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ISOLATION AND CHARACTERIZATION OF *Proteus* spp. FROM DOMESTIC DISPOSAL EXPRESSING EXTRACHROMOSOAL EXTENDED SPECTRUM β-LACTAMASE TRAIT

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Abstract- From a long time waterborne diseases have been major concern to living being. Handful countries have successfully implemented recycling of water. Recycling of water is under consideration in India. Indian rural region is yet to establish domestic disposal and sewage treatment plant. Number of villages due to insufficient rain depends largely on bore well water, a type of hard water. Pumped water off the bore well is coming from underground depth of which varies from village/town to town. We have isolated and characterized an enteric bacterium from domestic disposal, which exhibits Extended Spectrum β-Lactamase (ESBL) trait. The domestic disposal was enriched with triple strength MacConkeys broth. Cultural and biochemical characterization of various isolates was carried as per Bergeys Manual of Systematic bacteriology. Based on Morphological, Cultural, and Biochemical characters these isolates were *Citrobacter spp, Proteus rettgeri, Proteus morganii, Providencia spp, Escherichia coli, Klebsiella spp* and *Hafnia alvei*. All of these isolates were screened to determine resistance to third generation cephalosporins such as; Cefotaxime (CTX), Cefepime (CPM) and Ceftazidime (CAZ). Amongst ten isolates, AKA10 strain of *Proteus spp,* exhibited resistance to β-lactms as Penicillin, Ampicillin, Oxacillin Coxacillin and third generation cephalosporins. Phenotypic classification indicated that ESBL trait belongs to OXA-48 type. Genetic manipulations A. Plasmid curing and B. plasmid transformation indicated that ESBL trait is extrachromosomal and contains a broad host range replicon, perpetuating not only in *Proteus* but also in *E. coli*. Implications of ESBL trait in domestic disposal are discussed.

Keywords- Domestic disposal, *Enterobacteriaceae*, Disc diffusion, ESBL, plasmid curing

Running Title: Isolation of ESBL strain from domestic disposal

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Introduction

From long back water born disease are most prevalent in under developed countries or developing world and treating them remains a challenge for clinicians [1-7]. Earlier studies have shown that major cause of these diseases was due to improper sanitary conditions and fecal contamination [8-14]. Researchers have also reported that surface water from under developed world is facing challenge of contamination with heavy metal and other chemicals at concentrations more than permitted [15-22]. This is known to augment risk of affected metabolism, carcinogenesis, stress on liver function [23,24]. Majority of rural area in India is still unable to follow sophisticated methods for domestic disposal. The domestic disposal hence is contaminated with number of house hold activity components, including traces of detergents, food material and spent water [23-25]. Although fecal contamination is almost unobvious, however, presence of food material/ or food particle in water are sufficed to support the growth of saprophytic, oligophilic organisms and opportunistic pathogens. Although multicellular organisms are known to die due to pollution, on the contrary micro-organisms are able to adapt quite efficiently and manage to grow pretty well. Where ever improvements were made towards improved hygiene and water treatment has been known to correlate health positively.

Over 8 decades the use of antibiotic to treat infection is a common practice worldwide. In the beginning applications of penicillin was practiced before Walksman coined term antibiotic. Medical associations have indicated conditions when prescription of antimicrobial should be favored. In developing world use of antimicrobials with bypass of these indications could not be ruled out. Prophylactic and sometimes imperical treatment with antimicrobials is in excess and unjustified. Surgical wards and number of hospitalizations at times are prescribed with antimicrobials primarily to avoid postsurgical as well as community acquired infections. Without antibiotics, these procedures would be unthinkable owing to augmented infection risk.

