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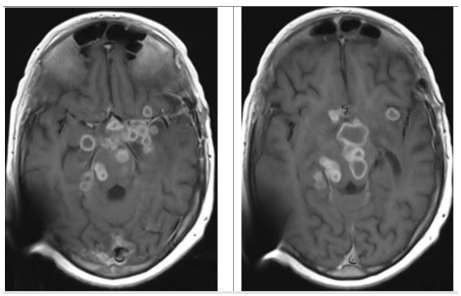

14 year/M, with headache and low GCS. Having a family history of treated pulmonary tuberculosis.
MRI brain showed multiple conglomerate and discrete abnormal signal intensity lesions in the supratentorial and infratentorial locations, predominantly involving the brainstem, hypothalamus, cerebral peduncles, cisterns, left frontal and bilateral temporal lobes. Findings are keeping with multiple tuberculomas.

Courtesy : Ali Faisal Saleem, Associate professor, Paediatric Infectious Diseases, Aga Khan University, Karachi, Pakistan

Typhoid Fever – Will we able to control this in Pakistan?

Salmonella typhi and *Salmonella paratyphi* causes typhoid fever and this is endemic in Pakistan. Every year there is consistency in cases reported across Pakistan. More than 20,000 cases were reported during 2017-2019 period.[1] Disease is associated with increasing morbidity and mortality. It is transmitted through oral-fecal route. Hand hygiene, water sanitation and good control on sewage practices can prevent its transmission from person to person. The disease fever lasts a little long compared to other pathogenic bacterial illnesses and mainly affects gastrointestinal and hepatic system of the body affecting appetite and linear growth of the affected person even post-cure. Cephalosporins were the treatment of choice in the past, however in last two years there is an increasing resistance reported across Pakistan. A new classification of non-resistant typhoid fever, multi-drug resistant (MDR) typhoid fever and extensive drug resistant (XDR) typhoid fever was established in 2018. XDR typhoid fever is sensitive against azithromycin and carbapenems; and currently are drug of choice in MDR typhoid.[2]

The presence of XDR Salmonella typhi in Pakistan is mainly due to poor WASH (water, sanitation and hygiene) practices across the country. Despite the endemicity there wasn't any approved official typhoid vaccination in EPI Pakistan till 2018. After the outbreak a conjugate typhoid vaccine (TCV) is licensed and given across the high-risk areas of the Sindh and Pakistan. Pakistan is the first country to introduce TCV in routine immunization program in November 2019.[3] The TCV was found to be effective and safe. There weren't any adverse events following immunization (AEFI) during vaccination campaign for children aged 6 months to 10-year-old in Hyderabad.[4] The potential strategies to control this disease in Pakistan is high level political commitment on water sanitation and sewage control. The Water and Sewage authority (SAWA) must put all the efforts to work on old line and seepage from lines, ensure the vacuum pumps are functional and there is no contamination of drinking water supply with sewage water. The commitment is crucial and require a lot of sustainability plan. There is also a need on control and check on drinking water supply across the country. The cane water is supplied across the cities without a government permit and audit. This may prevent a future outbreak. Community awareness sessions and educational activities on the outbreak and prevention strategies in continuous basis are needed. These require intervention and education particularly targeted around hygiene, water sanitation, boiling of drinking water, a complete and thorough washing of raw vegetables and fruits, and the risks of eating out from street vendors. Local/district and provincial stakeholders can play an integral and pivotal role in the execution. A public-private partnership is also essential to help a continuous engagement of all team members.

There has to a periodic check and bacterial culture or running polymerase chain testing particularly of water transmission pathogen on the clean line water supply. All drinking water should be check for its drinkability and should have chlorine as per Pakistan law.

As, the TCV is now the part of routine EPI. The availability and accessibility is not a major question but all children should receive it. Our routine immunization is lowest among our neighboring countries and in Eastern Mediterranean region. There are provincial, gender, urban and rural disparities. This need urgent actions.

In conclusion, TCV availability in Pakistan is a major success of public-private partnership in pediatric health care. Vaccine is one component in control. Other major component including WASH and water and sewage treatment need urgent and comprehensive monitoring and planning.

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ORIGINAL ARTICLE

Concordance Between Phenotypic Resistance to Fluoroquinolones and *gyrA* Mutations among Rifampin-Resistant Isolates of *Mycobacterium Tuberculosis* Complex from Pakistan

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Abstract

Background
Fluoroquinolones (FQ) are the cornerstone of treatment for Rifampicin Resistant (RR TB). Here we investigate whether FQ resistance detected by line probe assay (LPA) shows good concordance with phenotypic susceptibility testing.

Methods
Mycobacterium tuberculosis isolates were collected from clinical samples received in clinical Microbiology Laboratory, Aga Khan University, Karachi between January 2016 and February 2017. RR TB isolates were grown in culture and those who were resistant to ofloxacin (OFX) were selected for the present study. Minimum inhibitory concentrations (MICs) were performed for levofloxacin (LEV), moxifloxacin (MXF) and OFX using pre-prepared frozen plates (Thermo Fisher Scientific Inc., 194 Waltham, MA, USA). DNA was extracted using Genolyse® extraction kit (HAIN life sciences, Germany). For the detection of mutations, line probe assay Genotype MTBDRsl version 2 (HAIN Lifesciences, Germany) was used.

Results
From the total of 51 MDR TB stains that were included, majority of patients (n=29, 56.9%) were from Punjab province, 35.3% from Sindh province (n=18), and 5.9 %(n=3), and 1.9% (n=1) from Khyber Pakhtunkhwa and Baluchistan respectively. Concordance between genotypic resistance detection by LPA, and the phenotypic resistance detection by MICs to FQ (any one of OFX, LEV, or MXF) was observed in 84.3% of the isolates (n=43). The most common mutation identified was D94G in the *gyrA* gene in 50.9% of isolates (n=26). No *gyrB* mutations were detected. MIC testing showed high level of cross resistance between LEV/OFX and MFX MICS, with only 11/51 (21.6%) LEV/OFX resistant strains demonstrating MFX MICS of <1 µg/ml.

Conclusion
LPA method is a rapid and reliable method to identify resistance to FQ in MTB. However, for determination of susceptibility to individual FQs, further testing should be performed via phenotypic methods for confirmation.

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Keywords
Rifampin resistant, *Mycobacterium tuberculosis*, Line probe assay, gyrA, Fluoroquinolones

Introduction
Tuberculosis, caused by *Mycobacterium tuberculosis* complex (MTB), is a significant cause of morbidity and mortality throughout world. As of the year 2018, it is estimated that 10 million people got infected with the infection and from them 1.5 million people died[1] Mortality rates are higher for rifampin resistant tuberculosis (RR TB)[2]. In Pakistan, the incidence of RR TB in 2018 was 13 cases per 100 000 population[1], resulting in a high expected mortality burden. Fluoroquinolones (FQ) remain the main treatment option for RR TB.[3] Detection of fluoroquinolone resistance through rapid tests such as line probe assay (LPA) is valuable to direct therapy in such patients, and to identify genetic mutations which confer resistance to all antituberculous fluoroquinolones.

DNA gyrase subunit A and B changes in the fluoroquinolone resistance determining gene (QRDR) brings about resistance. Studies have shown multiple types of changes in the *gyrA* and *gyrB* mutation are correlated to the fluoroquinolone resistance.[4,5] However, the concordance of genetic drug resistance markers with the minimum inhibitory concentrations of FQ among clinical isolates from Pakistan has not been widely studied. Minimum inhibitory concentrations (MICs) of levofloxacin/ofloxacin (LEV/OFX) and moxifloxacin (MFX) are necessary to optimize therapeutic regimens used for RR TB.[3] Critical concentrations for these drugs by MGIT may also be used or even genotypic methods. Here, we have correlated the FQ MIC of LEV/OFX and MFX with *gyrA* and *gyrB* mutations in randomly selected RR MTB strains from Pakistan.

Materials & Method
Clinical strains of MTB isolated from samples received at the Clinical Microbiology Laboratory of the Aga Khan University in Karachi were included in the study. Culture was performed on pulmonary and extra pulmonary specimens with methods

described previously.[6] Briefly, culture was set up using *Mycobacterial* Growth Indicator Tube (MGIT) and 7H10 (Middlebrook) agar after digestion-decontamination 5 %N-acetyl-L-cysteine (NALC)/ (Sodium hydroxide) NaOH high speed centrifugation.

The susceptibility testing was carried out using the agar proportion method, as described previously.[6] Rifampin and ofloxacin resistance was determined using final concentrations of 1 µg/ml of rifampin and 2 µg/ml of ofloxacin in 7H10 agar, respectively. n=51 randomly selected RR and OFX resistant isolates form the year 2016-2017 were included in the study.

MIC testing for fluoroquinolones: MIC testing was performed for ofloxacin, levofloxacin, and moxifloxacin at concentration ranges of 0.12-8, 0.12-4, and 0.06-4 µg/ml using frozen and dried plates for broth microdilution in 7H9 medium (Thermo Fisher Scientific Inc., 194 Waltham, MA, USA) as part of the Bedaquiline DREAM Bedaquiline Drug Resistance Emergence Assessment in MDR-TB (Bedaquiline DREAM Program) project, as recently described by Kone *et al*.[7] FQ MICs were categorized as susceptible or resistant according to drug susceptibility criteria proposed by the Clinical Laboratory Standards Institute M62.[8] FQ MICs were categorized as susceptible or resistant according to drug susceptibility criteria proposed by the Clinical Laboratory Standards Institute M62 for commercial shorter incubation period liquid media systems[8] The critical concentration for LVX, MXF are 1.5 µg/mL and 0.25 µg/mL respectively. Ofloxacin testing is not recommended as it is not used for MDR TB, and specific fluoroquinolone has to be tested. But during the transition period, the recommended critical concentration in liquid system is 2 µg/mL.

Line Probe Assay: DNA was extracted from bacterial growth from isolated colonies that were inoculated into MGIT. The purity of isolates were ensured as these were initially collected from purity plate of agar sensitivity reporting. The DNA extraction was performed using GenoLyse®kit (Hain Lifesciences, Germany) as per manufacturer's instructions (https://www.hain-lifescience.de/en/products/dna-isolation/genolyse.html). The wild type probes used were wild type 1(codon 88), wild type 2(codon 90, 91) and wild type 3(codon 94).

