ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF MULTIDRUG RESISTANT *ESCHERICHIA COLI* RECOVERED FROM PIGS OF ARUNACHAL PRADESH, INDIA

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Development and circulation of multidrug resistant (MDR) enteric bacteria is increasingly posing a global threat in the field of therapeutic approaches in human and animals. Information on prevalence of MDR enteric bacteria in pig population is scarce in India in general and in North Eastern hilly states of India in particular. The present study was formulated to understand the prevalence of MDR *Escherichia coli* in organized and unorganized pig population of Arunachal Pradesh, India with special emphasis on detection of antimicrobial resistance gene profiles of the isolates. A total of 100 fecal samples from pigs of organized (n=49) and unorganized (n=51) farms in Arunachal Pradesh were collected randomly with or without any symptoms of diarrhoea. Samples were processed for isolation and identification of *E. coli* by phenotypic and genotypic methods. All the isolates were subjected to antimicrobial susceptibility assay by disc diffusion method followed by ESBLs production by double discs synergy test (DDST), detection of major ESBLs genes, non-ESBLs genes and integrons by specific PCR assays. A total of 292 *E. coli* was isolated, of which 155 and 137 were from organized and unorganized farms, respectively. The highest resistance was exhibited by cefalexin (83.56%), whereas imipenem (0.34%) was recorded with least resistance. Overall, isolates from organized farms showed higher level of resistance against majority of the antimicrobial agents compared to the isolates from unorganized farms. By DDST method, a total of 121 (41.44%) *E. coli* isolates were recorded as ESBLs producer, of which 80 (51.61%) and 41 (29.93%) were from organized and unorganized farms, respectively. A total of 41 (14.04%), 10 (3.42%), 15 (5.14%) and 6 (2.05%) isolates were found to be positive for *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{CMY} and *bla*_{SHV} gene, respectively. In addition, 81 (27.74%), 15 (5.14%), 75 (25.68%), 54 (18.49%), 51 (17.46%) and 15 (5.14%) were found to be positive for *tetA*, *tetB*, *sul1*, *sul2*, *aadA* and *dfra* genes, respectively. A total of 66 (22.60%) isolates were found to be positive for *intI1*, of which 42 (14.38%) and 24 (8.22%) were from organized and unorganized farms, respectively. Class 2 integrons (*intI2*) were not detected in any of the isolates. MDR *E. coli* are highly prevalent in pig population of Arunachal Pradesh and isolates from organized farms were more resistant than isolates from unorganized farms. Presence of such kind of MDR bacteria in pig population may pose a serious public health concern.

Key words: Arunachal Pradesh, *Escherichia coli*, India, Multidrug resistant, Pig

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Problems associated with the development and spread of antibiotic resistance have been increasing since the early 1960s and are currently viewed as a major threat to global public health (World Health Organization, 2001). The ability of bacteria to acquire and disseminate exogenous genes via mobile genetic elements considered to be the major factor in the development of MDR strains (Rowe-Magnus *et al.*, 2002). At present, the emergence of MDR bacteria is a global public health concern. In organized mass poultry and livestock production facilities, antimicrobial agents may be used for therapeutic and prophylactic purposes or as growth promoters at sub-therapeutic levels (Bacquero *et al.*, 1997), which puts selective pressure on commensals and pathogenic bacteria contributing to clonal expansion of MDR (Bacquero *et al.*, 1997). MDR bacteria can persist in the environment and can transmit the resistance determinants among the other close or distantly related bacteria as well as commensals bacteria. Therefore, there is an urgent need to understand the ecology of those strains and their resistance determinants.

E. coli is common gastrointestinal flora to be capable of accepting and transferring plasmids, which under stress readily transfers it to other species and is, therefore, considered an important reservoir of transferable antibiotic resistance (Lim *et al.*, 2007). The frequency of resistance in *E. coli* to β -lactam is increasing worldwide (Kallen *et al.*, 2010). Resistance in *E. coli* and other *Enterobacteriaceae* has continuously developed with decreasing susceptibility to first-line antimicrobials, viz., ampicillin, nitrofurantoin, co-trimoxazole as well as the fluoroquinolones like ciprofloxacin and levofloxacin (Sahm *et al.*, 2000). In

Europe, 30 to 50% clinical isolates are resistant to amoxicillin (Luzzaro *et al.*, 2002) and 6 to 17% to fluoroquinolones, which is still increasing (Kurt *et al.*, 2008). The huge quantity of inappropriately selected antibiotics is considered the most significant factors for the development of bacterial resistance to antimicrobial drugs (Goossens *et al.*, 2005).

