ANTIBIOTIC RESISTANCE (ABR) PROFILE OF *ENTEROBACTER CANCEROGENUS* (NS3-E-8) ISOLATED FROM HOSPITAL ASSOCIATED SAMPLES AT KHAIRPUR, PAKISTAN

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ABSTRACT

Resistance to a variety of frequently used antimicrobials in hospital-associated pathogen is one of the biggest challenge which have significant impact on patient's outcome and health care cost. Present study relates with the antibiotic resistance profile and frequency distribution of *Enterobacter cancerogenus* (NS3-E-8) isolated from hospital associated samples at Khairpur, Sindh, Pakistan. The hospital-associated samples in total 280 (162 male, 118 female) were randomly collected from the basic health care vicinities and their associated laboratories of Khairpur and Sukkur cities. *Enterobacter colonies* were separated and subjected to identification using microbial methods like cultural, morpho-microscopic and biochemical characteristics followed by the molecular biography using the 16SrRNA sequence-based homology. After all the antimicrobial resistance profile was determined according to the Kirby-Bauer's disc-diffusion assay using panel of antibiotics (Amoxicillin clavunic acid, Sparfloxacin, Gentamycin, Azomax, Enoxacin, Piperacillin Tazobactam, Moxifloxacin, Fosfomycin, Fusidic acid, and Sulbactam). Overall, the nine 9 (3% of 280) strains of genus *Enterobacter* were isolated from stool 100% (6 of 6) and blood 21% (3 of 14) while it was completely absent in rest of the samples like high vaginal swab, Urine, Pus, ear swab, throat swab, ascitic fluid, Cerebrospinal fluid and pleural fluid. *Enterobacter* in gender wise prevalence was 66.7% prevalent in stools and blood samples of female while 33.3% in respective samples of male patient. The results of antimicrobial resistance profile revealed that isolated strains were completely resistant against used panel of antibiotics. Phylogenetic correlation of amplified 16S rRNA gene sequence of *Enterobacter* isolate (*NS3-E-8)* shared 99% similarity with *Enterobacter cancerogenus* strain (LMG 2693) available at NCBI GenBank. Prevalence of antibiotic resistant pathogen *Enterobacter cancerogenus* in clinical specimens calls for timely control measures to reduce hospital associated infection, health care cost and developing resistance.

Keywords: *Enterobacter cancerogenus*, Antibiotic, Resistance, Prevalence, PCR.

INTRODUCTION

The antibiotic resistance (ABR), a major public health threat linked with morbidity and mortality continues to increase worldwide. Historically, the patients are affected in hospital settings, where factors like (hospitalization, exposure to antibiotics and use of in-dwelling devices) provide risks for acquisition (Siegel *et al*., 2007). Genera of family Enterobacteriaceae are frequently causing community-and hospital-acquired infections and thus have become the resistant to first- and second-line antibiotics (Nordmann *et al.,* 2011). The most commonly reported genera of family Enterobacteriaceae include *Escherichia*, *Proteus*, *Klebsiella*, and *Enterobacter*, (Foudaa *et al.,* 2015; Dange *et al.,* 2016). Enterobacter species are frequently associated with hospital acquired infections in outbreaks and are also capable of acquiring plasmids mediated ABR mechanism (lee *et al*., 2015). Thus the *Enterobacter* species are either very resistant to many antibiotics or develop resistance during antimicrobial treatment (empirical), hence the choice of treatment is relatively complicated. Therefore, the infections of these antibiotic resistant strains pose a serious therapeutic challenge (Blair *et al*., 2015).

Enterobacter cancerogenus (formerly known as *Erwinia cancerogena*, and *Enterobacter taylorae*) is a gram-negative bacillus, lactose-fermenter, most frequently isolated from human blood and spinal fluid. *Enterobacter cancerogenus* was found to be etiological agents of wound and urinary tract infections (UTI), sepsis and osteomyelitis (Abbott *et al.,* 1997). Little is known regarding the epidemiology and clinical significance of this organism although worldwide bacteraemia, osteomyelitis, wound infection, UTI, pneumonia, skin and soft tissues infections have been reported (Tena *et al.,* 2015). The rapid emergence of ABR in *Enterobacter* spp. have been documented in individual patients getting empirical treatment and in community under strong selective pressure of antimicrobial agents (Paterson *et al*., 2006; Pitout *et al*., 2008; Siedner *et al.,* 2014). The same trend of ABR is also

present in *E. cancerogenus* and reported as resistant or intermediate to amoxicillin, amoxicillin/clavulanate, cefaclor, cefazoline, loracarbef and cefoxitin (Stock, and Wiedemann, 2002).

