Original Article

Phylogenetic Analysis And Molecular Characterization Of Antibiotic Resistant Genes Of Proteus Mirabilis Recovered From Infected Patients Of Skin And Soft Tissue Wounds

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Abstract

Proteus mirabilishas the ability to infiltrate and colonize woundscausing wound infections in both community and hospitalized patients. Due to the production of extended-spectrum-lactamases, P. mirabilis has developed resistance to beta-lactam antibioticsas a result of conventional use of antibioticslimitingtreatment options for clinicians.The current study is aimed to determine the phylogenic analysis and molecular characterization of antibiotic resistant genes of P. mirabilisrecovered from infected patients of skin and soft tissue wounds.A total of 800wound samples were obtained from infected wound patientsat Khyber Teaching Hospital (KTH) and Hayatabad Medical Complex (HMC),Peshawar.The collected samples were identified phenotypically using API 20E strips and antibiogram was determined using standard procedures. Molecular identification was done through sequencing of 16sRNA and phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA-7) software. The antibiotic resistant genes of P. mirabilis were detected by PCR using specific primers.Out of 800 samples,84 isolatesyielded the growth ofP. mirabilis.Phylogenetic tree revealed that 100% of samples No. A-1 and No. A-2 are closely related to P. mirabilis to NR 043997.1:1-1503 P. mirabilis strain NCTC 11938 and 99%to (NR 114419.1:1-1495 P. mirabilis strain ATCC 29906).The results of antibiogram revealed that most of the isolates were resistant against antibiotics; cephalosporins, ampicillin, quinolones, chloramphenicol and aminoglycosides while showed good results against MEM, TZP and SCF.Of the isolates, 72.6% were found positive for ESBLs production. Molecular analysis showed that 40%of isolates were positive for blaTEM, blaCTX-M 14 (20%) and blaCTX-M (5%).The current study showed that the prevalence of blaTEM, blaCTX-M and blaCTX-M-14 genes carrying P. mirabilis in a tertiary care hospital Peshawar Pakistan. Phylogenetic analysis shows that sequences are closely related with the reference sequences ofP. Mirabilisamong infected patients of skin and soft tissue woundsand is resistant to the commercially available antibioticsresulting in a therapeutic challenge for clinicians.

Keywords: Proteus mirabilis, Antibiogram, PCR, ESBL genes, sequencing, Phylogenetic analysis.

Introduction

A member of the Enterobacteriaceae family, Proteus sp. (P. mirabilis) is a gram-negative rod (GNR), facultative anaerobe, and lactose-fermenting bacterium. Proteus mirabilis, which causes around 90% of Proteus infections in people, is the most significant species in this genus[1]. Proteus mirabilis, an opportunistic pathogen also known as

Proteus, is 90% of all Proteus infections in humans. Numerous illnesses, such as otitis media, bacteremia, wounds, and urinary tract infections, can be brought on by P. mirabilis (UTIs) [2]. P. mirabilis capable of infecting wounds severely. Generally speaking, extended spectrum cephalosporins (ceftriaxone and cefpodoxime) are the best option for treating a variety of bacterial illnesses. Wild-type strains of Proteus mirabilis are vulnerable to β-lactams [3]. The synthesis of acquired extended-spectrum β-lactamases is the main reason for the gradual/constant rise in the frequency of -lactam resistance of P. mirabilis documented throughout time [4]. Bacterial diverse β-lactams exposure has led to ongoing creation and modification of β-lactamases, increasing their activity even against contemporary -lactam antibiotics. The term "extended-spectrum β-lactamases" refers to these enzymes (ESBLs) [5, 6].

These enzymes prevent the third generation cephalosporins, such as ceftriaxone and cefotaxime, or aztreonam (monobactam), from working properly [7]. Due to these strains' variable levels of activity against numerous cephalosporins, they are also challenging to identify, which restricts the best course of treatment [8]. Extended Spectrum Beta Lactamases are thought to be responsible for P. mirabilis' resistance to Extended Spectrum Beta Lactams [9]. In order to offer clinically meaningful antibiotic testing in hospitals, healthcare-related laboratories must have the necessary infrastructure. It is clear that in order to overcome the resistance, thorough research on the topic of precise and quick identification of ESBL and their resistance pattern is necessary. The goal of the current work is to identify the resistance pattern and molecular detection of certain antibiotic resistance genes of P. mirabilis in clinical isolates of wound samples, taking into consideration the importance of wound infections for healthcare and patient health.

