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ORIGINAL ARTICLE

Pig farm environment as a source of beta-lactamase or AmpC-producing *Klebsiella pneumoniae* and *Escherichia coli*

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Abstract

The present study was undertaken to detect the occurrence of beta-lactamase-/AmpC-producing *Klebsiella* and *Escherichia coli* in healthy pigs, feed, drinking water, and pen floor or surface soil. The study also intended to detect the clonal relationship between the environmental and porcine isolates to confirm the route of transmission. Rectal swabs and environmental samples were collected from apparently healthy pigs kept in organized or backyard farms in India. The pigs had no history of antibiotic intake. Production of phenotypical beta-lactamase, associated genes, and class I integron gene was detected in *E. coli* and *Klebsiella* isolates. The phylogenetic relationship among the isolates was established on the basis of Random amplification of polymorphic DNA banding pattern. Beta-lactamase-producing *Klebsiella* were isolated from healthy pigs (20.0%), pen floor swabs/surface soil swabs (14.0%), and drinking water (100%). *Escherichia coli* isolated from healthy pigs (14.4%), pen floor/surface soil (8.0%), and drinking water (33.3%) were detected as beta-lactamase producers. Majority of beta-lactamase-producing isolates possessed $bla_{CTX\text{-}M\text{-}9}$. Further, 35 (81%) *Klebsiella* and all the *E. coli* isolates were detected as AmpC beta-lactamase ACBL producers and possessed bla_{AmpC}. Sixteen beta-lactamase-producing *Klebsiella* (37.20%) and 13 *E. coli* (86.67%) possessed class I integron. Few resistant isolates from environmental sources (surface soil swab and drinking water) and the studied pigs were detected within the same cluster of the dendrogram representing their similarities. The study indicated about the possible role of contaminated environment as a source of beta-lactamase/AmpC-producing *Klebsiella* and *E. coli* in pigs.

Keywords ESBL · *E. coli* · Environment · India · *Klebsiella* · Pig

Introduction

Environment plays a significant role in emergence and spread of antibiotic resistance determinants. The environmental "resistome" is created with exposure of environmental bacteria to antibiotics, shedding of resistant bacteria into environment from human, livestock or other resources, and transfer of resistance genes from donor to recipient bacteria (Wright 2010). Anthropogenic activities since industrialization released bulk amount of antibiotics into the environment through wastewater effluents, farm animal manures, treatment of crop diseases, and aquaculture (Czekalski et al. 2012). The environmental bacteria although carried genes for antibiotic resistance even before the "antibiotic era," such that evolution of class A β-lactamase was detected 2.4 billion years ago with emergence of active on Cefotaxime, first isolated in Munich CTX-M progenitors around 200–300 million years ago (D'Costa et al. 2011).

Rapid dissemination of antibiotic resistance mostly depends on horizontal gene transfer between competent bacterial

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population, i.e., either from fecal bacteria to environmental isolates or vice versa (Baquero et al. 2008). Extended spectrum beta-lactamases (ESBLs) are well documented resistance determinants acquired through horizontal gene transfer (Carattoli 2013). ESBLs belong to clinically important class A β-lactamase enzymes, found in *Enterobacteriaceae*, frequently in *Klebsiella pneumoniae* and *Escherichia coli*. It can confer resistance to a variety of β-lactam antibiotics, including penicillins, 2nd-, 3rd-, and 4th-generation cephalosporins and monobactams (e.g., aztreonam), but usually not the carbapenems or the cephamycins (e.g., cefoxitin) (EFSA Panel on Biological Hazards 2011). Moreover, AmpC β-lactamase-producing organisms can produce resistance against cephalosporins, penicillins, cephamycins, monobactams, and also against β-lactamase inhibitors such as clavulanic acid. There are three classical ESBLs, i.e., TEM (except TEM-1), sulphydryl variable SHV (except SHV-1 and 2), and CTX-M (EFSA Panel on Biological Hazards 2011). Among them, CTX-M is observed as the most prevalent type in clinically infected humans throughout the world (Carattoli 2013). The origin of CTX-Ms was detected in *Kluyvera* species, an environmental bacterium, present abundantly in water, soil, sewage, hospital sinks, and animal originated food items (Forsberg et al. 2012). Four major groups of CTX-M enzymes (1, 2, 8, and 9) identified in clinical isolates were originated from different species of *Kluyvera* (Humeniuk et al. 2002). AmpC β-lactamases were initially detected as intrinsic cephalosporinase found in the chromosome of gram-negative bacteria ("chromosomal") as well as acquired by plasmids, known as "plasmidic" AmpCs (Liebana et al. 2012).

In human, ESBL or AmpC-producing *Klebsiella pneumoniae* or *E. coli* causes nosocomial or community acquired infections associated with increased morbidity and mortality, prolonged hospital stays, and subsequent economic burden (Schwaber and Carmeli 2007). Role of environment was evidenced earlier in transmission of human infection with antimicrobial resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (Hower et al. 2013). Similarly, transmission of ESBL-producing *Klebsiella* or *E. coli* in a community is also dependent on environmental "resistome," in which livestock farm plays a significant role (Gao et al. 2015; von Salviati et al. 2015). Earlier works documented the spread of ESBL-producing organisms from farms to the community through air, water, and crops irrigated with contaminated water and flies (Søraas et al. 2013; Njage and Buys 2015; Solà-Ginés et al. 2015).

