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Antibiotic-resistant *Escherichia coli* isolated from dairy cows and their surrounding environment on a livestock farm practicing prudent antimicrobial use

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ABSTRACT

On a livestock farm where antimicrobial administration and its history had been managed for prudent use of antimicrobials, we surveyed antibiotic-resistant *Escherichia coli* strains isolated from cow feces and the surrounding environment (i.e., rat and crow feces, and water samples from a drainage pit and wastewater processing tank) every month for 1 year. Two strains (1.7%) in cow feces were resistant to tetracycline, whereas all other strains were susceptible to all other antimicrobials. Among 136 strains isolated from cows and wild animals, only one ampicillin-resistant strain was identified. The antibiotic resistance rate in the drainage from the barn was 8.3% (10/120), and all strains showed susceptibility for 8 months of the year. Tetracycline resistance was common in all resistant strains isolated from animal feces and water samples; all tetracycline-resistant strains carried *tet*A. These results strongly support the proper use and management of antibiotics on farms to minimize the outbreak and spread of antibiotic-resistant bacteria.

1. Introduction

Antibiotics are indispensable for the life support and health management of humans and animals and have been widely used for the treatment of infectious bacterial diseases. However, antibiotic-resistant bacteria can arise by administration of antibiotics to humans and animals, and they are excreted and discharged into the environment as hazardous microbes (Sawant et al., 2007; Jia et al., 2017; Menz et al., 2019; Tullo et al., 2019; Dafale et al., 2020). Currently, there are worldwide concerns regarding the outbreak and spread of infectious diseases caused by antibiotic-resistant bacteria. Indeed, the annual death toll worldwide from antibiotic-resistant bacteria is reported to be 700,000, but this number could exceed 10 million by 2050 (O'Neil, 2014). The World Health Organization and Centers for Disease Control and Prevention (CDC) have selected antibiotic-resistant bacteria that pose a threat to the world and published research data that warn of the seriousness of the problem (CDC, 2019; Willyard, 2017). The spread of antibiotic-resistant bacteria is also serious in Japan. According to a report by the National Center for Global Health and Medicine, 8000 deaths occur annually due to methicillin-resistant *Staphylococcus aureus* and fluoroquinolone-resistant *Escherichia coli* (Tsuzuki et al., 2020).

Antibiotics are used on livestock farms to treat animal diseases and to effectively utilize the nutritional components in feed. In fact, more antibiotics are used on farms than are used in humans. The annual amount of antibiotics used in Japan is 581.3 ton/year for humans and 915.5 ton/year for livestock animals including feed additives (AMR Clinical Reference Center, 2018). The most important meat-producing countries, such as China, the USA, and Brazil, all use large amounts of antibiotics during meat production, while Japan and countries in Europe also use antibiotics on a large scale (Center for Disease Dynamics, Economics and

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Policy, 2015). Consequently, livestock farms are recognized as an important source of antibiotic-resistant bacteria, which may even be transmitted to humans via meat (Asai, 2008). In the Netherlands, methicillin-resistant *S. aureus* (MRSA) derived from pigs has also infected livestock industry personnel (van Loo et al., 2007). In addition, transmission of antibiotic-resistant bacteria from livestock-derived compost to fields (Sengeløv et al., 2003; Zhang et al., 2020) and vegetables (Marti et al., 2013) has been confirmed. Furthermore, studies have shown that antibiotic-resistant bacteria can spread from livestock wastewater to natural rivers (Wei et al., 2011) and may be transported into the natural environment via small animals (Furness et al., 2017; Zanardi et al., 2020; Nishimura et al., 2021).

Managing the amount and careful administration of antibacterial substances used will suppress the spread of antibiotic-resistant bacteria (Fujimoto et al., 2021; Nicola et al., 2021). However, maintaining the productivity of livestock farms is often difficult while properly using antibiotics. Thus, to promote the proper use of antibiotics in livestock animals, it is necessary to accumulate and share data on the actual conditions and antibiotic resistance rates from practical cases.

