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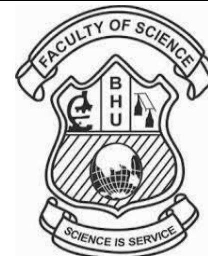

Molecular Characterization of Multi Drug Resistant (MDR) Extended Spectrum β-lactamase (ESBL) Producing *Klebsiella spp.* from Pond Water in West Bengal

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***Abstract*: Bacterial antibiotic resistance is an emerging global healthcare threat. Enterobacteriaceae family is eminent for development and transfer of antibiotic resistance (AR's). To control the spread of drug-resistant bugs, the environmental microflora should be screened for the presence and spread of antibiotic resistant genes (ARG's). In order to determine the possibility and frequency of antibiotic resistant organisms in genus *Klebsiella,* 226 *Klebsiella* colonies were isolated from pond water (n=113). In total, 16.81% *Klebsiella* isolates were found MDR, of which, 76.19% carried *bla* CTX-M, 19.04% *bla* TEM, and 71.42% AmpC type beta-lactamase. However, 42.10% *Klebsiella* were both ESBL & AmpC type beta lactamase (ACBL) co-producer. In addition, 19.04% of the isolates emerged as carbapenemase producers. This study indicates that urban ponds may act as natural reservoir for antibiotic resistant bacterium.**

***Index Terms*: Antibiotic resistance markers; ESBL; ACBL; Enterobacteriaceae; *Klebsillea oxytoca; K. pneumoniae;* MDR.**

I. Introduction

MDR ESBL expressing Enterobacteriaceae family represents worldwide threat to human health due not only because it seriously impairs the recovery of hospitalized patients, but also for its presence and consistent spread in the community, animals and environment (Upadhyay & Joshi, 2015), (Bradford, 2001), (Kojima et al., 2005). Thanks to exorbitant and unscrupulous use of the antibiotics coupled with its excessive unplanned discharge in environment led to the development and ever-increasing problem of AMR in environmental settings (Henriot et al., 2019; Wilkinson et al., 2017). Alarmingly, even non pathogenic bacteria acquired ARG's through mutation or horizontal gene transfer, due to stringent environmental selection pressure (Robicsek et al., 2006), (Ruppé et al., 2015; Vaidya, 2011; Warnes et al., 2012;). Antimicrobial resistance will becoming a serious health hazard (Lachmayr et al., 2009) which is also supported by World Health Organization and they warned it as one of the most dreaded public health issue for this 21st century. This study was undertaken to investigate the prevalence and occurrence of ESBL producing *Klebsiella* belonging to Enterobacteriaceae in ponds of West Bengal.

II. II. Material and Methods

A. *Sampling and isolation of bacteria*

One hundred and thirteen water samples (n=113) were collected aseptically from fresh water ponds from North 24 Paraganas (n=39), Nadia (n=57) and Kolkata (n=17) districts and immediately transported to the laboratory maintaining proper cold chain. A 0.2 mm pore-size membrane filter (Sartorius Stedim, Goettingen, Germany) were used to filter water samples (1000 ml), and the filter-papers were then inoculated in MacConkey Agar media (BD DIFCO USA) after brief enrichment for selective isolation of *Klebsiella*. Two Pink mucoid colonies for each sample were further streaked in slant for biochemical assays for identification of *Klebsiella spp* following the prescribed biochemical tests (Hansen et al., 2004).

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B. Species level confirmation by PCR

Biochemically confirmed *Klebsiella sp*. were subjected to PCR confirmation targeting some highly conserved regions of *Klebsiella* genus as *gyrA*; *pehx* and *rpoB* genes species specific identification of *K. oxytoca* and *K. pneumoniae*, respectively (Koovapra et al., 2016).

C. Antibiotic susceptibility assay

Antibiotic susceptibility assay were performed by an agar diffusion test using antibiotic paper discs (BD Difco/ Himedia) as described by the Clinical Laboratory Standards Institute (2018) using *E. coli* strain (ATCC 25922) as negative control. The following antibiotics were used in this study - ampicillin (10 mcg), amoxyclav (30 mcg), ceftriaxone (30 mcg), amikacin (30 mcg), chloramphenicol (30 mcg), trimethoprim-sulfamethoxazole (30 mcg), imipenem (10 mcg), cefotaxime (30 mcg), aztreonam (30 mcg) and ceftazidime (30 mcg).

