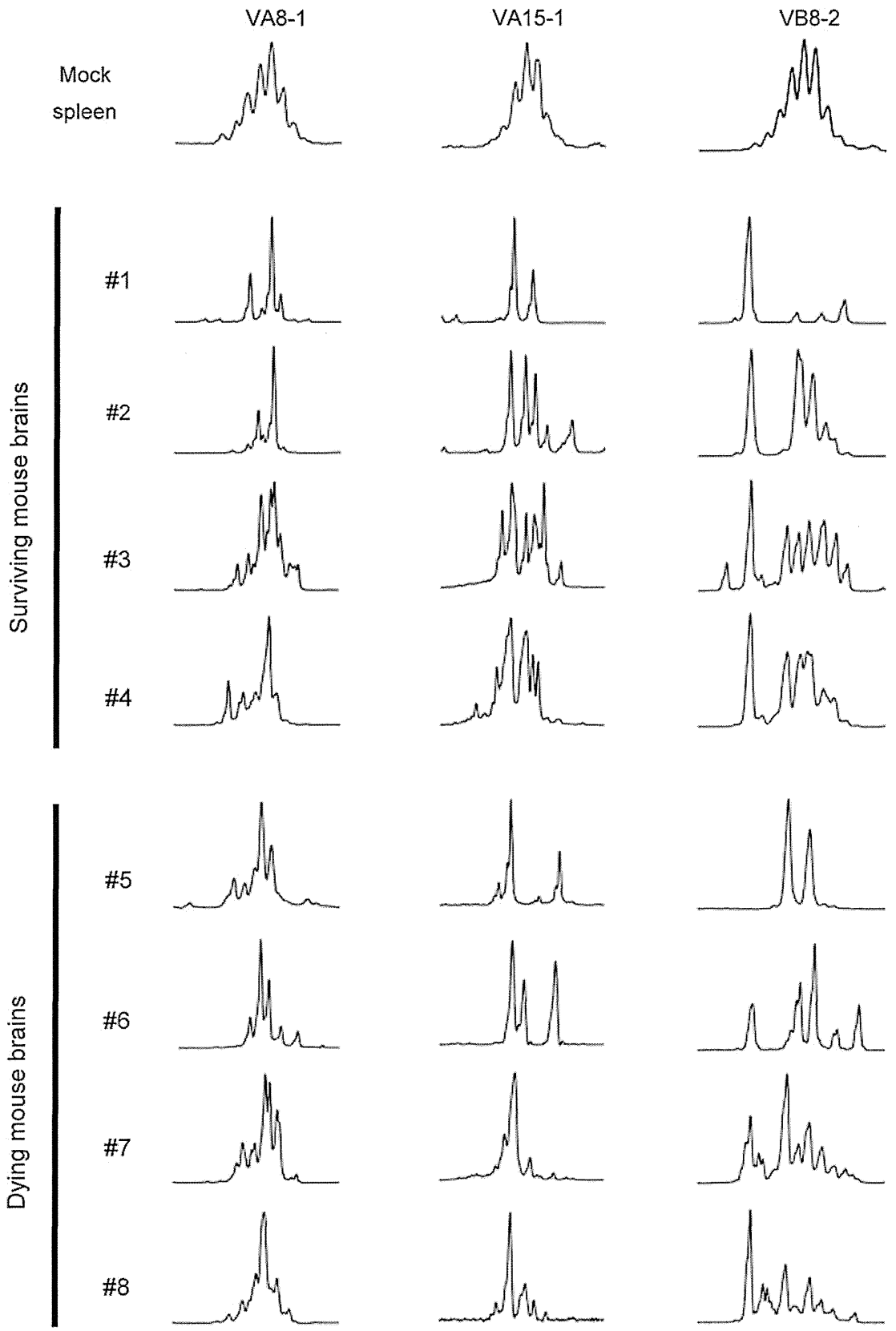

FIG. 3. CDR3 size spectratyping revealed different T-cell clonality patterns between surviving and dying mice. T-cell clonality for VA8-1, VA15-1, and VB8-2 families in individual mouse brains are shown. Peak numbers indicate the size variation of CDR3. Nucleotide size becomes longer at the right side. Generally, multiple peaks indicate the existence of polyclonal T cells, and one or a few peaks indicate the existence of monoclonal and oligoclonal T cells, respectively. Mock-infected spleen was used as a control to indicate polyclonal pattern.

A

AV8-1 (surviving mice)

mouse	freq.	CDR3 V	N	J	J gene	
#1	3/12	CALS	DLVT	NTGKLTF	AJ27	[§]
	3/12	CALR	AHY	NQGKLIF	AJ23	†
	3/12	CALT	PNY	NQGKLIF	AJ23	[‡]
	1/12	CALS	PNY	NQGKLIF	AJ23	[†]
	1/12	CALR	PNY	NQGKLIF	AJ23	†
	1/12	CALG	PNY	NQGKLIF	AJ23	†
#2	8/11	CALS	HYQ	GGRALIF	AJ15	[*]
	3/11	CAL	KGS	SGNKLIF	AJ32	
#3	2/15	CALS	LAY	NQGKLIF	AJ23	†
	2/15	CALS	ASSS	GSWQLIF	AJ22	
	2/15	CALR	GQ	GGRALIF	AJ15	*
	2/15	CAL	KGY	NQGKLIF	AJ23	†
	1/15	CAC	KGY	NQGKLIF	AJ23	†
	1/15	CALS	EGY	NQGKLIF	AJ23	†
	1/15	CALS	PNY	NQGKLIF	AJ23	[†]
	1/15	CALT	PNY	NQGKLIF	AJ23	[‡]
	1/15	CALS	DLVT	NTGKLTF	AJ27	[§]
	1/15	CALS	GTSG	GNYKPTF	AJ6	
	1/15	CAL	IRGS	ALGRLHF	AJ18	
#4	6/13	CALS	HYQ	GGRALIF	AJ15	[*]
	3/13	CAL	W	MGYKLTF	AJ9	
	1/13	CALS	EPS	GSWQLIF	AJ22	
	1/13	CALS	VTG	SGGKLTF	AJ44	
	1/13	CAL	AGY	NQGKLIF	AJ23	†
	1/13	CA	ST	GYQNFYF	AJ49	

AV8-1 (dying mice)

mouse	freq.	CDR3 V	N	J	J gene	
#5	5/11	CA	RN	SNNRIFF	AJ31	
	3/11	CALS	PNY	NQGKLIF	AJ23	[†]
	2/11	CALS	LN	YAQGLTF	AJ26	
	1/11	CA	STTA	SLGKLQF	AJ24	
#6	5/13	CALS	EGG	SNAKLTF	AJ42	
	5/13	CAL	YTE	GADRLTF	AJ45	
	2/13	CALS	GY	NQGKLIF	AJ23	†
	1/13	CALS	DHSYQ	GGRALIF	AJ15	
#7	3/15	CALS	DGG	TGSKLSF	AJ58	
	2/15	CALR	P	GYQNFYF	AJ49	
	2/15	CALS	PNY	NQGKLIF	AJ23	[†]
	1/15	CALS	QNY	NQGKLIF	AJ23	†
	1/15	CALS	Y	NQGKLIF	AJ23	†
	1/15	CALS	DGT	GGYKVVF	AJ12	
	1/15	CALS	GT	GGYKVVF	AJ12	
	1/15	CALS	VG	SNYQLIW	AJ33	
	1/15	CALR	LTSG	GNYKPTF	AJ6	
	1/15	CALS	DQTA	SLGKLQF	AJ24	
	1/15	CALR	SSYQ	GGRALIF	AJ15	
#8	3/12	CALS	ERN	YAQGLTF	AJ26	
	2/12	CALS	EGG	MGYKLTF	AJ9	
	2/12	CALR	RSP	GYQNFYF	AJ49	
	1/12	CAL	VNY	NQGKLIF	AJ23	†
	1/12	CAL	NTGG	LSGKLTF	AJ2	
	1/12	CALR	GS	AGNKLTF	AJ17	
	1/12	CA	ATS	SGQKLVF	AJ16	
	1/12	CA	WN	SNNRIFF	AJ31	

B

VA15-1 (surviving mice)

mouse	freq.	CDR3 V	N	J	J gene	
#1	4/15	CAAS	MDY	NQGKLIF	AJ23	
	11/15	CAAG	N	AGAKLTF	AJ39	
#2	5/14	CAAS	TGA	NTGKLTF	AJ52	
	3/14	CAAK	EA	SNYQLIW	AJ33	[§]
	3/14	CAAS	IG	SNYQLIW	AJ33	
	2/14	CAAS	T	GYQNFYF	AJ49	
	1/14	CAAS	IWGA	NTGKLTF	AJ52	
#3	6/15	CAPG	GATA	SLGKLQF	AJ24	
	4/15	CAAT	N	YAQGLTF	AJ26	
	1/15	CAAS	WAS	GSWQLIF	AJ22	¶
	1/15	CAAS	WSS	GSWQLIF	AJ22	¶
	1/15	CAAS	TSS	GSWQLIF	AJ22	[¶]
	1/15	CAAS	S	NYNVLYF	AJ21	
	1/15	CAAG	T	GGYKVVF	AJ12	*
#4	10/14	CAAR	D	MGYKLTF	AJ9	[†]
	1/14	CAAS	P	MGYKLTF	AJ9	[‡]
	2/14	CAAK	EA	SNYQLIW	AJ33	[§]
	1/14	CAAS	TSS	GSWQLIF	AJ22	[¶]

VA15-1 (dying mice)

mouse	freq.	CDR3 V	N	J	J gene	
#5	11/13	CAAR	T	GGYKVVF	AJ12	[×]
	2/13	CAAS	GA	GGYKVVF	AJ12	[*]
#6	5/10	CAAS	GA	GGYKVVF	AJ12	[*]
	5/10	CAAS	TSS	GSWQLIF	AJ22	[¶]
#7	5/13	CAAS	GA	GGYKVVF	AJ12	[*]
	4/13	CAAS	TA	GGYKVVF	AJ12	*
	3/13	CAAS	KA	GGYKVVF	AJ12	*
	1/13	CAAR	T	GGYKVVF	AJ12	[×]
#8	7/13	CAAR	D	MGYKLTF	AJ9	[†]
	2/13	CAAS	GA	GGYKVVF	AJ12	[*]
	1/13	CAAS	DGT	GGYKVVF	AJ12	*
	1/13	CAAS	P	MGYKLTF	AJ9	[‡]
	1/13	CAAS	TN	SGTYQRF	AJ13	
	1/13	CAAT	PPSS	GSWQLIF	AJ22	¶

FIG. 4. Amino acid sequences of TCR CDR3 regions of cDNA clones derived from TBEV-infected mouse brains. For the VA8-1 (**A**), VA15-1 (**B**) or VB8-2 (**C**) families, predicted amino acid sequences are shown with their frequencies of cDNA clones in each individual mouse. J gene usage is shown at the right side of each sequence. V, N (N-D-N), and J gene segments are not strictly divided. Each symbol indicates a group of identical or similar sequences, and brackets indicate an identical sequence among individuals.

