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Anti-microbial resistance of *Salmonella* isolates from raw meat-based dog food in Japan

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Abstract

Background: *Salmonella* contamination of raw meat-based diets (RMBDs) for pets poses a major public health concern but has not been investigated in Japan.

Objective: To investigate *Salmonella* contamination in RMBDs for dogs marketed in Japan and the anti-microbial resistance profiles of the *Salmonella* isolates.

Methods: Sixty commercial RMBD samples were collected in the Okayama and Osaka Prefectures, Japan, between December 2016 and March 2017. The obtained *Salmonella* isolates were serotyped, their anti-microbial resistance patterns were determined, and the anti-microbial-resistant isolates were screened for the presence of resistance genes by polymerase chain reaction.

Results: *Salmonella enterica* subsp. *enterica* was detected in seven of the 60 RMBD samples. Among them, five isolates were identified as *S.* Infantis (n = 3), *S.* Typhimurium (n = 1) and *S.* Schwarzengrund (n = 1), while the serotypes of two isolates were unable to be identified. All isolates were susceptible to ampicillin, cefazolin, cefotaxime and gentamycin. Two isolates were resistant to more than one anti-microbial agent; one of the *S.* Infantis isolates was resistant to streptomycin, kanamycin, tetracycline and trimethoprim, while the *S.* Typhimurium isolate was resistant to nalidixic acid, ciprofloxacin and chloramphenicol. The *S.* Schwarzengrund isolate was resistant to tetracycline. Additionally, the *S.* Typhimurium isolate harboured the anti-microbial resistance gene *gyrA* with a mutation corresponding to Ser-83→Phe amino acid substitution.

Conclusion: The study findings suggest that RMBDs for dogs marketed in Japan can be a potential source of *Salmonella* infection for dogs and humans including infections caused by quinolone-resistant isolates.

KEYWORDS
dog food, raw meat-based diet, *Salmonella*

1 | INTRODUCTION

Salmonella spp. are Gram-negative bacilli belonging to the family *Enterobacteriaceae* that can colonize the intestinal tract of most vertebrates. Non-typhoidal *Salmonella* is an important food-borne pathogen that causes gastroenteritis, bacteraemia and focal infections in humans and animals (Behravesh et al., 2010; Cavallo et al., 2015; Freeman et al., 2013; Kępińska-Pacelik & Biel, 2021; Lambertini et al., 2016). Transmission to humans typically occurs by ingesting meat, dairy products and other foods contaminated with *Salmonella*, but zoonotic transmission can also occur by direct exposure to the faces of reptiles, pets and other animals (Antunes et al., 2016; Behravesh et al., 2010; CDC, 2006, 2012; Cherry et al., 2004; Lambertini et al., 2016; Mizoguchi et al., 2011; Sato, Mori, et al., 2000; Tauni & Osterlund, 2000; Toyofuku, 2008). Importantly, case-control studies have reported that direct contact with an infected pet plays a major role in human salmonellosis, with frequent reports of direct transmission (Hoelzer et al., 2011; Lefebvre et al., 2008; Sato, Mori et al., 2000). Moreover, several outbreaks of salmonellosis have been reported in humans due to exposure to contaminated dry dog food and dog treats (Behravesh et al., 2010; Cavallo et al., 2015; Imanishi et al., 2014; Selmi et al., 2011).

