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EMERGENCE OF β-LACTAMASE PRODUCING AND MACROLIDE RESISTANT CLINICAL SPECIMENS OF PNEUMONIA IN PAKISTANI POPULATION; LIFE THREATENING PUBLIC HEALTH CONCERN

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Abstract

Pneumonia is life threatening public health issue in developing countries. The emergence of resistance genes is an increasing challenge, lead to morbidity and mortality in Pakistani population. The purpose of this research was to assess potential risk factors, characterization, antibiogram and screening of resistance genes among clinical isolates of pneumonia. From November 2021 to January 2024; a cross-sectional study was carried out. Socio-demographic data was collected at tertiary care hospitals of Lahore, Punjab, Pakistan. From various clinical specimens, bacterial samples were collected. Bacterial strains were characterized on the basis of biochemical and molecular characterization. Antibiotic testing was performed using disk diffusion method. The frequency of β-lactamase (*bla*CTX-M*, bla*TEM and *bla*OXA*)* and macrolide resistant genes (*erm* (B) and *mef* (A)) in pneumonia isolates were screened and amplified by PCR. Data was analyzed statistically. Total of n=261 samples were collected and cultured, in which 128(49.0%) were positive. Pneumonia was more prevalent among pediatrics 133 (51.0%) and older adults 95 (36.4%). The dominant pathogens were *K. pneumoniae* (33.6%) and *S. pneumoniae* (21.8) while frequency of other isolates *P. aeruginosa, S.aureus, E.coli* and *A.baumannii* were less. The majority of bacterial strains showed resistance to penicillin, ampicillin and amoxicillin-clavulanate. Among all pneumonia isolates, the percentage of resistant genes *bla*CTX-M and *bla*TEM was greater than *bla* OXA while macrolide resistant genes *mef* (A) and *erm*(B) was more predominant in *S. pneumoniae*. It is concluded that β-lactam antibiotic is not first line drug for treatment of pneumonia. However, macrolide is better option for treating pneumonia in Pakistani population.

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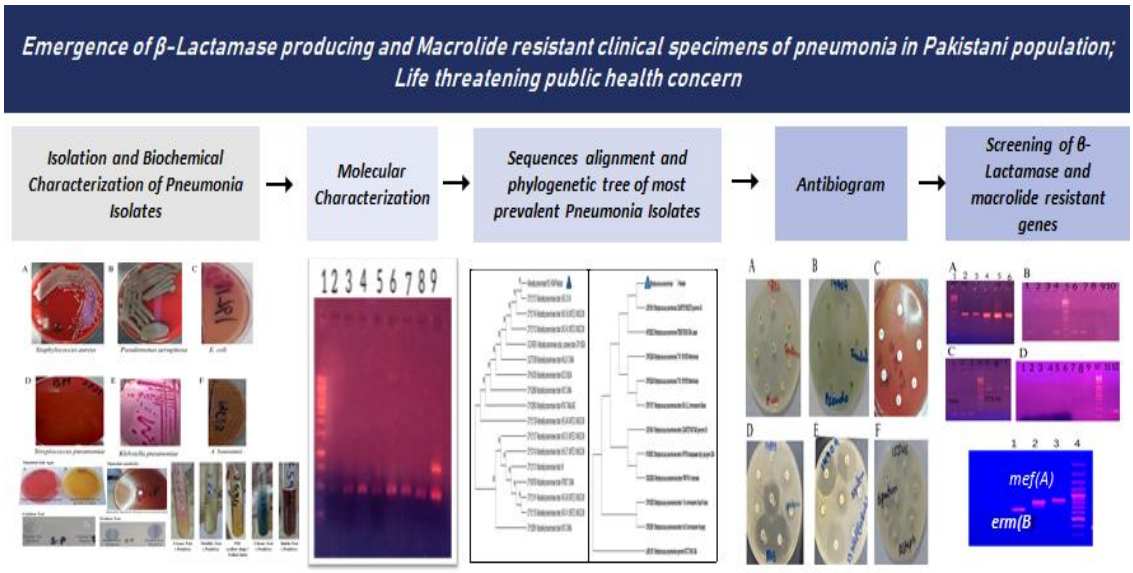
Graphical Abstract



Research Highlights

- Pneumonia is an emerging heath issue due to antimicrobial resistance in developing countries like Pakistan.
- From November 2021 to January 2024, Socio-demographic and clinical data was collected to check the association of pneumonia with its environmental and genetic risk factors. Isolation and characterization of pneumonia causing bacteria and there antibiogram was investigated by Kirby disk diffusion method.
- Phylogenetic tree was constructed by using bootstrapped Maximum-Likelihood (ML) method MEGA-11software to check the origin of bacterial pneumonia isolates. Screening of β-lactamase and macrolide resistance genes was also investigated.
- Our results showed that *K. pneumoniae* and *S. pneumoniae* was more prevalent while others bacterial strains were relatively less prevalent. Mostly bacteria were resistance to penicillin, ampicillin and amoxicillin-clavulanate whereas sensitive to colistin and imipenem.
- Resistance to β-lactamase drugs was more prevalent in Pakistani population than macrolide. So, it's inferred from study that β-lactam antibiotic is not first line drug for treatment of pneumonia. However, macrolide is better option for treating pneumonia in Pakistani population.

