13	2.55
Helminths		
Ancylostoma duodenale	05	0.98
Ascaris lumbricoides	06	1.18
Strongyloides stercoralis	08	1.57
Hymenolepis nana	02	0.39
Taenia spp.	02	0.39
Total	**87**	**17.06%**

Table 3: Various intestinal parasites detected by formol ether centrifugal sedimentation technique (n=510)

Intestinal Parasites	Single	%	Mixed	%	Total	%
Intracellular Protozoans						
Cryptosporidium parvum	39	7.65	10	1.96	49	9.61
Isospora belli	11	2.16	07	1.37	18	3.53
Extracellular Protozoans						
Entamoeba histolytica	18	3.53	02	0.39	20	3.92
Giardia lamblia	08	1.57	04	0.78	12	2.35
Helminths						
Ancylostoma duodenale	10	1.96	01	0.20	11	2.16
Ascaris lumbricoides	07	1.37	04	0.78	11	2.16
Strongyloides stercoralis	05	0.98	05	0.98	10	1.96
Hymenolepis nana	02	0.39	00	0.00	02	0.39
Taenia spp.	03	0.59	00	0.00	03	0.59
Total	103	20.20	33	6.47	136	26.67

Table 4: Various intestinal parasites detected by zinc sulphate centrifugal floatation technique (n=510)

Intestinal parasites	Single	%	Mixed	%	Total	%
Extracellular protozoa						
Entamoeba histolytica	18	3.53	00	0.0	18	3.53
Giardia lamblia	12	2.35	01	0.20	13	2.55
Helminths						
Ancylostoma duodenale	10	1.96	00	0.0	10	1.96
Ascaris lumbricoides	07	1.37	01	0.20	08	1.57
Hymenolepis nana	03	0.59	00	0.0	03	0.59
Total	**50**	9.80	02	0.40	52	10.20

Table 5: Coccidian parasites detected by Sheather's sucrose floatation technique (n=510)

Coccidian Parasites	No. of Isolates	%
Cryptosporidium parvum	56	10.98
Isospora belli	22	04.31
Total	**78**	**15.29**

Table 6: Comparative results of formol ether centrifugal sedimentation and zinc sulphate centrifugal floatation technique (n=510)

Intestinal parasites	Formol ether centrifugal sedimentation technique		Zinc sulphate centrifugal floatation technique	
	Total	%	Total	%
Intracellular Protozoans				
Cryptosporidium parvum	49	9.61	0	0
Isospora belli	18	3.53	0	0
Extracellular Protozoans				
Entamoeba histolytica	20	3.92	18	3.53
Giardia Lamblia	12	2.35	13	2.55
Helminths				
Ancylostoma duodenale	11	2.16	10	1.96
Ascaris lumbricoides	11	2.16	08	1.57
Strongyloides stercoralis	10	1.96	0	0
Hymenolepis nana	02	0.39	03	0.59
Taenia spp.	03	0.59	0	0
Total	136	26.67	52	10.20
Z= 10.083, P= <0.001, significant at p <0.005				

Table 7: Comparative findings of formol ether centrifugal sedimentation and Sheather's sucrose floatation technique for detection coccidian parasites (n=510)

Coccidian parasites	Formol ether Centrifugal sedimentation Technique		Sheather's sucrose floatation technique	
	Total	%	Total	%
Cryptosporidium parvum	49	9.60	56	10.98
Isospora belli	18	3.53	22	4.31
Total	67	13.13	78	15.29
X^2 =0.003 p< 0.05, not significant				

Discussion

Acquired immunodeficiency syndrome (AIDS) ranks among the most dreaded diseases affecting mankind, causing dysfunction of both limbs of the immune system, resulting in overwhelming and fatal opportunistic infections. Gastrointestinal involvement primarily in the form of diarrhoea is a universal problem affecting almost 90% of HIV infected patients in developing countries [8]. The etiological spectrum of the enteric pathogens which cause diarrhoea includes bacteria, parasites, fungi and viruses [9]. The parasites can cause self-limiting diarrhoea of short duration in healthy individuals, but in the immunocompromised host including AIDS patients, the diarrhoea is usually chronic and sometimes, life-threatening [10]. The coccidian parasites are foremost among the enteric parasites in HIV seropositive patients with diarrhoea [11].

In our study, prevalence of intestinal parasites among HIV seropositive patients was 25.88% (132/510). The results comparable to our study were shown

by Mehta K et al [12], Gupta M et al [13], Gupta S et al [14] and Mohandas K et al [15] while Babatunde S K et al [16], Dwivedi KK et al [8], and Basak S et al [17] have documented higher prevalence. The prevalence of intestinal parasitic infections among HIV seropositive patients ranged from 17.3% to 87.8% in different parts of the world. The difference in the pattern of prevalence of intestinal parasites seen in the present study and others may be attributed to the regional variability (demographic and ecological factors) of the pathogen, behavioural activities, diagnostic methods used, asymptomatic shedding of oocysts and the use of prophylactic drugs etc.

The diagnosis of intestinal parasites in stool was established by identification of ova and cysts by variety of techniques including direct wet mount preparation, sedimentation and floatation method.

In our study, the detection rate of intestinal parasites by saline and iodine mount was 17.06% while Parameshwarappa KD et al [18] (38%) and Mergani MH et al [19] (28.19%) found higher detection rate by saline and iodine mount in their study. If the number of organisms in the stool specimens is low, examination of a direct wet mount may not detect parasites, hence the stool should be concentrated. Eggs, cysts and larvae are recovered after concentration procedures whereas trophozoites get destroyed during the concentration procedure. This makes direct wet mount examination obligatory as the initial phase of microscopic examination [7].

We used different concentration techniques i.e. formol ether centrifugal sedimentation technique, zinc sulphate centrifugal floatation technique and Sheather's sucrose floatation technique to detect the maximum number of intestinal parasites from patients.

In our study, the detection rate of intestinal parasite by formol ether centrifugal sedimentation technique was 26.67%. Comparable detection rates were reported by Abbas M et al [20] (19.35%), Puri J et al [21] (26.75%) and Mergani H et al [19] (30.16%) while higher rate of detection was reported by Parameshwarappa KD et al [18](56.88%) and Balakrishna J et al [22] (83%).

In the present study, 116 HIV seropositive patients showed single parasite and 16(3.14%) showed mixed infections. Similar to our study, Anand B et al [23] and Gupta S et al [14] in their study reported mixed infection in 3% and 3.53% of patients respectively, whereas Awole M et al [24], Kotgire S et al [25], Fekadu S et al [26] and Kashyap B et al [27] showed 14.06%, 8%, 10.6% and 11% mixed infections among HIV seropositive patients respectively. Mixed infections is a common observation in areas where various types of parasites are prevalent and also due to poor hygienic practices.

The combination of *Cryptosporidium parvum* and *Isospora belli* was most frequently observed. Overall *Cryptosporidium parvum* was most frequently observed parasite in mixed infections. Similar to our study, Amatya R et al [28] noted *Cryptosporidium parvum* as most commonly observed parasite in mixed combination. While Anand B et al [23] and Gupta S et al [14] in their study showed *Isospora belli* was most frequently observed parasite in mixed combination.

In the present study, by zinc sulphate centrifugal floatation technique, 52 intestinal parasites were detected from 51 patients and mixed infection was seen in only one patient with a combination of *Ascaris lumbricoides* and *Giardia Lamblia*. The detection rate of intestinal parasites by zinc sulphate centrifugal floatation technique was 10.20%. Mergani H et al [19] (26.88%), Abbas M et al [20] (36.64%) and Parameshwarappa et al [18] (55%) noted a higher detection rate of intestinal parasites as compared to our study. Oocysts of *Cryptosporidium parvum*, *Isospora belli*, eggs of *Taenia spp* and larvae of *strongyloides stercoralis* are not detected by zinc sulphate centrifugal floatation technique. These parasites were maximally detected in our study, so this may be the reason for low detection rate of intestinal parasites in zinc sulphate centrifugal floatation technique.

In the present study for detection of coccidian parasites, we performed Sheather's sucrose floatation technique, as it is recommended for the detection of coccidian parasites [7]. Sheather's sucrose floatation technique detected 78 (15.29%) coccidian parasites amongst which 56 (10.98%) were *Cryptosporidium parvum* and 22 (4.31%) were *Isospora belli*. Mergani H et al [19] and Scott et al [29] detected 3.60% and 18.1% *Cryptosporidium parvum* by Sheather's sucrose floatation technique respectively.

In our study, 67 coccidian parasites were detected by formol ether centrifugal sedimentation and 78 parasites were detected by Sheather's sucrose floatation technique. The Sheather's sucrose floatation technique detected slightly more number of coccidian parasites compared to formol ether centrifugal sedimentation technique. However, this difference was not statistically significant. So we can say that for the detection of coccidian parasites, formol ether centrifugal sedimentation technique followed by modified Z-N staining is as effective as Sheather's sucrose floatation technique. Similar findings were shown by Mergani H et al [19] and Scott et al [29] in their study for detection of *Cryptosporidium parvum*.

In the present study, oocysts of Cyclospora were not detected. This can be attributed to varying geographical distribution of parasites.

No intestinal parasites were detected in 166 patients with diarrhoea in the present study which may be attributed to other diarrheaogenic agents like bacteria, viruses and fungi. The identification of which was not done in our study. In our study, significant number of intestinal parasites were also seen in HIV seropositive patients without diarrhoea thus indicating that there may be asymptomatic infections which may be going undiagnosed, thereby increasing the morbidity and mortality which are associated with them.

Conclusion

Detection of intestinal parasites in HIV seropositive patients will help in proper management of these patients as drugs are available for the treatment of most of the infections. Hence, routine screening of the stool samples of all HIV seropositive patients with diarrhoea and without diarrhoea should be done for prompt patient care, to prevent the fulminant form of the disease. Along with saline and iodine mount, formol ether centrifugal sedimentation technique and Sheather's Sucrose floatation technique can be used to increase diagnostic sensitivity.