It is therefore necessary to determine the antibiogram and molecular characteristics of resistant bacteria in the hospital settings to take infection control measures and empirical treatment for infections. The current study is aimed to determine the phylogenic analysis, antibiotic susceptibility patteren and molecular characterization of antibiotic resistant genes of P. mirabilis recovered from infected patients of skin and soft tissue woundsin tertiary care hospital Peshawar. This will be helpful for clinicians to overcome the resistance mechanisms.

Material & Methods

2.1. Sample collection and processing

This cross-sectional study was designed at Pathology Department, Khyber Teaching Hospital (KTH), Peshawar, Hayatabad Medical Complex (HMC), Peshawarand the Center of Biotechnology and Microbiology (COBAM), University of Peshawar (UoP). A total of 800 samples were obtained from infected patients of skin and soft tissue woundsvisiting KTH and HMC,Peshawar. However, the patients having any parasitic, or fungal infections or on antibiotics were excluded from the study.Initially, the samples were inoculated on MacConkey and Blood agarand incubated for bacterial growth for 24 hours at 37ºC. The colonies were identified as lactose and non-lactose fermenters based on its characteristics. In order to identify their gram status, the Gram staining was performed. Analytical Profile Index (API) strips were used to characterize and identify theP.mirabilis isolates.Tryptone soya broth (TSB) media was used to preserve all the clinical isolates of P. mirabilis and stored at -80°C for further processing [10].

Antibiotic Susceptibility Testing

Bauer disc diffusion technique was performed in order to identify the isolates, using Muller Hinton Agar (MHA) media for antibiogramusing standard protocols. The discs of selected antibiotics were placed on the plates and subjected to incubation for 24 hours at 37ºC [11].According to recommendations from the Clinical and Laboratory Standards Institute (CLSI), the results were classified as resistant, sensitive, and intermediate [12].

Phenotypic detection of ESBLs production

To detect ESBLs production, phenotypic synergy test was used. The MHA plate was evenly covered with a 0.5 McFarland turbid 18–24 hour broth culture. The cephalosporin discs (CTX, FEP, CAZ, and ATM) were positioned at least 20-25 mm apart, starting with an AMC disc in the center of the inoculated plate and then incubated for 24 hours at 37°C. If the zone of inhibition from cephalosporins or ATM extends to the side of the AMC, the bacteria is assumed to be an ESBL producer [13].

DNA Extraction

The Genomic DNA was isolated from 24-48 hour-old broth cultures using Isolate II Genomic DNA Kit (Cat # BIO-52066). The DNA was extracted and purified as per the manufacturer's protocol. The purified DNA was then stored at -20ºC for further analysis

Molecular Identification

The 16sRNA universal primers were used to identify the isolates. The samples were sent to Macrogen Korea for sequencing.The obtained sequence were subjected to BLAST search and phylogenetic analysis using MEGA 11.0 software.

Molecular characterization of ESBLs gene (s) and sequencing

The antibiotic-resistant genes were amplified using primers: Bla CTX-M[14], Bla CTX- M14[15],Bla TEM[16]and Bla SHV[17], (oligonucleotides, Macrogen Korea). The sequence of these primers is shown in **Table 1**. The PCR reactions were prepared for each sample by mixing 12.5 µl of Taq Master mix (Bioron, life sciences), 0.5 µl of each reverse and forward primers (oligonucleotides, Macrogen Korea), 11.5 µl of Nuclease- free water and 2 µl of sample DNA. To validate the credibility of the experiment, positive and negative control was also run. The amplicon was fragmented by using 2% agarose gel electrophoresis whereas bands were visualized through gel documentation system (BIO-RAD Gel Doc™ XR+). The size of amplicon was determined by matching it with DNA ladder of about 100bp.The randomly selected resistant genes of PCR products were then sent toMacrogen Korea for sequencing. According to NCBI database, the FASTA sequences of these PCR products showed similar results with original GenBank gene sequences. The nucleotide and amino acid sequences were analyzed carefully by using softwares like BioEdit and BLAST.

Table No 1: Oligonucleotide primer sequences used for the molecular detection of ESBLs genes.