Several scientific articles are citing the role of ESBLs for development of MDR bacteria in man and animals. Moreover, most of the organisms are capable of crossing the species barrier (the so called zoonotic agents) and therefore, becoming a serious threat in treatment of infectious diseases. Although, extensive studies were carried out in USA and Europe, the developing countries are still lagging behind, especially, India. So far prevalence of ESBLs in India has reached in epidemic proportions ranging from 62-100%, of which majority are in *E. coli* and *K. pneumoniae* isolates (Walsh *et al.*, 2007). Apart from TEM and SHV types, isolates from India additionally produce CTX-M enzymes (Mathai *et al.*, 2009), in which CTX-M-15 appeared to be the predominant ESBLs in Northern India (Walsh *et al.*, 2007). Studies conducted in North Eastern India confirmed the presence of ESBLs (*bla*_{CTX-M} and *bla*_{SHV}) through isolation of *E. coli* from faecal samples of pigs in different districts of Mizoram (Lalzampaia *et al.*, 2013). An ESBL-producing *E. coli* possessing *bla*_{CTX-M} and *bla*_{SHV} associated with human diarrhoea has been reported in India (Dutta *et al.*, 2013). Lalruatdiki (2013) also reported the prevalence ESBL producing genes (*bla*_{TEM}, *bla*_{CTX-M} and *bla*_{CMY}) in the enteric bacteria of pig population in Meghalaya and Assam. Although few sporadic reports are available, no efforts have

been made till date to detect the MDR enteric bacteria in food animals and their subsequent characterization from Arunachal Pradesh of India. Detailed molecular characterization of multidrug resistant strains largely remains unknown. The present study was undertaken to understand the prevalence of multidrug resistant *E. coli* in pig population of Arunachal Pradesh and their subsequent characterization.

MATERIALS AND METHODS

Sample collection: Fresh faecal samples were collected randomly from pigs of Arunachal Pradesh. A total of 100 faecal samples were collected randomly from pigs maintained under organized (n=49) as well as unorganized (n=51) farming system irrespective age, sex and with or without history of diarrhea during the study. All the samples were collected using a sterilized adsorbent cotton swab. However, for collection of samples from distant locations, a sterilized swab dipped in brain heart infusion broth was used as transport medium and transported to the laboratory under cold chain for further processing.

Isolation and identification of *Escherichia coli*: The collected faecal samples were processed for isolation and identification of *E. coli* using standard bacteriological techniques. Five single pure colonies were picked from each inoculated plate for further confirmation. All the isolates were further confirmed by BD Phoenix automated bacterial identification system and 16S rRNA based species specific PCR assay. All the isolates were stored as pure culture in semi-solid agar at 4°C as well as in Luria Bertani (LB) Broth containing 25% glycerol (v/v) at -80°C for further use.

Antibiotic susceptibility test and confirmatory test for ESBLs: Antimicrobial susceptibility test was done on Mueller-Hinton

agar (Hi-media) plate as per the recommendation of Clinical Laboratory Standard Institute (CLSI, 2014) using the following commercially available antibiotic discs: Amoxicillin (Ax, 30 mcg), Ampicillin (AMP, 10 mcg), Aztreonam (Az, 30 mcg), Cefalexin (CN, 30 mcg), Cefexime (CFM, 30 mcg), Cefotaxime (CTX, 30 mcg), Ceftazidime (CAZ, 30 mcg), Ceftriaxone (CTR, 30 mcg), Ciprofloxacin (CIP, 5mcg), Co-Trimoxazole (COT, 1.25/23.75 mcg), Gentamicin (GEN, 10mcg), Imipenem (IPM, 10mcg), Nalidixic acid (NA, 30 mcg), Piperacillin (PI, 100 mcg), Streptomycin (S, 10 mcg), Sulphafurazole/sulfisoxazole (SF, 300 mcg), Tetracycline (TE, 30 mcg) and Trimethoprim (TR, 30 mcg). Isolates exhibited inhibition zone of ≤ 22 mm for ceftazidime (30 μ g), ≤ 25 mm for ceftriazone (30 μ g) and ≤ 27 mm for cefotaxime (30 μ g) were identified as potential ESBLs producer.