ABR pattern of microbes varies from place to place, hospital to hospital, community to community and state to state. In Pakistan, the emergence of ABR is due to empirical- or inappropriate use (misuse and overuse) of antibiotics aiming to increase the resistance. (Tanvir *et al.,* 2012; Gholam-Mostafaei *et al.,* 2017) There is no systematic national surveillance of ABR and insufficient data is available to quantify the problem (Abdul *et al.,* 2008). Contaminated foods served in hospital settings, are the major source of nosocomial infections due to potential pathogenic strains (Gholam-Mostafaei *et al.,* 2017). Similarly the faecal pollution of drinking water sources due to improper management of sewerage systems, lack of proper spill ways and water storage capabilities are some of the key factors linked with dissemination of AB Resistant pathogens.

Currently, the infections caused by AB resistant pathogens especially enteropathogens are significantly increasing in Pakistan. These are mostly invading humans through drinking contaminated water, un-hygienic practices and food. Moreover, there has been an extensive and empirical use of antibiotics that also plays major role in the development of ABR. Therefore, the present study relates with the ABR pattern of *E. cancerogenus* strains isolated from hospital associated infections at District Khairpur Mir's, Pakistan.

MATERIALS AND METHODS

Materials (media, test reagent and chemicals)

The present study was carried out in the Postgraduates Research Laboratory (PGRL), Institute of Microbiology, Shah Abdul Latif University Khairpur Mir's, Pakistan. The growth substrates like Nutrient agar (NA), Mac Conkey agar, Eosin Methylene Blue (EMB), Mueller Hinton Agar (MHA), Simon Citrate Agar (SCA), and commercial antimicrobial disks such as Amoxicillin clavunic acid (AMC 30µg), Sparfloxacin (SPX 5µg), Gentamycin (GM 10µg), Azomax (AZM 15µg), Enoxacin (EN 10µg), Piperacillin Tazobactam (TZP 110µg), Moxifloxacin (MXF 5µg), Fosfomycin (FOS 50µg), Fusidic acid (FD 10µg), and Sulbactam (SCP 105µg) were purchased from Oxoid (Oxoid, UK). The chemicals, test reagents and sugars were purchased from Sigma Aldrich (Sigma-Aldrich USA). Glassware of present study was purchased from Borosil (Borosil, USA).

Sample collection and isolation of *Enterobacter*

The hospital-associated clinical sample such as urine, pus, blood, high vaginal swab (HVS), stool, ear, throat, cerebrospinal fluid (CSF), ascitic fluid and pleural fluid were collected randomly from the patients regardless of their age and gender. Subsequently samples were processed for isolation of hospital-associated pathogenic bacteria. The isolates based on their distinct cultural characteristics (macroscopic), similar morpho-microscopic and biochemical characteristics were designated to different group like NS1, NS2, NS3, NS4 and NS5. Among these groups, the *Enterobacter* strains were assigned to NS3 group. The cultural characteristics were recorded from the surface of nutrient agar, Mac conkey agar and EMB agar in order to record their features for the confirmation.

Characterization of *Enterobacter*

The hospital-associated isolates kept in group NS3 were preliminary characterised using morpho-microscopic (Cultural, Gram staining, hanging drop technique, capsule and spore staining) and biochemical characteristics such as Citrate Utilization, Oxidase, Catalase, Methyl Red (MR), Voges-Proskauer, Indole, and sugar fermentation pattern. After all the parameters, isolates were preliminary identified as Enterobacter. The isolate of interest exhibiting maximum resistance was confirmed using 16S RNA gene Sequence homology.

