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Phenotypic and molecular characterization of extended spectrum β-lactamase, AmpC β-lactamase and metallo β-lactamase producing *Klebsiella spp.* from farm animals in India

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ABSTRACT

Animal populace has attained less attention in antimicrobial resistance research than human sector resulting in limited information available on animal origin isolates. The study aimed to investigate the occurrence of ESBL, AmpC and MBL genes, plasmids and integrons in *Klebsiella spp*. Fecal samples were collected from healthy livestock (cattle, pig, sheep and goat) and poultry between 2012-2015. Preliminary identification of isolates was done by conventional phenotypic methods and confirmed by genotypic methods. Antimicrobial susceptibility testing was performed by disk diffusion method. Molecular characterization by PCR was conducted for 17 antimicrobial resistance genes, 3 integrons and 18 Plasmid replicons. A total of 48 *Klebsiella* isolates were identified. Multidrug resistance was observed in 23% of isolates. ESBL, AmpC and MBL resistance genes were detected in 21%, 6% and 4% of isolates, respectively. Integrons [*Int2* gene] and plasmids [*Y* and *IncI* genes] were identified in 6% and 8% of isolates.The study highlights the existence of ESBL, AmpC and MBL producing *Klebsiella* isolates with certain strains carrying mobile genetic elements in healthy livestock and poultry as reservoirs and probable disseminators of resistance, thus imposing public health threat. Prudent use of antimicrobials and continuous intensified surveillance in animal sector is crucial to limit the spread of such emerging resistant traits.

Key words: Antibiotic resistance, *Klebsiella spp*., Livestock, Mobile genetic elements.

INTRODUCTION

There is a growing concern all over the world about the emergence and spread of antimicrobial resistance (AMR) rendering available antibiotics ineffective. Experts opine that use of antibiotics in agriculture and allied sectors pose serious threat of emergence and dissemination of AMR, making it a global public health issue (Carattoli 2008). Though antibiotic application in livestock has largely contributed toward health and productivity, it has also played significant role in evolution of resistant strains (Sharma *et al.*, 2018). This emphasizes the need to understand the problem under one health perspective with surveillance being an important step towards it.

The food producing animals are widely recognised primary reservoirs of resistant zoonotic pathogens. In this growing antimicrobial resistance crisis, the rise in multidrug resistant *Klebsiella spp.* is a major concern to human as well as veterinary medicine. In animals, *Klebsiella pneumoniae* can cause a wide spectrum of diseases from mastitis in cattle, bacteremia in calves, cervicitis in mares, pneumonia in foals, etc. (Valentina *et al.*, 2014). In a recent report from India, 80-90% of *Klebsiella spp.* isolates were resistant to third-generation cephalosporins and fluoroquinolone resistance increased from 57 to 73% (CDDEP 2015). Carbapenem resistance among *K. pneumoniae* increased from 2% in 2002 to 52% in 2009 (Datta *et al.*, 2012). Moreover, it was observed that the resistance pattern among pathogens differs regionally and data from various studies when combined and evaluated revealed that there is definite resistance to commonly used antibiotics in the *Klebsiella spp.* (WHO 2010). Delineating the scope of the problem is necessary for mapping and monitoring an active response to AMR. Effective and creditably linked molecular based surveillance studies at multidisciplinary level can contribute to better understand and minimize the emergence of resistance.

At present, more systematic reports exist on antibiotic resistance surveillance in humans than animal populace, resulting in poor understanding of β-lactamase

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mediated resistance in animal sector (Carattoli 2008, Cao *et al*., 2014, Pitout *et al*., 2012). In the backdrop of the finite information about the antimicrobial resistance trends in *Klebsiella spp*. the present study investigated the occurrence and characteristics of ESBL/AmpC/MBL producing *Klebsiella spp*. isolated from livestock and poultry.

MATERIALS AND METHODS

Sample collection and isolation of *Klebsiella spp*: A total of 78 fecal samples were collected from healthy livestock (Cattle, Pig, Sheep and Goat) and poultry from different farms located in south and north eastern part of India during the period 2012-2015. The samples were inoculated on MacConky agar plates and incubated at 37°C for 18-24 hrs. *Klebsiella spp.* identification was done based on colony morphology, gram staining and standard biochemical tests.

DNA extraction: DNA was extracted from the overnight cultures grown on nutrient agar at 37ºC using QIAmp DNA mini kit (Qiagen, Duesseldorf, Germany) as per manufacturer's instructions. The quality and quantity of the extracted DNA was determined spectrophotometrically using Nanodrop 2000c (Thermofischer Scientific Inc., USA).

Genus specific PCR: *Klebsiella* genus specific PCR targeting *gyrA* gene (441 bp) was carried out as described previously (Chander *et al.*, 2011).