Confirmatory test for ESBLs production was carried out using cefotaxime (30 mcg), amoxicillin (30 mcg) and ceftazidime (30 mcg) alone as well as cefotaxime/clavulanate (30/10 mcg), amoxicillin/clavulanate (30/10 mcg) and ceftazidime/clavulanate (30/10 mcg) combination as per the recommendation of CLSI (2014). Both the discs were placed at least 25 mm apart, centre to centre, on a lawn culture of the test isolate in Mueller-Hinton agar plate and incubated overnight at 37°C. Difference in zone diameters with and without clavulanic acid was measured. When there was an increase of ≥ 5 mm in inhibition zone diameter around antimicrobial agent tested in combination with clavulanic acid versus its inhibition diameter zone when tested alone was confirmed as potent ESBLs producing isolates.

Genotypic detection of antibiotic resistance profile: Presence of antimicrobial resistance

Table 1. Details of the oligonucleotide primers used in the present study

Primer name	Sequence (5'→3')	Expected amplicon size (bp)	Annealing temperature (°C)	Reference
<i>bla_{TEM}</i>	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	1080	53	Weill <i>et al.</i> , 2004
<i>bla_{SHV}</i>	CTTTCCCATGATGAGCACCT CGCTGTTATCGCTCATGGTA	206	60	This study
<i>bla_{CTX-M}</i>	CAATGTGCAGCACCAGTAA CGCGATATCGTTGGTGGTG	540	58	Perez and Hanson, 2002
<i>bla_{CMY}</i>	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	60	Perez and Hanson, 2002
<i>tetA</i>	GTAATTCTGAGCACTGTCGCT CTGCCTGGACAACATTGCT	937	57	Guardabassi <i>et al.</i> , 2000
<i>tetB</i>	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659	57	Fonseca <i>et al.</i> , 2006
<i>Sul1</i>	TGGTGACGGTGTTTCGGCATTC GCGAGGGTTTCCGAGAAGGTG	789	63	Mazel <i>et al.</i> , 2000
<i>Sul2</i>	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	722	58	Maynard <i>et al.</i> , 2003
<i>AadA</i>	GCAGCGCAATGACATTCTTG ATCCTTCGGCGCGATTTTG	282	58	Madsen <i>et al.</i> , 2000
<i>Dfr1a</i>	GTGAACTATCACTAATGG TTAACCCTTTTGCCAGATTT	474	53	Navia, 2003
<i>Int11</i>	GGGTCAAGGATCTGGATTTTCG ACATGGGTGTAAATCATCGTC	483	60	Mazel <i>et al.</i> , 2000
<i>Int12</i>	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	788	60	Mazel <i>et al.</i> , 2000
5'-CS 3'-CS	GGCATACAAGCAGCAAGC AAGCAGACTTGACCTGAT	variable	52	Zhang <i>et al.</i> , 2004
Ti-F Ti-B	ACCTTTTTGTGCGCATATCCGTG CTAACGCTTGAGTTAAGCC	variable	55	Su <i>et al.</i> , 2006

(AMR) genes was analysed by PCR using bacterial lysate as template DNA. Detection of AMR genes were performed for sulfamethoxazole resistant isolates (*Sul* and *Sul2*), ampicillin resistance isolates (*bla_{TEM}*,

bla_{SHV}, *bla_{CTX-M}* and *bla_{CMY}*), tetracycline resistant isolates (*TetA*), streptomycin resistant isolates (*aadA*) and trimethoprim resistant isolates (*dfr1a*) using specific oligonucleotide primers (Table-1). PCR conditions were

followed as per the method described by Nazik *et al.* (2011) with suitable modifications. The repeatability of the assay was checked by repeating the PCR for three times.

Detection of antibiotic resistance integrons:

The MDR isolates were also screened for the presence of class 1 and 2 integrons, viz., *intI1* and *intI2* as well as its gene cassettes 5CS/3CS and TiB/TiF. PCR amplification was used to detect class 1 and class 2 integrase genes (*intI1* and *intI2* respectively) using specific primers (Table-1). The condition used for amplification of the two integrase genes were as follows: initial denaturation at 94°C for 5min, 30 amplification cycles consisting of 50 sec at 94°C, 50 sec at 60°C and 1 min at 72°C and final extension for 6 min. Amplification of variable region of class 1 and class 2 integrons were performed using the primers 52 -CS/32 -CS and Ti-F/Ti-B, as per the conditions and procedures described previously by Zhang *et al.* (2004).