Line Probe Assay was performed using Genotype MTBDR sl 96 version 2(Hain life sciences, Germany)[9] according to manufacturer kit instructions.

Quality control: Susceptible H37Rv MTB strain was used as quality control for all procedures, including agar proportion, broth microdilution MIC testing, and LPA.

Ethical review: The study approved by the ethical review committee of Aga Khan University, Karachi (no: 2019-2178-7005), and exempted from patient informed consent. All isolates used were delinked from patient identifiers and used.

Results

Description of isolates: Of the 51 RR MTB isolates, a total of n= 29 were isolated from samples received from Punjab province, n=18 from Sindh province, n=3 from Khyber Pakhtunkhwa, and n=1 from Balochistan province.

The age range from which samples were collected include patients from 6 years to a maximum age of 75 years of age. The median age range was of 30 years (interquartile range of 23.5- 44). There were a total of 4 extra pulmonary (3 pus and 1 cervical lump) and remaining were from pulmonary sites (n=41 sputum and n=6 bronchial wash). The male-female ratio was 30:21.

Correlation of FQ phenotypic resistance by MIC with genotypic resistance by LPA:

All isolates tested by MIC were resistant to at least 1 FQ tested. Concordance between genotypic resistance detection by LPA, and phenotypic resistance detection by MICs to FQ (any one of OFX, LEV, or MXF) was observed in 84.3% isolates (n=43). Of the 51 isolates, n=48/51 were resistant to LEV and n=49 were resistant to MXF. The OFX MIC=1 was seen in 3 isolates, OFX MIC 2-4 in n=4, OFX MIC =8 in n=44.

No mutations were detected in 8 (15.7%) isolates. Based on MIC results, the concordance between genotypic resistance detection by LPA and OFX resistance at 2 µg/mL was 87.5% (42 of 48 isolates), while for LEV at ≥1 µg/mL concordance was 89.6% (43 of 48 isolates), and for MXF at ≥ 1 µg/mL concordance was 86.7% (39 of 45 isolates).

In 4 isolates (7.8%), genotypic resistance was detected only on the basis of the absence of wild type probe binding, while specific mutations were identified in 39 isolates (76.5%). These specific mutations corresponded to the detected mutation in 33 (84.6%) isolates, while in 6 (15.4%) isolates the results were considered to be due to possible heteroresistance in the MTB strain. Heteroresistance in MTB isolates is a well know phenomenon, defined as the coexistence of different subpopulations with varying genetic resistance mechanisms within one strain.[10]

Asp94Ala substitution (D94G) was the most frequently observed mutation among these isolates, detected in 26 of 51 isolates (51%).[11] Table 1 shows the results of line probe assay on 51 study isolates, as well as distribution of mutations detected, in relation to MICs of OFX, LEV, MXF.

High level of cross resistance between LEV/OFX and MFX MICS was observed, with only 11/51 (21.6%) LEV/OFX resistant strains demonstrating MFX MICS of <=1 µg/ml.

No specific mutation correlated with MXF MICs of <1 µg/mL;

Table 1: Distributions of mutations and fluoroquinolone MICs in 51 clinical isolates of rifampin-resistant Mycobacterium tuberculosis from Pakistan

Mutation	Aminoacid substitution at locus	Number of isolates MIC (µg/mL)											TOTAL
		OFX			LEV			MXF					
		≤1	2-4	≥8	≤1	2	≥4	0.25	0.5	1	2	≥4	
D94G	Asp94Gly	-	1	22	-	-	23	-	1	5	13	4	23
A90V	Ala90Val	-	2	4	-	-	6	-	2	2	2	-	6
D94A	Asp94Ala	-	-	1	-	-	1	-	-	1	-	-	1
D94N/Y	Asp94Asp/Tyr	-	-	4	-	-	4	-	-	-	1	3	4
S91P	Ser91Pro	-	-	1	-	1	-	-	-	-	1	-	1
D94H	Asp94His	-	-	1	-	-	1	-	-	-	-	1	1
D94G, A90V	Asp94Gly, Ala90Val	-	-	1	-	-	1	-	-	-	1	-	1
D94N/Y, D94G	Asp94Asp/Tyr, Asp94Gly	1	-	1	-	-	2	-	1	-	-	1	2
Absence of WT¶	-	-	-	4	-	-	4	-	-	-	4		4
WT Pattern	-	2	1	5	3	2	3	2	-	-	5	1	8
													51

¶Absence of Wild Type sequence only, no specific mutation detected

in 2 isolates no mutation was detected, while in 4 isolates, D94G (n=1), A90V (n=2) and both D94G with D94N/Y (n=1) were detected.

Discussion

As FQ are the antituberculous agents of choice in the treatment of rifampin resistant tuberculosis, rapid and reliable identification of resistance can further guide treatment regimens. We observed that in RR MTB clinical isolates testing resistant to any one fluoroquinolone, LPA detected resistance in a substantial proportion of isolates. This shows that LPA testing on samples will be able to identify FQ drug resistance and predict phenotypic FQ resistant results in majority of patients. However, susceptibility to individual FQs should be confirmed with phenotypic testing methods on isolates obtained in culture. This is because the line probe assay has been shown to give raise to false resistance if there were synonymous mutations present that could lead to the non- binding of the probes.[12, 13]

We identified D94G to be the most common detected mutation in our study. This mutation is also the most prevalent globally, and has also been reported with high frequency from genomic sequencing databases from Pakistan.[14] The D94G mutation is associated with a low cure rate and higher MICs.[10]

We also observed a high rate absent wild type with no mutations detected. This implies that isolates from Pakistan have additional specific mutations that are not identified by LPA. Genomic

analysis of the QRDR region on these isolates will identify these additional specific mutations.

Heteroresistance in MTB isolates is a well know phenomenon, defined as the coexistence of different subpopulations with varying genetic resistance mechanisms within one strain.[10] LPA is not the ideal method to detect heteroresistance. However, presence of wild type gene mutation along with detection of a mutation signifies the presence of variants which point to heteroresistance. Since we performed LPA on pure MTB isolates in culture, we concluded that the results are due to heteroresistance and not a mixed infection.

Newer generation FQ such as MXF have greater activity against RR MTB strains. The ability of LPA results to predict susceptibility to MFX is therefore of interest. We found a high proportion of FQ resistant isolates to be resistant to MFX(n=49/51, 86.7%), perhaps due to greater population use of MXF. The overall levofloxacin resistance has been shown to be higher among studies from multiple countries including Pakistan i.e. : 87% moxifloxacin (72%) from among 282 tested isolates.[15] Nevertheless, to determine whether a higher dose of MXF can be used in RR MTB infections, more studies are needed with pharmacokinetic and pharmacodynamic data to determine the optimal dosing in correlation with MIC cutoffs.

This study has several limitations. The sample size is small. Clinical isolates were cultured from samples received at a reference laboratory center and therefore likely to have a higher rate of resistance. Isolates selected for the study were resistant to ofloxacin and therefore results do not apply to fluoroquinolone sensitive isolates. This study used the critical concentrations for MGIT for interpretation although this cannot be implemented accurately for clinical application. However, studies have attempted to establish clinical epidemiological cut offs for broth microdilution methods.[16] We also did not perform genome sequencing of isolates to confirm the resistance detected only in the basis of absence of wild type *gyrA*.

Conclusion

This study shows that genotypic testing by line probe assay for concordance between *gyrA* and fluoroquinolone is reliable and can be used to guide therapy in patients with MDR TB. It shows 84.3% concordance with the MICs. A high level of cross resistance between LEV/OFX and MXF MICs was also noted in this study.

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ORIGINAL ARTICLE

Frequency of Chikungunya, Dengue and Zika Viruses in Acute Non-localizing Febrile Illness via RT-PCR

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Abstract

Introduction
Chikungunya and zika are two new emerging threats across the globe. Like dengue, these two viruses also present with acute non-localizing fever and rash, and their route of transmission is the Aedes aegypti mosquito. The hallmark of chikungunya is arthralgia/arthritis which can become chronic, while zika has *in utero* transmission, resulting in congenital anomalies (microcephaly), low birth weight or still births. It is difficult to differentiate these infections on clinical grounds and must be confirmed by PCR or serology. Due to cross reactivity of ELISA serology among flaviviruses, PCR is the gold standard. Pakistan is endemic for dengue, and due to presence of the same vector, chikungunya outbreak was suspected after India's epidemic in 2016. The epidemic in Pakistan was declared in November 2016 in the metropolitan city of Karachi. We conducted this study to know the prevalence of chikungunya, presence and prevalence of zika, and to assess the associated clinical and laboratory parameters.

Methodology
A total number of 183 participants fulfilling the case definition of dengue (Dengue Expert Advisory Group), chikungunya and zika (World Health Organization) were included. Patients with other viral exanthematous illness like chicken pox, measles or an obvious source of infection were excluded. The patients were tested for dengue, chikungunya and zika via QiaAmp Viral RNA Mini kit (Qiagen Inc.), dengue IgM serology via Elisa.

Results
51% were male. Overall frequency of arboviruses was 100 (54.6%), 95 (52%) were PCR positive for one or two arboviruses; serology for DENV was positive in 5 (2.7%). The over all frequency of CHIKV and DENV were 91(49.7%) and 13(7%) respectively. CHIKV/DENV co-infection was found in 4 (2.2%). Zika virus was not isolated in any sample. In CHIKV patients, triad of fever, arthralgia/arthritis and rash was found in 45.1%, joint involvement was predominantly symmetrical and polyarticular. Hemoglobin, hematocrit, TLC, platelets and ALT were normal in majority. No mortality related to CHIKV was noted.

Conclusion
Chikungunya is a newly emergent arboviral illness in this territory. The disease is self limiting with negligible mortality and high morbidity due to joint involvement. Pakistan is a high risk area for zika virus as well. Vector control and improvement in sanitation is vital for avoiding future epidemics.

