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Occurrence of Extended Spectrum Beta-Lactamase Gram-Negative Bacteria from Non-Clinical Sources in Dubai, United Arab Emirates

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Abstract: Extended-spectrum beta-lactamase (ESBL) producing bacteria of the *Enterobacteriaceae* family are a significant threat to public health, posing a challenge for health authorities worldwide. In the UAE, very little information is available about ESBL producing bacteria from non-clinical sources. In this study, 206 pure cultures belonging to the *Enterobacteriaceae* family were isolated from food and wastewater sources in Dubai, UAE. All the isolates were tested against third-generation cephalosporin antibiotics by the disc diffusion method and screened on ESBL chromogenic agar. Among all isolates (n = 86), 41.7% were potential ESBL producers belonging to *E. coli*, *Klebsiella*, *Enterobacter*, *Shigella*, and *Citrobacter* (KESC group), and Proteus. Of all the potential ESBL producing isolates, 19 (22%) were confirmed as ESBL producers by a double-disc diffusion test with the fourth generation cephalosporin–Cefpirome. The multiplex polymerase chain reaction was used for the detection of ESBL *bla* genes in the screened isolates. Out of a total of 86 isolates, 52.3% possessed only the *bla*TEM gene; 39.5% contained both *bla*TEM and *bla*SHV genes, while only 3.5% contained the *bla*CTX-M gene. The carbapenemase resistance test showed eight isolates resistant to imipenem, and only one isolate with metallo-beta-lactamase activity. This study highlights the occurrence of ESBL *bla* genes among non-clinical isolates from food and wastewater sources in the UAE and emphasizes the importance of food and wastewater surveillance programs in controlling the spread of antibiotic resistance.

Keywords: antibiotic resistance genes; cephalosporin; Gram-negative; *Escherichia coli*; extended-spectrum beta-lactamase (ESBL); *Enterobacteriaceae*; food; wastewater; UAE

1. Introduction

Antimicrobial resistance (AMR) has emerged as a significant concern to public health with several reports alerting of the substantial risk and increasing threat of antibiotic-resistant bacteria (ARB) causing common infections that could enhance the worldwide mortality rate by 2050 [1,2]. AMR is defined as the ability of microorganisms to survive and be viable under the influence of antimicrobial agents [3]. AMR occurs naturally over time, usually through genetic changes; however, the misuse and overuse of antimicrobials are increasing the prevalence of antibiotic resistance in bacteria [3,4].

The World Health Organization (WHO) Global Antimicrobial Surveillance System (GLASS) recently reported higher levels of resistance in bacterial strains, which are known to cause several dangerous and frequent infections in many countries [5]. A list of bacteria recently identified by the WHO includes mainly the AMR bacteria divided into three categories: critical, high and medium priority, according to their impact on human health and the urgency for the development of new antimicrobial drugs to treat resistant infections [6]. Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* members are included under the critical category associated with severe and, often deadly, infections, including bloodstream infections and pneumonia [7].

ESBL producing bacteria evolved because of the excessive use of beta-lactam antibiotics, which have been the most prescribed drugs for the treatment of bacterial infections in humans and animals for the past six decades [8]. The synthesis of beta-lactamase enzymes is one of several mechanisms by which different bacteria develop multi-drug resistance. The extended-spectrum beta-lactamase enzymes in bacteria belonging to *Enterobacteriaceae* families are regulated by a group of several genes, prominent among them being *bla*TEM, *bla*SHV, and *bla*CTX-M types [9] In gram-negative bacteria, *bla*TEM and *bla*SHV genes encoded the earliest known beta-lactamases enzymes hydrolyzing penicillin and first-generation cephalosporin [10,11]. Genes from the *bla*CTX–M group encode third generation cephalosporin hydrolyzing enzymes [12]. A high prevalence of ESBL producing bacteria has been reported extensively in clinical settings worldwide, including in the United Arab Emirates and the Gulf Cooperation Council (GCC) countries [13,14]. The *Enterobacteriaceae* family resistance rates to third generation cephalosporins and carbapenems were found to be highest in clinical settings according to the 2017 UAE National AMR Surveillance report [15]. A high occurrence of ESBL *Enterobacteriaceae* clinical cases suggest potential widespread resistance challenges in the UAE. So far, efforts to tackle AMR have focused on clinical settings through a dedicated surveillance action plan and several other initiatives outlined in the UAE AMR action plan, but not much attention has been paid to non-clinical environments. A recent discussion article indicates that AMR in non-clinical settings in the GCC region probably has been underestimated [16].

The ESBL producing *Enterobacteriaceae* are becoming increasingly prevalent not only in hospital environments but also in water, wastewater, soil, and food such as fresh vegetables and meat [17–19]. Furthermore, meat is considered a reservoir of ESBL producing bacteria, as reported in recent studies [20–22]. As per the report published by the Glasgow consulting group, UAE meat consumption per capita rate is very high with poultry contributing 48.9% to per capita meat consumption, followed by lamb and goat (13.5%), beef (12.1%), and other meats [23]. A high rate of meat consumption patterns warrants the need for a high level of systematic monitoring and control of antibiotic-resistant bacteria in meat products. It is well established that the ESBL type gram-negative bacteria are the most common cause of severe infections globally. The detection and characterization of ESBL types of bacteria in the UAE is increasingly important. Due to the lack of documented information about ESBL producing bacterial species in the Gulf region, it is essential to determine the occurrence of ESBL bacteria in wastewater and meat samples collected from various supermarkets and butcheries in Dubai, UAE. The city of Dubai is the fourth most visited city in the world receiving over 15 million international overnight visitors in 2016 [16]. Thus, very strict regulations are in place to monitor, and control, the spread of microbial agents through the food and wastewater environment by the Dubai Municipality. We therefore hypothesized that the occurrence of ESBL producing *Enterobacteriaceae* is likely to be lower in non-clinical environments such as meat and wastewater than in clinical settings.

The present study attempted to investigate the occurrence of ESBL producing Gram-negative bacteria belonging to the *Enterobacteriaceae* family in retailed meat and in wastewater and to characterize the distribution of ESBL genes among ESBL types of bacteria.

2. Materials and Methods

2.1. Isolation, and Characterization of Pure Cultures

Isolation of Gram-negative bacteria from meat samples was carried out as follows: twelve meat samples were collected from a local butcher's shop, kept in plastic zip lock bags and stored in the refrigerator at 4 °C before transportation to the laboratory. Of these twelve samples, there were four each of chicken, lamb, and beef. All samples were processed within 24 h of collection. The following procedure was used for isolation of bacteria from meat samples: briefly, 1 g of each meat sample (i.e., chicken, lamb, and beef) was homogenized in sterile buffered peptone water using a mortar and pestle. The homogenate was then incubated for 24-h at 37 °C for pre-enrichment. Following this, 0.1 mL of the homogenate was spread onto MacConkey Agar and Hektoen Enteric Agar (HE) plates and was incubated for an additional 24-h at 37 °C [24].