References

1. Fauci SA . Human Immunodeficiency Virus Disease: AIDS and related disease. In: Kasper LD, Braunwald E, Fauci SA, Hauser SL, Lango DL JL, editor. Harrison's Principle of Internal Medicine. 17 th. New York: Mc Graw-Hill Medical Publishing Divison,; 2008.p.1506–87.
2. UNAIDS report on the global AIDS epidemic 2013. Geneva; 2013.
3. Okodua M, Age HO. Age and Sex Distribution of Intestinal Parasitic Among HIV Infected Subjects in Abeokuta, Nigeria. Online J Heal Allied Scs. 2003; 2(4):1–5.
4. Kulkarni S V., Kairon R, Sane SS, Padmawar PS, Kale V , Thakar MR, et al. Opportunistic parasitic infections in HIV/AIDS patients presenting with diarrhoea by the level of immunesuppression. Indian J Med Res. 2009; 130(1):63–6.
5. Zali MR, Mehr AJ, Rezaian M, Meamar AR, Vaziri S, Mohraz M. Prevalence of Intestinal Parasitic Pathogens among HIV-Positive Individuals in Iran. Jpn J Infect Dis. 2004; 57:268–70.
6. Vyas N, Sood S, Sharma B. The Prevalence of Intestinal Parasitic Infestation and the Related Profile of the CD 4+ Counts in HIV / AIDS People with Diarrhoea in Jaipur City. J Clin Diagn Res. 2013; 7(3):454–6.
7. Ichhpujani R., Baveja R. Practical Parasitology. Medical Parasitology. 1 st. New Delhi: Jaypee Brothers; 1994.p.353–80.
8. Dwivedi KK, Prasad G, Saini S, Mahajan S, Lal S, Baveja UK. Enteric Opportunistic Parasites among HIV Infected Individuals/ : Associated Risk Factors and Immune Status. Jpn J Infect Dis. 2007; 60:76–81.
9. Sachin D, Ruchi K, Santosh S. The Prevalence of Intestinal Parasitic Infections in HIV Infected Patients in a Rural Tertiary Care Hospital of Western Maharashtra. (A 5 year study). J Clin Diagn Res. 2011; 5(2):210–2.
10. Raytekar NA, Saini S, Deorukhkar S. Intestinal Parasitic Prevalence in HIV Infected Patients With & Without Diarrhoea and Its Association With CD4 T cells Counts. Int J Biomed Adv Res. 2012; 3(11):853–7.
11. Mohanty I, Panda P, Sahu S, Dash M. Prevalence of isosporiasis in relation to CD4 cell counts among HIV-infected patients with diarrhea in Odisha, India. Adv Biomed Res. 2013; 2(61).
12. Mehta KD, Vacchani A, Mistry MM, Kavathia GU, Goswami YS. To Study the Prevalence of Various Enteric Parasitic Infections Among HIV Infected Individuals in the P.D.U. Medical College and Hospital, Rajkot, Gujarat, India. J Clin Diagn Res. 2013 Jan; 7(1):58–60.
13. Gupta M, Sinha M, Raizada N. Opportunistic Intestinal Protozoan Parasitic Infection in HIV Positive Patient in Jamnagar, Gujarat. SAARC J Tuberc Lung Dis HIV/AIDS. 2008; 1:1–4.
14. Gupta S, Narang S, Nunavath V, Singh S. Diarrhoea In HIV Patients: Prevalence Of coccidian Parasites. Indian J Med Microbiol. 2008; 26(2):172–5.
15. Mohandas K, Sehgal R, Sud A, Malla N. Prevalence of Intestinal Parasitic Pathogens in HIV-Seropositive Individuals in Northern India. Jpn J Infect Dis. 2002; (75):83–4.
16. Babatunde SK, Salami AK, Fabiyi JP, Agbede OO, Desalu OO. Prevalence of intestinal parasitic infestation in HIV seropositive and seronegative patients in Ilorin, Nigeria. Ann Afr Med. 2010; 9(3):123–8.
17. Basak S, Bose S, Mallick SK, Ghosh AK. Intestinal Parasitic Infections In HIV Seropositive Patients –. J Clin Diagn Res. 2010; 4:2433–7.
18. Parameshwarappa K, Chandrakanth C, Sunil B. The Prevalence of Intestinal Parasitic Infestations and the Evaluation of Different Concentration Techniques of the Stool Examination. J Clin Diagn Res. 2012;

6(7):1188–91.

19. Mergani MH, Mohammed MA, Khan N, Bano M, Khan AH. Detection of intestinal protozoa by using different methods. Dent Med Res. 2014; 2(2):28–32.
20. Faraj AM. A Coprological Diagnostic Comparison Between Zinc Sulphate Floatation and Formalin/: Ether Sedimentation with Two Natural Extracts (Pomegranate Molasses and Honey). Diyala J Med. 2011; 1(2).
21. Puri J. Comparison of stool concentration methods for detection of prevalence of enteroparasitic infection in rural tertiary care teaching hospital of Maharashtra. Indian J Basic Apllied Med Res. 2014; 3(4):158–62.
22. Balakrishna J, Venkateswarlu S, Bai K. Comparison of Modified 1 % Potassium Hydroxide Formol-Ether Concentration Technique with Direct Wet Mount Preparation and Standard Formol-Ether Concentration Technique for Detection of Parasites in Stool. Pharma Sci Res. 2013; 5(7):147–9.
23. Janagond AB, Sasikala G, Agatha D, Ravinder T, Thenmozhivalli PR. Enteric Parasitic Infections in Relation to Diarrhoea in HIV Infected Individuals with CD4 T Cell Counts <1000 Cells/ìl in Chennai, India. J Clin Diagn Res. 2013 Oct; 7(10):2160–2.
24. Awole M, Solomon G, Kassa T, Kibru G. Prevalence of Intestinal parasites in HIV - infected adults in Southwestern Ethipia. Ethiop J Health Dev. 2003; 17(1):71–8.
25. Santosh K, Nilima T. Evaluation of Parasite Causing GIT (Gastro-intestinal Tract) Iinfestations in HIV / AIDS Patients. Int J Basic Appl Med Sci. 2011; 1(1):61–5.
26. Fekadu S, Taye K, Teshome W, Asnake S. Prevalence of parasitic infections in HIV-positive patients in southern Ethiopia: a cross-sectional study. J Infect Dev Ctries. 2013 Nov; 7(11):868–72.
27. Bineeta K, Sanchaita S, Shukala D, Nitesh R, Rajat J. Efficiency of diagnostic methods for correlation between prevalence of enteric protozoan parasites and HIV/AIDS status––an experience of a tertiary care hospital in East Delhi. J Parasit Dis. 2010; 34(2):63–7.
28. Amatya R, Shrestha R, Poudyal N, Bhandari S. Opportunistic intestinal parasites and CD4 count in HIV infected people. J Pathol Nepal. 2011; 1:118–21.
29. McNabb SJN, Hensel DM, Welch DF, Heijbel H, McKee GL, Istre GR. Comparison of sedimentation and flotation techniques for identification of Cryptosporidium sp. oocysts in a large outbreak of human diarrhea. J Clin Microbiol. 1985; 22(4):587–9.

Subscription Form

I want to renew/subscribe international class journal **"Journal of Microbiology and Related Research"** of Red Flower Publication Pvt. Ltd.

Subscription Rates:
• Institutional: INR8000/USD751

Name and complete address (in capitals): _____

_____

Payment detail:
Ch/Dd No.
Date of Ch/DD
Amount paid Rs./USD

1. Advance payment required by Demand Draft payable to Red Flower Publicaion Pvt. Ltd. payable at Delhi.
2. Cancellation not allowed except for duplicate payment.
3. Agents allowed 10% discount.
4. Claim must be made within six months from issue date.

Mail all orders to
Subscription and Marketing Manager
Red Flower Publication Pvt. Ltd.
48/41-42, DSIDC, Pocket-II
Mayur Vihar Phase-I
Delhi - 110 091(India)
Phone: 91-11-45796900, 22754205, 22756995, Fax: 91-11-22754205
E-mail: sales@rfppl.co.in

Original Article

Journal of Microbiology and Related Research
Volume 3 Number 1, January - June 2017
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.3117.5

Co-Selection of Multiple Heavy Metals Resistance and Multiple Antibiotics Resistance in Coliform Bacteria Present in Hasdeo River in Korba and Champa Region

Rajshree Singh*, Sweta Sao**

Author Affiliation
*Assistant Professor, Dept. of Microbiology, Shri Agrasen Girls College, Korba, Chhattisgarh, India. **HOD, Dept. of Microbiology, Dr. C.V. Raman University, Kota, Bilaspur, Chhattisgarh, India.

Reprint Request
Rajshree Singh, Assistant Professor, Dept. of Microbiology, Shri Agrasen Girls College, Korba, Chhattisgarh–495678, India.
E-mail: princessrajshree2011@gmail.com

Received on 13.02.2017,
Accepted on 23.02.2017

Abstracts

Prevalence of multiple antibiotics resistance in microorganisms was well known but also represents a serious threat to public health. Pollution of Hasdeo river due to heavy metals and Coliform bacteria increases with industrialization, urbanization and continuous discharge of domestic waste from livestock facilities. Pollution of river water was one of the greatest concerns for the water consumers with respect to the quality of river water of Hasdeo in Korba and Champa region. The present study deals with isolation identification and enumeration of Coliforms bacteria isolated from Hasdeo river in Korba and Champa region, and examine pattern of their multiple heavy metals resistance capacity and multiple antibiotic resistance together.

Keywords: Coliform Bacteria; Microorganisms; Antibiotics.

Introduction

Ecosystem of any region deal with the quantitative interaction of species population and their communities in complex system and resources with various biotic and abiotic factors affecting directly or indirectly. Water plays crucial role in the entire ecosystem as it was most important necessity of life which has been exploited most as compared to any other resource for the sustenance of his life; hence the quality of Water contaminated by various pathogenic microorganisms, Coliforms and various industrial effluents was considered to be one of the greatest concerns. Heavy metals contamination of river water pose severe threat to the biodiversity by entering into the food chain thus effecting health of human as they persists for long time in food chain and causes irreversible damage to health. With the increasing industrialization and urbanization and various anthropogenic activities such as industrial discharge, human activities, agricultural waste disposal and sewer drained in to river water causing heavy metals pollution and microbial pollution of water which are mostly enteric origin (Bonetta et al., 2011; Bayoumi Hamuda and Patko, 2012) Hasdeo river which was tributary to Mahanadi and most important river of Chhattisgarh was found to be most polluted at Korba region, due to industrial drainage of various power plants and small scale industries located in Korba, (Vaishnav and Hait, 2013; Rajshree and Shweta, 2015). Microbial contaminationof water due to pathogenic Coliforms was one of the most important problems for the water quality management and important factor for water pollution for Hasdeo river water. Like wise microbial pollution, pollution of the Hasdeo River water due to toxic heavy metals was also the most persistent problem and was increases continuously with industrial progress although levels of pollution and its impact on environment differ from place to place.

The abilities of bacteria species to adapted and become metal tolerant to toxic concentrations of heavy

metals through various resistance mechanisms (Avezzu et al., 1995; Hemme et al., 2010; Davis et al., 2003; Lee et al., 2006) can be used as a tool in bioremediation for the treatment of effluents contaminated. Studies suggest that *Staphylococcus* was found to resistant against Cd, Cu, Pb, Zn, As and Fe. *Acinetobacter radioresistens* was resistant towards As and Ni (Raja et al., 2009; Bisht et al.,2012), ability of *Bacillus pumilus* and *Staphylococcus sp.* to reduce Cr was reported by Farah et al., 2010. Which was also supported by Nanda and Abraham, 2011).

The discharge of domestics waste such as pharmaceutical waste and antibiotics were also discharged in river water causing Development of multiple antibiotic resistances in bacterial species present in water resources was serious threat for safety of environment and human health as it carries a negative impact for public health (Fick et al., 2009), exposure on microorganisms to the multiple drug residues and their constant exposure may lead to mutations and development of new strains for better survival (Calomiris et al., 1994) which pose a great threat to human health management. Identification of antibiotic resistance bacteria also reveals about the source of pollution (Middleton and Salierno, 2013; Shah et al., 2012) and had added a new dimension to the risk posed by the presence of antibiotics and drug residues.

Materials and Methods

Description of Sampling Sites

Water samples were collected from different locations of Korba and Champa in four replicates aseptically in sterile 500mL Duran Schott glass bottles, labeled properly and transported on ice bucket to the laboratory for analysis. Aliquots of the samples were used for heavy metals and selective isolation of different bacterial species based on standard microbiological procedures.

Analysis of Heavy Metal Concentration

Contents of the heavy metals in water samples were analyzed by AAS (Thermo Scientific, UK) following the method of American Public Health Association (APHA). Concentration of all heavy metals in water expressed as mg/L and all the samples were tested in four replicates.

Isolation of Heavy Metal Resistant Bacteria from Hasdeo River Water

Bacterial species resistant to Cd, Cu, Fe, As and Pb were isolated from water samples by diluting them in sterile phosphate buffer saline solution and 200µl from each dilutionswere plated on Nutrient Agar supplemented in the form of metal salts as $CdCl_2$, $CuCl_2$, $PbCl_2$, $Fe_3SO_4$and $AsCl_2$ at the concentration of 50ppm. The plates were incubated at 37° C for 24-48 hours. Bacterial colonies were isolated and were sub-cultured to obtain pure culture on Nutrient agar. The isolates were partially identified according to Bergey's Manual of Systematic Bacteriology (Bergey et al., 1994).