In this study, we focused on barn-reared dairy cows at the Sumiyoshi Livestock Science Station (known as Sumiyoshi Farm) attached to University of Miyazaki, Japan, where the administration of antibiotics and their history for all individuals has been recorded and managed. Antibiotic-resistant *E. coli* (AR-*E. coli*) isolated from cows and the surrounding environment was surveyed every month for 1 year. The strains of *E. coli* were isolated not only from the feces of dairy cows but also the feces of wild rats and wild crows living around the barn on the livestock farm. In addition, water samples were collected from the drainage pit and wastewater processing tank. The actual state of AR-*E. coli* on this livestock farm was then examined based on the resistance rate of strains collected from samples, the antibiotic resistance profile, and antibiotic administration history.

2. Materials and methods

2.1. Survey outline

The reasons for focusing on dairy cows were as follows: (1) dairy cows are extremely important industrial animals; (2) their rearing conditions are maintained and managed; (3) they are directly administered antibiotics for the treatment of mammitis and the dose is strictly controlled; (4) the rearing period of dairy cows is longer than that of beef cattle and/or swine; and (5) since the cows excrete a large amount of feces in the barn and surrounding area during rearing, they continuously affect the environment surrounding the farm. In addition, we assumed that rats and crows were vectors for antibiotic-resistant bacteria. The black rat (*Rattus rattus*) nests in the dairy barn, eats livestock feed and spilled feed mixed with cow feces, and drinks water from the water dispenser. The large Japanese field mouse (*Apodemus speciosus*), which lives in copse areas outside the barn, was also investigated as a contrast to the black rat. Carrion crows (*Corvus corone*) have been observed flying into the barn and pecking at the cows' feed and feces. Drainage is mixed with dairy cow manure, waste milk, and washing water from the barn. In addition, *E. coli* was targeted because it exists in the intestinal tract of warm-blooded animals and has the characteristic of easily acquiring antibiotic resistance depending on the antibiotics used in the host (Looft et al., 2012); *E. coli* can also adapt and survive in the natural environment (Ishii et al., 2006); several types of *E. coli* strains can cause diverse intestinal and extraintestinal diseases in healthy humans by means of individually acquired virulence factors, including Shiga toxins (Kaper et al., 2004). *Escherichia coli* are one of the most important bacteria because of the fear that antibiotic-resistant strains could spread from livestock farms.

2.2. Sampling

The Sumiyoshi Farm is the first facility in Japan to obtain GAP certification (certified in July 2014), which is an international initiative, in the field of livestock. As of March 2020, the number of farms with Global GAP certification for livestock farms in Japan is three management bodies (the total number of management bodies in Japan 63,790) (Ministry of Agriculture, Forestry and Fisheries, Japan, 2020; 2021). The farm manages to ensure various components of sustainability, including food safety and environmental conservation. In total, 12 surveys were conducted, once per month, from July 10, 2018 to June 25, 2019 for 1 year at the dairy barn in the Sumiyoshi Farm and in the surrounding area. At this farm, the dairy cow feed is self-mixed and does not contain antibacterial substances as feed additives. In addition, the administration of antibiotics for treatment is performed under the direction of a farm management veterinarian and the administration history (administration date and dose) is recorded. The farm has been prudently using antimicrobials for >10 years. Images of the dairy barn and each sampling point are shown in Fig. 1. The number of dairy cows reared during the survey period was 32–37, with an average of 34 per month.

The feces of dairy cows, black rats, and crows were collected in the barn and surrounding area. Ten fresh fecal samples excreted from each individual dairy cow were randomly selected and collected in a sterilized 50-mL polyethylene tube with a sterile spatula. All cow feces samples could be collected in 12 surveys, for a total of 120 samples. Black rats were captured by setting an adhesive mouse sheet (Sankyo-Shodoku Co., Tokyo, Japan) in the barn. As a result of conducting a survey 12 times, we captured three individual black rats in October 2018, two in December 2018, one in January 2019, and two in February 2019 (eight individuals in total). In the other survey months, we could not capture black rats. Rat feces were collected from the anus of each captured individual with a sterile cotton swab and placed in a sterile 15-mL polyethylene tube together with the sterile swab. During sampling, we observed crows flying to the trees near the dairy barn where their feces fell to the ground. Therefore, a survey of crows' feces was additionally conducted during the period from November 2018 to June 2019. Crows' feces could be collected each time in eight monthly surveys from November 2018 to June 2019. Fresh feces confirmed to be excreted from crows flying to the barn area were collected with a sterile cotton swab and placed into a 15-mL polyethylene tube. Finally, field mice were captured by setting up a live trap (Sherman Trap; H.B. Sherman Traps Inc., FL, USA) at a copse 300–500 m away from the Sumiyoshi Farm dairy barn. We conducted a survey to capture field mice in October 2018 and captured five individuals. The feces excreted from individual field mice were collected with tweezers and placed into 15-mL polyethylene tubes.