For isolation of Gram-negative bacteria of the *Enterobacteriaceae* family, six activated sludge samples were collected from a municipal sewage treatment plant, transported to the laboratory at a temperature of 4 °C and processed on the day of collection. The mixed liquor samples after homogenization were serially diluted from 10^{-1} to 10^{-8}, using 0.85% saline (NaCl) [25]. Aliquots of 0.1 mL from the final three dilutions (i.e., 10^{-6}, 10^{-7}, and 10^{-8}) were spread onto MacConkey Agar and HE Agar plates. All plates were incubated for 24 h at 37 °C. All isolates were purified on nutrient agar by streak plating several times. After purification, purified colonies were preserved in 10% glycerol stock at −80 °C in Eppendorf tubes and cultivated on nutrient agar medium as described earlier [25]. Biochemical characterization of bacterial cultures was performed using the standard Indole test, Methyl Red test, Voges-Proskauer test, and Citrate (IMViC) series of biochemical tests, including *E. coli* ATCC 25922, as a control culture [26]. The oxidase test for differentiating members of the *Enterobacteriaceae* family was performed by a drop of Kovac's oxidase reagent, consisting of 1% dimethyl-p-phenylenediamine, aseptically placed onto a smear made of overnight culture [25]. A purple/dark blue or black color reactant, appearing within 30 s or so, is positive for cytochrome oxidase, while no color development indicates a negative test for cytochrome oxidase. All bacteria isolation experiments were performed in triplicate for each sample. Molecular identification of all bacterial isolate pure cultures was performed by a fluorescence in situ hybridization technique (FISH) using oligonucleotide probes specific to the *Enterobacteriaceae* family and *E. coli* species described earlier [27]. The detailed procedure for FISH is described in Appendix A.

2.2. Antimicrobial Susceptibility of Isolated Bacteria

Antimicrobial susceptibility of all 206 isolates to third generation cephalosporin antibiotics cefpodoxime (10 µg), ceftazidime (30 µg), cefotaxime (30 µg) and ceftizoxime (30 µg) was determined by the Kirby-Bauer disc diffusion method [24]. The results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI) and recorded as susceptible (S), intermediately susceptible (I), or resistant (R) based on a standard protocol (Table S2 and Figure S1). *E. coli* (ATCC 25922) was included as a control culture throughout the antimicrobial susceptibility tests recommended by CLSI [28]. The susceptibility of *E. coli* ATCC 25922 to third generation cephalosporins is shown in supplementary Table S3.

2.3. Screening of ESBL Producers

ESBL activity of isolates and their classification into various groups of bacteria was determined by streaking onto ESBL chromogenic agar plates (HiMedia, India) incubated at 37 °C for 24 h. The chromogenic media enabled the grouping of isolates into coliform and *Proteus* groups. A double-disc diffusion test was performed to further confirm ESBL bacteria using fourth generation cephalosporin cefpirome (30 µg) discs alone and in combination with clavulanic acid (30/7.5 µg) (Figures S2 and S3) as recommended by CLSI [28]. *E. coli* ATCC 25922 was used as a negative control throughout the tests as a non-ESBL culture. The ESBL positive bacterial isolates were used to inoculate 5 mL of Tryptone Broth and incubated at 37 °C for 24 h. By using a sterile spreader, 0.1 mL of the inoculum

was spread onto Muller Hinton (MH) agar plates. Cefpirome (CFP) and cefpirome/clavulanic acid (CPC) discs were placed approximately 25–30 mm apart. The culture plates were then incubated at 37 °C for 24 h, after which the diameters of the clear plaques surrounding the discs were measured to the nearest mm. The zone size interpretation is shown in supplementary Table S4. A more than or equal to 5 mm increase in cefpirome/clavulanic acid zone versus clear zone, when tested with cefpirome disc alone, indicated positive confirmation of the ESBL isolate. The ESBL isolates were further evaluated by the combined disc test for carbapenem resistance based on the inhibition of MBL (Metallo-Beta-Lactamase) activity by Ethylenediaminetetraacetic acid (EDTA) according to the CLSI guidelines [28]. Two imipenem discs containing imipenem (IPM 10) alone and the other containing imipenem-EDTA (IE 10/750), were placed 25–30 mm apart (Figure S4). The ESBL positive bacterial isolates were inoculated in 5 mL of Tryptone Broth and incubated at 37 °C for 24 h. Using a sterile spreader, 0.1 mL of the inoculum was then spread onto MH agar plates. An isolate producing a zone diameter of >4 mm around the disc with IE (Imipenem-EDTA), and not around the disc with IPM (Imipenem) alone, was considered positive for MBL *E. coli* ATCC 25922 was used as a negative control throughout the test (Table S5).

2.4. Molecular Detection of ESBL Genes

Molecular typing of the potential ESBL producers screened earlier by phenotypic characterization was performed by multiplex Polymerase Chain Reaction (PCR) using previously reported ESBL *bla* primers [29,30]. The multiplex PCR was performed for *bla*TEM, SHV, and CTX-M genes using a previously reported primer (Table 1). Genomic DNA from bacterial strains was extracted using the Bacterial Genomic DNA Isolation Kit as per the manufacturer's instruction (Norgen Biotek Corp.) The multiplex PCR reaction mixture consisted of 1× Buffer (10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, 500 mM KCl), 0.2 mM each dNTP, 1 µM of each of the primers, 1.25 U Taq DNA polymerase and 50–100 ng DNA in a total volume of 25 µL. Initial denaturation was performed for 5 min at 94 °C, followed by 35 cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, followed by a post-extension hold at 72 °C for 7 min. The PCR products were then analyzed by gel electrophoresis using 2.0% agarose gel in a 1× TAE buffer (0.5% of Tris-acetate-EDTA). The gels were stained with 5 µg/mL Ethidium bromide in a 1× TAE buffer, and the PCR products were visualized by UV transillumination [30].

Table 1. List of primers used for Extended-spectrum beta-lactamase (ESBL) genes detection.

ESBL Detection Primer	Nucleotide Sequence	Amplicon Size (bp)	Reference
*bla*TEM	5′-GTG CGG TAT TAT CCC GTG TT-3′ 5′-AAC TTT ATC CGC CTC CAT CC-3′	416	[29,30]
*bla*SHV	5′-GGA AAC GGA ACT GAA TGA GG-3′ 5′-ATC CCG CAG ATA AAT CAC CA-3′	301	[29,30]
*bla*CTX-M	5′-CGC TTT CCA ATG TGC AGT AC-3′ 5′-TCG CCG CTG CCG GTC TTA TC-3′	510	[29,30]

2.5. Statistical Analysis

The results were subjected to statistical processing with SPSS 25 software (IBM Corp. Chicago, IL, USA) applying Fisher's exact test for comparing ESBL isolates from meat and wastewater with a level of significance of $p < 0.05$. The comparison was made for antibiotic resistance patterns between chicken and combined beef and lamb isolates, and all meat versus wastewater isolates. Statistical correlation was done between *Enterobacteriaceae* and *E. coli*, *Klebsiella*, *Enterobacter*, *Shigella*, and *Citrobacter* (KESC), and *Proteus* isolates (recovery based on ESBL chromogenic agar growth) from wastewater and meat sources. The statistical analysis was performed for distribution *bla*TEM and *bla*SHV/blaCTX-M for

E. coli versus KESC for the presence of TEM alone; *Proteus* sp. versus KESC for the presence of TEM alone, and *E. coli* versus *Proteus* sp. for the presence of TEM alone.