RESULTS

Isolation of *E. coli*: A total of 292 *E. coli* was isolated, of which 155 and 137 were from organized and unorganized farms, respectively.

Antibiotic susceptibility of the isolates:

Amongst the 292 isolates obtained from both organized and unorganized farms of Arunachal Pradesh, imipenem (99.66%) was found to be the most sensitive antibiotic, which was followed by ceftriaxone (96.23%), cefotaxime (90.75%), streptomycin (89.04%), nalidixic acid (84.59%), co-trimoxazole (82.19%), trimethoprim (81.85%), ceftazidime (81.51%), ampicillin (79.79%), tetracycline (77.74%), gentamicin (75.00%), aztreonam (70.21%), cefexime (60.62%), sulphafurazole

(54.45%), piperacillin (51.71%), amoxicillin (21.58%) and cefalexin (16.44%). Conversely, the maximum level of resistance was exhibited by cefalexin (83.56%) and imipenem (0.34%) was recorded with least level of resistance (Table 2).

As depicted in (Table 2), all the 155 isolates from organized farms of Arunachal Pradesh showed 100.00% sensitivity to imipenem but lowest sensitivity to cefalexin (10.32%). On the other hand, the isolates showed maximum level of resistance to cefalexin (89.68%) followed by amoxicillin (70.97%), piperacillin (60.65%), aztreonam (46.45%), cefexime (42.58%), sulphafurazole (41.29%), tetracycline (32.26%), gentamicin (25.81%), trimethoprim (19.35%), streptomycin (18.71%), co-trimoxazole (17.42%), ampicillin (12.90%), nalidixic acid (10.97%), ceftazidime (9.68%) and cefotaxime (3.23%).

Similarly, all the 137 isolates from unorganized farms of Arunachal Pradesh, highest level of sensitivity was observed against imipenem (99.27%) and lowest level of sensitivity was exhibited by amoxicillin (13.14%) (Table 2). Conversely, the maximum level of resistance was recorded against amoxicillin (86.86%) followed by cefalexin (76.64%), sulphafurazole (50.36%), cefexime (35.77%), piperacillin (34.31%), ampicillin (28.47%), ceftazidime (28.47%), gentamicin (24.09%), nalidixic acid (20.44%), co-trimoxazole (18.25%), cefotaxime (16.06%), trimethoprim (16.79%), aztreonam (10.95%), tetracycline (10.95%), ceftriaxone (5.84%), streptomycin (2.19%), ciprofloxacin (1.46%) and imipenem (0.73%).

By DDST method, a total of 121 (41.44%) *E. coli* isolates were recorded as ESBLs producer, of which 80 (51.61%) and 41 (29.93%) were from organized and unorganized farms, respectively.

Table 2. Antibiotic susceptibility profile of *E. coli* isolated from fecal samples of pig from organized and unorganized farms of Arunachal Pradesh, India

ANTIBIOTIC	No. of isolates			No. of isolates			No. of isolates					
	Organized farm			Unorganized farm			Total (Organized + Unorganized)					
	S	%	R	S	%	R	S	%	R			
Amoxicillin (AMX)	45	29.032	110	70.97	18	13.14	119	86.86	63	21.58	229	78.42
Ampicillin (AMP)	135	87.097	20	12.90	98	71.53	39	28.47	233	79.79	59	20.21
Aztreonam (AT)	83	53.548	72	46.45	122	89.05	15	10.95	205	70.21	87	29.79
Cefalexin(CN)	16	10.323	139	89.68	32	23.36	105	76.64	48	16.44	244	83.56
Cefexime (CFM)	89	57.419	66	42.58	88	64.23	49	35.77	177	60.62	115	39.38
Cefotaxime (CTX)	150	96.774	5	3.23	115	83.94	22	16.06	265	90.75	27	9.25
Ceftazidime (CAZ)	140	90.323	15	9.68	98	71.53	39	28.47	238	81.51	54	18.49
Ceftriaxone(CTR)	152	98.065	3	1.94	129	94.16	8	5.84	281	96.23	11	3.77
Ciprofloxacin (CIP)	154	99.355	1	0.65	135	98.54	2	1.46	289	98.97	3	1.03
Co-rimoxazole(COT)	128	82.581	27	17.42	112	81.75	25	18.25	240	82.19	52	17.81
Gentamicin (GEN)	115	74.194	40	25.81	104	75.91	33	24.09	219	75.00	73	25.00
Imipenem (IPM)	155	100.000	0	0.00	136	99.27	1	0.73	291	99.66	1	0.34
Nalidixic acid (NA)	138	89.032	17	10.97	109	79.56	28	20.44	247	84.59	45	15.41
Piperacillin (PI)	61	39.355	94	60.65	90	65.69	47	34.31	151	51.71	141	48.29
Streptomycin (S)	126	81.290	29	18.71	134	97.81	3	2.19	260	89.04	32	10.96
Sulphafurazole (sulfisoxazole)(SF)	91	58.710	64	41.29	68	49.64	69	50.36	159	54.45	133	45.55
Tetracycline (TE)	105	67.742	50	32.26	122	89.05	15	10.95	227	77.74	65	22.26
Trimethoprim(TR)	125	80.645	30	19.35	114	83.21	23	16.79	239	81.85	53	18.15