A variety of commercial raw meat-based diets (RMBD) and brands are available in most pet shops and supermarkets (Freeman & Michel, 2001; Freeman et al., 2013). RMBDs, which are not heat treated before they are refrigerated or frozen (Mack & Kienzle, 2016; Morgan et al., 2017; Stogdale, 2001), usually consist of uncooked meat, edible bones and organs from various types of animals. In recent years, RMBDs have gained increasing popularity among dog owners who prefer not to feed their pets conventional dry or canned pet food for several reasons (Empert-Gallegos et al., 2020; Morelli et al., 2019; Morgan et al., 2017; Thomas & Feng, 2020; Viegas et al., 2020). However, the health benefits attributed to RMBDs are mostly anecdotal and not supported by research. Conversely, several studies have reported the risks associated with RMBDs (FDA, 2020; Morley et al., 2006), including their microbiological load since the constituent meats do not undergo treatment to reduce or eliminate possible pathogenic microorganisms. Indeed, contaminated RMBDs have been identified as a source of gastroenteritis in greyhounds and diarrhoea in young puppies, sometimes leading to the animal's death (Morley et al., 2006). Specifically, *Salmonella* have been recovered from RMBDs and canine faecal samples (Finley et al., 2007; Morley et al., 2006). While faecal shedding of salmonellae is generally thought to last for up to 1 week after a single feeding with contaminated RMBD, shedding may last for up to 8 months if animals are fed the contaminated product over a longer period (Finley et al., 2007; Lefebvre et al., 2008). Because RMBDs for dogs have been linked to salmonellosis in humans (FDA, 2018b) and dogs (FDA 2020), the US Centers for Disease Control and Prevention (CDC) and US Food and Drug Administration (FDA) do not recommend feeding RMBDs to dogs (CDC, 2021; FDA, 2018a).

Consequently, *Salmonella* contamination of RMBDs is currently being evaluated in several countries (Bacci et al., 2019; Bottari et al., 2020; Finley et al., 2008; Hellgren et al., 2019; Lenz et al., 2009; Nemser et al., 2014; Van Bree et al., 2018; Weese et al., 2005). However, *Salmonella* contamination in RMBDs has not been investigated in Japan, even though *Salmonella* has been isolated from certain raw meat products intended for human consumption in Japan. For example, one study reported that 0.2% of raw beef, 12.7% of raw chicken and 0.4% of raw horse meat samples intended for human consumption in Japan were contaminated with *Salmonella* (Hara-Kudo et al., 2013). Moreover, several cases of multi-drug-resistant *Salmonella* have been recently reported in Japan (Hu et al., 2018; Miriagou et al., 2004; Su et al., 2004; Viana et al., 2019), as well as being detected in foods (Ahmed et al., 2009; Duc et al., 2019; Mori et al., 2018; Noda et al., 2015; Osawa et al., 2014).

Therefore, the aim of the present study was to determine the current prevalence of *Salmonella* contamination in commercial RMBDs for dogs available in Japan and to investigate anti-microbial resistance among the obtained *Salmonella* isolates.

2 | MATERIALS AND METHODS

2.1 | *Salmonella* isolation and identification

Based on a previous study by Hellgren et al. (2019), 60 RMBDs for dogs were collected in the Okayama and Osaka Prefectures, Japan, between December 2016 and March 2017, comprising 50 domestic and 10 imported products. All products were sold frozen, transported to the laboratory and stored at −20°C until analysis.

Salmonella was isolated following procedures described in the Bacteriological Analytical Manual of US Food and Drug Administration (Andrews et al., 2016). Briefly, 25 g sample was mixed with 225 mL sterile lactose broth (Difco, Detroit, MI, USA) and blended for 2 min. The homogenised mixture was then transferred to a sealed, sterile jar for 60 ± 5 min at room temperature. Blending was omitted for powdered, ground or comminuted products. The pH of the sample was adjusted to 6.8 ± 0.2, if necessary. Then, 2.25 mL steamed (15 min) Triton X-100 (Thermo Fisher Scientific, Waltham, MA, USA) was added to the sample to minimize foaming, followed by mixing and incubation for 24 ± 2 h at 35°C.