Keywords
Chikungunya, Dengue, Zika, RT- PCR, Fever, Rash, Triad, Arthritis, Co-infection, mosquito –borne infections

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Introduction

Globalization has led to rapid travel and with it, a parallel increase in spread of diseases in areas which once were considered "disease free". Rapidly emerging arboviral infection, particularly chikungunya (CHIKV) and zika (ZIKV), are examples of mosquito –borne infections resulting in outbreaks across the globe. Dengue (DENV), CHIKV, and ZIKV are arthropod transmitted viruses, classified as flaviviruses (DENV and ZIKV) and alphavirus (CHIKV), sharing certain common features such as vector and clinical presentation.

DENV is a worldwide growing problem. Bhatt *et al* estimate 390 million DENV infections per year, of which 96 million (average 67–136 million) manifesting clinically with varying degree of disease severity[1], and the number of cases doubling every decade, from 8·3 million in 1990, to 58·4 million in 2013[2]. Pakistan is hyperendemic for DENV, Lahore being the epicenter. The country experienced its first outbreak in 1994 and the largest epidemic in 2011[3] resulted in 20,000 cases and 300 deaths. Out of four DENV serotypes, 2 and 3 are most prevalent in Pakistan.[4]

CHIKV is another rapidly spreading arboviral infection. The causative virus is a positive sense, single stranded RNA member of genus Alphaviridae, family Togaviridae[5], first described during an outbreak in a Swahili village in the Newala district of Tanzania, Africa in 1953. Phylogenetic analysis on partial sequences of NS4 and E1 genes reveals 3 distinct groups: the West African, the East-Central-South African (ECSA), and the Asian.[6,7] Asia experienced its first epidemic in 1954 in Bangkok,

Thailand, and continued till 1964. The presence of the virus in Pakistan dates back to 1980s when Darwish *et al* confirmed the presence of antibodies to CHIKV and ZIKV along with other arboviruses in rodents, domestic animals and humans[8] and in 2011, antibodies were detected by Afzal *et al* in a pediatric population.[9] Outbreaks continued across the globe: in 2016 India experienced a large outbreak that was followed by the current outbreak in Pakistan, starting from mid of September 2016, and confirmed by NIH-Islamabad in December 2016. According to WHO, Italy and France are facing the epidemic of 2017.[10,11]

ZIKV, another emerging threat for Pakistan, is the enveloped, single stranded RNA virus, described first in rhesus monkeys in Zika Forest, Uganda. Only a few outbreaks have been noted due to asymptomatic clinical course and close resemblances with other flaviviruses. WHO reported active circulation of zika virus in 38 countries in 2016.[12] The presence of ZIKV virus antibodies were confirmed in sera of 2.4% of all samples from rodents, domestic animals and humans.[8] Pakistan is at increased risk for ZIKV after confirmation of 3 cases in Ahmedabad, India in 2017.[13]

The common route of transmission in all three viral infections is the mosquito vector - Aedes aegypti and Albopictus. Some features of these infections are in common; however, there are specific features for each infection: arthritis and debilitating arthralgia are the hall mark for CHIKV; bone pain and hemorrhage in DENV fever; and neonatal microcephaly and other pregnancy -related complications with ZIKV. Signs and symptoms come in close differential of fever presenting with rash arthralgia/myalgia and thrombocytopenia, making it difficult to distinguish on clinical grounds alone. Antibodies against these viruses have shown cross reactivity with each other and the only gold standard for diagnosis of each is RT-PCR during acute febrile phase. Malaria is another close clinical differential, but is easily diagnosed on smear or rapid ICT malaria test.

We have observed that a significant number of patients presenting with acute febrile illness are negative for malaria or DENV. This observation of serologically negative cases for DENV/malaria, absence of serological evidence of ZIKV,[8] and confirmed existence of vector and recent outbreak of CHIKV and ZIKV in neighboring country India[13] led us to conduct the study to unmask the frequency of other two viruses. The purposes of this study were confirmation of the CHIKV and subsequent analysis of its clinical and laboratory parameters and early detection of ZIKV. Also, the study will be helpful in identifying and approaching the areas of major epidemics for effective vector control measures and public awareness sessions on prevention in future.

Materials and Methods

This was a descriptive, cross sectional study. Total 183 participants were included after non-probability, consecutive sampling. The study was conducted at The Indus Hospital, Karachi. Indus Hospital is 150 bed, charity based tertiary care hospital providing state of art facilities. Samples were collected from December 27, 2016 till June 12, 2017 from cohort fulfilling inclusion criteria. Patients were recruited from The Indus Hospital (Korangi), Star General Hospital and Al-Tibri Hospital (Malir), Patel Hospital (Gulshan-e-Iqbal), Ziauddin Hospital (North Nazimabad), and directly from laboratories in Malir. Majority of our participants belonged to Malir town, located in the eastern part of Karachi, Sindh, Pakistan, with a multi-ethnic population, bordered by the Jinnah International Airport and the Malir River. Patients of either gender, presenting to the Emergency Department or Out-patient clinics and referred from different hospitals with non-localizing acute febrile illness of less than 14 days duration were included. Patients with obvious source of fever, such as upper respiratory tract, urinary tract etc. or with rash suggestive of any other cause of viral fever e.g. varicella, measles, etc. were excluded.

Sample collection

After hospital institutional review board (IRB) approval and informed consent, information was noted on a performa. Pediatric population was evaluated by a pediatrician to exclude other causes of fever. Blood samples were collected and sent for complete blood picture, ALT, malarial parasite (thick and thin films with Giemsa stain), MP-ICT, dengue serology IgM (fever >5 days) and RT-PCR for dengue, chikungunya and zika (fever =5 days), and blood cultures where indicated. Viral RNA from serum samples was extracted through QiaAmp Viral RNA Mini kit (Qiagen Inc.) according to manufacturer's instructions. Extracted RNA was employed in real-time multiplex PCR (RT-PCR) mix containing probes and primers to detect dengue, chikungunya and zika viral RNA (RT-PCR kit that was kindly provided by Center for Disease Control, USA).

Operational Case Definition

Standard definitions for suspected, probable and confirmed cases have been used for DENV[14], CHIKV[15] and ZIKV[16] fever.

DENV[14]:

Confirmed Case: Detection of viral ribonucleic acid (RNA) by PCR or IgM antibodies in serum.

CHIKV[15]:

Confirmed case: Presence of viral ribonucleic acid (RNA) in acute-phase sera as determined with RT-PCR.

ZIKV[16]:

Confirmed case: Presence of viral ribonucleic acid (RNA) in serum determined with RT-PCR

Data Analysis

Data was entered and analyzed using SPSS version 24. Mean ± SD or median (IQR) was computed as appropriate for all the quantitative variables. Frequencies and percentages were calculated for all the categorical data. Pearson chi square and Fisher exact test were applied to assess significant association of various categorical variables with chikungunya status.

Furthermore, Independent sample T-test and Mann Whitney-U test were applied as appropriate to assess significant differences in quantitative variables between chikungunya status. P-value<0.05 was considered statistically significant.

Results

Out of 183 samples collected, 140 (76.5 %) were from Indus hospital and 43 (23.5%) from different hospitals in Karachi and laboratories from Malir. The overall frequency of arboviruses was 100 (54.6%). 95 (52%) were PCR positive for one or two arboviruses; serology for DENV was positive in 5 (2.7%). The frequency of CHIKV and DENV were 91 (49.7%) and 13 (7%) respectively (Figures 1& 2).

Male to female ratio was almost 1:1, while age ranged between 2-81 years with a mean of 31.7 ± 14.6 years with most of the patients falling between 14-44 years. 40 patients had associated comorbids, while hypertension and diabetes mellitus were most frequent, as it is in the general population. One patient was hemophiliac with HIV/HCV, co infection. 10 (13%) females were pregnant. None of the participants was on immunosuppressive agents.

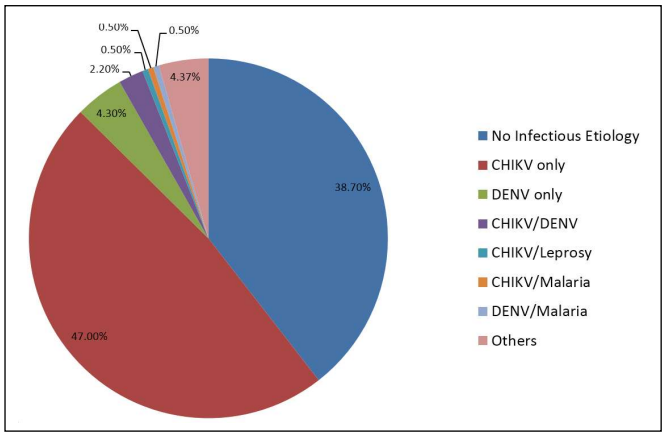

Figure 1: Infectious etiology among participants

CHIKV= Chkiungunya virus, DENV= Dengue virus, ZIKV= Zika Virus, PCR= Polymerase Chain Reaction, /=Coinfection

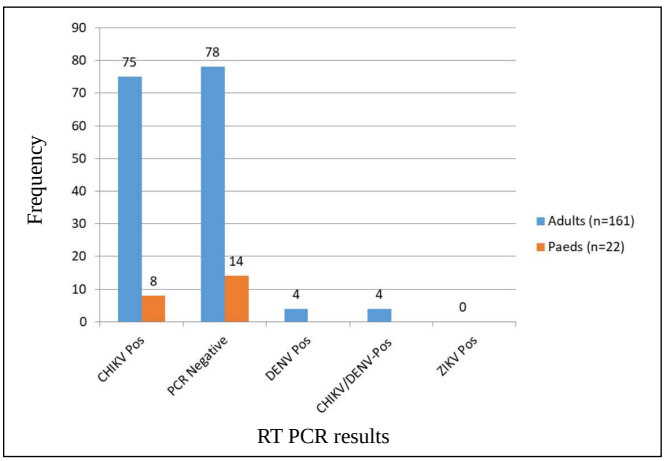

Figure 2: Age Group Based Frequency of Arboviruses via RT-PCR Only.

CHIKV= Chkiungunya virus, DENV= Dengue virus, ZIKV= Zika Virus, PCR= Polymerase Chain Reaction, Pos=Positive

CHIKV was found to be the predominant virus, showing two peaks (figure 3). The degree of maximum temperature noted was higher in PCR positive patients (p=0.028) than in PCR negative. The over all duration of fever ranged 1-90 days (6.4 ± 11.6). Percentages for symptoms were calculated (See Table 1). Only 4 patients were hypotensive, 7 had relative bradycardia but this was not a consistent feature.