Keywords: Pneumonia, Antimicrobial Susceptibility Pattern, MDR, Gram-Negative Bacteria, β-lactamase, Macrolide.

INTRODUCTION

Pneumonia has largest burden in developing countries due to emergence antimicrobial resistance (AMR) acquired through resistance genes of various bacterial pathogens that has a detrimental impact on public health. Multidrug resistance leads to several bacterial infections such as urinary tract infections, gastrointestinal tract, pneumonia and respiratory tract (Shaaban *et al*., 2019). Pneumonia causes inflammation of lung tissues. It can be acquired from community as well as from hospital environment and may be

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transmitted by inhalation and aspiration of pathogenic microorganisms (Mackenzie, 2022). It accounts about 2 to 3 million mortality each year in developing countries India, Pakistan and Bangladesh etc. Several studies reported in Pakistani population that aging is an important risk factor that is associated with bacterial pneumonia. Infants that are <5 years and older adults that are >65 years are more prone of having bacterial pneumonia due to various risk factors such as poor healthcare standards, socioeconomic factors, malnutrition, smoking, mother unawareness, poor educational status lack of immunization, weak immune system and other comorbid conditions diabetes, hypertension, pulmonary disease etc. (Aftab *et al*., 2016; Ahmad *et al*., 2017). However, in developed countries, cases of pneumonia is less due to pneumonia specific interventions, better surveillance system and effective vaccine against pneumonia pathogens (Shabban *et al*., 2019; Metlay *et al*., 2019).

Klebsiella pneumoniae and *Streptococcus pneumoniae* are the two major bacterial pathogens that cause infections in hospitalized or immune-compromised patients and are mostly occurred in older adults and children. However, in other studies prevalence of other bacterial pneumonia *E. coli, P. aeruginosa* and *A. baumannii* are also reported (Seligman *et al.,* 2013; Jean *et al*., 2020). *Klebsiella pneumoniae* are opportunistic nosocomial pathogen that accounts 10% of all bacterial infections. It acquired resistance through multiple β-lactam antibiotics penicillin, ampicillin cephalosporins, carbapenems and amoxicillin-clavulanate etc (Ur Rahman *et al*., 2018). Resistance attained by these microorganisms due to presence of resistance genes bla_{TEM}, $bla_{CTX\text{-}M15}$, $bla_{OXA\text{-}48}$, and bla_{SHV} (Gondal *et al*., 2023). The plasmid born enzymes such as β-lactamases that hydrolysis the β-lactam ring present in cephalosporins and aztreonam (third generation antibiotics). Multi-drug resistance may also develop due to over expression of cephalosporinases and β-lactamases, metallo β-lactamases and oxacillinases β-lactamases (Shnaiderman–Torban *et al*., 2019). In several studies *Enterobacteriaceae* family *Klebsiella pneumoniae* and *Escherichia coli* have highest β-lactamases resistance genes $bla_{CTX\text{-}M}$ and bla_{TEM} (Dirar *et al*., 2020; Mohammedkheir *et al*., 2024). In many findings, β-Lactamase and Metallo lactamase resistance genes in *P.aeruginosa* was also detected in which bacterial isolates were more resistance to cefotaxime and less resistance to imipenem and meropenem, also emergence $bla_{CTX\text{-}M}$ and bla_{SHV} genes in *Klebsiella pneumoniae* was also reported in various tertiary care hospitals of Lahore, Pakistan (Ali *et al*., 2022; Gondal *et al*., 2023). Among *Enterobacteriaceae* family carbapenems (imipenem and meropenem), colistin and ceftazidime/avibactum might be effective choice of drug for the treatment of Pneumonia (Norgaard *et al*., 2019).

The 2nd most occurring bacterial pathogen that cause pneumonia is *S. pneumoniae* that has become a major global health concern because no. of variables have been identified are the root cause of the antibiotic resistance and irrational use of antibiotics, self-medication and non-compliance by patients. *S. pneumoniae* is the most challenging pathogen that cause acute respiratory infections in children and mostly isolated from pediatric pneumonia. *S. pneumoniae* is hard to manage because of spreading of several resistant clones, serotype replacement, capsular switching and ability to horizontally transfer of resistant genes (Cilloniz *et al*., 2023). In earlier ages, the backbone for the