Aliquots of the sample mixture were then transferred to various media: 0.1 mL sample mixture was added to 10 mL Rappaport-Vassiliadis (RV) medium (Oxoid, Hampshire, UK) and 1 mL sample mixture was added to 10 mL tetrathionate (TT) broth (Oxoid). The inoculated RV medium and TT broth were incubated for 24 ± 2 h at 42 ± 0.2 and 35 ± 2.0°C, respectively. Subsequently, 3 mm loopfuls (10 μL) of incubated TT broth or RV medium were streaked on bismuth sulphite agar (Oxoid), xylose lysine desoxycholate agar (Merck Millipore, Burlington, MA, USA), and Hektoen enteric agar (Merck Millipore). The plates were incubated for 24 ± 2 h at 35°C, after which two or more *Salmonella* colonies were selected from each agar plate. Irrespective of whether or not colonies were selected after the first 24 h incubation, the agar plates were incubated for an additional 24 ± 2 h. After the second incubation, two or more typical colonies were selected, if present. The isolates were identified using API 20E identification kits (bioMérieux, Marcy-l'Étoile, France). According to

TABLE 1 *Salmonella* isolated from raw meat-based diets (RMBDs) for dogs in Japan

Country of origin	Animal material	No. of samples	No. of *Salmonella*-positive samples	*S. enterica* subsp. *enterica* serotype (no. isolates)
Japan	Deer	15	1	Typhimurium (1)
	Horse	13	1	Infantis (1)
	Chicken	7	3	Infantis (1); Schwarzengrund (1); Untypable (1)
	Cow	5	0	
	Duck	3	0	
	Ostrich	1	0	
	Pig	1	0	
	Kangaroo	1	1	Untypable (1)
	Miscellaneous	4	1	Infantis (1)
United States	Turkey	3	0	
	Ostrich	2		
Canada	Horse	2	0	
New Zealand	Sheep	2	0	
Mexico	Horse	1	0	
Total		60	7	

the Kauffmann-White scheme, the isolates were serotyped using slide and tube agglutination tests with commercially available antisera (Denka Seiken Co., Ltd., Tokyo, Japan) (Grimont & Weill, 2007).

The serotypes of the isolates were verified using polymerase chain reaction (PCR). DNA templates were prepared using the boiling method as previously described (Matayoshi et al., 2015). Briefly, bacterial cells were suspended in 200 μL distilled water and boiled for 10 min. The cells were then pelleted by centrifugation for 1 min at 2000 *g*. PCR reactions were performed with 5 μL supernatant in a final volume of 25 μL using 2 × GoTaq Green Master Mix (Promega, Madison, WI, USA), according to the manufacturer's instructions. The primer sequences were previously described (Alvarez et al., 2004). Amplification was performed using a LifeECO Thermal Cycler (Hangzhou Bloer Technology Co., Ltd., Zhejiang, China) with the following thermal cycling protocol: initial denaturation (95°C, 2 min) followed by 30 cycles of denaturation (95°C, 1 min), annealing (57°C, 1 min) and extension (72°C, 2 min) with a single final extension (72°C, 5 min). The PCR products were electrophoresed on 2.5% agarose gels (wt./vol) to obtain 50–800 bp fragments (Nacalai Tesque Inc., Kyoto, Japan), stained with 2 μg/mL ethidium bromide (Nacalai Tesque), and photographed under UV light. A 100 bp DNA Ladder (Takara Bio, Shiga, Japan) was used as a molecular size marker.

2.2 | Anti-microbial susceptibility testing

Escherichia coli ATCC 25922 was used as the quality-control strain in the experiments. Minimum inhibitory concentration (MIC) values were determined using a broth microdilution method on Eiken dry plates (Eiken Kagaku, Tokyo, Japan), following the manufacturer's instructions. Resistance of the isolates and *E. coli* was assessed for the following anti-microbial drugs: ampicillin (ABPC), cefazolin (CEZ), cefotaxime (CTX), chloramphenicol (CP), tetracycline (TC), gentamicin (GM), kanamycin (KM), nalidixic acid (NA), ciprofloxacin (CPFX) and trimethoprim (TMP). MIC breakpoints were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013). Susceptibility to streptomycin (SM) was determined using the standard disk diffusion method with Sensi-Discs (Becton Dickinson Company, Tokyo, Japan) (CLSI, 2013).