3. Results

3.1. Isolation and Biochemical Characterization

A total of 206 pure cultures were isolated from wastewater and meat samples by serial dilution and spread plating techniques. Among all meat samples, the highest number of isolates were obtained from chicken meat (n = 95), followed by beef (n = 29) and lamb (n = 21). A total of 61 isolates were recovered from an activated sludge unit of a municipal sewage treatment plant. All the bacterial isolates were biochemically characterized by standard IMViC series of biochemical tests, including *E. coli* ATCC 25922 as quality control test culture. All pure cultures were found to be Gram-negative, with 155 isolates with bacilli and 51 isolates with coccobacilli morphology. Oxidase test and IMViC Tests were performed using log-phase cultures of bacteria. The series of biochemical reactions allowed differentiation among members of the *Enterobacteriaceae* group. The oxidase test for cytochrome oxidase was performed on all 206 isolates, and 19 isolates were oxidase positive, while 187 isolates were oxidase negative, thereby confirming that they belonged to the family Enterobacteriaceae. A positive Indole reaction in 121 isolates was indicated by forming a red ring at the medium's surface. A negative reaction in 85 isolates showed the original yellow color of the Kovacs reagent, which remained unchanged. The positive MR reaction was shown by 180 isolates for the presence of an acid, as indicated by a distinct red color, whereas 26 isolates showed negative (yellow) MR reactions after adding the reagent. A positive VP reaction in 46 isolates was indicated by the development of a cherry red color throughout the culture medium while a VP negative reaction was shown by 160 isolates where the color of the culture medium remained unchanged (yellow). A positive citrate reaction in 106 isolates was indicated by bacterial growth along the streak accompanied by a color change of the medium from its initial green to deep blue. One hundred isolates were citrate negative as the cultures either did not grow or left the color of the medium unchanged. The results of morphological and biochemical characterization of 206 pure cultures are shown in supplementary Table S1. FISH assay confirmed all 206 pure cultures that were isolated from wastewater and meat samples belonged to the *Enterobacteriaceae* family. All isolates showed excellent hybridization with both ENTBAC (specific to the family *Enterobacteriaceae*) and universal eubacterial (EUB)oligonucleotide probes and displayed strong fluorescent signals without a false-positive reaction (Appendix A Table A1 and Figures A1 and A2).

3.2. Antibiotic Susceptibility of Isolates

The sensitivity pattern of 206 isolates belonging to the *Enterobacteriaceae* family against third generation cephalosporin antibiotics is shown in Table 2. Out of 206 strains, 86 were found resistant to one or more third generation cephalosporin antibiotics, with high resistance to cefpodoxime (39%) followed by cefotaxime (33%), ceftizoxime (32.5%), and ceftazidime (29%).

Table 2. Distribution of isolates resistant to third generation cephalosporins.

Resistance to Third Generation Cephalosporins			
Cefpodoxime (CPD^{10})	**Ceftazidime (CAZ^{30})**	**Cefotaxime (CTX^{30})**	**Ceftizoxime (CZX^{30})**
39% (n = 80) *	29% (n= 60) *	33% (n = 68) *	32.5% (n = 67) *

* Out of total number of Gram-negative isolates (n = 206).

The antibiotic susceptibility test results of 86 potential ESBL producing isolates against third generation cephalosporins are shown in Table 3. All potential 86 ESBL isolates found to be resistant to one or more third-generation cephalosporins, with 93% of the isolates, have shown resistance to cefpodoxime followed by cefotaxime (79%), ceftizoxime (78%), and ceftazidime (70%). The phenotypic confirmation

of potential ESBL producing isolates was conducted by testing both cefotaxime and ceftazidime, alone and in combination with clavulanic acid, as recommended by CLSI [28]. Out of 86 potential ESBL isolates, only 22% (n = 19) were confirmed as ESBL producers by phenotypic characterization using double disc diffusion assay, including cefpirome (the fourth generation cephalosporin). These 19 isolates showed a zone of enhancement >5 mm in diameter between the cefpirome discs and clavulanic acid (Figure 1).

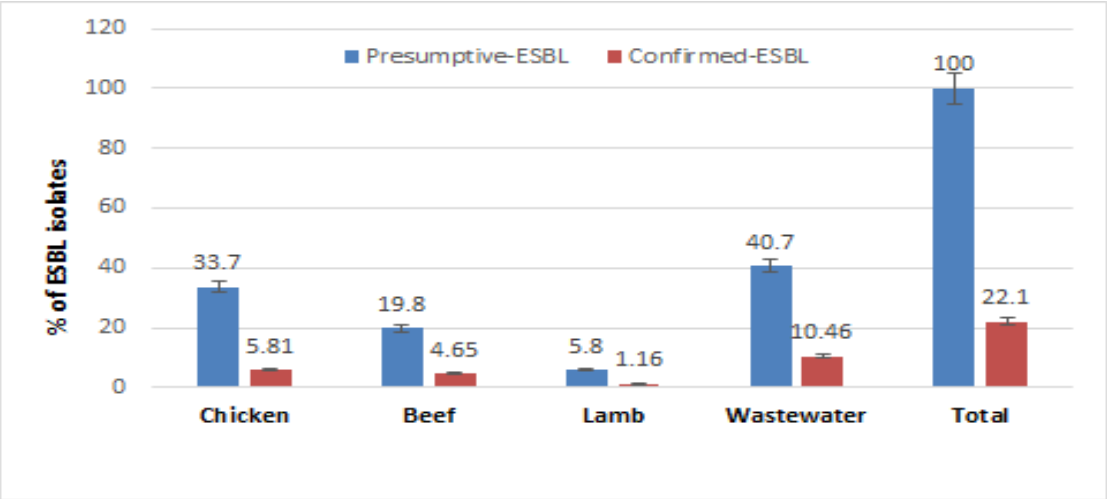


Figure 1. Number of confirmed ESBL isolates by double disc diffusion method.

Table 3. Antibiotic resistance pattern of ESBL-*Enterobacteriaceae* isolates (n = 86).

Antibiotic Class	Antibiotic	Meat Types (%) *			p **	Wastewater (n = 35)	p ***	All Isolates (n = 86) (%)
		Chicken (n = 29)	Beef (n = 17)	Lamb (n = 5)				
Cephalosporins (3rd generation)	Cefpodoxime (CPD^{10})	86.2 (n = 25)	94 (n = 16)	100 (n = 5)	NS	97.1 (n = 34)	NS	93 (n = 80)
	Cefotaxime (CTX^{30})	65.5 (n = 19)	88.2(n = 15)	80 (n = 4)	NS	86 (n = 30)	NS	79 (n = 68)
	Ceftizoxime (CZX^{30})	52 (n = 15)	82.3 (n = 14)	60 (n = 3)	NS	100 (n = 35)	0.0001	78 (n = 67)
	Ceftazidime (CAZ^{30})	62 (n = 18)	65 (n = 11)	80(n = 4)	NS	77.1 (n = 27)	NS	70 (n = 60)
Cephalosporin (4th generation)	Cefpirome+ Clavulanic acid	17.2 (n = 5)	23.5 (n = 4)	20 (n = 1)	NS	26 (n = 9)	NS	22 (n = 19)
Carbapenems	Imipenem (IMP)	6.9 (n = 2)	5.9 (n = 1)	0	NS	14.3 (n = 5)	NS	9.3 (n = 8)
	Imipenem-EDTA	0	(n = 1)	0	NS	0	NS	1.1 (n = 1)
Multi drug resistance (MDR)		79.3 (n = 23)	100 (n = 17)	100 (n = 5)	0.0305	100(n = 35)	NS	93 (n = 80)

* Isolates with susceptibility (S) and intermediate (I) resistance to antibiotics are excluded; ** Fisher's exact test: Chicken versus beef plus lamb isolates; *** Fisher's exact test: All meat versus wastewater isolates; NS: Statistically nonsignificant at significance level $p > 0.05$.

