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Article

Occurrence and Characteristics of Extended-spectrum β-Lactamase-Producing *Escherichia coli* from Dairy cattle, milk and farm environment in Peninsular Malaysia

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Abstract: The emergence and spread of antimicrobial resistance genes and resistant bacteria does not recognized animal, human or geographic borders. Addressing this threat requires a combination of multidisciplinary approach involving human, animal and environmental health (One Health). Because antimicrobial agents used in veterinary medicine maybe the same or like those in human medicine. Extended-spectrum beta lactamase (ESBL) *E. coli* is a growing public health problem worldwide, and the Agri-Food industry is constantly becoming sources of clinically important ESBL bacteria. Accordingly, the aim of this study was to investigate the occurrence and characteristics of ESBL-producing *E. coli* from dairy cattle, milk, and the farm environment. *E. coli* isolates were identified by their 16sRNA and their ESBL production was confirmed by ESBL-CHROMagar media and double disk diffusion method. Genotypes of ESBL producers were characterised using mPCR assay. Among the examined samples, 18(4.8 %) were positive for ESBL-producing *E. coli*. Of these, 66.7% were from milk, 27.8% and 5.5% were from farm environment and faecal samples respectively. Predominant ESBL Genotype identified were a combination of both TEM and CTX-M in eight out of 18 (44.4%) isolates. Four (22.2%) isolates produced CTX-M gene, two (11.1%) isolates produced TEM gene and four (22.2%) remaining isolates produced ESBL genes other than TEM, SHV, CTX-M and OXA. The SHV and OXA gene were not detected in all 18 isolates. The occurrence of these genotype in indicator organisms from dairy cattle, milk, and farm environment further re-enforced the potentials of food-animals as sources of infection for humans via the food chain. Thus, consolidating the need for the adoption of tripartite One Health approach in surveillance and monitoring antimicrobial resistance.

Keywords: Characterisation; Epidemiology; ESBL; *Escherichia coli*)

1. Introduction

Despite being a significant public health problem, the remote cause and actual cost of antimicrobial resistance (AMR) in most part of the world, remains unclear. As a complex global health problem, tackling the menace of resistant bacteria requires a holistic transdisciplinary

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approach involving human, animal and environmental health sectors, collectively tagged One-Health approach. The concept of One Health in tackling resistant bacteria through active surveillance and monitoring has been recognized as priority action, that facilitate better understanding of AMR. AMR surveillance program in the Agri-food sector considers commensal bacteria such as *E. coli* as target pathogen, because AMR profiles in *E. coli* almost accurately mirrors the use of antimicrobial agents in food animals [1, 2].

Escherichia coli producing narrow and extended spectrum beta-lactamases (ESBL) are continuously becoming a public health problem worldwide [3, 4]. ESBL are plasmid-mediated β-lactamase enzyme recognized for their remarkable ability to hydrolyse, penicillin, 3rd and 4th generation cephalosporins and monobactams except for carbapenem and cephamycin [5]. These enzymes emerged from *bla*TEM-1, *bla*TEM-2, and blaSHV a narrow-spectrum parent. Recently, blaCTX-M, a new class of ESBL genes appeared have gained global traction, because of the burden it placed on environment health. Amino acid sequence analysis of CTX-M variant grouped these enzymes into five distinct clusters including CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 [4, 5]. The success of the genes in the ecosystem may likely be associated with the spread of bacterial strains carrying ESBL genes and horizontal transfer of these genes on transmissible plasmids [6-8]. Thus, making identification of sources and routes of transmission of ESBL-producing *E. coli* difficult. The mechanism of resistance to β-lactams in *E. coli* is majorly based on the inactivation of the β-lactams antibiotics via hydrolysis of their β-lactam rings catalysed by β-lactamase enzymes. *E. coli* isolates that carry ESBL genes can hydrolyse almost all cephalosporins and penicillin [8-10]. ESBL enzymes are mostly found in *Enterobacteriaceae* and often exhibit multi-drug resistance against non-β-lactams antimicrobial agents [4, 11].