First generation β-lactams are well known narrow spectrum antibiotics targeting bacterial peptidoglycon. Interestingly third generation cephalosporins are known to control Gram negative infection in addition to Gram positive if applied during active phase. This fact has made third generation cephalosporins as the drug of choice for many clinicians world wide [26-29]. First β-lactam resistance was reported during early 60s [31]. Since 1964 antibiotic resistant bacte-

ria have been recovered from various sources [32]. Interestingly, bacteria conferring resistance not only to primary β-lactams but also to third generation cephalosporins have been recovered from patients suffering with various illnesses [35-42]. The ESBL strains are also recovered from surgical ward, burn ward and through hospital born infections [43-47]. The ESBL traits have also been found to be present in nasocomial infection causing organisms [48-52]. Multidrug resistance or resistance to third generation cephalosporin by bacterial pathogen demanded search for fourth generation cephalosporins [53-57]. Wealth of information is available on classification of ESBL trait bearing organisms [58,59]. In majority cases ESBL has been known to be extrachromosomal and is known to be transferred not only vertically but also horizontally; across species & genera [60]. Infections are passed from one person to other through aerosols, water, milk, food and air.

Recently rain patterns have been changed in parts of world including India, water table level is going deeper gradually. Although provision of treated potable water is a need of time, however, it is not possible for all rural parts. An open well or bore well are common options for agricultural and domestic purpose in rural parts of India. Bore wells are dug up to 300 feet below. Chemicals percolated from natural soil and stone capillaries serve nutrients for oligophilic bacteria. Regions with poor water facility largely rely on bore-well water at least for domestic purpose if not for drinking. Domestic disposal containing minerals, biological ingredient is known to support bacterial growth. The domestic waste passed into sever lines is also known to account a great portion of sewage in addition to industrial effluent. Therefore while estimating water pollution domestic disposal coming from household source should also be addressed along side industrial waste. Domestic disposal may contaminate reservoir through percolation. In present study we focused our attention to isolate enteric bacteria exhibting ESBL trait from domestic disposal and characterize genetically as to trait is chromosomal or extrachromosomal.

Materials and Methods

Sample Collection

For isolation of enteric bacteria water samples were collected aseptically from different locations. Disposed water that is being sent in common sever line were collected from about 1 feet distance. Also household disposed water (domestic disposal) through small gutters were also collected from a feet depth, specifically from the region where bore well water is primary source for domestic purpose. For isolation of the bacteria three various spots were considered from Osmanabad city (MS, India). In a sterile 20 ml vials water was collected, immediately upon arrival to the laboratory an aliquot of one ml was spun through sterile 1.5 ml eppendorff tubes at 5000 xg for 1 minute. About 0.1 ml of supernatant was inoculated for enrichment.

Enrichment and Isolation

It is anticipated that domestic waste generated from treated water or potable water is less likely to have enteric bacteria. Also various different types of saprophytic bacteria may exist in soil contaminated domestic disposal. We designed a strategy to avoid non enteric bacteria with the inclusion of selective medium for enrichment. Although number of enrichment media for different *Enterobacteriaceae* genera are likely, use of MacConkey's broth is however known to inhibit growth non- enteric bacteria. For enrichment a slight modifications were made in MacConkey's composition. The Na.taurocholate concentration was increased from 0.5% to1.0%, 1.5% and 2.0% in four independent enrichment trials. By increasing higher amount of bile salt it was expected to have total absence of growth of non-enteric bacterium those may tolerate 0.5% routine amounts. Modified sterile MacConke's broth (5ml) containing 1.5% and 2.0% Na. taurocholate were inoculated with 0.1% of water sample collected from different spots [Table-1]. Tubes were subjected for incubation at 37ºC for 24 hrs. Enrichment was confirmed with turbidity in inoculated tubes when compared to un-inoculated clear tubes.

Table 1- Water samples used for enrichment and isolation of enteric bacteria from domestic disposal

Sr. No.	Quantity (ml)	% of Na taurocholate			LB broth (ml)
Sample1	0.1	1%	1.50%	2%	5
Sample2	0.1	1%	1.50%	2%	5
Sample3	0.1	1%	1.50%	2%	5

A loopfull growth was then streaked on to MacConkey's agar supplemented with 0.5% Na. taurocholate. Colonies appeared after 24 hours of incubation at 37ºC were considered for further studies. Lactose fermenting pink colonies, non Lactose fermenting pale colonies and green fluroscent and pyocynin pigment producing colonies were selected and were used for biochemical analysis.