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treatment of *S. pneumoniae* is β- Lactam antibiotics. In later, empirical treatment shifted towards macrolide drugs. However, in present era, *S. pneumoniae* showed resistance against clindamycin, tetracycline, penicillin, sulfamethoxazole and erythromycin (Purwanto *et al*., 2024). The most common macrolide resistance genes are; erythromycin ribosomal methylase *erm* (B) gene and macrolide efflux gene *mef* (A) (Diawara *et al*., 2016). Macrolide resistance may vary geographically ranges from less than 10% to greater than 90% of isolates (Xiao *et al*., 2015). Resistance in pneumococcal was mediated by *erm* (B) gene that encoding enzymes which undergoes methylation of 23S rRNA, thus inhibit the macrolide binding. Macrolide efflux protein, efflux pump encode by the *mef* (A) gene and mutation in 23S rRNA, cause macrolide resistance in *S. pneumoniae* (Schroeder *et al*., 2016). Hence, macrolide (e.g., erythromycin) vancomycin, lincosmides (e.g., clindamycin) may be used to treat *S. pneumoniae* reported in different studies (Sallam *et al*., 2019; Kulkarni *et al*., 2023). Life threatening disease prevention that develops through antimicrobial resistance is an important concern in Pakistan. Therefore, development of vaccines against these infectious diseases decreases the medical cost as well as the post-infection complications. It could also mitigate the need of antibiotics (1st and 2nd line of treatments), thus prevent the multi-drug resistance.

In Asian country like Pakistan, AMR is increasing tremendously. Hence, in bacterial pneumonia pathogens β-lactamase and macrolide resistance is spread empirically. Thus, the study was aimed to identification of most prevalent bacterial pathogens that causing pneumonia and determination of antibiogram pattern of pneumonia isolates. Also, it was designed to investigate the pneumonia causing bacterial strains and its associated risk factors in Pakistani population and screening of bacterial resistance genes (β-Lactamase and macrolide) from bacterial strains isolated from tertiary care hospitals of Lahore, Pakistan.

MATERIALS AND METHODS

Samples collection

From November 2021 to January 2024, the cross-sectional study was carried out at tertiary care hospitals of Lahore, Punjab, Pakistan. Socio-demographic data was collected from pneumonia suspected patients by reviewing medical lab reports. Total (n=261) samples were collected from nasal and ear swab, sputum, tracheal and pleural fluid. Samples were isolated onto selective media plates Mac-Conkey, chocolate and blood agar respectively and then further incubated for 18-24 hours at 37 °C. Colonies were sub-cultured for further identification.

Biochemical Characterization

Bacterial isolates were characterized depending upon gram staining and other biochemical characteristics. *S. pneumoniae* isolates were initially characterized by optochin (5µg) sensitivity test and other bacterial isolates along with *S. pneumoniae* were identified by catalase, coagulase and mannitol salt agar test while gram negative isolates were identified by API 20 E (Cheesbrough *et al*., 2006). All the isolates were initially

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cultured on nutrient broth for 24hrs at 37°C. The genomic DNA from bacterial pathogens were extracted by organic (Phenol: Chloroform) method (Sambrook *et al*., 1989). By using Nano drop, the quality and quantity of extracted DNA was determined (Thermo Scientific, USA).

Molecular Identification

The bacterial pneumonia isolates were amplified using 16S universal primers. Each PCR reaction was set in a final volume of 25µl that contained 2µl DNA template, PCR buffer (2.5µl), each PCR primer (1.25µl), 4µl of dNTPs, Taq DNA polymerase (0.3µl), $MgCl_2$ (4.5µl) and PCR water (9.2 µl). The reaction mixture was amplified by PCR (thermo fisher scientific). First, DNA was denatured at 94°C for 1minute followed by denaturation 30 cycles at 94°C; 45s annealing at temperature 52°C, 45s extension at 72°C. Final extension was made at 72°C for 5 min. Further, electrophoresis was done of PCR purified products by preparing 2 % agarose gel and took picture with UV illuminator (Bio-Rad, USA).

Characterization by species- specific Primers

The most frequently occurring bacterial isolates *Klebsiella pneumoniae* and *Streptococcus pneuomniae* were characterized by species-specific primers are indicated in table I. The PCR conditions of *Klebsiella pneumoniae* was initial PCR activation at 95°C for 5 min, 30s denaturation of 30 PCR cycles proceed at 95°C having 30s annealing temperature at 53°C and extension of PCR at 72°C for 30s. The final extension continued at 72°C for 10 min while for *Streptococcus pneuomniae* amplification of *cpsA* gene was performed at annealing temperature 57.5°C.

Table I: Specie-specific Primer sequence of *K. pneumoniae and S. pneumoniae.*

Primers	Primer Sequence	PCR Product size (bp)	References
KP-27F3 KP27B3	5'GGATATCTGACCAGTCGG3 5' GGGTTTTGCGTAATGATCTG3'	176	Dong *et al.,* 2015
cpsA-382 F *cpsA*-735 R	5'ACGCAACTGACGAGTGTGAC3' 5'GATCGCGACACCGAACTAAT3'	353	Park *et al.,* 2010

Sequences alignment and phylogenetic tree

The PCR amplified products were sent to the First Base Laboratories, Selangor, Malaysis for DNA sequencing. The data were compared to the reference 16SrRNA sequences by using nBLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was made by bootstrapped Maximum-Likelihood (ML) method by MEGA-11software (https://doi.org/10.1093/molbev/msab120).