Joints involved were predominantly symmetrical and multiple (Table 2). Though not statistically significant, joint pain lasted longer (12 months v/s 6 months) in PCR positive patients.

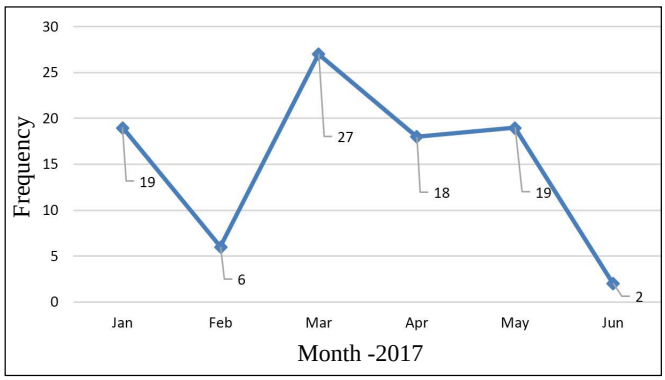

Figure 3: Month wise distribution of CHIKV cases

Table 1: Descriptive Analysis and Comparison of Frequency of Symptoms in CHIKV Positive and Negative Cases (n=160)

Symptom	Total	Positive	Negative	P- Value
Vomiting	48(30)	25(30.1)	23(29.9)	
Rash	78(48.8)	43(51.8)	35(45.5)	
Joint pain	138(86.25)	78(94)[b]	60(77.9)	
Minor bleed (e,g gum bleed, epistaxis)	5(3.1)	1(1.2)	4(5.2)	
Major bleeding (e.g hemetemsis, malena)	2(1.3)	1(1.2)	1(1.3)	
Rigors	38(23.8)	26(31.3)[b]	12(15.6)	
Shivering	50(31.3)	22(26.5)	28(36.4)	0.003[*t I]
Chills	76(47.5)	47(56.6)[b]	29(37.7)	
Muscle pain	70(43.8)	38(45.8)	32(41.6)	
Headache	75(46.9)	39(47)	36(46.8)	
Nausea	46(28.8)	31(37.3)[b]	15(19.5)	
Diarrhea	14(8.8)	6(7.2)	8(10.4)	
Itching	11(6.9)	7(8.4)	4(5.2)	
Abdominal pain	6(3.8)	3(3.6)	3(3.9)	
Redness of eye	5(3.1)	2(2.4)	4(5.2)	
Cough	10(6.3)	2(2.4)	8(10.4)[a]	
Other	42(26.3)	21(25.3)	21(27.3)	

*P-value<0.05, I Pearson Chi Square test, -For significant pair, the key of the category (a=positive, b=negative) appears in the superscript

Table 2: Comparison of Joint Manifestation in CHIKV Positive and Negative Patients

Characteristic	Positive n(%)	Negative n(%)	Total n(%)	P-Value
Arthralgia (n=160)	**78(94)[b]**	**60(77.9)**	**138(86.25)**	**0.003*[I]**
Arthritis (n=139)	**50(60.9)**	**32(39)**	**82 (58.99)**	
Restricted movements	27(54)	16(50)	43(42.4)	0.449 I
Joints involved				
Ankle	14(29.8)	15(50)	29(37.7)	
Wrist	22(46.8)	12(40)	34(44.2)	
Elbow	13(27.7)	6(20)	19(24.7)	
Knee	39(83)[b]	18(60)	57(74.0)	
Small joints of Feet (IP and MTl)	6(12.8)	3(10)	9(11.7)	0.209 [I]
Back	3(6.4)	2(6.7)	5(6.5)	
Small joints of Hand (IP and MCP)	17(36.2)	10(33.3)	27(35.1)	
Only Toes (MTP or IP only)	1(2.1)	2(6.7)	3(3.9)	
Hip	1(2.1)	1(3.3)	2(2.6)	
Shoulder	4(8.5)	0(0)	4(5.2)	
Fingers(IP only)	2(4.3)	0(0)	2(2.6)	
Type				
Large joint pain	27(60)	18(40)	45(100)	
Small joint pain	3(50)	3(50)	6(100)	0.771
Both large and small joints	18(64.3)	10(35.7)	28(100)	
Total	48(60.8)	31(39.2)	79(100)	
Symmetry				
All symmetrical	39(73.6)	19(63.3)	58(69.9)	
All asymmetrical	11(20.8)	8(26.7)	19(22.9)	0.518
Both symmetrical and asymmetrical	3(5.7)	3(10)	6(7.2)	
Total	53(100)	30(100)	83(100)	
Number of Joints Involved (arthralgia/arthirits)				
Mono (single joint)	5(9.4)	2(6.5)	7(8.3)	
Oligo (2-4)	16(30.2)	16(51.6)	32(38.1)	0.177
Poly (=5)	32(60.4)	13(41.9)	45(53.6)	
Total	53(100)	31(100)	84(100)	
Duration of joint pain lasted				
<1month	6(14.3)	2(6.7)	8(11.1)	
1-3months	26(61.9)	23(76.7)	49(68.1)	0.437
>3months	10(23.8)	5(16.7)	15(20.8)	
Total	42(100)	30(100)	72(100)	
Presence of Fever, joint pain and rash (n=183)				
Fever only	11(12.1)	29(31.5)	40(21.9)	
Rash + Fever	2(2.2)	3(3.3)	5(2.7)	0.010*
Joint pain + Fever	37(40.7)	28(30.4)	65(35.5)	
All three	41(45.1)	32(34.8)	73(39.9)	
Total	91(100)	92(100)	183(100)	

*P-value<0.05, **P-value<0.0001, I Pearson Chi Square test, Fisher's Exact test. IP Interphalangeal, MCP metacarpophalyngeal, MTP metatarsophalyngeal

35% cases with CHIKV had hematocrit >40. Mean hemoglobin, hematocrit, TLC, platelet count values were found to be 12.4 (± 2.3) v/s 12.6 (±2.4), 38.2 (±6.1) v/s 39.5 (±7.8), 6.9(±3.3) v/s 7.4(±4.2), 222.7(±86) v/s 233.6(±138.7) in PCR positive v/s negative patients (statistically insignificant). Only 8 patients manifested thrombocytopenia and anemia. SGPT was within normal range in CHIKV positive cases in all, except 4 patients. There was no statistically significant difference in terms of age, gender, visit to outbreak area, family history of similar illness and employment status between the two groups.

Two mortalities recorded were not due to CHIKV or related complications.

Discussion

Acute febrile illness is one of the most frequent reasons for patients seeking healthcare. The entity comprises of various infectious and noninfectious causes. Due to lack of diagnostic facilities, most cases are treated clinically, sometimes resulting in unnecessary antimicrobial administration despite high suspicion of viral illness.

The study confirms the presence of CHIKV in Pakistan. Frequency of chikungunya in our study (49.7%) is higher than that found by Kaur[17] (24.1%) and Marlen[18] (30%) who found higher rates of DENV and co-infections.

In the six months of sample collection, CHIKV outnumbered other viruses with two peaks (figure 3), in January (20.9%) and then in March-May 2017 (29.7%). The reason for fall in cases in February could be a climatic change affecting vector breeding. Frequency of DENV Virus (n=8) and DENV/CHIKV co-infections (n=4) were too low to show any seasonality. A study of longer duration is required to establish behavior of the virus. Our observation is entirely different from Marlen[18], who noted an even distribution of CHIKV throughout the period under review and two peaks of DENV. Comparing with DENV and malaria in Pakistan, malaria peaked significantly from May to October, while dengue cases occurred more frequently between September to December and declined afterwards.[19]

The demographic analysis between RNA PCR positive v/s negative groups was statistically insignificant in terms of gender (p=0.713), age (p=0.168), employment status (p=0.108) and history of mobilization outside or within Karachi in areas of ongoing epidemic (p=0.193). The frequency of involvement of other family members with CHIKV like illness was similar in both groups (78.6 v/s 76%) but number of household members involved in CHIKV +ve was higher (range 1-20 v/s 1-4). Anish[20] also found 377 households in which 71.4% (67.5-74.3%) had at least one member affected by chikungunya. Since we could not do CHIKV serology we may have missed this diagnosis in some patients.

An interesting observation was that out of the 72 CHIKV PCR +ve cases 18 had become afebrile at the time of sample collection, indicating that viremia may persist even after fever resolution. In contrast to this, afebrile patients were sero positive in one study.[21] We found positive CHIKV PCR after 14 days of fever (n=1), suggesting viremia may persist up to 2 weeks (biphasic nature of disease). In a study done in travellers returning from India, RT-PCR remained positive till day 10.[22] Temperature more than >40ºC was noted in one patient with positive PCR. Waggoner[23] and Lee[24] noted mean temperature of 37.4ºC (±0.9) and 39ºC (T -max) with CHIKV respectively.

CHIKV is known for its debilitating course due to joint involvement which may persist for>3 months, few may require steroids or disease modifying therapy. There is disagreement between Chang *et al*[25] and Brad *et al*[26] regarding persistence of virus in synovial membrane as the cause of chronic joint involvement, but both have consensus on autoimmunity and exacerbation of preexisting joint disease. In our study, the shortest duration of joint involvement was 3 days, and maximum up to 12 months in CHIKV positive patients. The finding is likely due to earlier (within 1 week of fever onset) presentation in CHIKV positive cohort, increasing the chances of PCR positivity. Symptoms persisted for > 3 months in only 10 PCR positive patients. Stratification done on the basis of symmetry, number of joints, presence of synovitis and type of joints involvement (Table 2) is comparable with international data.[25,27] Kaur *et al*[17] found more frequent joint restriction (97%). The presence of inflammatory arthritis in CHIKV chronic arthritis can be confused with other inflammatory arthritides. Further studies are required to establish differentiating points.

Like Cunha[28], we also found blanchable erythema/macular or morbiliform rash as a common presentation, generalized in most cases and pruritic in a few cases. The rash was found in 43 out of 78 (51%) CHIKV positive cases which is higher as compared to Kaur *et al*[17] (30%). One patient developed rash after 6 days of fever. Petechiae were seen in CHIKV PCR negative group (11 v/s 125). Unlike Cunha[28], we did not notice other skin manifestations (e.g. photosensitivity, erythema nodosum, vesicles, blisters hyperpigmentation etc.).

