





## Article

# Prevalence of Antibiotic-Resistant *Escherichia coli* Isolated from Beef Cattle and Dairy Cows in a Livestock Farm in Yamagata, Japan

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**Abstract:** Antimicrobials are used on livestock farms to treat and prevent infectious animal diseases and to promote the growth of livestock. We monitored the prevalence of antibiotic-resistant *Escherichia coli* (AR-EC) isolates from beef cattle (BC) and dairy cows (DCs) on a livestock farm in Yamagata, Japan. Fecal samples from 5 male BC and 10 male DCs were collected monthly from October 2022 to November 2023. In total, 152 and 884 *E. coli* isolates were obtained from the BC and DC fecal samples, respectively. Notably, 26 (17.1%) and 29 (3.3%) *E. coli* isolates in the BC and DC groups, respectively, were resistant to at least one antibiotic. The resistance rates to tetracycline, ampicillin, gentamicin, and chloramphenicol of the isolates were significantly higher than those to the other antimicrobials. The tetracycline resistance genes *tetA* (70.6%) in DCs and *tetB* (28%) in BC were identified, along with the *bla*<sub>TEM</sub> gene in ampicillin-resistant isolates (BC: 84.2%, DCs: 42.8%). Despite significant variations in the monthly detection rates of AR-EC isolated from BC and DCs throughout the sampling period, the judicious use of antimicrobials reduced the occurrence of AR-EC in both BC and DCs, thereby minimizing their release into the environment.

**Keywords:** *Escherichia coli*; antibiotic-resistant; feces; beef cattle; dairy cow



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## 1. Introduction

Since the last century, the use of antibiotics has significantly increased life expectancy. However, the overuse and misuse of these antimicrobials have led to the development of antibiotic-resistant bacteria (ARB). ARB are a growing concern for global public health and are recognized by the World Health Organization [1] as one of 21st century's biggest threats. According to estimates, the annual death toll from ARB was 700,000 worldwide in 2014 and is projected to exceed 10 million by 2050 [2]. By 2050, the mortality rate due to ARB per 10,000 people in Africa and Asia is expected to be approximately twice as high as that in North America, Europe, and Australia [3]. Conditions such as tuberculosis and gonorrhea and bacteria such as *Escherichia coli* are becoming increasingly resistant to treatment [3].

Inappropriate antibiotic use, both in excessive and in limited quantities, in humans and livestock causes ARB. Globally, more than 60,000 tons of antibiotics are consumed annually by large-scale livestock operations, leading to the evolution of ARB that can be transmitted to humans [3]. Antibiotic use in livestock farming is higher than in humans, making livestock a hotspot for ARB emergence because of the frequent use of antimicrobial agents. The top five antimicrobial consumers in 2020 were China, Brazil, India, the United States of America, and Australia [4]. In Japan, the amount of antibiotics used for livestock,



Table 1. Cont.

Name	CTX	CAZ	CPX	ABP	CXM	CFX	IPM	AZT	ACV	T/P	ST	GM	AMK	CIP	TC	TGC	FOM	CP	Injection History
D8 (n = 78)	0	0	0	6.4	0	0	0	0	0	1.3	5.1	5.1	0	0	6.4	0	0	0	-
D9 (n = 106)	0	0	0	0	0	0	0	0	0	0	0	0	0	1.9	0	0	0	0	SE, PK, C
D10 (n = 60)	0	0	0	0	0	0	0	0	0	1.7	0	0	0	0	0	0	0	0	SE, C
DCs (n = 884)	0	0	0	4.6	0.7	0	0	0	0	1.3	2.6	0	3.3	2.6	11.2	0	0	0	
BC (n = 152)	0	0.7	0	12.5	0.0	0	0	0	0	0	0	11.8	0	0	16.5	0	0	9.9	

Abbreviations: CTX, cefotaxime; CAZ, ceftazidime; CPX, cefpodoxime; ABP, ampicillin; CXM, cefuroxime; CFX, cefoxitin; IPM, imipenem; AZT, aztreonam; ACV, amoxicillin and clavulanic acid; T/P, tazobactam/piperacillin; ST, sulfamethoxazole/trimethoprim; GM, gentamicin; AMK, amikacin; CIP, ciprofloxacin; TC, tetracycline; TGC, tigecycline; FOM, fosfomycin; CP, chloramphenicol; SE: ceftazolin; PK: kanamycin; P: pirlimycin hydrochloride hydrate; C: cephalonia.

