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Original Article

Hepatitis B Virus Infection & Socioeconomic Status in Females of Rural Population of North India: An Observation (A Three and Half Year Study)

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Abstract

Background and Objective: Health status of rural females is the most neglected in India. Hepatitis B is one of the highly infectious and sexually transmitted diseases which have a direct impact on the health of rural females as well on their siblings and family. So this study was undertaken to know the burden of Hepatitis B viral (HBV) infection in rural females so a prevention strategy can be made to curtail such dreaded infection in rural society. *Method:* A total number of 5035 female subjects of different age ranging from neonate to 90 years were grouped in 8 different groups designated as A to H. They were screened for detecting HBsAg both by Rapid Diagnostic tests and ELISA technique *Results:* Maximum number of subjects belonged to age group 21-30 yrs of group C, followed by group D(31-40yrs) and B(11-20 yrs). Of 5035 subjects, 256(5.08%) were positive for HBsAg. The positivity was maximum in both the extremes of life, 7.8%% & 21.9 t & 19.5% percent respectively. In other group the HBs Antigen positivity varied from 2.5 to 5.8 percent. *Interpretation and Conclusion:* The high incidence of Hepatitis B Viral infection clearly reflects the uneducated and neglected health status of rural females. Prevention strategy and education can reduce the further transmission and thereby reducing morbidity and mortality with HBV infection.

Keyword: HBsAg; HBV; HBV in Females.

Introduction

Hepatitis B is a common but also serious infectious disease of the liver because of its severe pathological consequences like chronic hepatic insufficiency, cirrhosis of liver and hepatocellular carcinoma. Infections usually occurs during early childhood, may be asymptomatic but often leads to chronic carrier state and are capable of transmitting disease for many years. More than 2000 million people are infected with HBV at some time of their life(WHO 2002) [1].In spite of such serious consequences HBV has been over shadowed by HIV, which it deserves and as a result HBV has taken back seat in the mind of medical administration all over the world specially in India, but the virus continues to play its natural history of disease. In India many reports are available about the incidence of HBV in general urban population .In females the carrier rate of HBsAg has been studied only from cities and that too in mothers or from tribal population, but there is

no report available in literature about incidence of HBV in general female population of rural India. Inspite of the fact that rural females are most neglected community of Indian society, irrespective of cast and religion. They have to carry out all the household and field work till they are completely bed ridden. Maximum they get is medical consultation from unregistered medical practitioners or quacks .The present work has been undertaken with a view to study the health status of rural female population in relation to systemic infections like Hepatitis B which affects future life of the subjects. The incidence of HBV was studied in the general female population among different age groups.

Materials and Method

The present work was carried out in Department of Microbiology, U.P. Rural Institute of Medical Sciences & Research(UPRIMS & R, Saifai, Etawah) from September 2006 to March 2010.This institute drain large rural population of western U.P. including Kanpur Dehat, Auriya, Etawah, Mainpuri, Farukhhaabad, Shikohabaad, Firozabad and even some parts of M.P. including Bhind .

Samples were collected from patients both from outpatient & indoor department. The test was carried out by commercially available kits including ELISA (S.D. make 3.0) & rapid (hepacard –immunopak and Viruchek-orchid) .Positive sample were stored at -20° C for further study.

Observation

In the present study 5035 female subjects grouped in 8 designated group A to H were studied for the presence of HBsAg (Table1). This included subject of

Table 1: HBsAg positivity in different age group

Group	Age Group (In Years)	Number of Subjects	%	HBsAg Positivity	% Positivity	Maximum Age	Minimum Age
A	0-10	64	1.3	5	7.8	1 month	10 years
B	11-20	787	15.6	34	4.3	11 year	20 year
C	21-30	2590	51.5	108	4.2	21 year	30 year
D	31-40	863	17.1	39	4.5	31 year	40 year
E	41-50	357	7.1	23	6.4	41 year	50 year
F	51-60	203	4.0	11	5.4	51 year	60 year
G	61-70	114	2.3	25	21.9	61 year	65 year
H	≥71	57	1.1	11	19.3	70 year	90 year
	Total	5035	100	256	5.084		

Table 2: Showing HBV positivity in female Population report by different investigators

Year	Investigator	Place of study	Sample size	No. of Positivity	Percentage
1980	Khatri etal	Bombay	1276	8	0.62
1989	Biswas et al	Chandigarh	1000	23	2.3
1991	Panda et al	Delhi	8431	191	2.26
1992	Gupta et al	Chandigarh	2337	58	2.48
1996	Sharma et al	Aligarh	157	16	10.19
1998	Prakash et al	Delhi	1112	106	9.5
2001	Abbas et al	Delhi	6910	70	1.01
2004	Varghese et al	Delhi	6341	52	0.82
2004	Sahani et al	Delhi	987	22	2.22
2005	Chakravorty et al	Delhi	400	17	4.25
2005	Banerjee et al	Kolkata	400	15	3.75
2012	Pande and Omar(present study)	Rural western UP	5035	256	5.084

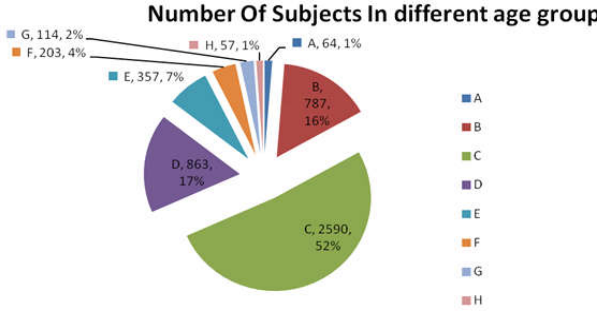

Fig. 1:

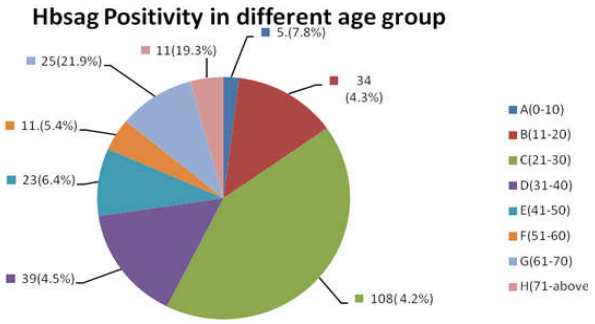

Fig. 2:

youngest neonate and old of 90 years .Maximum no. of subjects belonged to age group C(21-30years), 2590(51.5%)followed by group D(31-40years) 863(17.1%) and B(11-20years) 787(15.6%). Positivity for HBsAg was maximum in group G (61-70years) 21.9% and group H (71yrs and above) 19.5%. The youngest positive case in present study was a neonate and oldest subject of 90 years old lady.

Discussion

Five thousand and thirty five female subjects belonging to rural population of western U.P. ,ranging between age of neonate to 90 years were screened for presence of HBsAg. Out of 5035 subjects screened for HBsAg, 256(5.084%) were found to be positive . The youngest subject positive for HBsAg was a neonate and oldest lady was of 90 years age. The maximum HBSAg positivity was found in the oldest group i.e. (61-70years) and group H (71 years and above) and it was 21.9% and 19.3% respectively. In the age group C (21-30 years) and Group D (31-40 years) to which highest numbers of subjects were screened showed a positivity of 4.2% and 4.5% respectively. Group C and D age groups most important for rural ladies not only because of its sexually active age group but also because they have shoulder full responsibilities of house, children, and husbands and in laws. These age groups are also important because mother to child transmission of HBsAg may also occur if the female is positive for HBsAg and later this will increase the problems of rural female and family both mentally and economically. Incidentally there is increase in the incidence of HBsAg positivity after 40 years also.

There is no report available in literature about incidence of HBsAg positivity in rural population of India. Various Investigators have detected HBV markers in mothers of urban population or in tribal population but not in rural population. Reports available from neighboring areas like Delhi [2-6], Aligarh [7] and Chandigarh [4]. The reports from Delhi itself showed wide variation about HBV positivity ranging from 0.82% in the study of Varghese et al(2004) [12], to 9.5% by Prakash et al (1998) [8] Table 2.

Other Investigators have reported figures ranging between the above two extremes, Abbas et al (2001) [1] found HBV positivity -1.01%, Panda et al (1991) [7] 2.26%, Shahini et al (2004) [10] 2.22% in the mothers only. From Aligarh Sharma et al (1996) [11] reported positivity of 10.19% among mothers. In contrast from Chandigarh, Biswas et a l(1989) [3] reported 2.3% positivity in 1000 cases while Gupta et al (1996) [5] reported 2,48% of positivity in a study in their 2337 subjects. From Bombay (now Mumbai) Khatri et al (1980) [6] reported only 0.62% and From Kolkata Banerjee et al (2003) [2] reported 2.81 % positivity. In the present study the HBV positivity in rural population in females of western U.P. is nearer and slightly on higher side to those reported from studies of urban population of Delhi by Chakravorty et al (1997)-4.25% and Banerjee et al -3.75%, but these data are from urban population who are educated and relatively better of economically in contrast to rural population which is by and large uneducated and relatively poor. Higher percentage of positivity clearly reflects the status of rural population.

Concluding, a good vaccination strategy, information and education may will definitely reduce the incidence of HBsAg positivity in them and thereby have a positive impact on female health status of rural India.

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Original Article

Blood Stream Infections in Intensive Care Units: A Study from North India

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Abstract

Title : Blood stream Infections in Intensive Care Units. A Study from North India. *Study Design:* Prospective study. *Place and Duration of Study:* Sher-i-Kashmir Institute of Medical Sciences, Srinagar Kashmir. (July 2012 and Dec 2014). *Methodology:* A prospective analysis of blood specimens from various intensive care units (ICUs) was done over a period of four years. Antimicrobial susceptibility of culture positive isolates to various antibiotics was performed as per Clinical Laboratory Standards Institute (CLSI) guidelines.Gram-negative bacteria (GNB) were screened for extended spectrum β-lactamase (ESBL) and metallo-β-lactamase (MBL) production; whereas methicillin and vancomycin resistance was searched in *staphylococci* and *enterococci* isolates respectively. *Results:* The frequencies of Gram-positive and Gram-negative bacteria were 16.83% with yeast recovered in 5.78% of the specimens. *Acinetobacter spp* and *K. pneumoniae* were the most common Gram-negative bacteria and *S. aureus* the most common Gram-positive one. High level resistance to all the antimicrobials was seen; with *Acinetobacter spp* being the most multidrug resistant GNB isolated in the ICU setting. ESBL production was highest in *K. pneumoniae* isolates (77.1%). Also 49.6% of *Acinetobacter* isolates were found to be MBL producers. Methicillin resistance was seen in 95% of S. aureus and 91% of coagulase negative *staphylococci* (CoNS) isolates with vancomycin resistance seen in 46% of enterococcal isolates. *Conclusion:* An increasing trend over the years in the antibiotic resistance of blood stream pathogens in ICUs of this north Indian state was seen that calls for urgent measures to limit their continued rise.

Keywords: Gram-Negative Bacteria (GNB); Coagulase Negative *Staphylococci* (CoNS); Metallo-β-Lactamase (MBL).

Bloodstream infections (BSIs) occur more frequently in patients hospitalized in intensive care units (ICUs) than in other units. It has been shown that these patients stay in clinics longer than others. BSIs also cause an increase in hospital mortality rates and also increase hospitals charges. Early initiation of appropriate antimicrobial treatment is critical in decreasing morbidity and mortality among patients with BSI [1,2,3].

In the intensive care unit (ICU) setting, the incidence of infection is often higher than in the less acute in-patient or ambulatory setting. In the ICU, central venous access might be needed for extended periods of time; patients can be colonised with hospital-acquired organisms, and the catheter may be manipulated several times daily for administration

of fluids, drugs, and blood products. Moreover, some catheters may be inserted in urgent situations, during which optimal attention to aseptic technique might not be feasible [4].

ICU-BSI can occur either secondary to the dissemination of pathogens from a primary focus of infection at a clinical site into the bloodstream, or can be primary where the source of infection is unclear. The common clinical site foci for secondary ICU-BSI are the respiratory, gastrointestinal and urinary tracts [5, 2, 6 and 7].

The frequency, epidemiology and microbiological profile of nosocomial BSIs vary among institutions and also among ICUs within hospitals. Drug resistance has rendered antimicrobial therapy difficult in India like everywhere else and highly resistant bacteria like the MBL producing Gram-negative bacteria are a common occurrence in the hospital settings especially the intensive care units [8, 9]. Recently the New Delhi metallo β-lactamase (NDM) producing multidrug resistant Gram-negative bacteria have been reported from Kashmir. (10) These have serious implications for the management of critically ill patients in ICUs, limiting the utilitiy of beta-lactam antibiotics, fluoroquinolones and aminoglycosides. The present study was designed to identify the microbiological profile and susceptibility pattern of the organisms isolated from Blood stream infections of patients admitted in the ICUs of our hospital which is the only tertiary care institute in the North Indian state of Jammu and Kashmir.

Design

This observational study was performed in the medical–surgical ICU of a teaching hospital with around 700 beds in SKIMS Srinagar J&K, during the years July 2012–Dec 2014. The intensive care units included the surgical ICU (13-bed ICU), neonatal ICU (8-bed ICU), medical ICU (8-bed ICU) and cardiac surgical ICU (6-bed ICU).

Data Collection

The patients hospitalized longer than 48 h in the ICU were included in this study. The diagnosis of BSI was based on the criteria of the Center for Disease Control (CDC). (11) Blood cultures were taken from patients in BacT/Alert FA bottles. Bar coded, inoculated bottles were loaded into the BacT/Alert Microbial Detection System. Flag positive bottles were taken out from the system and streaked on Blood agar and MacConkey agar to be incubated at 37^0C overnight. Isolates were identified by VITEK 2 system Antimicrobial resistance patterns of isolated microorganisms were determined by VITEK 2 system according to the recommendations of the Clinical Laboratory Standards Institute (CLSI) (12) Descriptive statistics (frequency and percentage) was used for the presentation and comparison of data.

Definitions

BSI was defined as the isolation of a pathogenic microorganism from at least one blood culture specimen. Organisms of the skin flora commonly associated with contamination were required to be isolated from two separate blood culture specimens. A BSI was classified as primary in the absence of an identified source of infection or if it was catheter related. A BSI was classified as secondary in the presence of an identified source infected with the same microorganism at another body

Results

A total of 3112 were received from the medical–surgical ICU during the $2^{1/2}$ year study period out of which 524(16.83%) were culture positive (bacteria), 180(5.78%) were culture positive for yeasts; 2139(68.73%) were sterile and 269(8.64%) grew organisms generally regarded as contaminants. Most of the samples were received from the neonatal ICU 1725 (55.43%) followed by the surgical ICU 990 (31.81%), the medical ICU 182 (5.84%) (Table 1, Fig 1). Patients enrolled in the study included 1908(61.3%) males and 1204(38.7%) females. Majority of the patients had underlying respiratory or neurological disorders.

Among the BSI episodes caused by bacteria, 224 (42.74%) were caused by Gram-positive organisms and 300 (57.26%) by Gram-negative organisms. The most frequently isolated microorganisms of BSIs among gram positive organisms was *Staphylococcus aureus* (19.45%) followed by Coagulase negative *staphylococcus* (14.2%) and among gram negative batcteria it was *Acinetobacter spp* (14.55%) followed by *Klebsiella pneumonia* (12%) (Table 2). The proportion of *Enterococcal species* isolated from BSIs increased over the years from 6.6% in 2012 to 10.2% in 2014 ($p = 0.4522$). However, there was negligible change in the proportion of Gram-negative bacteria isolated from BSI, during this period ($p = 0.320$).