Altered level of consciousness was present in 3 patients: only one had CHIKV PCR positive in blood. Unlike Kaur *et al*[17] who confirmed CHIKV encephalitis via positive CHIKV PCR in cerebrospinal fluid (CSF), we did not check CHIKV PCR in CSF of these patients. None of our patients developed neurological sequelae. We did not find visceromegaly or lymphadenopathy specifically associated with CHIKV.

In contrast to Kaur[17] and Torres[29] who correlate hypotension with complicated cases of CHIKV, hypotension (Systolic BP<90, diastolic BP<60) was seen in only four patients complicated by other severe infections or co morbidities: an HIV/HCV +ve, hemophilia with XDR Klebsiella bacteremia, a pregnant female in her third trimester and CHIKV with complicated malaria.

46(73%) PCR positive cases had total leukocyte count within normal limits with leftward shift (neutrophilia>monocytosis) but leukopenia (n=9) and leukocytosis (n=8) were also found. The finding has also been noted in other studies.[24,29] Leukocytosis or leukopenia in the face of high fever and tachycardia mimicked sepsis, and despite negative blood cultures, led to injudicious use of antibiotics.

There were only 12 cases of DENV, too small a number to compare with CHIKV positive patients. DENV presenting as abdominal pain, ascites, pleural effusion, visceromegaly, neurological involvement, but no joint manifestations in our as well as international studies [30,31]. Another study found similar hematological and biochemical parameters.[24]

8 out of 10 pregnant females (80%) were PCR positive for CHIKV only, 1 had CHIKV/DENV co-infection and 1 was negative for any arbovirus. 50% were in their third trimester. In contrast to Kaur,[17] no intrauterine death or adverse pregnancy outcomes were subsequently noted. Follow up of neonates for manifestation was out of scope of this study.

Majority of our patients responded well to initial non-steroidal anti-inflammatory drugs (NSAIDs) and hydration in case of CHIKV and hydration and acetaminophen in DENV infections.

There were no cases of ZIKV but we are at high alert for this emerging disease after the 2018 outbreak of the virus in India. CDC categories Pakistan as a risk area for ZIKV. Serology has also been reported positive in Pakistan by Darwesh *et al* in rodents.[8] In 71(38.7%) cases, we did not find any alternate diagnosis. This could be explained either by lack of availability of serological test for CHIKV; or infections with other viruses known for causing fever with exanthems (adenovirus, rubella, rubeola, coxsackie, parvovirus B19) leading a new path for research.

The transmission of viruses can be halted with adequate vector control, improved sanitation and personal protective measures e.g. mosquito repellants, nets etc. Public awareness sessions should be arranged in areas of epidemic. CHIKV related debilitating arthritis has a financial impact on daily wage earners due to absenteeism.

To the author's best knowledge, no other study has been published so far from Pakistan with descriptive analysis of multiple variables and spectrum of disease. Previously published studies were either done with serology[9] or did not consider comparative analysis.[32] This study can be a stepping stone for future studies from the region.

Study Limitations

The duration of study was short to assess seasonality. The sample size was small and thus head to head comparison of DENV and CHIKV manifestations was not possible. Samples collected directly from labs lacked uniform clinical data. We were unable to find cross reactivity among viruses due to unavailability of serology kits for CHIKV and ZIKV, and were unable to identify serology positive cases in late cases.

Conclusion

Chikungunya is a newly emergent problem in Pakistan, while we are at high risk for zika virus as well. CHIKV can be co-infected with other viruses or malaria, and may mimic more severe illness such as sepsis and rheumatoid arthritis. Laboratory tests are usually nonspecific in CHIKV. Non-infectious diseases can co-exist with CHIKV. The hall mark triad of fever, joint pains and skin rash in CHIKV was significantly higher, and if present, can help in clinically differentiating from other illnesses. CHIKV is a self-limiting, though temporarily disabling disease, and does not require specific therapy, while supportive care for fever and pain control are sufficient. There is a great need to improve diagnostic tests, and most importantly, conditions for prevention of mosquito breeding need urgent attention.

Future Directions

In future, studies can be done on arthritis and its correlation with other parameters.

Acknowledgements

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Abbreviations

CHIKV Chikungunya Virus
DENV Dengue Virus
RA Rheumatoid Arthritis
RT-PCR Reverse Transcriptase Polymerase Chain Reaction
TLC Total Leucocyte count
ZIKV Zika Virus

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ORIGINAL ARTICLE

Isolation of Extensively Drug Resistant S*almonella Typhi* in Blood Culture from Tertiary Care University Hospital

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Abstract

Background
Typhoid fever is a serious systemic infection which affects all age group from children to elderly. It has multiple systemic effects as untreated infections may cause prolonged morbidity and hospitalization. The treatment modalities range multiple antibiotics like co- trimoxazole, ciprofloxacin, chloramphenicol and ampicillin. The treatment of choice in multidrug resistant *Salmonella* has been third generation cephalosporins like Ceftriaxone.

Methodology
The total number of Blood culture received was 464, collected from January to December in year 2018 at Jinnah Sindh Medical University laboratory. The sample was received in BACTEC blood culture bottle. When a culture bottle signaled positive, the bottle was removed and an aliquot of the broth was Gram stain and processed for culture for organism identification and antimicrobial susceptibility testing

Results
During 2018 from January till December 464 blood cultures came to JSMU Diagnostic laboratory and blood bank out of which 31 cases of enteric fever were diagnosed in automated blood culture Bactec. All the isolated *Salmonella* belonged to *Salmonella typhi* subgroup and there was no case of *salmonella paratyphi* A or B. out of 31, 25 *Salmonella* were resistant to Ceftriaxone sensitive only to azithromycin and Meropenem. All the 31 positive *Salmonella* cases were from patient of pediatric age group with range of 11 months to 14 years.

Conclusion
The rise of extensively drug resistant *Salmonella* is alarming and leaves limited treatment options with carbapenems which are costly as well as require administration by injectable route. Preventive measures like improved sanitation and vaccination may be adapted in order to curtail spread of such resistant strains of typhoid.

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Key Words
XDR**,** *Salmonella typhi*, extremely drug resistant enteric fever

Background
Typhoid fever caused by *Salmonella typhi* and *paratyphi* is one of the several diseases causing disease burden of developing countries. In 2000, over 2 million people were diagnosed to have this preventable infection worldwide. In 2004, typhoid fever caused over 200,000 deaths globally, of which majority of cases occurred in Asia.[1] Despite advancement in this modern era, recent statistics shows that typhoid fever is very common in developing countries accounting for an estimated 120 million infections and 700,000 annual deaths occurring worldwide.[2] Enhanced water quality and proper sanitation constitute cornerstone solutions to this preventable infectious disease, vaccination in high-risk areas is a possible control strategy recommended by the World Health Organization (WHO) for the short-to medium-term management of high risk group like children.[3]

Typhoid has been historically treated by antibiotics like ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol; hence these are termed as first line treatment antibiotics.[4] Those strains of *Salmonella typhi*, which show resistance to these three antibiotics, are considered multidrug resistant (MDR), and resistant isolates were first observed in the late 1970s to early 1980s. These resistant strains were treated with fluoroquinolones (ciprofloxacin, ofloxacin) which constitute the second line treatment. Non-susceptibility to the second-line antibiotics (the fluoroquinolones) or fluoroquinolones resistance has also been frequently reported since these became the treatment of choice in regions with MDR infections. Ceftriaxone, a third-generation injectable cephalosporin, and azithromycin, a macrolide, are employed as the treatment for typhoid fever when other options cannot be used as typhoid strains show resistance to first and second line antibiotics.[4] However, periodic cases of ceftriaxone- or azithromycin resistant typhoidal *salmonella* infection have recently been reported. Over the last twenty years, a dominant, commonly MDR, haplotype of *S. Typhi* called H58 has been scattering all over the world.[5] It is rampant across South and Southeast Asia. Several local outbreaks of typhoid have been linked to various sublineages of H58.[6-8]

Fluoroquinolones like ciprofloxacin are considered treatment

of choice in treating typhoid fever caused by multidrug resistant strains of *Salmonella typhi* but with increasing antibiotic resistance there are numerous reports of treatment failures with fluoroquinolones.[9] Multidrug resistance (MDR), defined as resistance to the three first-line classes of antimicrobial agents (chloramphenicol, ampicillin, and trimethoprim/ sulfamethoxazole [TMP/SMX]), has become prevalent in most of South Asia, with figures reaching 15% in India and 45% in Pakistan.[10] There has been previous research in Pakistan demonstrating increase in resistance against Ciprofloxacin. The trend of antibiotic resistance was already on the rise with emerging MDR strains of *Salmonella*.[11] Extensively drug resistant *Salmonella* is defined as in addition to being Multi drug resistant isolates are resistant to third generation cephalosporins (cefixime / ceftriaxone). Previously reports of emerging resistance in *Salmonella typhi* and *paratyphi* against third generation cephalosporins which is termed as XDR strains was seen first reported by outbreak in Hyderabad Sindh. Subsequently it was sporadically reported by cases seen in Karachi followed by widespread uprising of reported cases of XDR *Salmonella typhi*.

Materials and Methods

This cross-sectional study was conducted in Jinnah Sindh Medical University laboratory Karachi. Ethical permission to use lab data was taken from JSMU laboratory as no patient confidential data was used and privacy was maintained. The total number of Blood culture received was 464 from January 1st to December 31 2018.The samples were received in BACTEC blood culture bottle. When a Bactec bottle gave a positive fluorescent signal, it was removed and an aliquot of the broth was Gram Stained and processed by sub culturing on blood agar and Mac conkey agar, antibiotic sensitivity was done on Mueller Hinton agar (MHA). After 24 hours of incubation, the grown bacterial colonies were identified by serological identification, done by Remel 9-0 antisera and biochemical identification was done by TSI, urea, SIM and citrate. Antibiotic sensitivity done on MHA was interpreted using CLSI 2018 break points for *Salmonella*.