Antibiogram

According to Kirby Bauer disk diffusion method, antibiotic susceptibility was performed of pneumonia causing bacterial strains. Following guidelines of Clinical laboratory standards institute (CLSI 2021) were used to assess antibiotic susceptibility using Piperacillin (PIP_110µg), Oxacillin (OXA_1µg), Ceftazidime-avibactam (CZA-50µg), Cefipime (FEP_5µg), Amikacin (AK_30µg), Trimethoprim/Sulphamethoxazole

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(SXT;TET_1.25+23.75µg) amoxicillin/clavulanate (AMC_25µg), Gentamicin (CN_10µg), Ciproflaxcin (CIP_5µg), Imipenem (IMP_10µg), Meropenem (MER_10µg), Colistin (CST_10 µg), Ampicillin (AMP_10µg), Cefatriaxone (CZA_30µg), Penicillin (P_10), Doxyoycline (DOX_30µg), Erthyromycin (E_30µg), Clindamycin (CN_2µg), Vancomyicin (VAN_30µg), Teicoplanin (TEC_30µg) and Linezolid (LZD_30µg). The bacterial suspensions were incubated for 24 hours. The bacterial suspension was swabbed on Muller- Hinton agar (Merk Co., Germany). Antibiotic disk was placed with sterile forceps at each distance 24mm. Then plates were left to dry for 30 mins. After incubation for 24 hours at 37 °C, diameter of zones was measured. The susceptibility pattern was noted as resistant and sensitive. The plates of Muller Hinton were prepared with 5% sheep blood for *Streptococcus pneumoniae* (Cheesbrough *et al*., 2006).

Screening of β-Lactamase and macrolide resistant genes

β-lactamase genes of bacterial pneumonia strains *bla*CTX-M*, bla* OXA and *bla*TEM and macrolide resistance genes *erm (B) and mef (A)*) were amplified by PCR to check the presence of resistance genes. For β-Lactamase, PCR reaction was performed at annealing 60°C-62°C. While for *Streptococcus pneumoniae*, macrolide resistant genes, PCR reaction was performed at annealing 56°C as mentioned in Table II.

Table II: Primer Sequence of β-Lactamase and Macrolide resistant genes.

Primers	Primer sequence	PCR Product size (bp)
*bla*TEM_F *bla*TEM_R	5'ATGGGGGATCATGTAACTCG3' 5'TTGCCGGGAAGCTAGATAA3'	153
bla OXA_F *bla* OXA_R	5'TTTGGCTCGATGGTGGTATT3' 5'CCCGTTTTAGCCCGAATAAT3'	163
*bla*CTX-M_F *bla*CTX-M_R	5'TCTTCCAGAATAAGGATCCC3' 5'CCGGTTTCCGCTATTACAAAC3'	909
*erm(B)*_F *erm(B)*_R	5'ACGAGTGAAAAAGTACTCAACCA3' 5'ACTTGCTCATAAGTAACGGTACT3'	656
*mef(A)*_ F *mef(B)*_R	5'AGTGGATCGTCATGATAGGAAGA3' 5'ACACGTCCTAAATATTCAGGCT3'	840

Statistical Analysis

By using SPSS version 22 (Armonk, NY), the data analysis was performed. Descriptive statistics was used to describe frequency and percentage of pneumonia suspected patient's. The Pearson's chi-square test was applied to check the relation of risk factors with different age groups. Level of significance (P-value) was determined to check the degree of association. P-Value with less than 0.05 was considered as statistically significant.

RESULTS

Socio-demographic Characteristics

The current study was conducted among n= 261 pneumonia suffered patients, from 133 (51.0%) were pediatric children (6 Months to 14 Years), 33 (12.6%) were adults and 95 (36.4%) were individuals in older age group (50-75). The most common risk factors

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associated in pediatrics were bottle feeding, which was found in 47.5% (n=124) while anemia and maternal education was 41.8 % (n=109) and 39.1 % (n=102) respectively. The common comorbidities in older adults were smoking 35.6% (n=93), hypertension 32.6 % (n=85), diabetes 28.4% (n=74), chronic kidney disease 25.3 (n=66) and chronic obstructive pulmonary disease 22.2 (n=58) as shown in Table III.

Table III: Socio-demographic Age-wise Risk Factors of pneumonia.

Characteristics	Frequency (N=261)	Percentage
Age		
Pediatric (6Months to 14Years)	133	51.0%
Adults (15-49)	33	12.6%
Older Age (50-75)	95	36.4%
Risk Factors : **Pediatric (6Months to 14Years) N=133**		
Bottle Feeding	124	47.5%
Immunization Status	100	38.3%
Nutritional Status	74	28.4%
Anemia	109	41.8%
Maternal education	102	39.1%
Risk Factors: Adults (15-49 Years) N=33		
Diabetes	5	1.9%
Hypertension	09	3.4%
Asthma	06	2.3%
TB	03	1.1%
HIV	02	0.8%
Smoking	14	5.4%
Risk Factors: Older Adults (50-75 Years) N=95		
Diabetes	74	28.4
Hypertension	85	32.6
Asthma	46	17.6
TB	18	6.9
HIV	08	3.1
Smoking	93	35.6
Ventilation Status	14	5.4
Chronic obstructive pulmonary disease (COPD)	58	22.2
Chronic Kidney disease	66	25.3