In the present study, 5% of *S. aureus* and 9% of Coagulase-negative *Staphylococci* (CoNS) were

sensitive to methicillin. All isolates were susceptible to vancomycin, teicoplanin and linezolid. All the isolates were resistant to Penicillin G. The rate of vancomycin resistance among *Enterococcus* spp. was 46% (n=22). Among these resistant isolates 20 were *Enterococcus faecium and 2 were Enterococcus fecalis.* Of 48 *Enterococcus* spp., only 10 (21%) isolates were susceptible to ampicillin. The effective antibiotics against the Gram-negative bacteria were amikacin, imipenem, gentamicin, levofloxacin and polymixin B. Among the Gram-negative bacteria, the rates of resistance to various antibiotics commonly used in the ICU were as follows: amikacin 52%, imipenem 51%, piperacillin-tazobactam 74%, ciprofloxacin 80%, ceftazidime 100%, levofloxacin 67%. None of the isolates of gram negative bacteria was resistant to polymyxin B. Piperacillin-tazobactam showed highest activity against *E.coli.*, whereas amikacin and gentamicin showed highest activity against *E.coli, Klebsiella and Enterobacter spp*. The rates of resistance to imipenem were 71% and 65% for *Acinetobacter* spp. and *P. aeruginosa*, respectively whereas imipenem showed good sensitivity for *E.coli* and *Enterobacter spp.*

Table 1:

	Total	Sterile	Contaminated	Yeast	Clinically Significant Bacteria
SICU	990	653	29	35	273
NICU	1725	1166	237	142	180
MICU	397	320	3	3	71
	3112	2139	269	180	524

Table 2:

Organism isolated	Cases(% isolated)
Enterobacteriaceae	133(25.45%)
Klebsiella pneumoniae	63(12%)
Enterobacter spp	29(5.45%)
Escherschia coli	26(5%)
Serratia spp	10(2%)
Salmonella Typhi	5(1%)
Acinetobacter spp	76(14.55%)
Coagulase negative Staphylococci	74(14.2%)
Staphylococcus aureus	102(19.45%)
Enterococcus spp	48(9.2%)
Stenotrophomonas maltophilia	28(5.4%)
Other (*Pseudomonas*, other non fermenters)	63(11.75%)

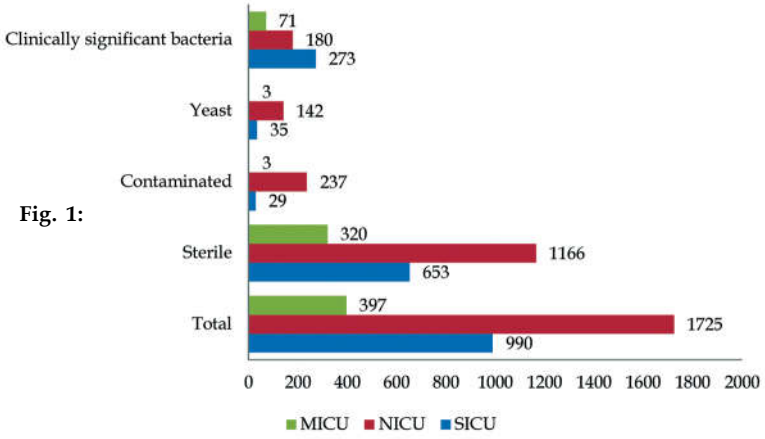

Fig. 1:

ESBL producing *K. pneuomoniae* accounted for 77.1% of the total number of *Klebsiella* strains isolated. Likewise, 37.4% of *E.coli*, were ESBL producers. Also 49.6% of *Acinetobacter* and 37.3% of *P. areuginosa* isolates were MBL producers. An increasing trend in the prevalence of these enzymes (ESBL, MBL) in the isolates was seen over the years.

Discussion

Nosocomial BSIs are associated with a high morbidity and mortality. Patients hospitalized in ICUs are at high risk of nosocomial BSIs because of their debilitated condition as a result of underlying

disease and frequent invasive diagnostic and therapeutic procedures [13,14]. Our data provides an understanding of the antibiotic resistance patterns of commonly isolated organisms in ICU patients in Kashmir. The scene is alarming and clearly demonstrates that drug resistance is on the rise and clinicians are left with very few options for treating patients with serious infections in the ICU.

Rise in the antimicrobial resistance among pathogens in ICU's due to inadvertent and non judicious administration of antibiotics generally before the availability of the culture results, is a matter of concern worldwide. Organisms causing nosocomial BSIs vary depending upon the location of patients within the institution [1].

In many studies, the dominance of Gram-positive pathogens has been documented. *CoNS, S. aureus* and *enterococci* were the three most common causes of nosocomial BSIs in many institutions [15, 16, 17 &18].

There has been a decrease in relative importance of infections as a result of Gram-negative bacteria over the past three decades [3,9]. In this study, the majority of ICU-acquired BSIs were as a result of *S. aureus* (19.45%). There was an increase in the proportion of *enterococci* among isolates from BSIs. This increase may be explained by extensive use of antibiotics and indwelling devices in this unit.

Increasing antimicrobial resistance rates among microorganisms isolated from BSIs are a significant problem worldwide. Methicillin-resistant *S.* aureus (MRSA), vancomycin-resistant enterococci(VRE), extended-spectrum beta-lactamase-producing *Klebsiellaspp.*, carbapenem-resistant enter obactericeae, *P.aeruginosa* and *Acinetobacter* spp. were seen more frequently in ICU patients than in non-ICU patients in many countries [1,20-27]. In the present study, 95% *Staphylococcus aureus* isolates were methicillin resistant. The rate of VRE was 46%. Vancomycin was used frequently in ICU. Amikacin, imipenem and polymyxin B were the most active compounds against Gram-negative bacteria.

In conclusion, this study demonstrates a high rate of antimicrobial resistance to several prescribed antibiotics among the microorganisms isolated from patients with BSIs. During this 3-year period, there was a tendency towards an increase in frequency of BSIs. The insufficient antibiotic-prescribing practices, especially the unnecessary use of broad-spectrum antibiotics together with the insufficient hospital infection prevention programme, are considered to be the cause of high antimicrobial resistance rate and an increased incidence of BSI.

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Original Article

Microbial Flora of Semen and Its Impact on Sperm Parameters

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Abstract

Various microorganisms have been found to colonise the male genital tract which may play an important role in altering seminal parameters and thereby reducing male fertilizing potential. A total of 35 semen samples, obtained from PGIMER, Sector-12, Chandigarh, India were subjected to routine semen analysis according to WHO guidelines. The volume of all the samples, motility of 88.5% samples, pH of 80% of the samples was optimum. On culturing these samples on Brain Heart Infusion agar plates, it was found that 80% of the isolates so obtained were Gram positive cocci, 18% Gram negatives and 2% were yeast. Out of these isolates, 4% showed complete immobilization whereas 12% led to agglutination of spermatozoa. Scanning electron microscopy showed morphological alterations in sperm head, neck and mid piece etc. when incubated with the sperm immobilizing and sperm agglutinating strains. Further when the enzymatic activity was looked for, the results showed that these organisms were able to produce either protease, phospholipase, lipase or all of these. Hemolysis on sheep blood agar showed that only 2% of the isolates were capable of causing complete hemolysis and 6% showed partial hemolysis. The Gram positive bacteria were maximally sensitive to Oxacillin and Gram negative were sensitive to tobramycin and gentamycin. In conclusion, various Gram positive and Gram negative bacteria inhabiting male reproductive tract might produce certain factors which may impair sperm parameters. Eradication of these microbes by use of antibiotics can be a probable cure of microorganism induced infertility.

Introduction

Amongst the leading causes of male infertility, genitourinary tract infection accounts for about 15% of the cases (Pellati *et al*., 2008). These infections not only deteriorate the quality of spermatozoa and sperm cell function but also the process of spermatogenesis. Different microorganisms have been reported to alter reproductive functions in a number of ways and to varied degrees. These microorganisms found in the male urogenital tract are associated with sperm abnormalities, especially a reduced sperm count, poor morphology, aberrant motility, deficient mitochondrial function, and loss of DNA integrity (La Vignera *et al*., 2011). These microorganisms include *Escherichia coli*, *Enterococcus faecalis, Ureaplasma urealyticum*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma hominis*, *Candida albicans*, and *Trichomonas vaginalis*. The possible mechanisms by which infertility occurs include attachment of bacteria to sperm, immobilizing factor

produced by some bacteria, immune system recruitment, alteration of glandular function, production of enzymes such as proteases, elastases, metabolic end products, apoptosis and necrosis (Cottell *et al*., 1996).

The alleged pathogenic bacteria are found not only in the reproductive tracts of infertile patients, but also in those of healthy men (Kiessling *et al*., 2008). Reports are at variance regarding the effect of indigenous bacteria on semen quality. Some investigators have reported that there was not any significant difference in sperm parameters of infectious and non-infectious ejaculates (Golob *et al*., 2014) whereas others have observed significant differences in sperm characteristics between fertile and infertile men (Weidner *et al*., 2013).

Therefore, it remains a controversy if the microorganisms found in semen necessarily signify infection and significantly contribute to male infertility (Hou *et al*., 2013).This discrepancy might be due to number of factors such as use of different collection procedures, differing definitions of bacteriospermia, contamination of semen by non-pathogenic commensals of skin, glans penis or lower urethra (Domes *et al*., 2012). Although, isolation of microorganisms in seminal fluid especially of infertile men has been widely reported, their pathophysiologic role in male infertility has not been established (Maciejewska *et al*., 2005).

Due to shortcomings in diagnostic standards and asymptomatic nature of the microbial infections, their exact role in the aetiology of infertility is not very certain, however, their possible effect on the properties of seminal fluid has been suggested (Bukharin, 2002). Though many studies agree that bacterial infection in the genital system could be possible reason for a significant number of cases of male infertility, yet, there is no consensus on how these microbes specifically affect seminal parameters (Khalili *et al*., 2000). Given that, it is one of the potentially preventable forms of infertility; thus, culture-positive patients must undergo antibiotic profiling of the associated microorganisms, before the initiation of the therapy. In this regard, studies conducted by Mogra *et al*., (1981) have shown that out of the most frequently isolated microorganisms from the semen of infertile men, all the strains of *S. aureus* were resistant to Penicillin, thereby, indicating the pervasiveness of penicillin-resistant Staphylococci in semen. Recently, a study conducted by Pajovic *et al*., (2013) has shown a clear effect of antibiotic therapy on the volume and pH of the seminal fluid. Moreover, on completion of the therapy, a significant improvement in sperm concentration and motility was observed.

Therefore, it is necessary to scrutinize seminal fluid for the presence of bacteria so as to fill the gaps in knowledge about infections as cause of infertility.

Materials and Method

Semen Sample

Semen samples were obtained from the patients attending infertility clinic, PGIMER, Chandigarh, India. On liquefaction (37^0C, 30-45min), various macroscopic parameters viz. colour, volume and pH and microscopic parameters viz. sperm count, motility, viability were evaluated as per the WHO standards (WHO, 2010).

Isolation and Presumptive Identification of Microorganisms from Semen

Semen samples were streaked on Brain Heart Infusion agar plates and incubated at 37^0C for 48h and observed for bacterial growth. The isolates so obtained were subjected to various tests for identification according to the characteristics laid down in the Bergey's Manual of Determinative Bacteriology.

Effect of cell culture and cell free supernatant of isolates on motility/ agglutination/viability of human spermatozoa.

The effect of cell culture and cell free supernatants of isolates was studied on motility/agglutination/ viability of human spermatozoa. Briefly, isolates were grown for 72h at 37°C under shaking and stationary conditions. The cultures were then centrifuged, supernatant was separated and the cells were washed with PBS (50mM, pH 7.2) and resuspended in same buffer. Human semen ejaculates that satisfied WHO criteria of normal standards were selected and the sperm count adjusted to 39×10^6 per ml with sterile PBS (pH 7.2) used as diluent. Equal volumes of sperm suspensions and cell culture/ cell free supernatant (1:1) were mixed and incubated at 37^0C. For control PBS/ BHI was added instead of cell culture/cell supernatant. At different time intervals, a 10 µl aliquot of the mixture was placed on a clean glass slide, covered with a coverslip and observed under X400 magnification using a bright-field microscope (Olympus).

Scanning Electron Microscopy

Scanning electron microscopy was used to investigate the morphology of the spermatozoa. The sample processing was done according to the

standard method (Hafez and Kanagawa, 1973) with slight modifications. For this, semen sample was washed twice with PBS at 700rpm for 10min and finally resuspended in same buffer so as to get a final count of 40 x 10^6/ml. 200µl of this sample was mixed with 200µl of bacterial culture and incubated for 2h for 37^{0}C. As control, 200µl of sample was mixed with 200µl of PBS. After incubation, 2.5% of gluteraldehyde was added and incubated at 37^{0}C for 30min. The samples were then washed thrice with PBS. One drop of fixed and washed spermatozoa was placed on a silver painted adhesive tape mounted on brass stubs and air dried. 100 A^0 gold coating was done using fine coat; Jeol ions sputter (JFC-1100). This gold coated stub was finally examined at different magnifications under the scanning electron microscope (model JSM-6100, SM-Jeol 20kV). SEM was carried out at sophisticated analytical instrumentation facility (SAIF), Panjab University, Chandigarh.

Screening of Isolates for Various Enzymatic Activities

Protease: Proteolytic activity was determined by the method described by Iida *et al*., 1982. For this, 1% milk casein agar plates were prepared and isolates were inoculated on by spotting and plates were incubated for 24-72h at 37^{0}C. The activity was revealed by the clear zone formation around the inoculum spot.

Phospholipase: Phospholipase activity was analysed by the procedure described by Collee and Miles (1989). Briefly, egg yolk was collected under sterile condition and was mixed with normal saline in equal volumes (1:1). 50% egg yolk agar plates were made by and the isolates were spotted on the plate and incubated at 37°C for 72h. Clear zone formation around the colonies were taken as phospholipase positive.

Lipase: Lipase activity was checked by using tributyrin agar plates (Collee and Miles, 1989). Tributyrin agar plates were prepared by adding 1% tributyrin (v/v) to nutrient agar medium. Isolates were spotted on the plates and incubated at 37^{0}C for 72h. Clear zone formation around the colonies indicated lipase positive.

Quantitative Assay for Extracellular Enzymatic Activity of Organisms

All the positive isolates were further used for quantitation of extracellular enzymes like protease, phospholipase, and lipase. 5mm steel wells were placed on 1% milk casein agar plates, 5% egg yolk agar plates, 1% tributyrin agar plates. 100µl of 72h old cell free supernatant was added to each well. Plates were kept at 4^{0}C for 2h and incubated at 37^{0}C for 24h.

Production of Haemolysin by the Isolates

Certain bacteria produce extracellular enzymes that lyse red blood cells in the blood agar (haemolysis). These haemolysins (exotoxin) radially diffuse outwards from the colony causing complete or partial destruction of the red cells (RBC) in the medium and complete denaturation of haemoglobin within the cells to colourless products. β-haemolysis (complete or partial haemolysis) was seen on sheep blood agar. For this 5% blood agar plates were prepared. The isolated colonies were streaked on plates and the plates were incubated at 37^{0}C for 24h. After incubation, the plates were observed for zone of haemolysis surrounding the colony.