Results

During 2018 from January till December 464 blood cultures came to JSMU Diagnostic laboratory and blood bank out of which 31 cases of enteric fever were diagnosed in automated blood culture Bactec 9050. All the isolated *Salmonella* belonged to *Salmonella typhi* subgroup. Out of 31 isolates 26 *Salmonella* were resistant to Ampicillin, chloramphenicol, co- trimoxazole, Cefixime and Ceftriaxone, with showing sensitivities only to azithromycin and meropenem. The antibiotic susceptibility of the xdr isolates showed, all but five were resistant to ampicillin, chloramphenicol, co-trimoxazole and third generation cephalosporin (cefixime and ceftriaxone). Remaining five *Salmonella* were resistant to cefixime but sensitive to ceftriaxone. None of the isolates were resistant to azithromycin, meropenem and imipenem.

The age group of all patients with *Salmonella* infection ranged from 6 months to 14 years. Out of thirty-one cases, 18 were male and 12 were female.

All but five patients with XDR *Salmonella* were treated with azithromycin for 5 days with complete recovery on follow up. The remaining five pediatric patients were treated with meropenem as they were clinical unstable to be treated with oral antibiotics.

Discussion

Typhoid fever is a life-threatening infectious disease caused by *Salmonella* enterica serovar *Typhi*. *Salmonella typhi* colonizes only humans, is transmitted through the fecal-oral route. Typhoid fever can be cured invariably by adequate and timely antimicrobial treatment. The rise of extremely drug resistant *Salmonella* is alarming and leaves limited treatment options with carbapenems which are costly as well as require administration by injectable. Preventive measures like improved potable water quality sanitation and vaccination in high-risk areas are means of potential control strategy recommended by the World Health Organization (WHO) for the short-to medium-term management.

Typhoid fever is a notifiable illness in the Sindh province of Pakistan. The cases of *Salmonella typhi* and para*typhi* identified in blood are reported to the Sindh health authorities with a special note to indicate the emergence of ceftriaxone resistance. Swift emergence and brisk spread of resistant isolates underline the significance of AMR surveillance for typhoid and other enteric Gram-negative bacteria and draw attention to the inadequacy of relying solely on non-culture-based serological methods for diagnosis of typhoid (such as Widal and *Typhi*dot tests), which do not provide susceptibility results. In view of the emergence of ceftriaxone resistance in *Salmonella typhi*, culture- and sensitivity-guided treatment becomes imperative as empirical treatment with ceftriaxone is no longer dependable in the region.[12] Following antibiotic resistance testing, cases were effectively treated with azithromycin and meropenem, resulting in recovery by all patients. Immediate control measures include education of the patients and household members and emphasis on hygiene and food safety.

The emergence and spread of XDR *S. Typhi* in Sindh, Pakistan, is a disquieting demonstration of how an omnipresent antibiotic resistance can be acquired by MDR *S. Typhi*, rendering it XDR and further narrowing treatment options. Antibiotics save millions of lives annually, but the evident ease and rapidity by which life-threatening bacteria such as *S. Typhi* can develop resistance severely limit their efficacy.

Small data from tertiary care hospital university laboratory propose that better strategies against typhoid are warranted, such as awareness of treating clinicians to send blood cultures when suspecting enteric fever and not rely on serological

typhoidal tests as they are not specific and cannot give information regarding antibiotic susceptibility pattern.

However, vaccination could not immediately be undertaken to prevent the spread of this highly resistant clone to patients and their household contacts due to unavailability free of cost vaccine and inability of these poor patients with limited resources to afford it privately. Patients' families were counseled to increase awareness about ease of spread among house hold contacts and preventive measures like hand washing, boiling drinking water, consumption of hygienic food and drinks. Handouts about awareness of proper sanitation and proper disposal of human wastes was also given to patients' attendants in order to drive knowledge and attention to take steps to break the chain of transmission of these infections.

Limitations

1. This study included only bacteremic isolates of XDR *Salmonella*. 21 of the patients were lost to follow up after reporting of positive culture and treatment history could not be taken via telephonic communication and only advice for treatment was sent through a comment in the blood culture report.

Table 1: Age group and resistance patterns of XDR *Salmonella*

Age group	ceftriaxone resistant	ceftriaxone sensitive	Male	Female
6month-14 years	26	--	16	10
1year-10years	--	05	3	2

Isolation of *Salmonella typhi* in different age group and gender

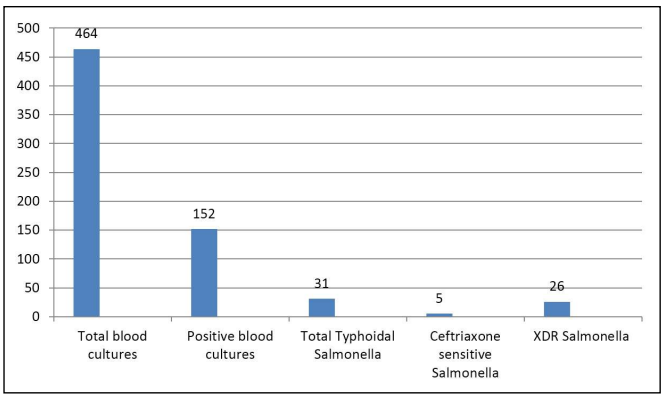

Figure 1. Graphical distribution of 464 blood cultures (total blood cultures during year 2018 from JSMU laboratory)

2. The study participants who were hospitalized in JPMC and NICH Provided relevant clinical history, but clinical details of 14 outpatients from various parts of Karachi could not be obtained.

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ORIGINAL ARTICLE

Central Line Associated Blood Stream Infection with Gram Negative Organisms: Clinical Features, Risk Factors and Mortality

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Abstract

Background
Gram negative (GN) central line associated blood stream infection (CLABSI) has high mortality. Sindh Institute of Urology and Transplantation (SIUT) have a large dialysis unit and CLABSI is a major source of bacteremia. The aim of this study is to determine the clinical and microbiological characteristics, risk factors, antibiotics usage and mortality between CLABSI and non-CLABSI patients.

Methods
It is a cross sectional study done at SIUT from May 2017 to March 2018. Patients >18 years, with GN bacteremia were included. Patients were divided into CLABSI and non-CLABSI groups. Age, ICU stay, mechanical ventilation, Pittsburgh (PITT) bacteremia score, comorbidities (diabetes mellitus, end stage renal diseases, hemodialysis, urinary catheters, recent surgery, stone disease etc.), clinical features (fever, hypotension, altered level of consciousness, leukocytosis, leucopenia, thrombocytopenia), appropriate antibiotic use were noted. Patients were followed till day 30.

Results
Out of 137, 78 (56.9%) were CLABSI and 59 (43%) non-CLABSI. The significant risk factors for CLABSI were end stage renal disease (ESRD) [71.8% vs 15.3% p=<0.001 CI 14.14(5.97-33.56)] and hemodialysis [88.5% vs 30.5% p=<0.001 CI 17.46(7.18-42.46)]. *Klebsiella species* was commonly found in CLABSI (p=0.007) and *Escherichia coli* in non-CLABSI (p<0.001). Only 31% received appropriate empirical antibiotics. Mortality in CLABSI group was significantly associated with PITT bacteremia score (p=0.004), mechanical ventilation (p=0.007) and acute renal failure (p=0.008).

Conclusion
ARF is major risk factor for CLABSI. Arterio-venous (AV) fistula formation should be expedited to prevent CLABSI associated bacteremia. Empirical antibiotics should be according to local antibiogram to avoid inappropriate use.

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Introduction

Central line associated blood stream infection (CLABSI) is the one of most common hospital acquired infection worldwide.[1]

Patients undergoing renal replacement therapy need temporary angioaccess till arteriovenous fistulas are created. It has been observed that the risk of infections due to dialysis catheters is 15 fold greater than arteriovenous fistulas.[2] CLABSI is found to be more common in middle to low income countries ranging from 7.4 to 12.2 per 1000 central line days as compared to 1.3-2.1 per 1000 central line days in high income countries.[3]

The most common organisms causing CLABSI are gram positives, however over the last decade infections with gram negative organisms have become more prominent. According to a study from Spain there is an increase incidence of gram negative (GNR) CLABSI from 4% to 40% over a period of 18 years.[4] Another study from Israel reported increase in the trend of GNR CLABSI over 15 years.[5] In patients on hemodialysis, an increased frequency of gram negative CLABSI has been reported from Saudi Arabia.[6]

Solid organ transplantation, prior use of penicillin and hospital stay longer than 11 days are found to be independent risk factors for GNR CLABSI.[4] GNR bacteremia in patients with central lines are associated with very high mortality.[5,7] Kiran et al studied gram negative bacteremia from our center, 50% of which were CLABSI, they reported infection with a multi drug resistant organisms, prolonged ICU stay of >48 hours and more than one positive blood culture for that organism as risk factors for mortality.[8]

The data on GNR CLABSI is sparse. There are limited comparative reports in terms of clinical and microbiological features and outcome between CLABSI and non- CLABSI. Cairo et al compared GNR CLABSI with non-CLABSI in cancer patients and concluded polymicrobial infections, stenotrophomonas bacteremia and high colony counts on blood cultures are strong predictors of CLABSI which should prompt physicians to remove the line.[9]

To the best of our knowledge, little work has been done on GNR CLABSI in our part of the world especially from Pakistan. Our aim is to determine the clinical characteristics, risk factors, microbiological characteristics, antibiotics used and mortality in gram negative central line blood stream infections.

Material and Methods

This is cross sectional study, conducted at Sindh Institute of Urology and Transplantation (SIUT) Karachi, Pakistan, from May 2017 to March 2018. SIUT is a 700 bedded tertiary care public sector hospital; it mainly caters to nephrology, urology, gastroenterology, oncology and solid organ transplantation. The hospital provides a large number of inpatient and outpatient renal replacement therapy.

The study included all patients >18 years of age, who were admitted at SIUT with documented bacteremia due to *E.coli, Klebsiella species, Psuedomonas aeruginosa, Psuedomonas species, Enterobacter* and *Acinetobacter baumanni*. Patients <18 years, solid organ transplant recipients, patients on out-patient hemodialysis and bacteremia due to organisms other than mentioned above were excluded.

We divided the patients into two groups based on source of bacteremia whether originating from central line (CLABSI) or originating from source other than central line (non-CLABSI). Data was collected after taking informed consent. Demographics, clinical features, co-morbid conditions, on hemodialysis or not, recent antibiotics exposure, ICU stay, on mechanical ventilation, recent surgery, causative microorganisms, antibiotics used were noted. Patients were again followed on day 30 to note whether alive or dead.