Gene	Primers Sequences (5'à3')	Product Size (bp)	References
Bla- CTX-M	F: ATGTGCAGCACCAGTAAAGT	545	[14]
	R: ACCGCGATATCGTTGGTGG		
Bla- CTX- M- 14	F: CTGATGTAACACGGATTGACC	871	[15]
	R:CGATTTATTCAACAAAACCAG		
Bla- TEM	F:CGCAGATAAATCACCACAATG	247	[16]
	R: GTCTATTTCGTTCATCCATA		
Bla- SHV	F: CACCACGATGCCATGTTCATCTGC	768	[17]
	R: TCGCCTGTGTATTATCTCCC		

Data analysis

Moreover, the scientific calculations of this study were carried out by using the latest version of IBM SPSS Statistics software.

Results

Prevalence of P. mirabilis

A total of 800 samples were obtained from infected patients of skin and soft tissue wounds. Among the samples, 84 isolates were identified as P.mirabilis using standard techniques. Of the isolates,52 were acquired frommale patients, while 32 isolates were obtained from females.

Antibiotic Susceptibility Testing

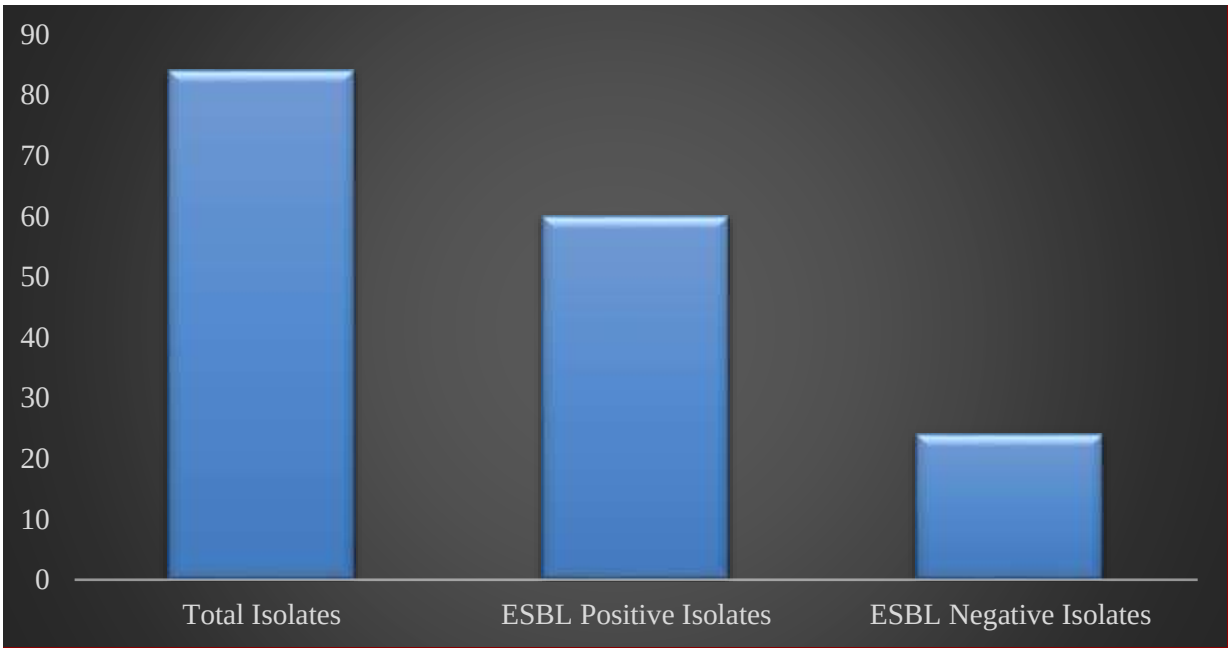
The P. mirabilisisolates were tested by using the disc diffusion method against 18 different antibiotics by using the CLSI-2021 recommendations. Antibiotic susceptibility testing revealed that that most of the isolates were resistant against antibiotics; cephalosporins, ampicillin, quinolones, chloramphenicol and aminoglycosides while showed good results against MEM, TZP and SCFas shown in **Table 2**.