Fecal samples (>10–50 g) were collected directly from the BC and DCs by rectal palpation and transported in a cooler box (4–8 °C) to the laboratory on the same day.

## 2.2. Bacterial Counting, Isolation, and Identification of *E. coli*

The number of *E. coli* in the fecal samples from both BC and DCs was determined using the plate dilution method. Specifically, 1 g of each fecal sample was diluted with 9 mL of sterile saline to achieve a  $10^{-5}$  dilution. Subsequently, 0.1 mL of a  $10^{-1}$ – $10^{-5}$  dilution was spread on an agar plate (Chromocult Coliform; Sigma Aldrich, Germany). For *E. coli* screening, the diluted samples were smeared on CHROMagar™ extended-spectrum  $\beta$ -lactamase (ESBL)-producing Enterobacterales, vancomycin-resistant Enterococcus (VRE), and carbapenem-resistant Enterobacterales (CRE) agar plates (Kanto Chemical, Tokyo, Japan). The plates were incubated at 37 °C for 24 h, and the positive colonies on the agar were counted. Colony counts were calculated as the average number of colony-forming units (CFUs) in triplicate. Up to 10 of the presumptively identified blue colonies of *E. coli* were picked from the Chromocult Coliform and streaked on Luria–Bertani agar (LB, Difco™ LB Broth, Lennox, USA, agar powder, Fujifilm Wako Pure Chemicals Co., Ltd., Osaka, Japan). After incubation at 37 °C for 24 h, the colonies grown on LB agar were purified by streaking onto the same agar. Purified colonies were isolated in LB broth and stored at –80 °C until further analysis.

DNA of the suspected positive *E. coli* isolates was extracted using the InstaGene Matrix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Presumptive *E. coli* isolates were identified by detecting a specific gene for *E. coli* (*uidA*) using a KAPATaq EXtra PCR kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) [11]. The PCR conditions were as follows: three minutes at 95 °C for initial denaturation, 35 cycles of 30 s each at 95 °C for denaturation, 30 s at 58 °C, and one minute at 72 °C for elongation, followed by 10 min at 72 °C for final elongation using a Bio-Rad T100™ Cyclor (USA) (Appendix A Table A1). The PCR products (2.5  $\mu$ L) were mixed with 0.5  $\mu$ L of 6  $\times$  GR Green Loading Buffer (Biocraft, Tokyo, Japan) and confirmed by 1.5% agarose gel electrophoresis using Tris–borate–EDTA buffer (Takara, Shiga, Japan). *E. coli* NBRC3301 was used as a positive control for PCR analysis.

## 2.3. Antibiotic Susceptibility Test

Single-well isolated colonies from Luria–Bertani agar were emulsified in 10 mL of sterile saline and adjusted to a McFarland standard 0.5 (bioMérieux SA Ltd., Marcy l'Etoile, France). A sterile cotton swab was dipped in a standardized bacterial suspension and evenly streaked over the entire surface of Mueller–Hinton agar. Impregnated paper discs with a constant concentration of antibiotics were placed on the surface of the agar and incubated at 37 °C for 16–18 h. Subsequently, *E. coli* isolates were tested for susceptibility to 18 antibiotics (cefotaxime [30  $\mu$ g], ceftazidime [CAZ, 30  $\mu$ g], cefpodoxime [10  $\mu$ g], ampicillin [ABP, 10  $\mu$ g], cefuroxime [CXM, 30  $\mu$ g], cefoxitin [10  $\mu$ g]; imipenem [10  $\mu$ g], aztreonam [30  $\mu$ g], amoxicillin and clavulanic acid [20:10  $\mu$ g], tazobactam/piperacillin (T/P, 100  $\mu$ g/10  $\mu$ g], sulfamethoxazole/trimethoprim [ST, 23.75  $\mu$ g/1.25  $\mu$ g], gentamicin [GM,

10 µg], amikacin [AMK, 30 µg], ciprofloxacin [5 µg], tetracycline [TC, 30 µg], tigecycline [15 µg], fosfomycin [200 µg], and chloramphenicol [CP, 30 µg]) that were selected based on the Clinical and Laboratory Standards Institute (CLSI) guidelines, using the disc diffusion method of Kirby–Bauer, and their resistances were determined following the standards of the CLSI M100-ED33:2023 [12]. *E. coli* ATCC 25,922 was included in all antimicrobial susceptibility tests (ASTs) for quality control.