The drainage from the barn was collected from the drainage pit (i.e., the pit water) using a dipper and placed into a sterilized 1-L polyethylene bottle. The drainage and washing wastewater generated from the entire rearing facility on the farm, including the pit water, are transported to a wastewater treatment facility where the water is processed in an aerobic batch-type tank. The wastewater treatment system sequentially stores wastewater in a terete reaction tank (capacity 45.5 m^3, diameter 13 m, height 3.5 m). The wastewater is treated under aerobic and anaerobic conditions by turning the aeration on and off. The mixed liquor suspended solids are not controlled in the reaction tank. When the reaction tank is full, the treated water is subsequently sprayed onto the fields. The stored wastewater in the tank (known as tank water) was collected in a 1-L polyethylene bottle. During the survey period, due to a breakdown of the treatment facility, it was not possible to sample the tank water during the period from October 2018 to January 2019.

All samples were placed in a cool box without a refrigerant after collection and taken back to the laboratory. *E. coli* isolation was conducted within 3 h after the survey.

Fig. 1. Images of the survey area and sampling points at the Sumiyoshi Livestock Science Station.

2.3. Collection of *E. coli* strains

Escherichia coli were isolated from each sample using the membrane filter method. For cow and field mouse feces, approximately 0.1 g of fecal sample was dispensed into a sterilized 15- mL tube using a sterile cotton swab, and then 10 mL of sterile physiological saline water was added to prepare a suspension. The physiological saline water was adjusted to 0.90% sodium chloride in ion-exchange distilled water and then sterilized. Similarly, 10 mL of saline water was added to the sample tubes containing cotton swabs with the feces of black rats or crows without weighing the feces. These suspensions were then serially diluted from 10- to 10^3-fold. The diluent was filtered through a membrane filter (diameter: 47 mm; pore size: 0.45 μm; Advantec, Tokyo, Japan) and the filters were placed on CHROMagar ECC agar plates (CHROMagar, Paris, France) for incubation at 37 °C for 24 h. After incubation, blue colonies putatively identified as *E. coli* were picked from the ECC agar plates and purified by repeated single-colony isolation on the same medium. The isolates were incubated on brain heart infusion agar plates (1.5% agar; Becton, Dickinson and Company, NJ, USA) at 37 °C for 18 h and then species were identified. By this series of isolations, 10 strains of *E. coli* were isolated and collected from each fecal sample. If less than 10 positive colonies were found, all positive strains were isolated. The pit water and tank water were serially diluted from 10- to 10^4-fold. Similarly, the diluents were filtered through membrane filters and placed on CHROMagar ECC agar plates. In the same manner as the fecal samples, 10 strains of *E. coli* were isolated from each water sample.

2.4. Identification of *E. coli*

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis was used for species identification (Suzuki et al., 2018). An aliquot (1.0 μL) of colony suspension was spotted directly onto a 384-well stainless-steel target plate (MTP 384; Bruker Daltonics, Billerica, MA, USA). Following air-drying for 10 min, a template was overlaid with 1.0 μL of the matrix solution. All samples were analyzed using an Autoflex III TOF/TOF (Bruker Daltonics, Billerica, MA, USA) operated in the linear positive mode within a mass range of 2000–20,000 Da based on the manufacturer's instructions. For database construction and validation, measurements were performed in the auto-execute mode using Flex Control 3.4 software (Bruker Daltonics). The software settings were as follows: linear positive: 3–20 kDa; detector gain: 2691 V; laser shots: 40–200; laser power: 30%. A Bruker bacterial test standard (part no. 8255343, Bruker Daltonics) was used for instrument calibration. Recorded mass spectra were analyzed with the MALDI Biotyper Compass (Bruker Daltonics) under standard settings. The MALDI Biotyper output is a log score value from 0.000 to 3.000; the *E. coli* identification score was >2.000.