Bacterial Characterization

For bacterial identification it is important to study their morphological, cultural and biochemical properties. Phenotypically different colonies observed as to pink, pale or with other pigment were collected. These colonies were processed for study with reference to their size, shape, color, margin, elevation, opacity, and consistency. From single colony bacterial suspension was prepared in 0.85% saline, a loop-full suspension was then used for knowing Grams' character and ability to perform motility with hanging drop preparation.

Few biochemical tests, those were helpful in discriminating popular genera and species within them were selected from Bergeys Manual of Systematic Bacteriology. As per prescription these biochemical tests were performed either in solid agar, agar butts, or in broth. Most of the biochemical reactions were made in specially designed medium for them. For each biochemical test appropriate controls were performed, inoculation of positive control and un-inoculated as blank control. Observations were recorded in tabular form and compared to that standards provided in Bergey's determinative bacteriology

Antibiotype Estimation with Agar Disc Diffusion Method

Stated herein is the list of antibiotic discs and their concentration in each disc. Discs containing Ampicillin 10 µg, Penicillin 10 units, Tetracycline 30µg, Oxacillin 1µg, Cloxacillin 30 µg, Cefotaxime 30 µg, Cefepime 30 µg, and Ceftazidime 30 µg. Whether these isolates exhibited susceptibility or resisted the above antimicrobials was tested with Kirby and Buayers agar disc diffusion method. Disk diffusion susceptibility test were performed on MacConkey´s agar plates. The test strains were pre-incubated in macConkey´s broth at 37ºC for 24 hrs. Selective dilution was used for spreading. An aliquot of 0.1 ml was spread inoculated on MacConkey´s agar plates, 4 antibiotic discs were placed in 4 sectors of each plate. Plates were subjected for antibiotic diffusion at 4ºC for 15 minutes. Plates

were then incubated at 37ºC for 24 hrs for appearance of inhibitory zone around the disc against matt growth in remaining parts of plate. Zone of inhibition around disc was measured and results were interpreted for susceptibility and resistance as per standards provided by Clinical Laboratory Standards Institute (CLSI). A clear cut extension of the inhibition zone of defined size around the antibiotic disc was interpreted as susceptible to a particular antibiotic. When inhibition zones around the disc were small or absent then result is interpreted as intermediate and resistant respectively.

Plasmid Curing

Enteric bacteria exhibiting multidrug resistance including third generation cephalosporins, were considered for further investigation and used in plasmid curing experiment. These multidrug resistant colonies were grown in MacConkey´s broth supplemented with 25 µg/ml Ethidium bromide overnight at 37ºC. Ten fold serial dilutions up to 10^{-5} to 10^{-7} were made from overnight grown cultures, aliquot of 0.1ml was spread inoculated on 24 hrs old MacConkey´s agar plates. Plates were subjected for incubation for 24 hrs at 37ºC for the appearance of colonies. Ten well isolated colonies from above were selected and inoculated individually in 3-5 ml of MacConkeys broth in separate test tubes and incubated for 24 hrs at 37ºC, were processed for antibiotic susceptibility along with its parent colony culture. Inhibition zones around antibiotic disc for these 10 colonies were compared to that of their parent strain. If one or more than one of these single individual culture now has lost resistance to cephalosporin or other antibiotic was considered to carry resistance gene on to plasmid rather than being on chromosome.

Results and Discussion

Isolation and Characterization of Pathogens

A total of 10 bacteria belonging to different genera were isolated from domestic disposal from selected site of Osmanabad city. Based on their morphological, cultural, and biochemical studies on IMViC, Triple Sugar Iron agar slant, and ability to utilize tested sugars results are shown in [Table-2], these strains belonged to the family *Enterobacteriaceae* and are *Escherichia coli* (n=3), *Proteus rettgerri* (n=1), *Proteus morganii* (n=1), *Proteus spp* (n=1) *Klebsiella edwardsii* (n=1), *Hafnia alvei* (n=1), *citrobacter spp* (1) *Providencia spp* (n=1) [Table-3]. Based on our observations domestic disposal were found to be contaminated with *E.coli, Proteus, Klebsiella, Citrobacter, Hafnia and Providencia*. Isolates were inoculated into tryptone broth, production of indole was determined with the use of Kovac's reagent upon xylene extraction. Ability to produce mixed acid or combination of mixed acid and neutral product when glucose was being fermented was tested by growing isolates into glucose phosphate broth. Grown cultures were subdivided in equal half and each of these half grown culture was used to detect mixed acid with a pH indicator methyl red and other for detection of neutral products by saturating alkalinity with KOH followed by use of α-naphthol. Whether or not these enteric bacteria could use citrate as a sole carbon source was tested by checking their growth on Simmon's citrate agar slant. Results of these biochemical tests are shown in the [Table-2]. Whether these isolates are able to use urea and produce ammonia by the action of enzyme urease was tested by growing bacteria on Christensen's urea agar with Phenol red as pH indicator.