*S- sensitive, R-resistant

Table 3. Details of detection of MDR *E. coli* isolated from faecal samples of pigs from organized and unorganized farms of Arunachal Pradesh, India

Antibiotics	Organized farm	Unorganized farm	Total	ND value
Amoxicillin	110 (70.97)	119 (86.86)	229 (78.42)	3.29**
Ampicillin	20 (12.90)	39 (28.47)	59 (20.205)	3.31**
Aztreonam	72 (46.45)	15 (10.95)	87(29.795)	6.62**
Cefalexin	139 (89.68)	105 (76.64)	244 (83.562)	3**
Cefexime	66 (42.58)	49 (35.77)	115 (39.384)	1.19 ^{NS}
Cefotaxim	5 (3.23)	22 (16.06)	27 (9.247)	3.78**
Ceftazidime	15 (9.68)	39 (28.47)	54 (18.493)	4.13**
Ceftriaxone	3 (1.94)	8 (5.84)	11 (3.767)	1.75 ^{NS}
Ciprofloxacin	1 (0.65)	2 (1.46)	3 (1.027)	0.69 ^{NS}
Co-Trimoxazole	27 (17.42)	25 (18.25)	52 (17.993)	0.16 ^{NS}
Gentamicin	40 (25.81)	33 (24.09)	73 (25.000)	0.34 ^{NS}
Imipenem	0 (0.00)	1 (0.73)	1 (0.342)	1.07 ^{NS}
Nalidixic acid	17 (10.97)	28 (20.44)	45 (15.411)	2.24*
Piperacillin	94 (60.65)	47 (34.31)	141 (48.288)	4.5**
Streptomycin	29 (18.71)	3 (2.19)	32 (10.959)	4.51**
Sulphafurazole	64 (41.29)	69 (50.36)	133 (45.548)	0.38 ^{NS}
Tetracycline	50 (32.26)	15 (10.95)	65 (22.260)	4.37**
Trimethoprim	30 (19.35)	23 (16.79)	53 (18.151)	0.57 ^{NS}

*Statistically significant ($P < 0.05$) between organized and unorganized farm; **Highly significant ($P < 0.01$) between organized and unorganized farm; ^{NS}Not significantly different between organized and unorganized farm. Figures in parentheses indicate percentages

Statistical analysis showed that the level of resistance against amoxicillin, ampicillin, aztreonam, cefalexin, cefotaxime, ceftazidime, piperacillin, streptomycin and tetracycline differed significantly ($P > 0.01$) between the organized and unorganized farms of Arunachal Pradesh. The resistance level against nalidixic acid was also differing significantly ($P > 0.05$) between the organized and unorganized farms. However, no significant difference was observed in the

level of resistance against the remaining antimicrobial agents (Table 3).

Genotypic detection of beta-lactamase genes: Amongst all the *E. coli* isolates from Arunachal Pradesh, 41 (14.04%) were found to be positive for *bla*_{TEM} gene. Likewise, a total of 10 (3.42%) isolates were found positive for *bla*_{CTX-M} genes. In the case of *bla*_{CMY} gene, a total of 15 (5.14%) isolates were recorded as positive. A total of 6

Table 4. Distribution of antimicrobial resistance genes among *E. coli* isolated from fecal samples of pigs from different organized and unorganized farms of Arunachal Pradesh, India