Estimation of Total Coliforms

Most Probable Number (MPN) test was performed to assess the domestic pollution level in the selected area for total Coliform count. The technique involves three successive steps namely, presumptive test, confirmatory test and completed test. This method has direct application in quantification studies for media and for alternate microbiological methods. The number of broth tubes producing gas used to determine the statistical range of coliform. Confirmed and positive test are used to calculate the MPN.

Cultural Characteristic of Bacteria

Bacterial isolates were identified based on their morphology, staining reaction and molecular marker. Microorganisms show diverse culture characters and the diversity depending upon the type of medium used for culture. Cultural characteristic such as size, colour, texture margin, elevation, consistency etc. were observed for the identification of the bacterial isolates. Thesecolony characters, morphology and staining reaction helps in preliminary identification.

Biochemical Characterization of the Isolates

The biochemical tests performed include Gelatin hydrolysis test, Starch hydrolysis test, Casein hydrolysis test, Catalase activity test, Glucose Fermentation test, Citrate utilization test, Nitrate reduction test, Oxidase Test, VP -Voges-Proskauer Test ("Vi"), Urease Test. These biochemical tests were performed as per standard Microbiological methods (Cappuccino and Sherman, 2007).

Antibiotic Sensitivity

Antibiotic resistance of the metal tolerant species was determined by Kirby-Bauer disc diffusion method (Barry and Thornsberry, 1981) with using 6 mm diameter discs antibiotics discs: tigecyline, terramycin, lactoclave, Amoxycillin, Penicillin,

Ecoflox, Ofloxacin and tetracyclin (Himedia, India). Inhibition zones formed around antibiotic discs were evaluated according to the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2011). Whereas antibiotics sensitivity of Ticarcillin. Piperacillin, Clavulanic acid, Ceftazidine, Cefoperazone/sulbactam, cefepime, aztreonam, doripenem, imipenam, meropenam, amikacin, gentamicin, ciprofloxacin, levofloxacin, minocyclin, tigercyclin, colistin, trimethoprim/sulpamethoxazole, kanamycin, erythromycin, streptomycin, oxytetracyclin and neomycin were tested in VITEK-2C (Biomerieux, France) as per manufacturers instruction. The bacterial isolates were scored assusceptible, intermediate or resistant to a given antibiotic bythe inhibition zone diameter around the antibiotic disc.

Multiple antibiotics resistance (MAR) index were calculated as A/B, where 'A' represents the number of antibiotics to which the isolate was resistant and 'B' represents the total number of antibiotics tested (Kruperman, 1983).

Results and Discussions

In the present study water samples were collected from Hasdeo river water at different locations of Korba and Champa, and bacterial species resistance to heavy metals were isolated, identification and characterization based on staining techniques, morphological examination, colony characteristics, biochemical tests reveals that the bacterial species were *E.coli, Klebsiella pneumonia, Salmonella typhi, Pseudomonasa aeruginosa, Shigellaflexneri, Yersinia entrocolitica, Staphylococcus aureus, Bacillus pumilus, Bacillus altitudinis, Bacillus subtilis , Pseudomonas stutzeri.* 200 colonies were screened from initial level of heavy metal supplemented LB medium. The bacterial isolates showed optimum growth at 30°C and pH 7.0. The morphological, colony characteristics and biochemical characteristics of sewage bacteria were shown in table 1, 2, 3.

Table 1: Table represents the morphological Characteristics of bacteria species isolates from river water samples of Hasdeo at different locations of Korba and Champa

S. No.	Names of bacteria	Gram staining	Shapes	Arrangement	Sizes in (μm)
1	*E.coli*	-ve	Rods	Single	3μm
2	*Enterobacterfaecalis*	-ve	Rods	Single	4 μm
3	*Staphylococcus aureus*	+ve	Cocci	Grapes like cluster	1 μm
4	*Pseudomonas sp.*	-ve	Cocci	Single/pair/short chain	1 μm
5	*Yersinia sp.*	-ve	Rod	Single/chain/cluster	-.
6	*Shigella sp.*	-ve	Rod	Single/pair/short chain	-
7	*Klebsiella pneumonia*	-ve	Rod	Single/pair/short chain	2 μm
8	*Salmonella typhi*	-ve	Rod	Single	2 μm
9	*Bacillus pumilus*	+ve	Rod	Single/cluster	1.0-5.0 μm
10	*Bacillus altitudinis*	+ve	Rod	Pair/Cluster	2.0-3.0 μm
11	*Bacillus subtilis*	+ve	Rod	Cluster	2 μm

Table 2: Data represent the cultural characteristics of bacteria species isolates from hasdeo river water samples at different locations ofKorba and Champa

S. No.	Names of Isolated organisms	Form	Color	Margin	elevation	Odour
1.	*E. Coli*	Circular	White/metallic sheen	Entire	Raised	Fecal odour
2.	*Enterobacterfaecalis*	Irregular, large	Lack of sheen	Entire	convex	Fecal odour
3.	*Staphylococcus aureus*	Circular	Golden yellow	Entire	convex	Unpleasant
4.	*Pseudomonas aeruginosa*	Circular	Flour yellow	Undulate	Raised	Fruity
5.	*Salmonella typhi*	Circular	Colourless	Entire	Raised	Unpleasant
6	*Yersinia sp.*	Circular	White	Entire	Raised	Fruity pineapple
7	*Klebsiella pneumonia*	Irregular	Blue colour	Entire	convex	Yeasty odour
8	*Shigellasp*	Irregular	Yellow colour	Lobate	convex	Fruity odour
9	*Bacillus pumilus*	Irregular	White	Regular	convex	Unpleasant
10	*Bacillus Subtillus*	Circular	Golden brown	Entire/Undulate	Convex	Foots odour
11	*Bacillus altitudinis*	Circular	creamy	Entire	Convex	Ammonia like

Table 3: Data showing the biochemical characterization of bacterial isolates isolated from the different location of river water Hasdeo in Korba and Champa

	Gelatin hydrolysis	Starch hydrolysis	Casein hydrolysis	Catalase activity	Glucose Fermentation tubes	VP -Voges-Proskauer Test	Citrate utilization test	Nitrate reduction test	Oxidase Test:
E. Coli	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	MR+VP-	**-ve**	**+ve**	**-ve**
Klebsiella pneumonia	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	MR-VP+	**+ve**	**-ve**	**-ve**
Pseudomonas sp.	**+ve**	**-ve**	**-ve**	**+ve**	**-ve**	MR-VP-	**+ve**	**-ve**	
Staphylococcus aureus	**+ve**	**-ve**	**+ve**	**+ve**	**+ve**	MR-VP-	**D**	**+ve**	**-ve**
Baciluspumilus	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	MR-VP	**-ve**	**+ve**	**+ve**
Bacillus altitudinis	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	MR-VP-	**-ve**	**-ve**	**+ve**
Enterococcus faecalis	**-ve**	**+ve**	**-ve**	**-ve**	**+ve**	MR-VP-	**+ve**	**-ve**	
Bacillus subtilis	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**	MR-VP-	**-ve**	**-ve**	**+ve**

Isolation and Identification of Metal resistant bacteria and evaluation metal tolerance

Table represents sample analysis from the river water of Hasdeo, Data reflects the presence of *E.coli, Pseudomonasa sp., Bacillus sp.*, and *Enterococcus faecalis* in the river water of Hasdeo and these are showing resistant to the heavy metal pollution.

Table 4: Table shows the average density (Percent) of microorganisms having heavy metal remediation capacity isolated from Hasdeo river water from different location of Korba and Champa

S. No	Name of organisms	Average density (%) of organism
1.	*Bacillus subtillus*	14%
2.	*Pseudomonas aeruginosa*	13%
3.	*Pseudomonas stutzeri*	12%
4.	*Enterococcus faecalis*	20%
5.	*Bacillus pumilus*	11%
6.	*E. Coli*	20%
7.	*Bacillus altitudinis*	10%

Maximum metal remediation capacity was observed in *Pseudomonas sp.* towards multiple heavy metals (Cd, Cu, Pb, Zn, As and Fe) with varying degree of removal capacity in optimal growth conditions. The results were also supported by various other researchers. Lin and Harichund, (2011) reported heavy metal resistance of *Pseudomonas areuginosa* towards Cd, Co, Cu, Pb and As. Metal tolerance capacity of *Pseudomonas fluorescens* towards Pb, Ni, Cu, Cr and Cd was also reported by Wasi et al., (2011) and Selvi et al., (2012). *Bacillus sp*. was also showing great degree of metal resistance towards various metals.

Antibiotic Resistance

An antibiotic is a kind of ubiquitous contaminant in the aquatic environment with industrial effluents and sewage discharge. All the isolates were showed significant resistant against all the tested antibiotics. E. coli showed highest degree of resistance against all the antibiotics than other isolated bacteria.

It was also reported that the metal resistance capacities of the microbes are mainly associated with antibiotic resistance. Antibiotics resistance was tested against *Pseudomonas sp.,* and *Bacillus sp.,* All the bacterial strains showed different degrees of sensitivity against Ticarcillin. Clavulanic acid, Ceftazidine, Cefoperazone/sulbactam, cefepime, aztreonam, doripenem, imipenam, meropenam, amikacin, gentamicin, ciprofloxacin, levofloxacin, minocyclin, tigercyclin, colostin, trimethoprim/ sulpamethoxazole, kanamycin, erythromycin, streptomycin, oxytetracyclin, lactoclave, amoxycillin, penicillin, ofloxacin and neomycin but all bacterial strains showed resistance against Ecoflox, terramycin, tetracycline, and bacitracin. *Pseudomonas sp., Bacillus sp.* were shown to sensitive against almost all the antibiotics used but maximum sensitivity was reported with doripenem, imipenam, meropenam (MIC<=0.25). All the species show were showing least sensitivity to trimethoprim/Sulfamethoxazole (MIC=20). It was previously reported that the metal resistance has been reported to hold an association with antibiotic resistance (Verma et al., 2001). It was assume that under metal stress, metal and antibiotic resistance in microorganisms possibly helps them to adopt faster by the spread of resistant factors rather than by mutation and/or natural selection (Silver and Misra, 1988).

Table 5: MAR Index of Sample Collection site (Korba, Champa) + sign indicates sensitive against antibiotics, whereas – sign represents resistance towards antibiotics

	Antibiotics	Korba	Champa
1	ticardin	+	+
2	piperacillin	+	+
3	ceflazidime	+	+
4	cefepime	+	+
5	Aztreonam	+	+
6	doripenem	+	+
7	doripenem	-	+
8	Imipenem	-	+
9	Amikacin	+	+
10	Gentamicin	+	+
11	Ciproflaxacin	+	+
12	minocycline	+	+
13	Colistin	+	+
14	trimethoprim	+	+
15	Sulphamethoxazole	+	+
16	tigecyline	+	+
17	terramycn	-	-
18	lactoclave	+	+
19	Amoxycillin	+	+
20	Penicillin	+	+
21	Ecoflox	-	-
22	Ofloxacin	-	-
23	Tetracyclin	+	+
25			
MAR Index		0.208	0.130

MAR index of Water samples collected from Champa region were recorded 0.16 whereas it was recorded 0.208; MAR index more than 0.2 indicated that the isolates were from high-risk contamination sources such as human wastes, commercial animal farms where antibiotics used often, MAR index less than 0.2 indicated that the strain was from animals in which antibiotics were seldom or never used (Matyar et al., 2008; Kruperman, 1983; Vivekanandhan et al., 2002). Based on MAR index and heavy metal analysis, River water at Korba was found to be most polluted among all the collection sites.