Antimicrobial resistance (AMR) in veterinary medicine is a growing problem, because it involves various species of animals and microorganisms, different breeding and animal rearing environment as well as resistance mechanisms [9, 12]. Farm animals including poultry, pigs, beef and dairy cattle have been reported as likely sources of antimicrobial resistant bacteria and resistance genes [7, 13]. These resistant bacteria may transfer resistance genes horizontally to humans via the food chain and thus may pose risk to public health. However, resistant bacteria are not only limited to food animals. Food animal-derived products namely meat, milk and cheese have been reported to harbour resistant zoonotic bacteria [4, 11, 14]. Farm environment including soils, water, pests and workers have also been reported to carry these harmful pathogens which contribute to the dissemination and maintenance of resistance genes in the environment [10, 15, 16]. This situation may cause serious bilateral implications to farm animals and human. Food producing animals have been recognized to harbour several zoonotic pathogens including beta lactamase-producing *E. coli* [17]. Hence, this study aimed to investigate the occurrence and characteristics of ESBL-producing *E. coli* from dairy cattle, milk, and farm environment in Malaysia.

2. Results

Out of the 377 samples collected and examined for ESBL production, 18 (4.8%) were positive for ESBL *E. coli*. Of these, only one cattle (0.4%) from a total of 229 faecal samples, was positive for ESBL-producing *E. coli* and seven farms had either faeces, environment and/or milk samples positive. ESBL-producing *E. coli* were not detected in faeces, environment or milk samples in three farms (farm 6, farm 8 and farm 10). Among all farms, farm 4 yielded the highest occurrence of ESBL-producing *E. coli* (15.9%). The highest occurrence of ESBL-producing *E. coli* in milk samples was also from farm 4, where six of seven (8.5%) isolates were detected (Table 1). For farm environment, ESBL-producing *E. coli* were detected in drinking water at 3/77 (3.9%), and one isolate (1.3%) each from water source and house flies respectively (Table 2). ESBL-producing *E. coli* was not detected from floor, feed and water trough swabs as well as feed samples. Genotypic detection of ESBL genes produced by isolates was dominated by gene combination of both TEM and CTX-M in eight out of 18 (44.4%) isolates. Four (22.2%) isolates produced CTX-M gene, two (11.1%) isolates produced TEM gene and four (22.2%) remaining isolates produced ESBL gene other than TEM, SHV, CTX-M and OXA. The

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SHV and OXA gene were not detected in all 18 isolates (Table 3). Figure 1 showed genes amplified from the multiplex PCR assay.

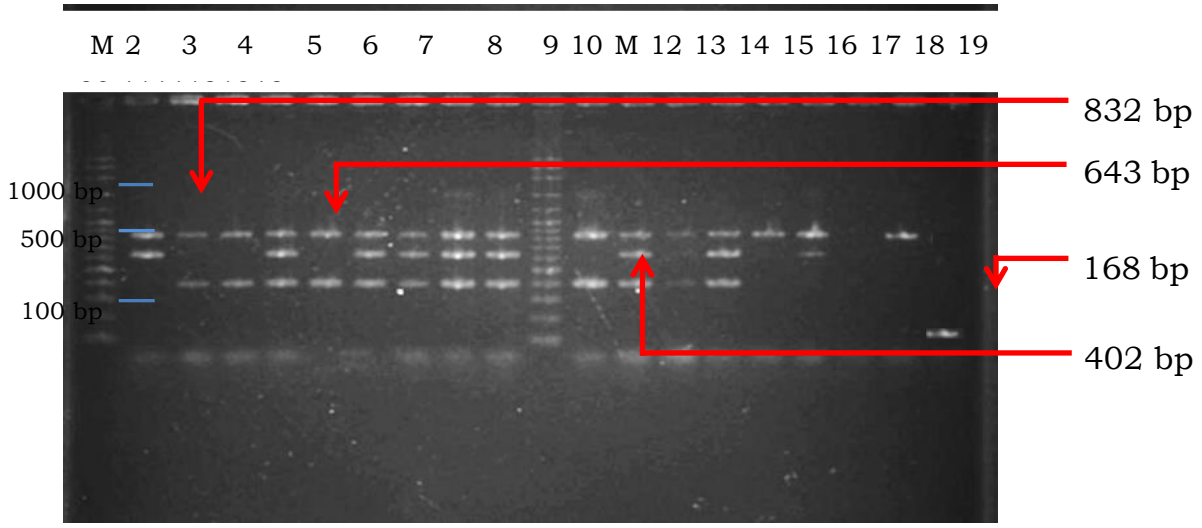

Figure 1. Extended Spectrum Beta Lactamase (ESBL) genes detected using multiplex PCR assay: Lanes M: marker 100 bp ladder; Lanes 2 to 10 and lanes 12 to 17: ESBL-producing *E. coli* isolates; Lane 18: negative control; Lane 19: *E. coli* ATCC 25922; Lane 20: *K. pneumonia* ATCC 700603.