Antimicrobial susceptibility testing

The ABR was determined using Kirby-Bauer disk diffusion susceptibility testing as mentioned in previous paper of Mangi, *et al*., (2016) and breakpoints for each antibiotic were interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2015). Antibiotics panel tested included, AMC (30µg), TZP (110µg), GM (10µg), FD (10µg), MXF (5µg), SPX (5µg), FOS (50µg), AZM (15µg), EN (10µg), and SCP (105µg).

16s rRNA gene sequencing and phylogenetic correlation

For molecular identification, the pure culture stock of selected bacterial isolate, i.e. *Enterobacter* strain, was sent to Genomic Division, Macrogen Inc., Seoul, Korea for amplification and partial sequencing of 16S rRNA gene using set of universal primers for amplification , i.e. 27F and 1492R primers (5'AGAGTTTGATCMTGGCTCAG-3' and 5'- TACGGYTACCTTGTTACGA CTT-3', respectively). After

amplification, the resulting amplicons were subjected to partial sequencing of 16S rRNA gene using ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystem, USA) by using universal sequencing primers, i.e. 518F (5' CCAGCAGCCGCGGTAATACG-3') and 800R (5'TACCAGGG TATCTAATCC-3'). Finally, the obtained partial sequences by each primer were then assembled using an online CAP3 sequence assembly program (Huang and Madan, 1999). Resulting contiguous sequences were then analysed and compared with existing GenBank nucleotide sequence databases at National Centre for Biotechnology Information (NCBI) website using Basic Local Alignment Search Tool (BLAST) program in order to confer the percentage sequence similarities. The phylogenetic correlations were obtained according to the Kumar *et al*., (2016) using MEGA7 software using the Maximum Likelihood method based on the model of Tamura (2004).

RESULTS

The antibiotic resistance determination is key to success for the recognition of significant treatment modalities and fighting against hospital-associated bacterial pathogen. In present study 280 (162 from male and 118 from female) hospital-associated clinical samples were randomly collected in order to trace the antimicrobial resistance and gender wise prevalence of Enterobacter pathogen. The uniqueness of present study was the unexplored area of Pakistan, (Khairpur and Sukkur) and good samples number in order to accomplish the promising results for the isolation of hospital-associated bacteria particularly Enterobacter.

Table 1. Morpho-microscopic, biochemical and sugar fermentation pattern of the bacterial isolates of group NS3 pertaining to hospital-associated pathogen.

Test type	Assay/Test	Group NS3 bacterial isolates
Microscopy	Gram's staining	Gram negative
	Shape	*Bacilli*
	Spore staining	Non-sporulating
	Capsule staining	Non-capsulated
	Motility	Motile
	Flagella staining	Peritrichous arrangement
Biochemical	Catalase	+
	Oxidase	-
	Nitrate reductase	+
	Indole	-
	Methyl red	+
	Voges Proskauer	+
	Urease	-
	Citrate Utilization	+
Sugar fermentation	Glucose	+ AG
	Lactose	+ AG
	Mannose	± AG
	Sucrose	± AG
Tentatively Identified as:		*Enterobacter spp*.

Note: + = Positive result; - = negative result; ± = variable; +AG = Positive acid and gas; ±AG = variable for acid and gas.

Isolation and Identification of *Enterobacter*

The nine (NS3=9) isolates were isolated with the maximum number of isolates from the stool 100% (6 of 6) followed by the blood 21% (3 of 14) while it was not found in rest of the hospital associated samples like HVS, urine, ear swab, throat swab, pleural fluid, ascitic fluid and CSF. All the isolates (Nine) were subjected to identification by in-vitro macroscopic and physical characteristics as aerobe glistening, smooth, translucent and irregular followed by the microscopic observations as gram negative, straight rod shape (bacilli), motile, non-spore former and non-capsulated. Table 1 describes the biochemical characteristics in addition to microscopic and macroscopic features of isolates. All the nine isolates were positive for the catalase, citrate utilization, nitrate reduction, methyl red and Voges-Proskauer reaction while the urease, indole, and oxidase were negative. The isolate

ferment sugars (glucose, lactose, mannose, and sucrose) into acid and gas. Thus based on cultural, morpho-microscopic and biochemical nature, isolates were presumed as *Enterobacter spp.* Table 2 describes the sample and gender wise prevalence as 66.7% in stools and blood samples of female while 33.3% in respective samples of male patients.