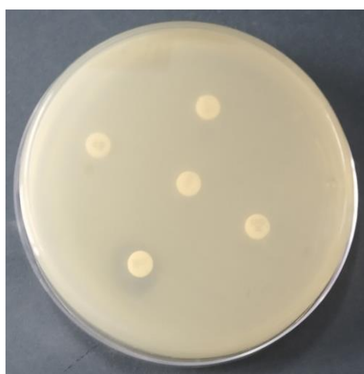

Fig 1: Representative picture for antibiotic sensitivity test.

D. Phenotypic determination of ESBL and ACBL

Isolates which were resistant or with low susceptibility to cephalosporin were further checked for ESBL expression by combination disc method using the discs containing 30 mcg of ceftazidime, cefotaxime and in combination with clavulanic acid (30 mcg /10 mcg) separately. The zone of inhibition more than 5 mm in diameter in presence of combination disc with clavulanate, indicate the ESBL producer. ACBL expression was evaluated among MDR isolates by cefoxitin disc test deploying cefoxitin (30 mcg) and cefoxitin-cloxacillin (30/20 mcg) discs. The inhibition zone more than 4 mm, in presence of cloxacillin consider as ACBL positive. (Koovapra et al., 2016).

E. Molecular diagnosis of antimicrobial resistance genes

Selected MDR isolates were subjected to molecular diagnosis by PCR for *bla* ESBL genes (*bla* $_{\text{TEM}}$, *bla* $_{\text{SHV}}$, *bla* $_{\text{CTX-M}}$), carbapenemase genes (*bla* $_{\text{OXA48}}$, *bla* $_{\text{KPC}}$ and *bla* $_{\text{NDM}}$) and ACBL gene. In addition, isolates were also screened depending on presence of genes for tetracycline resistance (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*), resistance phenomenon in mobile genetic element such as quinolone resistance genes (*qnrA*, *qnrB* and *qnrS*), integron gene (*int-1*), sulphonamide resistance genes (*sul1*).

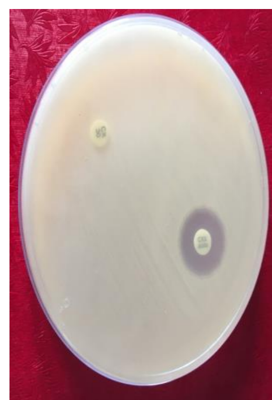

Fig 3: ACBL detection by using Cefoxitin and cefoxitin- cloxacillin disc .

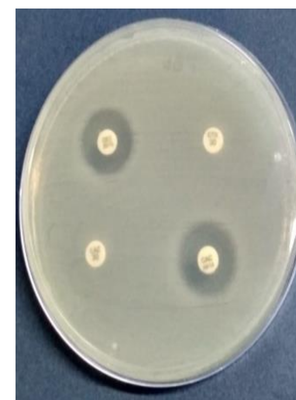

Fig 2: ESBL detection by combination disc diffusion method.

F. Cloning and sequencing

Amplicons for selected genes were cleaned up through nucleospin gel and PCR clean up kit (MN Takara) and cloned in pMD20-T (Mighty TA-cloning Kit,Takara) or in pTZ57/R (Fermentas, USA). The plasmids with expected insert were further subjected to sequencing in the Big Dye Terminator_ v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) on an automated sequencer machine (Applied Biosystems 3130 Genetic Analyzer) following the manufacturer's instructions. After successful sequencing homology were analyzed using BLAST algorithm available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