2.3 | Detection of anti-microbial resistance genes

Isolates demonstrating anti-microbial resistance were prepared for PCR analysis as described above, with sequences screened for the presence of class 1 and class 2 integron genes (*intI1* and *intI2*) (Sáenz et al., 2004), as well as 16 anti-microbial resistance genes. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Tokyo, Japan) and sequenced by Macrogen Japan Corp. (Tokyo, Japan). The resulting DNA sequencing data were compared with sequences deposited in the GenBank database using the BLAST algorithm. All anti-microbial-resistant isolates were tested in triplicate.

The aminoglycoside-resistant phenotype was identified by the presence of *aadA1* and *aadA2* (Chuanchuen & Padungtod, 2009). The TC-resistant phenotype was identified by the presence of *tet*(A) and *tet*(B)

(Chuanchuen & Padungtod, 2009). The CP-resistant phenotype was identified by the presence of *floR*, *cmlA1* and *catA1* (Bolton et al., 1999; Keyes et al., 2000; Maynard et al., 2003). The TMP-resistant phenotype was identified by the presence of *dfrA1* and *dfrA12* (Chuanchuen & Padungtod, 2009). The quinolone-resistant phenotype was identified by the presence of the plasmid-mediated quinolone resistance genes *qepA*, *aac(6′)-Ib-cr*, *qnrA*, *qnrB* and *qnrS* (Park et al., 2006; Robicsek et al., 2006; Yamane et al., 2008). This isolate was also screened for the presence of mutations in the quinolone resistance-determining regions of *gyrA*, *gyrB*, *parC* and *parE* (Matayoshi et al., 2015).

3 | RESULTS

3.1 | Isolation and serotyping of *Salmonella* from RMBDs

Salmonella enterica subsp. *enterica* was isolated from 7 (12%) of the 60 samples, of which all were domestic products. In terms of the raw materials used for RMBD production, the contaminated food samples were derived from chicken (n = 3), deer meat (n = 1), kangaroo meat (n = 1) and miscellaneous meat (n = 2) (Table 1). The isolates were identified as serotypes *S.* Infantis (n = 3), *S.* Typhimurium (n = 1) and *S.* Schwarzengrund (n = 1). Two isolates could not be serotyped.

3.2 | Anti-microbial susceptibility profiling of *Salmonella* isolates obtained from RMBDs

All seven *Salmonella* isolates were susceptible to ABPC, CEZ, CTX and GM. Four isolates were susceptible to all anti-microbial agents tested (Tables 2 and 3). Additionally, the *S.* Typhimurium isolate was resistant to NA, CPFX and CP, the *S.* Schwarzengrund isolate was resistant to TC, and one of the *S.* Infantis isolates was resistant to SM, KM, TC and TMP.

3.3 | Detection of anti-microbial resistance genes harboured by *Salmonella* isolates from RMBDs

PCR screening revealed that none of the *Salmonella* isolates harboured the class 1 or class 2 integron genes. The resistance genes identified in the isolates are shown in Table 3. The SM- and KM-resistant isolate (*S.* Infantis) harboured *aadA1*. Meanwhile, *tet*(B) was identified in the two TC-resistant isolates (*S.* Infantis and *S.* Schwarzengrund). *floR* was detected in the CP-resistant isolate (*S.* Typhimurium). *dfrA12* was detected in the TMP-resistant isolate (*S.* Infantis). The CPFX- and NA-resistant isolate (*S.* Typhimurium) did not harbour any other plasmid-mediated quinolone resistance genes, but a mutation corresponding to the amino acid substitution Ser-83 →Phe was identified in the quinolone resistance-determining regions of *gyrA* in the *S.* Typhimurium isolate.