Table 2- Biochemical Tests and performance

Test	AKA01	AKA02	AKA03	AKA04	AKA05	AKA06	AKA07	AKA08	AKA09	AKA10
Indole	+	+	+	-	+	+	+	+	+	+
Methyl Red	+	-	-	+	+	+	+	-	-	-
Vogues Proskauer	+	-	-	-	-	-	-	+	-	-
Citrate	+	-	-	+	-	-	-	+	+	+
Urease	-	+	+	-	-	-	-	+	-	-
Phenyl Alanine Deamination	+	+	+	-	-	-	+	-	-	-
H2S in TSI	+	+	+	+	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
Mannitol	+/-	+/-	-	+/-	+/+	+/+	+/+	+/+	-	-
Dulcitol	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Salicin	-	-	-	-	-	-	-	-	-	-

+ indicates positive test, - indicates negative test, +/- indicates sugar fermentation with acid but no gas (anaerogenic), +/+ indicate sugar fermentation acid and gas (aerogenic)

Table 3- Identification of enteric bacteria isolated from domestic disposal

Sr. No.	Isolate	Name of the isolate
1	AKA01	*Citrobacter spp*
2	AKA02	*Proteus rettgerri*
3	AKA03	*Proteus morganii*
4	AKA04	*Hafnia alvei*
5	AKA05	*E. coli*
6	AKA06	*E.coli*
7	AKA07	*E.coli*
8	AKA08	*Klebsiella edwardsii*
9	AKA09	*Providencia spp*
10	AKA10	*Proteus spp*

Magenta colored slants of strain AKA02, AKA03 and AKA08 are the only three of the ten strains could produce Urease enzyme. When tested for ability to deaminate phenylalanine it was apparent that AKA02, AKA03 and AKA08 were able to produce phenyl-alanine-deaminase enzyme shown in [Table-2]. Growth of these ten isolates in Triple Sugar Iron agar slant indicated that AKA01, AKA02, AKA03 and AKA04 could produce H_2S gas.

Ability to ferment Inositol, Salicin, Mannitol and Dulcitol aerobically or anaerobic was tested in sugar fermentation medium supplemented with inverted durmas tube within. Results recorded after 24 hrs incubation shown in [Table-2], indicated that none of these ten isolates could ferment Inositol or Salicin (aryl β-glucoside). On the contrary all of the ten isolates could ferment Dulcitol. Fermentation of Mannitol by AKA05, AKA06, AKA07 and AKA08 followed respira-

tion pattern for facultative anaerobe. Strains AKA03, AKA09 and AKA10 could not utilize mannitol while the AKA01 and AKA02 could metabolize mannitol by anaerogenic mode as shown in [Table-2].

Determination of ESBL Phenotype to Enteric Bacteria from Domestic Disposal

One of the major objectives of this exercise was to determine as to whether these isolates exhibit ESBL anitbiotype phenotype. The ESBL phenotype to these isolates was attributed by performing antimicrobial assay towards selected antibiotics largely from β-lactam such as; penicillin, ampicillin, Oxacillin, Coxacillin, partially broad spectrum and third generation β-lactam members namely Ceftazidime, Cefepime and Cefotaxime along with a broad spectrum antibiotic Tetracyclin. Sensitivity was processed as per Kirby, Buayer 1966 disc diffusion assay, inhibitory zones obtained around disc were compared with the standard recommended zones for Gram negative Enteric bacteria CLSI. Isolates showing ESBL positive phenotype rendered resistance to the third generation cephalosporins and sometime to all the antibiotics used in this study. Results shown on sensitivity pattern in [Table-4] suggest that most of these isolates exhibited resistance to penicillin, ampicillin, coxacillin and oxacillin however with an exception of AKA09 and AKA10 they remained sensitive to tetracycline. The AKA09 was resistant to tetracycline but sensitive to cefotaxime and cefepime. The AKA10, strain of *Proteus spp* was found to be resistant to all cephalosporins shown in [Fig-1]. Interestingly AKA10 though exhibited resistance to β-lactams and cephalosporins tested, it remained sensitive to Tetracycline. The AKA10 was thus confirmed to exhibit ESBL antibiotype phenotype and was considered for further studies.