Table 1. Occurrence of ESBL-producing *E. coli* in dairy cattle, farm environment and milk.

Farms	No. of samples collected	Sample type			Occurrence ESBL-producing *E. coli* (%)
		Faecal samples *n* = 229 (%)	Farm Environment *n* = 77 (%)	Milk *n* = 71 (%)	
1	**38**	0	0	1	1 (2.6)
2	42	0	1	-	1 (2.4)
3	44	0	2	1	3 (6.8)
4	44	1	0	6	7 (15.9)
5	26	0	1	1	2 (7.7)
6	44	0	0	0	0 (0)
7	28	0	1	0	1 (3.6)
8	35	0	0	0	0 (0)
9	39	0	0	3	3 (7.7)
10	37	0	0	0	0 (0)
TOTAL	**377**	**1 (0.27)**	**5 (1.32)**	**12 (3.18)**	**18 (4.8)**

Table 2. Occurrence of ESBL-producing *E. coli* in farm environment.

Sample Type	No. of samples collected	Occurrence ESBL-producing *E. coli* (%)
Floor, feed and water trough swabs	20	0 (0)

 doi:10.20944/preprints202009.0485.v1

Drinking water	16	3 (18.6)
Source of drinking water	10	1 (10)
Feed	17	0 (0)
House flies (*Musca domestica*)	14	1 (7.1)
TOTAL	**77**	**5 (6.5)**

Table 3. ESBL genotypes detected in ESBL-producing *E. coli.*

Farms	sample ID (18 isolates)	Sample Type	ESBL Genotype
1	M3	Milk	TEM
2	WS	Source of drinking water	CTX-M
3	M8	Milk	CTX-M
	DW1	Drinking water	TEM, CTX-M
	DW2	Drinking water	CTX-M
4	Milk 1	Milk	Not detected
	Milk 3	Milk	TEM, CTX-M
	Milk 4	Milk	TEM, CTX-M
	Milk 4 (ii)	Milk	TEM, CTX-M
	Milk 5	Milk	TEM, CTX-M
	Milk 6	Milk	CTX-M
	M4	Faeces	TEM, CTX-M
5	Milk 4	Milk	Not detected
	Hf 2	House flies	Not detected
7	DW2	Drinking water	TEM, CTX-M
9	Milk 5	Milk	TEM, CTX-M
	Milk 7	Milk	Not detected
	Milk 8	Milk	TEM

3. Discussion

The emergence and spread of ESBL *E. coli* and genotypes ESBL has become a public health concern, because of its association with increased morbidity and mortality, reduced treatment options and prolonged hospital admission. The present study was designed to investigate the occurrence and characteristics of ESBL *E. coli* from dairy cattle, milk and farm environment in Malaysia. Of the 229 faecal samples collected, only one (1) (0.4%) was positive for ESBL-producing *E. coli*, that is, isolated from a lactating cow; five out of 77 (6.5%) samples that were positive, were isolated from the farm environment. Milk samples yielded the highest occurrence of ESBL-producing *E. coli* which was 12/77 (16.9%). There was a statistically significant difference (χ^2=32.94, p<0.00) in the occurrence of ESBL-producing *E. coli* among dairy cattle, farm environment and milk. Five (5) out of eight (8) (62.5%) samples that produced gene combination of TEM and CTX-M were from milk, two from drinking water and one from faecal sample. The TEM gene alone was identified from two (2) milk samples, while the CTX-M gene alone was identified from milk, water source and drinking and faecal sample. ESBL genotypes were not detected in four (4) of 18 (22.2%) isolates. However, according to Schmid et al. [13], the phenotypically positive ESBL-producing *E. coli* but genotypically negative ESBL genes could also be regarded as ESBL producers because the performed m-PCRs screened for the most common resistance genes and did not include all existing resistance genes. In Malaysia, the occurrence of ESBL-producing *E. coli* in dairy cattle has not been reported prior to this study. A large percentage (>60%) on the prevalence of ESBL-producing organisms in food animals

and their products have been extensively reported by various countries particularly in the European region [13, 14,15,18]. Those studies reported occurrence of ESBL-producing organisms in food animal including dairy cattle, poultry and beef cattle, and in animal-based products. However, reports on ESBL-producing organisms in Asia were only limited to Japan, China and Korea [16, 19-21].