There is always scarcity of data for identification of environmental risk factors associated with transmission of ESBL-/AmpC-producing *Enterobacteriaceae* in livestock farms such as pig farms. A few reports are available where the studies confirmed emission of ESBL-producing bacteria from the pig farms to the surrounding environment but excluded the possibility of transmission *from* the environment (Gao et al. 2015; von Salviati et al. 2015). The present study was undertaken to detect the occurrence of ESBL-/AmpC-producing *Klebsiella* and *E. coli* in healthy pigs kept in organized or backyard farms, their feed, drinking water, and pen floor swabs or surface soil swabs from roaming arena. The study was also intended to reveal the occurrence of class I integron genes in *Klebsiella* and *E. coli* isolates along with their clonal relationship. The study was conducted with the samples from different agro-climatic zones of West Bengal, a major pig rearing state in India. The pigs are reared due to high consumption of pork observed among the northeast inhabitants and Chinese people living in the state (Tiwari and Arora 2005).

Materials and methods

Collection of samples

The rectal swabs were collected from apparently healthy pigs of either sex kept in farms (organized or backyard) at three different agro-climatic zones of West Bengal, India during 2017. The organized farms were selected on the basis of production level, whereas the backyard farms were selected on the basis of convenient location and willingness of the farmers to participate in the study. The rectal swabs were collected from the pigs selected randomly from the farms. The pigs of organized farms were 1–2 years old with no history of antibiotic intake with feed. The indigenous pigs belonged to 5–6 months age, and all the studied backyard farms had no history of costly antibiotic intake (cephalosporins) either with feed or with during therapy. Occasionally the pigs were treated with tetracycline or gentamicin by local veterinarian or paraveterinarians. The organized piggeries kept the pigs in brick-made pens with concrete floors and asbestos shed. The backyard pigs were kept in houses during night only made of bamboo and jute-stick with earthen floors. The organized piggery and backyard farm offered the feed and water in cement-casted and earthen mangers, respectively. Properly treated water was not used in both the types of farms. The pigs kept in backyard farms roamed in a fenced place during daytime.

The rectal swabs ($n = 120$) and pen floor swabs/swabs from surface soils of roaming arena (backyard pigs) ($n = 48$) were collected with the help of sterile cotton swab sticks (HiMedia, India) in sterile vials containing transport medium (HiMedia, India) as per the standard guideline (OIE 2002). Drinking water ($n = 10$) and feed samples ($n = 6$) were collected randomly from the studied pig farms into sterile vials containing transport medium (Table S1, supporting information). Feed and drinking water samples were collected in replicate in consecutive days from three different agro-climatic zones. All the samples collected were brought into the laboratory maintaining the cold chain for further examination. The study was approved by Institutional Biosafety Committee.

Isolation and Identification of *Klebsiella* spp. and *E. coli* from collected samples

The samples collected from the pigs and environment were inoculated into *Klebsiella* selective agar (HiMedia, India) and incubated at 37 °C for overnight. Next day, characteristic colonies (more than one colony per sample) were picked and streaked into nutrient agar (HiMedia, India) slant for further morphological and biochemical confirmation as per the standard methods (Quinn et al. 1994).

Similarly, for isolation of *E. coli*, the samples collected in transport medium were inoculated into MacConkey's agar (HiMedia, India) and incubated at 37 °C for overnight. Next day, rose pink colonies were transferred into EMB agar (HiMedia, India) and again incubated overnight at 37 °C. The colonies were observed after incubation and single colony was streaked into nutrient agar (HiMedia, India) slants for further morphological and biochemical confirmation (Quinn et al. 1994).

PCR-based confirmation of *Klebsiella* spp. and *E. coli*

For PCR-based confirmation of *Klebsiella* spp. and *E. coli* isolates, DNA was extracted from all the morphological and biochemically confirmed *Klebsiella* spp. and *E. coli* isolates. For confirmation of *Klebsiella* spp. and *E. coli*, all the suspected samples including positive (ATCC 27736, ATCC 4157, HiMedia, India) and negative controls (nuclease free water) were subjected to PCR (Table 1). All the PCR positive *Klebsiella* spp. isolates including positive (ATCC 27736, HiMedia, India) and negative control (nuclease free water) were further tested for the identification of *Klebsiella pneumoniae* by specific PCR (Table 1). The amplified products were visualized by gel documentation system (UVP, UK) after electrophoresis in 2% (*w*/*v*) agarose (SRL, India) gel containing ethidium bromide (0.5 μg ml^{-1}) (SRL, India).