Antibiogram of Proteus mirabilis obtained from different types of Wound Samples

Abbreviation	Antibiotics	Sensitive n (%)	Resistant n (%)
AMC	Augmentin	14 (17%)	70 (83%)
FEP	Cefepime	54 (64%)	30 (36%)
ATM	Azeteronem	12 (14%)	72 (86%)
CTX	Cefotaxime	6 (7%)	78 (93%)
CAZ	Ceftazidime	6 (7%)	78 (93%)
TGC	Tygacil	26 (30%)	58 (69%)
SCF	Sulzone	78 (92%)	6 (7%)
TZP	Tazocin	70 (83%)	14 (17%)
AK	Amikacin	24 (28%)	60 (71%)
DO	Doxycycline	14 (16%)	70 (83%)
FOS	Fosfomycin	58 (69%)	26 (31%)
AMP	Ampicillin	20 (23%)	64 (76%)
CIP	Ciprofloxacin	30 (35%)	54 (64%)
MEM	Meronem	66 (78%)	18 (21%)
CH	Chloramphenicol	26 (30%)	58 (69%)
SXT	Cotrimoxazole	68 (80%)	16 (19%)

Phenotypic detection of ESBLs production

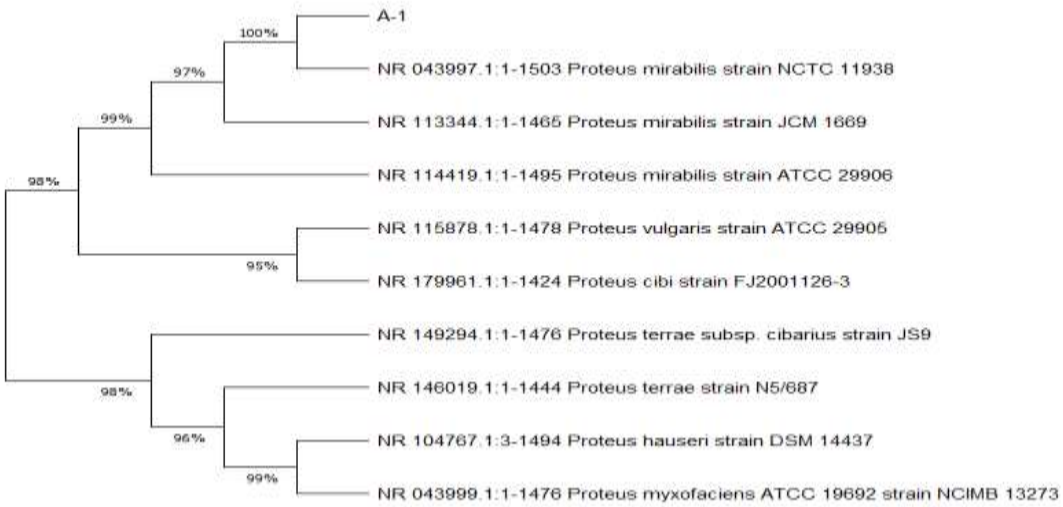
The synergy test was used to determine the phenotypic expression of Extended Spectrum Beta Lactamases (ESBLs). The zone of inhibition was created by 59(70.23%) out of the 84 P. mirabilis isolatesas illustrated in **Figure 1**.



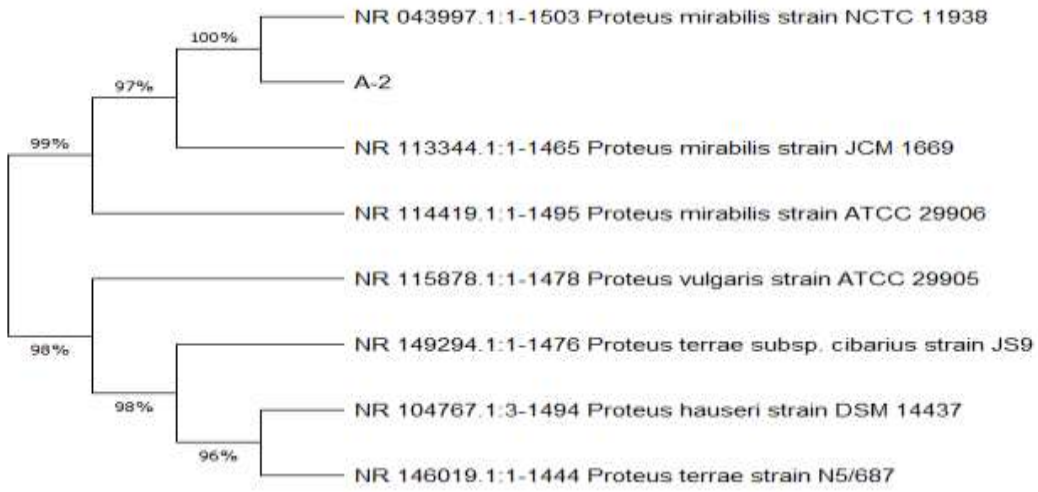
Prevalence of ESBL Producing Proteus mirabilis in Wound Samples.