C

VB8-2 (surviving mice)

mouse	freq.	CDR3 V	CDR3 N-D-N	CDR3 J	J gene	
#1	8/17	CASGD		EQYF	BJ2.7	[*]
	4/17	CASSD	APPNTG	QLYF	BJ2.2	
	3/17	CASGD	LGGRGNYA	EQFF	BJ2.1	[§]
	1/17	CASRA	PWGEGTG	QLYF	BJ2.2	
	1/17	CASGD	ASFY	EQYF	BJ2.7	
#2	4/15	CASGD	ATGY	EQYF	BJ2.7	[¶]
	3/15	CASGE	LGAY	EQYF	BJ2.7	
	3/15	CASGD	MGQKD	TQYF	BJ2.5	[£]
	2/15	CASGD	AQLSY	EQYF	BJ2.7	
	2/15	CASGD		EQYF	BJ2.7	[*]
	1/15	CASGD	AGDRGV	EVFF	BJ1.1	
#3	2/10	CASGD	LGGRGNYA	EQFF	BJ2.1	[§]
	1/10	CASGD	LWGALNQD	TQYF	BJ2.5	
	1/10	CASGV	PGQGTTG	QLYF	BJ2.2	
	1/10	CASSG	QGAGNQ	APLF	BJ1.5	
	1/10	CASSD	ATISNE	RLFF	BJ1.4	
	1/10	CASGG	TGVAE	TLYF	BJ2.3	
	1/10	CASGE	GGVGN	TLYF	BJ1.3	
	1/10	CASGS	R	EQYF	BJ2.7	*
	1/10	CASGD		EQYF	BJ2.7	[*]
#4	2/10	CASGD	VRGDS	DYTF	BJ1.2	
	1/10	CASGD	MGQKD	TQYF	BJ2.5	[£]
	1/10	CASGK	LANQD	TQYF	BJ2.5	
	1/10	CANSE	WGADHD	TQYF	BJ2.5	
	1/10	CARGD	AGGSAE	TLYF	BJ2.3	
	1/10	CASKS	IQD	TQYF	BJ2.5	
	1/10	CASGD	ATGY	EQYF	BJ2.7	[¶]
	1/10	CASGD	G	GEYF	BJ2.7	*
	1/10	CASGD		EQYF	BJ2.7	[*]

VB8-2 (dying mice)

mouse	freq.	CDR3 V	CDR3 N-D-N	CDR3 J	J gene	
#5	5/14	CASGT	PGGNT	EVFF	BJ1.1	
	3/14	CASGD	IRV	EVFF	BJ1.1	[†]
	3/14	CASGD	VRV	EVFF	BJ1.1	†
	2/14	CASGD	AWGKD	TQYF	BJ2.5	
	1/14	CASSD	APTSAE	TLYF	BJ2.3	
#6	8/13	CASGD	ATAIT	EVFF	BJ1.1	
	1/13	CASGG	DWGANTG	QLYF	BJ2.2	
	1/13	CASSD	LGVNYA	EQFF	BJ2.1	
	1/13	CASGD	NRV	EVFF	BJ1.1	[‡]
	1/13	CASGD		TQYF	BJ2.5	*
	1/13	CASGD		EQYF	BJ2.7	[*]
#7	2/13	CASGD	AGTG	EQYF	BJ2.7	
	2/13	CASGA	G	LGIF	BJ2.7	*
	1/13	CASGD	IRV	EVFF	BJ1.1	[†]
	1/13	CASGA	GST	EVFF	BJ1.1	†
	1/13	CASGG	GQKNS	DYTF	BJ1.2	
	1/13	CASGE	VGGRSA	EQFF	BJ2.1	
	1/13	CASGD	ALV	EQYF	BJ2.7	
	1/13	CASSD	AGW	EQYF	BJ2.7	
	1/13	CASGE		SEYF	BJ2.7	*
	1/13	CASGD		GPYF	BJ2.7	*
	1/13	CASGA		LEYF	BJ2.7	*
#8	3/15	CASGV	PY	EQYF	BJ2.7	
	2/15	CASGD		EQYF	BJ2.7	[*]
	1/15	CASGD	ARGNQD	TQYF	BJ2.5	
	1/15	CASGD	NRV	EVFF	BJ1.1	[‡]
	1/15	CASGE	AW	GGAF	BJ2.5	
	1/15	CASGD	AGHSY	EQYF	BJ2.7	
	1/15	CASGD	LGAD	EQYF	BJ2.7	
	1/15	CASGD	G	GEYF	BJ2.7	*
	1/15	CASGD		FQYF	BJ2.7	*
	1/15	CASGD		TLYF	BJ1.3	
	1/15	CASGD		EVFF	BJ1.1	

FIG. 4. (Continued).

reagent kit (Takara Bio Inc., Shiga, Japan), and the PCR reaction was performed using SYBR® Premix Taq™ (Takara Bio) for SYBR Green I according to the manufacturer's instructions. The expression level of each gene was measured by qPCR as demonstrated previously (10). Expression levels were normalized based on the housekeeping gene GAPDH copy number. Relative quantification was expressed as a ratio between TBEV-infected and mock-infected brains.

Viral RNA levels of TBEV were examined with NS1-specific primers (forward: 5′-CGGCTAGCCACACTATCGACAA-3′, reverse: 5′-GGCGAGTACTTCCATGGTCCTT-3′). Reverse transcription and PCR reactions were conducted as described above. Viral RNA was quantified as copy number per 1 ng of total RNA. Copy number in each sample was determined on the basis of a standard curve.

Statistical analysis

Student's *t*-test was used for statistical analysis to assess significant differences in weight change ratio, the degree of the relative expression in TCR repertoire analysis, and the expression levels of genes in qPCR analysis. The Mann-Whitney *U* test was used to evaluate frequencies of CDR3 sequences. A p value <0.05 was determined to be statistically significant.

Results

Discrimination of surviving and dying mice

Thirteen C57BL/6 mice were subcutaneously inoculated with 10^3 PFU of TBEV and weighed daily (Fig. 1A). Four mice from each of the surviving and dying groups were used in the experiments. Dying mice were defined as those exhibiting more than 25% weight loss at 13 dpi, and surviving mice were defined as those with less than 10% weight change. Mock-infected mice (n = 4) exhibited negligible weight change (data not shown). Weight percentage at 13 dpi, compared with that on day 0, was significantly different between the surviving and dying mouse groups (Fig. 1B). However, the amounts of viral RNA in the brains at the 13 dpi were no different between these two groups of mice (Fig. 1C). These data indicate that the virus propagation level is not the main factor that determines the fatality of TBEV-infected mice. ◀F1

TCR repertoire analysis

TCRAV and TCRBV repertoires were analyzed using brains and spleens collected from TBEV-infected dying mice, surviving mice, or mock-infected mice at 13 dpi (Fig. 2). Using TCR repertoire analysis, significant expressions of TCRAV and TCRBV were detected in TBEV-infected brains ◀F2

both in surviving and dying mice. Frequencies of T cells bearing VA8-1, VA15-1, and VB8-2 were significantly increased in brains compared with mock-infected spleens. However, there was no significant difference between surviving and dying mice. VA14-1, known as the family expressed on NKT cells (3), was expressed at very low levels in virus-infected brains. No difference between mock-infected and TBEV-infected mouse spleens was observed for both TCRAV and TCRBV repertoires, and this suggests that systemic T-cell response changes were below the detectable level in our analysis. In mock-infected mouse brains, the expression of TCRAV and TCRBV was not detected (data not shown) due to the low lymphocyte numbers.

CDR3 size spectratyping

We performed CDR3 size spectratyping analysis to confirm the clonalities of T cells expressing VA8-1, VA15-1, and VB8-2 families in TBEV-infected mouse brains (Fig. 3). Different patterns were observed between TBEV-infected brains and mock spleens for all three families. Furthermore, VA15-1 clonalities for dying mice were higher than those for surviving mice. A short VB8-2 size peak was also observed in surviving mice.