2.5. Determination of minimum inhibitory concentration

An antibiotic susceptibility test was performed on one strain from each fecal sample identified. Ten isolated strains were randomly numbered. Then, *E. coli* isolates were identified using MALDI-TOF-MS. Among the identified *E. coli* isolates, the isolate with the lowest number of colonies was selected for the antibiotic susceptibility test. In addition, 10 identified *E. coli* strains isolated from pit and tank water were tested. The minimum inhibitory concentration (MIC) of each antimicrobial agent was determined via the microliquid dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). The *E. coli* isolates were cultured at 37 °C for 18 h in Mueller–Hinton broth (Becton Dickinson, Sparks, MD, USA) and then diluted to a final concentration corresponding to the 0.5 McFarland turbidity standard with fresh Mueller–Hinton broth. Inocula were then applied to the microplate surface containing graded concentrations of each antimicrobial agent in a microplate well (Eiken Chemical Co., Tokyo, Japan). The plates were incubated at 37 °C for 18 h before MICs were determined. MIC breakpoints for resistance (susceptibility: S, intermediate resistance: I, resistant: R) were based on the CLSI criteria.

The antimicrobials used in the current study (all from Wako Pure Chemical Industries, Ltd., Osaka, Japan, unless otherwise stated)

included ampicillin (ABPC; breakpoints concentrations; S ≤ 8, I = 16, R ≥ 32 μg/mL) as a representative penicillin; cefazolin (CEZ; S ≤ 2, I = 4, R ≥ 8 μg/mL) and cefotaxime (CTX; S ≤ 1, I = 2, R ≥ 4 μg/mL) as representative cephem antimicrobials; imipenem (IMP; S ≤ 1, I = 2, R ≥ 4 μg/mL) as a representative carbapenem; gentamicin (GM; S ≤ 4, I = 8, R ≥ 16 μg/mL) and kanamycin (KM; S ≤ 16, I = 32, R ≥ 64 μg/mL) as representative aminoglycosides; tetracycline (TC; S ≤ 4, I = 8, R ≥ 16 μg/mL) as a representative tetracycline; nalidixic acid (NA; S ≤ 16, R ≥ 32 μg/mL) and ciprofloxacin (CPFX; S ≤ 1, I = 2, R ≥ 4 μg/mL) as representative fluoroquinolones; sulfamethoxazole/trimethoprim (SMX/TMP; S ≤ 2/38, R ≥ 4/76 μg/mL) as a compound; and chloramphenicol (CP; S ≤ 8, I = 16, R ≥ 32 μg/mL; Sigma-Aldrich) as a representative phenicol. The reference *E. coli* strain ATCC25922 was used for quality control.

2.6. Detection of the tetracycline resistance gene tet by PCR analysis

For the strains that were resistant to tetracycline, the types of tetracycline resistance gene, *tet*, were detected by PCR analysis. The target types of *tet* gene were *tetA*, *tetA* (P), *tetB*, *tetB* (P), *tetD*, *tetH*, *tetL*, *tetM*, *tetT*, and *tet37* (Aminov et al., 2001; Jin et al., 2002; Call et al., 2003; Diaz-Torres et al., 2003). The sequence information of each *tet* was referred to in the comprehensive antibiotic resistance database (CARD, Alcock et al., 2020). The primers and probes specific for each *tet* gene were designed using Primer3 web tool (Untergasser et al., 2012; Table S1) and purchased from Integrated DNA Technologies (IDT). DNA was extracted using the InstaGene matrix (Bio-Rad, Laboratories Inc., USA) according to the manufacturer's recommendations. The reaction was conducted with 20-μL volume containing 10 μL of SsoAdvanced Universal Probe Supermix (Bio-rad Laboratories Inc., USA), 2-μL of primer probe mix (primer: 5 μM; probe: 2.5 μM), 3 μL of nuclease-free water, and 5 μL of template DNA. A thermal cycler (CFX-96 Touch, Bio-Rad Laboratories Inc., USA) was used for the PCR reaction. The reaction conditions for PCR were 95 °C (30 s), and reactions at 95 °C (10 s) and 60 °C (30 s) for 40 cycles. The specificities of the *tet* assays were compared using a standard DNA (Table S2) that was designed based on sequence information in CARD (Alcock et al., 2020) and were purchased from IDT. The endpoint fluorescence of the sample and standard DNA at each thermal cycle was measured. When a fluorescence signal from sample confirmed until 40 cycles, the sample DNA was considered positive. Nuclease-free water was used as a negative control. Reactions for the DNA template and control DNA were run in two replicates to detect *tet*.