Sample and isolate details				Genes										
	No. of samples	No. of isolates	M	<i>blaTEM</i>	<i>blaCTX-</i>	<i>blaCMY</i>	<i>blaSHV</i>	<i>tetA</i>	<i>tetB</i>	<i>sul1</i>	<i>sul2</i>	<i>aadA</i>	<i>dfp1a</i>	<i>Int11</i>
Organized farms	49	155	31 (20.00)	4 (2.58)	2 (1.29)	-	72 (46.45)	15 (9.68)	69 (44.52)	42 (27.09)	42 (27.09)	5 (5.80)	9 (5.80)	63 (40.65)
Unorganized farms	51	137	10 (7.30)	6 (4.38)	13 (9.48)	6 (4.38)	9 (6.57)	-	6 (4.38)	12 (8.76)	9 (6.57)	6 (4.38)	6 (4.38)	3 (2.19)
Total	100	292	41 (14.04)	10 (3.42)	15 (5.14)	6 (2.05)	81 (27.74)	15 (5.14)	75 (25.68)	54 (18.49)	51 (17.46)	15 (5.14)	15 (5.14)	66 (22.60)

Figures in parentheses indicate percentages

(2.05%) isolates were recorded as positive for *bla*_{SHV} genes.

Among the isolates from organized farms, 31 (20.00%) were positive for *bla*_{TEM}, whereas, *bla*_{CTX-M} and *bla*_{CMY} were detected in 4 (2.58%) and 2 (1.29%) isolates, respectively. *bla*_{SHV} gene was not detected in any of the isolates. Similarly, from the unorganized farms, 13 (9.48%), 10 (7.30%), 6 (4.38%) and 6 (4.38%) isolates were found as positive for *bla*_{CMY}, *bla*_{TEM}, *bla*_{CTX-M} and *bla*_{SHV} genes, respectively (Table 4)

Genotypic characterization of Non-ESBLs genes: In Arunachal Pradesh, of the 292 isolates from both organized and unorganized farms, 81 (27.74%), 15 (5.14%), 75 (25.68%), 54 (18.49%), 51 (17.46%) and 15 (5.14%) were found to be positive for *tetA*, *tetB*, *sul1*, *sul2*, *aadA* and *df*_{rla} genes, respectively. Amongst the isolates from the organized farms, 72 (46.45%), 15 (9.68%), 69 (44.52%), 42 (27.09%), 42 (27.09%) and 9 (5.80%) were positive for *tetA*, *tetB*, *sul1*, *sul2*, *aadA* and *df*_{rla} genes, respectively. On the other hand, amongst the isolates from the unorganized farms, only 9 (6.57%), 6 (4.38%), 12 (8.76%), 9 (6.57%) and 6 (4.38%) were positive for *tetA*, *sul1*, *sul2*, *aadA* and *df*_{rla} genes, respectively. No isolates were found to be positive for *tetB* gene (Table 4)

Detection of antibiotic resistance Integrations and gene cassettes: In the present study, a total of 66 (22.60%) *E. coli* isolates were found to be positive for Class 1 integrons (*int11*), of which 42 (14.38%) were from organized farms and 24 (8.22%) were from unorganized farms. However, Class 2 integrons (*int12*) were not detected in any of the isolates.

DISCUSSION

In India, the information regarding ESBLs producing enteric bacteria in animal population is very limited. A very limited study has been done in this laboratory on prevalence of ESBLs producing enteric bacteria in man and animals in Mizoram state only. But the data from other parts of the country including NER remains unexplored. Therefore, the present study was conducted to investigate the prevalence of ESBLs producing *E. coli* isolates from pigs of Arunachal Pradesh in India with the broad objectives to improve the practice of antimicrobial's use in clinical practice, farm biosecurity, epidemiological studies and also safeguarding against the zoonotic outbreaks by MDR bacteria in human and animal population.

In the present study, a total of 292 *E. coli* were isolated and identified from 100 faecal samples collected from pigs of organized and unorganized farms of Arunachal Pradesh in India with or without the history of diarrhoea. *E. coli* is a commensal in the intestinal tract of man and animals. The population in the gut may vary depending upon the physiological and/or pathological status of the host. A similar or little variable rate of *E. coli* isolation from various animals is reported from India and abroad. Earlier, from the same laboratory, Lalzampuia (2012) isolated 102 *E. coli* from 53 faecal samples collected from pigs of different districts of Mizoram. On the other hand, Lalruatdiki (2013) isolated 867 *E. coli* from 228 fecal samples collected from Meghalaya and Assam. In another study, Wani *et al.* (2003) reported 249 *E. coli* from 391 bovine and 60 *E. coli* from 101 ovine faecal samples in