Molecular identification

The 5ul of extracted DNA was sent to Macrogen Korea for 16sRNA sequencing against universal primers.The sequences ofthese bacterial species were subjected to BLAST search and the phylogenetic trees was created using the obtained sequences for bacterial isolates using MEGA 11.0.Phylogenetic tree revealed that 100% of samples No. A-1 and No. A-2 are closely related to P. mirabilis to NR 043997.1:1-1503 P. mirabilis strain NCTC 11938 and 99% to (NR 114419.1:1-1495 P. mirabilis strain ATCC 29906) as shown in **Figures 2-3**.



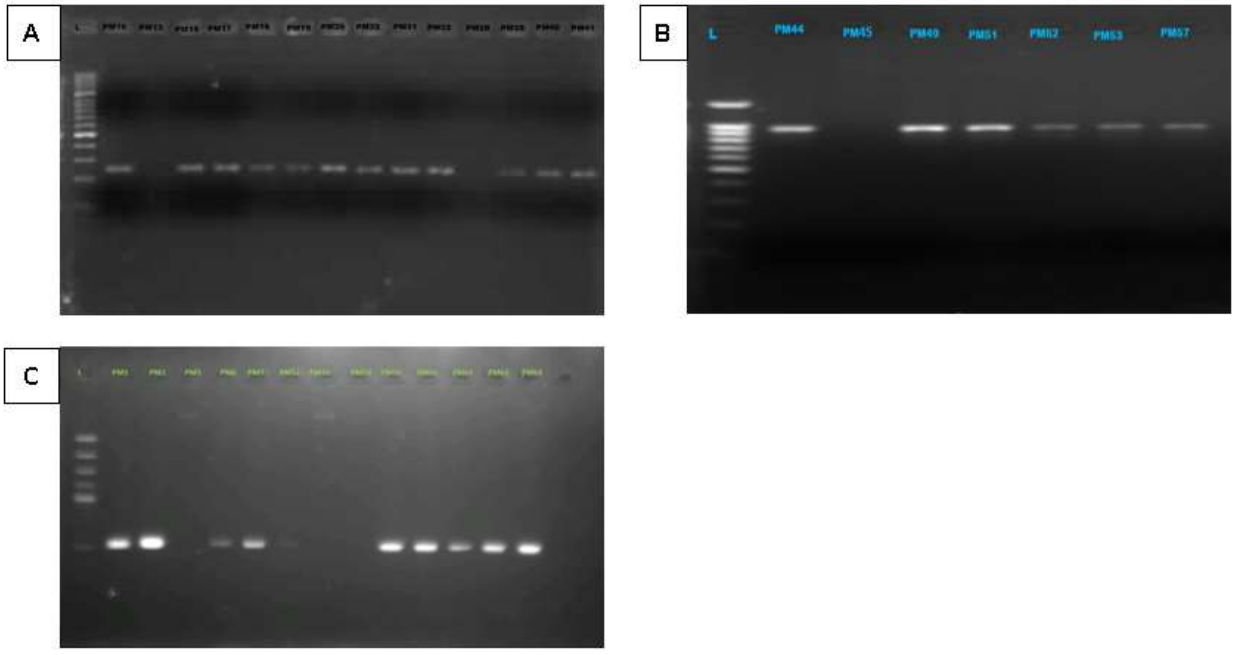
Phylogenetic tree of P. mirabilis (A-1).



Phylogenetic tree of P. mirabilis (A-2).

Molecular characterization of ESBLs gene (s)

Molecular analysis showed that 40% of isolates were positive for blaTEM, blaCTX-M 14 (20%) and blaCTX-M (5%) while blaSHV gene was not detected.The sequences of the ESBL genes were compared with similar data present at NCBI data base through blast program. The sequence analysis through blast further confirmed the presence of blaTEM, blaCTX-M, and blaCTX-M14 as shown in **Figure 3-4**.