III. RESULTS

A. Phenotypic determination:

In the present study, 226 presumptive *Klebsiella* colonies were picked up from 113 water samples and 21 isolates were confirmed as *Klebsiella* by PCR of which 4 were *K. pneumoniae* and 17 were *K. oxytoca* (2 *K. pneumoniae*, 5 *K. oxytoca* from North 24 Parganas, 2 *K. pneumoniae*, 3 *K. oxytoca* from Kolkata and 9 *K. oxytoca* from Nadia respectively). A total of 19 (16.81%) isolates were categorized as MDR as they exhibited resistance to three or more group of antibiotics, whereas remaining 2 (*K. pneumoniae*) isolates were represented their resistivity to only Ampicillin and Amoxyclav. Among 19 MDR *Klebsiella*, only 8 (42.10%) *K. oxytoca* isolates exhibited ESBL and ACBL production; though, all of them were phenotypically resistant to Ampicillin, Amoxyclav Ceftriaxone, Cefpodoxime, Cefotaxime, Ceftazidime and Aztreonam. These isolates were frequently sensitive to Cotrimoxazole (89.47%), Amikacin (84.21%), Imipenem (78.94%) and Chloramphenicol (57.89%) respectively.

B. Genotypic screening:

The *bla* $_{\text{CTX-M}}$ was the most abundant gene found [16 (76.19%)] followed by AmpC (15) and *bla* $_{\text{TEM}}$ (4). Carbapenem-resistant gene *bla* $_{\text{NDM}}$ was found in 6 (28.57%) isolates and 4 out of 6 were *K. pneumoniae* and 2 *K. oxytoca*.

Other carbapenemase genes *bla* $_{KPC}$ or *bla* $_{OXA-48}$ was not found in any of the isolates. Other resistance markers such as *qnrA, qnrS, tetB, tetC, tetD, tetE* were found in none while *qnrB* and *tetA* was found in 2 (9.52%) *K. oxytoca* isolates. Sulfonamide resistant determinants, *sul-1*gene was found in 8 (38.09%) *K. oxytoca* candidates.

IV. Discussion

Although innumerable studies have been conducted on emergence and spread of MDR bacteria in India, those were mostly focused towards hospital-borne infection and studies on AMR in environmental setting is truly limited. This study tried to explore the status of such pathogens, in aquatic environment of West Bengal, India as occurrence of these resistant markers in ecosystem may further be transmitted to human and animal hosts. Recently, it was documented by some researchers that the healthy individuals may get infected with carbapenemase producing enterobacteriaceae during their travel to Indian subcontinent without any contact with health care premises (Ruppé et al., 2015). During our study, we found *bla* $_{CTX-M}$ (76.19%) as the most prevalent ESBL gene in the MDR isolates from water as recorded in a previously conducted study in Sweden (Hessman et al., 2018) followed by *bla* $_{TEM}$ (19.04%) and AmpC (71.42%). The *bla* $_{TEM}$ gene was detected only among 19% of the isolates; however previous workers reported it frequently in environment even after processing of the waste water (Lachmayr et al., 2009). Prevalence of metallo-beta-lactamase is 19.04% in our study which is low in comparison to a similar kind of study performed in Bangladesh where they found 50% of waste water samples were positive for carbapenem-resistance enterobacteriaceae (Islam et al., 2017),(Shah & Zahra, 2014) but persistence of any kind of carbapenemase in fresh water ecosystem is a serious public health issue (Sivalingam et al., 2019). Other resistant determinants that were detected in this study were *sul-1* (38.09%), *tetA* and *qnrB* (9.52% each). This observation is much lower than what was noted by other workers in estuarine ecosystem and in livestock-farm environment in China (Ou et al., 2015), (Liu et al., 2019). Among the qnr gene(s), only *qnrB* was detected in the collected water samples. However, the *qnrB* is not very frequently detected genes, although it was reported in some countries from environmental waste water samples (Chen et al., 2019). Our finding indicates low sulphonamide and tetracycline resistant genes in our pond ecosystem which is an indication of less exposure of these antibacterial substances in environment. The present investigation was a preliminary pilot study, and further intensive investigation should be carried out to determine the possible gene transfer mechanisms. Thus, it may be concluded that an appropriate planning is required to reduce the load of antimicrobial substances in our ecosystem, simultaneously, proper treatment required for municipal and hospital waste water to improve the water quality (Saba Riaz, 2012).