Table 2. The Gender-wise frequency distribution and % prevalence of Enterobacter species in hospital.

Sample source	Male Samples	Female Samples	Total Samples	Gender-wise frequency distribution of Enterobacter species (NS3) %Prevalence (No. of distinct isolates)		Total Isolate NS3 and % prevalence
				Male	Female	
Urine	45	33	78		0	0
Pus	75	61	136		0	0
Stool	3	3	6	33.3(2)	66.7(4)	6 (100%)
HVS		10	10	0	0	0
Ear swab	2	1	3	0	0	0
Throat swab	7	1	8	0	0	0
Ascitic fluid	2	1	3	0	0	0
Blood	11	3	14	33.3(1)	66.7(2)	3 (21%)
CSF	4	0	4	0	0	0
Pleural fluid	13	5	18	0	0	0
Total	162	118	280			9 (3%)

Note: HVS = high vaginal associated samples swab, CSF = cerebrospinal fluid and % = Percentage.

Antimicrobial sensitivity profile (ASP) of Enterobacter

According to CLSI (2015) the results were interpreted and it was found that antimicrobial panel used was ineffective and not displayed any satisfactory results (zone of inhibition) against *Enterobacter* isolates (NS3). Fig. 1 presents data on the isolate wise resistance (A.) and antibiotic wise resistance pattern (B.) of Enterobacter isolates (NS3). The 5 of 10 antibiotics such as AMC 30µg, SPX 5µg, FOS 50µg, FD 10µg, and AZM 15µg, were not exhibiting zone of inhibition while MXF (5µg) displayed maximum (11.5mm) zone of inhibition, followed by the TZP 110µg (10.5mm), EN 10µg (10mm), SCP 105µg (10mm) and GM 10µg (9mm) as mentioned in Fig. 2.

Phylogenetic correlation analysis

The isolate (NS3-E-8) from blood has displayed complete resistance against all the tested antibiotics and was subjected to molecular characterization using 16S rRNA sequence homology. The phylogenetic correlation studies revealed that the amplified 16S rRNA gene sequence of NS3-E-8 isolate shared 99% similarity with *Enterobacter cancerogenus strain* (LMG 2693) available at NCBI GenBank (Fig. 03).

DISCUSSION

The worldwide increasing ABR among bacterial pathogens causing both hospital- and community-acquired infections is one of the most serious vulnerable public health problems. Resistance pattern among pathogens pertaining to Enterobacteriaceae are especially troubling one due to their hosts and ubiquity nature. To combat this serious problem, determination of ABR pattern is need of hour for the recognition of significant treatment regime and fighting against them. Therefore, present study relates with the frequency distribution (Gender wise) and ABR profile of *Enterobacter* in hospital associated pathogens at District Khairpur Mir's, Pakistan. In present study for the optimistic results, total 280 samples (118 from female and 162 from male) were randomly collected from the private and public health care hospitals and their associated laboratories. ABR among bacterial isolates has been known as growing clinical problem and a serious threat to public health. In present study, similar threat was evident from antibiotic resistant *Enterobacter* which showed complete resistance to all the tested antibiotics.