Srinagar, Jammu & Kashmir. But from the same laboratory, Bhat *et al.* (2008) isolated 284 *E. coli* from 236 samples from lambs with or without diarrhoea. The rate of isolations of *E. coli* from faecal samples may vary depending upon the nature of samples, types of media used, laboratory practices of the researchers, treatment status of the host and so on. Number of *E. coli* colonies picked up from a particular sample is also variable. It may lead to variation in the frequency of *E. coli* isolates from a particular number of samples. In this study, a minimum of 5 suspected colonies were picked up randomly from primary culture plate for further confirmation by morphological and biochemical characteristics. All the isolates obtained in this study revealed typical biochemical and cultural characteristics as mentioned by Ewing (1986). No isolates exhibited any atypical biochemical or cultural properties in the laboratory.

Antimicrobial resistance is a very complex phenomenon involving a large variety of antimicrobial agents, bacterial species, resistance genes and mechanism of resistance. Different countries in the world use different antibiotics for treatment of same kind of infections. In this regard, β -lactam antibiotics are widely used for treatment of infectious diseases in human and animals. Many pathogenic organisms encoding multiple antibiotic resistance genes are increasing day by day throughout the world (Perez *et al.*, 2007). In this study, all the isolates showed resistance to at least three antimicrobial agents; majority was multi-resistant. The resistance pattern of *E. coli* isolates against all the antimicrobial agents are variable within the isolates obtained from different

farms of the state. In this study, the resistance pattern of *E. coli* isolates against all the four third generation cephalosporins showed variable resistance. The NARMS report also mentioned that resistance to ceftriaxone was ranging from 6.3-13.5% among the *E. coli* recovered from chickens during 2000–2008. In contrast to our result, Sasirekha *et al.* (2010) reported that 84%, 75% and 85% *E. coli* were resistant to cefotaxime, ceftriaxone and ceftazidime, respectively. On the other hand, Rosengren *et al.* (2008) reported no resistant isolates against ceftriaxone and less than 1% resistant to cefoxitin and ceftiofur. Resistant strains were isolated from the pigs in the production system that used beta-lactam antimicrobials including tetracycline class more often than from pigs in another type of large production system (Gebreyes *et al.*, 2004).

In the present study, the level of resistance against streptomycin and tetracycline is significantly low in case of unorganized farms compared to the organized farms of Arunachal Pradesh, which may be due to less usage of these drugs by backyard farmers in this state. We could not compare our data on antibiotic resistance in pigs with different farming practices because of non-availability of similar information in this region. A relatively low frequency of resistance was also observed against gentamicin, nalidixic acid and ciprofloxacin (Table 2). In a retrospective study on antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950–2002, Tadesse *et al.* (2012) stated that tetracycline resistance was the most common type of resistance observed and the most prevalent resistance phenotype

in animal isolates (71.1%). This finding was not surprising because tetracycline has been widely used in therapy and to promote feed efficiency in animal production systems since its approval in 1948. Persistence of tetracycline resistance was reported in animal coliforms a decade after it was no longer used in feed or for treatment. We commonly found co-resistance for tetracycline with streptomycin, sulfonamide, ampicillin, and chloramphenicol, as in other studies. A pan-European monitoring study on susceptibility to human-use antimicrobial agents in enteric bacteria isolated from healthy food producing animals (de Jong *et al.*, 2012) specifically pointed out the relationship of antimicrobials used and development of resistance. The food animals including pigs showed very low level of resistance against the older antimicrobials, which are not very frequently in use for human or veterinary practices. With the availability of third and fourth generation of cephalosporins and without any restriction upon their use in veterinary and human health practice in this country, a high level of resistance against them are increasingly evidenced. At the same time, a decrease in level of resistance against older antimicrobials is also evidenced. Although, some antimicrobial agents are not in use for veterinary practices in India, a high level of resistance against them is recorded in the fecal *E. coli* of swine. The persistence of resistance might result from the process of co-selection by other antimicrobials being used in pigs (Binch *et al.*, 2008). This occurs when genes encoding for these antimicrobials are linked with those encoding for other antimicrobials being used (Witte, 1998). Another pathway for

persistence of antimicrobial resistance is the linkage of chromosomally located antimicrobial resistance genes that are not readily lost by bacteria (Holzel *et al.*, 2010). Most of the ESBL producing organisms were co-resistant to flouoroquinolones, aminoglycosides and co-trimoxazole, which corroborates with the study of various other workers (Denholm *et al.*, 2009; Jabeen *et al.*, 2005). A nearly similar kind of information, where the ESBLs producing enteric bacteria was also recorded as resistant to other group of antibiotics (Perez *et al.*, 2007). Development of such co-resistance against other classes of antibiotics including β -lactam antibiotics by the ESBLs producing organisms generally appeared in the large plasmids, where most of the resistance genes may co-exist.