Resistance to  $\geq 3$  antibiotic classes was used to classify the bacterial isolates as multidrug-resistant (MDR).

#### 2.4. Detection of Antibiotic Resistance Genes

To characterize the TC- and ABP-resistant *E. coli* isolates, the *tet* and *bla* genes corresponding to the TC and ABP resistance genes were identified using PCR. DNA was extracted from the isolates. The primers used for each targeted antibiotic resistance gene and the PCR amplification program are described in Appendix A Table A1 [13–15]. The enzyme KAPATaq Extra was used for PCR amplification of the ARGs. PCR amplification was confirmed by electrophoresis as described above.

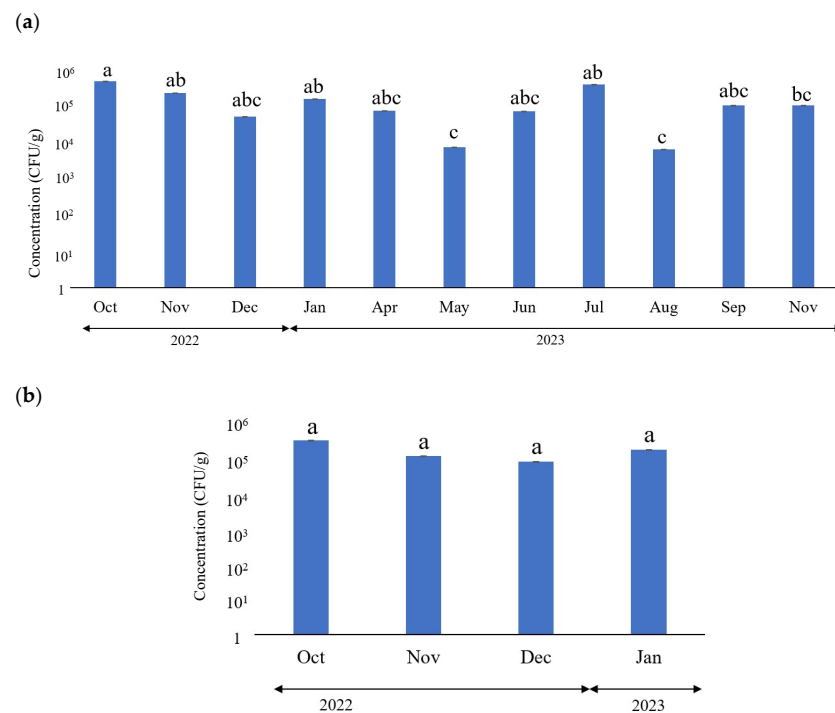
#### 2.5. Statistical Analysis

To examine statistical differences in the proportions of AR-EC isolates, we used the Z-test and the correlation coefficient in Excel. Additionally, one-way analysis of variance (ANOVA) and Tukey's honestly significant difference analyses were performed using SPSS (version 22) statistical software.

### 3. Results and Discussion

#### 3.1. *E. coli* Concentrations in Feces

Figure 1 shows the monthly *E. coli* concentrations in the fecal samples obtained from BC and DCs. According to the WHO, carbapenem- and  $\beta$ -lactam-resistant bacteria and vancomycin-resistant enterococci are classified as serious ARB. None of these three resistant strains (ESBL, CRE, and VRE) were detected on any of the media for AR bacteria, indicating that the farm effectively managed antibiotic usage.



**Figure 1.** Monthly *Escherichia coli* concentrations in the feces of (a) dairy cows (DCs) and (b) beef cattle (BC).

No significant differences were observed in *E. coli* concentration between BC ( $3.33 \times 10^3$ – $2.7 \times 10^6$  CFU/g) and DCs ( $1.7 \times 10^2$ – $7.9 \times 10^6$  CFU/g). Furthermore, the *E. coli* concentrations in BC and DCs did not depend on seasonal changes. However, the *E. coli* concentrations were significantly higher in October and November 2022 and January and July 2023 than in May and August 2023 (Figure 1). This may be associated with lower concentrations of antimicrobial medications or lower quality sanitation facilities in May and August.

### 3.2. Antibiotic Susceptibility Test

A total of 152 and 884 *E. coli* isolates, identified by the presence of the *uidA* gene using PCR, were obtained from fecal samples of BC and DCs, respectively. All isolates harbored the *uidA* gene and were subsequently identified as *E. coli*.