Antimicrobial susceptibiity testing: All the *Klebsiella* isolates were tested by the Kirby-Bauer disc diffusion method according to the 2014 Clinical and Laboratory Standard Institute guidelines (CLSI 2014). The details of antimicrobials tested are depicted in (Table 1). Confirmation of ESBL, AmpC and MBL determinants of resistance was done by their respective Double Disc Synergy Test (DDST), Inhibitor Potentiated Disc Diffusion (IPDD) and Epsilometer strip test (E-test) (Khatiyar *et al.*, 2016, Shah *et al*., 2014, Sachdeva *et al*., 2017, Khari *et al*., 2016). *Klebsiella pneumoniae* ATCC 700603 was used as a refrence strain for antibiotic susceptibility testing. Isolates exhibiting resistance to at least three different classes of antimicrobials were classified as Multidrug resistant (CLSI 2014).

Antimicrobial resistance gene profiling: A combination of 2 multiplex and 6 uniplex PCR assays were performed to screen 17 antimicrobial resistance genes comprising ESBL genes (*TEM, SHV, CTXM-I, II, III* and *IV*) (Kojima *et al*., 2005, Pitout *et al*., 2004), MBL genes (*IMP, VIM, SIM, GIM, SPM*) (Mendes *et al*., 2007) and AmpC genes (*FOX, MOX, ACC, EBC, CMY, DHA*) (Perez *et al*., 2002). PCR protocol was followed as published earlier (Kojima *et al*., 2005, Pitout *et al*., 2004) with modifications in the reaction set-up for *CTXM* group (Table 2). Primer sequences were synthesized from Eurofins Scientific, Bangalore.

Plasmid replicon typing: Plasmid replicon typing was carried out to investigate the presence of 18 replicons by three multiplex PCR assays, using primer sequences as described earlier (Valentina *et al.*, 2014). Selection of genes in multiplex panels and reaction conditions were followed according to Johnson *et al*., (2007).

Detection of integrons: To investigate the occurrence of integrons in the isolates, a multiplex PCR assay targeting Class 1, 2 and 3 was performed (Cao *et al*., 2014).

Ethics: The study was approved by the independent institutional ethics committee of ICAR-NIVEDI and was conducted according to the good laboratory practices.

Statistical analysis: Data were analysed statistically using SPSS version 18 software (SPSS, IBM, SOMARS, NY, USA). Univariant analysis was performed for calculation of drug resistance by using the χ^2 test.

Table 1: β-lactam antibiotics with classes and concentration.

β-lactamase	B-lactam antibiotics	Concentration(mcg)
3rd generation cephalosporins	Cefotaxime	30
	Ceftazidime	30
	Ceftriaxone	30
2nd generation cephalosporins	Cefoxitin	30
	Cefotetan	30
Carbapenem β lactams	Imipenem	10
	Meropenem	10
Monobactum	Aztreonam	30
ESBL-inhibitor	Piperacillin/Tazobactum	100/10
Amino penicillin	Ampicillin	10

**Antibiotics Source : (Himedia, Mumbai)*

Table 2: Modifications in the reaction setup for CTXM group.

Gene target	Kojima *et al.*, (2005)	Pitout *et al.*, (2004)	Present Study	
	Annealing temperature	$MgCl_2$ Conc	Annealing temperature	$MgCl_2$ Conc
CTXM-I	60°C	1.5mM	64°C	1mM
CTXM-II,III,IV	60°C	1.5mM	62°C	2mM

Table 3: Distribution pattern of 17 antimicrobial resistance genes (AGRs), plasmid replicons types (n=18), integron types (n=3) and their antibacterial resistance phenotypes in livestock and poultry origin *Klebsiella* isolates.