Antibiotic Susceptibility Test

To check the antibiotic susceptibility, bacterial isolates were inoculated in 10ml BHI broth under shaking conditions at 37^{0}C for 24h.100µl of culture was spread plated on sterile Muller Hinton Agar plates. Antibiotic disk was removed from container under sterile conditions with the help of sterile forceps and carefully placed on the surface of the medium. The disk was slightly pressed with the help of forceps to make complete contact with surface of medium. The plates were incubated at 37^{0}C for 24h. After incubation, diameter of zone of inhibition was recorded in mm.

Results

Semen Analysis

A total of 35 semen samples were obtained from patients attending infertility clinic PGIMER, Chandigarh, India. The samples were macroscopically and microscopically examined according to WHO standards. Macroscopic parameters such as colour, pH and volume of the semen sample were looked for. All the samples were homogeneous and had grey-opalescent appearance. The volume of the sample was in the normal range of 1.5 to 6 ml. The pH of all the samples was also in optimal range i.e. 7.2 to 7.6 except for 20% samples where pH range (pH 6- 6.8) was below optimum.

The microscopic parameters such as % motility, agglutination, % viability, morphology and presence of other cells were also assessed. The results revealed

that percentage motility of 88.5% of the samples was normal (lower reference limit 40) whereas 25.7% of the samples showed agglutination. The percentage viability of 68.6% of the samples was above 58% which was the reference limit of WHO. All the samples had normal sperm morphology however, 34.3% of the samples showed presence of round cells.

Isolation of Microorganisms from Semen Samples

For isolation of microorganisms, semen samples were cultured on Brain Heart Infusion (BHI) agar plates and the plates were incubated aerobically at 37^0C for 24 to 48 h. In total 50 isolates were obtained and Gram staining of these isolates showed the presence of Gram positive (80%), Gram negative (18%) microorganisms and also yeast 2% (Figure 1).

Presumptive Identification of Microorganisms

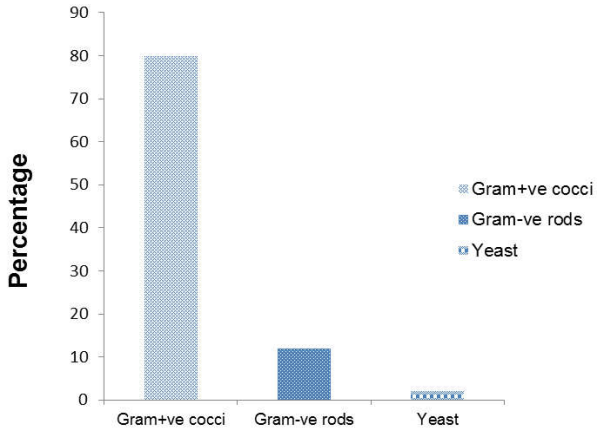

Fig. 1: Distribution of Gram positive, Gram negative and yeast in semen samples

Gram Positive

For the presumptive identification, Gram positive microorganisms were subjected to catalase test and the results showed that all the isolates were catalase positive indicating that they belonged to either *Micrococcus* or *Staphylococcus*. These isolates were further tested for their ability to ferment mannitol under aerobic and anaerobic conditions. It was observed that 80% of the isolates could ferment mannitol under both aerobic and anaerobic conditions revealing these isolates to be staphylococci whereas 20% of the isolates which fermented mannitol aerobically only were identified as micrococci.The different species of *Staphylococcus* were further identified by coagulase test. 45% of the total gram positive isolates were positive for coagulase leading to their presumptive identification as *Staphylococcus aureus* and rest 35% of the total isolates were coagulase negative staphylococci. *Staphylococcus aureus* was confirmed by presence of yellow zones around the colonies on mannitol salt agar (MSA) (Figure 2).

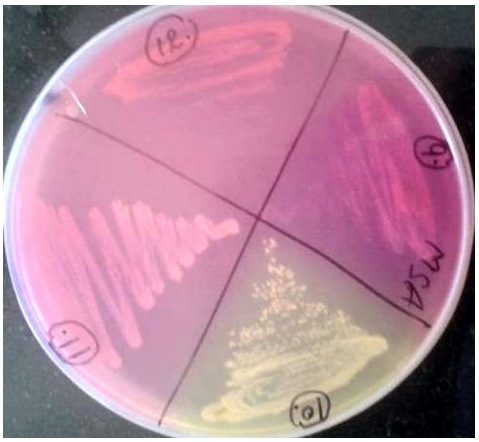

Fig 2: Representative photograph of *Staphylococcus aureus* showing yellow colonies on MSA and pink coloured colonies indicating coagulase negative staphylococci.

Gram Negative Microorganisms

Gram negative organisms so obtained were subjected to oxidase test. The result showed that 55.6% of the total gram negative isolates were oxidase negative indicating these isolates to be belonging to *Enterobacteriaceae*. These isolates were further biochemically identified. Based on biochemical characterization, it was observed that out of total isolates, 44.4% were *Escherichia coli* whereas 11.1% were *Serratia*. *E. coli* was further confirmed by the presence of green metallic sheen on eosin-methylene blue agar *Serratia* was also found to give red pigmented colonies on BHI (Figure 3).

The oxidase positive isolates (44.4%) were identified to be *Pseudomonas* by their ability to utilize various biochemicals. They were also found to produce green pigmented colonies on BHI.

Effect of cell culture and cell free supernatant of the isolates under stationary and shaking conditions on motility, agglutination and viability of Human spermatozoa.

All the 50 isolates obtained from semen samples were grown in BHI for 72h (shaking and stationary conditions) and the interaction of culture and cell free supernatant with human spermatozoa was studied with respect to change in motility, agglutination and viability of spermatozoa.

Cell Culture

Motility and Agglutination

When semen samples were mixed with 72h old cell culture, it was observed that 4% of the isolates under stationary as well as shaking conditions could cause 100% immobilization of spermatozoa. 4% and 12% of the isolates grown under stationary and shaking conditions resulted in ≥ 50% sperm immobilization, respectively. However, immobilization in 80% of isolates grown under stationary conditions and 72% of the isolates in shaking conditions was not significant. The 12% of the isolates in both the conditions could result in agglutination of spermatozoa however, the size of agglutination clumps were larger under shaking conditions (Figure 4)

Cell free Supernatant

The effect of cell free supernatant from 72h old cultures of semen sample isolates on motility of spermatozoa was studied. The results showed that 4% of supernatants from the isolates under stationary as well as shaking conditions could cause 100% immobilization of spermatozoa. ≥ 50% of sperm immobilization was observed in 8% and 24% of the isolates grown under stationary and shaking conditions respectively. However immobilization in 88% of isolates grown under stationary conditions and 72% of the isolates in shaking conditions was insignificant. No agglutination was observed in any of the supernatants.

Percentage Decrease in Viability

The decrease in motility was more pronounced with cell culture and supernatant under shaking conditions, therefore viability of the spermatozoa was studied with cell culture and supernatant under shaking conditions only. Results showed that cell culture as well as supernatant of only 18% of the isolates could lead to ≥ 50% death of spermatozoa (Figure 5).

Fig. 3: Representative photographs showing (A) Green metallic sheen of *E. coli* on EMB agar plate(B) Red pigmented colonies of *Serratia* on BHI agar plate (C) Green pigmented colonies of *Pseudomonas* on BHI agar plate.

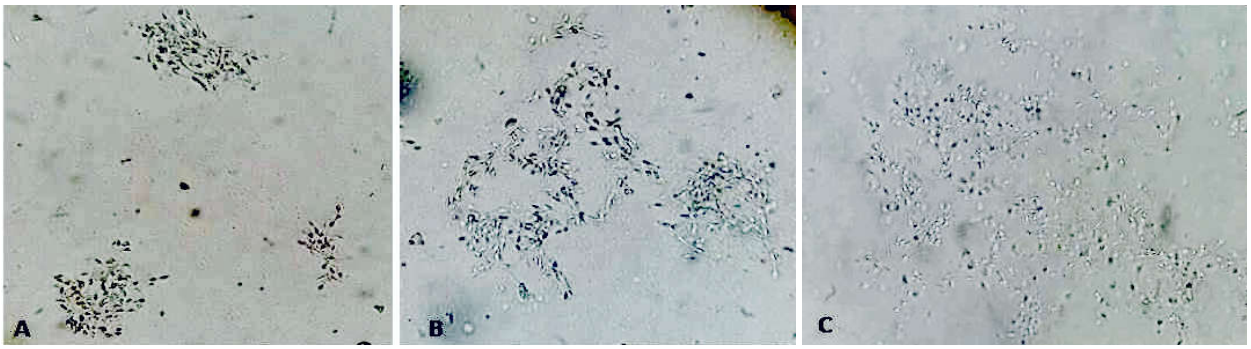

Fig. 4: Photomicrograph showing grades of agglutination of human spermatozoa (A) Grade 2; (B) Grade 3; (C) Grade 4

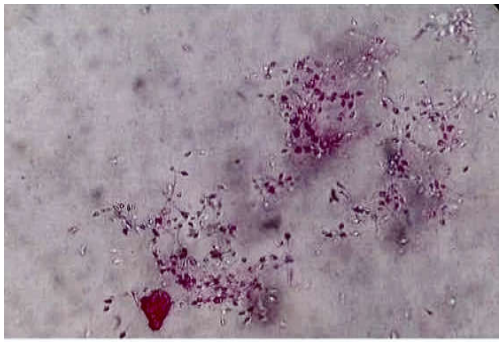

Fig. 5: Representative photograph of eosin staining of human spermatozoa after incubation with isolates showing live spermatozoa (unstained) and dead spermatozoa (pink stained)

Scanning Electron Microscopy

The effect of cell cultures of sperm agglutinating *E. coli, Serratia* and sperm immobilizing *Pseudomonas* sp. on human spermatozoa was studied by scanning electron microscopy. Cell cultures were incubated with 200µl of sperm suspension (40×10^6 sperms ml^{-1}). BHI was added to the sperm suspension in case of control.

Normal human spermatozoa were observed in control which was characterized by flattened ovoid heads covered anteriorly by rough rigid surface and posteriorly by smooth surface (Figure 6A).

From the results, it was observed that sperm agglutinating *E. coli* and *Serratia* could adhere to spermatozoa. Further, *E. coli* resulted in morphological alterations in head, neck and midpiece region due to loosening and disruption of membrane (Figure 6B). *Serratia* also led to loosening and disruption of membrane causing damage in head and tail. Breakage in the neck region and coiling of end piece of tail was also observed with *Serratia* (Figure 6C). However, decrease in thickness around neck region was observed with sperm immobilizing *Pseudomonas* sp. (Figure 6D).

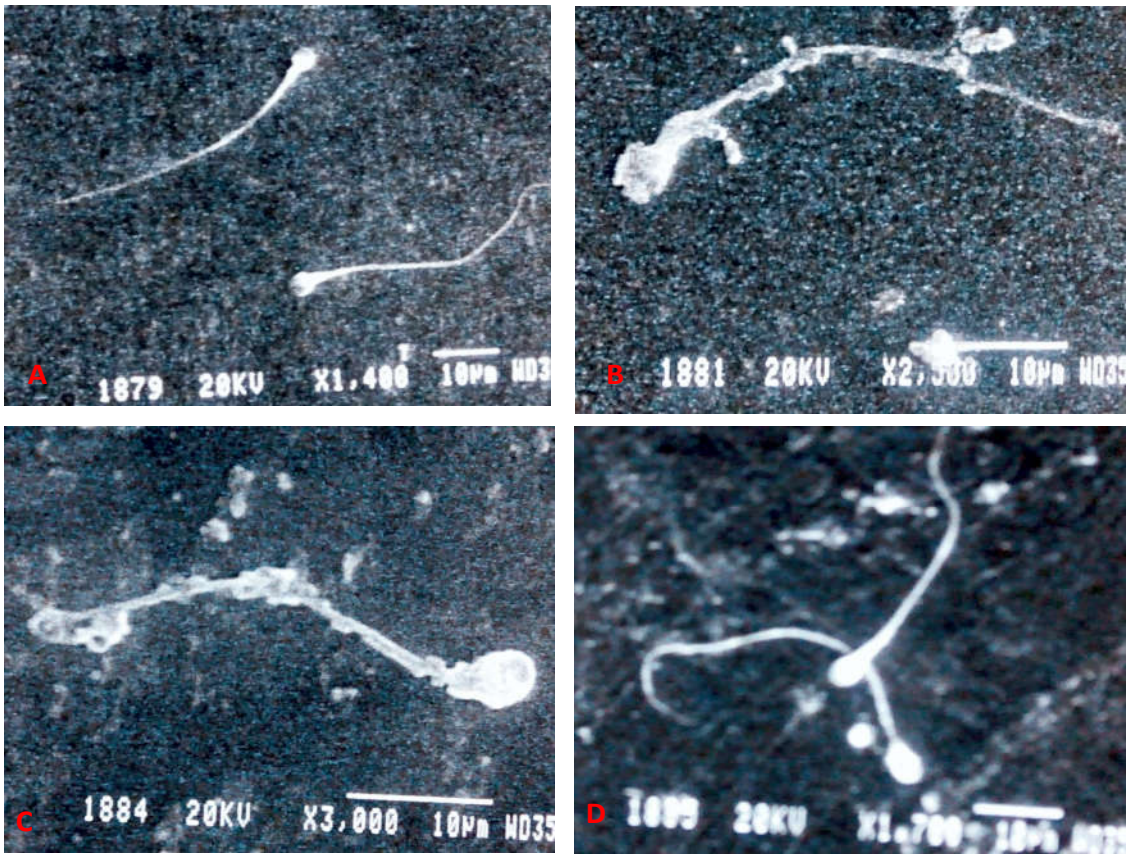

Fig. 6: Scanning electron micrograph of (A)control showing normal spermatozoa, (B) spermatozoa showing morphological alteration on treatment with sperm agglutinating *E. coli*, (C)spermatozoa showing loosening and disruption of membrane on treatment with spermag glutinating *Serratia* (D)spermatozoa showing decrease in thickness around neck region on treatment with sperm immobilizing *Pseudomonas* sp.

Enzymes Produced by Microorganisms

All the 50 isolates from semen samples were screened for their abilities to produce various enzymes like protease, phospholipase, and lipase on milk agar, egg yolk agar and tributyrin agar plates, respectively (Figure 6). From the results, it could be observed that out of total isolates, 40% hydrolysed casein indicating protease activity, 4% showed phospholipase activity and 56% isolates possessed lipase activity (Figure 7).

Quantitation of Extracellular Enzymatic Activity of Microorganisms

All positive isolates were further used for quantitation of extracellular enzymatic activity like protease, phospholipase and lipase by means of quantitative test following the well diffusion technique. From the results, it was observed that 29% of the protease positive isolates, all the phospholipase positive isolates and 22% of the lipase positive isolates produced enzymes extracellularly(Fig:8).

Protease, Phospholipase and Lipase activity in terms of clear zone (mm) produced by 100µl of cell free supernatant on milk agar, egg yolk agar and tributyrin agar plates after 48h of incubation at 37°C.