Amino acid sequences of CDR3

Because differences in T-cell clonality were observed between surviving and dying mice, nucleotide sequences of the CDR3 were determined for the above-mentioned TCR families, using PCR-amplified and randomly-selected cDNA clones. Predicted amino acid sequences are shown along with the frequencies of cDNA clones derived from the brains of individual mice infected with TBEV (Fig. 4). Cysteine (C) at the N-terminal portion and phenylalanine (F) or tryptophan (W) at the C-terminal portion are not contained within CDR3. Despite the fact that we analyzed more than 30 clones in mock-infected spleens, we did not find any clones with identical sequence (data not shown). Meanwhile, in TBEV-infected brains, many clones with identical sequences were found. VA8-1 (Fig. 4A) surviving mice were divided into two groups: individuals with high frequency of AJ23 gene usage (#1 and #3) or AJ15 gene usage (#2 and #4). Although dying mice also produced some clones bearing AJ23 or AJ15, the frequency of these clones was lower than in surviving mice. For VA15-1 (Fig. 4B), a high frequency of AJ12 gene usage was observed in dying mice. In addition, clones with identical amino acid sequences to CDR3 (CAAS GA GGYKVVF) were detected in all four dying mice. Identical clones bearing AJ9 and AJ22 were detected in both surviving and dying mice. For VB8-2 (Fig. 4C), a high frequency of BJ2.7 genes with short CDR3 consisting of seven amino acids (aa) was characteristic for both surviving and dying mice. Clones with such a short CDR3 were rare in TCR β chains. Some clones bearing BJ1.1 with 10 aa CDR3 (CASGD XRV EVFF, X = I, V or N) were found in every dying mouse. As shown for VA8-1, VB8-2 surviving mice were also divided into two groups: individuals (#1 and #3) that obtained clones with BJ2.1 (CASGD LGGRGNYA EQFF), or individuals (#2 and #4) that obtained clones with BJ2.7 (CASGD ATGY EQYF) and BJ2.5 (CASGD MGQKD TQYF). The frequencies of these three clones were not high in each individual, but were specific for each group. In mouse #5, there was no clone bearing BJ2.7 or short CDR3, therefore the frequency of these clones might be high but not necessary in TBEV-infected mice. As illustrated by mice #7 and #8, a high single peak in CDR3 size spectratyping analysis does not necessarily indicate monoclonal expansion. Clones were also sometimes included that were the same size as CDR3, yet had different sequences. However, several reports have shown that the β-chain J region of CDR3 does not specifically interact with antigen peptides (7,11,13,14,43). The sequence of the N-terminal half of CDR3 (V segment, D segment, and N addition) rather than J gene usage might therefore be important, as potentially each clone with a short CDR3 recognizes an identical antigen peptide. To organize sequence data, clone frequencies with more than 10% J gene usage in the total number of clones analyzed for each V family were individually plotted in Fig. 5. CDR3 length was considered for the β chain. Using the Mann-Whitney *U* test, the frequency of VA8-1/AJ15 was significantly higher in surviving mice, and the frequencies of VA15-1/AJ12 and VB8-2/BJ1.1 (10 aa) were significantly higher in dying mice. This suggests that these T-cell clones might be closely associated with disease severity in TBEV-infected mice.

Levels of cell surface markers and cytokines determined by qPCR

Differential patterns of T-cell clones can indicate that brain-infiltrating cells may play different roles between surviving and dying mice. To investigate the active state of the infiltrating cells, we studied the expression levels of T-cell antigen markers, lymphocyte activation markers, and apoptosis-related genes: CD3, CD4, CD8, and CD25 (expressed on activated T cells and B cells) (28), CD69 (expressed rapidly after lymphocyte activation) (47), and Gzm A, Gzm B, perforin, and FasL using qPCR analysis (Fig. 6). The resulting expression levels for all genes in TBEV-infected brains studied were significantly increased when compared with mock infection. This was especially the case for the expression of CD69, Gzm A, and Gzm B, which were increased more than 100 times. No significant differences were observed between mouse groups, indicating that equivalent $CD8^+$ T-cell infiltration into TBEV-infected brains occurs in surviving and dying mice with similar activation state levels.

Discussion

Understanding the clinical variability caused by encephalitic flavivirus infection is important in explaining differences between severe and subclinical human cases. It may also further elucidate the mechanism of pathogenesis for viral encephalitis. Previous reports showed that the TBEV Oshima strain elicited dose-independent mortality following peripheral infection in some mouse strains (6,21). We therefore distinguished surviving and dying mice by their degree of weight loss after TBEV infection according to our previous study (21), as a simple and effective method to evaluate the severity.

Based on the amount of viral RNA in brain tissue, we found no difference in virus replication between surviving and dying mice. This result suggests that direct virus-induced neuronal injury cannot completely explain the

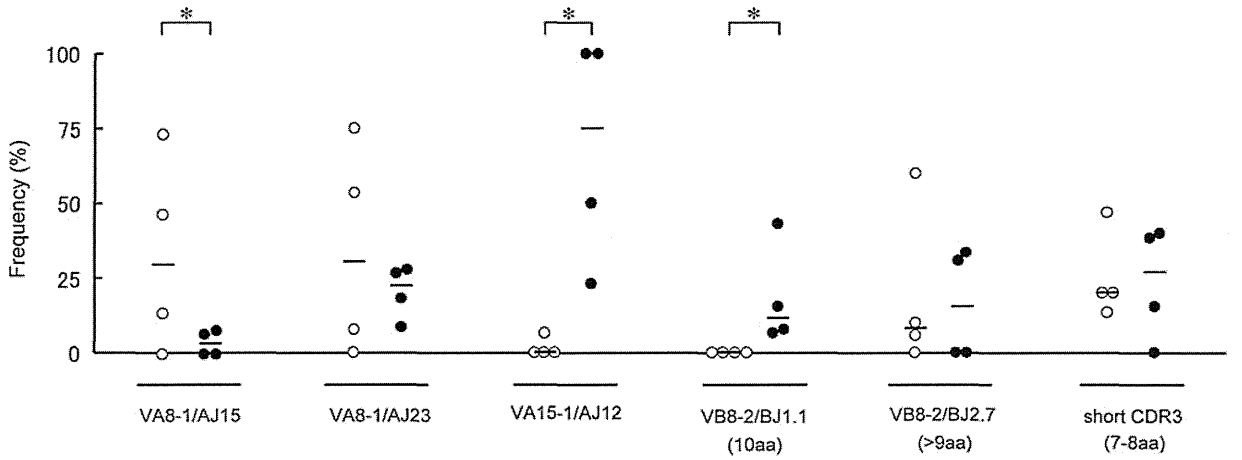

FIG. 5. Differential patterns of TCR gene usage between surviving and dying mouse brains. Frequencies for characteristic combinations of V and J gene usages were individually plotted. CDR3 length was taken into consideration for β chain: BJ2.7 (long) indicates CDR3 consisted of more than 9 aa, and short CDR3 indicates CDR3 consisted of 7 or 8 aa. Open circles indicate surviving mice, and closed circles indicate dying mice. Bars indicate the median for each group. Asterisks denote statistically significant ($p < 0.05$) differences between surviving and dying mice using the Mann-Whitney U test.

severity of TBEV infection. In our previous study (21), large numbers of $CD8^+$ T cells infiltrated the brains of TBEV-infected mice, yet no significant difference was observed between surviving and dying mice. We therefore investigated the individual characteristics of brain-infiltrating T cells in this study.

TCR repertoire analysis revealed that the frequencies of T cells bearing VA8-1, VA15-1, and VB8-2 were significantly increased in TBEV-infected mouse brains compared with those of spleens. However, there was no significant difference between surviving and dying mice. This indicated that once a certain amount of virus was inoculated, T cells with selected TCR V families accumulate in the brain regardless of disease severity. In contrast, clonality results and CDR3 sequencing analysis indicated a distinct difference between mouse groups, with frequencies of VA15-1/AJ12 and VB8-2/BJ1.1 gene usage higher in dying mice. One clone with an identical CDR3 sequence (CAAS GA GGYKVVF) was detected in all four dying mice, strongly suggesting that this clone was associated with severe encephalitis. High frequencies of VA8-1/AJ15 gene usage were characteristic of surviving mice, while high frequencies of VB8-2/BJ2.7 gene usage and clones with short CDR3 were observed in both surviving and dying mice. We therefore observed a unique increase of T-cell clones for each mouse group. Specific clones were found only in dying mice, while other clones were frequently found in surviving mice or were commonly found in every infected mouse. Thus, there are several T-cell clones that may be associated with the severity of TBEV infection, while the remaining clones may be different in

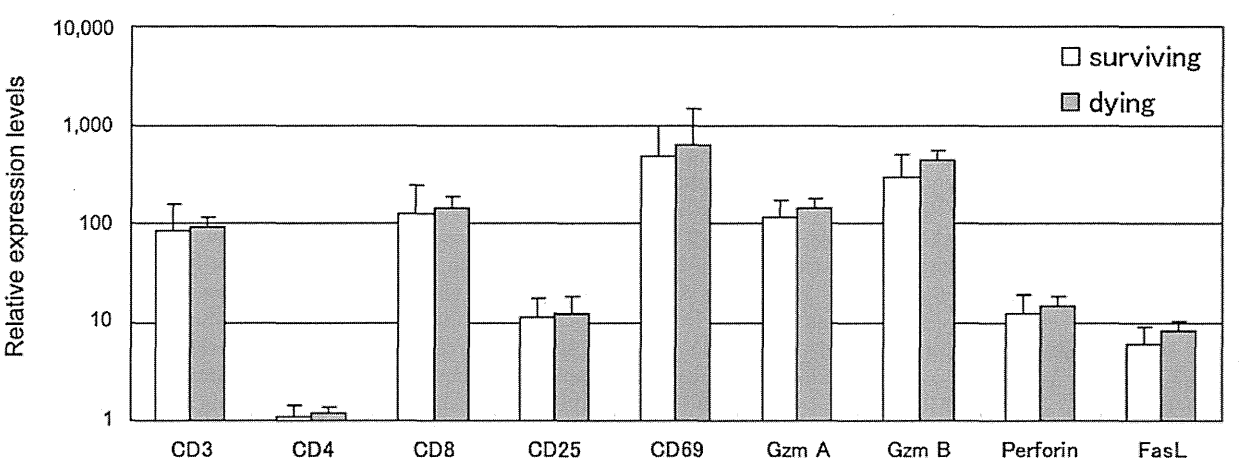

FIG. 6. Quantification of mRNA expression of T-cell-related antigens, activation markers, and apoptosis-related genes in brains using qPCR. Sample RNAs were extracted from brains of mock-infected and TBEV-infected surviving and dying mice at 13 dpi (n = 4). The mRNA expression levels in TBEV-infected brains were normalized by GAPDH expression and are shown as the relative expression levels compared with mock-infected brains. Vertical error bars indicate the standard deviation of three independent experiments.

each mouse. T cells associated with surviving mice may also be variable and were divided into two groups based on CDR3 sequence patterns for VA8-1 and VB8-2. Thus, specific mechanisms to overcome TBEV infection may exist. It is therefore further worthwhile to investigate the roles of the distinct clones identified in our study, especially clones with VA15-1/AJ12, VB8-2/BJ2.1 (10 aa), and VA8-1/AJ15, that exhibited different patterns between surviving and dying mice.