All isolates underwent ASTs using 18 antimicrobial agents. Notably, 26 (17.1%) and 29 (3.3%) *E. coli* isolates from BC and DCs, respectively, exhibited resistance to at least one antibiotic. The highest resistance rates were observed for TC (BC, 16.5%; DCs, 11.2%), ABP (BC, 12.5%; DCs, 4.6%), GM (BC, 11.8%; DCs, 0%), and CP (BC, 9.9%; DCs, 0%), which are commonly used for disease treatment. The resistance rates of the isolates to other antimicrobials were generally low, as follows: AMK (BC, 0%; DCs, 3.3%), ST (BC, 0%; DCs, 2.6%), CIP (BC, 0%; DCs, 2.6%), T/P (BC, 0%; DCs, 1.3%), CXM (BC, 0%; DCs, 0.7%), and CAZ (BC, 0.7%; DCs, 0%). According to reports from other countries, the most frequently observed antimicrobial resistance in *E. coli* isolates is to tetracycline [16,17]. The resistance rates to TC were 6% lower than those reports, and that to ABP was similar to that reported in the Nippon Antimicrobial Resistance (AMR) One Health Report 2022 [5]. In 2018, Japan's national drug resistance statistics reported a 26.5% tetracycline resistance rate in healthy cattle on livestock farms [10]. However, studies conducted in Asia, the U.K., and the U.S.A. have revealed much higher resistance rates in dairy cows, ranging from 33.3% to 93% [18–20]. These variations in resistance rates can be attributed to factors such as infectious diseases incidents, treatment protocols, and geography. The high rates of tetracycline resistance are linked to its extensive use in both human medicine and animal husbandry. Tetracycline is favored for its affordability and minimal side effects. The correlation coefficient was employed to assess the correlation between time and AR rate. However, no significant correlation was observed between time and AR rates in either animal type. The monthly detection rates of AR-EC isolated from the BC and DCs varied considerably throughout the sampling period (Table 2).

The antibiotic resistance rates of the *E. coli* isolates were not correlated with head or injection history (Table 1). The antimicrobials and injections used for the DCs were different, except for CXM. The prevalence of AR-EC can be attributed to many factors such as drug administration, feed additives, farm management, farm hygiene, and farm size. The emergence of AR involves the administration of antibiotics and other poorly understood factors [21]. One reason for the prevalence of AR-EC is that farm hygiene problems such as forage and haylage can lead to fecal matter contamination from wild animals [22]. Farm size is crucial for the prevalence of AR-EC. Increasing the number of cattle increases the incidence of AR-EC. Furthermore, farms that buy more cattle have a higher likelihood of detecting ESBL-producing *E. coli* [23]. The prevalence of antimicrobial resistance among isolates collected from calves is thought to be greater than that among those collected from cows, as calves receive antimicrobial treatment more frequently than lactating dairy cows [24].

**Table 2.** Antibiotic-resistance rates (%) of *E. coli* isolates to the tested antimicrobials.

Name	BC				DCs										
	Oct-22 n = 36	Nov-22 n = 46	Dec-22 n = 40	Jan-23 n = 30	Oct-22 n = 100	Nov-22 n = 100	Dec-22 n = 90	Jan-23 n = 97	Apr-23 n = 81	May-23 n = 52	Jun-23 n = 80	Jul-23 n = 80	Aug-23 n = 64	Sep-23 n = 74	Nov-23 n = 66
CTX	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CAZ	0	2.2	0	0	0	0	0	0	0	0	0	0	0	0	0
CPX	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ABP	22.2	23.9	0	0	1	1	1.1	0	0	0	0	0	0	0	6.1
CXM	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CFX	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IPM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AZT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACV	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T/P	0	0	0	0	0	0	1.1	0	0	1.9	0	0	0	0	0
ST	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.1
GM	19.4	23.9	0	0	0	0	0	0	0	0	0	0	0	0	0
AMK	0	0	0	0	1.0	0	3.3	0	0	0	0	0	1.6	0	0
CIP	0	0	0	0	2.0	0	1.1	0	0	0	0	0	0.0	0	0
TC	27.8	32.6	0	0	3	0	0	0	1.2	15.4	0	0	1.6	0	6.1
TGC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FOM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CP	8.3	23.9	0	0	0	0	0	0	0	0	0	0	0	0	0