Definitions

Central line associated blood stream infection (CLABSI): is defined as when a patient had 1 or more blood cultures growing gram negative bacteria with clinical signs and symptoms consistent with infection and no other site of infection other than central line identified. [10]

Non-central line associated blood stream infection (non-CLABSI): is defined as when a patient had 1 or more blood cultures growing gram negative bacteria with clinical signs and symptoms consistent with infection and central line is not the cause of infection.

Acute renal failure (ARF): acute derangement of renal failure diagnosed and labeled by nephrologists at presentation on basis of KDIGO criteria.[11]

End-stage renal disease (ESRD): Chronic kidney disease (CKD) is defined as the presence of kidney damage or an estimated glomerular filtration rate (eGFR) less than 60 ml/min/1.73 mt2, persisting for 3 months or more, irrespective of the cause.[12]

Fever: having an axillary temperature above 101^0F. Altered level of consciousness: defined as any measure of arousal other than full orientation in time, place and person.

Hypotension: It is defined as systolic blood pressure ≤90 mmHg and diastolic blood pressure ≤60 mmHg on 2 different occasions and/or requirement of vasopressor agents within 48 hours of onset of bacteremia.

Leukocytosis/ leucopenia: Leukocytosis is defined as total leukocyte count ≥ 12000 cells/mm^3 and leucopenia as count ≤4500 cells/mm^3 within 48 hours of onset of bacteremia.

Thrombocytopenia: platelets count ≤$100*10^9$/L within 48 hours of onset of bacteremia.

Complications of bacteremia: gram negative bacteremia disseminate to different organs resulting to meningitis, brain abscess, empyema, hydropneumothorax etc.

PITTs bacteremia score: Includes clinical variables (range 0–14 points): temperature of 35.1?36.0°C or 39.0?39.9°C (1 point), temperature of ≤35°C or ≥40°C (2 points), mental status (alert, 0 points; disoriented, 1 point; stuporous, 2 points; comatose, 4 points), hypotension (2 points), receipt of mechanical ventilation (2 points) and cardiac arrest (4 points).

Appropriate antibiotics: Antibiotics which has been started before culture report and later found to be appropriate according to culture and then continued.

The study has been approved by ethical review committee of SIUT.

Statistical analysis: SPSS version 20 was used to analyze the data. Continues variables were reported as mean + SD and categorical variables were presented as frequencies and percentages.

To compare the mean difference between groups for continues variables two sample t-test was used whereas chi square independent test or fisher exact test was used to determine proportion difference between groups. P value <0.05 was considered as significant for categorical variables.

Results

A total of 137 cases with gram-negative bacteremia were included. 78 (56.9%) had CLABSI and 59 (43%) had non-CLABSI.

The age and sex distribution were similar. Significantly more patients with CLABSI presented with high grade fever as compared to non-CLABSI [n=66(84.6%) vs n=31 (52.9%) p=0.001 CI 4.97(2.23-11.05)]. Acute renal failure was seen

more in non-CLABSI patients. Regarding risk factors, CLABSI was found to be significantly associated with ESRD and hemodialysis. However, there is no difference regarding ICU stay [43.6% vs 35.6% p=0.34 CI 1.40 (0.69-2.80)], previous antibiotics exposure [55.1% vs 49.2% p=0.49 CI 1.27(0.64-2.50)], being on mechanical ventilation [17.9% vs 11.9% p=0.33 CI 1.62(0.61-4.32)] and diabetes [17.9% vs 15.3% p=0.68 CI 1.22(0.49-3.04)]. (table1)

Source of bacteremia in non-CLABSI patients are: urinary tract infection 35/59 (59%), pneumonia 5/59 (8.4%), cholangitis 7/59 (11.8%) and others 12/59 (20.33%).

Polymicrobial bacteremia is more seen in CLABSI (20.5% vs 13.6% p=0.29), however not statistically significant. Regarding individual organisms, *Klebsiella spp.* was found more frequently in CLABSI than in non-CLABSI (p=0.007) and *Escherichia coli* was found more commonly in non-CLABSI (p<0.001). Carbapenem resistant organisms were isolated in 38.5% of CLABSI and 49% of non-CLABSI bacteremia. (Table 2)

Overall empirical antibiotics were started in 124/137 (90.5%) of patients (Table 2). Piperacillin-tazobactam was the most commonly used antibiotic 83/124 (67%), 37/68 (54%) in CLABSI group and 46/56 (82%) in non-CLABSI group. Around

Table 1: Comparison of demographics, clinical characteristics, risk factors and mortality between CLABSI and non-CLABSI gram negative bacteremia. n=137

	CLABSI n=78	non-CLABSI n=59	p-value	OR (95%CI)
Age	42.5 (±16.1)	45.6 (±16.3)	0.277	NA
Male	54(69.2%)	41(69.5%)	0.97	0.98 (0.47 – 2.05)
Risk factors				
ICU stay >48hrs	34 (43.6%)	21 (35.6%)	0.34	1.40 (0.69-2.80)
Mechanical ventilation	14(17.9%)	7(11.9%)	0.33	1.62(0.61-4.32)
Diabetes Mellitus	14(17.9%)	9(15.3%)	0.68	1.22(0.49-3.04)
ESRD56	(71.8%)	9(15.3%)	<0.001	14.14(5.97-33.56)
Hemodialysis	69(88.5%)	18(30.5%)	<0.001	17.46(7.18-42.46)
Recent Antibiotics exposure	43(55.1%)	29(49.2%)	0.49	1.27(0.64-2.50)
Surgery	6(7.7%)	12(20.3%)	0.03	0.33(0.12-0.93)
Malignancy	2 (2.6%)	8 (13.6%)	0.02	0.17(0.03-0.82)
Stone diseases	8(10.3%)	12(20.3%)	0.09	0.45(0.17-1.18)
PCN	5(6.4%)	10(16.9%)	<0.001	0.34(0.11-1.04)
Foleys	29(37.2%)	40(67.8%)	<0.001	0.28(0.14-0.57)
Clinical features				
Fever	66 (84.6%)	31 (52.9%)	<0.001	4.97(2.23-11.05)
Hypotension	23 (29.5%)	14 (23.7%)	0.45	1.34(0.62-2.91)
ALOC	29 (37.2%)	17 (28.8%)	0.31	1.46(0.71-3.03)
Leukocytosis	55 (70.5%)	45 (76.3%)	0.45	0.74(0.34-1.61)
Leucopenia	5(6.4%)	2(3.4%)	0.35	1.95(0.37-10.43)
Thrombocytopenia ($<100*10^9$/L)	16(20.5%)	13(22.0%)	0.83	0.91(0.40-2.08)
Cardiac arrest	6 (7.7%)	1 (1.7%)	0.15	4.8(0.56-41.3)
PITT bacteremia score >4	14 (17.9%)	7 (11.9%)	0.32	1.62(0.61-4.32)
Complications	3(3.8%)	4(6.8%)	0.35	0.55(0.12-2.56)
Acute renal failure	20(25.6%)	44(74.6%)	<0.001	0.12(0.05-0.25)
Mortality				
30 Day mortality	24 (30.8%)	13 (22%)	0.25	0.63(0.29-1.39)

ICU=Intensive Care Unit, PITTs =Pittsburgh ESRD=End stage renal disease PCN= Percutanous nephrostomy ALOC= Altered level of consciousness

Table 2: Comparison of empirical antibiotics used and microorganisms isolated between CLABSI and non-CLABSI gram negative bacteremia

	CLABSI N=78	non-CLABSI N=59	p-value	OR (CI)
Empiric antibiotics	68(87.17%)	56 (94.9%)	0.13	0.36 (0.9-1.39)
Appropriate antibiotics received empirically	21(31%)	22(39%)	0.19	0.62 (0.29 – 1.28)
Micro-organisms isolated				
Polymicrobial	16 (20.5%)	8 (13.6%)	0.29	0.61(0.24-1.53)
Pseudomonas aeruginosa	11(14.1%)	8(13.6%)	0.93	1.05(0.39-2.79)
Acinetobacter	14(17.9%)	11(18.6%)	0.92	0.95(0.40-2.29)
Klebsiella species	36(46.2%)	14(23.7%)	0.007	2.75(1.30-5.81)
E.coli	11(14.1%)	25(42.4%)	<0.001	0.22(0.10-0.50)
others	6(7.7%)	1(1.7%)	0.12	4.83(0.57-41.29)
Carbapenem resistant organisms	30 (38.5%)	29 (49.2%)	0.21	0.65 (0.33-1.29)

43(31%) received appropriate antibiotics and 94(68.6%) received inappropriate antibiotics empirically.

Out of a total of 137 patients, 24 (30.8%) in CLABSI and 13 (22%) in non-CLABSI were died. There was no significant difference in 30 days' mortality between CLABSI and non-CLABSI patients. When clinical features and risk factors were compared, the PITT bacteremia score ≥4 was found to be significantly associated with mortality in CLABSI group as compared to non-CLABSI group (10/14 vs. 0/7, p value= 0.004). There is also a significantly high mortality in patients with CLABSI who had altered level of consciousness (p=0.07), on mechanical ventilation (p=0.007) and had acute renal failure (p=0.008). Regarding bacteria isolated, *Klebsiella* bacteremia in non-CLABSI patients is more fatal than in CLABSI patients however not statistically significant (p=0.089) (Table 3). Overall out of 37 patients who died, 28 (76%) patients were on inappropriate antibiotics and 9 (24%) were on appropriate antibiotics, however the difference was not statistically significant (p=0.27).