The large sequence variation observed in our data can be explained by multiple reasons. First, TCR-peptide MHC (pMHC) recognition is flexible (1), and different TCRs with similar binding capabilities can recognize identical antigen peptides. Such flexibility may therefore produce variations in induced T-cell clones in TBEV-infected brains. Another possible reason relates to the presence of quasispecies. Noncloned RNA viruses generally exist as a quasispecies (22,25), and it has been reported that the virus stock used in our study was a complex of quasispecies (19). Consequently, the different amino acids for the antigen peptides among the quasispecies might produce T-cell clone variations in TBEV-infected brains.

We must also consider why different characteristics between surviving and dying mice were observed despite of an absence of differences between these groups in TCR repertoire analysis. Common V gene usage means that the TCR could recognize identical or similar antigen peptides (12,33). However, as described above, TCR-pMHC recognition can allow for some cross-reactivity; for example, different TCRs can bind to the same pMHC, and a single TCR can bind to a different pMHC (29,46). Therefore, TBEV infection can induce multiple T-cell clones with certain V genes that recognize specific antigens, yet only a few may play a critical role.

Different roles may also exist for the distinct T-cell clonotypes found in surviving and dying mice. However, qPCR analysis failed to identify any difference in T-cell function-associated markers between these groups, and apoptosis-associated genes were almost equivalently expressed in both surviving and dying mice. As this was the result for total brain expression, further studies of each T-cell clonotype are needed. We also need to investigate if the difference in target antigens recognized by T cells has an impact on severity and fatality rather than activation level. Or perhaps the difference in T-cell clonotype resulted from encephalitis progression and requires further investigation.

We must exercise care, because virulent and host immune responses can be very different in experimental models, depending upon the subtype and strain of virus, the mouse strain, and so on. In addition, because our data are based on analysis at 13 dpi only, we may need to investigate immune responses at earlier time points post-infection. We previously reported increased levels of serum corticosterone in dying mice (21). Glucocorticoids are known to exert immunomodulatory effects by activating the hypothalamic-pituitary-adrenal axis and/or cytokine expression (2,31). Thus, the relationship between T-cell clone bias and the level of corticosterone is interesting and warrants further investigation.

In conclusion, we have revealed an association between brain-infiltrating T-cell clones and severity in TBEV-infected mice, although the cause of this relationship is still unclear. Specifically, as the brain is originally free from adaptive immunity, it is unknown whether particular T-cell accumulation determines disease severity, or if a certain type of disorder induces particular T-cell accumulation by changing antigen presentation patterns. Further experiments are needed to elucidate which factor causes the difference in induced T-cell clones. Although our results are complicated, we believe our data are an initial step in better understanding of the mechanisms of viral encephalitis.

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Author Disclosure Statement

No competing financial interests exist.

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FULL PAPER *Public Health*

Bartonella Species in Wild Rodents and Fleas from Them in Japan

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ABSTRACT. The purpose of this study was to assess the role of fleas for transmission of *Bartonella* species among wild rodents in Japan. Flea samples were collected from wild rodents and examined genetically for *Bartonella* infection. *Bartonella* DNA was detected from 16 of 40 (40.0%) flea samples. Sequence analysis demonstrated that 3 of 16 (18.8%) of the *Bartonella*-positive animals were infested with fleas from which the closely related *Bartonella* DNA sequence was detected, indicating that the fleas acquired *Bartonella* from the infested rodents. The DNA was detected in hemolymph, the midgut and the ovary (only in female), indicating that *Bartonella* might be colonized through the midgut and distributed into the body.

KEY WORDS: *Bartonella*, flea, rodent, transmission, zoonosis.

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The genus *Bartonella* is comprised of aerobic, fastidious, gram-negative, slow-growing bacteria that can be isolated from the blood of many mammalian species. A variety of *Bartonella* species have been isolated from wild rodents. Several reports have indicated that *Bartonella* species are widely distributed at a high frequency among wild rodents around the world [4, 12–14, 28]. Notably, four rodent-associated *Bartonella* species, *B. elizabethae, B. grahamii, B. vinsonii* subsp. *arupensis* and *B. washoensis*, have been suspected to cause an endocarditis [9], neuroretinitis [16], bacteremia, fever and endocarditis [27] and cardiac disease [5, 18] in humans, respectively.

Bloodsucking arthropods are involved in the transmission of *Bartonella* species among their host animals. The sandfly, human body louse and cat flea play roles in the transmission of *B. bacilliformis* [15], *B. quintana* [24] and *B. henselae* [8], respectively. The DNA of *Bartonella* species has been detected in fleas on wild rodents, suggesting a potential role as a vector in the transmission of *Bartonella* species among animals [3]. Supporting this, experimental transmission of *B. grahamii* and *B. taylorii* by rodent fleas (*Ctenophthalmus nobilis*) has also been reported with a vole model [6]. Effective transmission of the organisms is thought to be essential to maintain the high prevalence of *Bartonella* among wild rodents; however, the mechanisms and participation of blood sucking arthropods have not been fully investigated.

In this study, fleas from wild rodents were assessed as a possible vector of *Bartonella* by detection and comparison of *Bartonella* DNA from the isolates of the host animals and fleas. Furthermore, we examined the distribution of the DNA in the flea organs and discussed the mechanisms of the transmission of *Bartonella* by fleas among wild rodents.

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MATERIALS AND METHODS

Wild rodents and Bartonella strains: Fifty-three wild rodents (Nos. 1–53) were captured using Sherman traps around Sapporo in Hokkaido Prefecture, Japan, in August 2005. *Bartonella* was isolated from 36 (67.9%) animals, and the species were identified as described in a previous study [12]. The prevalences of *Bartonella* species among the wild rodents used in this study are summarized in Table 1.

Flea samples and DNA extraction: A total of 40 rodent fleas (n=15 and n=25 for males and females, respectively) were collected from 27 of 53 rodents and kept in 70% ethanol for further investigations. The species of fleas were morphologically identified as *Ctenophthalmus congener truncus* (n=32), *Neopsylla sasai* (n=5) and *Hystrichopsylla microti* (n=3; Table 2). The lymph, midgut, and ovaries were carefully separated under a stereoscopic microscope with aseptic needles, and the DNA from the organs was extracted using a DNeasy tissue kit (Qiagen, Inc., Valencia, CA, U.S.A.) [7].

PCR of gltA and rpoB in flea samples: Nested PCR was applied for the detection of *rpoB* and *gltA* genes in flea samples, respectively. The primers used for the first amplification of the *rpoB* gene (966 bp) of bartonellae were 1350F (5'-GGCAATCGTCGCGTTCGTTC-3') and 2350R (5'-CTACCCGATCACCAACATGC-3'), and those for *gltA* (1,018 bp) were CS.139f (5'-TTTACTTATGATCCKG-GYTTTA-3') and CS.1162r (5'-AWTGCAAAAAG-WACAGTRAACA-3'). The first PCR was performed in a 20-μl mixture containing 20 *ng* of the extracted DNA, 20 μl of 2× Ampdirect Plus (Shimazu, Kyoto, Japan), 0.5 U Ex *Taq* Hot Start Version (Takara Bio Inc., Otsu, Shiga, Japan), and 1 pmol of each primer. Amplifications were performed under the following conditions: 1 cycle for 2 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 56 °C and 90 sec at 72

Table 1. Prevalence of *Bartonella* spp. among wild rodents used in this study

Rodent spp.	No. of animals	Percentage of culture-positive animals carrying			
		B. grahamii	*B. taylorii*	*B. japonica*	Total
Apodemus speciosus	31	23 (74.2)	0	6 (19.4)	29 (93.6)
Apodemus argenteus	5	1 (20.0)	0	2 (40.0)	3 (60.0)
Myodes rufocanus bedfordiae	17	0	4 (23.5)	0	4 (23.5)
Total	53	24 (45.3)	4 (7.5)	8 (15.1)	36 (67.9)

Table 2. Detection of *Bartonella* DNA from fleas found on wild rodents

Flea spp.[a)]	Rodent spp.[b)]	No. of fleas tested	Number (%) of fleas positive for the DNA of				
			B. grahamii	*B. taylorii*	*B. japonica*	Subtotal	Total
Cc truncus	As	25	3 (12.0)	8 (32.0)	0	11 (44.0)	14 (43.8)
	Mrb	7	0	3 (42.9)	0	3 (42.9)	
Ns	As	4	2 (50.0)	0	0	2 (50.0)	2 (40.0)
	Aa	1	0	0	0	0	
Hm	Mrb	3	0	0	0	0	0
Total		40	5 (12.5)	11 (27.5)	0	16 (40.0)	

a) Cc truncus: *Ctenophthalmus congener truncus*. Ns: *Neopsylla sasai*. Hm: *Hystrichopsylla microti*. b) As: *Apodemus speciosus*. Aa: *Apodemus argenteus*. Mrb: *Myodes rufocanus bedfordiae*.

Table 3. Detection of *Bartonella* DNA in tissues (midgut, hemolymph and ovary) of fleas

Flea ID[a)]	Sex	Detection of *Bartonella* DNA[b)] from		
		Midgut	Hemolymph	Ovary
8 Cc truncus	Female	+	+	–
12 Cc truncus	Male	+	+	NA
14 Cc truncus	Male	+	–	NA
18 Cc truncus	Male	+	–	NA
19 Cc truncus	Male	+	–	NA
22 Cc truncus	Male	+	+	NA
26 Cc truncus	Female	+	–	+
27 Cc truncus	Female	+	–	+
28 Cc truncus	Female	+	–	+
33 Cc truncus	Female	+	–	–
34 Cc truncus	Male	+	–	NA
36 Ns	Female	+	+	+
42 Cc truncus	Male	+	–	NA
43 Cc truncus	Female	+	–	–
45 Cc truncus	Female	+	–	+
47 Ns	Female	+	–	+
Total		16	4	6

a) Number of host rodents and flea spp. (Cc truncus: *Ctenophthalmus congener truncus*. Ns: *Neopsylla sasai*). b) The results of PCR targeting for *gltA* and *rpoB* are the same and are shown as "*Bartonella* DNA". +: PCR positive. –: PCR negative. NA: not applicable.