3.3. Profiling of AR-EC Multidrug Resistance

Based on their AR patterns, 152 and 884 *E. coli* isolates from BC and DCs belonged to five and seven different phenotypes, respectively. These ranged from resistance to a single antimicrobial to a combination of four phenotypes (Table 3). The most frequently observed phenotype was ABP-GM-TC, with a prevalence of 11.8% (18 isolates), followed by ABP-GM-TC-CP (9.2%; 14 isolates), TC (3.9%; 6 isolates), ABP-TC-CP (1.3%; 2 isolates), and CAZ (0.7%; 1 isolate) in BC. In 2020, antimicrobial resistance rates exceeding 50% were observed for ABP, streptomycin, and TC in deceased cattle [5]. The most frequently observed phenotype in DCs was TC, with a prevalence of 1.5%, followed by ABP-ST-TC, CIP, and AMK at 0.5% each, and ABP-T/P-AMK, ABP, and T/P at 0.1% each. In general, multidrug AR-EC in BC was higher than that in DCs. This may be because antibiotics are mainly used to treat mastitis on dairy farms; however, ARB in mastitis-causing pathogens are relatively infrequent [25]. The TC resistance rates were almost four times lower than those in the U.S.A. (25%) and five or more times lower than those in other countries (Canada, 31%; Germany 36%; France 52%; Italy 79%), according to data from the National Action Plan on AMR (2023–2027) [26]. Furthermore, MDR strains with resistance to as many as four antibiotics in this study were fewer than those reported in a Japanese report [27], with more than six antibiotics, and in other countries. The U.S.A. reported MDR to more than six antibiotics [28], Canada to five [29], and Korea to six [30]. MDR strains are more frequently found in calves than in growing or mature cattle in various research studies [24,31]. These findings suggest that calves are an optimal source of ARB. This notion is further supported by the negative correlation between the prevalence of resistant *E. coli* and the age of the source animal [32]. Furthermore, Gow et al. indicated that ARB in calves could be transmitted among cattle, given the similarity in AMR patterns between cows and calves [31]. The low prevalence of MDR strains in the studied farm may be associated with well-managed drug administration.

**Table 3.** Patterns of antimicrobial resistance phenotypes for *E. coli* strains isolated from BC and DCs in the study, with pattern codes.

	Antibiogram Patterns	Number of Isolates	Percentage of Resistance (%)
BC (n = 152)	TC	6	3.9
	CAZ	1	0.7
	ABP + GM + TC	18	11.8
	ABP + TC + CP	2	1.3
	ABP + GM + TC + CP	14	9.2

Table 3. Cont.

	Antibiogram Patterns	Number of Isolates	Percentage of Resistance (%)
DCs (n = 884)	TC	13	1.5
	CIP	4	0.5
	AMK	4	0.5
	ABP	1	0.1
	T/P	1	0.1
	ABP + ST + TC	4	0.5
	ABP + T/P + AMK	1	0.1

### 3.4. Detection of Antibiotic-Resistant Genes

Based on the AST results, 42 isolates were resistant to TC (BC: 16.5%, DCs: 11.2%), and 26 isolates were resistant to ABP (BC: 12.5%, DCs: 4.6%). These numbers were higher than those for the other tested antibiotics. Nine TC resistance-related genes and five ABP resistance-related genes were identified.

Notably, *tetC*, *tetD*, *tetE*, *tetG*, *tetJ*, *tetM*, and *tetW* were not detected in any of the monthly samples from BC or DCs. However, *tetA* and *tetB*, associated with efflux pumps, were detected in 11.7% and 3.4% of the isolates, respectively. Furthermore, *tetA* (BC: 0% [0/25], DCs: 70.6% [12/17]) was detected in all TC-resistant strains, with a significant difference between the two groups (Table 4). The *tetB* gene (BC: 28% [7/25], DCs: 0% [0/17]) was detected in all TC-resistant strains, and the result was not statistically different between the two types of animals. The prevalence rates of *tetA* and *tetB* reported by Shin et al. were 24.1% lower and 17.1% higher, respectively, in BC compared to those in this study [33]. Furthermore, *tetA* and *tetB* were detected in BC in Japanese studies [27]. The high prevalence of TC resistance in *E. coli* is probably due to the horizontal transfer of *tet* determinants from *E. coli* isolates carrying *tet* genes that survive the selective pressure exerted by TC derivatives [33]. Therefore, understanding the origin and transmission route of *E. coli* in cattle is crucial to mitigate its prevalence.

