Table II. Alignment of genus *Pestivirus* species genotypes variable loci 5' UTR RNA secondary structure sequences from prototype strains, segregated according to types of base pair combinations. The different types are ordered according to increasing divergence in the genus (*), expressed in number of divergent base pairs, with reference to most common base pairs in the prevalent positions. Highly conserved base pair positions are excluded. Y: G or U. HV: highly variable.

Variable locus	V1																			V2								V3										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)
Prevalent base pairs	**GY**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GY**		**CG**		**UA**					—	—	—		**YG**	**YG**	**UA**	**GY**			**GC**	**AU**		**CG**		**GY**	**UA**				**A**	
BVDV1	**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		—	—		**CG**	**CG**	**UA**	**GC**			**GC**	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**	
Genotype BVDV-1a																																						
BVDV1 Singer	.										CG		UA		GU	AA	—	—	—	AU					AC	GC	.	.	GU		AU			UG	CC	UC	AA	1
BVDV1 NADL	.										CG		UA		GU	AA	—	—	—	AU					AC	GC	.	.	GU		AU			UG	UC	UC	AA	1
BVDV1 SD-1	.										CG		CA		GU	UA	—	—	—	AC					AC	GC	.	.	GU		AU		AU		CA	GC	AC	1
BVDV1 Oregon	.										CG		UA	AG	AU	CA	—	—	—	GC	CA		CG		AU	GC	.	.	AC		AU				UC	GU	.	2
BVDV-1b1																																						
BVDV1 Sanders	.					AA					UA		UU		GG	AG	—	—	—	AU					GU	AU	.	.	AC		GC				UC	AC	.	1
BVDV1 CD89	.										UA		UC		GG	AG	—	—	—	AU					GU	AU	AC	.	AC		GC				UC	AC	.	1
BVDV1 Draper	.										UA		CC		GG	AG	—	—	—	AU					GU	AU	.	.	AC		GC				UA	GU	AC	1
BVDV-1b2																																						
BVDV1 NY-1	.										UA		CC		GG	AG	—	—	—	AU					AU	GU	.	.	AC		GC				UC	AC	.	0
BVDV1 Osloss	.				G						UA		CC		UG	GU	—	—	—	AU					AU	GU	.	.	GC		GC				UC	AC	.	1
BVDV-1c																																						
BVDV1 Europa	.								AU		CG		CA	GG	GA	AA	—	—	—	AU					GC	AC	.	.	GU		AU				UU	GA	.	0
BVDV1 SE5726	.								AU		CG		CA	GG	GA	AA	—	—	—	AU					GC	AC	.	.	GU		AU				UC	AC	.	0
BVDV-1d																																						
BVDV1 438/02	.										CG		CC		GG	AG	—	—	—	AU					AU	AU	.	.	GC		GC				UC	AC	.	0
BVDV1 Massimo 4	.										CG		AA		GG	AG	—	—	—	AU					GU	AU	.	.	AC		GC				AC	AC	.	0
BVDV-1e																																						
BVDV1 23-13	.										CG		UA		GA	UC	—	—	—	GU			CA		GU	GC	.	.	AC		GC			UG	UU	GU	.	1
BVDV1 23-15	.										CG		UA		GC	U	—	—	—	GU					GU	GC	AC	.	AC		GC			UG	UU	GU	.	1
BVDV-1f																																						
BVDV1 CRFK	.								AC		CG		UA	GC	GA	GA	AG	—	—	AU					GU	AU	.	.	AU		AU			CG	UC	AC	.	1
BVDV-1g																																						
BVDV1 so CP/75	.								AU		CG		AU	UG	GC	GG	U	—	—	GU					GU	GC	.	.	GC		GU				UC	AC	.	1
BVDV-1h																																						
BVDV1 KM	.										CG		UU	AC	AA	UA	GA	—	—	AU					CU	GC	.	.	GU		GC			AG	UU	AA	.	1
BVDV1 G	.										CG		UU	AC	AA	GA	GA	—	—	AU					CU	GC	.	.	GU		GC		CA	AG	UU	GA	.	2
BVDV-1i																																						
BVDV1 10-84	.								AU		CG		AU	GG	GG	AU	—	—	—	GU					AC	GC	.	.	GC	GC				AA	AA	AC	GA	1
BVDV1 L256	.								AU		CG		AU		GG	AU	—	—	—	GU					AC	GC	.	.	GC	UA	GC			AG	AA	AC	GA	2
BVDV-1j																																						
BVDV1 22146/81	.										CG		GC	UA	AC	GG	—	—	—	AU					AU	GC	AC	.	GU		GC		GC		AU	AC	.	2
BVDV1 4998/89	.										CG		UU	AU	GU	AG	—	—	—	AU					GC	GU	AC	.	GU		GC		GA	AA	AC	AC	.	2
BVDV-1k																																						
BVDV1 Deer	.			UU							CG		CG		GU	AA	A	—	—	AU					AU	GC	.	.	GU		GC				CC	AC	.	2
BVDV1 M557A/90	.			UU							CG		CA		GU	AA	A	—	—	AU		CU			AU	GC	.	.	GU		GC				CC	AC	.	3