°C; and a final extension cycle for 2 min at 72°C for *rpoB*. For *gltA*, the conditions were 1 cycle for 3 min at 94°C; 45 cycles for 60 sec at 94°C, 60 sec at 56°C and 90 sec at 72 °C; and a final extension cycle for 10 min at 72°C. The second PCR was performed by using the same protocol used in previous studies [22, 26].

Ten microliters of each PCR product was run on a 2% agarose gel (Agarose S; Nippon Gene, Tokyo, Japan), and was visualized by staining with ethidium bromide on an UV transilluminator (Image Saver AE-6905C; ATTO, Tokyo, Japan). Each PCR included negative (distilled water) and positive (DNA extracted from *B. doshiae* R18^T) controls.

Sequencing and phylogenetic analysis of rpoB and gltA: The *rpoB* and *gltA* PCR products were sequenced with specific primers for *rpoB* (1400F and 2300R as described above, and 1600R, 5'-GGRCAAATACGACCATAATGSG-3', 2000R, 5'-CGYGGYRCCATRAAAACTTCWCC-3', and 2000F, 5'-GGWGAAGTTTTRATGGYRCCRCG-3') and *gltA* (BhCS.781p and BhCS.1137n as described above) using an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The sequences were aligned with those of type strains of *Bartonella* by using Clustal X. The MEGA 3.1 software was used for the phylogenetic analysis of the obtained sequences. The neighbor-joining (N-J) method by Kimura's 2-parameter distance method and bootstrap calculation were carried out for 1,000 resamplings.

Statistical analysis: The positive rates were analyzed by 2×2 tables, and the chi-square test was used to determine statistical significance. $P<0.05$ was considered to be significant.

RESULTS

Detection of Bartonella DNA from the organs of fleas from wild rodents: In total, 40.0% (16/40) of the fleas tested were positive for *Bartonella* DNA in either of the organs tested by PCR (Table 2). Of the flea species found, 43.8% (14/32) of *C. congener truncus*, 40.0% (2/5) of *N. sasai* and 0% (0/3) of *H. microti* were positive for *Bartonella*, respectively. No significant difference was observed in the positive rates between male and female fleas (data not shown).

Eleven out of twenty-five (44.0%) *C. congener truncus* fleas and two out of four (50.0%) *N. sasai* fleas collected from *A. speciosus* were positive for *Bartonella* DNA. The DNA of *B. grahamii* and *B. taylorii* were detected from three and eight of *C. conger truncus* fleas collected from *A. speciosus*, respectively. Two of four *N. sasai* fleas found on *A. speciosus* were positive for only *B. grahamii* DNA, but one *N. sasai* fleas collected from an *A. argenteus* was negative for *Bartonella* DNA. Although 7 *C. congener truncus* and 3 *H. microti* fleas were collected from *M. rufocanus bedfordiae*, the DNA of *B. taylorii* was detected only from 3 *C. congener truncus* fleas. No *Bartonella* DNA was found in *H. microti* fleas found on *M. rufocanus bedfordiae* (Table 2).

Bartonella DNA from flea organs was examined using 16 positive fleas (7 male, 9 female). The DNA was detected from the midgut of all 16 fleas. Furthermore, in the organs of the fleas, DNA was in found in the midgut only (7/16), midgut and ovary (5/16), midgut and hemolymph (3/16), and midgut, hemolymph and ovary (1/16), respectively. The DNA sequences of *gltA* and *rpoB* detected from different organs were completely identical in the individual fleas (data not shown).

Phylogenetic analysis of Bartonella *spp. isolated from wild rodents and fleas*: Phylogenetic trees were constructed based on the DNA sequences of the *gltA* (Fig. 1-1) and *rpoB* (Fig. 1-2) genes from the rodent isolates and fleas. All the type strains of 12 *Bartonella* species (includes 2 subspecies) isolated from rodents were included in the phylogenetic analysis. *Agrobacterium tumefaciens* strain C58 and *Brucella melitensis* strain 16M^T were also included as outgroups. The DNA sequences of *gltA* were classified into 3 genotypes from *B. grahamii* (Bg-g1, Bg-g2 and Bg-g3), 2 genotypes from *B. taylorii* (Bt-g1 and Bt-g2) and a genotype from *B. japonica* (Bj-g1; Table 4). The DNA sequences of *rpoB* were classified into 6 genotypes from *B. grahamii* (Bg-r1 to Bg-r6), 6 genotypes from *B. taylorii* (Bt-r1 to Bt-r6) and a genotype from *B. japonica* (Bj-r1; Table 4). The DNA sequences of the *gltA* and *rpoB* genes of *Bartonella* spp. isolated from rodent No. 36 were identical to those from the fleas found on it. Both of the *Bartonella* species isolated from the rodents (Nos. 8, 12, 33 and 47) and the corresponding DNA from the fleas found on them were classified as *B. grahamii*, although their genotypes were not identical to each other. Different species of *Bartonella* DNA were detected from rodent Nos. 14, 18, 19, 22, 27, 28, 43 and 45 and the fleas found on them. The novel *gltA* and *rpoB* genotypes have been submitted to nucleotide databases of GenBank, EMBL and DDBJ with the following accession numbers: AB469818 to SB469821.

DISCUSSION

Bartonella species can cause a latent and relapsing infection in their host animals [17], and this may result in the high prevalence of *Bartonella* among wild rodents. Furthermore, effective transmission of the organisms is thought to be indispensable to maintain the high prevalence of *Bartonella* in natural environments.

In the present study, 40.0% (16/40) of the rodent fleas tested were positive for *Bartonella* DNA by PCR. Previous studies have reported the detection of *Bartonella* DNA from several species of flea with positive rates ranging from 2.2% (21/937) in Egypt to 26.0% (20/77) in Afghanistan [10, 20, 21, 23, 25]. In particular, *B. grahamii*, a causative agent of neuroretinitis in humans, was reported to be the most dominant species among wild rodents in Japan [12]. In this study, the DNA was also detected from 5 of 40 fleas on the rodents. Assessment of the possibility that the fleas carrying *B. grahamii* could be a vector for infection of humans is required.

The DNA of the isolates from rodent No. 36 was identical to that from each of the infested fleas, suggesting that the rodent flea acquired bartonellae from the infested rodents and that the fleas may play a potential role in the transmission of *Bartonella* among wild rodents. Supporting this hypothesis, Bown *et al.* [6] reported that 75% (21/28) of naive bank voles (*Clethrionomys glareolus*) housed with wild-caught fleas for 4 weeks became bacteremic with *Bartonella*. However, it is important to note that PCR detection of *Bartonella* in fleas does not necessarily mean active infection in the host.

The *Bartonella* DNA was detected in hemolymph and the midguts of the positive fleas, indicating the possibility that the organisms might accompany blood cells and be

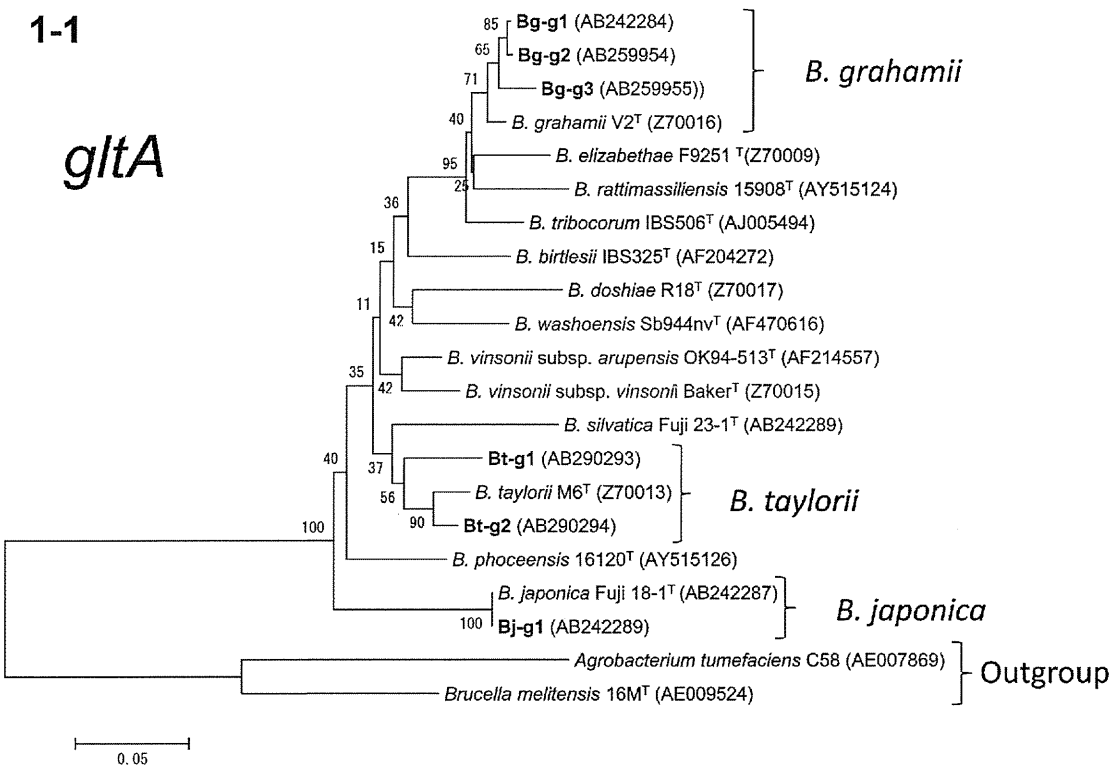

Fig. 1. Phylogenetic analysis of the DNA derived from the *Bartonella* isolates of wild rodents and the fleas found on them based on the sequences of *gltA* (312 bp) and *rpoB* (825 bp). The tree was constructed by the N-J method based on *gltA* (Fig. 1-1) and *rpoB* (Fig. 1-2, right page). All the type strains of 13 *Bartonella* species, including 2 subspecies, namely, *B. birtlesii* IBS325^T, *B. doshiae* R18^T, *B. elizabethae* F9251^T, *B. grahamii* V2^T, *B. japonica* Fuji 18-1^T, *B. phoceensis* 16120^T, *B. rattimassiliensis* 15908^T, *B. silvatica* Fuji 23-1^T, *B. taylorii* M6^T, *B. tribocorum* IBS506^T, *B. vinsonii* subsp. *arupensis* OK94-513^T, *B. vinsonii* subsp. *vinsonii* Baker^T, and *B. washoensis* Sb944nv^T, isolated from rodents were included in the phylogenetic analysis. *Agrobacterium tumefaciens* strain C58 and *Brucella melitensis* strain 16M^T were also included as outgroups. Genotypes for *gltA* found in this study, including three genotypes from *B. grahamii* (Bg-g1 to Bg-g3), two genotypes from *B. taylorii* (Bt-g1 and Bt-g2) and a genotype from *B. japonica* (Bj-g1), are indicated by boldface (Fig. 1-1). Genotypes for *rpoB* found in this study, including six genotypes from *B. grahamii* (Bg-r1 to Bg-r6), six genotypes from *B. taylorii* (Bt-r1 to Bt-r6) and a genotype from *B. japonica* (Bj-r1), are indicated in boldface (Fig. 1-2). Bootstrap values were obtained with 1,000 replicates. Corresponding accession numbers for each genotype are indicated in parentheses.