2.7. Statistical analysis

To examine the statistical differences in the proportions of antibiotic-resistant strains in the different sampling environments, we used Fisher's exact test following Holm's multiple comparison test; we used fisher. multcomp in theRVAideMemoire package under R ver. 3.6.3. In this process, only the Cow, Pit, and Tank environments were compared owing to the smaller sample size for the other environments.

3. Results and discussion

3.1. Antibiotic resistance rate and resistance profiling

An antibiotic susceptibility test was conducted on 341 strains of *E. coli* (120, 8, 5, 8, 120, and 80 from cows, black rats, Japanese field mice, crows, pit water, and tank water, respectively) isolated and identified from each sample (Table S3). Fig. 2 shows the resistance rate to the antibiotic agent(s) in the strains from each type of sample. Fisher's exact test results showed no significant differences in the resistance rate among the Cow, Pit, and Tank samples (p = 0.102–785). In addition, the ratio of susceptible strains and strains resistant to 1–4 antibiotics is shown in Fig. S1. Tetracycline resistance was common to all resistant

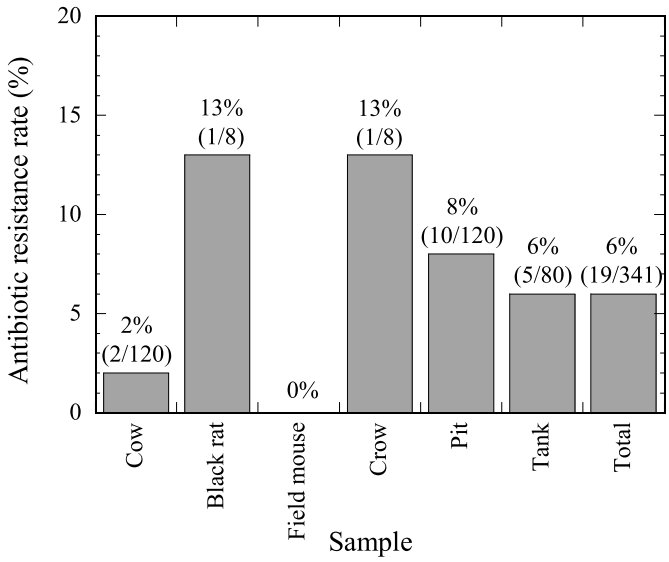

Fig. 2. Resistance (%) to one or more antibiotics in the *Escherichia coli* strains isolated from each sample.

