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First Detection of Extended-Spectrum ß-Lactamase Producing *Escherichia coli* in Breeder Pigs in Bhutan

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Abstract: *This study investigated the occurrence of extended-spectrum ß-lactamase (ESBL) producing Escherichia coli (E. coli) in a strain collection of E. coli originating from faecal samples of pigs from three breeding farms in Bhutan. Screening for ESBL producers was done using chromogenic selective agar plates. ESBL genes (bla_{ESBL}) were identified using polymerase chain reaction (PCR) and sequencing. Isolates with $bla_{CTX\text{-}M\text{-}15}$ were classified according to their phylogenetic group and multilocus sequence type (MLST). Antimicrobial susceptibility profiles were determined by the agar diffusion method. Two (2.4%) of the 83 E. coli strains were ESBL producers (CTX-M-15). The two isolates were multidrug resistant (MDR) and belonged to sequence type (ST) ST156 and 4173, respectively. This is the first study to detect and characterize ESBL-producing E. coli in breeding pigs in Bhutan.*

Keywords: *Extended-spectrum ß- lactamases; CTX-M-15; Escherichia coli; pigs; Bhutan.*

Abbreviations:

AM, ampicillin

bla, ß-lactamase encoding gene

$bla_{CTX\text{-}M\text{-}15}$, CTX-M-15 encoding gene

bla_{ESBL}, Extended-spectrum ß-lactamaseencoding gene

$bla_{TEM\text{-}1}$, TEM-1 gene

C, chloramphenicol

CC, clonal complex

CF, cephalothin

CIP, ciprofloxacin

CTX, cefotaxime

CLSI, Clinical and Laboratory Standards Institute

E. coli, *Escherichia coli*

ESBL, Extended-spectrum ß-lactamase

K, kanamycin

MLST, multilocus sequence type

NA, nalidixic acid

PCR, polymerase chain reaction

S, streptomycin

SHV, Sulfhydryl variable

SMZ, sulfamethoxazole

TE, tetracycline

TEM, Temoneira

TMP, trimethoprim.

1. INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) are bacterial enzymes that catalyse the hydrolysis of ß-lactam antibiotics, including third generation cephalosporins (e.g. ceftazidime or cefotaxime) and monobactams (aztreonam) [1]. ESBLs represent the most common mechanism of antibacterial resistance in Gram-negative bacilli. With regard to their primary sequence homology [2] and their substrate profiles [3], ESBLs are categorized into classes and groups, whereby the majority belong to Ambler class A and to the Bush group 2be. The classical plasmid-mediated TEM- and SHV-ESBLs, which derived from point mutations in the structural genes of their precursors TEM-1, TEM-2, and SHV-1, were originally detected in the early 1990s in human clinical isolates associated with nosocomial infections [4]. Today, the most important ESBLs belong to the CTX-M family of enzymes which are cefotaximases that originated through mobilization of chromosomal $bla_{\text{CTX-M}}$ genes from environmental *Kluyvera* spp. into mobile genetic elements such as transposons and plasmids [5]. The CTX-M enzymes are classified according to their amino acid similarities into the five major groups CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 [6]. In recent years the rapid and global spread of CTX-M enzymes throughout the human population, food animals, wildlife and the environment has been recognized as a major threat to human and animal health [5]. Food producing animals may serve as a reservoir for ESBL producing *Escherichia coli* (*E. coli*), which can then be transmitted to humans either through direct contact or through meat contaminated during the slaughtering process. This represents a risk to human health, as *E. coli* is not only an important opportunistic pathogen for humans, but may also colonize the human gut as a commensal and transfer resistance genes to pathogenic bacteria [7]. In the EU, CTX-M-1 is the most frequently reported ESBL subtype in *E. coli* originating from food producing animals (poultry, cattle and pigs) and food [8]. By contrast, in Asia, CTX-M-2, CTX-M-14 and CTX-M-15 are found in cattle and pigs [9].

