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Challenge of Multidrug-resistant Strains of Enterobacteriaceae Isolated from Clinical Samples

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ABSTRACT

Background: The rising level of antimicrobial resistance among bacterial pathogens is one of the most significant public health problems worldwide. Antibiotic resistance of clinically important bacteria, the types and levels of resistance and multidrug resistance (MDR) among pathogens is extremely important. With the rise of infections caused by ESBL-producing *Enterobacteriaceae* (ESBL-E) and with their co-resistance to many other antibiotic classes, carbapenems have been considered to be the last line of defence against these life-threatening infections. The current study was carried out to determine the frequency, disease burden and therapeutic challenge of infections caused by multidrug resistant strains of *Enterobacteriaceae* with particular reference to Extended-Spectrum Beta-lactamase-producing *Enterobacteriaceae* (ESBL-E), Carbapenem-resistant *Enterobacteriaceae* (CRE) and the emerging infections caused by Extended-Spectrum Beta-lactamase-producing Carbapenem-resistant *Enterobacteriaceae* (ESBL-CRE)

Methodology: This cross-sectional study was carried out in the Microbiology Department of Islamabad Diagnostic Centre over a period of two years, from January 2018 to December 2020**.** *Enterobacteriaceae* isolated on culture from clinical samples were identified using appropriate characterization tests including the selective use of API 20E. Antimicrobial susceptibility testing (AST) and ESBL detection was performed on Vitek 2 compact system by Minimum Inhibitory Concentration (MIC) methodology. Isolates that were resistant to more than one carbapenem were identified as Carbapenem-Resistant *Enterobacteriaceae* (CRE).

Results: Out of 7270 specimens that yielded the growth of *Enterobacteriaceae*, 2943 (40.5%) were ESBL positive (ESBL-E) and 487 (6.7%) were carbapenem resistant (CRE). Further analysis of CRE revealed 247/487 as non-ESBL-CRE and 240/487 as ESBL-producing CRE (ESBL-CRE). Maximum number of CRE isolates - both non-ESBL and ESBL CRE - were from urine specimens. *Klebsiella* species followed by *Escherichia coli* and *Enterobacter* were the dominant ESBL-CRE isolates. Admission to a health care facility was the major risk factor followed by advancing age.

Conclusion: Besides ESBL-E, Carbapenum-resistant *Enterobacteriaceae* (CRE), particularly those co-producing Extended-Spectrum Beta-lactamase (ESBL-CRE), (wherein resistance mechanisms to both carbapenems as well as to beta-lactam antibiotics are concomitantly expressed in the same organism), have emerged as the major pathogens of concern.The later appears to have introduced a new dimension in the resistance profile of infections caused by multidrug-resistant *enterobacteriaceae.*

Keywords: ESBL-producing *Enterobacteriaceae*, Carbapenem-resistant *Enterobacteriaceae* (CRE), ESBL-producing Carbapenem-resistant *Enterobacteriaceae* (ESBL-CRE), *Escherichia coli, Klebsiella*, Multi-drug Resistant *Enterobacteriaceae*.

Authors' Contribution:
[1,2]Conception; Literature research; manuscript design and drafting; [2,3]Critical analysis and manuscript review; [5,6]Data analysis; Manuscript Editing.

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Introduction

Ever since the emergence of infections caused by ESBL-producing *Enterobacteriaceae* (ESBL-E), along with their genetically determined co-resistance to many other antibiotic classes, carbapenems have traditionally been considered to be the last line of defence and virtual life-saving agents against these life-threatening infections.[1]Emergence of strains that had developed resistance to carbapenems was, therefore, a serious development. First reported from Japan in 1994,[2]carbapenem-resistant *Enterobacteriaceae* (CRE), with their marked propensity for nosocomial spread, have emerged as one of the most serious threats to the management of infections in health care facilities all over the world.[3,4]Ominously, we are now faced with the more formidable challenge of emerging infections caused by ESBL-producing carbapenem-resistant *Enterobacteriaceae* (ESBL-CRE). These strains, reported sporadically worldwide in recent years, harbour both ESBL and carbapenemase genes in the same organism that confer a high level of resistance to both the carbapenems as well as to higher generation cephalosporins, thus leaving extremely limited therapeutic options. [5-7]