On the basis of preliminary screening of seminal isolates with respect to their enzyme production, organisms were grouped under $P^+P_L^+L^+$, $P^+P_L^+LÉ$, $P^+P_L^ÉL^+$, $P^ÉP_L^+L^+$, $P^+P_L^ÉLÉ$, $P^ÉP_L^+LÉ$, $P^ÉP_L^ÉL^+$, $P^ÉP_L^ÉLÉ$ (P-protease, P_L-phospholipase, L- lipase). From the results, it was observed that 4% were $P^+P_L^+L^+$, 26% were $P^+P_L^ÉL^+$, 10%were $P^+P_L^ÉLÉ$, 26% were $P^ÉP_L^ÉL^+$ and 19%were $P^ÉP_L^ÉLÉ$ whereas none of the isolates fell under the groups $P^+P_L^+LÉ$, $P^ÉP_L^+L^+$, and $P^ÉP_L^+LÉ$.

When the organisms were grouped on the basis of enzyme activity and their ability to impair sperm motility, it was observed that all the isolates of group $P^+P_L^+L^+$(2/2) could cause 100% immobilization of spermatozoa.

7.6% of the isolates from group $P^+P_L^ÉL^+$, 20% of the isolates belonging to $P^+P_L^ÉLÉ$ group, 7.6 % of the isolates of group $P^ÉP_L^ÉL^+$ and 17.6% isolates of the group $P^ÉP_L^ÉLÉ$resulted in agglutination of spermatozoa.

Thus protease and lipase seemed to be important enzymes affecting sperm motility. However, immobilization of spermatozoa was also observed in the 17.6% of isolates belonging to group $P^ÉP_L^ÉLÉ$, producing none of the above enzymes, indicating that some other factors might be causing sperm impairment.

Production of Haemolysin by Isolates

The isolates were further checked for haemolysin activity on sheep blood agar plates. It was observed that only 2% of the isolates showed complete haemolysis (transparent zone was observed due to lysis of red blood cells), 6% of the isolates showed partial haemolysis whereas all the remaining isolates failed to lyse sheep RBCs (Figure 9).

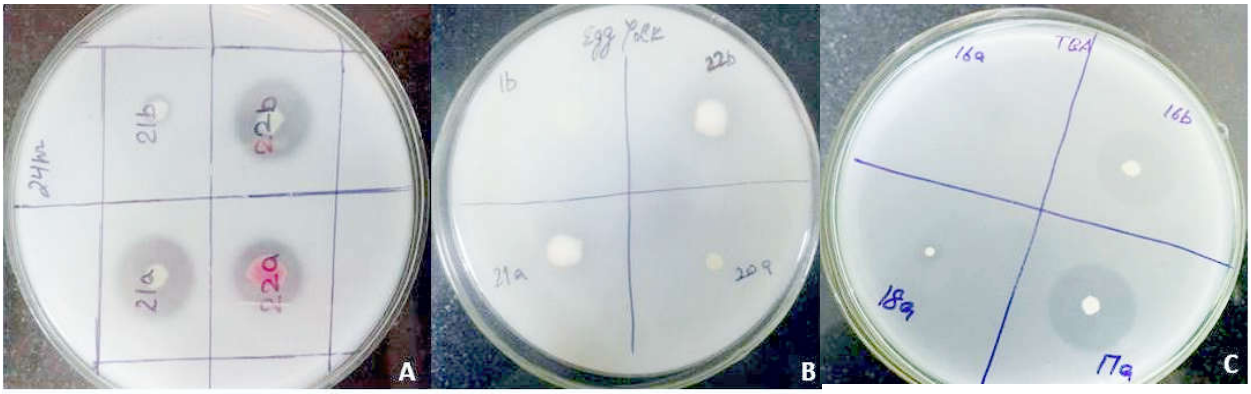

Fig. 7: Representative photographs showing (A) protease, (B) phospholipase and (C) lipase activity in terms of clear zone produced by isolates on milk agar, egg yolk agar and tributyrin agar plates respectively

Fig. 8: Quantitative assay of protease, phospholipase and lipase activity in terms of clear zone size (mm) produced by isolates on milk agar, egg yolk agar and tributyrin agar plates by plate method

Antibiotic Susceptibility

In vitro, antimicrobial susceptibility was determined for clinical isolates by disk diffusion method, recommended and interpreted according to CLSI (Clinical and Laboratory Standard Institute) guidelines.

11 commonly used antibiotics belonging to different classes were chosen for Gram positive

organisms and zone of inhibition in mm was measured (Figure 10). Most of isolates (96%) were found to be sensitive to Oxacillin, only 3.4% isolates were resistant, 79% of isolates were resistant and 21% were sensitive to Penicillin G and 48% isolates were found to be intermediate, 3.4% were resistant and 48% were sensitiveto cefotaxime (Figure 11).

For Gram Negative organisms, 6 antibiotics belonging to different classes were chosen and zone of inhibition in mm was measured (Figure 12). All isolates were sensitive to Tobramycin and Gentamycin and all isolates were seen to be resistant to Ampicillin and Amoxyclav. 22.2% of isolates were seen to be intermediate, 11.1% were resistant and 67% were sensitive to cefotaxime whereas 55.5% were resistant and 44.4 % were sensitive to co-trimaxazole (Figure 13).

Fig. 9: Representative photograph showing clear zone indicating haemolysis on sheep blood agar

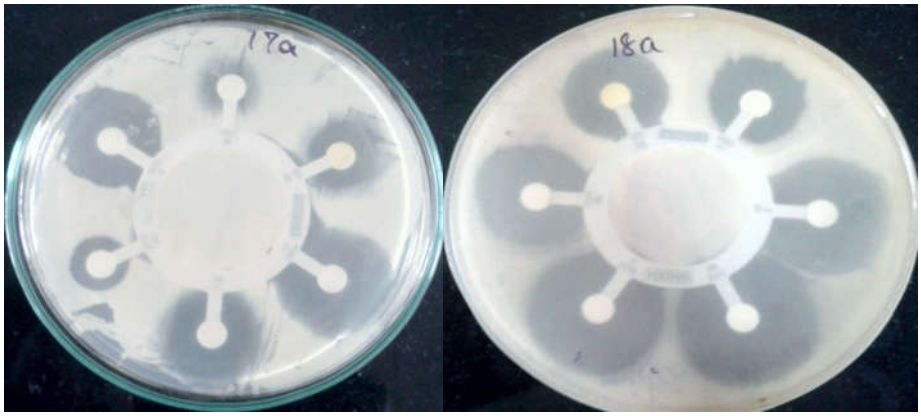

Fig. 10: Susceptibility of Gram Positive Organisms to different antibiotics. A disk diffusion test with an isolate of semen sample. The diameters of all zones of inhibition are measured and those values translated to categrories of susceptible, intermediate or resistant using the latest tables published by the CLSI.

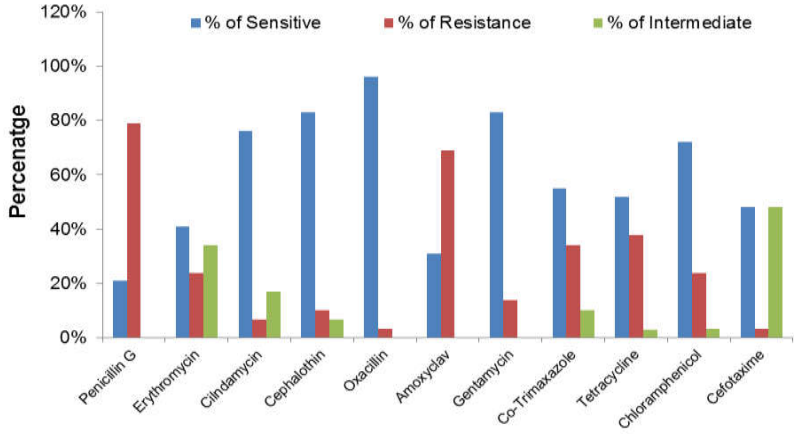

Fig. 11: Percentage susceptibility of Gram positive organisms

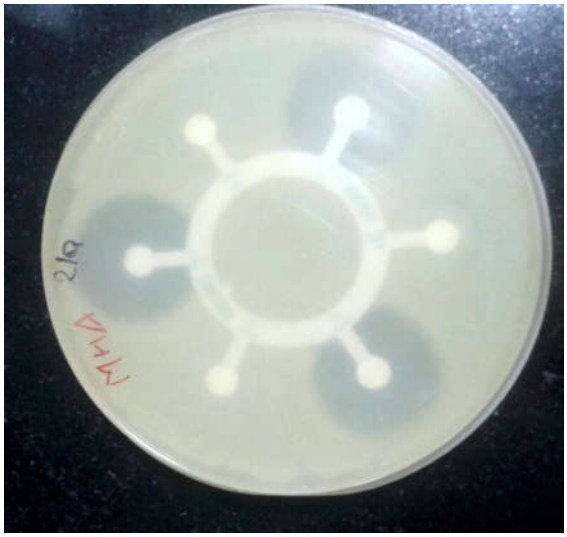

Fig. 12: Representative photograph shows antimicrobial susceptibilty of Gram negative organisms. A disk diffusion test with an isolate of semen sample. The diameters of all zones of inhibition are measured and those values translated to categories of susceptible, intermediate or resistant using the latest tables published by the CLSI.

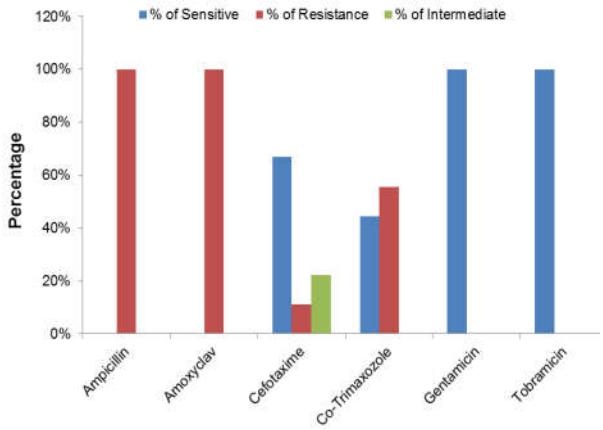

Fig. 13: Percentage susceptibility of Gram negative organisms

Discussion

Semen analysis is considered as an important step to examine various disorders distressing the genital tract of males (Andrade-Rocha, 2003). As the ejaculate is a blend of secretions from the urogenital tract and the male accessory glands, therefore, analysis of semen can offer a clear understanding in terms of sperm parameters viz. count, motility (ability to move), vitality and morphology (size and shape) and presence of non-sperm cells, if any (Samplaski *et al*., 2010). Investigation of these parameters helps in gaining important clinical information regarding spermatogenesis, the functional competence and the secretory pattern of glands (Moazzam *et al*., 2015). Also, evaluation of these factors may help us uncover the reasons for infertility.

Recently, role of microorganisms in male infertility has been constantly gaining consideration. Various microorganisms commonly found in the male genital tract include *Escherichia coli*, *Mycoplasma hominis*, *Enterococcus faecalis, Neisseria gonorrhoeae*, *Ureaplasma urealyticum*, *Chlamydia trachomatis*, *Candida albicans*, and *Trichomonas vaginalis* (Nelson *et al*., 2010). They may lead to altered semen parameters in different ways. Therefore, the present study was carried out with an aim to assess the frequency of occurrence of different microorganisms in semen and to study their effect on semen quality.

A total of 35 semen samples were obtained and were primarily checked for the macroscopic parameters viz. colour, pH and volume. All the parameters were found to be in normal ranges except in 20% of the samples where pH was lower than the reference limit. Further, when the microscopic seminal parameters like motility, viability and agglutination were evaluated, the results showed that morphology was normal in all the samples; % motility in 88.5% of the samples was normal (i.e. above 40%) and rest 11.5% of the samples showed motility below 40%. The % viability of 68.6% of the samples was normal (i.e. above 58%) whereas the remaining 31.4% samples showed viability less than 58%, which was lesser than the WHO standards. Furthermore, only 34.3% samples also showed the presence of other cells like pus cells and macrophages.

As the incidence of microorganisms in semen can be linked to infertility, therefore, semen samples were cultured on growth media to check the presence of any microbial isolates. It was found that out of all the isolates obtained, Gram positive bacteria were the predominant flora (80%) as compared to Gram negative bacteria (18%) and yeast (2%). These isolates were further identified based on their ability to utilize various biochemicals. The results showed that coagulase positive Staphylococci, coagulase negative staphylococci and micrococci constituted the Gram positive organisms. The major Gram negative bacteria identified were *E. coli, Serratia* sp. and *Pseudomonas* sp.

Similar results have been obtained by Momoh *et al*., (2011) wherein *S. aureus* was found to be the major organism with 75% prevalence in the semen samples studied whereas *E. coli* and *Pseudomonas* were lesser in number. These results are also in concordance with the work of Ekhaise and Richard (2014), who also reported the occurrence of *S. aureus* (77%) and *E. coli* (11.1%) in semen samples of men complaining of infertility. Similar findings have been documented by Ikechukwu *et al*., (2007) showing *S. aureus* (37.1%) as the highest bacterial isolate and *E. coli* (8.9%) as

the lowest.

A number of microorganisms are capable of interacting directly or indirectly with spermatozoa (Golshani *et al*., 2006) resulting in immobilization, agglutination phenomena and morphological alterations of the spermatozoa. In the present study, when the effect of these isolates was checked on sperm parameters *in vitro* under stationary conditions, it was found that12% of the isolates (comprising of *E. coli* and *Serratia* sp.) were capable of impeding sperm motility via agglutination of spermatozoa. On the other hand, only 4% of the isolates (belonging to *Pseudomonas* sp. and *S. aureus*) could cause 100% immobilization of spermatozoa while 50-100% of sperm immobilization was observed in another 4% of the isolates (belonging to *Pseudomonas* sp. and *S. aureus*). However, yeast isolates failed to agglutinate or immobilize spermatozoa. Moreover, the size of clumps of agglutinated spermatozoa was bigger and inhibition of motility was better when the cultures were grown under shaking conditions rather than stationary conditions.

Seminal parameters such as count, percent motility and percent viability play a vital role in fertility potential of men (Dohle *et al*., 2005). Hence, with an aim to find out the effect of these isolates on the viability of spermatozoa, the supra-vital staining was done. From the results, it was observed that cell cultures of only 18% of the isolates could lead to death of more than 50% of spermatozoa. Similar findings have been reported by Liu *et al*., (2002) wherein they have reported spermicidal effect of *S. aureus* when co-incubated with human spermatozoa. Similarly, Teague *et al*., (1971) has reported that upon incubation of *E. coli* with human spermatozoa, there was a significant inhibition of motility and viability.

Similar findings have been made by Diemer *et al*., (2003) wherein *E. coli* was seen to adhere to human spermatozoa *in vitro*, resulting in agglutination of spermatozoa. In another study, Vander and Prabha (2015) have also shown that *Serratia marcescens* causes agglutination of human spermatozoa. Also, while studying the effect of *Pseudomonas* on sperm motility parameters, Rennemeier *et al*., (2009) has reported that it could lead to reduction in motility of spermatozoa in a dose dependent manner. The results obtained by Huwe *et al*., (1998) are in concordance with our study, who also checked the influence of various uropathogens on human sperm parameters by means of CASA and reported that *S. aureus* retards the sperm motility. However, our results are in contrast to the work done by Tian *et al*., (2007) who have reported that yeast, has an inhibitory effect on human sperm motility.

Further, in order to detect the ultrastructural anomalies of spermatozoa caused by these isolates, scanning electron microscopy (SEM) was carried out. The results showed the adherence of sperm-agglutinating *E. coli* and *Serratia* sp. to spermatozoa, thereby, causing prominent morphological defects. However no adherence was observed in case of spermatozoa upon incubation with sperm immobilizing *Pseudomona s*sp. or PBS.