Table II. Continued.

Variable locus	V1																			V2								V3										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)
BVDV1	**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		—	—		**CG**	**CG**	**UA**	**GC**			**GC**	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**	
BVDV-1l																																						
BVDV1 ZM-95	.										CG		UG	AU	GC	GU	A	—	—	UA	UA		CG		GC	AC	.	.	GU		GC				GU	UC	.	3
BVDV-1m																																						
BVDV1 J	.										CG		AU	UG	AU	GA	A	—	—	AU					AC	GC	AC	.	GU		GC		AA		AA	A	-	4
BVDV1 S	.										CG		UU	AA	GU	GA	—	—	—	AU					AC	GC	AC	.	GU		GC		AA	UU	AA	AA	-	3
BVDV-1n																																						
BVDV1 A	.										CG	CG	CC	AG	GU	AA	—	—	—	AU				AU	GU	GC	AC	.	GU		GC		GA	AA	GU	AC	.	4
BVDV1 L	.							AU			CG	CG	CC	GG	GU	AA	—	—	—	AU				AU	GU	GC		.	GU		GC			UG	GC	AC	.	3
BVDV-1o																																						
BVDV1 Rebe	AU								AU		CG		AU	CG	GC	GA	A	—	—	AU		UA		UA	GC	GC	AC	.	GC	UA	GC			CA	UC	AC	.	6
BVDV1 SuwaCp	AU								AU		CG		AU	UG	GC	GA	A	—	—	AU		UA		UA	GC	GC	AC	.	GC	UA	GC		CA	CA	UC	AC	.	7
Variable locus	V1																			V2								V3										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)
BVDV-2	**AU**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**AU**	**CG**	**UA**	**CG**	**CG**				**AU**				**CG**	**CG**		**AU**	**UA**	**GC**	**GU**	**AU**	**GU**	**CG**		**CG**	**CG**	**AU**	**UG**	**UC**	**—**	
BVDV-2A group																																						
BVDV-2a1.1																																						
BVDV-2 713-2	.													AA	UG	CC		G	—	AU			UA				.	.			GC					UU	—	2
BVDV-2 97/730	.													AA	UG	CC	AC	A	—	AU			UG				.	.			AU						—	2
BVDV-2a1.2																																						
BVDV-2 BD-78	.			G										AA	UG	UC		G	—	AU			CG			CA	.	.			GC						—	3
BVDV-2 C413	.													AA	UG	UC		G	—	AU			CG			CA	.	.			GC						-	2
BVDV-2a1.3																																						
BVDV-2 Lees	.													AU	UA	CC		U	—	AU			UG				.	.			GC						-	2
BVDV-2a1.4																																						
BVDV-2 AZ Spl	GU													AA	UG	CU	AC	G	—	AU			UG				.	.			GU						—	1
BVDV-2 NY93	.													AA	UG	UC		G	—	AU			CG				.	.			GC						-	2
BVDV-2a1.5																																						
BVDV-2 890	.													UA	UG	UC		G	—	AU			CG			CG	.	AC			GC						—	3
BVDV-2a1.6																																						
BVDV-2 CD87	.													AA	UG	CU		A	—	GU			UA	GU			.	.			GU						-	2
BVDV-2 Munich 1	.													AA	UG	CU		G	—	GU			UA	GU			.	.			GU						-	2
BVDV-2a2																																						
BVDV-2 Munich 3	.													UA	CG	CU		G	—	AC			UA				.	.			AU			AC	CA		—	2
BVDV-2 Giessen-1	.													AA	UG	CU		G	—	AC			UA				.	.			AU			AC	CA		—	2
BVDV-2e																																						
BVDV-2	AU		GC	UG			UA	GC	AU		UA	CG	CG	GA	GA	UU	CC	A	—	GC			UA	AU	UA		GU	.	GU					GC	UA	CU	—	1