The present study, conducted at the Microbiology Department of Islamabad Diagnostic Centre (IDC) over a period of two years, was essentially a comparative analysis of the etiological roles played by these three strains of multidrug resistant (MDR) *Enterobacteriaceae* in the causation of clinical infections namely Extended-spectrum Beta-Lactamase-producing *Enterobacteriaceae* (ESBL-E), Carbapenem-resistant *Enterobacteriaceae* (CRE), and, importantly, the newly emerging ESBL-producing Carbapenem-resistant *Enterobacteriaceae* (ESBL-CRE), and to determine their frequency, clinical significance, risk factors and therapeutic options available within our healthcare set-up.

Methodology

Clinical specimens were collected with full aseptic precautions using transport media where necessary and were processed according to standard protocols. Clean-catch midstream urine collected in sterile containers was cultured within one hour of collection or stored at 4°C until inoculated. Blood cultures were performed on Versa TREK automatic Microbial Detection System (Thermo-Fisher Scientific USA). CLED agar (Oxoid Ltd, Basingstoke, UK) was used for quantitative urine culture; a colony count of ≥ 10^5 Colony-Forming Units (CFU) per ml having been taken assignificant. All other routine specimens were inoculated on blood agar, MacConkey agar and chocolate agar (Oxoid Ltd, UK) and incubated at 37°C for 24-48 hours. Appropriate characterization tests were used to identify the isolates, including the selective use of API-20E and/or Vitek 2 ID card. All gram-negative rods (GNRs) characterized as *Enterobacteriaceae* were included in the study. All oxidase positive and non-fermenting GNRs were excluded.

Antimicrobial susceptibility testing (AST) and ESBL detection was performed on Vitek 2 compact system (bioMérieux, Marcy-l'E'toile, France) by Minimum Inhibitory Concentration (MIC) methodology. Appropriate Vitek 2 Standard Susceptibility Cards were used throughout the study to test susceptibility against Amikacin, Amoxiclav, Ceftriaxone, Ciprofloxacin, Imipenem, Meropenem, Ertapenem, Cotrimoxazol, Piperacillin/tazobactam, Minocycline and Tigecycline. ESBL positive strains were detected by Vitek 2 Advanced Expert System (AES).Isolates identified as ESBL-positive by Vitek 2 AES were further confirmed by double disc diffusion (disc approximation) method performed on Mueller-Hinton agar (Oxoid Ltd, UK) ; results were interpreted as previously described.[8]

Clinical Laboratory Standards Institute's (CLSI) performance standards for AST were followed

throughout, including the reduced MIC breakpoints for carbapenems susceptibility that were used to interpret the susceptibility or resistance to imipenem, meropenem and ertapenem. This approach effectively obviated the need for carbapenemase testing[9]. EUCAST breakpoints were used for interpreting susceptibility to colistin / polymyxin B and tigecycline.[10]

CDC's 2015 definition of CRE, primarily meant for use in USA health care facilities, was slightly modified and all the GNR isolates that were resistant to more than one carbapenem in our series were identified as Carbapenem-Resistant *Enterobacteriaceae* (CRE).[11]Relevant demographic and clinical data including age, gender, hospital or community infection, ICU admission (wherever available) were recorded. Site of infection was determined from the nature of clinical samples received.

Findings were analysed by calculating frequencies and percentages. Pearson's Chi Square test was applied as test of significance for categories of variables; p-value of ≤ 0.05 (95% CI) was taken as statistically significant

Study was duly approved by the IDC Ethical Committee. Since all patient data was analysed anonymously from the lab records, requirement for informed consent of participants was waved by the approval committee.