Table 4. Detection of antibiotic resistance genes by head.

Name	BC				DCs			
	B1 (n = 6)	B3 (n = 6)	B5 (n = 7)	Total (n = 19)	D1 (n = 0)	D3 (n = 2)	D8 (n = 5)	Total (n = 7)
<i>bla</i> <sub>CTX-M-1</sub>	0	0	0	0	0	0	0	0
<i>bla</i> <sub>CTX-M-2</sub>	0	0	0	0	0	0	0	0
<i>bla</i> <sub>CTX-M-8</sub>	0	0	0	0	0	0	0	0
<i>bla</i> <sub>CTX-M-9</sub>	0	0	0	0	0	0	0	0
<i>bla</i> <sub>TEM</sub>	5 (83.3%)	6 (100%)	5 (71.4%)	16 (84.2%)	0	0	3 (60%)	3(42.8)
	n = 10	n = 8	n = 7	n = 25	n = 12	n = 0	n = 5	n = 17
<i>tetA</i>	0	0	0	0	8 (66.7%)	0	4 (80%)	12 (70.6%)
<i>tetB</i>	5 (50%)	2 (25%)		7 (28%)	0	0	0	0
<i>tetC</i>	0	0	0	0	0	0	0	0
<i>tetD</i>	0	0	0	0	0	0	0	0
<i>tetE</i>	0	0	0	0	0	0	0	0
<i>tetG</i>	0	0	0	0	0	0	0	0
<i>tetJ</i>	0	0	0	0	0	0	0	0
<i>tetM</i>	0	0	0	0	0	0	0	0
<i>tetW</i>	0	0	0	0	0	0	0	0

Notably, the *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-2</sub>, *bla*<sub>CTX-M-8</sub>, and *bla*<sub>CTX-M-9</sub> genes were not detected in the monthly samples (Table 5). However, the antibiotic-resistant gene *bla*<sub>TEM</sub> was detected in 16.3% of the livestock. Furthermore, *bla*<sub>TEM</sub> (BC: 84.2% [16/19], DCs: 42.8% [3/7]) was detected in all ABP-resistant strains, and the result was not significantly different between the two groups. The extended-spectrum  $\beta$ -lactamase (ESBL) genes *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> were initially identified in the 1980s and were the most prevalent genes until 2000 [33]. Currently, ESBL production, particularly associated with *bla*<sub>TEM</sub>, is considered one of the most significant ARB mechanisms from both clinical and epidemiological perspectives [33].

Previous studies indicated that *bla*<sub>TEM</sub> was detected in 78.9% of the isolates from dairy cattle farms in the Nile Delta, Egypt, whereas *bla*<sub>SHV</sub> and *bla*<sub>OXA</sub> were only detected in 0.87% of the isolates [34]. Notably, *bla*<sub>TEM</sub> was detected in dairy cows [35] and remains the most common ARG in China and other countries, regardless of whether the isolates are from dairy cows or BC [36].

**Table 5.** Detection of antibiotic resistance genes monthly.

Name	BC				DCs										
	Oct-22 n = 8	Nov-22 n = 11	Dec-22 n = 0	Jan-23 n = 0	Oct-22 n = 1	Nov-22 n = 1	Dec-22 n = 1	Jan-23 n = 0	Apr-23 n = 0	May-23 n = 0	Jun-23 n = 0	Jul-23 n = 0	Aug-23 n = 0	Sep-23 n = 0	Nov-23 n = 4
<i>bla</i> <sub>CTX-M-1</sub>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>bla</i> <sub>CTX-M-2</sub>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>bla</i> <sub>CTX-M-8</sub>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>bla</i> <sub>CTX-M-9</sub>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>bla</i> <sub>TEM</sub>	7 (87.5%)	9 (81.8%)	0	0	0	0	0	0	0	0	0	0	0	0	3 (75%)
	n = 10	n = 15	n = 0	n = 0	n = 3	n = 0	n = 0	n = 0	n = 1	n = 8	n = 0	n = 0	n = 1	n = 0	n = 4
<i>tetA</i>	0	0	0	0	0	0	0	0	0	8 (100%)	0	0	0	0	3 (75%)
<i>tetB</i>	3 (30%)	4 (26.7%)	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>tetC</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>tetD</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>tetE</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>tetG</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>tetJ</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>tetM</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>tetW</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