This observation is supported by the study made by Wolff *et al*., (1993) wherein they have shown the attachment of *E. coli* to both heads and tails of spermatozoa. In another study, Diemer *et al*., (2000) have revealed multiple and intense alterations in the ultrastructure of spermatozoa, such as membrane defects as well as cytoplasmic vacuoles, when incubated with *E. coli*. Morphological alterations involved all of the superficial structures of sperm, in particular the plasma membrane of the mid-piece and neck and the acrosomal membrane, showing that these morphological defects might be responsible for the immobilization of spermatozoa. Similar results were obtained by Ohri and Prabha (2005) wherein SEM of sperm samples incubated with *Staphylococcus aureus* showed spermatozoal structural abnormalities.

Moreover, to check whether the immobilizing activity was the property of supernatant or cells, the supernatants of the isolates were checked for their effect on spermatozoa *in vitro*. From the results, it could be seen that supernatants from 4% of the isolates were capable of causing 100% immobilization of spermatozoa whereas 8% of the isolates rendered 50% of the sperms immotile. However, no agglutination was observed in any of the supernatants. Our results are in agreement with findings of Rennemeier *et al*., (2009), who have reported deleterious effects on sperm motility by quorum sensing signalling molecule, a secretory factor from *P. aeruginosa*. The decrease in sperm motility and viability was also observed earlier by Schluz *et al*., (2013), when *E. coli* supernatant was incubated with spermatozoa. Also, Paulson and Polakoski (1977) have reported a soluble, heat-stable factor of low molecular weight isolated from *E. coli* filtrates as being the causative agent for the immobilization of human spermatozoa. These results further corroborate earlier findings of our laboratory wherein sperm immobilization factor isolated and purified from supernatant of *E. coli* (Prabha *et al*., 2010) and *S. aureus* (Prabha *et al*., 2009) were shown to cause sperm immobilization *in vitro*.

Since the effect of various microorganisms on sperm parameters under *in vitro* conditions is multifactorial, therefore microbial products such as

enzymes were looked for their negative influence on spermatozoa. Some researchers have reported different pathogenetic mechanisms exerted by microorganisms upon spermatozoa, such as production of proteases, phospholipases A and C, and lipases, may injure sperm parameters (Fraczek and Kurpisz, 2007). On the similar grounds, screening of all the isolates for production of various enzymes viz. protease, phospholipase and lipase was done. From the results, it was observed that out of 50 isolates, 56% were found to be positive for lipase production, 40% for proteases, and rest 4% for phospholipases indicating that production of these enzymes may, in part, be responsible for the various detrimental effects on human spermatozoa. Further, the isolates positive for enzymatic activity associated with cell culture were screened for extracellular enzymatic activity. From the results, it was observed that 29% of the protease positive isolates, all the phospholipase positive isolates and 22% of the lipase positive isolates produced enzymes extracellularly. Based on the above grouping, it was observed that 100% of the isolates possessing all the three enzymatic activities could cause 100% immobilization of spermatozoa. However, 7.6% of the isolates which were positive for both protease and lipase activity; 17.6% negative for both; 20% and 7.6% positive for protease and lipase activity, respectively, resulted in agglutination of spermatozoa. Our results are in consistence with those of Villegas *et al.,* (2005) who have demonstrated that *E. coli* could cause sperm deterioration by activating several proteases, responsible for alterations in membrane symmetry. However, the isolates which lacked all the three enzymatic activities were also found to impair spermatozoa via agglutination which paved the way for the involvement of some other factor which might be responsible for the same.

A few researchers have described, in an *in vitro* study, a negative influence of hemolysin, a virulence factor of Enterococci, on membrane integrity of sperm head, neck and mid piece (Qiang *et al*., 2007). Similar observations were made by Boguen *et al*., (2007) who have reported that damaging effect of *E. coli* on sperm could be attributed to alpha hemolysin production. In view of these findings, in the present study, all the isolates were tested for haemolysin production, and the results revealed that 2% of the isolates showed complete hemolysis, 6% showed incomplete hemolysis whereas 92% of the isolates showed no hemolysis. The results in the present study indicate that haemolytic condition might not be important in pathogenicity affecting human sperm parameters; rather some other factor might be more relevant.

Antibiogram typing is a traditional epidemiological typing method used to distinguish between individual strains. The organisms exhibit remarkable versatility in their behaviour towards antibiotics (Uwaezuoke *et al*., 2004), with some strains have overcome most commonly used drugs. Globally there is an increasing concern about the spread of antibiotic resistance in various strains of microbes. Hence, for specific therapy for infertility resulting from bacterial infection, sensitivity of microorganisms to an array of antibiotics must be determined.

With this aim, in the present study, antibiogram typing of all the isolates was done by disc diffusion method and zone of inhibition was measured. From the results, it was observed that out of all the gram positive isolates tested,96%of the isolates were sensitive to most of the antibiotics tested except Penicillin G (to which 79% were resistant), whereas intermediate sensitivity was seen in case of Cefotaximine. In line with our findings, Mogra *et al*., (1981) have also shown that all the strains of *S. aureus* isolated from infertile patients were resistant to Penicillin, thus indicating the high prevalence of penicillin resistant Staphylococci in seminal fluid. This development of bacterial resistance to Penicillin G could be attributed to their frequent and indiscriminate use. In case of gram negative isolates, all the isolates were sensitive to Tobramycin and Gentamycin but were resistant to Ampicillin and Amoxyclav. Intermediate sensitivity was seen in 22.2% of the isolates.

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Original Article

Bacteriological Profile of Unclean Ultra Sonography Probes with Antibiogram

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Abstract

Ultrasonography machines are ideal vectors for cross infections. A busy machine may be used to scan many patients a day. The infection can be transmitted via ultrasound probes and coupling gel. Staphylococcus aureus, Enterococci, Klebsiella, Pseudomonas, E. coli are frequent cause of infections in both community and hospital. Organisms isolated from unclean US probe are important nosocomial pathogens and infections due to it are difficult to manage due to resistance to multiple antibiotics. So this study aimed to determine the percentage of bacteria isolated from unclean US probes and to determine the antibiotic sensitivity pattern.

Keywords: Ultrasonography; Bacteria; Antibiogram.

Introduction

Ultra sonography machines are ideal vectors for cross infections. Busy machines may be used to scan many patients a day including both patients who may act as a source of infection and those who are vulnerable. Probe of US machines could act as a vector between these groups unless there is effective cleaning. To our knowledge best practice is yet to be established [1]. Radio-diagnosis department can be source of transmitting nosocomial infection as it is a integral part of medical services for admitted as well as for walk in patients particularly in ultrasound (including bed side portable scan) and intervention division. Many studies have shown that US probe are ideal vector for transmitting pathological organism from one patient to other patient unless there is effective cleaning methods [2-9]. The infection can be transmitted via ultrasound probes and coupling gel. Connection cord comes in contact with patient's skin and often due to length it is contact with floor [10]. Gel left on probe for prolonged periods can harbor bacteria [11]. Best practice are yet to be established however lack of effective cleaning methods for the probes may place the patients at risk[12]. Paper wipes and alcohol wipes have been recommended as sufficient to clean USG probes hence reducing the cross infections. Use of dry wipes is effective for abdominal scanning where as alcohol wipes are recommended for the axillary and the inguinal regions [13].

Materials and Methods

Prospective observational study was carried out in dept of microbiology in PDVVPF'S Medical College & Hospital, Ahmednagar from Aug 2015 to Dec 2015. Total 120 Swabs were taken from unclean ultrasound probes of patients attending in radio diagnostic department. After the ultrasound was carried out samples were send to microbiology laboratory which were obtained from USG probes after scanning the patients. Gram stain of swab was done followed by

culture on blood agar and MacConkey agar at 37degree Celsius for 24 hrs. Organisms obtained were subjected for biochemical tests for identification. Study group includes minimum of 120 patients presenting the department of radiology for USG of various body parts. Antibiotic sensitivity testing was carried out using the kirby- Bauer disc diffusion technique on Muller Hinton agar as per CLSI guidelines [14]. Antibiotic disc were obtained from high media company. Turbidity of the broth was compared to 0.5 macfarlands standards. Control strains used were Staphylococcus aureus ATCC 25923, E. coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853.

An inclusion criterion was probes used for USG of IPD and OPD patients.

Exclusion criteria was probes which are used for USG of immuno compromised patients and neonates.

Results

Table 1 Shows total no of bacteria isolated from the120 specimens that is 68 (56.6%) & 52(43.3%) samples were sterile.

Table 2 shows type of the organisms isolated before cleaning probes. Total number of bacteria isolated was 68 [56.6%]. Staphylococcus aureus was the commonest bacteria isolated 18 (26.5%), followed by coagulase negative Staphylococcus 30 (44.1%). Klebsiella species was the commonest amongst gram negative bacteria 7 (10%).

Table 3 shows Antibiotic sensitivity pattern of gram positive isolates. In our study Staphylococcus aureus showed maximum sensitivity towards Linezolid (83.3%). Enterococci & Cons were 100% sensitive to Linezolid.

Table 4 shows Antibiotic sensitivity pattern of gram negative isolates. All the gram negative isolates (Klebsiella, Pseudomonas, E. coli) were 100% sensitive to Imepenem. Pseudomonas also showed maximum sensitivity towards Pepracilin. All the three isolates showed maximum sensitivity towards Gentamycin.

Table 1:

S. No.	Total number of samples (from probe) before cleaning	No of isolates obtained before cleaning	Sterile Samples
	120	68 (56.6%)	52 (43.3%)

Table 2: Type of organisms isolated before cleaning

Organisms	Total No
Staphylococcus aureus	18 (26.5%)
Pseudomonas aeruginosa	2 (2.9%)
Enterococcus species	5 (7.3%)
E. coli	6 (8.8%)
Coagulase negative staph	30 (44.1%)
Klebsiella species	7 (10%)
Total	**68**

Table 3: Antibiotic sensitivity pattern of gram Positive isolates

Antimicrobial	Staphylococcus aureus (n =18)		Enterococcus (n = 5)		Cons (n = 30)	
	No of isolates sensitive	Percentage sensitivity	No of isolates sensitive	Percentage sensitivity	No of isolates sensitive	Percentage sensitivity
Amoxyclav	8	44%	3	60%	24	80 %
Ceftazidime/ clav	9	50%	2	40%	20	66.7 %
penicillin	10	55.6%	3	60%	20	66.7%
Cefazolin	5	27.8%	2	40%	12	40%
Cefoxitin	8	44%	2	40%	12	40%
Linezolid	15	83.3%	5	100%	30	100%
Vancomycin	13	72.2%	4	80%	20	66.7%
Azithromycin	12	66.7%	1	20%	8	26.7%
Tetracycline	13	72.2%	1	20%	10	33.3%
Cotrimoxozole	12	66.7%	2	40%	15	50%
Ofloxacin	9	50%	1	20%	13	43.3%
Erythromycine	4	22.2%	1	20%	7	23.3%

Table 4: Antibiotic sensitivity pattern of gram negative isolates

Antimicrobial	Klebsiella species (n =7)		E. coli (n =6)		Pseudomonas (n = 2)	
	No of isolates sensitive	Percentage sensitivity	No of isolates sensitive	Percentage sensitivity	No of isolates sensitive	Percentage sensitivity
Ampicilin	0	0%	0	0%	1	50%
Pepracilin	1	14.2%	1	16.7%	2	100%
Ceftazidime/ Clav	5	71.4%	5	83.3%	1	50%
Cefipime	3	42.9%	2	33.3%	1	50%
Cefoperazone	4	57.1%	1	16.7%	1	50%
Gentamycin	5	71.4%	5	83.3%	2	100%
Amikacin	5	71.4%	4	66.7%	1	50%
ciprofloxacin	3	42.9%	1	16.7%	1	50%
Cloramphinicol	4	57.1%	5	83.3%	1	50%
Cotrimoxozole	5	71.4%	3	50%	1	50%
Imepenem	7	100%	6	100%	2	100%
Meropenem	5	71.4%	5	83.3%	1	50%

Discussion

In our study total percentage of the organisms obtained from unclean US probes were 68 (56.6%). 52 (43.3%) out of 120 specimens before cleaning were sterile. Spencer and Spencer has found that 66% of swabs taken at random from US machines showed growth of bacteria which is in accordance with our study 56.6%. Similar observations were seen in study conducted by Tesh c Froschiea and Spencer. In a study Moradeli concluded that single paper wipe was effective as immersion in chlorhexidine. Similar observations were seen by Spencer and spencer. In our study commonest gram positive a bacterium isolated was staphylococcus aureus 18(26.5%) followed by CONS 30(44.1%) & Enterococcus 5(7.3%). Amongst Gram negative rods maximum isolation was of Klebsella species 7(10%) followed by Ecoli 6(8.8%) and Pseudomonas aeruginosa 2 (2.9%).

The study carried out in 1998 confirmed that it was apparent that ultrasound procedures transferred colonizing staphylococci from patient's skin on to the ultra sound instruments [10]. It has been also demonstrated that bacterial colonization of probes with pathogenic bacteria occurs under in-use conditions [15]. Study conducted by Hutchinsun etal has incriminated the ultrasound gel as a potential source of infections [16].

Paper wipe & alcohol wipes have been recommended as sufficient to clean the ultrasound probe, hence reducing risk of cross infections [13]. Paper wipe followed by normal saline wipe is 76% effective and appear to be better as compared to simple paper towel cleaning. However soap wipe technique was found to be most effective of the cleaning methods tested with effectiveness of 98% & this is comparable to the alcohol effectiveness of 99%. as per the study conducted by Schabrun etal & Abdullah etal [17,18]. In our study Staphylococcus aureus showed maximum sensitivity towards Linezolid (83.3%). Enterococci & Cons were 100% sensitive to Linezolid. In Enterococcus & Cons showed maximum resistance to Azithromycin, Tetracyclin, and Ofloxacin & Erythromycin. All the gram negative isolates were 100% sensitive to Imepenem. All the three isolates showed maximum sensitivity towards Gentamycin. Appropriate cleaning method needs to be tailored for clinical situation to prevent transmission of bacteria.

Conclusion

It has been found that Bacteria isolated from unclean US probe are Important nosocomial pathogens and infection due to it can be hazardous. Bacteria can be transmitted by ultrasonographic probes and coupling gel, it is highly recommended that ultrasound departments must revive their probe cleaning and sterilization procedures to assess whether they are a safe in particular environment. And practitioners should ensure that risk of cross infection should minimize. Applying simple cleaning methods can

Prevent nosocomal infections from ultrasound probes. Special infection control measures should also be taken in high risk group of patients.

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Original Article

Inducible Clindamycin Resistance (ICR) in *Staphylococcus Aureus* Among Various Clinical Samples

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Abstract

Introduction: Staphylococcus aureus is increasingly recognized as a cause of hospital associated (HA) and community associated (CA) infections. The Macrolide-lincosamide-StreptograminB (MLS_B), family of antibiotics serves as one such alternative, clindamycin being the preferred agent due to its excellent pharmacokinetic properties. However widespread use of clindamycin led to increase in resistance due to target site modification mediated by erm genes which can be expressed either constitutively or inducibely so use of D-test in a routine laboratory enables us to guide clinicians in judicious use of clindamycin. *Aims and Objective:* To study prevalence of inducible and constitutive clindamycin resistance among *Staphylococcus aureus* and to compare in between MRSA and MSSA isolates. *Material and Methods:* A total of 107 *Staphylococcus aureus* isolates were subjected to routine antibiotic susceptibility testing including cefoxitin (30mcg) by Kirby Bauer disc diffusion method. Inducible clindamycin resistance was detected by using D test, as per CLSI guidelines on erythromycin resistant isolates. *Results:* A total of 67 isolates were resistant to erythromycin. Among 67 isolates, 17(25.37%) showed inducible Clindamycin resistance, 27(40.2%) showed MS phenotype and Constitutive resistance was seen in 23(34.3%) isolates. Constitutive and inducible clindamycin resistance was found to be higher in MRSA as compared to MSSA. *Conclusion:* For efficient use of clindamycin, D-test should be used as a mandatory method in routine disc diffusion testing to detect Inducible clindamycin resistance.