Bhutan is a small developing country located in the eastern Himalayas. There is a scarcity of data regarding the occurrence of antibiotic resistant bacteria among the human population as well as among animals. In 2013, a study investigating human clinical isolates originating from urinary tract infections revealed that 31.8% of the *E. coli* isolates were resistant to cefotaxime [10]. A very recent report confirmed the presence of CTX-M-15 in a collection of multidrug resistant human clinical isolates in Bhutan [11]. No data has been available so far to document the occurrence of ESBL producing *E. coli* in food producing animals in Bhutan. The aim of this study was to evaluate the presence of ESBL producing *E. coli* in suckling pigs and weaners on three pig breeding farms in Bhutan, and to characterize the isolated strains by (i) antibiotic susceptibility testing, (ii) identification of the extended-spectrum ß-lactamase genes (bla_{ESBL}), (iii) classification of the isolates into phylogenetic groups and multilocus sequence types (MLST).

2. MATERIALS AND METHODS

2.1. Collection of Samples

The investigation involved three governmental pig-breeding farms (farm 1: Yusipang; farm 2: Gelephu; and farm 3: Lingmethang, respectively) in Bhutan. A total of 83 faecal samples were collected (farm 1: n = 11; farm 2: n=34; and farm 3: n=38). The samples were sent to the National Centre for Animal Health, Thimphu, Bhutan for the isolation of *E. coli.*

2.2. Microbiological Procedures

E. coli was isolated according to standard procedures on MacConkey agar. From each sample, one suspected colony with typical *E. coli* morphology was selected for further analysis. Identification of isolates was performed using in-house classical biochemical methods at the National Centre for Animal Health, Bhutan. Thereafter, all *E. coli* isolates were sent to the Institute for Food Safety and -Hygiene, Zürich, Switzerland for further characterization.

The detection of ESBL producers was performed using chromogenic Brilliance ESBL agar plates (Oxoid, Hampshire, UK). Plates were incubated at 37°C for 24 h under aerobic conditions. Colonies were picked from the selective plates and subcultured on Mueller Hinton agar plates (Becton Dickinson, Allschwil, Switzerland) at 37°C for 24 h. Isolates were subjected to susceptibility testing against 13 antimicrobial agents by the disc diffusion method according to CLSI protocols and evaluated according to CLSI criteria [12]. The panel included ampicillin (AM), amoxicillin-clavulanic acid (AMC), cephalothin (CF), cefotaxime (CTX), nalidixic acid (NA), ciprofloxacin (CIP), gentamicin (GM), kanamycin (K), streptomycin (S), sulfamethoxazole (SMZ), trimethoprim (TMP) tetracycline (TE), and chloramphenicol (C) (Becton Dickinson, Heidelberg, Germany).

Multidrug resistance (MDR) was defined as such for isolates that were resistant to three or more classes of antimicrobials, counting ß-lactams as one class.

2.3. Molecular Biological Analysis of ß-Lactamase Genes

Isolates identified as potential ESBL producers were selected for further analysis. DNA was extracted by a standard heat lysis protocol and analysed by using the polymerase chain reaction (PCR) technique for the presence of *bla* genes. Synthesis of primers and DNA custom sequencing was carried out by Microsynth (Balgach, Switzerland). Purification of amplicons was done using a PCR purification kit (Qiagen Courtaboeuf, France).

Screening for $bla_{CTX\text{-}M}$ alleles belonging to CTX-M groups 1, 2, 8, 9, and 25 was performed as described by Woodford et al. [13]. Amplicons for sequencing individual open reading frames were generated using primers described by Geser et al. [14] Screening for bla_{TEM} and bla_{SHV} was carried out using primers described previously [15].

2.4. Phylogenetic Classification of *E. coli* Isolates

DNA from isolates was subjected to triplex PCR targeting the *chuA* gene, the *yjaA* gene and an unspecified DNA fragment termed TspE4.C2, as described by Clermont et al. [16]. Each isolate was assigned to one of the four phylogenetic groups designated A, B1, B2 or D. Group A and B1 typically contain commensal *E. coli* strains while groups B2 and D consist of virulent extra-intestinal strains [17].