Conclusion

This study revealed the prevalence of multiple antibiotics resistant and multiple heavy metal resistance in bacterial species present in Hasdeoriver in Korba and Champa region. Presence of multiple antibiotic resistances in bacteria; species showing multiple heavy metal resistance was most likely due to the spread and evolution of antibiotic resistance may be triggered by anthropogenic pollutant such as heavy metals (Baker-Austin et al., 2006) and co-selection of both antibiotics and heavy metal resistance in bacterial species.

It was very clear that maximum discharge of domestic waste and settled effluent were deposited at river in Korba region, which was considered to be the reason for high level of pollution of river water at that region. The feacal coliform bacteria are numerous in this riverine water might be due to the various anthropogenic activity particularly sewage. These microorganisms may be repeated from the total coliform group by their capability to grow and proliferate at elevated temperature. This study was thus pertinent to the present situation of environmental pollution and climate change. Due to these plenty of reasons, importance has been put on the antibiotic resistance status of such bacterial isolates and causing various bacterial species resistance to multiple antibiotics resistance.

References

1. Avezzu, F., Bissolotti, G., Collivignarelli, C., Ghirardini, A.V. Behavior of heavy metals in activated sludge biological treatment of landfill leachate. J. Waste Manage. Res. 1995; 13:103-121.
2. Baker-Austin, C., Wright, M.S., Stepanauskas, R., and McArthur, J.V. Co-selection of antibiotic and metal resistance.Trends Microbiol. 2006; 14:176–182.
3. Barry, A.L., and Thornsberry, C. Susceptibility testing. In: Manual of clinical microbiology, Lennette E.H., Balows A.W., Hausler J., Truant J.P. (Eds.) Washington, American Society for Microbiol. 1981;

561–574.

4. Bauer, A. W., Kirby, W. M., Sherris, J. C., and Turck, M. Antibiotic susceptibility testing by a standardized single disk method. American J. of Clinical Pathol. 1966; 45(4):493–496.
5. BayoumiHamuda, H. E. A. F., Patko, I. Ecological monitoring of Danube water quality in Budapest region. Am. J. Environ. Sci. 2012; 8:202–211.
6. Bergey, D.H., Holt, G.H., Krieg, N.R., and Peter, H.A.S. Bergey's manual of determinative bacteriology. 9th edition.Lippincott Williams and Wilkins. ISBN 0-683-00603-7.
7. Bisht, S.S., Praveen, B., Rukmini, M., and Dhillon, H. Isolation and Screening of Heavy Metal Absorbing Bacteria from the Industry Effluent Sites of the River Nagavali. Inter. J Pharm. Sci., Res. 2012; 3(5):1448-1451.
8. Bonetta, S., Borelli, E., Bonetta, S., Conio, O., Palumbo, F. and Carraro, E. Development of a PCR protocol for the detection of *Escherichia coli O157:H7 and Salmonella spp.* in surface water. Environ. Monit. Assess. 2011; 177: 493–503.
9. Calomiris, J.J., Armstrong, J.L., Seidler, R.J. Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Appl. Environ. Microbiol. 1994; 47: 1238.
10. Cappuccino, J., and Sherman, N. Microbiology-A laboratory manual 8th Edition. Benjamin-Cummings Publisher. 2007.
11. CLSI. Performance standards for antimicrobial susceptibility testing: twenty-first Informational Supplement, Clinical and Laboratory Standards Institute. 2011; 31.
12. Davis, A.T., Volesky, B., Alfonso, M. A review of the biochemistry of heavy metal biosorption by brown algae. Water Res. 2003; 37:4311-4330.
13. Farah, R., Tabassum, S.S., Rehman, A., and A. R. Shakoori, A.R., Isolation and Characterization of Cr6+Reducing Bacteria and Their Potential Use in Bioremediation of Chromium Containing Wastewater. Pakistan J. Zool. 2010; 42(6):651-658.
14. Fick, F., Hanna, S. M., Lindberg, R.H., Phan, C., Tysklind, M., and Larsson D.G.J. Contamination of surface, ground, and drinking water from Pharmaceutical production. Environ. Toxicology and Chem. 2009; 28(12):2522–2527.
15. Hemme, C.L., Deng, Y., Gentry, T.J., Field, M.W., Wu, L., Barua, S., Barry, K., Tringe, S.G., Watson, D.B., He, Z., Hazen, T.C., Tiedje, J.M., Rubin, E.M., Zhou, J. Metagenomic insights into evolution of a heavy metal contaminated groundwater microbial community. ISME, 2010; 4:660-672.
16. Krumperman P.H. Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol. 1983; 46(1):165.
17. Lee, C. M., Lin, T. Y., Lin, C.-C., Kohbodi, G. A., Bhatt, A., Lee, R., et al. Persistence of fecal indicator bacteria in Santa Monica Bay beach sediment. Water Res. 2006; 40(14):2593–2602.
18. Lin, J., and Harichund, C., Isolation and characterization of heavy metal removing bacterial bioflocculants.African J. of Microbiol. Res. 2011; 5(6): 599-607.
19. Matyar, F., Kaya, A., and Dinçer, S. Antibacterial agents and heavy metal resistance in Gram-negative bacteria isolated from seawater, shrimp and sediment in Iskenderun Bay, Turkey. Sci. Total Environ. 2008; 407:279.
20. Middleton, J.H., and Salierno, J.D. Antibiotic resistance in triclosan tolerant fecal coliforms isolated from surface waters near waste water treatment plant outflows (Morris County, NJ, USA). Ecotoxicol. Environ. Saf. 2013; 88:79–88.
21. Nanda, S., and Abraham, J. Impact of heavy metals on the rhizosphere microflora of Jatrophamultifida and their effective remediation. Afr. J. Biotechnol. 2011; 10:11948-11955.
22. Raja, C.E., Edward, C., Selvam, G.S. and Kiyoshi, O. Isolation, identification and characterization of heavy metal resistant bacteria from sewage. In: Proceedings of the International joint symposium on geodisaster and geo-environment in Asia, JS Fukuoka, Japan, 2009; 205-211.
23. Rajshree, S., and Shweta, S. Evaluation of water quality by physicochemical parameters, heavy metal and use of metal resistant property of bacteria for bioremediation of heavy metals. World J. of Environ. Pollution 2015; 5(2):23-28.
24. Selvi, A. T., Anjugam, E., Devi, A. R., Madhan, B., Kannappan S. and Chandrasekaran, B. Isolation and characterization of bacteria from Tannery effluent treatment plant and their tolerance to heavy metals and antibiotics. Asian J. Exp. Biol. Sci. 2012; 3(1):34-41.
25. Shah, S.Q., Colquhoun, D.J., Nikuli, H.L., Sørum, H. Prevalence of antibiotic resistance genes in the bacterial flora of integrated fish farming environments of Pakistan and Tanzania. Environ. Sci. Technol. 2012; 46:8672–8679.
26. Silver, S. and Misra, T.K. Plasmid mediated metals resistance. Annual Review of Microbiol. 1988; 42:717-743.
27. Vaishnav, M.M. and Hait, M. Effect of Paper Mills Effluents on ground and surface water bodies of some selected areas of JanjgirChampa, CG, India. Indian J. of Science and Res. 2013; 4(2):119-126.
28. Verma, T. Srinath, T., Gadpayle, R.U., Ramtake, P.W., Hans, R.K., and Garg, S.K. Chromate tolerant bacteria isolated from tannery effluent. Bioresource Technol. 2001; 78:31-35.

29. Vivekanandhan, G., Savithamani, K., Hatha A.A.M., Lakshmanaperumalsamy, P. Antibiotic resistance of Aeromonashydrophila isolated from marketed fish and prawn of South India. Int. J. Food Microbiol. 2002; 76:165.

30. Wasi, S., Tabrez, S. and Ahmad, M. Detoxification potential of Pseudomonas fluorescens SM1 strain for remediation of major toxicants in Indian water bodies.Water, Air, and Soil Pollution. 2011; 222(1-4):39-51.

Original Article

Journal of Microbiology and Related Research
Volume 3 Number 1, January - June 2017
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.3117.6

Colonization of Aspergillus Species in COPD Patients and Their Antifungal Suceptibility

Priyanka Choudhary*, Pooja Singh Gangania*

Author Affiliation
*Research Scholar, Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad, U.P.

Reprint Request
Priyanka Choudhary, Research Scholar, Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh 201009.
E-mail: priyankateotia2013@gmail.com

Received on 06.02.2017,
Accepted on 23.02.2017

Abstract

Objectives: To isolate *Aspergillus* species from lung infection in COPD patients and to determine Antifungal Susceptibilty against Amphotercin B by E-Test Strip method. *Materials and Method:* Sputum sample collected from total 54 patients visiting TB and Chest department of Santosh Hospital. All are above 18 year of Age and suffering from COPD. *Aspergillus* species were isolated by culturing samples on SDA which were confirmed by conventional method. Antifungal Susceptibilties were determined by E-Test strip method on Muller Hinton Agar with Methylene Blue Dye . E-Test minimum inhibitory concentrations (MIC) of Amphotericin B determined. *Result:* 33% of *Aspergillus* species were isolated from sputum samples of COPD patients. *Conclusion:* The analysis of the present study concludes that *Aspergillus* is one of the major cause of colonization in COPD patients,especially males within age group(41-60) years, chronic smoking increases the rate of *Aspergillus* colonization and Amphotericin B gives a poor result in treatment.

Keywords: Chronic Obstructive Pulmonary Disease; Aspergillus Fumigatus ; Aspergillus Flavus ; Aspergillus Niger; Aspergillus Terreus; Lactophenol Cotton Blue; Sabourad's Dextrose Agar; Potato Dextrose Agar; Minimum Inhibitory Concentration; Elipsometer Test.

Introduction

Members of the genus *Aspergillus* are ubiquitous moulds widely distributed in the environment. About 185 different species of *Aspergillus* have been identified, out of which 20 are declared pathogenic.

Aspergillus spores, upon inhalation, can lead to colonization, allergic manifestations or invasive infection depending on host immunity. Invasive aspergillosis is the second most common invasive fungal infections in humans [1].

COPD is a common , preventable lung disorder characterized by progressive, poorly reversible air flow limitation often with systemic manifestation, in response to tobacco smoke and other harmful inhalation exposures. Patients with severe copd who often receive broad-spectrum antibiotics and corticosteroids are becoming one of the main risk groups for Invasive pulmonary aspergillosis [2].

Majority (80%) of invasive infections caused by *A. fumigatus* and the second most frequent (15-20%) pathogenic is *A. flavus* and to a lesser extent *A. niger* and *A. terreus* but now *A.* flavus is overcoming

A.fumigatus. *A. flavus*, with its unique ability to survive at higher temperatures is making it, most predominant pathogen in arid dry weather countries like India [1].

Materials and Methods

Expectorated morning sputum samples were collected from each patient in a wide mouth sterile disposable plastic container, total 60 patients sputum sample were taken, who belongs to above 18 year of age having history of cough with sputum production, shortness of breath, wheezing sound, smoking from long time and those who have conformed COPD history.

All sputum samples were cultured on SDA (Sabourad's dextrose agar) with Chlorhexidine and incubated at 32-37^{0}c for 3 to 4 days or a week for isolation of *Aspergillus* species. Identification and confirmation is done on the basis of Conventional method such as colony characterstics, LPCB (Lactophenol cotton blue) preparation and Slide culture.

After seven days, filamentous colonies were examined and *Aspergillus* spp. Identified based on macroscopic and microscopic methods. Species are differentiated by morphological characterstics and colour. Macroscopically, colonies are flat, granular, downy to powdery in texture often with radial grooves. Colony surface is yellow initially but turns dark yellowish green with age on SDA agar.

Microscopically,hyphae are septate and hyaline branching at 45^0 angle. The conidiophores originating from supporting hyphae and terminate in vesicles at the apex [3].