3.3. Presumptive Identification of Potential ESBL Producing Isolates

All bacterial isolates were screened for ESBL production using ESBL chromogenic agar. A total of 206 pure cultures streaked on ESBL chromogenic agar formed various types of colorful growth. Based on the developed color, bacterial isolates were categorized into potential *E. coli*, *Klebsiella*, *Enterobacter*, *Shigella*, and *Citrobacter* (KSEC), and proteus groups. A total of 41.7% (n = 86) out of 206 strains showed growth on ESBL chromogenic agar. ESBL producing *E. coli* showed pink or purple colony growth. The KESC group produced bluish-green colonies; *Proteus*, *Morganella*, and *Providencia* that do not utilize any chromogen resulted in colorless to light brown colonies on ESBL chromogenic agar (Figure 2).



Figure 2. Presumptive identification of Gram-negative bacterial isolates on ESBL chromogenic agar.

The grouping of isolates based on their growth characteristics on ESBL chromogenic agar are shown in Table 4. Out of a total of 86 potential ESBL isolates, 38 were grouped as possible *E. coli*, 25 (KESC), and 23 (*Proteus*). The highest percentages of ESBL positive isolates found among meat samples were from beef (n = 29, 58.6%) followed by chicken (n = 95, 31.6%) and lamb (n = 21, 24%). In contrast, total ESBL positive isolates (n = 61) from a wastewater source were found to be 56% (Table 4). The combined disc test for carbapenemase resistance based on the inhibition of Metallo-Beta-Lactamase activity by EDTA was conducted for potential 86 ESBL isolates. The carbapenemase resistance assay showed only eight (9.3%) isolates resistant to imipenem, and one (1%) isolate from beef meat source was confirmed as MBL positive, as it showed a zone of enhancement >4 mm in diameter between the imipenem and imipenem-EDTA discs.

Table 4. Screening of *Enterobacteriaceae* isolates based on ESBL chromogenic agar growth pattern.

Sample Source	No. of Isolates Analyzed	KESC *	*p*	*E. coli* *	*p*	*Proteus* *	*p*	Number of ESBL Producing *Enterobacteriaceae*	*p*
Wastewater (n = 6)	61	17	0.0001	11	NS	7	NS	35 (57.3%)	0.0001
Beef (n = 4)	29	3		10		4		17 (58.6%)	
Lamb meat (n = 4)	21	1		3		1		5 (24%)	
Chicken meat (n= 4)	95	4		14		11		29 (30.5%)	
Total	206	25		38		23		86 (41.7%)	

* Groups of *Enterobacteriaceae* based on growth on ESBL chromogenic agar; All p values for Fisher's exact test: Wastewater versus meat isolates at the level of significance $p < 0.05$; NS: Statistically not significant at significance level $p > 0.05$.

3.4. Molecular Typing of Potential ESBL Isolates

The presence of ESBL genes in all 86 isolates was determined by multiplex PCR. The PCR results provided an expected band size of 510, 416, and 301 bp for ESBL *bla*CTX-M, TEM, and SHV genes, respectively (Figure 3).

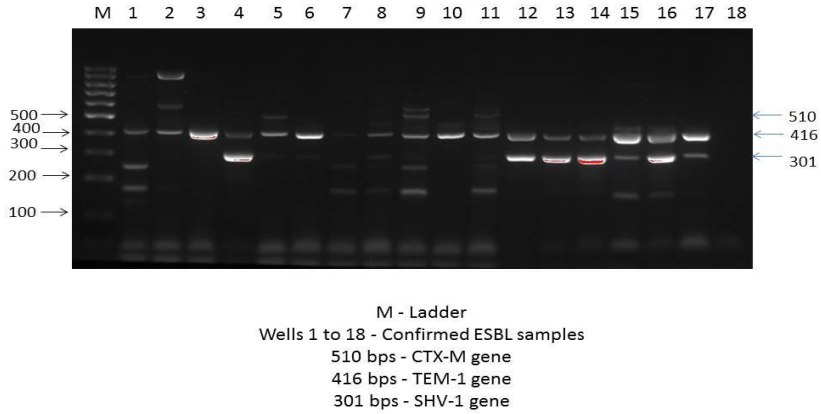


Figure 3. Detection of ESBL *bla* genes of the CTX-M, TEM, and SHV families by multiplex Polymerase Chan Reaction (PCR).

All the 86 pure culture isolates were found to contain *bla*TEM, *bla*SHV, and *bla*CTX-M ESBL genes by molecular typing (Table 5). Of these 86 isolates, 52.3% of isolates were found to possess only TEM genes, 39.5% contained both TEM + SHV genes, while only a small number (5.8%) of isolates contained the *bla*CTX-M gene along with *bla*TEM or *bla*TEM/*bla*SHV. Among all ESBL genes containing isolates, 44% were *E. coli*, 29% KESC (*Klebsiella*, *Enterobacter*, *Shigella*, and *Citrobacter* species) and 26.7% *Proteus* group. The percentages of extended-spectrum beta-lactamase genes among ESBL *Enterobacteriaceae* isolates from meat and wastewater samples are shown in Figure 4.

Table 5. Distribution of *bla*TEM, *bla*SHV and *bla*CTX-M ESBL types among the 86 isolates.

ESBL Type	*E. coli*	* *p*	KESC *	** *p*	*Proteus* sp.	*** *p*	Total Isolates
TEM	16		13		16		45
TEM + SHV	17		10		7		34
TEM + CTX-M	3		0		0		3
SHV	1	NS	1	NS	0	NS	2
SHV + CTX-M	0		0		0		0
CTX-M	0		0		0		0
CTX-M + TEM-SHV	1		1		0		2
Total	**38**		**25**		**23**		**86**

KESC *: *Klebsiella*, *Enterobacter*, *Shigella*, and *Citrobacter;* * Fisher's exact test *E. coli* vs. KESC for the presence of TEM alone; ** Fisher's exact test: *Proteus* spp; versus. KESC for the presence of TEM alone; *** Fisher's exact test: *E. coli* versus *Proteus* spp. For the presence of TEM alone; NS-Statistically nonsignificant at significance level $p > 0.05$.

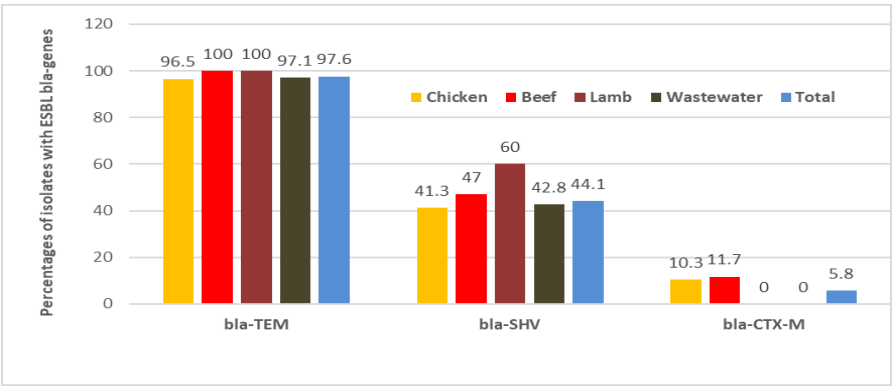


Figure 4. Percentage of beta-lactamase gene types detected in *Enterobacteriaceae* isolates ($n = 86$) from meat and wastewater samples.