Double disc diffusion test

PCR confirmed *Klebsiella* spp. and *E. coli* isolates were subjected to screening for extended spectrum beta-lactamase production by antibiotic sensitivity test containing cefotaxime (30 μg, HiMedia, India) and ceftazidime (30 μg, HiMedia, India) antibiotic discs with or without clavulanate (10 μg, HiMedia, India). A difference of ≥ 5 mm between the zone diameters of either of the cephalosporin discs and their respective cephalosporin/clavulanate discs was considered to be phenotypically positive for ESBL production (CLSI 2014). Further, cefoxitin-cloxacillin double disc synergy (CC-DDS) was performed with all *Klebsiella* spp. and *E. coli* isolates for phenotypic confirmation of ACBL production (Tan et al. 2009).

Detection of beta-lactamase genes (bla_{CTX-M}, bla_{TEM}, bla_{SHV}) in *Klebsiella* spp. and *E. coli*

All the phenotypically ESBL-producing *Klebsiella* spp. and *E. coli* isolates including controls were subjected to PCR for

Table 1 Oligonucleotide sequences and PCR conditions used in the study

	Oligonucleotide sequence(5′-3′)	Annealing temperature (°C)	Product size (bp)	References
Klebsiella sp.	CGCGTACTATACGCCATGAACGTA ACCGTTGATCACTTCGGTCAGG	50	441	Brisse and Verhoef (2001)
E. coli 16s rDNA	GACCTCGGTTTAGTTCACAGA CACACGCTGACGCTGACCA	58	585	Wang et al. (1996)
K. pneumoniae	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	57	130	Liu et al. (2008)
bla_{TEM}	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	53	1080	Weill et al. (2004)
bla_{SHV}	TTATCTCCCTGTTAGCACC GATTTGCTGATTTCGCTCGG	52	792	Weill et al. (2004a)
bla_{CTX-M}	CAATGTGCAGCACCAAGTAA CGCGATATATCGTTGGTGGTGGTG	53	540	Weill et al. (2004)
bla_{CMY}	GACAGCCTCTTTCTCCACA TGGAACGAAGGCTACGTA	50	1000	Van Kessel et al. (2013)
bla_{ampC}	CGCATCAAATGCCATAAGTG CCCCGCTTATAGAGCAACAA	60	634 bp	Féria et al. (2002)
Int1	GGTCAAGGATCTGGATTTCG ACATGCGTGTAAATCATCGTC	61	481 bp	Mazel et al. (2000)
Klebsiella RAPD	TCACGATGCA	36	Variable	Gori et al. (1996)
E. coli RAPD	AGGGAACGAG	36	Variable	Lim et al. (2009)

detection of bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes using the primers and the cycle conditions as described in Table 1. The positive controls used in the study were provided by Department of Veterinary Microbiology, CAU, Aizawl, India. The PCR products were sequenced from commercially available sources (Xcelris Genomics, India). The sequence homology searches were conducted using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), and selected sequences were submitted into DDBJ.

Detection of chromosomal bla_{ampC} and plasmid-mediated ampC β-lactamase gene (bla_{CMY}) in *Klebsiella* spp. and *E. coli*

All the phenotypically ACBL-producing *Klebsiella* spp. and *E. coli* isolates including controls were subjected to PCR for detection of bla_{ampC} and bla_{CMY} genes using the primers and the cycle conditions as described earlier (Table 1). The positive controls used in the study were provided by Department of Veterinary Microbiology, CAU, Aizawl, India. The PCR products were sequenced from commercially available sources (Xcelris Genomics, India). The sequence homology searches were conducted using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), and selected sequence was submitted into DDBJ.

Detection of class I integron gene in *Klebsiella* spp. and *E. coli* isolates

All the beta-lactamase-producing *Klebsiella* spp. and *E. coli* isolates were subjected to PCR for detection of class I integron as described earlier (Table 1).

Serotyping of ESBL-producing *E. coli* isolates

All the beta-lactamase-producing *E. coli isolates* were sent to Central Research Institute, Kasuli, HP, India for serotyping and storage in the repository.

Antimicrobial sensitivity of beta-lactamase-producing *Klebsiella* spp. and *E. coli* isolates

All the beta-lactamase-producing *Klebsiella* spp. and *E. coli* isolates were further tested for their sensitivity/resistance to different common antibiotics such as amikacin, gentamicin, tetracycline, chloramphenicol, levofloxacin, amoxicillin/clavulanic acid, ceftriaxone, piperacillin/tazobactam, imipenem, cotrimoxazole, ertapenem, cefpodoxime, cefoperazone, cefepime, and ceftriaxone/tazobactam by disc diffusion method (CLSI 2014).

Clonal relationship of beta-lactamase-producing *Klebsiella* spp. and *E. coli*

The molecular typing of all the beta-lactamase-producing *Klebsiella* spp. and *E. coli* isolates were done by relative afferent pupillary defect (RAPD)-PCR (Table 1). All the images taken by the gel documentation system were analyzed by using the Doc-itLs image analysis software supplied with the system as per manufacturer's instruction (UVP, UK). By comparing the difference in the RAPD-PCR banding pattern, phylogenetic relationship among the isolates was established. An unrooted phylogenetic tree was made by using neighbor joining method.