Prevalence of Pneumonia causing clinical Isolates

The percentage distribution of clinical samples in pneumonia suspected patients was highest in sputum followed by nasal and ear swab, tracheal and pleural fluid 140, 78, 31 and 12 respectively. The positive bacterial pneumonia isolates were identified n=128 (49.0%) from n=261. The frequently isolated pathogens were *Klebsiella pneumoniae* 43 (33.6%) and *Streptococcus pneumoniae,* 28 (21.8) whereas *P. aeruginosa, S. aureus, E. coli and A. baumannii* relative frequencies are 25(19.5%), 14 (10.9%), 13 (10.6%) and 5 (3.90%) respectively as indicated in Table IV.

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Table IV: Prevalence of bacterial Isolates in clinical specimens.

Bacterial Pneumonia Isolates	Nasal and Ear swab	Sputum	Tracheal	Pleural fluid	Frequency of Positive Isolates	Percentage of positive Isolates
Klebsiella pneumoniae **(n=67)**	9	43	11	4	**43**	**33.6**
Streptococcus spneumoniae **(n=64)**	9	55	-	-	**28**	**21.8**
Staphylococcus aureus **(n=41)**	20	9	9	3	**14**	**10.9**
Pseudomonas aeruginosa **(n=38)**	19	17	2	-	**25**	**19.5**
Escherichia coli **(n=28)**	13	11	3	1	**13**	**10.6**
Acinetobacter baumannii **(n=23)**	8	5	6	4	**5**	**3.90**
Total=261	**78**	**140**	**31**	**12**	**128**	**49.0**

Characterization of clinical Isolates

The pneumonia causing bacterial strains was characterized on the basis of morphological and biochemical features. *Streptococcus pneumoniae* strains was optochin (5µg) sensitive and showed green hemolysis on blood and chocolate agar while *Staphylococcus aureus* showed hemolysis having spherical and opaque colonies whereas all other gram negative isolates were characterized by API 20 E Kit. All positive bacterial isolates were further confirmed by 16Sr RNA primer yielding a band size 500bp while characterization of most prevalent bacterial species *K. pneumoniae* and *S. pneumoniae* by species-specific primers was done that produced the amplicon size 176 and 353bps respectively.

Sequencing and Phylogenetic analysis

Sequence homology showed that all bacterial pneumonia isolates have highest homology approximately 99% between the obtained sequences and the expected targets, specifically within the designated regions of the rRNA gene sequences. Among them *Klebsiella pneumoniae* isolates showed 100% sequence homology with target sequences whereas *Streptococcus pneumoniae* represented 92% homology with reference sequences isolated from different regional area of different countries. The phylogenetic tree was built by using MEGA-11software as shown in Figure 1.

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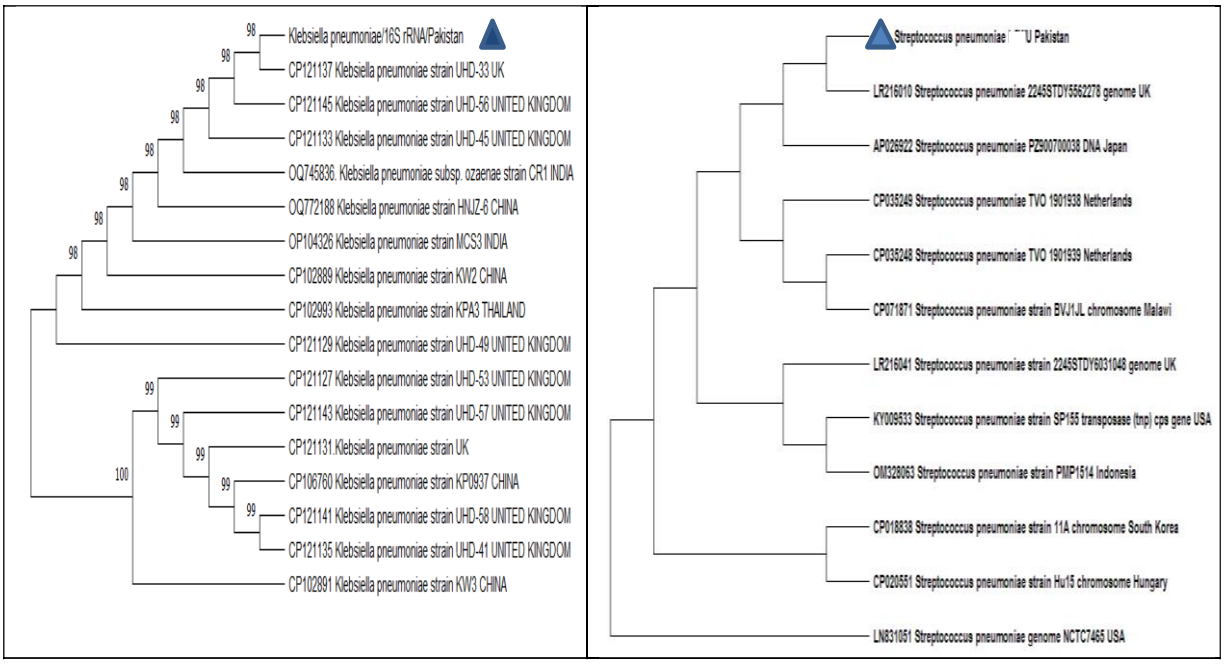