strains sampled from animal feces and water, except for one strain in the tank water. The antibiotic resistance rate of *E. coli* from cows, which play a leading role as source from which the strains spread, was 1.7% (2 resistant strains of 120 strains). From the March 2019 samples, only two strains were resistant to tetracycline while the other strains were susceptible to all antibiotics. According to national drug resistance statistical data from Japan in 2018 (AMR Clinical Reference Center, 2018), the resistance rate of tetracycline in *E. coli* from healthy cattle in livestock farms is 26.5% on average, which is 13-fold higher than the resistance rate detected in this study. In addition, referring to data from the AMR Clinical Reference Center (2018), the resistance rates of specific antibacterial drugs were as follows: ABPC: 11.6%; CEZ: 0.5%; CTX: 0.0%; GM: 0.0%; KM: 0.0%; CPFX: 0.5%; NA: 2.1%; CP: 4.8%; and SMX/TMP 5.3%. Data from the AMR Clinical Reference Center (2018) were based on the test results of the isolated *E. coli* strains cultured in a regular medium (without antibacterial agents) (Kijima-Tanaka et al., 2003), which was similar to the data analyzed in this study. Consistent with this survey result and AMR report, CTX-, GM-, and KM-resistant *E. coli* were not detected, and it is considered that farms in Japan are not the source of these antibacterial-resistant bacteria. Notably, these resistance rates largely differ from the results obtained in this study, which showed susceptibility to each antibiotic. Indeed, the antibiotic resistance rates of dairy cows on the studied farm were extremely low in comparison to the rates in other cattle in Japan and overseas. (DeFrancesco et al., 2004; Sawant et al., 2007; Cheney et al., 2015). High resistance rates of 33.3%–93% have been reported for TC-resistant *E. coli* from dairy cows in many farms in Asia, the UK, and the USA (DeFrancesco et al., 2004; Sawant et al., 2007; Cheney et al., 2015, Hennessey et al., 2020; Sobur et al., 2019), and farms would be one of the sources of TC-resistant *E. coli*. In addition, resistant strains have not been detected in Japanese farms; CTX-, GM-, KM-resistant strains have been detected as follows: CTX, 3.1% (Cheney et al., 2015); GM, 0.3%–12.76% (DeFrancesco et al., 2004; Cheney et al., 2015, Sobur et al., 2019); and KM, 42.55% (Sobur et al., 2019). It is inferred that there are differences in the use and management of antibacterial agents on farms between Japan and overseas countries in Asia, the UK, and the US.

The antibiotic resistance rate of *E. coli* from black rats captured in the barn was 12.5% (1 of 8 samples); the resistant strain showed resistance to ampicillin and tetracycline. The resistance rate of *E. coli* from crows was also 12.5% (1 of 8 samples), with the only resistant strain showing resistance to tetracycline. The antibiotic resistance rate of *E. coli* from wild animals living around the barn was lower than that in livestock in

domestic farms in Japan based on a large-scale study that compared antimicrobial-resistant bacteria from different regions in Japan (Yoshizawa et al., 2020). Our data confirm that the acquisition and transmission of AR-*E. coli* to wild animals around the barn from the feces of dairy cows had not occurred to a great extent at Sumiyoshi Farm. In addition, antibiotic-resistant strains of *E. coli* were not detected in Japanese field mice caught in the copse area away from the barn.

The antibiotic resistance rate of samples from the pit water was 8.3% (10 of 120 samples). Among 12-month samples, resistant strains were observed in 4 months. Manure and waste milk were mixed in the pit water, and the pollutants had extremely high solid content. Nevertheless, antibiotic-resistant strains were rarely detected throughout the year. Of the survey months, the detection of resistant strains was concentrated in June, July, and August (Table S3). During this period, four strains of multidrug-resistant (i.e., resistant to four antibiotics) *E. coli* were detected from pit water. Two resistance patterns were observed for the four antibiotics: ABPC– TC– NA–CPFX (three strains) and ABPC–GM–KM–TC (one strain). From June (mean daily maximum temperature: 27.1 °C) to the early part of July in the subtropical rainy season, conditions were hot and humid, and cefazolin was frequently administered to treat mammitis (Table 1). In the midsummer from July (30.8 °C) to August (31.6 °C), doses of benzylpenicillin alone and kanamycin–benzylpenicillin were increased for the treatment of mammitis. ABPC-resistant strains were detected in the pit water from June to August. One KM-resistant strain was detected in the pit water in August, when the dose of kanamycin was the highest (Table S4). The increased antibiotic doses used for the treatment of mammitis likely gave rise to the resistant strains detected in the pit water; however, these resistant strains were not retained in this water, with the resistance rate shown to be extremely low in September. The resistance rate of the tank water was 6.3% (5 of 80 samples), similar to the resistance rate of the pit water. In April, one strain resistant to three antibiotics was detected with a resistance pattern of ABPC–TC–CP. However, AR-*E. coli* was rarely detected in the tank water during the survey period, which was in agreement with the data obtained from the pit water. In Miyazaki City, in which Sumiyoshi Farm is located, the antibiotic resistance rates of *E. coli* in sewage and the urban river water were previously reported as 59.5% and 28.5%, respectively (Ogura et al., 2020). Therefore, the antibiotic resistance rates of the pit water and treated tank water on the studied farm were much lower than the rates found in water bodies in the urban city.