TABLE 2 Minimum inhibitory concentration (MIC) values of *Salmonella* isolates from raw meat-based diets (RMBDs) for dogs

Product No.	Animal materials	Serotype	MIC (measured range; MIC interpretive criteria as resistant) (μg/mL)								
			ABPC (1–128:32)	CEZ (1–128:8)	CTX (0.5–64:4)	GM (0.8–64:16)	KM (1–128:64)	TC (0.5–64:16)	NA (1–128:32)	CPFX (0.03–4:1)	CP (1–128:32)	TMP (0.25–16:16)
1	Horse	Infantis	≤1	≤1	≤0.5	≤0.5	4	2	4	0.06	4	2
2	Miscellaneous	Infantis	2	2	≤0.5	≤0.5	2	2	4	0.06	2	2
3	Kangaroo	Untypable	≤1	2	≤0.5	≤0.5	4	2	8	0.06	4	0.5
4	Deer	Typhimurium	≤1	≤1	≤0.5	≤0.5	4	2	>128	4	>128	0.25
5	Chicken	Untypable	≤1	≤1	≤0.5	2	4	2	4	≤0.03	8	≤0.25
6	Chicken	Infantis	≤1	2	≤0.5	≤0.5	>128	64	8	0.06	2	>16
7	Chicken	Schwarzengrund	≤1	2	≤0.5	≤0.5	2	>64	4	0.06	4	≤0.25

ABPC, ampicillin; CEZ, cefazolin; CTX, cefotaxime; GM, gentamycin; KM, kanamycin; TC, tetracycline; NA, nalidixic acid; CPFX, ciprofloxacin; CP, chloramphenicol; TMP, trimethoprim.

TABLE 3 Summary of resistance profiles of *Salmonella* isolates from raw meat-based diets (RMBDs) for dogs

Product No.	Animal material	Serotype	Resistance phenotype	Resistance genes
1	Horse	Infantis		
2	Miscellaneous	Infantis		
3	Kangaroo	Untypable		
4	Deer	Typhimurium	NA, CPFX, CP	*floR*, *gyrA* (Ser-83→Phe)
5	Chicken	Untypable		
6	Chicken	Infantis	SM, KM, TC, TMP	*aadA1*, *tetB*, *dfrA12*
7	Chicken	Schwarzengrund	TC	*tetB*

CPFX, ciprofloxacin; CP, chloramphenicol; KM, kanamycin; NA, nalidixic acid; SM, streptomycin; TC, tetracycline; TMP, trimethoprim.

4 | DISCUSSION

RMBDs are a potential source of pathogenic bacteria. In the current study, we analysed the prevalence of *Salmonella* in RMBDs for dogs sold in Japan. Among them, 12% (7/60) of products were contaminated by *Salmonella* and some strains displayed anti-microbial resistance. Contamination of animal material was considered one of the possible sources of *Salmonella* contamination in the tested products. In Japan, *S.* Typhimurium was previously detected in deer (Sato, Kobayashi et al., 2000), while *S.* Infantis and *S.* Schwarzengrund have been detected in chickens (Duc et al., 2019; Ishihara et al., 2020; Noda et al., 2015). Additionally, although uncommon, *S.* Infantis can infect horses (Soza-Ossandón et al., 2020; Tillotson et al., 1997; Van Duijkeren et al., 1995). Possible contamination of the factory line is likely, but further investigation is needed to ascertain the source of contamination.

Although the sample size was small, the *Salmonella* contamination rate in this survey was similar to previously reported findings. Specifically, studies from the United States and Canada have reported *Salmonella* in 5–21% of RMBD samples (Finley et al., 2008; Lenz et al., 2009; Nemser et al., 2014; Strohmeyer et al., 2006; Weese et al., 2005). In Europe, Hellgren et al. (2019) identified *Salmonella* in 7% (4/60) of RMBD samples for dogs in Sweden, while Van Bree et al. (2018) identified *Salmonella* in 20% (7/35) of RMBD samples for dogs and cats in the Netherlands. Further, Bottari et al. (2020) identified *Salmonella* in 71% (15/21) of RMBD samples for pets in Italy. These findings contradict those of dry, semi-moist and canned pet foods, which are rarely contaminated with pathogens (Nemser et al., 2014; Strohmeyer et al., 2006; Wojdat et al., 2004).