4. Discussion

Antimicrobial resistance (AMR) represents a critical public health problem worldwide [2,3], and in GCC countries [13,14]. Antibiotic resistance in bacteria, including extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-E), has been reported in the UAE in clinical settings [15]. However, information about ESBL-E in non-clinical environments, especially in food and wastewater environments, has so far been limited in the UAE. To our knowledge, this is the first study to document data on the occurrence of ESBL bacteria from non-clinical (meat and wastewater) sources in Dubai, UAE. Among all meat samples, the highest number of Gram-negative isolates were recovered from chicken (n = 95) followed by beef (n = 29) and lamb (n = 21). As reported in earlier studies, the high degree of bacterial contamination in meat samples (100% recovery rate) from butcheries could be due to unhygienic meat handling, improper processing practice of raw meat, and cross-contamination [31,32]. Several reports indicate that the high prevalence of ESBL *E. coli* has resulted from growing reservoirs in food animals such as chickens due to the use of antimicrobials in poultry animals [33,34]. A recent study in China found ESBL producing *Enterobacteriaceae* in 23.8% and 13.3% of raw retailed chicken and beef meat samples, respectively [22]. However, in our study, we found a higher proportion of ESBL producing *Enterobacteriaceae* in chicken (31.6%) and beef meat (58.6%) samples. The ESBL-*Enterobacteriaceae* in retailed chicken in this study is, however, lower than 50%, as reported in closely related studies in other countries [21,24,35,36]. In this study, we found a higher number of ESBL-*Enterobacteriaceae* isolates in retailed minced beef as compared to chicken and lamb meat samples. Our results are dissimilar to other studies where ESBL-*Enterobacteriaceae* were found to be in lower percentages in beef than chicken meat [24,36,37]. In another study in Southwest Ethiopia, the proportion of ESBL-producing isolates in minced meat retailer shops was only 23.8% [17], which is lower than observed in this study.

In our study, 56% of Gram-negative isolates from wastewater sources belonged to ESBL-*Enterobacteriaceae*, less than in other studies conducted in Spain [38]. Several recent studies reported a high prevalence of ESBL-*Enterobacteriaceae* from hospital wastewater [18]. In another study, only 28.3% of the beta-lactamase-producing-*Enterobacteriaceae* isolates were detected in a wastewater treatment plant, with the most common microorganisms included *Escherichia coli* (83%), *Citrobacter freundii* (11%), and *Enterobacter cloacae* complex (4%) [39]. However, the lower recovery of ESBL-*Enterobacteriaceae* might be due to the type of wastewater sample used. For example, in this study, wastewater samples were obtained from a domestic municipal wastewater treatment plant rather than hospital wastewater. In our study, recovery of all ESBL producing *Enterobacteriaceae* and KESC isolates was statistically higher from wastewater than meat sources (p = 0.0001). However, recovery of *E. coli* and *Proteus* species from wastewater and meat sources was statistically insignificant at level $p > 0.05$ (Table 4).

Overall, all ESBL positive isolates (n = 86) have shown very high levels of resistance to cefpodoxime (93%), followed by cefotaxime (79%), ceftizoxime (78%) and ceftazidime (70%). Among meat isolates, the highest resistance to cefpodoxime was found in lamb (100%) followed by beef (94%) and chicken (86.2%). A recent study reported resistance of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* isolates from chicken samples to third generation cephalosporins in the following pattern: cefpodoxime, CPD (100%); cefotaxime, CTX (97.1%); ceftazidime, CAZ (70.6%), with the lowest resistance to imipenem, IMP (5.9%) [21]. However, in our study, the resistance to third generation cephalosporins was lower (CPD; 86.2%; CTX 65.5%, CAZ; 62%) but slightly higher for imipenem, IMP (6.9%) than observed in that study. The resistance to cephalosporins found in ESBL isolates from retailed chicken meat in this study was higher than reported in a study in China where only 16.0% of *Enterobacteriaceae* isolates from retail foods showed cefotaxime (CTX) and/or ceftazidime (CAZ) cephalosporin resistance [22]. Furthermore, only 79.3% of ESBL isolates from chicken meat were found to be multi-drug resistant, which is lower than reported (97%) by a study in Spain [21]. An increase in cefotaxime resistant *Enterobacteriaceae* family bacteria such as *E. coli* has also been reported in poultry meat in the Netherlands since 2005 [34]. This study reported a rapid increase

in third generation cephalosporin-resistant *E. coli*, particularly in poultry, not only in Europe but worldwide. To estimate more accurately the associated increased deaths among persons resulting from third-generation cephalosporin use in poultry, detailed data from more countries is essential [40].

In this study, we observed, the highest resistance to all third generation cephalosporin for ESBL *Enterobacteriaceae* isolates in lamb meat (100%) followed by beef (94%) and chicken (86.2%). The results are higher than a study conducted in Egypt that showed that the antimicrobial resistance of *E. coli* isolated from poultry was more than from beef to most tested antibiotics [41]. Another recent study in Germany reported a lower prevalence of cefotaxime-resistant *E. coli* in the range of 4.2%–11.9% in beef meat [20]. However, in the current study, all ESBL *E. coli* isolates from beef and lamb meat were found resistant to cefotaxime. In contrast, only 71.4% of *E. coli* from retailed chicken samples were resistant to cefotaxime. Besides, all ESBL *Enterobacteriaceae* isolates (100%) from beef and lamb meat were found to be multi-drug resistant as compared to 79.3% isolates from chicken meat. The resistance pattern of chicken and beef/lamb isolates to different antibiotics (third and fourth generation cephalosporins and carbapenems) was not statistically significant. However, the multi-drug resistance pattern of beef/lamb isolates was found to be statistically higher than chicken isolates (p value = 0.0305, Table 3).

ESBL *Enterobacteriaceae* isolates from wastewater showed a very high level of resistance to third generation cephalosporins in the following order: CZX (100%), CPD (97%), CTX (86%), CAZ (77%). All wastewater isolates (10%) were found to be multi-drug resistant with higher statistically significance than the meat isolates' MDR pattern (p = 0.0001). While comparing meat isolates with wastewater, the resistance pattern to all antibiotics except ceftizoxime (CZX^{30}) was statistically insignificant at $p > 0.05$. The resistance profile of wastewater isolates to ceftizoxime was higher with more statistical significance than all meat isolates (p = 0.0001, Table 3). Our resistance rates of ESBL-E isolates from activated sludge samples are higher for CAZ (65%) and CPD (58%) but similar to CTX (85%) measures observed in a study from Poland [25]. A study in Portugal revealed, the highest incidence of ESBL-E in hospital sewage, and the lowest in urban wastewater with 51.9% of the isolates potentially extended-spectrum beta-lactamase positive. Frequency of resistance to the beta-lactam group antibiotics was as follows: cefotaxime (22.7%), cefpirome (19.2%), and ceftazidime (16.2%) with none of the isolates resistant to imipenem (IPM) [42]. When comparing our results to a study performed in Portugal, the resistance rate of ESBL-E isolates to cefotaxime (80%), cefpirome (26%), and ceftazidime (77.1%) was much higher, and 14.3% isolates were found resistant to Imipenem (IPM). In another study, out of 310 ESBL-producing *Enterobacteriaceae* strains isolated from hospital sewage effluents, 295 (95.2%), 253 (81.6%), and 228 (73.5%) isolates were resistant to cefotaxime, ceftazidime, and cefpodoxime, respectively [43]. However, in comparison, the resistance patterns of ESBL-E isolates from this study is slightly lower for cefotaxime (86%) and ceftazidime (77.1%) but much higher for cefpodoxime (97.1%).