Results

During the study period, a total of 7270 specimens yielded growth of *Enterobacteriaceae.* Initially the isolates were divided into two main study groups; 2943 (40.5%) were ESBL positive E*nterobacteriaceae* (ESBL-E) and 487(6.7%) were carbapenem-resistant E*nterobacteriaceae* (CRE). On the basis of co-production of ESBL, CRE were further divided into two sub-groups, with more or less equal distribution; 247/487 were non-ESBL-CRE as opposed to 240/487 that were ESBL-CRE (being ESBL producers as well as carbapenem-resistant). The frequencies of the three major groups of "pathogens of concern" that have emerged from this analysis, namely ESBL-E, non-ESBL-CRE and ESBL-CRE, are shown in Tables I, II and III according to clinical specimens/site of infection, species of organisms isolated and the demographic characteristics, respectively.

Table I: CRE (non-ESBL and ESBL-CRE both), from different clinical samples as compared to total ESBL (n : 7270)

Specimens / site of infection	GNRs* No. of isolates (%)	ESBL (n:2943)	CRE (n : 487)		
		No (% of GNR in the samples)	Total (%)	Non-ESBL CRE	ESBL - CRE
Urine (UTI)	4652(64)	2094 (45)	185 (3.4)	87	98
Pus/wound swab (SSSI, SSI)	852(11.6)	239 (28)	123 (14.4)	64	59
Catheter Tips (Inv Med Dev)	426(6)	207 (49)	59 (13.8)	28	31
Sputum/BAL (Lower Resp Tract Inf)	392(5.4)	165 (42)	49 (12.5)	24	25
Blood (BSI)	630(8.7)	93 (15)	17 (2.7)	13	04
Others (Misc)	318(4.3)	145 (45.6)	54 (16.9)	31	23
Total	7270	2943 (40.5)	487 (6.7)	247(3.4%)	240(3.3%)

*GNR – Gram Negative Rods (For the sake of brevity this term is used synonymously with *Enterobacteriaceae*)

Table II: Frequency according to species of isolates – both non-ESBL CRE as well as ESBL-CRE, as compared to total ESBL frequency (n: 7270)					
Organisms	No. of isolates	ESBL (% of total isolates)	CRE (n:487)		
			Total (% Of total isolates)	Non-ESBL CRE (%)	ESBL – CRE (%)
Klebsiellaspp	1351	405 (30)	235 (17.4)	133 (9.8)	102 (7.5)
E. coli	4778	2384 (49.9)	179 (3.7)	76 (1.6)	103 (2.1)
Enterobacterspp	239	52 (22)	60 (25)	28 (11.7)	32 (13.4)
Others (Misc.)	902	102 (11.8)	13 (1.4)	10 (1.1)	03 (0.33)
Total	7270	2943(40.5)	487 (6.7)	247 (3.4)	240(3.3)

Table III: Demographic distribution of CRE (n : 487)					
Variables	C R E				
	Total %	Non-ESBL CRE	P - value*	ESBL-CRE	P - value*
Healthcare versus community associated					
Healthcare associated	322 (66.1)	149	**0.0009**	173	**< 0.0001**
Community associated	165 (33.8)	98		67	
Gender-based Frequency					
Male	295 (60.6)	150	**< 0.0001**	145	0.0625
Female	192 (39.4)	77		115	
Age related Frequency					
Age < 1 - 40 yrs	152 (31.2)	80	**< 0.0001**	72	**< 0.0001**
Age 40 - 100 yrs	335 (68.8)	167		168	
Mean age		52.35 yrs		56.31 yrs	0.7003
Age distribution		1d – 100 yrs		2d – 100 yrs	

(p values that have been highlighted are statistically significant)*

* Pearson's Chi Square test has been applied as test of significance for categories of variables;

* p-value of ≤ 0.05 (95% CI) has been taken as significant.

Table IV: Antibiograms showing antimicrobial susceptibility profiles of the two most frequent CRE isolates (both ESBL and non-ESBL)									
E. coli (n : 179)									
CRE	No. of isolates	Antibiotics (Percentage susceptible)							
		AK	Cip	SXT	TZP	MH	TGC	Cephs	CT/PB
Non-ESBL- CRE	76	61	3.8	5.2	2.6	40	40	0	100
ESBL-CRE	103	62	03	7.7	7.7	36	37	0	100
Klebsiella (n : 235)									
CRE	No. of isolates	Antibiotics (Percentage susceptible)							
		AK	Cip	SXT	TZP	MH	TGC	Cephs	CT/PB
Non-ESBL -CRE	133	16.5	5.2	6.7	1.3	10.8	47.4	0	100
ESBL-CRE	102	24.5	3.9	6.8	2.9	33	47	0	100

AK: Amikacin; **Cip**:Ciprofloxacin; **SXT**: Cotrimoxazol; **TZP:** Pipracillin-Tazobzctum; **MH**: Minocycline; **TGC**: Tigecycline ; **Cephs** : 3rd gen Cephalosporins (ceftriaxone used as class representative) **; CT**: Colistin; **PB**: Polymyxin B.