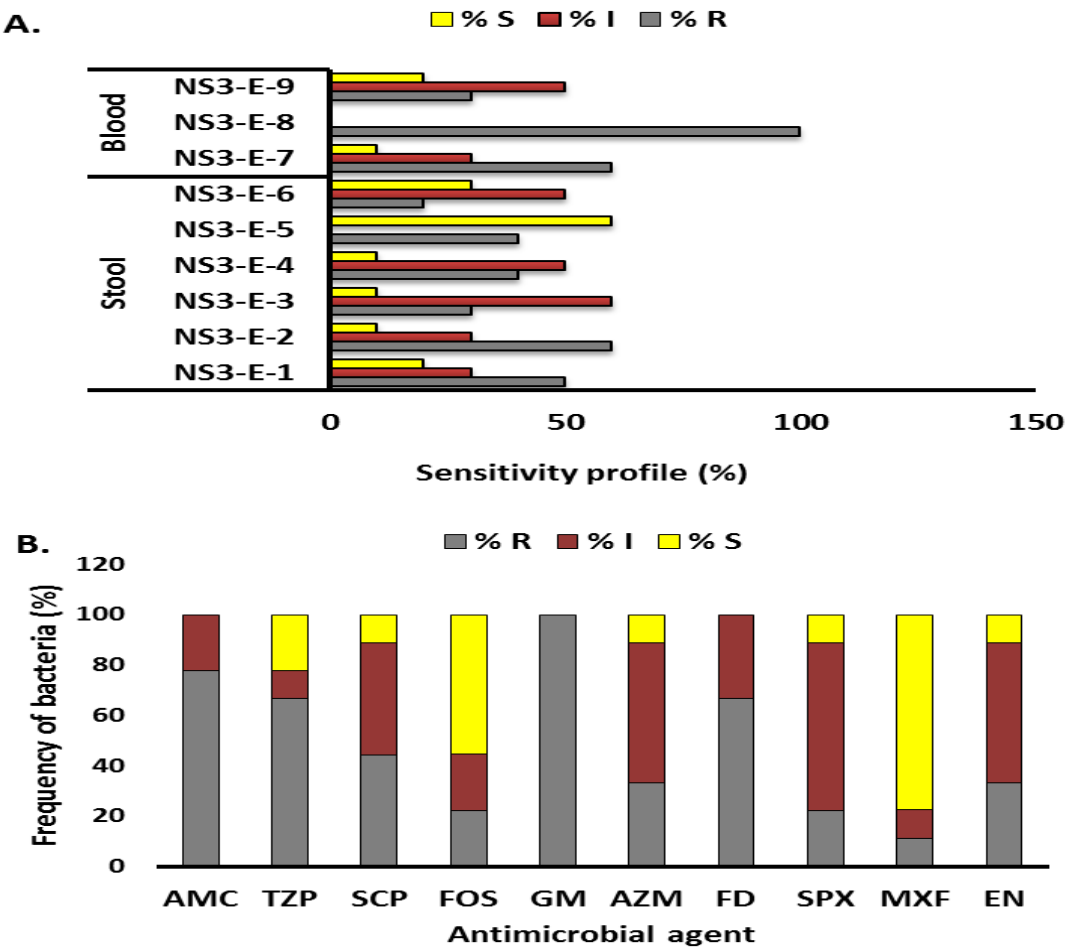


Fig. 1. The isolate wise cumulative antibiotic-sensitivity pattern of all the *Enterobacter* strains (NS3) (A.) and antibiotic wise resistant pattern of NS3 (B.) against panel of test antibiotics.
Note: Commercial Antibiotic Disc codes: AMC = Amoxicillin-Clavulanate, SPX = Sparfloxacin, GM = Gentamicin, FOS = Fosfomycin, MXF = Moxifloxacin, FD = Fusidic acid, EN = Enoxacin, AZM = Azithromycin, TZP = Piperacillin-Tazobactam, SCP = Sulbactam. Alphabetical letter "R" in each data label stands for "Resistant", I for Intermediate and S for sensitive.

In the study of Paterson et al., (2005), the prevalence of Enterobacteriaceae account for 84% of the total isolates from intra-abdominal infections worldwide with *E. coli* (46%), *Klebsiella* spp. (17%) and *Enterobacter* spp. (8%). The *E. cancerogenus* was the very less prevalent (0.2%) while *Enterobacter cloacae* was the most common (72%) species among the Enterobacter spp. isolates. In present study three percent of *Enterobacter* isolates 3% (9 of 280) were isolated from the total clinical samples. In sample wise distribution, the maximum 100 % from the stool (6 of 6) and 21% from blood (3 of 14), while in rest of the hospital-associated samples like HVS, urine ear swab, throat swab, pleural fluid, ascitic fluid and CSF were not found prevalent. In gender wise prevalence of *Enterobacter* isolates, female patients predominate in prevalence than the male patients. Among the isolates, (NS3) isolated from blood exhibiting maximum resistance against all the tested antibiotics was subjected to 16S rRNA sequence homology. The phylogenetic correlation studies revealed that the amplified 16S rRNA gene sequence of NS3-E-8 isolate shared 99% similarity with *Enterobacter cancerogenus* strain (LMG 2693) available at NCBI GenBank database as mentioned in figure 2. Abdul-Ridha, and Al-Abbas, (2016) have isolated *E. cancerogenus* from cancer patients having bacteraemia. Similarly, the isolate (NS3-E-8) of present study was from blood.