Figure 1: The Phylogenetic tree, constructed using neighbor-joining MEGA-11 software of most prevalent bacterial strains with already reported sequences, revealing a close relationship (100% with *K. pneumonia* and 92 % with *S. pneumoniae* (Note: ▲ Blue color indicates the study isolate).

Antibiotic testing

Total (n=43) *Kaleibsella pneumoniae* were 100% resistant to Co-trimaxazole, Doxycycline, amoxicillin/clavulanate and penicillin while *Streptococcus pneumoniae* (n=28) isolates were 100% resistant to trimethoprim-sulfamethoxazole, ciprofloxacin (78.5%) and oxacillin (60.7%) and showed 100% sensitivity to teicoplanin, linezolid and vancomycin. Others bacterial pneumonia isolates *Staphylococcus aureus* showed resistance to amikacin (100%) and doxycycline (78%) whereas *Pseudomonas aeruginosa* showed more resistance to Co trimaxazole (88%) and piperacillin (76.0%) while susceptible to amikacin, colistin and imipenem 72%, 84.1% and 68% respectively are depicted in Table V.

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Table V: Antibiotic resistance pattern of bacterial Isolates

GP Isolates	E	P	OXA	CIP	DOX	VA	TEC	LZD	AMC	MER	AK	CN	COT	SXT	
***S.pneumoniae* (n=28)** *%Resistance*	19 (67.8)	12 (42.8)	17 (60.7)	22 (78.5)	17 (60.7)	28 (0)	28 (0)	28 (0)	17 (60.7)	NT	NT	NT	22 (78.5)	28 (100)	
% Susceptible	9 (32.1)	16 (57.1)	11 (32.7)	6 (21.4)	11 (32.7)	28 (100)	28 (100)	28 (100)	11 (32.7)				6 (21.4)	28 (0)	
***S. aureus* (n=14)**															
%Resistance	5 (35.0)	14 (100)	NT	10 (71)	11 (78.0)	14 (0)	14 (0)	14 (0)	8 (57.1)	9 (64.2)	14 (100)	4 (28.0)	3 (21..4)		
% Susceptible	9 (64.0)	14 (0)		4 (28.0)	3 (21.4)	14 (100)	14 (100)	14 (100)	6 (42.8)	5 (35.7)	14 (0)	10 (71.0)	11 (78.5)		
GN Isolates	**AMP**	**AMC**	**PIP**	**CRO**	**AK**	**CN**	**CIP**	**DOX**	**COT**	**FEP**	**CST**	**CZA**	**MER**	**IMP**	**P**
***K. pneumoniae* (n=43)** *%Resistance*	43 (100)	43 (100)	32 (74.4)	36 (83.7)	31 (72.0)	31 (72.0)	35 (81.3)	43 (100)	43 (100)	35 (81.3)	34 (79.0)	25 (58.1)	32 (74.4)	32 (74.4)	43 (100)
% Susceptible	43 (0)	43((0)	11 (25.5)	7 (16.2)	12 (27.9)	12 (27.9)	8 (18.6)	43 (0)	43 (0)	8 (18.6)	9 (20.9)	18 (41.8)	11 (25.5)	11 (25.5)	43 (0)
***P.aeruginosa* (n=25)**															
%Resistance	NT	NT	19 (76.0)	16 (64.0)	7 (28.0)	14 (56.0)	17 (68.1)	NT	22 (88.0)	18 (72.0)	4 (16.0)	14 (56.2)	9 (36.3)	8 (32.0)	19 (76.0)
% Susceptible			6 (25.3)	9 (36.0)	18 (72.0)	11 (44.2)	8 (32.4)		3 (12.4)	7 (28.2)	21 (84.1)	11 (44.4)	16 (64.0)	17 (68.1)	6 (25.0)
***E.coli* (n=13)**															
%Resistance	13 (100)	13 (100)	11 (84.6)	13 (100)	10 (76.9)	5 (38.4)	10 (76.9)	11 (84.6)	12 (92.0)	13 (100)	13 (0)	8 (61.5)	4 (30.7)	4 (30.7)	NT
% Susceptible	13(0)	13 (0)	2 (15.3)	13 (0)	3 (23.2)	8 (61.5)	3 (23.2)	2 (15.3)	1 (7.64)	13 (0)	13 (100)	5 (38.4)	7 (69.2)	9 (69.2)	
***A.baumannii* (n=5)**															
%Resistance	3 (60.0)	5(100)	4 (80.0)	4 (80.0)	4 (80.0)	3 (60.0)	5 (100)	4 (80.0)	4 (80.0)	4 (80.0)	1 (20.0)	4 (80.0)	3 (60.0)	4 (80.0	NT
% Susceptible	2 (40.0)	5(0)	1 (20.0)	1 (20.0)	1 (20.0)	2 (40.0)	5 (00.0)	1 (20.0)	1 (20.0)	1 (20.0)	4 (80.0)	1 (20.0)	2 (40.0)	1 (20.0)	