Antifungal Susceptibility Testing

Preparation of Inoculum

All isolates were freshly sub–cultured on potato dextrose agar (PDA) slants to obtain good sporulation. The culture tubes were flooded with 1ml of 0.9% saline and vortexed for 15 seconds to dislodge the conidia. The growth suspensions were transferred to another sterile tube containing 1.5 ml saline and 0.2% Tween 80. A conidial suspension containing approximately 1x10^6-5x10^6 cells was used as inoculum [4].

E-TEST

(MHA) Mueller- hinton agar with 2% glucose and methylene blue (0.5 ug/ml) (MH-GMB) were used for Etest. Etest was performed according to manufacturer's instructions. Briefly, each 150-mm petri plate containing 60 ml of medium was inoculated by streaking the swab over the entire surface of the medium. Before apply the E strips, the plates were allowed to dry for 15 min. Etest antifungal susceptibility strips for Amphotericin B were stick on the plate. MIC reading were taken after 24 hour of incubation at 35^0 C. The Etest MIC was define as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip, microcolonies within the zone were ignored [4].

Result

Distribution of Aspergillus Species

Total no of *Aspergillus* species	*Aspergillus fumigatus*	*Aspergillus flavus*	*Aspergillus niger*	other isolates
18(25%)	12(23%)	3(6%)	3(6%)	5(9%)

A total of 54 possible patients with severe exacerbation of COPD were evaluated. Of these most of belong to age group 41-60(64%) years. Isolation of *Aspergillus* species was higher in males compared to females due to their addictive habit of smoking. Total 18(25%) *Aspergillus* species isolated, 12(23%) were identified as *A.fumigatus*, 3 (6%) as *A.flavus* and 3(6%) were *A.niger*. Antifungal susceptibility by Etest method showed only 1 strain to be sensitive for Amphotericin B had MIC range 2-3mcg/ml while other 17 were resistant.

Sensitivity Pattern

Antibiotic Pattern	No of Patients
Senstive	1
Resistant	17

Discussion

One of the first question that arises when physicians face cultures positive to *Aspergillus* from lower respiratory samples in non immunocompromised: Is there colonisation or infection?, should the patient be treated with antifungals?, and what should be is the prognosis?, that is, how to interpret and manage patients from which *Aspergillus* is obtained. The answer to the question is important since an early diagnosis is crucial to improve prognosis. It has been postulated that isolation of an *Aspergillus* species from respiratory samples in critically ill patients (even when immunocompetent) should not be routinely discarded as colonisation, but in elderly patients (commonly having underlying diseases) isolation is usually interpreted as colonization [5].

In our study a total of 54 COPD patients were included of which 43(80%) were males and 11(20%) were females. The prevalence of males having COPD was higher than females due to their frequent smoking habits, field work, labour work and higher outside exposure etc. while females were only infected by bio mass fuel cooking, as less smoking habits are found in females compared to males [2].

In the present study we have observed that most of the COPD patients belonged to age group between 41-60 years due to their continuing smoking habits.

Of the 54 COPD patient a total number of 18(33%) *Aspergillus* species were isolated. Of these 17(94%) were isolated from males while only one(6%) was isolated isolated from a female patient. The result of our study was similar to a study by AM khurhade et.al, in which 16.26% of *Aspergillus* species were isolated from COPD patients [6]. Recently a large, retrosepective study conducted by Guinea et.al analyzed the incidence of *Aspergillus* species isolation from lower respiratory tract samples in patients admitted for AECOPD in tertiary hospital, the authors found 22% *Aspergillus* isolation [7].

In our study we isolated most of the *Aspergillus* species from the males, which was 39%. This was in contrast to a study by Mahesh et. al, in which they found 11.1% males to be were infected by *Aspergillus* species. This is due to the fact that males are highly involved in addictive habits like smoking, alcoholism etc and some are infected due to their occupations like diary farms, farmers, labour etc. as a result they are highly exposed to dust, smoke, hazardious chemicals etc which lead to respiratory infections [8].

A study by Arturo Huerta et. al, reported that *A.fumigatus* was isolated in 25(17%) cases out of 144, *A.niger* in 1(0.69%) and *A.flavus* in 1(0.69%) [7]. A study by Kurhade et. al is also showed similar result in which *Aspergillus fumigatus* is isolated in 16(13%) cases out of 123 cases, *A.niger* in 3(2.4%) and *A.flavus* in 1(0.81%) [47]. Another study by Barberan et. al showed 16(15%) cases positive for *A.fumigatus* of the 106 samples, 1(0.94) isolated positive for *A.niger* and 1(0.94) positive for *A.flavus [9]*. In the present study also of the 18 *Aspergillus* species isolated, 12(66%) were identified as *A.fumigatus*, 3(16%) as *A.flavus*, 3(16%) as *A.niger* and 6(33%) were other isolates like Candida species, Rhizopus and Penicillum.

The prevalence of *Aspergillus* spp. isolation may have been higher if we had used bronchoscopic techniques and specific culture media. However, in real-life settings, clinicians often only have access to sputum samples. In a recent study, Phasley et al [10] reported that the isolation of *A.fumigatus* in sputum culture was significantly higher using a research approach compared to the standard method for mycological investigations. Previous studies, which have not focused solely on *Aspergillus spp.*, have found different prevalence rates of fungi isolation in respiratory samples from patients with cystic fibrosis, COPD and asthma. Recently, a large, retrospective study conducted by Guinea et al [11], analyzed the incidence of *A.fumigatus* isolation from lower respiratory tract samples in patients admitted for AECOPD in a tertiary hospital. The authors found 239 isolations of *Aspergillus* species (16.3 per 1000 admissions), but only 53 (22%) patients had probable IPA. However, unlike our prospective study, the fungal isolations were detected retrospectively by the microbiology laboratory. There is no doubt that COPD patients are a population at risk for *Aspergillus spp.* colonization. In a previous study of critically ill patients, *Aspergillus* spp. isolation from respiratory secretions was significantly associated with both an underlying diagnosis of COPD and treatment with corticosteroids [12]. These findings have been confirmed by other authors, and have strengthened the relationship between pulmonary infection with *Aspergillus spp.* and the use of intravenous corticosteroids in COPD patients admitted to the ICU for severe exacerbation. In contrast, a study conducted by Afessa et al [13] reported no isolation of *Aspergillus spp.* in the respiratory specimens from 250 COPD patients admitted to the ICU because of acute respiratory failure, although no report on corticosteroid therapy was performed.

Antifungal susceptibility testing has become an important tool for physician faced with making

difficult treatment decisions regarding treatment of patients with fungal infections.

In present study we determined antifungal susceptibility of isolated *Aspergillus* strains of which only one strain was found to be sensitive for Amphotericin B with MIC range 2-3mcg/ml and the remaining strains were found to be resistant for Amphotericin B with MIC range >32mcg/ml. The result of our study are similar to a study by Khurhade et al who showed that only 2 *Aspergillus* strains were found sensitive for Amphotericin B (MIC range 0.5-2ug/ml) of the 20 *Aspergillus* strains isolated. A study by Barberan et al however showed all fungal isolates from 65 patients to be resistant against Amphotericin B.

The results of our study were in contrast to a study by Al wathiqui et.al in which from the total 92 patients, 69 isolates were inhibited by Amphotericin B (0.064-4 to 3ug/ml).

Susceptibility testing are carried out by a broth microdilution test and disc diffusion. MICs are determined after 48 h by the reference broth microdilution method, and after 24 and 48 hours by disc diffusion. As others studies have shown, the broth microdilution and disc diffusion produced comparable MICs and a good level of agreement for all *Aspergillus spp*.

The medium employed for the disc diffusion method was Mueller–Hinton agar (Difco) supplemented with 2% glucose and Methylene Blue (0.5 mg/L). This medium is recommended in the document M44-P for disc diffusion susceptibility testing for yeast because of its enhanced growth and simplified reading relative to the broth microdilution method. 31 Zone size measurements are subjective, and this adds an important source of variability to the test; however, our isolates showed zone diameters with very clear border edges in the Mueller-Hinton agar.

Our finding may serve to purpose future more comprehensive studies, with biological basis that includes pulmonary and systemic markers of the immune and inflammatory response, in order to determine the role of this fungus in COPD exacerbations. On basis of our results, it appears that the E- test method is a useful method for testing the activity of drugs against *Aspergillus species* and Amphotericin B were found to be highly resistant against the *Aspergillus family*.

Conclusion

The analysis of the present study concludes that *Aspergillus* is one of the major cause of colonization in COPD patients,especially males within age group (41-60) years, chronic smoking increases the rate of *Aspergillus* colonization and Amphotericin B gives a poor result in treatment.

References

1. Dheeraj Gupta, Ritesh Aggarwal, Ashutosh Nath Aggarwal, VN Mathur for the COPD guidelines work in group guidelines for diagnosis and management of COPD disease.
2. American Thoracic Society/ Europian Respiratory Society 2004: Standards for the Diagnosis and management of patients with COPD, New York.
3. Elmer W.Koneman, Color Atlas and Text book of diagnostic microbiology 5th edition.
4. Reference Method for Broth Dilution Susceptibilty Testing of Yeasts; Third Informational Supplement. CLSI document M27-S3 2008; 28:15.
5. Huerta A, Torres A:The importance of Aspergillus species infection in Chronic Obstructive Pulmonary Disease. Clinical Pulmonary Med 2011; 18:161–168.
6. AM Kurhade, JM Deshmukh, RP Fule, C Chande, S Akulvar. Mycological and serological study of pulmonary Aspergillosis in central India, Indian Journal of Medical Mycology (2002).
7. J Guinea, M Torres Morpone, P Giyon. Pulmonary Aspergillosis in patient with COPD : Incidence, Risk, Factors and Outcome.Indian Journal of Medical Micology (2006).
8. Anzueto A, Sethi S, Martinez FJ: Exacerbations of chronic obstructive pulmonary disease. Proc Am Thorac Soc 2007; 4(7):554–564.
9. Barberan J, Alcazar B, Malmierca E, Garcia dL, Dorca J, Del CD, Villena V,Hernandez-Febles M, Garcia-Perez FJ, Granizo JJ, Israel DA, Salama N, Arnold CN, Moss SF, Ando T, Wirth HP, Tham KT, Camorlinga M, Blaser MJ, Falkow S, Peek RM Jr:Repeated Aspergillus isolation in respiratory samples from non-immunocompromised patients not selected based on clinical diagnoses: colonisation or infection? BMC Infect Dis 2012; 12:295.
10. Pashley CH, Fairs A, Morley JP, Tailor S, Agbetile J, Bafadhel M, Brightling CE, Wardlaw AJ:Routine processing procedures for isolating filamentous fungi from respiratory sputum samples may underestimate fungal prevalence. Med Mycol 2012; 50(4):433–438.
11. Guinea J, Torres-Narbona M, Gijon P, Munoz P, Pozo F, Pelaez T, de Miguel J, Bouza E:Pulmonary aspergillosis in patients with chronic obstructive pulmonary disease: incidence, risk factors, and outcome.Clincal Microbiology Infection 2010; 16 (7):870–877.

12. Garnacho-Montero J, Amaya-Villar R, Ortiz-Leyba C, Leon C, Alvarez-Lerma F, Nolla-Salas J, Iruretagoyena JR, Barcenilla F. Isolation of Aspergillus spp. from the respiratory tract in critically ill patients: risk factors, clinical presentation and outcome. Crit Care 2005; 9(3):R191–R199.

13. M. A. S. H. Mennink-Kersten, J. P. Donnelly, and P. E. Verweij. "Detection of circulating Galactomannan for the diagnosis and management of Invasive Aspergillosis," the Lancet Infectious Diseases, 2004; 4(6):349–357.