Keywords: Constitutive; Inducible Clindamycin Resistance; MRSA; MSSA.

Introduction

Staphylococcus aureus is increasingly recognized as cause of hospital associated (HA) and community associated (CA) infections. Emergence of methicillin resistance in *Staphylococcus aureus* has left us with very few therapeutic alternatives such as vancomycin and linezolid to treat such methicillin resistant Staphylococcus aureus (MRSA) infections [1]. But recently emergence of vancomycin resistance was reported in few studies. So there is strong need to use alternative antimicrobial agents to treat such infections keeping vancomycin as reservoir drug [2].

The Macrolide-lincosamide-Streptogramin B (MLS_B) family of antibiotics serves as one such alternative, with clindamycin being the preferred agent due to its excellent pharmacokinetic properties [3]. Macrolides such as erythromycin, roxithromycin,

clarithromycin and lincosamides (clindamycin and lincomycin) are most commonly used in treatment of Staphylococcal infections. However, widespread use led to increase in resistance to these antibiotics especially clindamycin. The most common mechanism for such resistance is target site modification mediated by *erm* genes which can be expressed either constitutively or inducibely [2]. Treatment of an infection using clindamycin, caused by a strain carrying inducible *erm* gene, can lead to clinical failure [4]. Detection of inducible clindamycin resistance, a disc approximation test can be performed by placing a 2mcg clindamycin disc, 15-26mm away from the edge of a 15mcg erythromycin disc on Mueller Hinton agar plate at 37^0C for 16-18 hrs [5].

So, the present study was conducted to know the prevalence of constitutive and inducible resistance pattern among methicillin sensitive *Staphylococcus aureus* (MSSA) and methicillin resistance Staphylococcus aureus (MRSA).

Aims and Objective

To study prevalence of inducible and constitutive clindamycin resistance among *Staphylococcus aureus* and to compare in between MRSA and MSSA isolates.

Materials and Methods

The present study was conducted in a tertiary care hospital from April to June 2015. A total of 107 isolates of *Staphylococcus aureus* isolated from various clinical specimens like pus, wound swab, aspirates, blood and body fluids. These isolates were identified as *Staphylococcus aureus* by using conventional methods [6]. Antibiotic susceptibility testing was done by Kirby-Bauer's disc diffusion method using various antimicrobial agents like Penicillin [10units], Cefoxitin[30mcg], Gentamycin [10mcg], Ciprofloxacin [5mcg], Erythromycin [15mcg] , Clindamycin [2mcg], Amoxicillin/ Clavulanicacid [20/10mcg], levofloxacin [5mcg], Netilmycin [30mcg], Linezolid [30mcg], Teicoplanin [30mcg] as per CLSI guidelines [7]. For detection of methicillin resistance, cefoxitin [30mcg] disc was placed and plates were incubated at 37^0C for 24 hrs. Isolates with zone diameters < 21mm were labelled as methicillin resistant. For detection of inducible clindamycin resistance, a disc approximation test was performed by placing a 2mcg clindamycin disc, 15-26 mm away from the edge of a 15mcg erythromycin disc on Mueller Hinton agar plate at 37^0C for 16-18 hrs [5].

Following overnight incubation at 37^0C, three different phenotypes were appreciated and interpreted as follow.

MS Phenotype

Staphylococcal isolates exhibiting resistance to erythromycin (zone size ≤ 13mm), while sensitive to clindamycin (zone size ≥ 21mm) and giving circular zone of inhibition around clindamycin (D test negative).

Inducible MLS_B phenotype

Staphylococcal isolates showing resistance to erythromycin (zone size ≤ 13mm) while being sensitive to clindamycin (zone size ≥ 21mm) and giving D-shaped zone of inhibition around clindamycin with flattening towards erythromycin disc were labelled as having this phenotype (D test negative).

Constitutive MLS_B Phenotype

This phenotype was labelled for those Staphylococcal isolates which showed resistance to both erythromycin (zone size ≤ 13mm) and Clindamycin (zone size ≤ 14mm) with circular shape of zone of inhibition if any around clindamycin.

Results and Observations

Among 107 isolates of *Staphylococcus aureus*, 67(62.61%) showed resistance to erythromycin. These isolates were subjected to D test which showed various phenotypes.

Among 67 isolates of *Staphylococcus aureus* resistant to erythromycin, 56(83.58%) were MRSA and 11(16.42%) were MSSA. Inducible and constitutive clindamycin resistance was 17(25.3%) and 23(34.33%) respectively (Table 1). Overall inducible and constitutive resistance was higher amongst MRSA isolates as compared to MSSA isolates but it was found to be statistically insignificant. (Feisher's exact test)

All the strains were sensitive to vancomycin, linezolid and teicoplanin and resistant to penicillin. D-test positive isolates showed more resistance to antibiotic like Gentamycin,ciprofloxacin and Netilmycin as compared to D Test negative isolates (Table 2).

Table 1: MLS_B Resistant phenotype of *Staphylococcus aureus*

	MRSA	MSSA	Total
Constitutive MLS_B Resistance	21 (91.30 %)	2 (8.70%)	23 (100%)
Inducible MLS_B Resistance	16 (94.11%)	1 (5.89%)	17 (100%)
MS Phenotype	19 (70.37%)	8 (29.63%)	27 (100%)
Total	56 (83.58 %)	11 (16.42 %)	67 (100%)

MLS_B –macrolid-lincosamide-streptogramin B

Table 2: Percentage of antimicrobial resistance in D test positive & negative isolates

Antibiotics	D test –ve (n=27)	D test +ve (n=17)
Penicillin	27(100%)	17(100%)
Gentamycin	16(59.26%)	16(94.11%)
Ciprofloxacin	14(51.85%)	14(82.35%)
Amoxycillin/Clavulanic acid	19(70.37%)	16(94.11%)
Levofloxacin	6(22.22%)	7(41.18%)
Netilmycin	0 (0)	3(17.65%)

Discussion

In the era of increasing multidrug resistance it is necessary to determine the antimicrobial susceptibility of a clinical isolate so that appropriate treatment can be given to infected patients. Few therapeutic options are available for treatment of MRSA. Clindamycin is rapidly absorbed after oral ingestion and widely distributed in body fluids and blood (including bones), also used as an alternative for patients allergic to penicillin [3]. However some strains carrying *erm* gene give rise to inducible phenotype of Staphylococcal isolates and such isolates give rise to spontaneous constitutively resistant mutants in vivo during Clindamycin therapy leading to clinical failure [4]. So use of D-Test in a routine laboratory enables us in guiding clinicians for judicious use of clindamycin.

Among 107 *Staphylococcus aureus* isolates studied, 62.61% were erythromycin resistant, which is similar to lyall et al (51.5% [2]) and higher compared to other studies (28.4% [8],32.4% [9]), ICR was observed in 25.37% of isolates, which was higher compared to studies conducted by Ciraj AM et al [4] (13%) and prabhu K et al [8] (10%).while other studies reported higher prevalence as compared to our study (45% [9], 50% [10], 49% [11]) .

In our study, ICR (23.88%) was much higher in MRSA than in MSSA (1.49%) similarly study conducted by Mohamed Rahabar et al reported 22.6% in MRSA and 4% in MSSA [12], while the percentage was almost equal among MRSA and MSSA (33.2% and 34.6% respectively) in a study conducted by Lyall et al [2].

Similar to our study Lyall et al [2] reported that resistance to different antibiotics was more among D-Test positive isolates as compared to D-Test negative isolates.

Conclusion

To conclude, reporting of staphylococcal isolates as susceptible to clindamycin without checking for inducible resistance may result in institution of inappropriate therapy while negative result for inducible clindamycin resistance confirms clindamycin susceptibility and provide a very good treatment option.

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Original Article

Susceptibility Pattern of Fosfomycin from Urinary Isolates in a Private Diagnostic Centre of Central Madhya Pradesh

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Abstract

Introduction: Urinary tract infection (UTI) in a very common bacterial infection in both sexes and all age groups and is also a major cause of hospitalization. E.coli is the most common causative organism in UTI with almost 85% isolates being E.coli . The main aim of antibiotic treatment is to completely eliminate the bacteria from the urinary tract. However, with the changing pattern of antibiotic sensitivity of urinary pathogens to commonly used antibiotics and the emergence of ESBL producing E.coli the situation has worsened. A new drug, Fosfomycin , has now been frequently used as an alternative drug in the treatment of uncomplicated UTI. The present study was carried out in our microbiology department with the aim of finding out the susceptibility pattern of Fosfomycin to frequently isolated urinary pathogens. *Materials and Methods:* This was a retrospective study and urine culture of patients from January to December 2015 were included in the study. A total of 2759 urine cultures of all ages and both sexes were included in the study. All urine specimens were inoculated on Blood and MacConkey agar plates and incubated at 37^0C for 24 hours .A growth of $>10^5$CFU/ml of bacteria was labelled as significant and was further processed.The bacteria were identified on the basis of Gram staining and colony morphology and accordingly the panel for identification & susceptibility was chosen to be processed on Vitek II ((Biomerieux). *Results:* Out of 2759 urine cultures ,1341 (48.6%) cultures showed growth of bacteria. 1418 (51.3%) cultures were sterile .E. Coli (86.7%) was the commonest organism in followed by Klebsiella (8.50%), Pseudomonas (2.53%), Staphylococcus (1.41%),Enterococci (0.67%) and Proteus (0.14%). Highest sensitivity(100%) was observed in Proteus,followed by Pseudomonas(82.3%), Klebsiella(73.6%), Enterococci(66.6%) E.Coli(57.6%),and least for Staph.aureus(51.8%). *Conclusion:* The presence study suggests that resistance to Fosfomycin is on the rise and should be used with caution and resistance pattern should be monitored periodically.

Keywords: Fosfomycin; UTI; Antibiotic Susceptibility.

Introduction

Urinary tract infection (UTI) in a very common bacterial infection in both sexes and all age groups with females accounting for greater incidence of UTI because of their short urethra. It is a also a major cause of hospitalization. It has been estimated that 1 in 5 women develop UTI during their lifetime and 34% adult over the age of 20 years develop UTI once in their life time [1,2,3]. Although UTI is a benign illness with no long term medical consequences but

it increases the risk of developing pyelonephritis ,premature delivery, increases fetal mortality among pregnant women and also impairment of renal function and end stage renal disease. E.coli is the most common causative organism in UTI with almost 85% isolates being E.coli. Other pathogens include enterobacteriaceae like Klebsiella, Proteus etc. Enterobacteriaceae are the most common organisms isolated from uncomplicated UTI in children [4-8]. The main aim of antibiotic treatment is to completely eliminate the bacteria from the urinary tract. However, with the changing pattern of antibiotic sensitivity of urinary pathogens to commonly used antibiotics and the emergence of ESBL producing E.coli the situation has worsened [9-12].

A new drug, Fosfomycin ,has now been frequently used as an alternative drug in the treatment of uncomplicated UTI. Fosfomycin is a broad spectrum phosphoenol pyruvate analog which acts by preventing the cell wall synthesis of bacteria by inhibiting UDP-N- acetyl glucosamine enolpyruvyl transferase (MurA) enzyme. The major advantage of Fosfomycin is that it is given as a single dose and has rare side effects and low resistance rates till date [12,13] .

The present study was carried out in our microbiology department with the aim of finding out the susceptibility pattern of Fosfomycin to frequently isolated urinary pathogens, specially enterbacteriaceae.

Materials and Methods

This was a retrospective study and urine culture of patients from January to December 2015 were included in the study. A total of 2759 urine cultures of all ages and both sexes were included in the study.

All 2759 urine specimens were collected by standard clean catch mid stream protocol and for catheterized patients samples were collected in sterile screw capped wide mouth container after clamping the catheter for 30 minutes. All urine specimens were inoculated on Blood and MacConkey agar plates and incubated at 37^0C for 24 hours. A routine urine examination was done to test for presence of white blood cells and bacteria in fully automated urine analyser (Beckman Coulter). Plates were observed for bacterial growth and a growth of $>10^5$CFU/ml of bacteria was labelled as significant and was further processed.

The bacteria were identified on the basis of Gram staining and colony morphology and accordingly the panel for identification & susceptibility was chosen to be processed on Vitek II (Biomerieux).

Results

The 2759 patients were divided into 0-20,21-40,41-60,61-80 And > 80 age groups.There were 40.30 % males and 59.7% females.Maximum females were in reproductive age group (700/2749) i.e. 21-40 years of age followed by 41-60 years (370/2759) and lowest in >80 years of age. The M:F ratio was 2:3. The demographic data of patients is shown in Table 1.

Out of 2759 urine cultures, 1341 (48.6%) cultures showed growth of bacteria. 1418 (51.3%) cultures were sterile. E. Coli (86.7%) was the commonest organism in followed by Klebsiella (8.50%), Pseudomonas (2.53%), Staphylococcus (1.41%), Enterococci (0.67%) and Proteus (0.14%).

Out of 1163 E.coli isolates, 57.6% isolates showed sensitivity to Fosfomycin while 42.1% were resistant.

Out of 114 Klebsiella isolates, 73.6%were sensitive to Fosfomycin and 26.3% were resistant.

82.3% Pseudomonas isolates were sensitive to Fosfomycin while 17.6 % were resistant.

66.6% Enterococci showed sensitivity to Fosfomycin and 33.3% were resistant.

51.8%Staphylococcus isolates were sensitive while 48.2% were resistant to Fosfomycin.

Proteus showed 100% sensitivity to Fosfomycin.No resistant case was observed in this isolate.The MIC value and sensitivity percentage is shown in Table 3 .