To the best of our knowledge for the first time we have found that domestic disposal which is not having access to hospital discharge is known to contain enteric bacterium exhibiting ESBL trait. Number of researchers around the globe has successfully identified their existence in hospital wards [7,14,17,36,41,53,58] in patients suffering from wide range of infections and in some cases post-operative surgical complications [35,52]. In most of these cases ESBL trait was known to be carried on episome in stead of bacterial genome, for recent review [12].

Table 4- Sensitivity pattern by agar well diffusion method

Strains	Isolate	TE	P	OX	AMP	CPM	COX	CAZ	CTX
Citrobacter spp	AKA01	S	R	R	R	S	R	I	S
Proteus rettgerri	AKA02	S	R	R	R	S	R	I	S
Proteus morganii	AKA03	S	R	R	R	I	R	R	R
Hafnia alvei	AKA04	S	R	R	R	I	R	S	R
E.coli	AKA05	S	R	R	S	S	R	I	I
E.coli	AKA06	S	S	R	S	S	R	S	I
E.coli	AKA07	I	R	R	S	S	R	S	R
Klebsiella edwardsii	AKA08	S	R	R	S	S	R	S	S
Providencia spp	AKA09	R	R	R	S	S	R	R	R
Proteus spp	AKA10	R	R	R	S	R	R	R	R

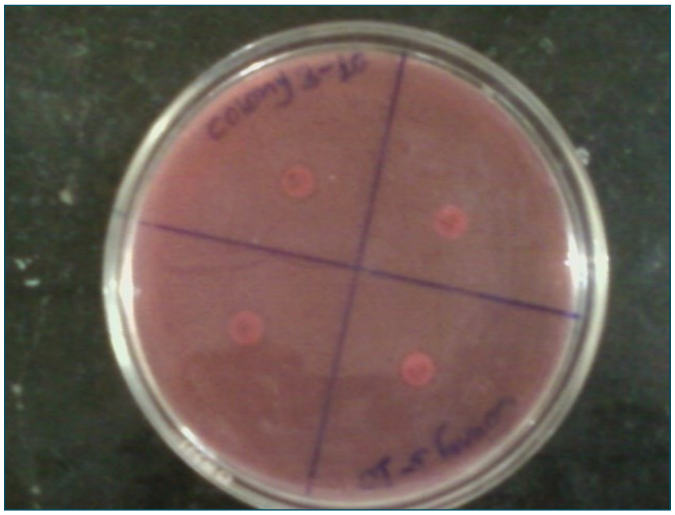

Fig. 1- Chosen dilution of AKA10 was spread inoculated on MacConkey's agar plate. Discs for Cefotaxime (CTX), Cefepime (CPM), Ceftazidime (CAZ) and Tetracyline (TE) were placed in four sectors, incubated to realize zone of clearance around the disc.