Species	No. of samples collected	*Klebsiella* positive samples by culture and PCR	Antimicrobial resistant *Klebsiella* isolates	Antimicrobial Resistance determinants			3rd generation Cephalosporins			2nd Generation Cephalosporins		Monobactam	ESBL-inhibitor	Amino-penicillin	Carbapenem β-lactam	
			Sample ID	ARGs (ESBL/MBL/ AmpC)	Integrons	Plasmid Replicons	Cefotaxime (30mcg)	Ceftazidime (30mcg)	Ceftriaxone (30mcg)	Cefoxitin (30mcg	Cefotetan (30mcg)	Aztreonam (30mcg)	Piperacillin/ Tazobactum (100/10mcg)	Ampicillin (10mcg)	Imipenem (10mcg)	Meropenem (10mcg)
Pig	20	15	NE-PG17	*bla* $_{FOX}$	-	*Y*	I	S	S	S	S	S	S	S	S	S
			NE-PG18	*bla* $_{FOX}$	-	-	S	S	R	R	S	R	S	R	S	S
			NE-PG 77	*bla* $_{FOX}$	-	-	R	S	R	S	S	S	R	R	S	S
			NE-PG 82	*bla* $_{IMP}$	-	-	S	S	R	R	S	S	S	S	R	S
			NE-PG-83	***bla*** $_{IMP}$	-	*Y*	**R**	**I**	**R**	**R**	**S**	**R**	**I**	**R**	**R**	**R**
			NE-PG-66	*bla* $_{TEM11}$	*Int2*	-	R	R	S	R	R	I	S	R	S	S
Poultry	20	13	NE-PL-104	*bla* $_{TEM29}$	-	-	S	S	R	S	S	R	R	S	S	S
Cattle	20	11	NE-CT-106	*bla* $_{TEM11}$	-	-	S	S	S	I	S	S	S	S	S	S
Sheep	8	3	NE-SH-107	*bla* $_{TEM11}$	-	-	S	S	R	R	S	I	R	S	S	R
Goat	10	6	KN-GO-01	*bla* $_{TEM12}$	-	-	S	I	S	S	R	S	R	R	S	S
			KN-GO-02	***bla*** $_{TEM 26}$	*Int2*	*IncI1*	**R**	**R**	**S**	**R**	**I**	**R**	**R**	**R**	**R**	**I**
			KN-GO-03	*bla* $_{TEM10}$	-	-	S	S	S	S	S	R	S	I	S	S
			KN-GO-04	*bla* $_{TEM11}$	-	-	S	S	S	S	S	S	S	S	S	S
			KNE-GO-05	***bla*** $_{TEM 29}$	*Int2*	*IncI1*	**R**	**I**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**I**
			KNE-GO-06	*bla* $_{TEM11}$	-	-	S	S	I	S	S	I	S	S	S	S

I - Intermediate resistance ; R – Complete resistance ; S – Susceptible

RESULTS AND DISCUSSION

Food animals are often exposed to antimicrobials to treat and prevent infectious disease or to promote growth. Antimicrobial resistance has emerged in zoonotic enteropathogens, commensal bacteria and bacterial pathogens of animals, however, the prevalence of resistance varies (Carattoli 2008). Since the last decade, studies on antimicrobial resistance have primarily targeted human population and comparatively meager studies focusing animal populace. The limited understanding of the role of animals contributing to the growing antibacterial resistance crisis emphasizes the need for effective surveillance accompanied with adequate characterization of antimicrobial resistant bacteria in the livestock and poultry population.

In the present investigation, a total of 48 (62%) *Klebsiella* isolates were identified from 78 fecal samples by conventional culture method and genus specific PCR (Table 3 and Fig 1). 100% correlation was observed between the two methods. ABST of 48 *Klebsiella* isolates showed 25% (12/48) resistance to 3rd generation cephalosporins, 19% (9/48) to 2nd generation cephalosporins, 23% (11/48) to monobactum and ESBL inhibitor and 10% (5/48)to carbapenem β-lactam (Table 3). Multidrug resistance was observed in 21% (10/48) of *Klebsiella* isolates. Further, 31% (15/48) of *Klebsiella* isolates were positive for one or the other ARGs tested by PCR. However, a discrepancy between phenotypic and genotypic methods was noted, which emphasizes the imperative need of molecular methods to obtain reliable interpretation of antimicrobial resistance profile. The difference observed in detection of resistance may be justified by the lower sensitivity of phenotypic method and the influence of environmental factors on the incidence of resistance (Alyamani *et al.*, 2017). The lack of constant sensitivity of different phenotypic methods has been emphasized by some studies (Ravi *et al*., 2011). In contrast, the genotypic method using specific PCR amplification of resistance genes seems to have 100% specificity and sensitivity (Veena *et al*., 2013).

ESBL genes were detected in 21% (n=10/48) of *Klebsiella* isolates with bla_{TEM-11} as the most common ESBL gene (50%, n=5) among livestock and poultry (Fig 2). AmpC beta lactamase genes were identified in 6% (n=3/48) of *Klebsiella* isolates recovered from pig population, appearing positive for bla_{FOX} family specific primers. 4% (n=2/48) of *Klebsiella* isolates were positive for bla_{IMP} gene of MBL class (Table 3).Two isolates of *Klebsiella* from pig, positive for AmpC and MBL genes carried plasmid replicon *Y*. Interestingly, these isolates were from two different geographical areas. This observation suggests the circulation of plasmids within strains from different environments (Carattoli *et al*., 2005).

Cao *et al*., (2014) highlighted *K. pneumoniae* possessing *TEM* and *SHV* types of ESBLs as major cause of hospital acquired infections. *Klebsiella* with ESBL phenotype were also described in dogs and cats from China and *CTXM*-15 positive *K. pneumoniae* was reported from hospital-acquired infections in pets from France (Dorina *et al*., 2016). The current study observed co-presence of bla_{TEM} and bla_{SHV} in two *Klebsiella* isolates obtained from goat, however, no *CTXM* groups were identified.