Discussion

P. mirabilis has progressed through time to a level of antibiotic resistance where people are confronted with significant difficulties in the form of severe wound infections. P. mirabilis is showing a rapid proliferation of the resistance-granting genes. The dominant genes responsible for producing ESBLs include blaCTX-M, blaTEM, and blaSHV. Such P. mirabilis strains, which produce ESBLs, have the capacity to decrease the effectiveness of extended-spectrum

antibiotics. As a result, the selection of medications has been constrained and it is now challenging to treat infections brought on by Proteus mirabilis. The primary defense mechanism of this species against beta-lactam drugs is ended spectrum-lactamases. In our investigation, bacterial growth was detected in 97.2% of accidental wound samples, 99.1 percent of pathological wound samples, and 99.3 percent of post-surgical wound samples. The study by Mordi and Momoh (2009), which found a 33.04 percent rate for trauma wounds and a 22.04 percent rate for surgery site wounds, supports the findings of this research. Gram staining was used to identify samples as gram-negative (78 percent) and gram-positive (21.9% of the wound samples) [18]. This study's findings matched those of Ali and Al-Jaff, in which gram-negative bacteria were present in 71% of the wound samples [19].

In our study, 84 isolates were recognized as P. mirabilisusing the API 20 E strips. The findings are supported by Olumuyiwa et al. (2017) study, in which 43.5 percent of the wounds were infected with P. mirabilis[20]. In present study, the 16s RNA region of bacterial isolates were sequencedand then confirmed as P. mirabilis by phylogenetic analysis using MEGA 11.0.The Same identification method was done byMukhtar, A.A., et al (2018) in khartoum state using 16S rRNA gene sequencing for molecular identification andusing BLAST for sequence similarity search, MEGA7 software for phylogenetic analysis and Clustal W program for multiple sequence alignment[21]. Our sequencing results showed the 100% identical match to the reference P. mirabilis 16S rRNA gene using BLAST. The phylogenetic tree was constructed to show the evolutionary relationships of the obtained sequence with similar sequences in the databases using MEGA7 software, and the closest strain was found to be P. mirabilis strain from India (EU411047).

In our study, P. mirabilis showed maximum susceptibility to Cefotaxime (92.8 %), followed by Ceftazidime (92.8%), Azeteronem (85.71%), Augmentin (83.33%), Doxycycline (83.33%), Ampicillin. A similar investigation into the molecular characterization of the multi-drug resistant Proteus mirabilis was conducted by Olumuyiwa et al. (2017). Their research revealed that TEM was the dominant ESBL gene among CTX-M and other ESBLs in 48.3 percent of the isolates [20]. The findings of this investigation, in which TEM was found in 40% of isolates, are supported by the results of this work. The presence of TEM, CTX-M 14, and both alone and in combination have been documented in earlier investigations by Wu et al. and Ojdana et al. [22, 23]. According to Wu et al., findings, 24 (70 percent) of the 34 Proteus mirabilis isolates carried both CTX-M 14 and TEM. This represents 97%isolates. According to Ojdana et al. (2014), 91.7 percent of the P. mirabilisisolates have TEM genes [22]. The findings of this investigation are supported by the two studies described above.

In our investigation, BlaCTX-M was discovered in P. mirabilis isolates. Aragon et al. (2008) have currently revealed the existence of the CTX-M ESBL gene, which verifies our discovery and further confirms the spread of ESBL genes among resistant bacteria [24]. There was no evidence of BlaSHV in the P. mirabilis isolates. The findings of our investigation contradict Jones et al. (2009) [25], who reported the presence of BlaSHV in P. mirabilis; however, Ojdana et al. (2014) support our findings, which show that no BlaSHV was found in P. mirabilis isolates from wound swabs [22]. By sequencing, the existence of these resistance genes was further verified. Antibiotic susceptibility results and the resistant pattern of ESBL-positive P. mirabilis isolates were in agreement. Cefotaxime and ceftazidime were resistant to the majority of ESBL-producing isolates.

Conclusion

Our studyconcluded that Proteus mirabilis is a well-known species which is involved in causing wound infections. It is also one of the predominant pathogens isolated from infected wounds. P. mirabilis, which was isolated from infected patients of skin and soft tissue wounds, was shown to be extremely resistant to ceftazidime and cefotaxime as well as susceptible to sulzone, according to tests on antibiotic susceptibility. The current study showed that prevalence of blaTEM, blaCTX-M and blaCTX-M-14 genes carrying Proteus mirabilis in a tertiary care hospital Peshawar Pakistan.The current study showed that the prevalence of blaTEM, blaCTX-M and blaCTX-M-14 genes carrying P. mirabilis in a tertiary care hospital Peshawar Pakistan. The Phylogenetic analysis revealed that the obtained 16s RNA sequences are closely related with the reference sequences of P. mirabilisamong infected patients of skin and soft tissue wounds and also found to be resistant to those antibiotics which are commercially available antibioticsresulting in a therapeutic challenge for clinicians.

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