### 4. Conclusions

Our study indicated that the prevalence of AR-EC isolated from BC and DCs at the Yamagata Prefecture Livestock Research Institute was low or similar to that reported in Japan and other countries. This is probably because the surveyed farms were well managed by the prefectural government. Generally, the antimicrobial resistance rates in BC are higher than those in DCs. This difference was attributed to the higher prevalence of antimicrobial resistance among isolates obtained from calves, as calves tend to receive antimicrobial treatments more frequently than lactating dairy cows. The prudent use of antimicrobials contributes to reducing the occurrence of AR-EC in BC and DCs and, consequently, their release into the environment. This study provides fundamental information about ARB and contributes to improving food safety and to promoting the careful use of antimicrobial agents.

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**Data Availability Statement:** The original data presented in the study are included in the article. If you need any further information, you can contact the corresponding author.

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## Appendix A



Figure A1. Location of the farm.

Table A1. Primers and amplification programs for the detection of *bla*<sub>CTX-M-1, 2, 8, 9</sub>, *bla*<sub>TEM</sub>, and *tetA*, B, C, D, E, G, J, M, and W.

Gene	Primer	Sequence	Product Size (bp)	Reference	Amplification Program		
<i>uidA</i>	F	TGGTAATTACCGACGAAAACGGC	162	[10]	Initial denaturation: 95 °C, 3 min Denaturation: 95 °C, 30 s Annealing: 58 °C, 30 s Elongation: 72 °C, 1 min 35 cycles Final extension: 74 °C, 10 min		
	R	ACCGGTGGTTACAGTCTTGCC					
<i>bla</i> <sub>CTX-M-1</sub>	F	TTAGGAARTGTGCCGCTGYA	688	[13]	Initial denaturation: 94 °C, 10 min Denaturation: 94 °C, 40 s Annealing: 60 °C, 40 s Elongation: 72 °C, 1 min 30 cycles Final extension: 72 °C, 1 min		
<i>bla</i> <sub>CTX-M-2</sub>	R	CGATATCGTTGGTGGTRCCAT	404				
	F	CGTTAACGGCACGATGAC					
<i>bla</i> <sub>CTX-M-8</sub>	R	CGATATCGTTGGTGGTRCCAT	326				
	F	AACRRCAGACGCTCTAC					
<i>bla</i> <sub>CTX-M-9</sub>	R	TCGAGCCGGAASGTGYAT	561				
	F	TCAAGCCTGCCGATCTGGT					
<i>bla</i> <sub>TEM</sub>	R	TGATTCTCGCCGCTGAAG	800				
	F	CAITTCGGTTCGCCCTIATTC					
<i>tetA</i>	R	CGTTCATCCATAGTTGCCTGAC	494	[14]	Initial denaturation: 95 °C, 2 min Denaturation: 96 °C, 30 s Annealing: 60 °C, 30 s Elongation: 72 °C, 30 s 30 cycles Final extension: 72 °C, 10 min		
	F	TTGGCATTCTGCATTCACCTC					
<i>tetB</i>	R	GTATAGCTTGCCGGAAGTCG	571				
	F	CAGTGCCTGTTGTGTCATTAA					
<i>tetD</i>	R	GCTTGGAAATACTGAGTGTA	546				
	F	GCAAACCATTACGGCATTCT					
<i>tetE</i>	R	GATAAGCTGCGCGGTAAAAA	544				
	F	TATTAACGGGCTGGCATTTC					
<i>tetM</i>	R	AGCTGTCAGGTGGGTCAAAC	536				
	F	ACACGCCAGGACATATGGAT					
<i>tetW</i>	R	ATTTCCGCAAAGTTCAGACG	549				
	F	GGGAAATGTTCGGACAGAC					
<i>tetC</i>	R	AACGGATACCATCCCTGACA	207			[15]	Initial denaturation: 94 °C, 5 min Denaturation: 94 °C, 5 s Annealing: 68 °C, 10 s 25 cycles Final extension: 68 °C, 7 min
	F	GCGGGATATCGTCCATTCCG					
<i>tetG</i>	R	GCAGAGCAGGTTCGCTGG	134				
	F	CCYGCAAGAGAAGCCAGAAG					
<i>tetJ</i>	R	CGAAAACAGACTCGCCAATC	184				
	F	TCCATAATGAGGTGGGGC					

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