In the current study, the most common beta-lactamase *bla* gene type in all meat and wastewater sample ESBL-E isolates was TEM-type (97.6%), followed by SHV-type (44.2%) and CTX-M (5.8%). Furthermore, 39 out of 86 isolated ESBL strains (45.3%) expressed two or more beta-lactamases. The distribution of the *bla*TEM gene alone as compared to the other two *bla* genes (*bla*SHV and *bla*CTX-M) was found to be statistically insignificant (at significance level $p > 0.05$) by comparing isolates of *E. coli* vs. KESC, *Proteus* vs. KESC and *E. coli* vs. *Proteus* (Table 5). Our findings are dissimilar to other studies in China, which showed that *bla*TEM (81.9%) was the most common gene, followed by *bla*CTX-M (68.1%) and *bla*SHV (38.9%) among retailed chicken and beef ESBL isolates [22]. Another study in Germany found ESBL resistant genes of the CTX-M-group (10.1% of all samples) were most frequently detected [20]. A study in Spain reported predominant that the beta-lactamase type producing *Enterobacteriaceae* in food products was SHV-12 (50.1%), followed by TEM-type (24.5%) and CTX-M (20.8%), and 30% of isolated strains expressed two or more beta-lactamases [21]. However, in our study, 45.3% of ESBL strains had two or more beta-lactamases. Also, CTX-M type was only detected in five ESBL-E isolates from chicken (n = 3) and beef (n = 2) meat products. A better understanding of the diversity of CTX-M enzymes' encoding gene is a key component of any comprehensive plan to mitigate the further spread of CTX-M ESBLs among clinically relevant bacteria [10]. A high prevalence

of ESBL drug-resistant genes was found in chicken in a study in Turkey but was lower in other meat types, including beef [34]. However, in our study, beef and lamb meat isolates were found to contain a higher percentage of *bla*TEM-type (100%) and *bla*SHV (40% and 60% respectively). The *bla*CTX-M gene type was detected in 10.3% of chicken ESBL isolates and 11.7% of beef isolates. Lamb isolates were found negative for the *bla*CTX-M gene.

In this study, 35 (57.3%) out of a total of 61 isolates from municipal sewage treatment plant were phenotypically ESBL-positive, and the most detected *bla* gene was *bla*TEM (97.1%) followed by *bla*SHV (42.8%). The *bla*CTX-M gene was not found in any of the wastewater isolates. The *bla*TEM and *bla*SHV detected in this study was higher than in the research conducted in Portugal, where the most common genes among the potential ESBL producers found were *bla*TEM (24.1%) and *bla*CTX-M (5.6%) [42]. In another study, in wastewater samples collected during biological treatment, the most prevalent gene was *bla*TEM, which occurred in all samples, including the treated wastewater, whereas the *bla*SHV gene was least prevalent in the tested samples [44]. This finding compares with the results of our study, where the *bla*TEM gene was found in 97% of ESBL isolates obtained from the secondary treatment stage, whereas only 42.8% isolates had the *bla*SHV gene. A recent study found very low percentages of the ESBL genes in 11.84% of isolates obtained from wastewater effluent samples, and of these, only 9.2% of isolates carried *bla*TEM, 1.4% *bla*SHV-12, 0.2% *bla*CTX-M-1 and 1% *bla*CTX-M-15 [45].

The role of environmental reservoirs in antibiotic-resistant bacteria and the dissemination and penetration of their resistance genes remains unclear [10]. Identifying ESBL genes in non-clinical isolates and their pattern of resistant bacteria provides useful scientific data and may help to create a clear strategy for controlling infections. It may also aid in mitigating the spread of multi-drug resistance. One of the limitations of this study was the smaller sampling size for both meat and wastewater sources; therefore, this study's results may not truly represent the prevalence of ESBL-E in non-clinical environments in Dubai, UAE. Nevertheless, we observed a high rate of resistance of ESBL-E isolates to multi-drug and third generation cephalosporins in the range of 80–100%. Furthermore, we detected carbapenemase resistance phenotypically with eight (9.3%) isolates showing resistance to imipenem, and only one (1%) of the isolates from a beef meat source confirmed with metallo-beta-lactamase activity (MBL). Given reports on high carbapenem resistance in clinical isolates of *Enterobacteriaceae* in the UAE and other GCC countries [46,47], the inclusion of New Delhi metallo-beta-lactamase 1(NDM-1) monitoring is highly recommended in the surveillance program for non-clinical environments. Overall, we demonstrated the occurrence of ESBL *Enterobacteriaceae* members, predominantly *E. coli*, suggesting possible introduction into the environment and thus emphasizing the importance of regular review of surveillance programs in controlling the spread of antibiotic resistance. However, a relative enrichment of ESBL *E. coli*, particularly during the wastewater and sludge treatment process, and a comparative study with clinical isolates within the region and their loads in sludge fertilizer after the end of a treatment cycle need to be further evaluated for a broader perspective.

5. Conclusions

The present study attempted to investigate the occurrence of Gram-negative *Enterobacteriaceae* that are resistant to the extended-spectrum beta-lactam class of antibiotics in wastewater and meat samples. The following are the main conclusions drawn from this study:

1. Extended spectrum beta-lactamase (ESBL) based antibiotic resistance was observed in non-clinical isolates of wastewater and meat origin. A total of 86 isolates (41.7%; n = 206) have shown resistance towards third generation cephalosporins such as cefpodoxime (93%), ceftazidime (70%), cefotaxime (79%) and ceftizoxime (78%). Hence, they may be classified as potential ESBL strains of bacteria.
2. A total of only 19 (22%) out of 86 isolates were phenotypically confirmed as ESBL by the double disc diffusion method.