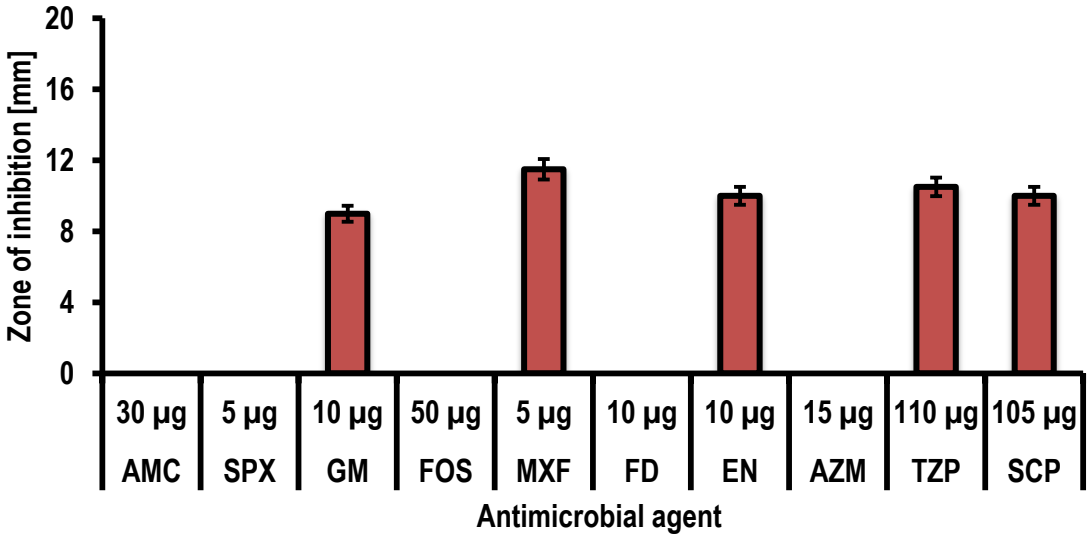
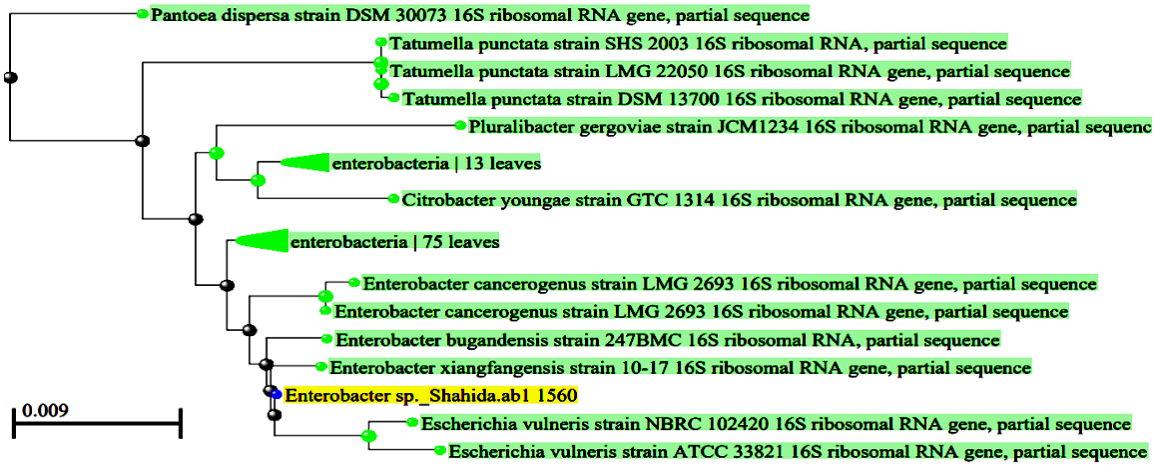