Statistical analysis

Occurrence of beta-lactamase-producing *Klebsiella* spp. and *E. coli* in different agro-climatic zones from which the samples were collected was compared by chi-square test using SPSS software version 17.0 (SPSS Inc.).

Results

In total, 227 *Klebsiella* spp. were isolated and identified by biochemical tests and PCR from healthy pigs (91/120, 75.8%), pen floor/surface soil (29/48, 60.4%), drinking water (1/6, 16.6%), and feed (1/6, 16.6%) samples. Each of the 227 *Klebsiella* spp. isolates showed typical biochemical tests results and generated a PCR amplicon of approximately 441 bp. Further, 113 (113/227, 49.7%) *Klebsiella* spp. isolates were identified as *Klebsiella pneumoniae* by specific PCR with characteristic amplicon.

Similarly, 114 *E. coli* were isolated and identified by biochemical tests and PCR from healthy pigs (83/120, 69.1%), pen floor/surface soil (25/48, 52.0%), drinking water (3/6, 50.0%), and feed (3/6, 50.0%) samples. Each of the 114 *E. coli* isolates showed typical biochemical tests results characteristics of *E. coli* and generated a PCR amplicon of approximately 585 bp.

In total, 43 *Klebsiella* spp. (43/227, 18.9%) and 15 *E. coli* strains (15/114, 13.1%) isolated from different samples were phenotypically detected as ESBL producers in double disc synergy test. *Klebsiella* spp. isolated from healthy pigs (35/175, 20.0%), pen floor swabs/surface soil swabs (7/50, 14.0%), drinking water (1/1, 100%), and feeds (0/1, 0%), respectively were detected as phenotypical ESBL producers (Table S1, supporting information). *Escherichia coli* isolated from healthy pigs (12/83, 14.4%), pen floor swabs/surface soil swabs (2/25, 8.0%), drinking water (1/3, 33.3%), and feeds (0/3, 0%), respectively were detected as phenotypical ESBL producers. Isolation rate varies significantly between different agro-climatic zones ($p < 0.05$; Table S1, supporting

Table 2 Genotype profile of ESBL- and ACBL-producing *Klebsiella* spp. isolated from pigs and farm environment in West Bengal, India

Isolate numbers (indent used in dendrogram)	Sources	*bla* $_{CTX\text{-}M}$	*bla*-$_{SHV}$	*bla*-$_{TEM}$	*bla*$_{AmpC}$	Class-I integron	*Klebsiella* spp. PCR	*Klebsiella pneumoniae*-specific PCR
K1	Pig	–	–	+	+	–	+	–
K2	Pig	+	–	–	–	–	+	–
K3	Pig	+	+	–	+	–	+	–
KP1	Pig	–	+	–	+	+	+	+
K4	Pen floor swab	–	+	–	+	+	+	–
KP2	Surface soil swab	+	–	+	+	–	+	+
KP3	Surface soil swab	–	+	–	+	+	+	+
KP4	Pig	+	+	–	–	–	+	+
K5	Pig	+	–	–	+	+	+	–
KP5	Pig	–	+	–	+	+	+	+
KP6	Pig	+	+	–	–	–	+	+
KP7	Pig	–	+	+	+	–	+	+
KP8	Surface soil swab	+	–	–	–	–	+	+
KP9	Pig	+	+	–	+	+	+	+
KP10	Pig	+	+	–	–	+	+	+
KP11	Pig	+	+	+	+	+	+	+
KP12	Pig	–	+	–	–	–	+	+
KP13	Pig	–	+	–	–	–	+	+
KP14	Pig	+	+	–	+	+	+	+
KP15	Pig	+	–	–	+	–	+	+
KP16	Pig	–	+	–	+	–	+	+
KP17	Pig	+	–	–	+	–	+	+
KP18	Pig	+	+	–	+	–	+	+
K6	Pig	+	–	–	+	+	+	–
K7	Drinking water	+	–	+	+	+	+	–
KP19	Pig	–	+	–	–	–	+	+
KP20	Pig	+	+	–	+	+	+	+
K8	Surface soil swab	–	–	+	+	+	+	–
K9	Surface soil swab	–	–	+	+	+	+	–
K10	Pig	–	–	+	+	–	+	–
K11	Pig	–	–	+	+	+	+	–
K12	Pig	+	–	–	+	–	+	–
K13	Pig	+	–	–	+	–	+	–
K14	Surface soil swab	+	–	–	+	–	+	–
K15	Pig	–	–	+	+	–	+	–
K16	Pig	+	–	–	+	–	+	–
K17	Pig	+	–	–	+	–	+	–
K18	Pig	+	–	–	+	–	+	–
K19	Pig	+	–	–	+	–	+	–
K20	Pig	+	–	–	+	–	+	–
K21	Pig	+	–	–	+	–	+	–
K22	Pig	+	–	–	+	–	+	–
K23	Pig	–	+	–	+	+	+	–

information). Among them, 27 *Klebsiella* spp. isolates (27/43, 62.79%) and six *E. coli* isolates (6/15, 40%) were phenotypically confirmed as CTX-M producers with cefotaxime and cefotaxime/clavulanate double disc. Majority of the beta-lactamase-producing *E. coli* isolates belonged to O88, O149, and O22 serogroups.