Note: GP: Gram Positive; GN: Gram Negative; E : erythromycin; OXA: Oxacillin; CIP: ciprofloxacin, DOX: Doxycycline; VAN: Vancomycin; TEC: Teicoplanin; LZD: linezolid; AMC: amoxicillin/clavulanate; AK: amikacin; CN: gentamicin; COT: Co-trimoxazole; AMP: ampicillin, PIP: piperacillin; CRO: ceftriaxone; FEP: cefepime; CST: Colistin; IMP: imipenem; ; MER: meropenem ;P: penicillin; CRO: ceftriaxone, CZA: cefatazidine Avibactum, NT, not tested

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Screening of resistant genes

Molecular characterization showed that frequency of resistance genes *bla*CTX-M and *bla*TEM in *Klebsiella pneumoniae* isolates 88.3 % and 95.3% respectively whereas in *S.pneumoniae* isolates these resistance genes were 100% while macrolide resistance of *erm*(B) and *mef*(A) was 78.0% and 96.4% respectively. In other bacterial isolates *P.aeruginosa, bla*TEM and *bla*OXA was 100% *and* 56.0% respectively and frequency of *bla*OXA was less and only reported in gram negative isolates such as in *E. coli* (38.4%) and *A. baumannii* (20.0%) as shown in Table VI.

Table VI: Screening of resistant genes in pneumonia Isolates.

Total bacterial Isolates (n=128)	Resistance encoding genes, n (%) *bla CTX-M*	*blaTEM*	*blaOXA*	*erm(B)*	*mef(A)*
Gram Positive Isolates					
S.pneumoniae (n=28)	28(100%)	28(100%)	0(0%)	22 (78%)	27(96.4%)
S. aureus (n= 14)	9(64.2%)	5(36.0%)	0(0%)		
Gram Negative Isolates					
K. pneumoniae (n=43)	38(88.3%)	41(95.3%)	0(0%)	-	-
P. aeruginosa (n=25)	16(64%)	25(100%)	14(56%)	-	-
E. coli (n=13)	6(46.1%)	11(84.6%)	3(38.4%)	-	-
A. baumannii (n= 05)	2(40%)	3(60%)	1(20%)	-	-

DISCUSSION

Pneumonia is the most important global health concern and high rate of mortality in older adults and childrens in developing countries. The current study describes the social burden, socio-demographic risk factors and emergence of antimicrobial resistance owing to resistant genes in bacterial pathogens causing pneumonia in Pakistani population. In a study, the overall prevalence of pneumonia was 49.0% while in other developing countries, such as in Ethiopia (38.7%), India (88.3%), Bangladesh (76.1%) and various districts of Pakistan (58% to 61.8%) the prevalence rate is varies due to socioeconomic differences, seasonal variation and self-medication (Dessie *et al*., 2021; Gupta *et al*., 2017). Many studies showed that age is a risk factor of developing pneumonia. The younger children and older adults are more at risk of having pneumonia due to various risk factors. In current study, the percentage of dominant risk factors in pediatrics were exclusively bottle feeding (47.5 %), anemia (41.8%), maternal education (39.1%), immunization status (38.3%) and poor nutritional status (28.4%). Our findings were consistent with the studies reported in India and Pakistan (Gothankar *et al*., 2018; Aftab *et al*., 2016). Pneumonia in young age may be associated with lack or partial immunization, malnurishment, weak immune system of infants, lack of breastfeeding and

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maternal low educational level. However, in our study, the major cause of older age pneumonia related with different comorbidities such as smoking (35.6%), hypertension (32.6%), diabetes (28.4%), chronic kidney disease (CRD) (25.3%) and chronic obstructive pulmonary disease (COPD) (22.2%). Similar outcomes were also reported in Japan and Pakistan (Morimoto *et al*., 2015; Ahmad *et al*., 2017). In older adults, pneumonia might be develop due to weak immune system, respiratory distress and pulmonary infections etc.

In current study, the dominant isolates that develop bacterial pneumonia was *Klebsiella pneumoniae* (33.6%) and *Streptococcus pneumoniae* (21.8%). In previous studies, similar findings were also reported in which prevalence of pneumonia due to these two bacterial isolates (Dessie *et al*., 2021; Temesgen *et al*., 2019; Perveen *et al*., 2018). The predominance nature of these isolates may be due to their capsular, emergence of antimicrobial resistance and self-medication (Paczosa *et al*., 2016). *K. pneumoniae* strains had high rate of resistance (80-100%) to different antibiotics amoxicillin/clavulanate, ampicillin, Co trimaxazole, ciprofloxacin and cefepime as indicated in our results. These results may be consistent with the results reported in previous studies that showed 90.2% and 71.7% multi-drug resistance (MDR) of *K. pneumoniae* (Temesgen *et al*., 2019; Rashid *et al*., 2020). MDR due to β-lactam is producing enzymes carbapenemase which provide resistance by inactivating the anti-bacterial properties of antibiotics (Vrioni *et al*., 2012). Therefore, β-lactam antibiotics are not the 1st line drug to treat pneumonia infections. However, tigecycline and imipenem may be recommended.