Classification of ESBL Trait from AKA10

Strain AKA10 was used to investigate the class of Extended Spectrum β-Lactamase trait expressed by it. Bacterial culture was processed to investigate for determination of presence of New Delhi Metalloprotease type Carbapenemase (NDM Class) with Ertapenem and Meropenem with sensitive strain *E. coli* ATCC 52922. In brief, sensitive strain of *E. coli* ATCC 52922 was spread inoculated on the agar plate, disc of Meropenem or Ertapenem was placed in the center of plate. Streak of AKA10 was drawn from periphery into center, incubated for 24 hrs at 37ºC. If NDM type carbapenemase is produced by test strain it would degrade Ertapenem or Meropenem allowing ATCC 52922 strain to grow close to the disc. Results obtained with AKA10 suggested that it did not express NDM type carbapenemase. The Metallo β-Lactamase (MBL type) ESBL trait was tested by placing the disc of Imipenem and Imipenem with EDTA. Strain AKA10 was also processed to determine whether the ESBL trait belongs to AmpC type by using discs of Cefoxitin and Cefoxitin with Phenylboronic acid. Sensitive of AKA10 was enhanced with combined application in a disc cefoxitin and Phenylbornic acid (data not shown). Because the AKA10 strain of *Proteus spp* found not to augment zone of clearance in AmpC testing or did not indicate existence of MBL type trait suggest that belongs to OXA-48 class β-lactamase.

Plasmid Curing with Ethidium Bromide

Acridine dye, ethidium bromide (EtBr) is an intercalating agent known to impart frameshift mutations in concentration dependent manner. As the concentrations of EtBr is increased then it is likely to increase number of hits within the genome and may produce large genetic rearrangements, which are at times difficult to repair

with the existing DNA safeguarding and repair machinery. On the other hand at sub mutagenic concentrations with reference to chromosome EtBr although is not likely impart mutations on the genome it will still be able to target low molecular weight linear and circular DNA including extrachromosomal genomes. Inhibitory effect for EtBr on AKA10, *P. morganii* was performed both by turbidometric and by total viable count (data not shown). When AKA10 strain was grown in LB supplemented with 20 times lower the inhibitory concentration of EtBr it was expected that it is less likely to induce mutations on the genome, however it may continue to hinder perpetuation of extrachromosomal genome. Bacteria grown for 18 hr in the presence of EtBr were spread inoculated well isolated ten colonies were used to determine antibiotic sensitivity assay as described in marials and methods. Observations recorded after 24 hours [Table-5] demonstrated that three out of 10 independent cultures had lost resistance capacity to cefotaxime, cefepime, ceftazidime as well as Tetracycline. [Fig-2] shows that parent strain AKA10 [Fig-2](A) is resistant to all third generation cephalosporins and tetracycline whereas AKA10-4, representative cured isolate exhibited sensitivity to the same cephalosporins [Fig-2](B). Loss of resistance to these above antibiotics in AKA10-4, AKA-10-8 and AKA10-9 clones indicate that resistance to third generation cephalosporin in AKA10 is located on extrachromosomal genome.