Particularly concerning, some of the *Salmonella* isolates obtained in the current study were resistant or multi-resistant to various anti-microbials. Increasing incidence of multi-drug-resistant *Salmonella* has been widely reported and is generally attributed to the extensive use of anti-microbial agents in human and veterinary medicine (Fluit, 2005; Foley & Lynne, 2008; Threlfall et al., 1993). Among the anti-microbial-resistant isolates in the current study, we detected anti-microbial resistance genes *aadA1*, *dfrA12*, *floR* and *tet*(B), which have been previously identified in *Salmonella* isolated from animals in Japan (Ahmed et al., 2009; Asai et al., 2007; Matayoshi et al., 2015). An American study reported that *Salmonella* isolates from dog treats harboured class 1 integron genes (White et al., 2003). Similarly, Pitout et al. (2003) reported that *Salmonella* isolates from dog treats expressed CMY-2, a type of beta-lactamase. Further, *Salmonella* strains isolated from RMBDs were reportedly resistant to up to seven anti-microbials (Finley et al., 2008). Although none of the isolates in the current study displayed resistance to CTX, a CTX-resistant *Salmonella* strain was previously detected in meat sold for human consumption in Japan (Furukawa et al., 2017; Noda et al., 2015). Moreover, one *Salmonella* isolate in the current study displayed resistance to CPFX, a fluoroquinolone that is considered critically important in human medicine to treat enteric diseases and septicaemia. Plasmid-mediated quinolone resistance genes were not detected in the isolates in the current study, although Ahmed et al. (2009) reported a plasmid-mediated quinolone resistance gene in *Salmonella* recovered from animals in Japan.

In light of previous reports, veterinary and public health organizations, including the CDC and the World Small Animal Veterinary Association (WSAVA), have published statements discouraging the use of RMBDs for dogs (WSAVA, 2020). Further, a *Salmonella* surveillance program in animal feed was established in the United States in 2002 (Li et al., 2012). In Japan, the Law for Ensuring the Safety of Pet Food came into effect in 2009, and the Ministry of Agriculture, Forestry, and Fisheries published a manufacturing manual for pet food in 2014 that includes RMBD standards for dogs. Nevertheless, the Japanese government has not conducted a *Salmonella* surveillance program in RMBDs. The study findings demonstrate that *Salmonella* contamination is present in RMBDs for dogs, supporting the implementation of stronger public measures to counteract the possible associated health threats. Considering possible pet and owner exposure and cross-contamination with human food, the study findings indicate that RMBDs for dogs in Japan should be routinely screened for *Salmonella*. Moreover, the size of the market for RMBD treats in Japan is unknown. Hence, efforts should be made to determine the distribution volume and sales of RMBDs in the country.

5 | CONCLUSIONS

In conclusion, our study findings demonstrate that some RMBDs for dogs sold in Japan are contaminated with *Salmonella* including

anti-microbial-resistant strains. *Salmonella* was detected in 12% (7/60) products purchased over a 4-month period. Among the five serotyped isolates of *S. enterica* subsp. *enterica*, three were identified as *S.* Infantis, one as *S.* Typhimurium, and one as *S.* Schwarzengrund. Since outbreaks of *Salmonella* in humans have been linked to RMBDs for dogs, exceptional care should be taken when handling RMBDs. Future research should include a larger number of samples collected across a wider geographic area over a longer time period.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Shoichiro Yukawa: Conceptualization, Investigation, Writing-original draft, Writing-review & editing—Ikuo Uchida: Formal analysis, Supervision, Writing-review & editing—Hiroshi Takemitsu: Formal analysis, Software, Writing-review & editing—Asako Okamoto: Investigation, Methodology, Writing-review & editing—Motomi Yukawa: Investigation, Resources, Writing-review & editing—Seinosuke Ohshima: Conceptualization, Validation, Writing-review & editing —Yutaka Tamura: Conceptualization, Formal analysis, Writing-review & editing.

ETHICS STATEMENT

This study was not conducted on human subjects. And this study was not conducted on animals.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.739

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