Fig. 2. The antibiotic-sensitivity pattern of *Enterobacter cancerogenus* strain (NS3-E-8) against panel of test antibiotics.
Note: Commercial Antibiotic Disc codes: AMC = Amoxicillin-Clavulanate, SPX = Sparfloxacin, GM = Gentamicin, FOS = Fosfomycin, MXF = Moxifloxacin, FD = Fusidic acid, EN = Enoxacin, AZM = Azithromycin, TZP = Piperacillin-Tazobactam, SCP = Sulbactam and µg denote microgram. The error bars indicate percentage error at 95% confidence interval *(P<0.05)*.



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterobacter cancerogenus strain LMG 2693 16S ribosomal RNA gene, partial sequence	2654	2654	99%	0.0	99%	NR_044977.1
Escherichia vulneris strain NBRC 102420 16S ribosomal RNA gene, partial sequence	2636	2636	99%	0.0	99%	NR_114080.1
Yokenella regensburgei strain CIP 105435 16S ribosomal RNA, partial sequence	2630	2630	99%	0.0	99%	NR_104934.1
Escherichia vulneris strain ATCC 33821 16S ribosomal RNA gene, partial sequence	2627	2627	98%	0.0	99%	NR_041927.1
Leclercia adecarboxylata strain CIP 82.92 16S ribosomal RNA, partial sequence	2625	2625	99%	0.0	99%	NR_104933.1
Enterobacter bugandensis strain 247BMC 16S ribosomal RNA, partial sequence	2625	2625	97%	0.0	99%	NR_148649.1
Enterobacter ludwigii strain EN-119 16S ribosomal RNA, partial sequence	2619	2619	99%	0.0	99%	NR_042349.1

Fig. 3. Neighbour-joining tree showing evolutionary relationship of isolate NS3-E-8 with closely related taxa.

Stock and Wiedemann, (2002) has investigated the natural susceptibility of 107 Enterobacter strains comprising *E. amnigenus* (n 18), *E. cancerogenus* (n 26), *E. gergoviae* (n 28) and *E. sakazakii* (n 35) against 69 antimicrobial agents. They found that *E. cancerogenus* was the only species showing natural resistance to amoxicillin, AMC, cefaclor, cefazoline, loracarbef and cefoxitin. *E. cancerogenus* was found susceptible to fosfomycin only. Contrarily

the fosfomycin was not found effective against *E. cancerogenus* of present study. Present study reveals that all the ten randomly tested antibiotics (AMC, TZP, GM, FD, MXF, SPX, FOS, AZM, EN, and SCP) were completely ineffective against the isolate *E. cancerogenus*. Based on a previous study of Stock and Wiedemann, (2002), the β-lactam sensitivity of *E. cancerogenus* seems to be similar to that of other common Enterobacter species, such as *E. cloacae* and *E. aerogenes.*

It is reported in previous literature that about 1 % of Enterobacter infections are due to *E. cancerogenus* and these infections seem to occur mostly in wounds contaminated setting. Moreover, in a statistic of the United States, it is mentioned in the case series that 59 percent of all published cases of *E. cancerogenus* have been found secondary to trauma with 11% mortality rate (Hall *et al.*, 2012). In addition, there have been case reports of *E. cancerogenus* causing osteomyelitis (Garazzino, 2005) bacteraemia (Bowles, 2006), urinary tract infection (Rubinstien *et al.*, 1993), and pneumonia (Demir *et al*., 2014; Hadano *at al.,* 2018).

Conclusion

The emerging ABR remains a concern worldwide. Present study relates with the isolation and ABR pattern of *E. cancerogenus.* This pathogen in previous studies has been isolated from wound, osteomyelitis, bacteraemia, urinary tract infection, pneumonia and cancer. Due to infectious nature, inadequate knowledge about pathogenicity, epidemiology and ABR pattern, *E. cancerogenus* may therefore be the more important human pathogen than the thought. Further studies are needed to clarify the ABR pattern, surveillance and clinical profile of *E. cancerogenus*

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