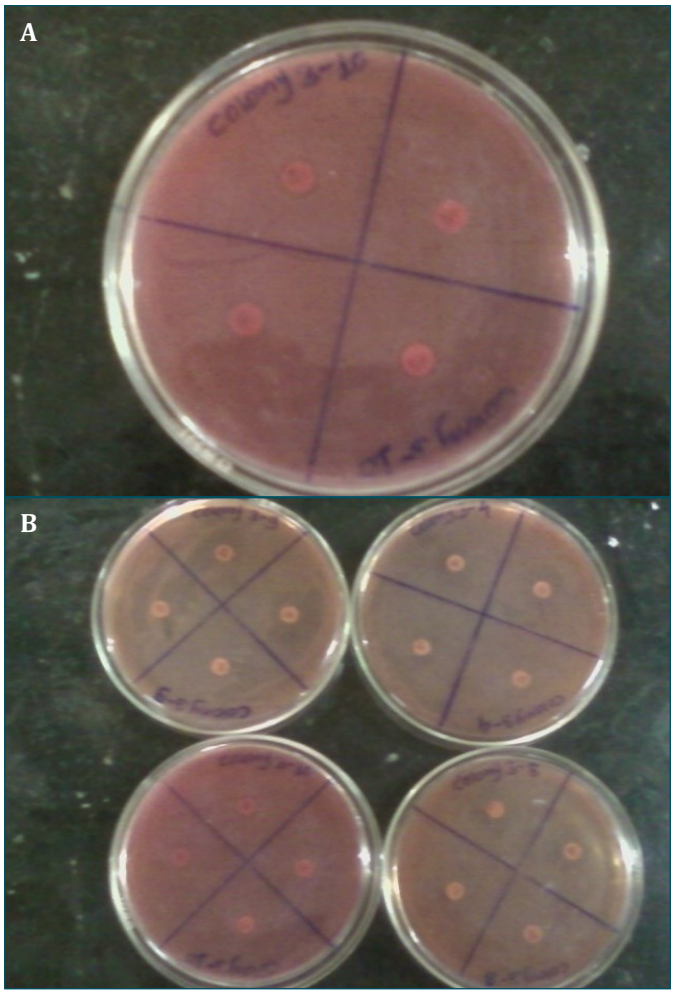

Fig. 2- Discs for CTX, CPM, CAZ and TE were placed in four sectors on spread inoculated MacConkey's agar plate. Panel A for AKA10 uncured domestic isolate. Panel B. AKA10-4 cured derivative of AKA10.

Table 5- AKA10 cured clones and sensitivity pattern (cephalosporin)

Isolate	TE	CPM	CAZ	CTX
AKA10-1	R	R	R	R
AKA 10-2	10	R	R	R
AKA 10-3	10	R	R	R
AKA 10-4	S	S	S	S
AKA 10-5	10	R	R	R
AKA 10-6	10	R	R	R
AKA 10-7	10	R	R	R
AKA 10-8	S	S	S	S
AKA 10-9	S	S	S	S
AKA 10-10	10	R	8	R

Transformation of Cephalosporin Resistance of AKA10 to as Laboratory Strain of *E. coli*

To substantiate our observations on Etbr mediated plasmid curing we sought test whether or not this plasmid could be horizontally mobilized from AKA10 to laboratory strain of *E. coli* DH5α. Plasmid DNA was isolated from AKA10 with alkaline lysis method and aliquot of plasmid DNA preparation was transformed to DH5a competent cells. Transformation mixture spread on LB agar supplemented with 30 mg/ml cefotaxime could recover a few ten colonies. One of these transformants AKA10-T1 was checked for its antibiotype on LB agar supplemented with desired concentrations of Cefotaxime, Cefepime and Ceftazdime independently. When streaked on LB Cefotaxime, LB Cefepime and LB Ceftazidime the transformant AKA10-T1 was able to grow on all plates, suggesting that cephalosoporin resistance was transferred from AKA10 to laboratory strain of *E. coli* (data not shown). This observation also indicates that origin or replication present on plasmid is of broad host range type, not limiting to *Proteus spp* but perpetuates in *E. coli* too.

Both these genetic experiments removal of plasmid from AKA10 parent strain resulting in sensitivity to third generation cephalosporin, cured strain AKA10-4 and secondly, plasmid prepared from AKA03 could be moved in to *E. coli* causing conferring transformant AKA10-T1 resistant to cephalosporins demonstrates existence of ESBL trait on the plasmid. These experiments are insufficient however to estimate if the same plasmid also has ability to engage conjugation.

Study of Growth Pattern of Plasmid Cured and Non-cured Strains

Energy is required for growth and metabolism. Maintenance of extrachromosomal genome with specified copy number requires enzymatic machinery. We sought to address growth pattern for parent strain and cured clones; AKA10 and AKA10-4, AKA10-8 and AKA10-9, respectively. Single colonies of these strains were grown overnight in LB broth. Fresh sterile LB medium was inoculated with overnight grown culture and followed for growth curve. When grown in LB broth it was observed that AKA10-4, AKA10-8 and AKA10-9 cured strains grew faster than AKA10, parent strain. Interestingly stationary phase for parent and cured strains was attained at the same optical density (data not shown). Albeit relatively slow growth of AKA10 to that of cured clones, cell density at the onset of stationary phase for parent and cured strain was same (data not shown). However, slight slow growth for AKA10 suggest that maintenance of extrachromosomal genome in this strain required more energy and cured clones could manage without additional energy expenditure.

For the first time we are reporting existence of ESBL trait in a *Proteus spp* from domestic disposal. Most of the ESBL strains as of now identified are reported from clinical background and sewage effluents. They are obtained from the samples/ specimens from or around the hospitals. Majority of the ESBL enteric bacteria are from *Klebsiella* and *E. coli*. A few reports of *Proteus* species are there from various clinical studies performed around the globe.

Conflicts of Interest: None declared.

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