3. Only 9.3% (n = 8) of strains have shown resistance towards imipenem, which means that they could be potentially producing carbapenemases. However, only one isolate was found to be MBL (metallo-beta-lactamase) positive by combined disc test assay. Hence, we conclude that the occurrence of metallo-beta-lactamase based resistance is insignificant among non-clinical isolates obtained in this study.
4. Through molecular characterization by PCR, all the 86 ESBL isolates were found to harbor one or the other *bla* ESBL genes tested of TEM, SHV, and CTX-M type. Most ESBL genes are found to be TEM and SHV types. The CTX-M gene was the least detected gene among all isolates.
5. The occurrence of ESBL producing Enterobacteriaceae in meat samples from farmed animals represents an obvious risk of contamination. Nevertheless, due to the high incidence of ESBL Enterobacteriaceae, prudent use of antibiotics in veterinary medicine and strict hygiene measures during slaughtering and retailing are essential. In addition, ESBL producing Enterobacteriaceae in wastewater also calls for the implementation of stringent decontamination procedures during the treatment or disposal process.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/9/2562/s1, Figure S1: Antibiotic susceptibility assay with 3rd-generation cephalosporins: CPD, CAZ, CTX and CZX, Figure S2: Confirmatory double disc diffusion test for ESBLs, Figure S3: Number of potential and confirmed ESBL isolates by double disc diffusion method, Figure S4. Zone of enhancement around imipenem plus EDTA, Figure S5: Number of imipenem resistant and MBL positive isolates, Table S1: Biochemical characterization of all 206 Isolates, Table S2: Antibiotics zones of inhibition diameter according to CLSI guidelines, Table S3: Susceptibility of *E. coli* ATCC 25922 to 3rd Generation Cephalosporins, Table S4: Susceptibility of *E. coli* ATCC 25922 to Cefpirome, Table S5: Susceptibility of *E. coli* ATCC 25922 to imipenem.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A. Materials and Methods

Appendix A.1. Fluorescence In Situ Hybridization (FISH)

The pure culture of 206 bacterial isolates were analyzed by FISH for identification of Enterobacteriaceae and *E. coli* isolates using three oligonucleotide probes fluorescently labeled at 5'end by Tetramethyl rhodamine (TRITC): Domain specific universal eubacterial oligonucleotide probe, Enterobacteriaceae family specific ENTBAC probe and *E. coli* specific EC probes described earlier (Jensen et al., 2000). For stringency of hybridization, a few isolates were analyzed with each probe by varying the concentration of formamide as 30, 35 and 40% in the hybridization buffer. The following is a brief description of the steps. (i) Fixation and dehydration: The isolates were fixed in 4% paraformaldehyde, 10 µL of the fixed sample was applied on a poly-L lysine coated slide and air dried. The samples were dehydrated with varying concentrations (50, 80 & 95%) of ethanol for 3 min each. (ii) Hybridization: Once the stringency of the hybridization probe was fixed to 35% for all the three probes, all the isolates were analyzed using a hybridization buffer with 35 % formamide concentration. On the dehydrated sample, 9 µL of hybridization buffer mixed with 1 µL of the probe was added. The slide was placed into a hybridization chamber and filled with hybridization buffer, sealed and hybridized at 46 °C for 1.5–3 h. (iii) Washing and observation: The washing buffer was pre-warmed at 48 °C for half an hour before the washing step was performed. The slides were

removed from the hybridization chamber and replaced the hybridization buffer with pre warmed washing buffer. The slides were incubated with the washing buffer at 48 °C for 10–15 min. Slides were rinsed with distilled water at room temperature. Slides were air dried and viewed under Olympus BX51 fluorescence microscope equipped with TRITC filter.

Appendix A.2. Results

Two hundred and six isolates were screened by FISH assay using three probes, one being the universal eubacterial oligonucleotide probe (EUB), probe specific to Enterobacteriaceae family (ENTBAC) for the identification of strains up to the family level. The hybridization stringency was obtained at a 35% formamide concentration for both oligonucleotide probe. Thus, the hybridization buffer and washing buffer were prepared for 35% formamide concentration. All 206 isolates showed excellent hybridization to both universal eubacterial oligonucleotide probe (EUB), and ENTBAC (Enterobacteriaceae) probe and displayed strong fluorescent signals without false positive reaction. The results of hybridization of potential ESBL 86 strains with eubacterial oligonucleotide probe (EUB), and ENTBAC (Enterobacteriaceae) probe is shown in Table A1. The FISH analysis images of an isolate *Escherichia coli* with TRITC–labeled with EUB, and ENTBAC at the magnification of 1000X under epifluorescent and phase contrast microscopy are shown in Figures A1 and A2.

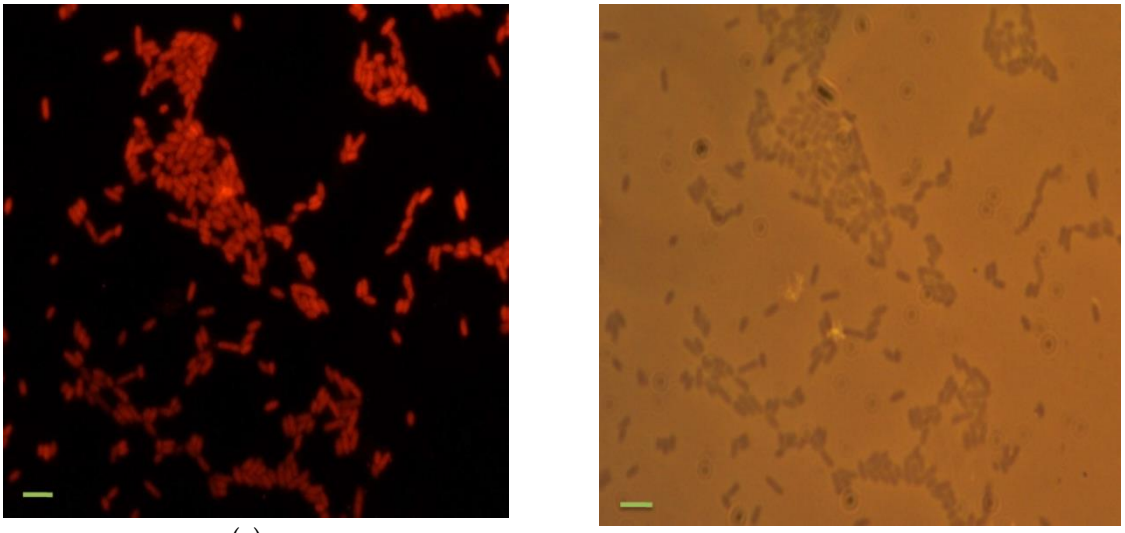
Table A1. Fluorescence *in situ* hybridization of strains with universal eubacterial (EUB), and *Enterobacteriaceae* (ENTBAC) family specific oligonucleotide probe.

S.N.	Strain	Source	Chromogenic Agar ID	EUB [1]	ENTBAC [2]
1	MUD15-01	Wastewater	*E. coli*	(+)	(+)
2	MUD15-03	Wastewater	Proteus	(+)	(+)
3	MUD15-06	Wastewater	*E. coli*	(+)	(+)
4	MUD15-07	Wastewater	*E. coli*	(+)	(+)
5	MUD15-08	Wastewater	Proteus	(+)	(+)
6	MUD15-10	Wastewater	*E. coli*	(+)	(+)
7	MUD15-11	Wastewater	*E. coli*	(+)	(+)
8	MUD15-13	Wastewater	KESC	(+)	(+)
9	MUD15-14	Wastewater	*E. coli*	(+)	(+)
10	MUD15-17	Wastewater	KESC	(+)	(+)
11	MUD15-18	Wastewater	KESC	(+)	(+)
12	MUD15-22	Wastewater	KESC	(+)	(+)
13	MUD15-23	Wastewater	KESC	(+)	(+)
14	MUD15-24	Wastewater	KESC	(+)	(+)
15	MUD15-25	Wastewater	KESC	(+)	(+)
16	MUD15-27	Wastewater	KESC	(+)	(+)
17	MUD15-28	Beef	*E. coli*	(+)	(+)
18	MUD15-32	Beef	KESC	(+)	(+)
19	MUD15-33	Beef	KESC	(+)	(+)
20	MUD15-34	Beef	*E. coli*	(+)	(+)
21	MUD15-35	Beef	*E. coli*	(+)	(+)
22	MUD15-36	Beef	KESC	(+)	(+)
23	MUD15-37	Beef	*E. coli*	(+)	(+)
24	MUD15-38	Beef	*E. coli*	(+)	(+)
25	MUD15-39	Beef	*E. coli*	(+)	(+)
26	MUD15-40	Lamb	KESC	(+)	(+)
27	MUD15-43	Lamb	Proteus	(+)	(+)
28	MUD15-46	Chicken	KESC	(+)	(+)
29	MUD15-47	Chicken	Proteus	(+)	(+)
30	MUD15-48	Chicken	Proteus	(+)	(+)
31	MUD15-49	Chicken	KESC	(+)	(+)
32	MUD15-50	Chicken	*E. coli*	(+)	(+)