From this study, the occurrence of ESBL-producing *E. coli* in dairy cattle in Malaysia was low. The dairy industry in the country is not a major livestock industry compared to poultry and swine industries. There are quite many commercial scale dairy farmers; however, most of the dairy farmers operated the farms at a small-scale level. The small scale dairy farmers had a small herd size which was less than 100 animals in a farm. High density of animals in a farm may provide a conducive environment for the transfer of resistant genes between animals as well as between bacterial species. Watson et al. [14], reported a high prevalence of CTX-M-15 producing *E. coli* which was observed in different cattle groups including heifers, dry cows, high and low milk yielding groups. However, in this present study, the occurrence was found in a lactating cow (0.4%). The other dairy cattle may not be shedding *E. coli* carrying ESBL enzymes at the time of sampling or they may be truly absent. Several other published studies reported low prevalence of ESBL-producing bacteria in cattle. In a study conducted in Japan, the prevalence of CTX-M-2 beta-lactamase among cattle was 1.5% (6 of 396 cattle sampled) [14]. In another study conducted by Reist et al . [22] in Swiss cattle population younger than two years old at abattoir level, the authors reported slightly lower (8.4%) prevalence of ESBL *E. coli*. In a Korean study, Tamang et al. [16] detected 0.2% ESBL-producing *E. coli* among healthy cattle. Furthermore, in Tunisia, Jouini et al. [23] found no ESBL-producing *E. coli* in cattle.

There was no association between the occurrence of ESBL-producing *E. coli* in the lactating cow group and the milk samples collected. This was also shown in a study by Geser et al. [24]. It was observed that although the animals were negative for ESBL-producing *E. coli;* however, the milk from these animals were positive. The reason could be that the *E. coli* carrying ESBL genotypes in the milk may have originated from the environment. Several factors have been reported to contribute to the presence of pathogens in milk, which included dairy farm environment hygiene, numbers of animals on the farm, farm management practices, farm workers, geographic location and season [25] .

In this study, CTX-M gene was predominantly detected in 66.7% of the isolates. This finding was consistent with the study conducted among healthy food animals in China [20] and cattle in Republic of Korea [16]. In a study conducted in a Malaysian hospital, it was reported that CTX-M-15-producing *E. coli* was the predominant CTX-M variant in paediatric patients [26]. However, Lim et al. [27] in their study on characterization of ESBL-producing *E. coli* isolates in a different Malaysian hospital found a high occurrence of TEM ESBL (87.5%). Farm management and practices may have contributed to the occurrence of ESBL-producing *E. coli* in the animal and environment. Frequency of farm cleaning might also be influenced the low prevalence of ESBL-producing *E. coli*. The floors of the animal stalls were cleaned at least two times per day which may help to reduce the risk of bacterial contamination to the animals and farm environment. Oliver et al. [25] reported farm management practices contribute to the prevalence of pathogenic microorganisms in the farm. The types of animal farming whether intensive, semi-intensive or free ranging can contribute to the development of antibiotic resistance due to inappropriate use of antibiotics. Mixing of animal feed with antibiotic for increasing feed efficiency and production level has been a common practice in livestock management particularly in poultry and swine industries. However, it is not a common practice to mix antibiotics in dairy cattle feed. Fresh, cut and carry grasses were given to the dairy cattle *ad. lib* supplemented with dairy cattle pellet and some agricultural by-product such as molasses. Hence, such a situation may result in the low prevalence of ESBL-producing *E. coli* in this study because of less use of antibiotics at sub therapeutic level. The presence of ESBL-producing *E. coli* in raw milk may pose food safety hazards to human if milk is not heat-treated. Such resistant organisms may colonize the human intestinal tract and contribute resistance genes to human endogenous flora [28]. Timofte et al. [29], reported the first case of bovine mastitis due to ESBL-producing *E. coli* with CTX-M-15 in Europe and due to *K. pneumoniae* subsp. *pneumoniae* SHV-12 in the United Kingdom.