absorbed through the midgut and distributed into the body. Furthermore, four out of the nine female fleas were positive for the DNA in their ovaries. *Rickettsia typhi* and *R. felis* have been shown to cause transovarial transmission in fleas [2, 11]. Further studies are needed to examine the possibility that *Bartonella* survives by vertical transmission in fleas.

Three species of fleas, i.e., *C. congener truncus*, *N. sasai* and *H. microti*, were obtained from the wild rodents in the present study. All of the DNA detected from *C. congener truncus* fleas found on *M. rufocanus bedfordiae* were from *B. taylorii*, indicating that specific transmission of *B. taylorii* was established between *M. rufocanus bedfordiae* and *C. congener truncus*. On the other hand, *N. sasai* was collected from *A. speciosus* and *A. argenteus*, but not from *M. rufocanus bedfordiae*. The only DNA detected from *N. sasai* was that of *B. grahamii*, which had been isolated from a rodent of the genus *Apodemus*. In addition, the DNA of *B. grahamii* and *B. taylorii* were detected from 3 and 8 of *C. congener truncus* fleas found on *A. speciosus*, respectively, but no *B. taylorii* were isolated from *Apodemus* mice used in this study. Although *C. congener truncus* fleas from *A. speciosus* harbored *B. taylorii* (32.0%) more frequently than *B. grahamii* (12.0%), *B. taylorii* was not isolated from the rodents examined in this study. These results suggest the possibility that host-parasite specificity exists between *Apodemus* mice and *B. grahamii* and that transmission may be accomplished by *N. sasai* and/or *C. congener truncus*.

This study indicated host-parasite specificity between *B. grahamii* and *Apodemus* mice and between *B. taylorii* and *M. rufocanus bedfordiae*, respectively, in Japan. However, *B. grahamii* and *B. taylorii* have been isolated from wild voles as well as *Apodemus* mice in the U.K. and Canada [5, 14]. The efficacy of *Bartonella* transmission may differ depending on the rodent and flea species combination [1]. Efficient transmission by favorable vectors in wild rodents and optimal host-parasite interactions could contribute to

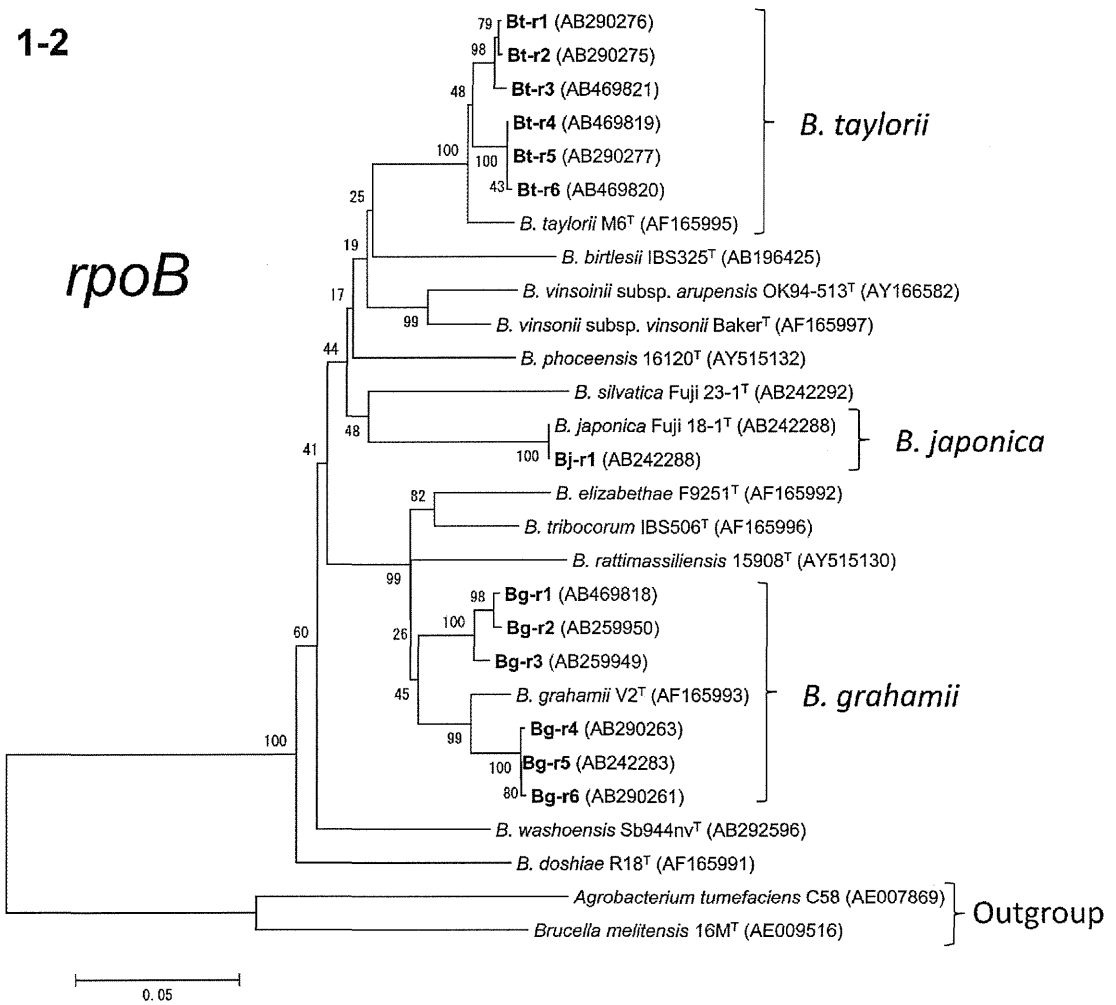

Table 4. Genotypes of *Bartonella* DNA in rodents and their fleas

Rodent No.	Rodent spp[a)]	Genotypes in rodents		Flea spp.[b)]	Genotypes in fleas	
		gltA	*rpoB*		*gltA*	*rpoB*
8	As	Bg-g2	Bg-r5	Cc truncus	Bg-g1	Bg-r5
12	As	Bg-g2	Bg-r5	Cc truncus	Bg-g1	Bg-r5
14	As	Bg-g2	Bg-r5	Cc truncus	Bt-g2	Bt-r3
18	As	Bj-g1	Bj-r1	Cc truncus	Bt-g2	Bt-r1
19	As	Bg-g1	Bg-r5	Cc truncus	Bt-g2	Bt-r3
22	As	Bg-g2	Bg-r5	Cc truncus	Bt-g2	Bt-r2
26	Mrb	NA[c)]	NA	Cc truncus	Bt-g1	Bt-r4
27	As	Bg-g2	Bg-r5	Cc truncus	Bt-g2	Bt-r2
28	As	Bg-g1	Bg-r5	Cc truncus	Bt-g2	Bt-r1
33	As	Bg-g3	Bg-r3	Cc truncus	Bg-g2	Bg-r5
34	Mrb	NA	NA	Cc truncus	Bt-g1	Bt-r6
36	As	Bg-g2[d)]	Bg-r5[d)]	Ns	Bg-g2[d)]	Bg-r5[d)]
42	Mrb	NA	NA	Cc truncus	Bt-g2	Bt-r2
43	As	Bg-g3	Bg-r3	Cc truncus	Bt-g2	Bt-r1
45	As	Bg-g1	Bg-r5	Cc truncus	Bt-g2	Bt-r1
47	As	Bg-g3	Bg-r3	Ns	Bg-g1	Bg-r1

a) As: *Apodemus speciosus*. Aa: *Apodemus argenteus*. Mrb: *Myodes rufocanus bedfordiae*. b) Cc truncus: *Ctenophthalmus congener truncus*. Ns: *Neopsylla sasai*. c) NA: not applicable. No *Bartonella* was isolated from the rodent. d) The genotypes from rodent No. 36 and its flea are identical.

the high prevalence and long-term infection of *Bartonella* species. Further studies are needed to confirm the host-parasite specificities between the *Bartonella* and rodent species.

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ORIGINAL ARTICLE

Prevalence of *Salmonella*, *Yersinia* and *Campylobacter* spp. in Feral Raccoons (*Procyon lotor*) and Masked Palm Civets (*Paguma larvata*) in Japan

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Impacts

- This is the first report on the prevalence of *Salmonella*, *Yersinia*, and *Campylobacter* spp. in feral raccoons and masked palm civets in Japan.
- Our results indicate that these animals are potential carriers of these pathogens and that these animals probably acquired their infections from human activities, other wild animals, and the environment.
- As these animals live near human habitations or livestock farms, their carrying the pathogens represents a serious public and animal health risk.