Plasmid replicon typing identified 2 different plasmid types- *IncI1* and *Y* (Table 3). These replicon types were detected in *Klebsiella* strains isolated from goat and pig hosts, respectively. Several studies have previously reported presence of plasmid *IncI1* majorly in *K. pneumoniae* of human origin and *E. coli* of pig origin (Abraham *et al.*, 2015, Carattoli 2009). Previous reports emphasized *IncI1* to be a hypervariable promiscuous resistance plasmids and strains carrying this plasmid is likely to acquire extended spectrum or AmpC β-lactamases if selection pressure is intensified by increased use of third generation cephalosporin's (Cavaco *et al*., 2008). Further, the resistance encoding plasmid also has the potential to horizontally transfer to other *Enterobateriaceae* (Abraham *et al.*, 2015, Carattoli 2009) e.g; pig gut which routinely enter the environment and food chain.

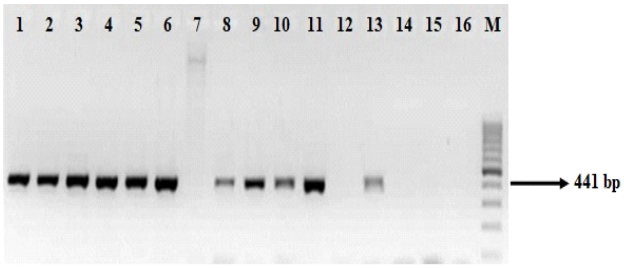

Fig 1: PCR amplification of *Klebsiella spp.*
Lane 1-6, 8-11 and 13 were representing samples positive for *Klebsiella genus* specific primers targeting *gyrA* gene
Lane 7, 12 and 14-16 were representing samples negative for *Klebsiella genus* pecific primers targeting *gyrA* gene
M: 1 kb DNA marker

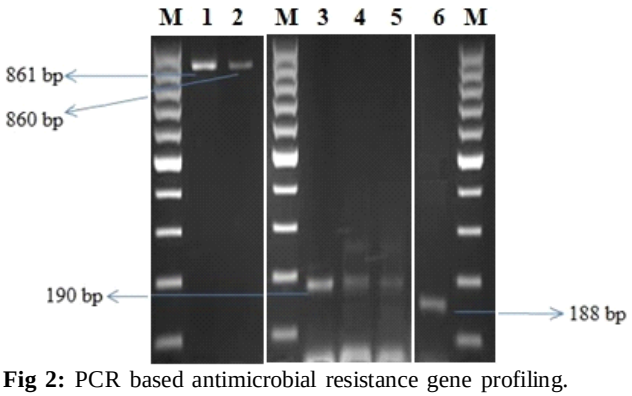

Fig 2: PCR based antimicrobial resistance gene profiling.
M: 1 kb DNA marker
Lane 1: *TEM*-861 bp (ESBL), Lane 2: *SHV*-860 bp (ESBL), Lane 3-5: *FOX*-190 bp (AmpC), Lane 6: *IMP*-188 bp (MBL)

In the present study, we identified 6% (n=3/48) *Klebsiella* isolates of pig and goat origin positive for Integron class 2. Class 1 and 3 were not identified in our study isolates. All the integron positive isolates were multi-drug resistant (Table 3). Independent studies conducted by (Kar *et al.,* 2015, Abraham *et al.,* 2015) reported higher percentage of Class 1 integron in *E. coli* isolates unlike in our study. Presence of integrons is a concern due to the potential for acquisition of resistance to new antimicrobials and horizontal transfer of resistance to sensitive bacterial populations.

The presence of plasmid replicon types and integrons in *Klebsiella* isolates from farms located in distant geographical areas in India suggests the successful spread of these genetic determinants in bacterial pathogens (Carattoli *et al.,* 2005). Previous studies have also reported that the ESBL/AmpC/MBL genes were carried equally on plasmid replicons and integrons in *Enterobacteriaceae* like *E. coli* isolates from cattle and that these were highly related to mobile genetic elements in human *E. coli* isolates (Timofte *et al.,* 2014). Such observations suggest the need for further understanding of the genetic constituents of drug resistant strains and their potential for horizontal transfer by characterization of integrons, associated MDR gene cassettes and plasmids.

CONCLUSION

The study identified relatively high prevelance of ESBL, AmpC and MBL positive *Klebsiella* isolates in pig population and the ESBL producing *Klebsiella* across the livestock and poultry. Monitoring of the epidemiology of ESBL producing bacteria in humans and livestock and the elucidation of possible transmission routes are needed. Extended surveillance report on animal populace originating from different geographical locations are required to capture the distribution pattern of specific genes, integrons and plasmid types.

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CONFLICT OF INTEREST: The authors declare that they have no competing interest.

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