Figures in the columns indicate percentage strains susceptible

Discussion

Global spread of ESBL-E and CRE infections, with their substantial morbidity and mortality, is presently one of the greatest challenges facing the healthcare authorities all over the world. Recent reports of infections caused by Extended-spectrum beta-lactamase–producing Carbapenem-resistant Enterobacteriaceae (ESBL-CRE), wherein both ESBL and CRE resistance mechanisms are expressed in the same species, appear to have introduced a new and more serious dimension in the natural history of infectious diseases.[5,7] WHO has included ESBL-CRE in its list of three top priority Multi-Drug Resistant (MDR) pathogens (priority 1- "critical"), requiring intensive Research and Development (R&D) efforts.[12]

Despite the fact that Piperacillin-tazobactam has been famously termed as carbapenem-sparing antibiotic against ESBL-E infections,[13] in actual clinical practice, carbapenems have been found to be the most effective and, by consensus, a preferred first line choice for severe ESBL-E infections, necessarily resulting in their substantial overuse.[14,15]Following the well-established historical pattern of genesis of antimicrobial resistance, carbapenem-resistant *Enterobacteriaceae* (CRE), therefore, did not take very long to appear and was soon followed by the emergence of ESBL-producing carbapenem-resistant strains (ESBL-CRE).

Overall ESBL-E prevalence of 40.5% in our study is more or less similar to those reported in other recent studies. In a systemic meta-analysis report, Ibrar et al have quoted a figure of 40% from Pakistan as against 46% and 42% from China and East Africa respectively.[16] On the other hand, our findings of 40.5% ESBL-E and 6.8% CRE differ from those reported by (i) Taqi M et al who reported 24.9% ESBL-E and 5.7% CRE in a sample size of 543 *Enterobacteriaceae* isolated from blood cultures [17] and (ii) from those reported by Legese et al - 78.6% ESBL-E and 12.12% CRE in a small sample size of 28 only from Ethiopia.[18]

Our findings of 6.7% overall CRE, with almost equal distribution between ESBL-CRE and non-ESBL-CRE, also appear to be at variance with another study from China wherein out of a total number of 149 CRE strains detected over a four-year period, 32 (21.5%) were non-ESBL CRE and 117 (78.5%) were ESBL-CRE.[5] In a study from Turkey, among 210 ESBL-producing *Enterobacteriaceae* isolated from blood, 23 (11%) were identified as ESBL-CRE, being resistant, as in our series, to all the three carbapenems tested.[7] Maximum yield of ESBL-CRE from urine samples in our series, however, compares favourably with the findings in the Chinese study making "urinary system disease as an independent predictor associated with the isolation of ESBL-CRE"[5]. Significant association with invasive medical devices in our study also concurs with that reported in the same Chinese study. However, there is a notable difference between the two studies in the most frequent ESBL-CRE and non-ESBL CRE species: our study shows *Klebsiella spp., E. coli* and *Enterobacter* frequencies in that order while the Chinese report mentions E*nterobacter, E. coli* and *Klebsiella in* the same order.