Out of 43 beta-lactamase-producing *Klebsiella* spp. and 15 beta-lactamase-producing *E. coli* isolates, 35 *Klebsiella* spp. (35/43, 81.3%) and all the *E. coli* isolates (15/15, 100%) were detected as ACBL producers with cefoxitin-cloxacillin double disc synergy.

All the CTX-M-producing *Klebsiella* spp. ($n = 27$) and *E. coli* isolates ($n = 6$) possessed $bla_{CTX\text{-}M}$ in PCR. Further, 19 *Klebsiella* spp. (19/43, 44.18%) and two *E. coli* isolates (2/15, 13.3%) were found positive for bla_{SHV} gene in PCR, whereas 10 *Klebsiella* spp. (10/43, 23.25%) and 10 *E. coli* (10/15, 66.7%) isolates were detected to possess the studied bla_{TEM} gene in PCR (Tables 2 and 3). The sequences of the PCR products were compared and found 98% cognate with $bla_{CTX\text{-}M\text{-}9}$, $bla_{SHV\text{-}12}$ and $bla_{TEM\text{-}1}$ in BLAST search. The sequences were published by DDBJ with accession numbers LC420321 ($bla_{CTX\text{-}M\text{-}9}$), LC421936 ($bla_{SHV\text{-}12}$), and LC421935 ($bla_{TEM\text{-}1}$).

All the ACBL-producing *Klebsiella* spp. and *E. coli* isolates possessed bla_{AmpC} in PCR. No plasmid mediated bla_{CMY} was found in any of the ACBL-producing *Klebsiella* spp. and *E. coli* isolates (Tables 2 and 3). The sequences of the PCR products were compared and found 99% cognate with bla_{AmpC} in BLAST search. The sequence was published by DDBJ with accession number LC421937 (bla_{AmpC}).

Sixteen beta-lactamase-producing *Klebsiella* spp. (16/43, 37.20%) and 13 beta-lactamase-producing *E. coli* isolates (13/15, 86.67%) were detected as positive for class I integron gene in PCR with desired product size of approximate 481 bp (Tables 2 and 3).

Phenotypical resistance of beta-lactamase-producing *Klebsiella* spp. was observed most frequently against cefotaxime (88.4%), cefepime (74.5%), cefpodoxime (88.4%), ceftazidime (79.1%), amoxicillin/clavulanic acid (83.7%), cefoxitin (65.2%), and ceftriaxone (60.5%). Majority of beta-lactamase-producing *E. coli* isolates were resistant to amoxicillin/clavulanic acid (80%), ceftazidime (86.8%), cefpodoxime (73.4%), cefotaxime (60%), and ceftriaxone (53.4%). Majority of the ESBL-producing *Klebsiella* and *E. coli* isolates were detected as sensitive to ertapenem, imipenem, amikacin, colistin, chloramphenicol, and levofloxacin.

In RAPD-PCR of beta-lactamase-producing *Klebsiella* spp., amplified fragments ranging from 112 to 3464 bp were detected (calculated by Doc-itLs image analysis software, UVP, UK). The phylogenetic analysis of ESBL-producing *Klebsiella* spp. generated a dendrogram where the strains isolated from environment and animals in same agro-climatic zone (K1, K2, K4; KP-1, KP-2, KP-3, KP-6, KP-9, KP-10, K3; K8, K13, K17; K14, K20) were detected in same cluster (Fig. 1).

Similarly, in RAPD-PCR of beta-lactamase-producing *E. coli*, amplified fragments ranging from 171 to 3965 bp were detected (calculated by Doc-itLs image analysis software, UVP, UK). The phylogenetic analysis of ESBL-producing *E. coli* generated a dendrogram, where the strains isolated from environment and animals in same agro-climatic zone (EC-18, EC-24; EC-6, EC-9; EC-13, EC-19) were detected in same cluster (Fig. 2).

Discussion

The present study detected *Klebsiella* spp. ($n = 227$) and *E. coli* ($n = 114$) in healthy pigs (75.8 and 69.1%, respectively) and farm environment in West Bengal, India. Earlier study in Thailand and India (West Bengal and Mizoram) detected lower occurrence of *Klebsiella* spp. (7%) and *E. coli* (28–

Table 3 Genotype profile of ESBL- and ACBL-producing *E. coli* isolated from pigs and farm environment in West Bengal, India

Isolate numbers (indent used in dendrogram)	Source	$bla_{\text{-}CTXM}$	$bla_{\text{-}SHV}$	$bla_{\text{-}TEM}$	bla_{AmpC}	*E. coli* 16S-PCR
Ec3	Pig	+	–	+	+	+
Ec5	Pig	+	+	–	+	+
Ec6	Surface soil swab	+	–	–	+	+
Ec12	Pig	+	–	–	+	+
Ec13	Pig	–	+	–	+	+
Ec15	Pig	–	–	+	+	+
Ec16	Pig	–	–	+	+	+
Ec17	Pig	–	–	+	+	+
Ec18	Surface soil swab	–	–	+	+	+
Ec19	Drinking water	–	–	+	+	+
Ec20	Pig	–	–	+	+	+
Ec22	Pig	–	–	+	+	+
Ec23	Pig	+	–	–	+	+
Ec24	Pig	+	–	+	+	+
Ec25	Pig	–	–	+	+	+