Conclusion

Drug resistant pathogens and their capability of spreading antibiotic resistance genes is a serious issue for mankind. Injudicious use of antibiotics is one of the major reasons. No therapeutics will be available in near future if such practices go on and such study of environmental microflora in aquatic ecosystem has an immense importance in aspects of human and animal health as all discharges are ultimately mixed up in the environment.

Acknowledgements

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Table-I: Identification of isolates through Biochemical test.

Isolate	Indole	M.R	V.P	Citrate	Glucose	Adonitol	Arabinose	Lactose	Sorbitol	Mannitol	Rhamnose	Sucrose	Remarks
PK1	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK2	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK3	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK4	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK5	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK6	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK7	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK8	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK9	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK10	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK11	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK12	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK13	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK14	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK15	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK16	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK17	+	+	+	+	+	+	+	+	+	+	+	+	*K.oxytoca*
PK18	-	+	+	+	+	+	+	+	+	+	+	+	*K.pneumoniae*
PK19	-	+	+	+	+	+	+	+	+	+	+	+	*K.pneumoniae*
PK20	-	+	+	+	+	+	+	+	+	+	+	+	*K.pneumoniae*
PK21	-	+	+	+	+	+	+	+	+	+	+	+	*K.pneumoniae*

Table II: Determination of resistance genes in *Klebsiella* isolates

ISOLATE	SPECIES	CTXM	TEM	NDM1	AMPC	QNRB	TETA	SUL1
PK1	K.O	+	-	-	+	+	+	+
PK2	K.O	+	-	-	+	+	-	-
PK3	K.O	+	-	-	+	-	+	-
PK4	K.O	+	-	-	+	-	-	+
PK5	K.O	+	-	-	+	-	-	+
PK6	K.O	+	-	-	+	-	-	+
PK7	K.O	+	-	-	+	-	-	+
PK8	K.O	+	-	-	+	-	-	-
PK9	K.O	+	+	-	+	-	-	-
PK10	K.O	+	+	-	+	-	-	-
PK11	K.O	+	-	-	+	-	-	-
PK12	K.O	+	-	-	-	-	-	-
PK13	K.O	+	-	-	-	-	-	-
PK14	K.O	+	-	-	+	-	-	-
PK15	K.O	+	-	-	+	-	-	-
PK16	K.O	+	+	+	+	-	-	+
PK17	K.O	-	+	+	+	-	-	+
PK18	K.P	-	-	+	-	-	-	-
PK19	K.P	-	-	+	-	-	-	-
PK20	K.P	-	-	+	-	-	-	-
PK21	K.P	-	-	+	-	-	-	-