Highest sensitivity(100%) was observed in Proteus, followed by PSeudomonas (82.3%), Klebsiella (73.6%), Enterococci (66.6%) E.Coli (57.6%), and least for Staph. aureus (51.8%)

Table 1: Showing demographic data of patients N= 2759

Age in years	Male	Female
0 - 20	300	350
21 - 40	200	700
41 - 60	310	370
61 - 80	248	200
> 80	55	26
Total	1113	1646
Percentage	40.30%	59.70%

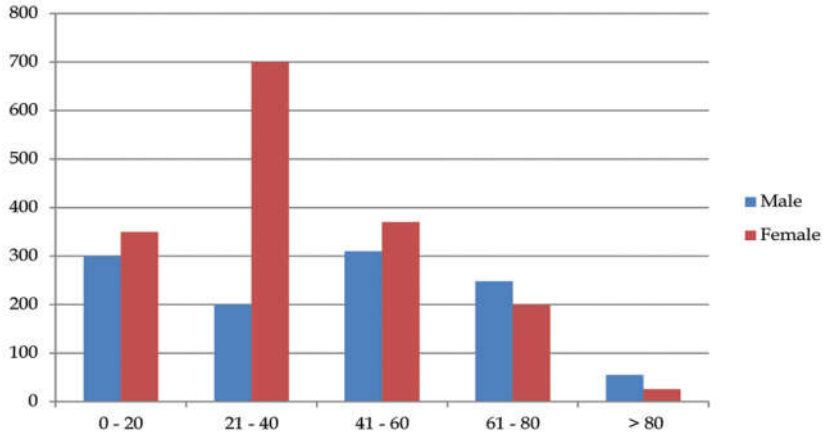

Fig. 1: Showing demographics of patients

Table 2: Showing percentage of different isolates N= 2759 Cultures

Organisms	Total Isolated	Percentage
E.coli	1163	86.70%
Klebsiella	114	8.50%
Pseudomonas	34	2.53%
Proteus	2	0.14%
Staphauerus	19	1.41%
Enterococci	9	0.67%
Sterile	1418	51.30%
Total positive	1341	48.60%

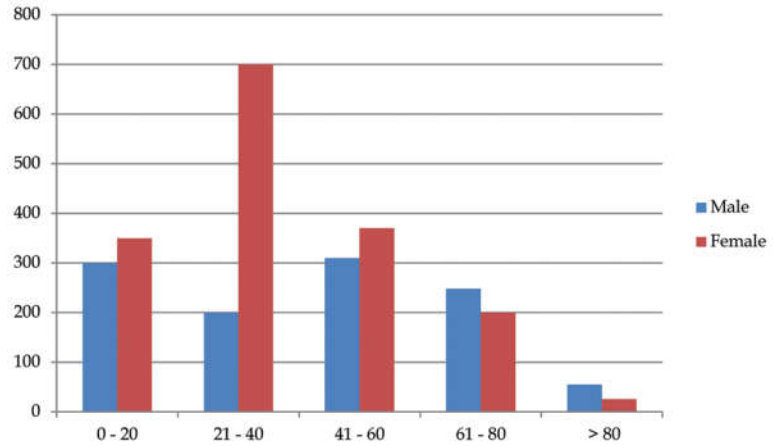

Fig. 2: Showing percentage of different isolates

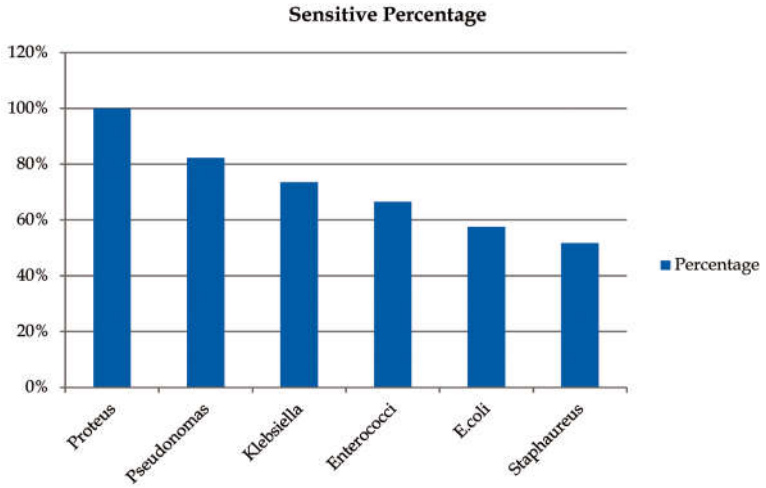

Fig. 3: showing percentage of fosfomycin sensitive isolates

Table 3: Showing sensitive and resistance percentage of isolates

Organisms	FOS- Sensitive	Percentage	Sensitive MIC	FOS- Resistant	Percentage	Resistant MIC	Total
E.coli	670	57.60%	<=16	493	42.30%	>=256	1163
Klebsiella	84	73.60%	<=16	30	26.30%	>=256	114
Pseudonomas	28	82.30%	<=16	6	17.60%	>=256	34
Enterococci	6	66.60%	<=16	3	48.20%	>=256	19
Staphaureus	11	51.80%	<=16	8	33.30%	>=256	9
Proteus	2	100%	<=16	0	0	>=256	2
Total	801	59.70%		540	40.20%		1341

Resistant Percentage

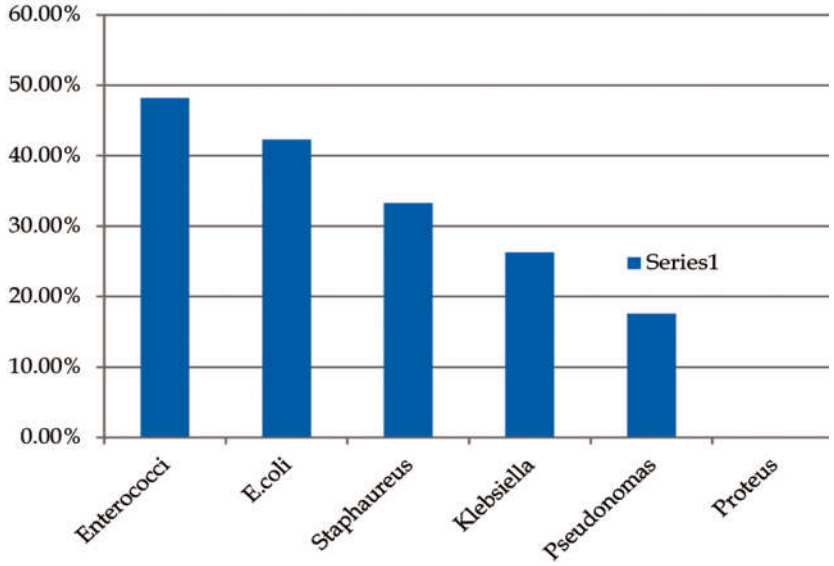

Fig. 4: showing percentage of fosfomycin resistant isolates

Discussion

Enterobacteriaceae are the most common isolates found in uncomplicated UTIs. UTIs have become increasingly difficult to treat and eradicate due to growing antibiotic resistance to commonly used antibiotics. TekinTas et al found a resistance rate of 1.9% to Fosfomycin in their study [15]. Other studies have revealed resistance to Fosfomycin between 1.2-4.5% [16-19].

Resistance to Fosfomycin rarely develops and is mostly chromosomal or plasmid mediated.The chromosomal resistance is caused by mutations in structural genes which code bacterial proteins helping to transport the agent in to the cell [20].

Fosfomycin is approved in many countries worldwide for the treatment of uncomplicated UTIs. It has a good in vitro activity against E. Coli,ESBL producing E. Coli,Proteus, Klebsiella and Staphylococcus. A single dose of Fosfomycin achieves good concentration in urine and has similar efficacy to 3-7 days regimen of other common urinary antibiotics like Norfloxacin,Nitrofurantoin Ciprofloxacin. The only known side effect of Fosfomycin is gastro intestinal disturbances like diarrhea and nausea. In our study, the resistance rate to Fosfomycin was quite high. 40.2% total isolates showed resistance to Fosfomycin with Staphylococci accounting for highest resistance (48.2%),followed by E.Coli(42.3%), Enterococci(33.3%), Klebsiella(26.3%) Pseudomonas(17.6%). This is an alarming sign.

Conclusion

The presence study suggests that resistance to Fosfomycin , an antibiotic of choice in UTIS because of its single dose regimen and very few side effects, is on the rise and should be used with caution and resistance pattern should be monitored periodically.

Conflict of Interest

none

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Original Article

Molecular Typing of Bluetongue Virus 16 From Karnataka State of India

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Abstract

Bluetongue disease (BT) is a vector borne infectious but non-contagious disease of wild and domestic ruminants. The BTV isolate (7bp) of sheep origin was inoculated to 9-11 day old chicken embryo followed by BHK-21 cell culture. Upon appearance of 75% cytopathic effect in cell culture viral nucleic acid was extracted. The viral nucleic acid showed BTV specific migration pattern of 3:3:3:1 in RNA-PAGE. The group specific ns1 gene RT-PCR confirmed the sample as BTV. The vp2 gene based serotype specific RT-PCR revealed the isolate as BTV16. The nucleic acid sequence of vp2 gene PCR products showed a high degree of identity (>99.0%) with other BTV16 isolates from different regions of India. It also showed maximum nucleotide identity of 99.7-96.4% with several other eastern BTV16 viruses from India, Israel, Japan, Cyprus, Greece etc. Sequence identity study also revealed that 7bp isolate only showed 75.5% identity with western isolate of BTV16 from Nigeria. The phylogenetic study also showed a close relation between isolate in study and BTV16 isolates from India Japan, Israel and Greece which form a separate eastern cluster. Thus, molecular study showed that the isolate in study is of eastern origin and closer to BTV16 isolates from India, Greece, Japan, and Israel.

Key words: Bluetongue Virus 16; Topotype; vp2 Gene; RT-PCR.

Introduction

Bluetongue (BT) is non-contagious and infectious viral disease of domestic and wild ruminants in several parts of the world. BT is caused by Bluetongue virus (BTV) of genus *Orbivirus* under family *Reoviridae*. BT is a vector borne disease and transmitted by *Culicoides* vector (MacLachlan, 1994). BT is characterized by high morbidity, mortality, still birth, foetal abnormality, abortion, weight loss, wool break, reduced meat and milk yield which lead to huge economic loss to farming community and livestock industry. The clinical form of BT is characterized by pyrexia, swelling of tongue and lips, coronitis, cyanotic discoloration of tongue and muzzle which may lead to death. However, subclinical infection of BT may also cause reduced milk yield, loss of condition and abortion leading to infertility in animals (Osburn, 1994). Therefore, due to severe economic losses mandatory disease surveillance and trade barrier have been imposed on movement of ruminant animals, their products and germplasm from BT endemic countries to BT free countries (Velthuis et al., 2009). The disease is seen in more severe form in sheep and white-tailed deer (Howerth *et al.*, 1988; Darpel *et al.*, 2007). Several other domestic animals such as buffalo, cattle and goats

act as silent reservoirs and may remain viraemic for several months post infection (Maclachlan *et al*., 2009). BT may infect several species of domestic and wild ruminanats. Therefore, it is listed as multi species disease by Office International des Epizooties (OIE, 2013).

BTV is icosahedral virus having ten segmented linear double-stranded RNA (dsRNA) genome. The BT genome segments encode 7 structural (VP1 to VP7) and 4 non-structural proteins (NS1, NS2, NS3/ NS3a and NS4). The structural proteins play essential role in viral nucleic acid replication and viral capsid assembly. However, non-structural proteins have role in egression of viral particle from infected cell (Mertens et al., 1989; Ratinier et al., 2011). They are produced in infected host cells only. The viral inner capsid is composed of two major proteins (VP3 and VP7) and three minor proteins (VP1, VP4, and VP6) (Roy, 1989). Similarly, outer capsid is consists of major and minor serotype specific VP2 and VP5 proteins for individual BTV serotype (Ghiasi et al., 1987).

Because of segmented nature of BTV genome, reassortment is a common phenomenon. BT viruses exchange genome segments with other BT viruses which lead to evolution of newer serotypes. There are twenty seven distinct BTV serotypes (BTV1 to BTV27) have been reported worldwide (Hofmann et al., 2008; Maan et al., 2011; Jenckel et al., 2015). A large number of BTV serotypes have also been reported from India. The serum neutralization assay and virus isolation in cell culture showed the prevalence of 22 distinct BTV serotypes in different geographical regions of India (Prasad et al., 2009; Susmitha et al., 2012). Several serotypes of BTV have been isolated from Karnataka state. In this study vp2 gene based molecular characterization of BTV isolate of sheep origin from Karnataka state has been reported.

Materials and Methods

Sample Preparation

The Blood sample was collected from a sheep suspected for BTV infection from Karnataka state in 2009. The blood sample was designated as 7bp. The sample was processed by ultrasonication followed by filtration. The filtrate was inoculated to 9-11 day old chicken embryo through intravenous route. On 7 day post inoculation, embryo showing embryopathic effect was harvested. The embryonic fluid was inoculated to one day old monolayer of BHK-21 cell culture.

Viral Nucleic Acid Extraction and RNA-PAGE

BHK-21 cells were harvested after appearance of about 75% cytopathic effect (CPE). The harvested BHK-21 cells were centrifuged at 2000Xg for 10 minutes (Remi, India). The supernatant materials were discarded and pellet was used for viral dsRNA extraction using Guanidinium isothiocynate method (Chomoczynski and Sacchi, 1987). The viral nucleic acid was subjected to 8% RNA-poly acrylamide gel electrophoresis (RNA-PAGE). The BTV specific nucleic acid was visualized using silver staining (Svensson et al., 1986).

cDNA Preparation and PCR

The viral nucleic acid was used for cDNA preparation using moloney murine leukemia (Mo-MuLV-RT) virus reverse transcriptase enzyme (Sibzyme, Russia) and random decamer primer (Ambion, USA) in thermal cycler (Biorad i-Cycler, USA) as per manufacturer's protocol. The cDNA was allowed for group specific ns1 gene based PCR to confirm the samples as BTV. The group specific PCR was performed using primer pairs F: 5'GTTCTCTAGTTGGCAACCACC3' and R: 5' AAGCCAGACTGTTTCCCGAT3' which produced an amplicon of 274bp size in agarose gel electrophoresis (Prasad et al., 1999).

The serotype of virus isolate was confirmed by vp2 gene based serotype specific RT-PCR using primers specific to all the BTV serotypes. The cDNA was allowed to PCR using individual serotype specific primers in a 20 µl reaction mixture having 20 µM of serotype specific primers, 2 µl cDNA, 3% DMSO, 0.4 µl of 10mM dNTPs mix (Finnzyme, Finland), 4 µl 5X HF buffer and 0.4 U (2U/ µl) phusion high- fidelity DNA polymerase (Finnzyme, Finland) in thermal cycler (Biorad iCycler, USA). The PCR amplification cycle was set as initial denaturation at 98°C for 2 minute, followed by 32 cycles of denaturation at 98°C for 10 second, primer extension at 72°C for 20 second and annealing for 20 second at 55°C. The final PCR extension was allowed at 72°C for 10 minute. The PCR products were visualized using gel documentation system (Biovis, USA) in 1% agarose gel (Sigma, USA) electrophoresis.

Nucleic Acid Sequencing and Sequence Data Analysis

The vp2 gene specific PCR products were purified using QIA quick gel extraction kit (Qiagen, USA). The purified PCR products were allowed for nucleic acid sequencing using serotype specific forward and reverse primers for final serotype confirmation. The

nucleic acid sequencing reaction was performed in Genetic Analyser ABI PREISM ™ 3130 XL machine using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) as per the manufacturer's instruction in our departmental laboratory.

The nucleic acid sequence data obtained was allowed for online available BLASTN+ 2.3.1 search (Zhang *et al.,* 2000) for serotype confirmation. The forward and reverse sequences of virus were aligned to generate contig sequences using Bioedit v7.2.5 software (Hall, 1999), which were used for further analysis. The percent nucleotide identity with global isolates of BTV16 was calculated using Bioedit v7.2.5 software (Hall, 1999). The phylogenetic analysis of vp2 gene sequences of our isolate (7bp) along with other global sequences were done using Mega 6 programme (Tamura *et al.,* 2013).