STATEMENT ABOUT OWNERSHIP AND OTHER PARTICULARS

"Journal of Microbiology and Related Research" (See Rule 8)

1. Place of Publication	:	Delhi
2. Periodicity of Publication	:	Quarterly
3. Printer's Name	:	**Asharfi Lal**
Nationality	:	Indian
Address	:	3/258-259, Trilok Puri, Delhi-91
4. Publisher's Name	:	**Asharfi Lal**
Nationality	:	Indian
Address	:	3/258-259, Trilok Puri, Delhi-91
5. Editor's Name	:	**Asharfi Lal**
Nationality	:	Indian
Address	:	3/258-259, Trilok Puri, Delhi-91
6. Name & Address of Individuals	:	**Asharfi Lal**
who own the newspaper and particulars of shareholders holding more than one per cent of the total capital	:	3/258-259, Trilok Puri, Delhi-91

I Asharfi Lal, hereby declare that the particulars given above are true to the best of my knowledge and belief.

Sd/-

(Asharfi Lal)

Revised Rates for 2017 (Institutional)

Title	Frequency	Rate (Rs): India		Rate ($):ROW	
Community and Public Health Nursing	3	5000	4500	357	300
Dermatology International	2	5000	4500	357	300
Gastroenterology International	2	5500	5000	393	340
Indian Journal of Agriculture Business	2	5000	4500	500	450
Indian Journal of Anatomy	4	8000	7500	571	500
Indian Journal of Ancient Medicine and Yoga	4	7500	7000	536	500
Indian Journal of Anesthesia and Analgesia	4	7000	6500	500	450
Indian Journal of Biology	2	5000	4500	357	300
Indian Journal of Cancer Education and Research	2	8500	8000	607	550
Indian Journal of Communicable Diseases	2	8000	7500	571	500
Indian Journal of Dental Education	4	5000	4500	357	300
Indian Journal of Emergency Medicine	2	12000	11500	857	800
Indian Journal of Forensic Medicine and Pathology	4	15500	15000	1107	1050
Indian Journal of Forensic Odontology	2	5000	4500	357	300
Indian Journal of Genetics and Molecular Research	2	6500	6000	464	400
Indian Journal of Hospital Administration	2	6500	6000	464	429
Indian Journal of Hospital Infection	2	12000	9000	857	800
Indian Journal of Law and Human Behavior	2	5500	5000	393	350
Indian Journal of Library and Information Science	3	9000	8500	643	600
Indian Journal of Maternal-Fetal & Neonatal Medicine	2	9000	8500	643	600
Indian Journal of Medical & Health Sciences	2	6500	6000	464	410
Indian Journal of Obstetrics and Gynecology	4	9000	8500	643	600
Indian Journal of Pathology: Research and Practice	4	11500	11000	821	780
Indian Journal of Plant and Soil	2	65000	60000	4623	4100
Indian Journal of Preventive Medicine	2	6500	6000	464	410
Indian Journal of Research in Anthropology	2	12000	11500	857	800
Indian Journal of Surgical Nursing	3	5000	4500	357	300
Indian Journal of Trauma & Emergency Pediatrics	4	9000	8500	643	600
Indian Journal of Waste Management	2	9000	8000	643	579
International Journal of Food, Nutrition & Dietetics	3	5000	4500	357	300
International Journal of Neurology and Neurosurgery	2	10000	9500	714	660
International Journal of Pediatric Nursing	3	5000	4500	357	300
International Journal of Political Science	2	5500	5000	550	500
International Journal of Practical Nursing	3	5000	4500	357	300
International Physiology	2	7000	6500	500	450
Journal of Animal Feed Science and Technology	2	78000	70000	5571	5000
Journal of Cardiovascular Medicine and Surgery	2	9500	9000	679	630
Journal of Forensic Chemistry and Toxicology	2	9000	8500	643	600
Journal of Geriatric Nursing	2	5000	4500	357	300
Journal of Medical Images and Case Reports	2	5000	4500	357	300
Journal of Microbiology and Related Research	2	8000	7500	571	520
Journal of Nurse Midwifery and Maternal Health	3	5000	4500	357	300
Journal of Organ Transplantation	2	25900	25000	1850	1700
Journal of Orthopaedic Education	2	5000	4500	357	300
Journal of Pharmaceutical and Medicinal Chemistry	2	16000	15500	1143	1100
Journal of Practical Biochemistry and Biophysics	2	5500	5000	393	340
Journal of Social Welfare and Management	3	5000	4500	357	300
New Indian Journal of Surgery	4	7500	7000	536	480
New Journal of Psychiatric Nursing	3	5000	4500	357	300
Ophthalmology and Allied Sciences	2	5500	5000	393	340
Otolaryngology International	2	5000	4500	357	300
Pediatric Education and Research	3	7000	6500	500	450
Physiotherapy and Occupational Therapy Journal	4	8500	8000	607	550
Psychiatry and Mental Health	2	7500	7000	536	490
Urology, Nephrology and Andrology International	2	7000	6500	500	450

Terms of Supply:

1. Agency discount 10%. Issues will be sent directly to the end user, otherwise foreign rates will be charged.
2. All back volumes of all journals are available at current rates.
3. All Journals are available free online with print order within the subscription period.
4. All legal disputes subject to Delhi jurisdiction.
5. Cancellations are not accepted orders once processed.
6. Demand draft / cheque should be issued in favour of "Red Flower Publication Pvt. Ltd." payable at Delhi
7. Full pre-payment is required. It can be done through online (http://rfppl.co.in/subscribe.php?mid=7).
8. No claims will be entertained if not reported within 6 months of the publishing date.
9. Orders and payments are to be sent to our office address as given above.
10. Postage & Handling is included in the subscription rates.
11. Subscription period is accepted on calendar year basis (i.e. Jan to Dec). However orders may be placed any time throughout the year.

Order from

Red Flower Publication Pvt. Ltd., 48/41-42, DSIDC, Pocket-II, Mayur Vihar Phase-I, Delhi - 110 091 (India), Tel: 91-11-22754205, 45796900, Fax: 91-11-22754205. E-mail: sales@rfppl.co.in, Website: www.rfppl.co.in

Original Article

Journal of Microbiology and Related Research
Volume 3 Number 1, January - June 2017
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.3117.7

Isolation of MTB Strains and Determining the Antibiotic Susceptibility Pattern via Bactec 320 from the Females of Child Bearing Age

P.S. Gangania*, D. Bisht, V.A. Singh*****

Author Affiliation
*Research Fellow **Professor & Head, Department of Microbiology, Santosh Medical College & University, Ghaziabad, Uttar Pradesh 201009.
***Professor & Head, Department of Microbiology, MMIMSR Mullana, Ambala, Haryana 133207.

Reprint Request
Dakshina Bisht, Professor & Head, Department of Microbiology, Santosh Medical College & University, Ghaziabad, Uttar Pradesh 201009.
E-mail: dakshinabisht@gmail.com

Received on 03.04.2017,
Accepted on 08.04.2017

Abstract

Background: The BACTEC MGIT 320 is a new, lower-capacity instrument for liquid culture developed for the growth and detection of *M. tuberculosis* and antimicrobial susceptibility testing. MGIT has an improved speed and sensitivity of MTB isolation and drug susceptibility testing, irrespective of the HIV status of the patient. This study was undertaken to find the appropriate antibiotic susceptibility pattern of confirmed positive MTB strains via MGIT 320 liquid culture technique. *Objectives*: Isolation of positive MTB strains and determination of their antibiotic susceptibility pattern using BACTEC 320 from the females of child bearing age group. *Material and Methods*: A total of 217 samples were processed involving the isolation of MTB strains along with the antibiotic susceptibility pattern of positive MTB's. Both techniques were used culture and RT-PCR to find the prevalence and AST via BACTEC 320 system. Analysis of the results was done at the end of the procedure. *Results*: Out of 217 samples, there were 29 positive MTB strains by RT-PCR technique whereas via liquid culture there were 11 positive MTB strains. The Antibiotic susceptibility pattern for MTB positive strains for both the first and second line was Levofloxacin, Kanamycin and PAS were found to be much sensitive whereas Isoniazid and Ethionamide were found to be more resistant than others. *Conclusion*: RT-PCR technique detects the total count of mycobacterial bacilli whereas via liquid culture only viable bacteria are detected i.e. true positive MTB strains. There were 2 MDR and 2 XDR TB detected, out of 11 confirmed MTB strains.

Keywords: AST; BACTEC 320; MTB Strains; RT-PCR Technique; Liquid Culture.

Introduction

Tuberculosis (TB) is a serious global public health problem. The diagnosis is made by detection of acid-fast bacilli on microscopy or culture, as Polymerase chain reaction may be false positive hence alone is not sufficient to make the diagnosis. To achieve early diagnosis and effective treatment of TB, rapid and accurate drug susceptibility testing (DST) methods must be used. The World Health Organization and the Centers for Disease Control and Prevention have recommended the use of liquid culture systems for DST and for improving time to detection [1,2,3]. Recent publications demonstrate the fundamental importance of liquid culture and phenotypic drug susceptibility testing (DST) as part of a complete strategy in the ongoing global efforts to combat

tuberculosis.

The BACTEC MGIT 320 is a new, lower-capacity instrument for liquid culture developed for the growth and detection of M. tuberculosis and antimicrobial susceptibility testing. MGIT has an improved speed and sensitivity of MTB isolation and drug susceptibility testing, irrespective of the HIV status of the patient. It is now employed for the diagnosis of female genital TB, a common cause of infertility in India. DST of *Mycobacterium tuberculosis* (MTB) with the BD BACTEC MGIT 320 system produces accurate results more rapidly than the conventional agar proportion method [4,5,6]. Multidrug resistant (MDR) and extreme-drug resistant TB (XDR) are a cause of serious concern [7,8].

In olden days before rifampicin, the antituberculous therapy (ATT) was given for 18–24 months with significant side effects and poor compliance but now a day's short-course chemotherapy for 6–9 months has been found to be effective for medical treatment of FGTB [9]. Surgery is only recommended after the continuous drug treatment of 12-18 months duration.

Material and Methods

The present study was done at Santosh Medical College and Hospital (Ghaziabad, Delhi NCR) in collaboration with Oncquest Laboratories Pvt. Ltd. (03 Factory Road, Safdarjung Delhi) in which a total of 217 samples were processed using different techniques like AFB smear microscopy, AFB liquid culture via BACTEC 320 and RT-PCR was done along with the DST of culture positive MTB strains for both first line and second line of drugs.

Sample Type

- Menstrual blood
- Endometrial tissue biopsy
- Tubal tissue biopsy
- Product of conception

Inclusion Criteria

1. Females willing to participate with their consent were included.
2. Study involved infertile and Tb suspected females with any of these symptoms like-

- Irregular menstrual cycle
- Pelvic pain
- Vaginal discharge that is stained with blood or which is persistent, heavy and discoloured.
- Bleeding after intercourse
- Infertility
- Abdominal mass
- Tubo-ovarian abscess
- Pregnancy loss
- Strong clinical suspicion of TB.

Exclusion Criteria

Eligible female patients not willing to participate and patients already on ATT and HIV positive were excluded for the study.

Methodology

Specimen Collection

Clinical specimens for female genital infections including endometrial and ovarian tissues along with menstrual blood were taken. Specimens were transported to the laboratory as soon as possible after collection. In case of delay, the specimens were refrigerated to inhibit the growth of unwanted micro-organisms.