Table A1. *Cont.*

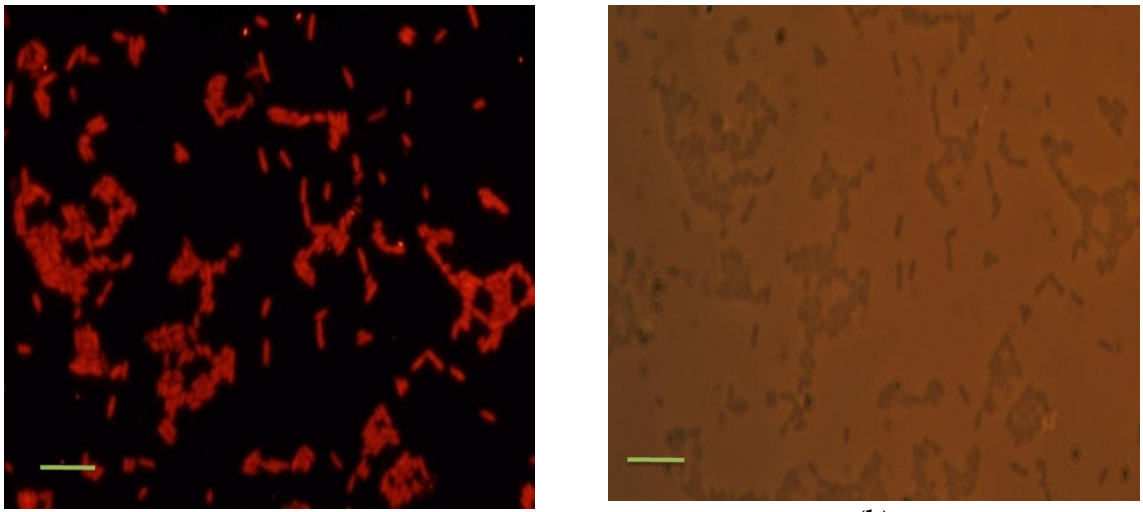
S.N.	Strain	Source	Chromogenic Agar ID	EUB [1]	ENTBAC [2]
33	MUD15-51	Chicken	Proteus	(+)	(+)
34	MUD15-52	Chicken	KESC	(+)	(+)
35	MUD15-53	Chicken	Proteus	(+)	(+)
36	MUD15-54	Chicken	*E. coli*	(+)	(+)
37	MUD15-55	Chicken	Proteus	(+)	(+)
38	MUD15-56	Chicken	Proteus	(+)	(+)
39	MUD15-58	Chicken	*E. coli*	(+)	(+)
40	MUD15-59	Chicken	*E. coli*	(+)	(+)
41	MUD15-60	Chicken	KESC	(+)	(+)
42	MUD15-61	Chicken	Proteus	(+)	(+)
43	MUD15-62	Chicken	Proteus	(+)	(+)
44	MUD15-63	Chicken	Proteus	(+)	(+)
45	MUD15-64	Chicken	*E. coli*	(+)	(+)
46	MUD15-65	Chicken	*E. coli*	(+)	(+)
47	MUD15-74	Chicken	*E. coli*	(+)	(+)
48	MUD15-82	Chicken	*E. coli*	(+)	(+)
49	MUD15-84	Chicken	*E. coli*	(+)	(+)
50	MUD15-89	Chicken	*E. coli*	(+)	(+)
51	MUD15-109	Chicken	*E. coli*	(+)	(+)
52	MUD15-110	Chicken	*E. coli*	(+)	(+)
53	MUD15-115	Chicken	*E. coli*	(+)	(+)
54	MUD15-133	Lamb	*E. coli*	(+)	(+)
55	MUD15-137	Beef	Proteus	(+)	(+)
56	MUD15-138	Beef	Proteus	(+)	(+)
57	MUD15-139	Beef	Proteus	(+)	(+)
58	MUD15-140	Beef	Proteus	(+)	(+)
59	MUD15-154	Lamb	*E. coli*	(+)	(+)
60	MUD15-156	Lamb	*E. coli*	(+)	(+)
61	MUD15-157	Beef	*E. coli*	(+)	(+)
62	MUD15-162	Beef	*E. coli*	(+)	(+)
63	MUD15-165	Beef	*E. coli*	(+)	(+)
64	MUD15-166	Beef	*E. coli*	(+)	(+)
65	ZU-04	Wastewater	Proteus	(+)	(+)
66	ZU-11	Wastewater	KESC	(+)	(+)
67	ZU-12	Wastewater	KESC	(+)	(+)
68	ZU-13	Wastewater	KESC	(+)	(+)
69	ZU-15	Wastewater	Proteus	(+)	(+)
70	ZU-16	Wastewater	*E. coli*	(+)	(+)
71	ZU-18	Wastewater	*E. coli*	(+)	(+)
72	ZU-19	Wastewater	KESC	(+)	(+)
73	ZU-21	Wastewater	*E. coli*	(+)	(+)
74	ZU-22	Wastewater	*E. coli*	(+)	(+)
75	ZU-24	Wastewater	*E. coli*	(+)	(+)
76	ZU-26	Wastewater	KESC	(+)	(+)
77	ZU-27	Wastewater	KESC	(+)	(+)
78	ZU-28	Wastewater	Proteus	(+)	(+)
79	ZU-29	Wastewater	KESC	(+)	(+)
80	ZU-30	Wastewater	KESC	(+)	(+)
81	ZU-31	Wastewater	Proteus	(+)	(+)
82	ZU-32	Wastewater	KESC	(+)	(+)
83	ZU-33	Wastewater	Proteus	(+)	(+)
84	ST-04	Chicken	Proteus	(+)	(+)
85	ST-05	Chicken	Proteus	(+)	(+)
86	ST-06	Chicken	*E. coli*	(+)	(+)

[1] Universal eubacterial probe, [2] Enterobacteriaceae family specific oligonucleotide probe. (+) = positive hybridization with the oligonucleotide probe.



(**a**) (**b**)

Figure A1. Epifluorescent micrographs (**a**): Tetramethyl rhodamine (TRITC) labeled EUB probe and phase contrast (**b**): TRITC–labeled EUB probe), Bar = 10 μm and applies to all photomicrographs. Original magnification: 1000X.



(**a**) (**b**)

Figure A2. Epifluorescent micrographs (**a**): Tetramethyl rhodamine (TRITC) labeled ENTBAC probe and phase contrast (**b**): ENTBAC probe, Bar =10 μm and applies to all photomicrographs. Original magnification: 1000X.

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