Nineteen per cent (19%) drinking water samples carried ESBL-producing *E. coli*. The drinking water may be contaminated with faeces of dairy cattle harbouring ESBL-producing *E. coli*. Another

 doi:10.20944/preprints202009.0485.v1

possible explanation is contamination of the cattle drinking water by birds' dropping. It was also found that flies carried ESBL-producing *E. coli*, which would spread the organism in the environment. Surface water comprised rivers, streams, lakes and ponds may be the source of hazardous biological contaminants. In a study conducted in Malaysian urban surface water, Tissera & Lee, [30] reported *E. coli* and *K. pneumoniae* were predominantly isolated (89.5%), with a relatively high occurrence of CTX-M genes (84.2%), followed by TEM genes (47.4%). Similarly, Lu et al.[31] found a high diversity of ESBL-producing bacteria, with CTX-M being the most dominant gene being isolated from an urban river sediment habitat . The finding in this present study was similar where CTX-M was isolated from sampled water source. Such water source if used for washing and drinking may lead to contamination of udder milking equipment and colonization in animals.

All dairy cattle farms in this study practiced open-house systems and wild birds were observed to freely flew to find food and water in those farms and hence they may have contaminated the house including feed and water. These birds were likely to disseminate resistance genes as they have been reported to shed ESBL-producing *E. coli* in the environment [32]. Migratory birds have been reported to play an epidemiological role in disseminating antibiotic resistance genes and as a potential reservoir of ESBL-producing organisms [28]. Food of animal origin may play a role in disseminating ESBL-producing *E. coli* implicating mastitis in dairy cattle which originate from the environment and were reported to occur more commonly in high producing cow at the first two weeks after calving. Cattle were most likely to get infected when they lie on faeces-contaminated-bedding [33]. *E. coli* may enter the teat orifice causing ascending infection of the mammary gland.

4. Materials and Methods

4.1. Study design and sample collection

A total of 377 samples were collected from ten dairy cattle farms located within Selangor and Negeri Sembilan states, and were examined for ESBL-producing *E. coli*. The samples included 229 faeces, 71 milk and 77 farm environment samples. Each faecal sample was collected using a sterile swab and placed in 9 mL of sterile buffered peptone water (BPW) (CM0509, Oxoid, Basingstoke, Hampshire, England). Milk samples were collected from the lactating cows from which faecal samples were taken. Approximately 20 mL of milk sample from each lactating cow were hand-milked directly into a sterile bottle. All cows were apparently healthy and did not exhibit any clinical signs of mastitis. Farm environment samples collected and used included, swabs of stall/pen floor, house flies, feed and water troughs. Floor swabs were collected randomly which included calves pen, areas covering dry and milking cow stall and working bull pen. Three swabs each were collected from the floors and feed and water troughs which were pooled as a sample. A scoopful of each leftover feed sample (n=17) approximately 100 g was collected and placed in a disposable bag. Live houseflies (*Musca domestica*) (n=14) were trapped by using adhesive fly trap placed at two spots in the farm. By using some sterile forceps, ten live houseflies were taken from the adhesive flytrap and put into a transport media and was considered as a pooled sample. Two pooled samples of houseflies were collected from each of the seven farms, and one pooled sample each for the remaining three farms. Drinking water (n=16) were taken directly from the dairy cattle water trough, while sources of drinking water (n=10) were taken if the sources were untreated pond or well. At least 100 mL of drinking water and source of drinking water were each collected in an individual sterile bottle. All swab samples were kept in BPW.

4.2. Isolation and identification of ESBL-producing E. coli

For primary isolation and identification of ESBL producing *E. coli*, sample were initially cultured on Chromocult® Coliform Agar (Merck KGaA, Darmstadt, Germany) and incubated at 37 °C for 18-24 h. The dark-blue to violet colonies which appeared on Chromocult® Coliform agar were presumptively identified as *E. coli.* These colonies were overlaid with a drop of Kovacs® Indole reagent. The presence of *E. coli* is positive and confirmed if a cherry-red colour appeared after a few seconds. Presumptive *E. coli* isolates from faeces, environment and milk samples were further

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cultured on CHROMagar™ ESBL (CHROMagar™, France) and incubated at 37 °C for 18 to 24 h. This medium is a selective and chromogenic media for phenotypic isolation of ESBL *E. coli*. Suspect ESBL-producing *E. coli* colonies were sub-cultured on Nutrient agar (CM0003, Oxoid Ltd, Basingstoke, Hampshire, England) prior to the phenotypic confirmatory tests.