Results and Discussion

India has several BTV serotypes reported from different geographical regions. In the present study, one of the BTV isolate (7bp) was isolated from sheep in Karnataka state, adapted in BHK21 cell line and used for vp2 gene based serotyping and molecular characterization. The 7bp isolate adapted to BHK-21 cell line produced BTV specific CPE such as vacuolation in cells, aggregation and rounding of cells, floating of dead cells in medium within 36 hours (Sekar *et al.* 2009) (Figure 1). The viral nucleic acid was extracted using Guanidinium isothiocynate method (Chomoczynski and Sacchi, 1987) from pelleted cell culture materials and screened by RNA-PAGE followed by silver staining. The RNA-PAGE analysis showed characteristics BTV specific migration pattern (3:3:3:1) of viral dsRNA (data not shown). The viral nucleic acid was subjected to group specific ns1 gene based RT-PCR. The ns1 gene PCR amplicon showed 274bp product size on agarose gel electrophoresis. This indicates the sample as BTV (Figure 2). The characteristic CPE in BHK-21 cell culture, specific migration pattern of viral nucleic acid (3:3:3:1) in RNA-PAGE and 274 bp amplicon of ns1gene group specific RT-PCR confirmed the samples as BTV.

Further, the cDNA of 7bp isolate was allowed for serotype specific RT-PCR using vp2 gene specific primers for all the BTV serotypes. The PCR amplicon showed 768bp amplification product on agarose gel electrophoresis, which is specific for BTV16 serotype (Figure 3). The remaining serotype specific primers did not show any amplification. Thus the 7bp isolate was serotyped as BTV16. For final confirmation of serotype of 7bp the vp2 gene PCR product was allowed for direct nucleic acid sequencing. The BLASTN+ 2.3.1 search of nucleotide sequence of vp2 gene of 7bp isolate showed the maximum identity only with several isolates of BTV16 from different regions of the world. Thus, vp2 gene specific RT-PCR followed by nucleotide sequencing confirmed the 7bp isolate as BTV16. The nucleotide sequence of 7bp isolate was deposited to GenBank database and accession number GU931316 was assigned. The contig sequence of 7bp isolate was generated and percent nucleotide sequence identity of 7bp isolate with several other BTV16 isolates from India and different regions of the world were calculated using Bioedit v7.2.5 (Hall, 1999) programme (Table 1).

The nucleotide sequence identity analysis showed that 7bp isolate (accession number GU931316) possessed 99.7% nucleotide identity with IND2010/cattle/16 (accession no JX007924), Sheep/2010/Ind/Hisar (accession no JQ904061) and IND/Goat/2010/16/HSR (Minakshi et al., 2012) isolate of BTV16 from India. However, 7bp isolate also showed nucleotide identity of 99.4-98.0% with several Indian BTV16 isolates such as G53/ABT/HSR, CU-NAU/IND/2010 (accession no JQ478482), INDAPMBNAP04/10 (accession no KC751423), G4/IND/2011 (Dadawala et al., 2013), VJW66/IND (Minakshi et al., 2015), GNT-27/IND (Minakshi et al., 2015), G53/IND/2011 (Dadawala et al., 2013) and MBN48/IND (Minakshi et al., 2015).

The 7bp isolate also showed nucleotide identity of 97.7-94.6% with several isolates from Israel (ISR2008/03, BTV16/ISR-2404/08 and BTV16/ISR-2228/08) (Nomikou et al., 2015), Greece (GRE2008/10, BTV-16/Gree1999/13(S-2) and GRE1999/13) (Nomikou et al., 2015), Cyprus (CYP2006/01) (Nomikou et al., 2015), Japan (MZ-1/C/01) (Shirafuji et al., 2012) and South Africa (RSArrrr/16) (Maan et al., 2004) (Table 1).

The BTV nucleotide sequence analysis from different geographical regions of globe broadly categorised BTV in to 'eastern' or 'western' topotypes (Maan et al., 2010). The sequence analysis of 7bp isolate showed an overall nucleotide identity of 99.7-94.6% with several eastern BTV16 viruses from India, Israel, Japan, South Africa, Greece and Cyprus. However, 7bp isolate showed only 75.5% nucleotide identity with Western isolate (NIG1982/10) of BTV16 from Nigeria (Mertens et al., 2013). Thus, it confirmed the eastern origin of 7bp isolate.

The phylogenetic study of BTV16 nucleotide sequences using Mega 6 software programme formed two separate major, western and eastern clusters. The isolate in study (7bp) formed a separate close cluster with several other Indian BTV16 viruses (IND2010/

cattle/16, Sheep/2010/Ind/Hisar, IND/Goat/2010/16/HSR, Cattle/2010/Ind/Hisar and INDAPMBNAP04/10) under eastern cluster. They were also found slightly distantly related to other eastern BTV16 isolates from India, Japan, Greece, Israel, South Africa and Cyprus. The BTV16 from Nigeria (Isolate NIG1982/10) was placed in western cluster (Mertens et al., 2013). Thus phylogenetic and sequence identity study revealed that 7bp isolate was found much closer to BTV16 isolates from Indian, Japanese, Israel or Greece.

India is a rainy tropical country which provides suitable environment for *Culicoides* vector growth and multiplication. Out of more than 1400 different *Culicoides* species globally reported, about 63 are identified from different geographical regions of India (Reddy et al., 2008; Halder et al., 2013; Archana et al., 2014). Thus, persistence of large numbers of *Culicoides* vectors explains the prevalence of 22 different BTV serotypes in India. The serological study revealed the presence of antibodies against BTV in several species of wild and domestic ruminants in India (Prasad et al., 1998).

Karnataka state is one of the major hubs of BT infection in India. Based on virus isolation and serum neutralization several BTV serotypes such as 1, 2, 4, 12, 16, 17, 18, 20 and 23 have been reported from Karnataka state (Prasad et al., 2009). Moreover, BTV16 was also reported from sheep population in adjoining states such as Tamil Nadu (isolate IND/Goat/2010/16/HSR) (Minakshi et al., 2012) and Andhra Pradesh (isolate, VJW66/IND, GNT-27/IND and MBN48/IND) (Minakshi et al., 2015).

The 7bp isolate showed a high degree of identity (99.7-98.0%) with several isolates of BTV16 from Tamil Nadu and Andhra Pradesh. Since Tamil Nadu and Andhra Pradesh states are neighbouring states to Karnataka and are also endemic for a known BTV vector (*Culicoides oxystoma*) in India (Minakshi, 2010). Thus, it may be assumed that BTV16 might be transmitted from neighbouring states to Karnataka either through vectors or migrating sheep population or through wind velocity. Moreover, due to serious BTV16 outbreaks in some states of India such as Andhra Pradesh, Karnataka, Gujarat and Tamil Nadu, it is included in inactivated Pentavalent vaccine formulation along with other serotypes such as 1, 2, 10 and 23 (Reddy et al., 2010). However, the knowledge about the molecular epidemiology of all the BTV serotypes is essential for a successful BTV control programme. The conventional serotyping methods along with molecular tests such as RT-PCR based typing and nucleic acid sequencing can be used for BTV surveillance in a particular geographical area. The surveillance information can be used for proper BT vaccine formulation.

Table 1: Percent nucleotide identity of vp2 gene of 7bp isolate with other bluetongue virus 16 from different regions of the world

S.N.	BTV16 vp2 gene sequences	7bp.GU931316.India Percent nucleotide identity
1	7bp.GU931316.India	100
2	IND2010/cattle/16.JX007924.India	99.7
3	Sheep/2010/Ind/Hisar.JQ904061.India	99.7
4	IND/Goat/2010/16/HSR.JQ924821.India	99.7
5	G53/ABT/HSR.KF664134.India	99.4
6	Cattle/2010/Ind/Hisar.JQ904063.India	99.2
7	Goat/2010/Ind/Hisar.JQ904062.India	98.9
8	CU-NAU/IND/2010.JQ478482.India	99.2
9	INDAPMBNAP04/10.KC751423.India	98.9
10	G4/IND/2011.JQ478483.India	98.6
11	VJW66/IND.JN106022.India	98.6
12	GNT-27/IND.JN106018.India	98.6
13	G53/IND/2011.JQ478486.India	98.0
14	MBN48/IND.JN106020.India	98.0
15	ISR2008/03.KP820992.Israel	97.9
16	CYP2006/01.KP820986.Cyprus	97.9
17	BTV16/ISR-2404/08.KP306785.Israel	97.9
18	BTV16/ISR-2228/08.KP306782.Isreal	97.9
19	GRE2008/10.KP820990.Greece	97.7
20	MZ-1/C/01.AB686220.Japan	97.6
21	GRE1999/13.KP820989.Greece	97.4
22	BTV-16/Gree1999/13(S-2).AM773709.Greece	97.4
23	GRE1999/13.AM773702.Greece	97.4
24	RSArrrr/16.AJ585137.South Africa	96.4
25	NIG1982/10.AJ585150.Nigeria	75.5

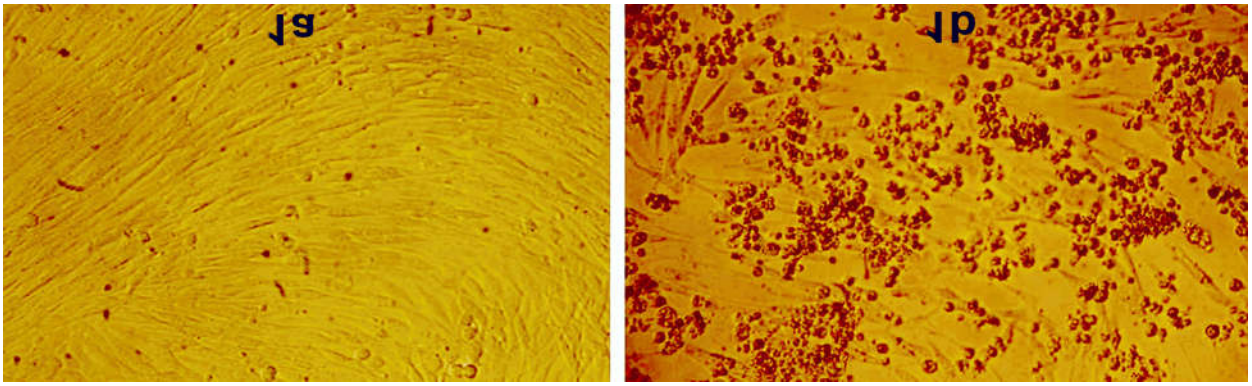

Fig. 1: Bluetongue virus isolation in BHK-21 cell line. (1a): Normal uninfected BHK-21 cell monolayer (48hours); (1b): BTV infected BHK-21 cells (48 hours) showing cytopathic effect characterized by degeneration and rounding of infected cells

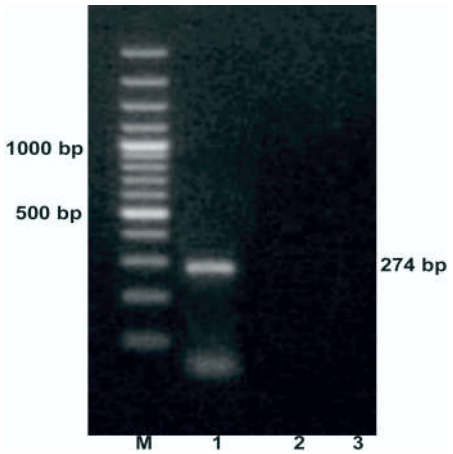

Fig. 2: 1% agarose gel electrophoresis of ns1 gene RT-PCR of Indian BTV16 isolate. Lane L: Ladder 100bp, 1: 7bp, 2: BHK21 cell control, 3: Nuclese free water control. The left side numbers indicate DNA marker and right side indicated size of PCR product.

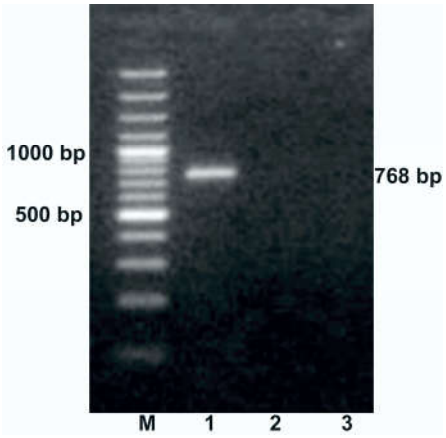

Fig. 3: 1% agarose gel electrophoresis of vp2 gene RT-PCR of Indian BTV16 isolate. Lane L: Ladder 100bp, 1: 7bp, 2: BHK21 cell control, 3: Nuclease free water control. The left side numbers indicate DNA marker and right side indicated size of PCR product.

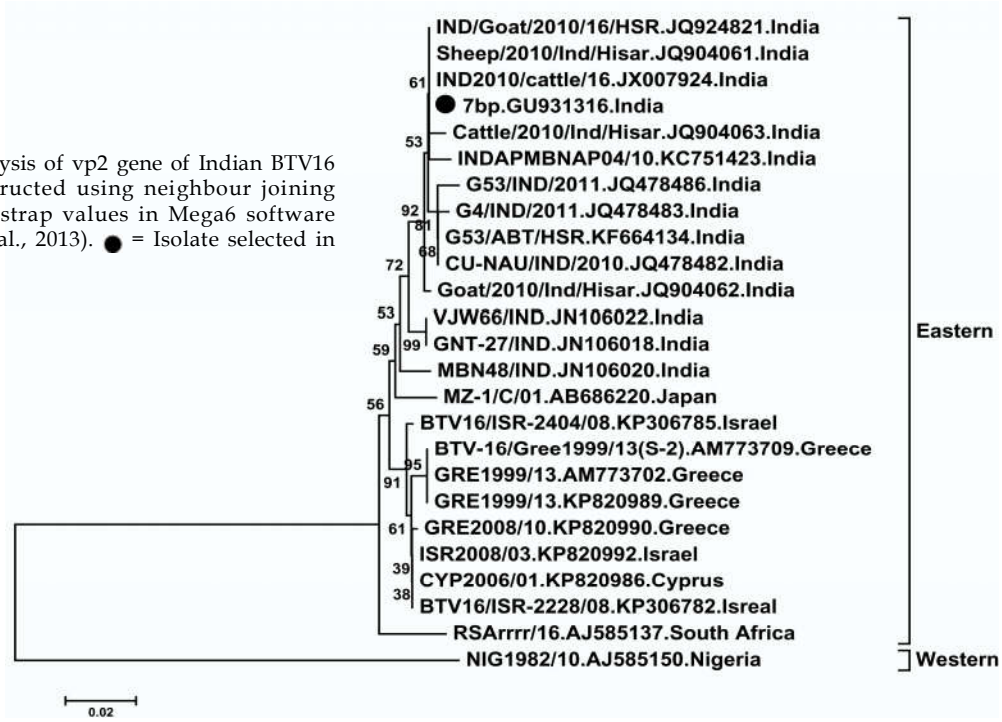

Fig. 4: Phylogenetic analysis of vp2 gene of Indian BTV16 isolate. Tree was constructed using neighbour joining method with 1000 bootstrap values in Mega6 software programme (Tamura et al., 2013). ● = Isolate selected in this study

Conclusion

BT is mostly a disease of sheep in India. However, several reports of BT outbreak in other ruminants such goat and cattle has also been reported from country. The BTV isolate (7bp) of sheep origin from Karnataka state was used for vp2 gene based serotyping and molecular characterization. The 7bp isolate was confirmed as BTV16 serotype based on RT-PCR, nucleic acid sequencing and vp2 gene sequence similarity search in GenBank data base. The nucleotide sequence identity and phylogenetic analysis revealed that 7bp isolate is much closer to other BTV16 isolates from India, Japan, Israel, Cyprus and Greece. Therefore, to control BT in India the close surveillance regarding import of live animal and its products should be initiated.

Competing Interest

All authors declare that they have no conflict of interest.

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