Table II. Continued.

Variable locus	V1																			V2								V3										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)
BVDV-2	**AU**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**AU**	**CG**	**UA**	**CG**	**CG**				**AU**				**CG**	**CG**		**AU**	**UA**	**GC**	**GU**	**AU**	**GU**	**CG**		**CG**	**CG**	**AU**	**UG**	**UC**	**—**	
BVDV-2B group																																						
BVDV-2b1.1																																						
BVDV-2 VS-63	.								GC				UA	AC	GG	CG		A	—	AU			UA		UG		.	.			AU					UU	U	2
BVDV-2b1.2																																						
BVDV-2 34b	.			UU					GC				UA	GA	GA	CG		A	—	GU			UA		UG		.	.			AU					CU	U.	3
BVDV-2b1.3																																						
BVDV-2 VS-123.4	.								GC				UA	GA	GA	CG	GU	G	—	AU			UA		UG		.	.			AU					CU	U	2
BVDV-2b1.4																																						
BVDV-2 LV96	GA								GC				UA	GA	GG	CG		A	—	AU			UA		UG		.	.		AU	AU					CU	U	2
BVDV-2b2																																						
BVDV-2 Soldan	.								GC				UA	GG	GA	CA	AC	A	—	AU			UA		UG		.	.			AU	UA				CU	U	2
BVDV-2c																																						
BVDV-2 i33283	.								GU		CG			GA	GA	CA	AA	GG	U	GU			UA		UG		.	.			AU					CU	C	3
BVDV-2d																																						
BVDV-2 354	.							AU					UA	GA	GA	CA	AA	G	—	AC			UA		CG		.	.		AU	AU				UA		U	3
Variable locus	V1																			V2								V3										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)
BVDV-3 (Hobi) * insertion G position 9r (divergence +1)																																						
Hobi	AU	UA	GC	UG	A	UA	CG	GC*	GC	CG	AU	UA	CA	AG	AG	—	—	—	—	AU	CG	UG	CG	AU	AU	GC	GC	AU	GC	GU	GC	UG	CG	AU	UA	UU	A	- 2
KhonKaen	AU	UA	GC	UG	A	UA	CG	GC	GC	CG	AU	UA	CA	AG	AG	—	—	—	—	AU	CG	CG	CG	AU	GU	GC	GC	AU	GC	GC	GG	UA	UA	GC	UA	UU	A	- 2
Variable locus	V1																			V2								V3										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)
BDV	**GC**	**UA**	**AU**		**A**	**UA**	**CG**	**AU**		**CG**	**AU**					**UG**		**—**	**—**	**AU**	**UG**	**CG**	**CG**		**GC**	**GC**	**GC**	**AU**	**AU**	**CG**	**AU**	**CG**	**CG**		**AA**	**—**	**—**	
BDV-a.1																																						
BDV A841/1	.			UU					AU			CU	AU	CG	AA		—	—	—	.				AU			.	.						UC		—	—	3
BDV V1414	.			UU					AU			AU	AU	UA	AA		—	—	—	.				AU			.	.						UC		—	—	3
BDV-a.2																																						
BDV Moredun cp	.			UG				CU	CC			GC	GU	CU	AG		—	—	—	.				UA			.	.						UU		—	—	2
BDV A1870	.			UG				CU	GC			GC	GU	CU	AG		—	—	—	.				UA			.	.						UU		—	—	2
BDV X818	.			UG					GC			GC	GU	CU	AG	CA	—	—	—	.			UA	CG			.	.						UC		—	—	2
BDV-a.3																																						
BDV BD31	.			UA				A.	GC			AC	AU	CC	AG	U	—	—	—	.			UA	CG			.	.						UC	AC	—	—	3
BDV-b.1																																						
BDV C27	.			UG				GC	AU			GC	GU	UA	GC	CU	GG	—	—	GU	UA	UG		CG		GU	.	.						UA	UA	—	—	2