4.3. Phenotypic confirmation of ESBL-producing E. coli

Confirmation of ESBL *E. coli* was carried out using double disk diffusion method [34]. All presumptive ESBL *E. coli* isolates were subjected to confirmation for ESBL production using combination disk diffusion method with ceftazidime (30 μg) and cefotaxime (30 μg) alone and in combination with clavulanic acid (30 μg/10 μg) (Difco/BD, Franklin Lakes, NJ, USA). The *E. coli* isolates were phenotypically considered as ESBL-producer, when an increase in the size of inhibition zone is greater than (≥ 5 mm) for antimicrobial agent with or without clavulanic acid was observed. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

4.4. Genomic DNA Extraction

Genomic DNA extraction was performed using boiling method as described by Li et al. [34] with slight modification (i.e., DNA was extracted at 98 °C instead of 100 °C). Suspension of overnight fresh cultures of ESBL *E. coli* isolates was prepared using a sterile distilled water in a 100 µL micro-centrifuge tubes (Eppendorf). Cells suspension were heated using a dry bath at 98 °C for 10 min followed by cooling at room temperature for 5-10 min prior to centrifuging at 13000 x g for 3 min at 25 °C. Total extracted DNA (200 *n*g equivalent to 5 µL) was then subjected to mPCR assay.

4.5. Genotypic detection of ESBL genes by multiplex Polymerase Chain Reaction (mPCR)

All the 18 ESBL *E. coli* isolates were investigated for the presence of ESBL genotypes including; TEM (643 bp), SHV (168 bp), CTX-M (402 bp) and OXA (250 bp) by mPCR assay. A list of primers used for mPCR assay is shown Table 4. The mPCR assay was performed in a 50 µL reaction mixture containing 10 µL of primers set (1µL each primer), 25 µL MyTaq™ HS Mix (Bioline, UK) and 10 µL RNAse free water (Qiagen, Germany). The multiplex-PCR cycling condition were as follows; initial denaturation at 95 °C for 1 min. followed by 30 cycles of denaturation at 95 °C for 15 secs, annealing at 60 °C for 15 secs, and extension at 72 °C for 10 secs and final extension at 72 ºC for 10 min [14]. Amplified mPCR products were resolved in 1.0% agarose gel containing ethidium bromide. Quality control organisms used in this study were *K. pneumoniae* ATCC 700603 as positive control and *E. coli* ATCC 25922 as negative control [34].

Table 4. Primer used for the detection of ESBL genes **and *E. coli.***

Gene	Primer sequence (5′ – 3′ direction)	Product size (bp)	Gene accession no.
TEM	Forward - TCCTTGAGAGTTTTCGCCCC Reverse - TGACTCCCCGTCGTGTAGAT	643	EU352903
SHV	Forward – CAATCACGACGGCGGAATCT Reverse – GTGGGTCATGTCGGTACCAT	168	AB731686
CTX-M	Forward – AAGCACGTCAATGGGACGAT Reverse – GTTGGTGGTGCCATAGCCA	402	JN411912
OXA	Forward – TTGCACTTGATAGTGGTGTGA Reverse – AGTGAGTTGTCAAGCCAAAAAGT	250	JN003412
E. coli	Forward – TGACGTTACCCGCAGAAGAA Reverse – CTCCAATCCGGACTACGACG	832	X80724

4.6. Data Analysis

Data were analysed using IBM SPSS Statistics version 21.0. The chi-square (χ^2) test was used to compare the occurrence of ESBL-producing *E. coli* in dairy cattle, farm environment and milk samples. Statistical significance was defined at 95% confidence interval ($p \leq 0.05$).

5. Conclusions

The occurrence of ESBL-producing *E. coli* in dairy cattle, farm environment and milk was low but in milk it was high, with CTX-M gene predominantly detected. Thus, it is of public health significance. It was also clearly indicated that healthy dairy cattle, farm environment and milk are the diverse reservoirs and sources of transmission of this potential zoonotic pathogen.

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