Keywords:
Alien species; epidemiology; *Salmonella*; *Yersinia pseudotuberculosis; Campylobacter*

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Summary

To estimate the public and animal health risk that alien species pose, the prevalence of *Salmonella*, *Yersinia*, and *Campylobacter* spp. in feral raccoons (*Procyon lotor*, $n = 459$) and masked palm civets (*Paguma larvata*, $n = 153$), which are abundant alien species in Japan, was investigated in urban and suburban areas of Japan. *Salmonella enterica* was detected from 29 samples [26 raccoons, 5.7%, 95% confidence interval (CI) 7.8–3.5%; three masked palm civets, 2.0%, 95% CI 4.2–0%]. Many of the isolates belonged to serovars that are commonly isolated from human gastroenteritis patients (e.g. *S.* Infantis, *S.* Typhimurium, and *S.* Thompson). The antimicrobial susceptibility test showed that 26.9 % of the isolates from raccoons were resistant to at least one antimicrobial agent, whereas none of the isolates from masked palm civets were resistant. *Yersinia* sp. was detected from 193 samples (177 raccoons, 38.6%, 95% CI 43.0–34.1%; 16 masked palm civets, 10.5%, 95% CI 15.3–5.6%). All virulent *Yersinia* strains belonged to *Yersinia pseudotuberculosis*, which was isolated from seven (1.5%, 95% CI 2.6–0.4%) raccoons and six (3.9%, 95% CI 7.0–0.8%) masked palm civets. According to the detection of virulence factors, all the *Y. pseudotuberculosis* isolates belonged to the Far Eastern systemic pathogenicity type. *Campylobacter* spp. was detected from 17 samples (six raccoons, 1.3%, 95% CI 2.3–0.3%; 11 masked palm civets, 7.2%, 95% CI 11.3–3.1%). Among these, three isolates from raccoons were identified as *C. jejuni*. These results showed that these pathogens can be transmitted by human activities, other wild animals, and the environment to feral raccoons and masked palm civets, and vice versa. As these animals have omnivorous behaviour and a wide range of habitats, they can play an important role in the transmission of the enteric pathogens.

Introduction

The raccoon (*Procyon lotor*) is a medium-sized mammal that is widely distributed in North America. Raccoons were introduced into Japan in the 1970s and have become naturalized in at least 42 of 47 prefectures (Ikeda et al., 2004). In urban areas, raccoons often use human houses for their dens. Raccoons use the feed stores of domestic animals as nests, and thus also have close contact with such animals (Zeveloff, 2002). It has been reported that the raccoon is a reservoir of various kinds of zoonotic pathogens in its place of origin, including the raccoon

roundworm (*Baylisascaris procyonis*) (Gavin et al., 2005), rabies virus (Finnegan et al., 2002), *Leptospira* spp. (Hamir et al., 2001), and *Francisella tularensis* (Berrada et al., 2006). Bigler et al. (1975) found that raccoons are so adaptive that they can bridge the gaps among avian, terrestrial, and aquatic environments, and thus they are appropriate as an indicator of the prevalence of various infectious diseases and pollutants. The masked palm civet (*Paguma larvata*) is also a medium-sized carnivore and is distributed widely in Asia. Masked palm civets are thought to have been introduced into Japan, although this contention is controversial (Abe, 2005). Masked palm civets often live in similar places as raccoons and can be carriers of human and animal pathogens, such as severe acute respiratory syndrome (SARS) virus (Tu et al., 2004) and canine distemper virus (Machida et al., 1992).

In Japan, attention has been focused on the role of these highly adaptive mammals in the transmission of zoonotic pathogens, including *Trichinella* T9 (Kobayashi et al., 2007), *Babesia microti*-like parasite (Kawabuchi et al., 2005), *Ehrlichia* spp., *Anaplasma phagocytophilum* (Inokuma et al., 2007), *Strongyloides procyonis* (Sato and Suzuki, 2006), and canine distemper virus (Machida et al., 1992). Pathogens carried by these animals can present serious public and animal health problems in the habitats to which they have been introduced. However, in spite of the fact that these two species often inhabit urban and suburban areas, there have been few reports on the prevalence of enteric pathogens among them.

The objective of this study was to determine the prevalence of the causal agents of enteric diseases including *Salmonella* spp., *Yersinia* spp., and *Campylobacter* spp. in feral raccoons and masked palm civets. We then assessed antimicrobial resistance in the isolates and analysed them using polymerase chain reaction (PCR) to elucidate their virulence factors and transmission routes.

Materials and Methods

Sample collection and transport

From March 2006 to May 2007, 459 feral raccoons and 153 feral masked palm civets were captured in Kanagawa, Gunma, and Tokyo Prefectures by each municipality as a part of the local governmental control and eradication programmes. The animals were caught using box traps and were killed by humanitarian methods (Japan Veterinay Medical Association, 2007). Of 459 feral raccoons, 229 (49.9%) were males, 211 (46.0%) were females, and 19 (4.2%) were of unknown sex. Of 153 feral masked palm civets, 72 (47.1%) were males, 79 (51.6%) were females, and two (1.3%) were of unknown sex. The age of the feral raccoons and masked palm civets was determined on the basis of tooth eruption and cranial suture obliteration by the method of Montgomery (1964) and Junge and Hoffmeister (1980). Of 459 feral raccoons, 71 (15.5%) were juveniles (<5 months), 357 (77.8%) were sub-adults or adults, and 31 (6.9%) were of unknown age. Of 153 feral masked palm civets, 47 (30.7%) were juveniles (<6 months), 104 (68.0%) were sub-adults or adults, and 2 (1.3%) were of unknown age. Faecal samples were collected and preserved in Cary and Blair transport medium (Eiken Chemical Co. Ltd., Tokyo, Japan) or sterile centrifuge tubes. The samples were then transported to the laboratory. The samples were suspended in 4 ml of sterile phosphate-buffered saline (PBS; pH 7.2) and tested within a week after collection.

Isolation and identification of *Salmonella*

One millilitre of each specimen was inoculated into 10 ml of buffered peptone water (BPW; Becton Dickinson, Franklin Lakes, NJ, USA). After incubation at 37°C for 24 h, 1 ml of BPW culture was transferred to 10 ml of Hajna-tetrathionate broth (Eiken Chemical). The broth was incubated at 37°C for 24 h, then one loopful of each tube was inoculated onto a plate of desoxycholate hydrogen sulfide lactose agar (DHL; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and mannitol lysine crystal violet brilliant green agar (MLCB; Nissui). These plates were incubated at 37°C for 24 h, and at least two suspicious colonies morphologically similar to *Salmonella* spp. from each plate were subcultured for biochemical examinations. Biochemical characteristics were examined on triple sugar iron medium (Nissui) and lysine indole motility medium (Nissui). The subspecies of *Salmonella* isolates were confirmed by biochemical examinations and the multiplex PCR assay (Popoff and Le Minor, 2005; Lee et al., 2009). Serotyping for *Salmonella* isolates was accomplished with commercial O and H antisera (Denka Seiken Co. Ltd., Tokyo, Japan) according to the method of Popoff and Le Minor (2001).

Isolation and identification of *Yersinia*

Specimens were incubated at 4°C for 4 weeks. After alkali treatment (Aulisio et al., 1980), a loopful of sample suspension was spread on virulent *Yersinia enterocolitica* (VYE) agar (Fukushima, 1987) and Irgasan-Novobiocin (IN) agar containing 2.5 mg/l of irgasan and novobiocin in Yersinia Selective Agar Base (Difco Laboratories, Detroit, MI, USA) (Schiemann, 1979). All plates were incubated at 25°C for 48 h. Colonies morphologically similar to *Yersinia* spp. were subcultured for biochemical examination. The identification of yersiniae was performed by the methods of Wauters et al. (1988). All isolates identified as yersiniae were subjected to autoagglutination tests to evaluate their potential pathogenicity (Laird and Cavanaugh, 1980).

Then, virulent isolates were subjected to further analysis. Serotyping of *Y. pseudotuberculosis* was accomplished by slide agglutination with commercial antisera (Denka Seiken). Isolates identified as *Y. pseudotuberculosis* were genotyped by the presence patterns of the gene encoding *Y. pseudotuberculosis*-derived mitogen typeA (YPMa) and high-pathogenicity island (HPI) so as to analyse their geographical origin using PCR assay (Fukushima et al., 2001).

Isolation and identification of *Campylobacter*

One millilitre of the specimen was inoculated into 5 ml of Preston enrichment broth (OXOID CM0067 + SR0117E + SR0232E) containing 5% defibrinated horse blood. After incubation at 37°C for 24 h, one loopful of each tube was inoculated onto a skirrow blood agar plate (OXOID CM0271 + SR0069E + SR0232E) containing 5% defibrinated horse blood. All skirrow blood agar plates were incubated for 48 h at 37°C under microaerobic conditions and examined for the presence of characteristic colonies of *Campylobacter*. Confirmation and characterization of the isolates were performed on the basis of microscopic morphology, an oxidase test, a catalase test, growth at 25°C and 42°C, and a multiplex PCR assay for *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* (Wang et al., 2002; Vandamme et al., 2005)

Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed according to the disc diffusion method (National Committee for Clinical Laboratory Standards, 2002; Luangtongkum et al., 2007). The following antimicrobial paper discs (Sensi-Disc; Becton Dickinson) were used: ampicillin (10 μg/disk), cefazolin (30 μg/disk), ceftriaxone (30 μg/disk), ciprofloxacin (5 μg/disk), chloramphenicol (30 μg/disk), gentamicin (10 μg/disk), kanamycin (30μg/disk), nalidixic acid (30 μg/disk), oxytetracycline (30 μg/disk), and streptomycin (10 μg/disk) for *Salmonella* and *Y. pseudotuberculosis*; and ampicillin, ciprofloxacin, clindamycin (2 μg/disk), erythromycin (15 μg/disk), gentamicin, kanamycin, nalidixic acid, norfloxacin (10 μg/disk), oxytetracycline, and streptomycin for *Campylobacter*.

Statistical analysis

Differences of the prevalence were analysed by the chi-squared test in SPSS software (SPSS Inc., Chicago, IL, USA).

Results

The raccoons and masked palm civets were essentially normal except for various external parasites. No discernible pathological evidence or signs of disease were observed. There were no significant sex- and age-specific differences in *Salmonella* spp., *Yersinia* spp., and *Campylobacter* spp prevalence in both animals (Table 1). There was no sample which was positive for more than two bacterial species or serotypes tested in this study, except nonpathogenic *Yersinia* spp.