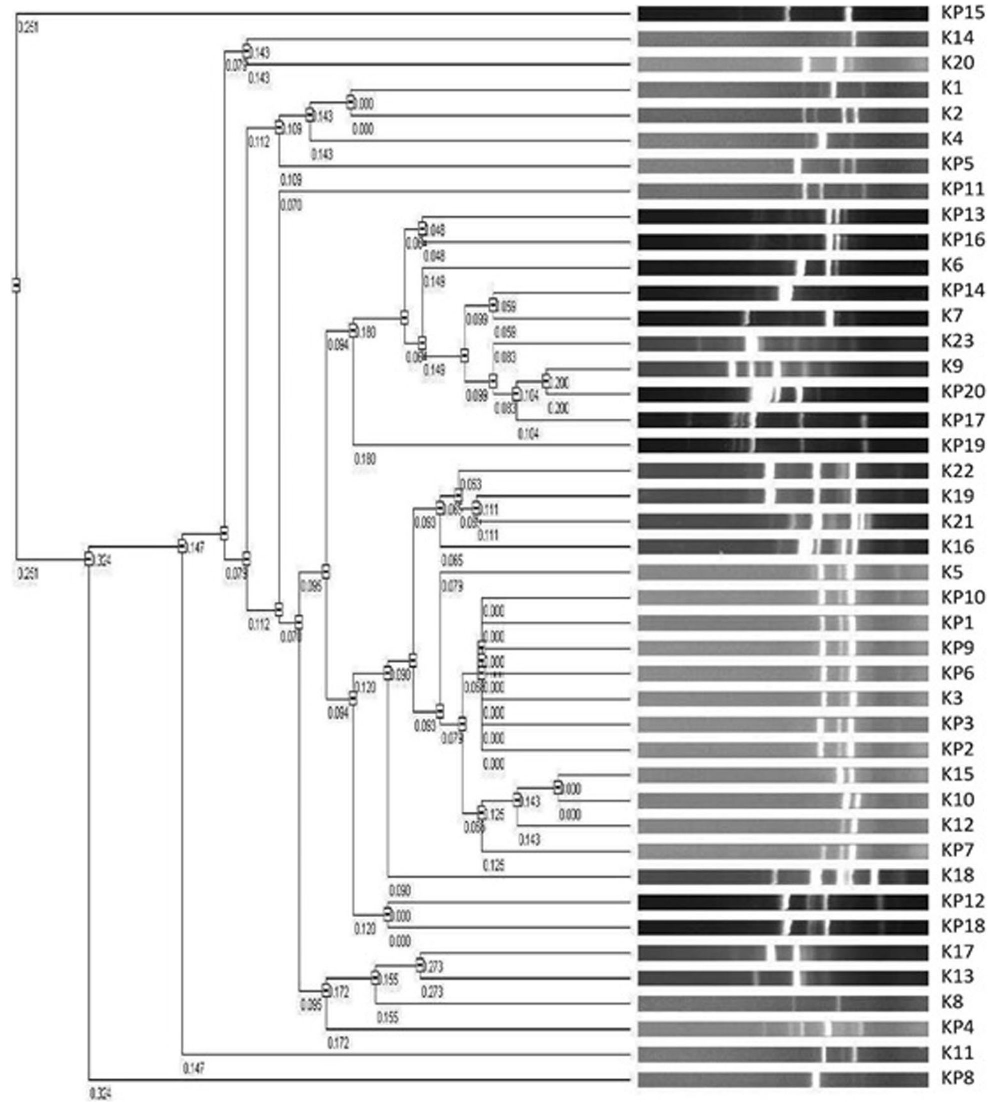

Fig. 1 Phylogenetic analysis of ESBL/AmpC-producing *Klebsiella* spp. strains isolated from healthy pigs and environment in West Bengal (India). The neighbor-joining method was used to summarize the similarity of RAPD-PCR profiles of ESBL/AmpC-producing *Klebsiella* spp. strains in a dendrogram

48%) in healthy pigs (Lalzampuia et al. 2013; Boonyasiri et al. 2014; Samanta et al. 2015). Use of selective medium and more than one colony was the probable reason for higher isolation rate of *Klebsiella* spp.