Table III: Primer and PCR reaction conditions

Gene	Primer sequence	Amplicon size(BP)	PCR cycle	Reference
bla TEM	F: ATG AGT ATT CAA CAT TTC CG R: CTG ACA GTT ACC AAT GCT TA	867	95 x 5 m/95 x1m – 55 x1m – 72 x 1m (35 Cycles)/72 xx10 m	(Kar et al., 2015)
bla CTXM	F: TTT GCG ATG TGC AGT ACC AGT AA R: CGA TAT CGT TGG TGG TGC CAT A	540	94× 2 m/ 95× 20 s – 51× 30 s – 72×30 s (35 Cycles) 72×3 m	(Kar et al., 2015)
bla SHV	F: AGG ATT GAC TGC CTT TTT G R: ATT TGC TGA TTT CGC TCG	393	94 × 5 m/94 × 30 s-60 × 30 s-72 × 30 s (20 Cycles)/94 × 30 s-58 × 30 s-72 × 30 s (15 Cycles)/72 × 7 m	(Kar et al., 2015)
AmpC	F: CCC CGC TTA TAG AGC AAC AA R: TCA ATG GTC GAC TTC ACA CC	631	94 × 5 m/94 × 30 s-58 × 2 m-72 × 60 s (10 Cycles)/94 × 30 s-55 × 1 m- 72 × 60 s (15 Cycles)/94 × 30 s-50 × 30 s-72 × 1 m (10 Cycles) 72 × 10 m	(Kar et al., 2015)
bla OXA48	F: TGTTTTTGGTGGCATCGAT R: GTAAMRATGCTTGGTTCGC	177	95 x 5 m/95 x 30 s-57 x 40s -72 x1 m (35 Cycles)/72 x 10 m	(Monteiro et al., 2012) 2012
bla NDM	F:GGGCAGTCGCTTCCAACGGT R:GTAGTGCTCAGTGTCGGCAT	475	95 x 5 m/95 x 30 s-52x 40s -72 x1 m (35 Cycles)/72 x 10 m	(Anjana Shenoy et al., 2014)
bla KPC	F: TCGCTAAACTCGAACAGG R: TTACTGCCCGTTGACGCCCAATCC	785	94 × 5 m/94 × 1 m-60 × 1 m-72 × 1 m (35 Cycles)/72 × 10 m	(Koovapra et al., 2016)
tetA	F:GCT ACA TCC TGC TTG CCT TC R:CAT AGA TCG CCG TGA AGA GG	210	95 x 5 m/95 x 30 s-55 x 1 m-72 x1 m (35 Cycles)/72 x 10 m	(Ng et al., 2001)
tetB	F:TTG GTT AGG GGC AAG TTT TG R:GTA ATG GGC CAA TAA CAC CG	659	95 x 5 m/95 x 30 s-55 x 1 m-72 x1 m (35 Cycles)/72 x 10 m	(Ng et al., 2001)
tetC	F:CTT GAG AGC CTT CAA CCC AG R:ATG GTC GTC ATC TAC CTG CC	418	95 x 5 m/95 x 30 s-55 x 1 m-72 x1 m (35 Cycles)/72 x 10 m	(Ng et al., 2001)
tetD	F:AAA CCA TTA CGG CAT TCT GC R:GAC CGG ATA CAC CAT CCA TC	787	95 x 5 m/95 x 30 s-55 x 1 m-72 x1 m (35 Cycles)/72 x 10 m	(Ng et al., 2001)
tetE	F:AAA CCA CAT CCT CCA TAC GC R:AAA TAG GCC ACA ACC GTC AG	278	95 x 5 m/95 x 30 s-55 x 1 m-72 x1 m (35 Cycles)/72 x 10 m	(Ng et al., 2001)
qnrA	F: ATT TCT CAC GCC AGG ATT TG R: GAT CGG CAA AGG TTA GGT CA	516	95 × 5 m/95 × 1 m-56 × 1 m-72 × 1 m (35 Cycles)/72 × 10 m	(Kar et al., 2015)
qnrB	F: GAT CGT GAA AGC CAGAAAGG R: ATG AGC AAC GAT GCC TGG TA	476	95 × 5 m/95 × 1 m-56 × 1 m-72 × 1 m (35 Cycles)/72 × 10 m	(Kar et al., 2015)
qnrS	F: GCA AGT TCA TTG AAC AGG GT R: TCT AAA CCG TCG AGT TCG GCG	428	95 × 5 m/95 × 1 m-56 × 1 m-72 × 1 m (35 Cycles)/72 × 10 m	(Kar et al., 2015)
Sul1	F: CGG CGT GGG CTA CCT GAACG R: GCC GAT CGC GTG AAG TTC CG	433	95 × 5 m/95 × 30 s-65 × 1 m-72 × 1 m (35 Cycles)/72 × 10 m	(Kar et al., 2015)
gyrA	F: CGC GTA CTA TAC GCC ATG AACGTA R: ACC GTT GAT CAC TTC GGT CAG G	441	95 × 5 m/95 × 30 s-55 × 1 m-72 × 2 m (35 Cycles)/72 × 10 m	(Koovapra et al., 2016)
pehX	F: GAT ACG GAG TAT GCC TTT ACGGTG R:TAG CCT TTA TCA AGC GGA TACTGG	343	95 × 3 m/95 × 30 s-55 × 1 m-72 × 2 m (35 Cycles)/72 × 10 m	(Koovapra et al., 2016)
rpoB	F: CAA CGG TGT GGT TAC TGA CG R: TCT ACG AAG TGG CCG TTT TC	108	95 × 3 m/95 × 30 s-55 × 1 m-72 × 2 m (35 Cycles)/72 × 10 m	(Koovapra et al., 2016)

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