Table 1. Prevalence of *Salmonella enterica*, *Yersinia pseudotuberculosis*, and *Campylobacter* sp. in raccoon and masked palm civets in each sex or age groups

			No. positive sample (%)		
Origin	Sex/age	Sample size	*Salmonella enterica*	*Y. pseudotubeculosis*	*Campylobacter* spp.
Raccoon	Female	211	11 (5.2)	2 (0.9)	2 (0.9)
	Male	229	15 (6.6)	5 (2.2)	3 (1.3)
	Unknown	19	0	0	1 (5.3)
	Juvenile	71	2 (2.8)	1 (1.4)	0
	Sub-, Adult	357	24 (6.7)	6 (1.7)	5 (1.4)
	Unknown	31	0	0	1 (3.2)
	Total	459	26 (5.7)	7 (1.5)	6 (1.3)
Masked palm civet	Female	79	1 (1.3)	3 (3.8)	7 (8.9)
	Male	72	2 (2.8)	3 (4.2)	4 (5.6)
	Unknown	2	0	0	0
	Juvenile	47	1 (2.1)	3 (6.4)	5 (10.6)
	Sub-, Adult	104	2 (1.9)	3 (2.9)	6 (5.8)
	Unknown	2	0	0	0
	Total	153	3 (2.0)	6 (3.9)	11 (7.2)

Table 2. Serovar and antibiotic resistance of *Salmonella* isolated from raccoons and masked palm civets

Origin	Species	Subspecies	Serovar	Antibiotic resistance	No. isolates
Raccoon	*Salmonella enterica*	*enterica*	*S.* Mbandaka	–*	5
			S. Infantis	ABPC, NA, OTC	1
				NA, OTC	1
				OTC	1
				–	1
			S. Typhimurium	ABPC, NA, OTC	1
				ABPC, KM, OTC	1
				–	2
			S. Nagoya	–	2
			S. Berta	–	1
			S. Manhattan	OTC	1
			S. Nigeria	OTC	1
			S. Rubislaw	–	1
			S. Thompson	–	1
		diarizonae	UT	–	6
Masked palm civet	*Salmonella enterica*	*enterica*	*S.* Enteritidis	–	1
			S. Nagoya	–	1
			4,12:i:-	–	1

UT, untypable; ABPC, ampicillin; NA, nalidixic acid; OTC, oxytetracycline; KM, kanamycin.
*Susceptible to all antimicrobial agents used in this study.

Salmonella

Salmonella enterica was isolated from the faecal samples of 26 of 459 raccoons (5.7%, 95% CI 7.8–3.5%) and three of 153 masked palm civets (2.0%, 95% CI 4.2–0%). Table 2 shows the serovars and antimicrobial resistance patterns of the isolates. Nine and three serovars were identified in the isolates from raccoons and masked palm civets, respectively. Those serovars included common serovars in gastroenteritis patients and domestic animals (e.g. *S.* Infantis, *S.* Typhimurium, *S.* Thompson, and *S.* Enteritidis) (Esaki et al., 2004; Ishihara et al., 2009; National Institute of Infectious Disease, 2009). Seven of 26 isolates (26.9%) from raccoons showed resistance to at least one antimicrobial agent used in this study, whereas all isolates from masked palm civets were susceptible to all of the antimicrobial agents.

Yersinia

Yersinia spp. were isolated from the faecal samples of 177 of 459 raccoons (38.6%, 95% CI 43.0–34.1%) and 16 of 153 masked palm civets (10.5%, 95% CI 15.3–5.6%). Among the isolates, seven strains from raccoons and six strains from masked palm civets showed positive reactions in autoagglutination tests and were subsequently identified as *Y. pseudotuberculosis*. Therefore, the prevalence of *Y. pseudotuberculosis* in raccoons and masked palm civets was 1.5% (95% CI 2.6–0.4%) and 3.9% (95% CI 7.0–0.8%), respectively. Four, one, and two *Y. pseudotuberculosis* isolates from raccoons belonged to serotypes 1b, 3, and 4b, respectively. Three, two, and one *Y. pseudotuberculosis* isolates from masked palm civets belonged to serotypes 1b, 3, and 4b, respectively. All *Y. pseudotuberculosis* isolates were susceptible to all of the antimicrobial agents used in this study. In all of the *Y. pseudotuberculosis* isolates, the gene of YPMa was detected and the gene of HPI was not detected by PCR assay.

Campylobacter

Campylobacter spp. were isolated from the faecal samples of six of 459 raccoons (1.3%, 95% CI 2.3–0.3%) and 11 of 153 masked palm civets (7.2%, 95% CI 11.3–3.1%). Three isolates from raccoons were identified as *C. jejuni* by multiplex PCR assay. The other *Campylobacter* isolates from raccoons and masked palm civets exhibited three different phenotypic patterns (Table 3). All *the Campylobacter* isolates were susceptible to all of the antimicrobial agents used in this study.

Table 3. Biochemical characteristics of *Campylobacter* spp. isolated from raccoons and masked palm civets

Origin	Species	No. isolates	Oxidase	Catalase	Growth at 25°C	Growth at 42°C
Raccoon	*Campylobacter jejuni*	3	+	+	–	+
	Campylobacter spp.	2	+	+	–	–
		1	+	+	–	+
Masked palm civet	*Campylobacter* spp.	9	+	+	–	+
		1	+	+	+	+
		1	+	+	–	–

Discussion

Salmonella

In this study, the prevalence of *S. enterica* in raccoons (5.7%) was almost concordant with the prevalence in Western Pennsylvania, USA (7.4%) (Compton et al., 2008). Investigations in other wild mammals also have revealed similar prevalence rates in UK (6.5%) and Spain (7.2%) (Euden, 1990; Millan et al., 2004). However, Morse et al. (1983) reported that *S. enterica* was isolated from 31.1% of feral raccoons. This finding may suggest that raccoons have the potential to harbor *S. enterica* at a high rate. The prevalence of *S. enterica* in masked palm civets was somewhat lower than that in raccoons. Differences in their behaviour could be responsible for the difference in the prevalence. Further analysis of their food habits and habitat choice may verify this hypothesis.

Some of the isolates were indicated to have originated from human activities, because many of the serovars isolated have been common in human gastroenteritis and in domestic animals, and the isolates from raccoons showed a relatively high resistance rate (26.9%). Interestingly, six strains isolated from raccoons were identified as *S. enterica* subsp. *diarizonae*. Because this *Salmonella* subspecies is not common in warm-blooded animals but is common in cold-blooded animals and the environment, it may be associated with their omnivorous behaviour and proclivity for wet habitats (Zeveloff, 2002; Bopp et al., 2003; Haley et al., 2009).

Yersinia

Yersinia pseudotuberculosis isolates belonged to serotypes 1b, 3, and 4b, which are predominant serotypes in human patients and wild animals in Japan (Hamasaki et al., 1989; Fukushima and Gomyoda, 1991; Hayashidani et al., 2002). The prevalence of *Y. pseudotuberculosis* in raccoons and masked palm civets was comparable with that of those studies. It is difficult to compare the prevalence with that in its place of origin, because there are few reports on isolation of pathogenic *Yersinia* from raccoons and masked palm civets (Hacking and Sileo, 1974). All of the *Y. pseudotuberculosis* strains showed the same genotypic pattern with the $YPMa^{+}$ HPI^{-} Far Eastern systemic-pathogenicity type. Fukushima et al. (2001) reported that most of the strains isolated in Far East Asia showed such a pattern and differed from the strains isolated in European countries. In addition to the information regarding geographical origin, this virulence characteristic has a clinical implication, because *ypmA* encodes YPMa, which contributes to the virulence of *Y. pseudotuberculosis* in systemic infection (Carnoy et al., 2000).

These results lead to the conclusion that raccoons and masked palm civets probably have acquired their infections in Japan and play a similar role to other indigenous animals on the ecology of *Y. pseudotuberculosis*. In previous studies, natural reservoirs of *Y. pseudotuberculosis* in Japan have been suggested to be wild mammals and birds, especially rodents and raccoon dogs (*Nyctereutes procyonoides*) (Hamasaki et al., 1989; Fukushima and Gomyoda, 1991; Hayashidani et al., 2002). The major transmission routes of the pathogen in wildlife are suggested as preying upon infected animals or ingesting environmental substances contaminated with *Y. pseudotuberculosis* rather than contact with human activities (Fukushima and Gomyoda, 1991). It is likely that raccoons and masked palm civets acquired their infection, as a result of sharing habitats with other reservoirs.

Campylobacter

The principal reservoirs of *Campylobacter* in the environment are wild mammals and birds (Mörner, 2001), with the occurrence of enteric *Campylobacter* higher in birds than in wild mammals. This tendency is consistent with our results. The prevalence rates in raccoons and masked palm civets were 1.3% and 7.2%, respectively, whereas the rate is often more than 10% in wild birds (Kapperud and Rosef, 1983; Matsusaki et al., 1986; Ito et al., 1988). However, wild mammals, especially species that have direct or indirect contact with human activities, are still important reservoirs of the pathogen. Workman et al. (2005) demonstrated that dogs were one of the most likely sources of human campylobacteriosis. Domestic animals, including dogs, are more likely to have opportunities to acquire infections from excretory substances or from environments contaminated by raccoons and masked palm civets.

Among the isolates, three strains were identified as *C. jejuni*. However, the other 14 isolates from both raccoons and masked palm civets could not be identified by the multiplex PCR assay. Additional biochemical tests suggested that these strains belonged to uncommon species of *Campylobacter* (Table 3). As such species (e.g. *C. hyointestinalis*, *C. larienae* or *C. rectus*) are isolated from healthy animals and enteritis patients, their pathogenicity and epidemiology have not been well known (Vandamme et al., 2005). Because there is little information about the carriage of uncommon *Campylobacter* species in wild mammals, further investigations are required to elucidate the real impact of these species in wild animals on public and animal health.

From these results, we concluded that raccoons and masked palm civets could be potential reservoirs of enteropathogenic *Campylobacter* (*C. jejuni*) and are more