3.2. Classification of tet tetracycline resistance genes

The types of *tet* gene, i.e., tetracycline resistance genes, were analyzed in all tetracycline-resistant strains (18 strains) in this study. Fig. 3 shows a comparison of the presence or absence of *tet* detected with the antibiotic resistance profile of strains. The *tet*A gene, which is the oldest known gene for encoding the tetracycline efflux protein, was detected in all tetracycline-resistant strains isolated from cow and crow feces and from pit and tank water. Additionally, *tet*B was detected in 17 strains excluding 1 strain from pit water. Conversely, *tet*M, which encodes a ribosomal protection protein detected in the feces of many domestic animals in Japan (Kobayashi et al., 2007), was found only in one strain from pit water. These results are consistent with a previous report that among the known tetracycline-resistant determinants, tetracycline efflux genes, especially *tet*A and *tet*B, are prevalent, but ribosomal protection genes, including *tet*M, are rarely detected in tetracycline-resistant *E. coli* strains (Chopra and Roberts, 2001), it has been detected in *E. coli* strains isolated from diverse human and animal sources (Bryan et al., 2004). Since the use of tetracycline was discontinued before 2013 on the entire farm including the barn, the low resistance rate of tetracycline and the possession of *tet*A and *tet*B genes likely indicate the positive implications for the environment around the farm. Tetracycline-resistant *E. coli* carrying *tet*A was below the detection limit in 1 week in the environment, and a correlation has been reported between the number of copies of tetracycline resistance gene in farm compost and the amount of tetracycline remaining (Yoshizawa et al., 2020). Thus, the tetracycline-resistant *E. coli* in this study was derived from outside the farm and may have been brought in by wild animals, but the details are unknown. It has been indicated that the feces of wild and migratory birds may be a potential factor in the spread of antibiotic-resistant *E. coli* in dairy farms (Fahim et al., 2019).

3.3. Milk production and antimicrobial doses

On Sumiyoshi Farm, the quality of milk (including residual antibiotics) produced by dairy cows administered antibiotics is strictly managed in accordance with “Food Sanitation Act” and “Japanese veterinary public health legislation: ministerial ordinance concerning compositional standards, etc. for milk and milk products, Ministry of Health and Welfare.” Thus, milk with a guaranteed quality is continuously produced every day from the farm (monthly production: 6762–13,371 kg; average = 9648 kg). Given that the tetracycline resistance rate of *E. coli* from the feces of cows was ≤2% and no other resistant *E. coli* isolate was found in cows during our 1-year study, we conclude that AR-*E. coli* are under control in cows reared on the farm. The monthly number of rearing cows, amount of antibacterial drugs used, and drug administration per number of cows are shown in Table 1. Indeed, if dairy cows are reared using the levels of antibiotics shown Table 1, it seems to be possible to control the expression of AR-*E. coli* on a farm. The number of treatment days using antibiotics per dairy cow per

Table 1
The head of cows per month, the amount of antibiotics used, and the amount of antibiotics used per head.

Antibiotics														
Sampling		Cow	Administration	Cephalonium		Cefazolin		Benzylpenicillin		Kanamycin and benzylpenicillin				Sulfamonomethoxyn
				Udder		Udder		Intramuscular		Udder				Intramuscular
Year	Month	heads		(mg, titer)	(mg/head)	(mg, titer)	(mg/head)	(mg, titer)	(mg/head)	(mg, titer)	(mg/head)	(mg, titer)	(mg/head)	(mg, titer)	(mg/head)
2018	July	32		0	0	12,000	375	3600	113	10,800	338	6480	203	0	0
	August	32		0	0	0	0	18,000	563	14,400	450	8640	270	0	0
	September	33		4000	121	0	0	1200	36	0	0	0	0	6000	182
	October	33		3000	91	4000	121	0	0	3600	109	2160	65	0	0
	November	34		1000	29	0	0	12,600	371	0	0	0	0	0	0
	December	37		1000	27	0	0	0	0	0	0	0	0	2000	54
2019	January	34		0	0	4000	118	0	0	4800	141	2880	85	2000	59
	February	34		1000	29	0	0	1800	53	0	0	0	0	0	0
	March	33		0	0	0	0	0	0	0	0	0	0	4000	121
	April	33		0	0	3000	91	1650	50	0	0	0	0	4000	121
	May	34		0	0	0	0	0	0	0	0	0	0	0	0
	June	33		1000	0	3000	91	0	0	0	0	0	0	0	0