2.5. Multilocus Sequence Typing

For multilocus sequence typing of the isolates, internal fragments of the seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA*, and *recA*) were amplified by PCR, as described by Wirth et al. [18]. Sequencing of the amplification products was performed by Microsynth (Balgach Switzerland). Sequences were imported into the *E. coli* MLST database website (http://mlst.ucc.ie/mlst/dbs/Ecoli) to determine MLST types.

2.6. Plasmid Incompatibility Typing

Plasmids were assigned to incompatibility groups on the basis of the presence of specific replicon sequences identified by PCR [19].

3. Results and Discussion

Of the 83 *E. coli* isolates investigated in this study, two (2.4%) were ESBL producers. PCR amplification and sequencing of the bla_{ESBL} genes revealed $bla_{CTX\text{-}M\text{-}15}$ in both isolates. In addition, both isolates harboured additionally the ß-lactamase $bla_{TEM\text{-}1}$ and both were MDR (Table 1). This is the first report of the detection of $bla_{CTX\text{-}M\text{-}15}$ harbouring *E. coli* in food producing animals in Bhutan. While none of the isolates belonged to extra-intestinal pathogenic phylogenetic groups B2 or D, B1 was identified in both strains. The detection of phylogenetic group B1 suggests that CTX-M-15 producers may have become established among commensal *E. coli* in the intestinal flora of pigs in Bhutan. As described in previous studies, this may be a consequence of the use of ß-lactam antibiotics in the swine industry [20;21]. The isolates contained plasmids which were assigned to plasmid incompatibility groups IncY((strainB2 L43 (S) and colE (strainB3 GS8 (S)). Strain B2 L43 (S) from farm 3 belonged to ST4173 and strain B3 GS8 (S) from farm 2 to ST156. Of particular interest is the description of CTX-M-15 producing *E. coli* belonging to ST156, which belongs to the clonal complex (CC) 156. This clone has been detected globally as a CTX-M-15 producer in sea gulls [22], water fowl and river water in Bangladesh [23], as well as in broilers and turkeys on farms in the United

Kingdom and Italy [24;25]. The detection of this clone in pigs provides further evidence for its dissemination among animals.

Table 1. *Characteristics of $bla_{CTX\text{-}M\text{-}15}$ harbouring E. coli isolated from faecal samples of breeding pigs in Bhutan.*

Strain ID	Origin	*bla* genes	Phylogenetic group	MLST	Antimicrobial resistance profile
B3 GS8 (S)	farm 2	CTX-M-15, TEM-1	B1	156	AM, CF, CTX, CIP, S, C, NA, SMZ, TMP
B2 L43 (S)	farm 3	CTX-M-15, TEM-1	B1	4173	AM, CF, CTX, TE, S, K, SMZ, TMP

Abbreviations: AM, ampicillin; *bla*, ß-lactamase gene; C, chloramphenicol; CF, cephalothin; CIP, ciprofloxacin; CTX, cefotaxime; K, kanamycin; MLST, multilocus sequence type; NA, nalidixic acid; S, streptomycin; SMZ, sulfamethoxazole; TE, tetracycline; TMP, trimethoprim.

Compared to other countries, ESBL producing *E. coli* appear to be rare in human clinical isolates and in animal isolates in Bhutan. However, an increase of ESBLs, including CTX-M-15 in bacteria is to be expected in future. In order to limit the dissemination of multidrug resistant bacteria, enhanced surveillance, transmission control strategies and antimicrobial stewardship in human and veterinary medicine are of major importance.

4. CONCLUSIONS

The results of the present study show that ESBL producing *E. coli* have emerged among breeding pigs in Bhutan. In addition, the isolates were found to be MDR. Monitoring for ESBL producing bacteria should be continued in humans and animals in order to protect public health.

Conflict of interest statement

None to declare.

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