Antimicrobial resistance in commensal *Enterobacteriaceae* from pigs may play an important role in the ecology of resistance, constituting an important reservoir for these transmissible resistance genes. In this study, isolates from organized farms showed highest prevalence of *bla*_{CTX-M} gene and least (0.46%) prevalence of *bla*_{CTX-M} gene. In case of unorganized farms, *E. coli* isolates recorded highest prevalence of *bla*_{CTX-M}. In this regard, our result is in corroboration with the report of other workers from abroad except one report from this laboratory. In a small scale study, Lalzampaia *et al.* (2013) reported that 7 (5.07%) and 3 (2.17%) *E. coli* isolated from pigs of Mizoram were positive for *bla*_{CTX-M-1} and *bla*_{TEM} gene, respectively, of which 3 (2.17%) isolates were positive for both the genes. Only 4 (2.90%) isolates carried *bla*_{CTX-M-1} gene alone.

Globally, the prevalence of CTX-M producing *E. coli* is varied between 0.8% in Europe to 3.4% in Western Pacific region as reported by Winokur *et al.* (2001). Li *et al.* (2005) reported 1.5% CTX-M producing *Salmonella* spp. in Taiwan. In contrast to our result, several workers from India and abroad reported a higher prevalence of CTX-M gene in enteric bacteria of human and animals (Adeleke *et al.*, 2012; Senthilkumaran, 2012). The variation of results in this study in comparison to the other reports may be due to lower expression of CTX-M genes. *bla*_{CTX-M} genes spread in the community mostly through the transmission of plasmids. It is also reported that animals may play as possible role for the dissemination to humans (Faure *et al.*, 2009). Indeed, from the same laboratory *bla*_{CTX-M} genes in *E. coli* from human and animals from Mizoram is already published (Dutta *et al.*, 2013; Lalzampaia *et al.*, 2013). These observations suggest the possibility of transfer of extended-spectrum cephalosporin resistance between animals and humans.

In the present study, the tetracycline resistance gene *tetA* was detected most frequently in organized farms, which is also in agreement with the study carried out on *E. coli* isolated from healthy pigs from various places of the world (Jurado-Rabadán *et al.*, 2014). The presence of *tet* genes has been reported as a prevailing mechanism for tetracycline resistance in *E. coli* isolates from pet animals and wild animals. In general, tetracycline resistance develops due to acquisition of genes involved mainly in three processes: antibiotic efflux through energy-dependent membrane associated proteins, ribosomal protection

and enzymatic inactivation of tetracycline.

Class 1 integrons are the most common antibiotic resistant genes found in the clinical isolates of gram-negative bacteria. Su *et al.* (2006) also reported the detection of three classes of integrons in 111 clinical isolates of *E. coli*, of which 85.6% and 3.6% of the isolates carried class 1 and class 2 integrons, respectively. Integrons are considered as a primary source of resistance genes and also are considered as reservoirs of antimicrobial resistance genes within microbial populations. Class 1 integron was observed in 43% *E. coli* strains recovered from animals and humans, while the class 2 integrons was recorded in only 1% isolated (Van Essen-Zandbergen *et al.*, 2007). Similarly, Goldstein *et al.* (2001) reported that 61.5% of *Salmonella* spp. isolated from poultry was positive for class 1 integron and only 4.6% were positive for class 2 integron. As far as

pig is concerned, Gebreyes and Thakur (2005) reported that out of the 28 isolates, 21 were multidrug resistant and all of them harboured the class 1 integron. Integron gene sequences contribute to the spread of antimicrobial resistance alleles by lateral gene transfer of gene cassettes in a variety of enteric bacteria including *Campylobacter* spp., *Escherichia coli* and *Salmonella enterica* sub sp. *enterica* serotype Typhimurium (Roe *et al.*, 2003). This finding suggests that the isolates with multiple ESBL genotypes have a greater opportunity to carry class 1 integron. Therefore, bacteria carrying both integrons and ESBL genes have much stronger multi-resistance activity. However, the location of ESBL genes in class 1 element was not studied and needs further investigation.

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