Discussion

SIUT mainly caters to renal and urological diseases with a large hemodialysis unit and a busy urology surgical service. We compared GNR CLABSI with non-CLABSI. The age and gender were comparable. When we looked into the risk factors, CLABSI is significantly associated with ESRD. It has been observed that around 80% of patients initiated dialysis via temporary central venous catheter access worldwide and there is three to fourfold higher risk of catheter related infections compared with either fistula or graft.[13] Mehmood *et al* from Pakistan also reported around 80% of patients presented with ESRD with acute need for hemodialysis through temporary access lines.[14] This patient population should have the arterio-venous (AV) fistula prepared in advance when they are in their stage 4 or 5 kidney disease in order to avoid dialysis lines. It has been observed that AV fistula is the best modality for hemodialysis with least infections.[13] Early diagnosis of chronic kidney disease (CKD) with prompt fistula formation has been associated with avoidance of central lines and hence infections.[15] The reason of late presentation may be the fact that majority of our patients come from low socioeconomic group with reduce access to diagnosis and management of CKD. Bokhari *et al* reported late diagnosis of CKD and lack of awareness among patients about seeking medical care for early fistula formation as the most frequent reasons of unavailability of AV fistula.[16]

At our center the central line infection rates in the dialysis unit are very high; around 40 per 100 patient months, according to our recent surveillance data. We observed the same trend in our study; CLABSI bacteremia is significantly more common in patients on hemodialysis than in non-CLABSI bacteremia. More focus has to be placed on stringent infection control measures in hemodialysis unit during insertion as well as handling of dialysis lines for the prevention of catheter infections.

In non-CLABSI patients bacteremia is significantly associated with foley's catheter or percutaneous nephrostomy tubes. It reflects our patient population, admitted mostly with urological problems including malignancies and urological surgeries. These patients require multiple urological interventions and requirement of indwelling catheters for long time posing them high risk of developing urinary tract infections and urosepsis.

We have found significant difference in microbiological characteristics of CLABSI and non CLABSI bacteremia. *Klebsiella spp*. infection is more common in CLABSI patients. Kiran *et al* from our center also reported *Klebsiella* bacteremia to be the most common organism among gram negative

Table 3: Comparison of clinical features, risk factors and microorganisms isolated with mortality between CLABSI and non-CLABSI gram negative bacteremia.

	30 day Mortality`` in CLABSI No/total no (%)	30 day Mortality in non- CLABSI No/total no (%)	p-value
Clinical features			
Pitt bacteremia score ≥4	10/14 (71.42%)	0/7	0.004
ICU 48 hrs.	13/34 (38.23%)	4/21 (19.04%)	0.229
Hypotension	11/23 (47.82%)	3/14 (21.42%)	0.166
Altered level of consciousness	15/29 (51.72%)	4/17 (23.52%)	0.073
Mechanical ventilation	9/14 (64.28%)	0/7	0.007
Cardiac arrest	5/6 (83.33%)	0/1	0.286
Complications	2/3 (66.66%)	2/4 (50%)	1.000
Acute renal failure	8/20 (40%)	5/44 (11.36%)	0.008
ESRD	14/56 (25%)	5/9 (55.55%)	0.108
Hemodialysis	21/69 (30.43%)	4/18 (22.22%)	0.572
Organisms			
Pseudomonas aeruginosa	5/11 (45.45%)	1/8 (12.5%)	0.177
Acinetobacter species	6/14 (42.85%)	2/11 (18.18%)	0.234
Klebsiella species	9/36 (25%)	7/14 (50%)	0.089
E.coli	4/11 (36.36%)	3/25 (12.0%)	0.167
Polymicrobial	4/16 (25%)	4/8 (50%)	0.363
Carbapenem resistant organisms	8/30 (26.66%)	6/29 (20.68%)	0.590
Inappropriate antibiotics	18/57 (31.5%)	10/37 (27%)	0.637

infections.[8] This finding is consistent with other studies done recently which report increase frequency of Klebsiella as the causative organism in CLABSI with high mortality.[4,5,6] *Klebsiella spp* has been reported as the third most common pathogen in hospital settings.[17] The possible reasons behind the rising trend of nosocomial *Klebsiella* infection are high rates of colonization in gastrointestinal tract of patients and increase propensity of biofilm formation by this organism.[18] Infection control measures with environmental cleaning and hand hygiene are the best possible way to prevent this pathogen to cause infections.

When we looked into antibiotic usage we found that around 90% of our patients received empirical antibiotics with more than half received pipracillin/tazobactam. However, if we assess the appropriateness of empirical therapy, only one third of the patients received appropriate antibiotics at the time of admission. Triffi *et al* reported empiric appropriate antibiotics is associated with early reduction of vasopressor requirement and hence reduce mortality.[19] The possible cause of inappropriate antibiotic can be explained by the increase prevalence of resistant organisms in our center. Around 40% of our patients in both CLABSI and non-CLABSI group had carbapenem resistant bacteremia. Kalam *et al* from our institute also reported the frequency of carbapenem resistant bacteremia as 42%.[8] We found a high mortality in patients who received inappropriate antibiotics, although not statistically significant. A regular communication between microbiologists and treating physicians regarding local susceptibility patterns may improve the prescription of appropriate empirical antibiotics.

There was no difference in all-cause mortality at 30 days, between CLABSI and non-CLABSI. However, in patients with CLABSI, mortality is significantly associated with high PITTs bacteremia score, being on mechanical ventilator and having renal failure. According to Cairo *et al* high bacterial load in blood culture is predictive of CLABSI.[9] We can infer that since CLABSI is associated with high bacterial load, it presents with more severe disease associated with high PITTs bacteremia score and more complicated clinical course before death.

The limitations of our study are the number of patients in non-CLABSI group is less than in the study arm, which may not represent the true associations. However, it is the first study to compare the CLABSI with non-CLABSI in Pakistan.

In conclusion CLABSI is a severe disease. We need to expedite

AV fistula formation in order to avoid central line infections. Good infection control practices in handling central lines as well as other devices should be emphasized. Empirical antibiotics in gram negative bacteremia should be according to the local antibiogram of each unit.

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Instructions to Authors

Scope

The Infectious Diseases Society of Pakistan sponsors the Infectious Disease Journal of Pakistan (IDJ). The Journal accepts Original Articles, Review Articles, Brief Reports, Case Reports, Short Communications, Letter to the Editor and Notes and News in the fields of microbiology, infectious diseases, public health; with laboratory, clinical, or epidemiological aspects.

Criteria for publication

All articles are peer reviewed by the IDSP panel of reviewers. After that the article is submitted to the Editorial Board. Authors may submit names and contact information of 2 persons who potentially could serve as unbiased and expert reviewers for their manuscript, but IDSP reserves the right of final selection.

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Manuscripts must be formatted according to submission guidelines given below, which are in accordance with the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (originally published in *N Engl J Med* 1997;336:309-15). The complete document appears at www.icmje.org. Please submit one complete copy of the manuscript and all enclosures to **The Managing Editors, Infectious Diseases Journal of Pakistan, Department of Pediatrics & Child Health, The Aga Khan University, Stadium Road, P.O. Box 3500, Karachi 74800, Pakistan.** An electronic copy of the manuscript must also be sent to pak_idj@yahoo.com**.** All manuscripts submitted to IDJP must be accompanied by an Authorship Declaration stating that *'The authors confirm that the manuscript, the title of which is given, is original and has not been submitted elsewhere. Each author acknowledges that he/she has contributed in a substantial way to the work described in the manuscript and its preparation'*. Upon submission a manuscript number will be assigned which should be used for all correspondence.

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Articles should report original work in the fields of microbiology, infectious disease or public health.The word limit for original articles is 2000.

Title page

This should list the (i) title of the article, (ii) the full names of each author with highest academic degree(s), institutional addresses and email addresses of all authors. (iii) The corresponding author should also be indicated with his/her name, address, telephone, fax number and e-mail address. (iv) A short running title of not more than 40 characters (count letters and spaces) placed at the foot end of the title page.(v) a conflict of interest statement should also be included in this section.

Abstract

Abstract should not exceed 250 words and must be structured in to separate sections headed *Background, Methods, Results and Conclusions*.

Please do not use abbreviations or cite references in the abstract. A short list of four to five key words should be provided to facilitate.

Background

The section must clearly state the background to the research and its aims. Controversies in the field should be mentioned. The key aspects of the literature should be reviewed focusing on why the study was necessary and what additional contribution will it make to the already existing knowledge in that field of study. The section should end with a very brief statement of the aims of the article.

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Please provide details of subject selection (patients or experimental animals). Details must be sufficient to allow other workers to reproduce the results. The design of study and details of interventions used must be clearly described. Identify precisely all drugs and chemicals used, including generic name(s) and route(s) of administration All research carried out on humans must be in compliance with the *Helsinki Declaration*, and animal studies must follow internationally recognized guidelines. The authors are expected to include a statement to this effect in the Methods section of the manuscript. A description of the sample size calculation and statistical analysis used should be provided.

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Present results in logical sequences in the text, tables and illustrations. Articles can have a maximum of 5 illustrations (in a combination of figures and tables) per article. The results should be in past tense and repetition of results presented in the tables should be avoided. Exact *P*-values should be reported along with reporting of OR and RR with their Confidence Intervals where applicable.

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Emphasize the new and important aspects of the study and conclusions that follow from them. Do not repeat the details from the results section. Discuss the implications of the findings and the strengths and limitations of the study. Link the conclusions with the goals of the study but avoid unqualified statements and conclusion not completely supported by your data.

Acknowledgments

Acknowledge any sources of support, in the form of grants, equipment or technical assistance. The source of funding (if any) for the study should be stated in this section. Please see below for format of **References, Figures and Tables**.

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Authoritative and state of the art review articles on topical issues are also published, with a word limit of 2000. It should consist of critical overview of existing literature along with reference to new developments in that field. These should be comprehensive and fully referenced. Articles should contain an Abstract; Main Text divided into sections, Conclusions and References.

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Number references consecutively in the order in which they are first mentioned in the text. Identify references in text, tables and legends by Arabic numerals (in superscript). References cited only in tables or in legends to figures should be numbered in accordance with a sequence established by the first identification of the particular table or illustration. Bibliography should be given in order. Authors, complete title, journal name (Abbr), year, vol, issue, page numbers. According to "Uniform Requirements of Manuscripts submitted to Biomedical Journals", as cited in N Engl J Med 1997; 336:309-15.

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Data reported either in a table or in a figure should be illustrative of information reported in the text, but should not be redundant with the text. Each table must be presented on a separate sheet of paper and numbered in order of appearance in the text. Table should be numbered consecutively in Arabic numerals. Tables and Figures legends should be self-explanatory with adequate headings and footnotes.Results which can be described as short statements within the text should not be presented as figures or tables.

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Instructions updated - April 2012.
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