Moderate occurrence of beta-lactamase-producing *Klebsiella* spp. (20.0%) and *E. coli* strains (14.4%) was observed in the studied healthy pig population. Similar occurrence of ESBL-producing *Klebsiella* spp. (19.2%) and *E. coli* (15.3%) in swine was reported from Republic of Korea and Switzerland, respectively (Rayamajhi et al. 2008; Geser et al. 2012). Earlier study in northeastern India (Mizoram) although could not detect any ESBL-producing *Klebsiella* spp. from pigs (Lalzampuia et al. 2013). Lower occurrence of ESBL-producing *E. coli* in healthy pigs was noted in Portugal (5.7%) (Machado et al. 2008), Hong Kong and Japan (2–3%) (Duan et al. 2006; Hiroi et al. 2012), and in our earlier study in West Bengal (6%) (Samanta et al. 2015). Presence of beta-lactamase-producing bacteria in healthy pigs in the present study with higher occurrence rate than European and Asian countries, even higher than our own study conducted 2 years ago in the same state, indicates either generation or transmission of beta-lactamase genes from the environmental resistome with an increasing trend. Mostly, the studied pigs did not receive higher generation cephalosporins and synthetic analog of thiamphenicol (florphenicol) through their feed for growth promotion or therapeutic purposes which are acknowledged risk factors for shedding of ESBL-producing bacteria (Paterson and Bonomo 2005; Meunier et al. 2010). Reduction of cephalosporin intake was recommended as a control measure for ESBL-producing *E. coli* in slaughter pigs (Hammerum et al. 2014).

The feed, drinking water, and pen floor/surface soil swabs from roaming arena were evaluated to detect the role of farm environment in transmission of beta-lactamase-producing bacteria. Beta-lactamase-producing *Klebsiella* spp. and *E. coli* were detected in drinking water (33.3–100%) and pen floor/surface soil swabs (8–14%). The water bodies

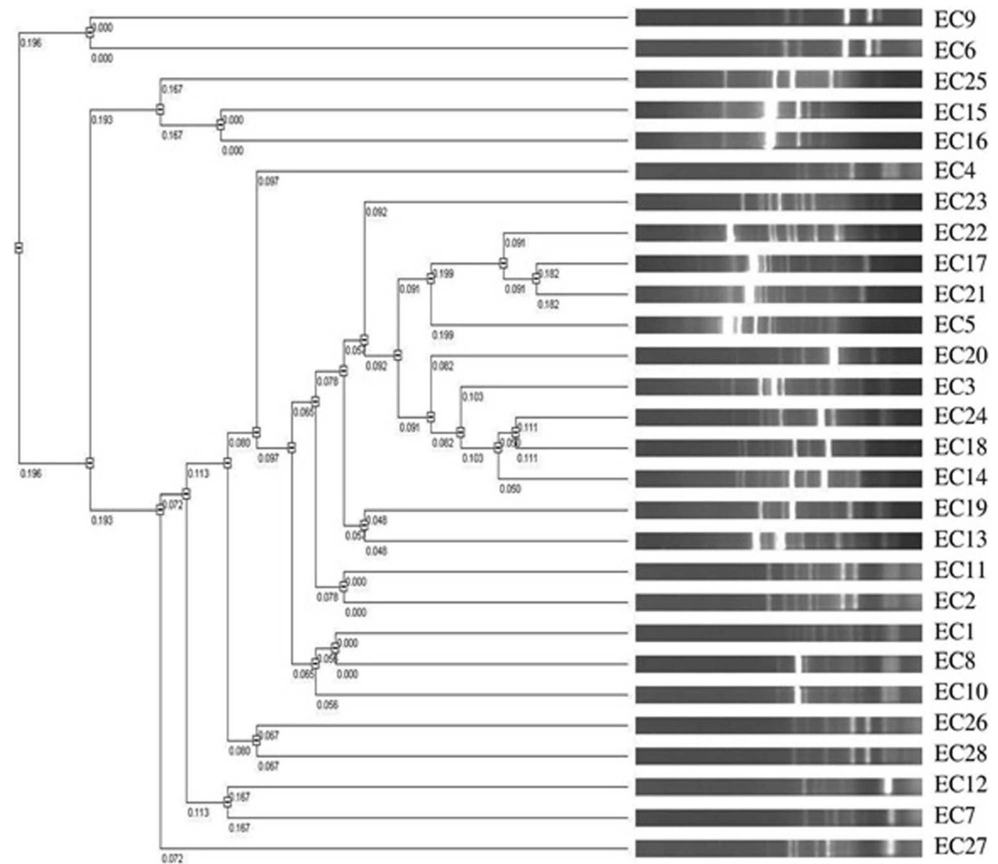

Fig. 2 Phylogenetic analysis of ESBL/AmpC/Shiga toxin-producing *E. coli* strains isolated from healthy pigs and environment in West Bengal (India). The neighbor-joining method was used to summarize the similarity of RAPD-PCR profiles of ESBL/AmpC/Shiga toxin-producing *E. coli* strains in a dendrogram. Figure 2 was constructed with both ESBL-and Shiga toxin-producing *E. coli* isolates, although the data of STEC was not included in the manuscript

present in the globe (e.g., rivers) acts as a source of ESBL-producing bacteria except the water present at an altitude of more than 1000 m, indicating the anthropogenic activities as responsible factor for the contamination (Zurfluh et al. 2013). In developing countries, even the drinking water is not an exception. Municipal drinking water was detected as a carrier of NDM-1 beta-lactamase in India (Walsh et al. 2011). Water samples adjacent to pig or duck farms in China were reported earlier as a source of ESBL-producing *E. coli* (Ma et al. 2012; Hu et al. 2013). Cultivated soil was also reported earlier as a reservoir of ESBL-producing *E. coli* in France, where application of manure, sewage sludge, or irrigation with wastewater was assumed as a source of infection (Hartmann et al. 2012). Sewage sludge was identified as a potential reservoir of ESBL-producing *E. coli* in Europe (Spain and Austria) (Mesa et al. 2006; Reinthaler et al. 2010). The surface soils, collected from different locations in India, irrigated with wastewater, showed the presence of AmpC-β-lactamase (Malik et al. 2007). Isolation of beta-lactamase-/AmpC-producing bacteria varied significantly between different agro-climatic zones in the present study which indicated differences in the environmental contamination level.