Strains code	Antibiotics											tet gene									
	ABPC	CEZ	CTX	GM	KM	TC	NA	IPM	CPFX	CP	SMX/TMP	*tet* A	*tet* A(P)	*tet* B	*tet* B(P)	*tet* D	*tet* H	*tet* L	*tet* M	*tet* T	*tet* 37
Cow 3_1	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Cow 3_6	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Black rat 6	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Crow 2	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 4_6	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 6_3	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	R≥32	S≤1	R≥4	S≤8	S≤1	+	+	+							
Pit 6_4	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	R≥32	S≤1	R≥4	S≤8	S≤1	+							+		
Pit 7_1	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 7_2	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 7_3	R≥32	I=4	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 8_3	R≥32	S≤2	S≤1	R≥16	R≥16	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+				+			
Pit 8_4	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	R≥32	S≤1	R≥4	S≤8	S≤1	+		+							
Pit 8_6	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Pit 8_7	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Tank 4_4	R≥32	I=4	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	R≥32	S≤1	+		+							
Tank 5_3	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+				+			
Tank 6_2	S≤8	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							
Tank 9_1	R≥32	S≤2	S≤1	S≤4	S≤4	R≥16	S≤16	S≤1	S≤1	S≤8	S≤1	+		+							

(μg/mL)

S : Susceptibility
I : Intermediate resistance
R : Resistance

Fig. 3. Detection of *tet* genes conveying antibiotic resistance in tetracycline-resistant *Escherichia coli* strains. Abbreviations: ABPC, ampicillin; CEZ, cefazolin; CTX, cefotaxime; GM, gentamicin; KM, kanamycin; TC, tetracycline; NA, nalidixic acid; IPM, imipenem; CPFX, ciprofloxacin; CP, chloramphenicol; SMX/TMP, sulfamethoxazole/trimethoprim.

year raised in Japan is estimated to be 15.5 days/year (Abe et al., 2021). In contrast, the number of treatment days for dairy cows on this farm was 2.4 days/year. Accordingly, we infer that this farm properly implements antibiotics administration compared with general domestic farms in Japan.

3.4. Conclusions

In a survey lasting 1 year with data collected monthly, we confirmed that the antibiotic resistance rate of *E. coli* in the animal feces and wastewater sampled from Sumiyoshi Farm, on which antibiotic use is strictly monitored and controlled, was maintained at extremely low levels compared with the levels of antibiotic resistance typically reported on domestic farms in Japan. Moreover, the problematic ESBL-producing and fluoroquinolone-resistant *E. coli* were not detected, despite 341 strains being analyzed. Antibiotic resistance may be kept low on Sumiyoshi Farm because antibiotics are used appropriately by the veterinarian supervisor, the administration history is recorded every day for all livestock individuals, and the rearing environment is strictly managed. In a previous study, Walk et al. (2007) analyzed *E. coli* strains from 30 conventional and 30 organic dairies and concluded that it takes a conventional farm approximately 8 years to acquire the lower resistance profile of an organic farm. The low rate of antibiotic resistance noted in this case study of the Sumiyoshi Farm, which has acquired GLOBAL G.A.P. and has been continuing to improve antibiotic use-related practices for >10 years, is consistent with the predictions from the previous study (Walk et al., 2007). From the results of our survey, we conclude that the outbreak and spread of antibiotic-resistant bacteria are markedly reduced in farms that practice the prudent use and management of antibiotics.

Credit authorship contribution statement

Yoshihiro Suzuki: Conceptualization, Methodology, Investigation, Writing – Original Draft, Writing — Review & Editing, Supervision. Hayate Hiroki: Investigation. Hui Xie: Investigation, Visualization. Masateru Nishiyama: Investigation, Validation. Shinsuke H. Sakamoto: Investigation, Validation. Ryoko Uemura: Validation — Review & Editing. Kei Nukazawa: Writing — Review & Editing. Yoshitoshi Ogura: Validation. Toru Watanabe: Investigation, Validation. Ikuo Kobayashi: Investigation — Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113930.

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