Sample Processing

1. The sample was divided into three parts. First part was subjected for ZN staining, second was used for isolating the mycobacterial species by culturing and third was being used for molecular detection via PCR.
2. Samples were smeared with Ziehl-Neelsen (ZN) staining to confirm Acid – fastness followed by Homogenization and decontamination by NAOH-NALC method.
3. Isolation of Mycobacteria was carried out by culturing on liquid media by BACTEC 320 using MGIT tubes.
4. Real time PCR was run to amplify the product using the proper gene for the detection of MTB strains. IS6110–specific primers for Mycobacterium tuberculosis complex was used.
5. Analysis of staining, molecular, and liquid culture diagnosis for MTB strains was done and the efficacy of the techniques was defined along with the prevalence rate of infected infertile females suspected to have genital tuberculosis.
6. Before doing DST of confirmed MTB strains they

were first subcultured on blood agar to check for any contamination and if it comes sterile then again they were subcultured on MGIT to get appropriate growth conditions for performing DST. (Incase, if, contamination is detected on blood agar the tube was again processed for decontamination procedure to obtain the pure growth of Mycobacterium tuberculosis)

7. The MGIT reagents which were used in this study were MGIT Mycobacteria Growth Indicator Tubes (7 mL), MGIT Growth Supplement, MGIT PANTA, MGIT 960 SIRE Susceptibility Test Kit and the MGIT PZA Susceptibility Test kit.
8. Drug Susceptibility Testing (DST) was done by using automated BACTEC for confirmed TB cases. First and Second line drugs were used for Drug Susceptibility Testing.
   - First Line Drugs
     - Rifampin
     - Pyrazinamide
     - Streptomycin
     - Ethambutol
     - Isoniazid
   - Second Line Drugs
     - Levofloxacin
     - PAS (Para-aminosalicylic Acid)
     - Ethionamide
     - Kanamycin
     - Rifabutin

Results

Out of 217, TB suspected infertile female's maximum were from the age group of 26-30 i.e. 34.10% (approximately 74 females) and the lowest were under the age group of 15-20 years i.e. 2.3% (5 females).

By liquid culture there were 11 positive MTB strains. The maximum numbers of females were from the age group of 41-45 years (16.67%) whereas the minimum number lied under the age group of 15-20 (0%) and 31-35 years (0%).

Table 1: Depicting Age Wise Distribution of Infertile females Due To MTB Infection diagnosed via Bactec 320 (liquid culture)

Age Range	Total Patients	MTB Culture Positive	Percentage
15-20	5	0	0 %
21-25	33	3	9.09 %
26-30	74	6	8.10 %
31-35	70	0	0 %
36-40	29	1	3.44 %
41-45	6	1	16.67 %
Total	217	11	5.06 %

Table 2: Depicting Age Wise Distribution of Infertile females Due To MTB Infection diagnosed via Multiplex RT-PCR

Age Range	Total Patients	MTB PCR Positive	Percentage
15-20	5	1	20 %
21-25	33	4	12.12 %
26-30	74	17	22.97 %
31-35	70	2	2.85 %
36-40	29	4	13.79 %
41-45	6	1	16.66 %
Total	217	29	13.36 %

Table 3: Depicting MTB positivity by RT-PCR and AFB Cultures considering sample type

Sample Type	MTB Positives by RT-PCR		MTB Positives by Culture	
	Total Number	Rate (%)	Total Number	Rate (%)
Endometrial Biopsy / Tissue	24	11.05 %	11	5.06 %
Menstrual Blood	4	1.84 %	0	0 %
Product of Conception	0	0 %	0	0 %
Tubal Biopsy	1	0.46 %	0	0 %
Total	29	13.36 %	11	5.06 %

By RT-PCR technique there were 29 positive MTB strains. The maximum numbers of females were from the age group of 26-30% (22.97%) whereas the minimum number lied under the age group of 15-20

(0%) and 31-35 (2.85%).

Total 217 cases of infertile TB suspected females were observed via RT-PCR technique and liquid culture. Out of those 217, Endometrial Biopsy / Tissue (195), 24 were found to be MTB positive via RT- PCR whereas 11 were found to be positive by liquid culture. Menstrual Blood (17), 4 MTB's by RT-PCR and 0 were detected by culture. POC (4), out of which there was no MTB strain detected by RT –PCR technique and culture too. Tubal Biopsy (1), which was MTB positive by RT-PCR and 0 were positive by liquid culture.

The Antibiotic susceptibility pattern for confirmed MTB culture positive strains for both the first and second line was performed via automated BACTEC 320 system. Levofloxacin (90.9%), Kanamycin (90.9%), Pyrazinamide (100%) and PAS (100%) were found to be much sensitive whereas Isoniazid (45.5%) and Ethionamide (36.36%) were found to be more resistant than others.

Table 4: Depicting the antibiotic susceptibility of MTB strains

S. No.	Antibiotics	No. Of Patients Sensitive (n = 11)		Number of Patients Resistant (n = 11)	
		Number	Rate (%)	Number	Rate (%)
1	Streptomycin	9	81.81%	2	18.18%
2	Rifampin	9	81.81%	2	18.18%
3	Pyrazinamide	11	100%	0	0%
4	Isoniazid	6	54.54%	5	45.45%
5	Ethambutol	9	81.81%	2	18.18%
6	Levofloxacin	10	90.9%	1	9.09%
7	PAS	11	100%	0	0%
8	Ethionamide	7	63.63%	4	36.36%
9	Kanamycin	10	90.9%	1	9.09%
10	Rifabutin	9	81.81%	2	18.18%

Discussion

Genitourinay tract is the frequent site for extrapulmonary tuberculosis after the pulmonary one. GTB is generally secondary to renal tuberculosis [10].

TB is still a major health problem hence it is important to predict the possibility of GTB in patients presenting with infertility [11]. Most of time it remain undiagnosed due to lack of symptoms and lack of diagnostic modalities because of which the disease is prone to false positive as well as false negative results.

In this prospective study, via RT-PCR technique, 29 positive MTB strains whereas via liquid culture 11 positive MTB strains were detected. RT-PCR test detected MTB with in 24 h, compared with average 24 days required for detection by conventional method, as supported by earlier studies [12]. But still the conventional culture and microscopy is considered as gold standard.

The RT-PCR technique is restricted by the need for an appropriate infrastructure and high cost of the test. Molecular diagnosis of TB by RT-PCR has a great potential to improve the ability of diagnosis of GTB as RT-PCR is a rapid, sensitive and specific technique that can be used for early diagnosis of GTB. Though culture is a time consuming method, early RT-PCR can enable the consultant to diagnose GTB and start early treatment. But in this study RT-PCR was negative for 2 cases which were found to be culture positive by BACTEC MGIT 320.

ZN smear examination and RT-PCR results were positive but culture was negative; this could be due to the existence of nonviable mycobacterial bacilli in the samples [13].

The only drawback is that sometimes there may be false positive results by PCR test which could be due to the ability to detect very low number and even dead bacteria in a sample which can be present in a symptomatic individual [14].

Therefore, to confirm the diagnosis of TB, either acid-fast staining or culture must be performed. Both of these tests have poor sensitivity than RT-PCR because of paucibacillary tissue samples [15,16]. RT-PCR technique detects the total count of mycobacterial bacilli whereas via liquid culture only viable bacteria are detected i.e. true positive MTB strains.

The Antibiotic susceptibility pattern for MTB culture positive strains using both the first and second line of drugs was evaluated by which Levofloxacin, Kanamycin, Pyazinamide and PAS found to be much sensitive whereas Isoniazid and Ethionamide were more resistant than others. There were 2 MDR and 2 XDR TB detected. There was no literature found, as per our knowledge, to support our results determining antibiotic susceptibility pattern of infertile females due to tubercular infections using BACTEC MGIT 320.

Conclusion

RT-PCR technique detects the total count of mycobacterial bacilli whereas via liquid culture only viable bacteria are detected i.e. true positive MTB strains. By RT-PCR technique, 29 positive MTB strains whereas via liquid culture 11 positive MTB strains were detected. The Antibiotic susceptibility pattern for MTB culture positive strains using both the first and second line of drugs was Levofloxacin, Kanamycin, Pyazinamide and PAS found to be much sensitive whereas Isoniazid and Ethionamide were more resistant than others. There were 2 MDR and 2 XDR TB detected.

Abbreviations

- MTB: Mycobacterium Tuberculosis
- FGTB: Female Genital Tuberculosis
- RT-PCR: Real Time Polymerase chain reaction
- AFB: Acid Fast Bacilli
- RIF: Rifampicin
- NTM: Non - Tuberculous Mycobacterium
- MGIT: Mycobacterium Growth Indicator Tube
- (ZN) staining: Ziehl-Neelsen staining
- AST: Antibiotic Susceptibility Testing
- ATT: Anti Tubercular Treatment

Acknowledgement

I am thankful to Dr. Dakshina Bisht (Professor & Head, Department of Microbiology) Santosh Medical College and University and Dr.Varsha A. Singh (Professor & Head, Department of Microbiology), MMU, Mullana, Ambala for standing by me while carrying out this study. I extend my earliest gratitude to each and everyone in the Department of Microbiology, who were very kind to me throughout my research.

References

1. World Health Organization. 2007. Use of liquid TB culture and drug susceptibility testing (DST) in low and medium income settings. Summary report of the expert group meeting on the use of liquid culture media. WHO, Geneva, Switzerland. 2007.
2. Tenover FC, Crawford JC, Huebner RE, Geit er LH, Horsburgh R Jr., Good R. The resurgence of tuberculosis: is your laboratory ready? J. Clin. Microbiol. 1993; 31:767–770.
3. Kent PT, Kubica GT. Public health myco-bacteriology: a guide for the level III laboratory. U.S. Dept. of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, GA. 1985.
4. Tortoli E, Benedetti M, Fontanelli A, Simonetti MT. Evaluation of automated BACTEC MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to four major antituberculous drugs: comparison with the radiometric BACTEC 460TB method and the agar plate method of proportion. J. Clin. Microbiol. 2002; 40:607–610.
5. World Health Organization. WHO report: global tuberculosis control 2011. The sixteenth global report of tuberculosis. WHO, Geneva, Switzerland. 2011.
6. Scarparo C, Ricordi P, Ruggiero G, Piccoli P. Evaluation of the fully automated BACTEC MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide, streptomycin, isoniazid, rifampin, and ethambutol and comparison with the radiometric BACTEC 460TB method. J. Clin. Microbiol. 2004; 42:1109–1114.
7. Dye C, Watt CJ, Bleed DM, et al. Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence and deaths globally. JAMA. 2005; 293:2790–2793.
8. World Health Organization. Global tuberculosis control: a short update to the 2009 report. WHO/ HTM/TB 2009, 426. Geneva: WHO; 2009.
9. Arora R, Rajaram P, Oumachigui A, et al. Prospective analysis of short course chemotherapy in female genital tuberculosis. Int J Gynecol Obstet. 1992; 38:311–314.
10. Rom W, Garay S. Tuberculosis. 1st ed. New York: Little Brown and Company (Inc.). 1996.
11. Luqman M, Mirza MD, Razzaq I. A histopathological study of endometrial curretage in cases of infertility in females. Pak Armed Forces Med J. 1997; 47:12–6.
12. Park DY, Kim JY, Choi KU, Lee JS, Lee CH, Sol MY, et al. Comparison of polymerase chain reaction with histopathologic features for diagnosis of tuberculosis in formalin-fixed, paraffin-embedded histologic specimens. Arch Pathol Lab Med. 2003; 127: 326–30.
13. Montenegro SH, Gilman RH, Sheen P, Cama R, Cavides L, Hopper T et al. Improved detection of M.tuberculosis in Peruvian children by use of heminested IS6110 PCR assay. Clin Infec Dis; 2003; 36:16-23.
14. Bechnoosh A, Lieberman JM, Duke MB, Stutman. Comparison of Quantitative polymerase chain reaction, therapy for pulmonary tuberculosis. Diag Microb Infect Dis. 1997; 29:73-79.