In their molecular based study of 46 CRE isolates (9 *E. coli* and 37 *Klebsiella*), Duangkaw W et al found that ESBL genes were co-harboured with carbapenemase genes in all but three CRE isolates,[19] an exceptionally high percentage of ESBL-CRE indeed. As for any reports of ESBL-CRE infections from Pakistan, a literature review, including google search, unfortunately returned no results. In that case, to the best of our knowledge and belief, this report would be the first published account of the prevalence of multidrug-resistant infections from Pakistan caused by ESBL-producing Carbapenum-resistant *Enterobacteriaceae* (ESBL-CRE). Coexistence of ESBL and CRE genetic elements in the same organism potentially makes a deadly combination as both strains often harbor plasmid mediated genes conferring resistance to

other antimicrobial classes.[15]Longer hospital stays, increased morbidity and mortality, propensity to spread widely within the health care settings, CRE both ESBL and non-ESBL, require extensive infection control measures and preventive interventions to limit their spread.[20] According to CDC, hospitalized patients with CRE including ESBL-CRE infections have one of the highest mortality rates, ranging from a low of 24 % to as high as 70 %. [3,10,11]

According to a standardized terminology, proposed by an international group of experts, to describe acquired resistance profiles of common pathogens,[21] majority of ESBL-E and CRE isolates (non-ESBL) in our series would be categorized as MDR (non-susceptible to at least one agent in three or more antimicrobial categories) and a significant number of ESBL-CRE as Extensively Drug Resistant (XDR - Non-susceptibility to at least one agent in all but two or fewer antimicrobial categories). Admission to a health care facility (table III) appears to be the single, most important independent risk factor for developing CRE infection, both non-ESBL ($p = 0.0009$) as well as ESBL-CRE ($p = < 0.0001$) followed by advancing age, maximum cases having been found in 40 plus individuals ($p = < 0.0001$). These findings are consistent with those of another study reported from Middle East.[22] Significantly no age was found to be exempt from CRE both non-ESBL as well as ESBL-CRE having been isolated even from day-old newborns.

As mentioned earlier, invasive medical devices, because of their enhanced infective potential due to bacterial adherence and biofilm formation, appear to be a significant risk factor.[5]Other risk factors include treatment and length of treatment with piperacillin-tazobactam, combination of carbapenems with fluoroquinolones[23], previous exposure to β-lactam antibiotics, transfer from another hospital, and some underlying diseases.[5]Based on susceptibility profiles of CRE isolates, our lab findings, as regards therapeutic options, are largely consistent with those reported in the literature. Combination therapy consisting of colistin and tigecycline would appear to be the best option in these infections, with fosfomycin replacing tigecycline in UTI.[24] This regimen however may have its own limitations; Tigecycline has not yet been approved for use in children and colistin has its own nephrotoxicity and neurotoxicity profiles. Despite the co-existence of ESBL and CRE genetic elements in the same species portraying a higher level of antimicrobial resistance, absence of any significant difference between the antibiograms of ESBL-CRE and non-ESBL-CRE in our study, appears to be somewhat paradoxical that defies a plausible explanation and arguably makes it a valid subject for further studies.

Limitations

One of the major limitations of the study is that it has been more or less entirely lab-based, with little clinical information available. Such information would be crucial to determine as to whether or not, or how far, the lab-reported susceptibility profiles are translated into matching clinical reality. There is a remote possibility of discordance between in-vitro susceptibility results and in-vivo clinical outcomes; as an example, ESBL-E infections based on in- vitro susceptibility to piperacillin-tazobactam and cefepime have been reported to have doubtful outcomes in clinical settings.[1]

Conclusion

Data presented in this study, spanning over a period of 2-years, shows that, besides the commonly encountered ESBL-producing *Enterobacteriaceae* (ESBL-E) in clinical samples, strains of Carbapenem-resistant *Enterobacteriaceae* (CRE), particularly those co-producing Extended-Spectrum Beta-lactamase (ESBL-CRE), wherein resistance mechanisms to both the carbapenems as well as to beta-lactam antibiotics are concomitantly expressed in the

same organism, have emerged as the major "pathogens of concern". The later appears to have introduced a new dimension in the resistance profile of infections caused by multidrug-resistant E*nterobacteriaceae.* Suggested therapeutic options for CRE, both ESBL and non-ESBL, would appear to be a combination of colistin and tigecycline, with fosfomycin replacing tigecycline as a priority in UTI. Relentless emergence of successive generations of resistant GNRs as highlighted herein calls for judicious restraints on the use of antibiotics under the WHO's stewardship programme.

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