In *K.pneumonia* isolates, highest percentage of *bla*CTX-M (88.3%) and *bla*TEM (95.3%) were detected whereas low frequency of *bla*OXA gene was identified. These results were similar with previous reported studies in Pakistan, where high prevalence rate of β-lactamase resistance genes (*bla*CTX-M and *bla*TEM) 80-90% (Mahmoudi *et al.*, 2017; Farhadi *et al*., 2021). While study reported in Africa and Tunisia, the percentage of these resistance genes was low, due to possible preventive measures adopted by different countries for spread of antibacterial resistance (Alibi *et al*., 2015). However, in other gram negative isolates, *bla*OXA gene was detected in *P. aeruginosa*, *E. coli* and *A. baumannii* (56%, 38.4% and 20%) respectively. Likewise, study reported in Iran, *bla* OXA resistant gene was present in *Acinetobacter baumannii* (Asadian *et al*., 2019). Similar findings were also reported in different studies of Iran and Netherland, showed carbapenem resistance in *A.baumannii* from ventilator associated pneumonia (VAN) harbored OXA resistance genes (Mohammadi *et al*., 2017; Krzysciak *et al*., 2017). Results of the study indicated that *bla*CTX-M and *bla*TEM resistance is a foremost health issue in developing countries because of their variant has been contributed to the transfer of genetic elements and give clonal expression of bacterial resistance microorganisms. Moreover, extensive utilization of self-administered antibiotics has contributed the development of resistance genes (Manyahi *et al*., 2017).

In our study, 2nd most life threatening and challenging bacterial pathogen that cause pneumonia in younger children is *Streptococcus pneumoniae* that showed antimicrobial

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resistance to trimethoprim-sulfamethoxazole (100%), erythromycin (67.8%) and oxacillin (60.7%) whereas highest sensitivity to teicoplanin and vancomycin was observed. Similar studies were also reported in Ehiopia, Europe and Tanzania (Temesgen *et al*., 2019; Manyahi, *et al*., 2023). Currently, the erythromycin and azithromycin are used as 1st and 2nd line of treatment to prevent pneumonia in different countries (Manyahi, *et al*., 2023; Kulkarni *et al*., 2023). In Asian countries, like India and Pakistan, macrolide resistant genes *erm* (B) and *mef* (A) are the most dominant genes in *Streptococcus pneumonia.* Our study showed that the highest resistance of *mef* (A) 96.4% as compared to *erm*(B) 78% in Pakistani population. Macrolide resistance was also reported in previous studies in Turkey, South Korea, China and South Africa whereas in Japan, Saudi Arabia, Germany, Italy and England, the resistance rate was less due to variability and geographical distribution of resistance genes (Cherazard *et al*., 2017; Talebi *et al*., 2016). Hence, emergence of macrolide resistance is increasing over the past decades in *S. pneumonia* due to over consumption of antibiotics in developing countries. Therefore, effective vaccine recommendation can mitigate the problem of pneumococcal infections. The national immunization program (NIP) was administered the pneumococcal vaccine to overcome the huge burden of pneumonia and to lessen the prevalence of *S. pneumoniae* serotypes that showed MDR to antibiotics and to prevent the respiratory illness along with pneumonia. PCV is 80 % effective in preventing the invasive pneumococcal illness; children who immunized have 27% lower risk of developing pneumonia but recently PPSV23 was most effective *S. pneumoniae* vaccine that have 40% lower risk of pneumonia than non-vaccinated individuals (Cafiero-Fonseca *et al*., 2017).

CONCLUSION

The conclusion of this study that in Pakistani population prevalence of pneumonia in children is high due to *Streptococcus pneumoniae* and in older adults owing to *Klebsiella pneumoniae* due to unawareness and irrational use of antibiotics. Also, emergence of resistance in bacterial strains causing pneumonia due to β-lactamase and penicillin spread empirically. Therefore, macrolide antibiotics are better option to prevent pneumonia. In future, there is a need for awareness compaigns and vaccine recommendations in developing countries to control this pandemic disease.

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Author Contributions

Dr. Rasheeda Bashir design the origianl concept of study, supervise and review the article. Iram waqar do collection of data, performed all experimental work, analyzed and wrote original manuscript. All authors review, revised and approved the manuscript for publication.

Statement of conflict of interest

The authors affirm they have no known competing financial interests and personal relationship in this paper.

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Data availability

All the clinical data collected and analyzed during the execuation of this study are included in the current research article.

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