15. Kaul KL. Molecular detection of Mycobacterium tuberculosis: Impact on patient care. Clin Chem. 2001; 47:1553–8.

16. Soini H, Musser JM. Molecular diagnosis of mycobacteria. Clin Chem. 2001; 47:809–14.

Guidelines for Authors

Manuscripts must be prepared in accordance with "Uniform requirements for Manuscripts submitted to Biomedical Journal" developed by international committee of medical Journal Editors.

Types of Manuscripts and Limits

Original articles: Up to 3000 words excluding references and abstract and up to 10 references.

Review articles: Up to 2500 words excluding references and abstract and up to 10 references.

Case reports: Up to 1000 words excluding references and abstract and up to 10 references.

Online Submission of the Manuscripts

Articles can also be submitted online from http://rfppl.co.in/customer_index.php.

I) First Page File: Prepare the title page, covering letter, acknowledgement, etc. using a word processor program. All information which can reveal your identity should be here. use text/rtf/doc/PDF files. Do not zip the files.

2) Article file: The main text of the article, beginning from Abstract till References (including tables) should be in this file. Do not include any information (such as acknowledgement, your name in page headers, etc.) in this file. Use text/rtf/doc/PDF files. Do not zip the files. Limit the file size to 400 Kb. Do not incorporate images in the file. If file size is large, graphs can be submitted as images separately without incorporating them in the article file to reduce the size of the file.

3) Images: Submit good quality color images. Each image should be less than 100 Kb in size. Size of the image can be reduced by decreasing the actual height and width of the images (keep up to 400 pixels or 3 inches). All image formats (jpeg, tiff, gif, bmp, png, eps etc.) are acceptable; jpeg is most suitable.

Legends: Legends for the figures/images should be included at the end of the article file.

If the manuscript is submitted online, the contributors' form and copyright transfer form has to be submitted in original with the signatures of all the contributors within two weeks from submission. Hard copies of the images (3 sets), for articles submitted online, should be sent to the journal office at the time of submission of a revised manuscript. Editorial office: Red Flower Publication Pvt. Ltd., 48/41-42, DSIDC, Pocket-II, Mayur Vihar Phase-I, Delhi – 110 091, India, Phone: 91-11-22754205, 45796900, 22756995. E-mail: author@rfppl.co.in. Submission page: http://rfppl.co.in/article_submission_system.php?mid=5.

Preparation of the Manuscript

The text of observational and experimental articles should be divided into sections with the headings: Introduction, Methods, Results, Discussion, References, Tables, Figures, Figure legends, and Acknowledgment. Do not make subheadings in these sections.

Title Page

The title page should carry

1) Type of manuscript (e.g. Original article, Review article, Case Report)
2) The title of the article, should be concise and informative;
3) Running title or short title not more than 50 characters;
4) The name by which each contributor is known (Last name, First name and initials of middle name), with his or her highest academic degree(s) and institutional affiliation;
5) The name of the department(s) and institution(s) to which the work should be attributed;
6) The name, address, phone numbers, facsimile numbers and e-mail address of the contributor responsible for correspondence about the manuscript; should be mentoined.
7) The total number of pages, total number of photographs and word counts separately for abstract and for the text (excluding the references and abstract);
8) Source(s) of support in the form of grants, equipment, drugs, or all of these;
9) Acknowledgement, if any; and
l0) If the manuscript was presented as part at a meeting, the organization, place, and exact date on which it was read.

Abstract Page

The second page should carry the full title of the manuscript and an abstract (of no more than 150 words for case reports, brief reports and 250 words for original articles). The abstract should be structured and state the Context (Background), Aims, Settings and Design, Methods and Materials, Statistical analysis used, Results and Conclusions. Below the abstract should provide 3 to 10 keywords.

Introduction

State the background of the study and purpose of the study and summarize the rationale for the study or observation.

Methods

The methods section should include only information that was available at the time the plan or protocol for the study was written such as study approach, design, type of sample, sample size, sampling technique, setting of the study, description of data collection tools and methods; all information obtained during the conduct of the study belongs in the Results section.

Reports of randomized clinical trials should be based on the CONSORT Statement (http://www.consort-statement. org). When reporting experiments on human subjects, indicate whether the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 2000 (available at http://www.wma.net/e/policy/l 7-c_e.html).

Results

Present your results in logical sequence in the text, tables, and illustrations, giving the main or most important findings first. Do not repeat in the text all the data in the tables or illustrations; emphasize or summarize only important observations. Extra or supplementary materials and technical details can be placed in an appendix where it will be accessible but will not interrupt the flow of the text; alternatively, it can be published only in the electronic version of the journal.

Discussion

Include summary of key findings (primary outcome measures, secondary outcome measures, results as they relate to a prior hypothesis); Strengths and limitations of the study (study question, study design, data collection, analysis and interpretation); Interpretation and implications in the context of the totality of evidence (is there a systematic review to refer to, if not, could one be reasonably done here and now?, What this study adds to the available evidence, effects on patient care and health policy, possible mechanisms)? Controversies raised by this study; and Future research directions (for this particular research collaboration, underlying mechanisms, clinical research). Do not repeat in detail data or other material given in the Introduction or the Results section.

References

List references in alphabetical order. Each listed reference should be cited in text (not in alphabetic order), and each text citation should be listed in the References section. Identify references in text, tables, and legends by Arabic numerals in square bracket (e.g. [10]). Please refer to ICMJE Guidelines (http://www.nlm.nih.gov/bsd/uniform_requirements.html) for more examples.

Standard journal article

[1] Flink H, Tegelberg Å, Thörn M, Lagerlöf F. Effect of oral iron supplementation on unstimulated salivary flow rate: A randomized, double-blind, placebo-controlled trial. J Oral Pathol Med 2006; 35: 540-7.

[2] Twetman S, Axelsson S, Dahlgren H, Holm AK, Källestål C, Lagerlöf F, et al. Caries-preventive effect of fluoride toothpaste: A systematic review. Acta Odontol Scand 2003; 61: 347-55.

Article in supplement or special issue

[3] Fleischer W, Reimer K. Povidone iodine antisepsis. State of the art. Dermatology 1997; 195 Suppl 2: 3-9.

Corporate (collective) author

[4] American Academy of Periodontology. Sonic and ultrasonic scalers in periodontics. J Periodontol 2000; 71: 1792-801.

Unpublished article

[5] Garoushi S, Lassila LV, Tezvergil A, Vallittu PK. Static and fatigue compression test for particulate filler composite resin with fiber-reinforced composite substructure. Dent Mater 2006.

Personal author(s)

[6] Hosmer D, Lemeshow S. Applied logistic regression, 2nd edn. New York: Wiley-Interscience; 2000.

Chapter in book

[7] Nauntofte B, Tenovuo J, Lagerlöf F. Secretion and composition of saliva. In: Fejerskov O, Kidd EAM,

editors. Dental caries: The disease and its clinical management. Oxford: Blackwell Munksgaard; 2003. p. 7-27.

No author given

[8] World Health Organization. Oral health surveys - basic methods, 4th edn. Geneva: World Health Organization; 1997.

Reference from electronic media

[9] National Statistics Online—Trends in suicide by method in England and Wales, 1979-2001. www.statistics.gov.uk/downloads/theme_health/HSQ 20.pdf (accessed Jan 24, 2005): 7-18. Only verified references against the original documents should be cited. Authors are responsible for the accuracy and completeness of their references and for correct text citation. The number of reference should be kept limited to 20 in case of major communications and 10 for short communications.

More information about other reference types is available at www.nlm.nih.gov/bsd/uniform_requirements.html, but observes some minor deviations (no full stop after journal title, no issue or date after volume, etc).

Tables

Tables should be self-explanatory and should not duplicate textual material.

Tables with more than 10 columns and 25 rows are not acceptable.

Table numbers should be in Arabic numerals, consecutively in the order of their first citation in the text and supply a brief title for each.

Explain in footnotes all non-standard abbreviations that are used in each table.

For footnotes use the following symbols, in this sequence: *, ¶, †, ‡‡,

Illustrations (Figures)

Graphics files are welcome if supplied as Tiff, EPS, or PowerPoint files of minimum 1200x1600 pixel size. The minimum line weight for line art is 0.5 point for optimal printing.

When possible, please place symbol legends below the figure instead of to the side.

Original color figures can be printed in color at the editor's and publisher's discretion provided the author agrees to pay.

Type or print out legends (maximum 40 words, excluding the credit line) for illustrations using double spacing, with Arabic numerals corresponding to the illustrations.

Sending a revised manuscript

While submitting a revised manuscript, contributors are requested to include, along with single copy of the final revised manuscript, a photocopy of the revised manuscript with the changes underlined in red and copy of the comments with the point to point clarification to each comment. The manuscript number should be written on each of these documents. If the manuscript is submitted online, the contributors' form and copyright transfer form has to be submitted in original with the signatures of all the contributors within two weeks of submission. Hard copies of images should be sent to the office of the journal. There is no need to send printed manuscript for articles submitted online.

Reprints

Journal provides no free printed reprints, however a author copy is sent to the main author and additional copies are available on payment (ask to the journal office).

Copyrights

The whole of the literary matter in the journal is copyright and cannot be reproduced without the written permission.

Declaration

A declaration should be submitted stating that the manuscript represents valid work and that neither this manuscript nor one with substantially similar content under the present authorship has been published or is being considered for publication elsewhere and the authorship of this article will not be contested by any one whose name (s) is/are not listed here, and that the order of authorship as placed in the manuscript is final and accepted by the co-authors. Declarations should be signed by all the authors in the order in which they are mentioned in the original manuscript. Matters appearing in the Journal are covered by copyright but no objection will be made to their reproduction provided permission is obtained from the Editor prior to publication and due acknowledgment of the source is made.

but no objection will be made to their reproduction provided permission is obtained from the Editor prior to publication and due acknowledgment of the source is made.

Abbreviations

Standard abbreviations should be used and be spelt out when first used in the text. Abbreviations should not be used in the title or abstract.

Checklist

- Manuscript Title
- Covering letter: Signed by all contributors
- Previous publication/ presentations mentioned, Source of funding mentioned
- Conflicts of interest disclosed

Authors

- Middle name initials provided.
- Author for correspondence, with e-mail address provided.
- Number of contributors restricted as per the instructions.
- Identity not revealed in paper except title page (e.g. name of the institute in Methods, citing previous study as 'our study')

Presentation and Format

- Double spacing
- Margins 2.5 cm from all four sides
- Title page contains all the desired information. Running title provided (not more than 50 characters)
- Abstract page contains the full title of the manuscript
- Abstract provided: Structured abstract provided for an original article.
- Key words provided (three or more)
- Introduction of 75-100 words
- Headings in title case (not ALL CAPITALS). References cited in square brackets
- References according to the journal's instructions

Language and grammar

- Uniformly American English
- Abbreviations spelt out in full for the first time. Numerals from 1 to l0 spelt out
- Numerals at the beginning of the sentence spelt out

Tables and figures

- No repetition of data in tables and graphs and in text.
- Actual numbers from which graphs drawn, provided.
- Figures necessary and of good quality (color)
- Table and figure numbers in Arabic letters (not Roman).
- Labels pasted on back of the photographs (no names written)
- Figure legends provided (not more than 40 words)
- Patients' privacy maintained, (if not permission taken)
- Credit note for borrowed figures/tables provided
- Manuscript provided on a CDROM (with double spacing)

Submitting the Manuscript

- Is the journal editor's contact information current?
- Is the cover letter included with the manuscript? Does the letter:

1. Include the author's postal address, e-mail address, telephone number, and fax number for future correspondence?
2. State that the manuscript is original, not previously published, and not under concurrent consideration elsewhere?
3. Inform the journal editor of the existence of any similar published manuscripts written by the author?
4. Mention any supplemental material you are submitting for the online version of your article. Contributors' Form (to be modified as applicable and one signed copy attached with the manuscript)