Majority of beta-lactamase-producing *Klebsiella* spp. and *E. coli* were phenotypically CTX-M producers and all of them possessed $bla_{CTX\text{-}M}$. Nucleotide sequencing of representative PCR products revealed the presence of $bla_{CTX\text{-}M\text{-}9}$, $bla_{SHV\text{-}12}$, and $bla_{TEM\text{-}1}$. The studies with ESBL characterization in *Enterobacteriaceae* revealed the global emergence of CTX-M in the last decade replacing TEM and SHVs. Among four main groups of CTX-M associated with clinical infection, CTX-M-9 and CTX-M-1 were detected as most prevalent throughout the world especially in Asia including swine feces and aquatic environment (Ewers et al. 2012). In China, maximum numbers of ESBL-producing *E. coli* strains isolated from pigs and local water bodies possessed CTX-M-9 alone or in combination with CTX-M-1 (Hu et al. 2013). Similarly, SHV-12 was reported from swine feces in Portugal (Machado et al. 2008) and pig slurry in Spain (Escudero et al. 2010). TEM-1 was prevalent in ducks and water samples collected from ponds adjacent to the duck farm in China (Ma et al. 2012).

All the ACBL-producing *Klebsiella* spp. and *E. coli* isolates possessed bla_{AmpC}, not plasmid mediated $bla_{CMY\text{-}2}$. Although, CMY-2 variant was detected as more prevalent than AmpC in United States (EFSA Panel on Biological Hazards 2011). In Asian countries such as in China and Japan, ESBL-producing bacteria isolated from pigs and aquatic environment did not possess AmpC gene (Hu et al. 2013; Norizuki et al. 2018). In India, our earlier study also revealed occurrence of AmpC in *E. coli* strains isolated from poultry and cattle with mastitis (Kar et al. 2015).

Transmission of ACBL-/ESBL-producing organisms is regulated by mobile genetic elements specially class 1 integrons. Sometimes class 1 integrons detected from animals and human ACBL-/ESBL-producing isolates belonged to homologous in nature which confirms their role in transmission (EFSA Panel on Biological Hazards 2011). The present study revealed higher occurrence of class 1 integron in beta-lactamase-producing *Klebsiella* spp. (16/43, 37.20%) and *E. coli* (13/15, 86.67%) isolates depicting their high transmission possibility.

Beta-lactamase-/AmpC-producing bacteria isolated from food animals including pigs possessed co-resistance against other common veterinary drugs such as aminoglycosides, sulfonamides, trimethoprim, fluoroquinolones, and tetracyclines (Deng et al. 2011; Liebana et al. 2012). Beta-lactamase-producing *Klebsiella* or *E. coli* isolates did not show cross-resistance against non-β-lactams such as gentamicin, tetracycline, levofloxacin, and cotrimoxazole, even though the studied pigs were infrequently treated with tetracycline or gentamicin. It seems that possession of antimicrobial resistance genes or phenotypes in the studied pigs was not correlated with antibiotic intake.

The clonal relationship of beta-lactamase-/AmpC-producing *Klebsiella* spp. and *E. coli* placed the isolates from environmental sources (surface soil swab and drinking water) and the studied pigs within the same cluster of the dendrogram. Presence of the strains within the same cluster of a dendrogram indicates their similarity. For example, K1 (pigs), K2 (pigs), and K4 (pen floor swab) *Klebsiella* isolates were in the same cluster indicating pen floor as a source of infection (Fig. 1; Table 2). Similarly, EC18 (soil surface) and EC 24 (pig) *E. coli* isolates were detected within same cluster indicating soil as a source of infection (Fog 2; Table 3). Earlier PFGE-based study showed limited similarities and indicated the cross-transmission possibility of ESBL-producing *E. coli* from pigs to environmental water (Hu et al. 2013).

The study indicated about the possible role of contaminated environment as a source of beta-lactamase-/AmpC-producing *Klebsiella* spp. and *E. coli* in healthy pigs. Possible explanation for the presence of beta-lactamase-/AmpC-producing bacteria in the studied environment (water and soil) is either persistence of bacteria which was excreted from earlier herd especially in organized farms. Horizontal transfer of ESBL genes through plasmid or other mobile genetic elements is another option which is possible in soil and water (Trevors and Oddie 1986).

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Compliance with ethical standards

The study was approved by Institutional Biosafety Committee.

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals (if applicable) Not applicable.

Informed consent Not applicable.

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