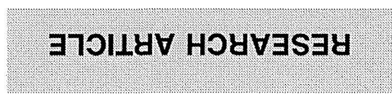

Table II. Continued.

Variable locus	V1																			V2								V3										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)
BDV-b.2																																						
BDV ZA1-1115	.			UG				GC	AU			GC	AU	CU	GG	UU	GU	G	—	GU	UA			CG		GU	.	.						UU	CA	—	—	2
BDV-c																																						
BDV 92-F-7119	.			UG				GC	AU			AU	AG	AU	UC	GU	GC	UU	—	AC	CG			UA			.	.						CC		—	—	1
BDV 90-F-6335	.			UG				GC	AU			AU	AU	AU	UC	GU	GC	UU	—	AC	CG			UA			.	.						CC		—	—	1
BDV-d.1																																						
BDV Rocco	.			UG				GC	AU			AU	AU	AU	GC		G	—	—	GU	UA			CG		GU	.	.						UU	CA	—	—	2
BDV C121	.			UG				GC	AU		UA	AU	AU	CU	AG		G	—	—	GU	UA			CG	AU	GU	.	.						UU	UA	—	—	2
BDV-d.2																																						
BDV AV	.			UG				GC	AU			AU	AU	CU	GG		—	—	—	GC	GU			CG			AC	.					CU	UC	CA	—	—	2
BDV 93-F-7289	.			UG				GC	AU			AU	AU	CU	GG		—	—	—	GU	GU			CG			AC	.					CU	UC	CG	—	—	2
BDV-d.3																																						
BDV 0502234	.			UU					AU		UA	AU	AU	CG	GU		G	—	—	GU	UA			CG	AU		.	.						AU		—	—	4
BDV 2112/99	.			UU					AU		UA	AU	AU	CG	GU		G	—	—	GU	UA		UA	CG	AU		.	.						AU		—	—	4
BDV M3	GU			UU				GC	AU		UA	AU	AU	CG	GU		G	—	—	GU	UA			CG	AU	GU	.	.						AC	UA	—	—	3
BDV-d.4																																						
BDV Chamois1	.			UG				GC	AU		UA	AU	AU	CC	UG		—	—	—	GU				CG	AU		.	.	AC				AG	UC	UA	—	—	1
BDV ARAN-1	.			UG				GC	AU		UA	AU	AU	CC	UG		—	—	—	GU	CG			CG	AU	GU	.	.	AC					UC	UA	—	—	1
BDV Orlu-R41	.			UG				GC	AU		UA	AU	AU	CU	UG	CG	—	—	—	CU	CG			UG	CG	AU	AC	.					CA	UU	UA	—	—	2
BDV CADI-1	.			UG				GC	AU		UA	AU	AU	CU	UG	CG	—	—	—	GU	CG	UG		CG	AU		AC	.					CA	UU	CA	—	—	2
BDV-e																																						
BDV Genzkow 701	.			UG				GC	GC			AU	UA	GA	CG	G	—	—	—	.	CG			UA			.	CU	GU					UU	AU	—	—	2
BDV Rentier Rudolph	.			UG					GC			AU	UG	GA	UG	G	—	—	—	.				UA			.	CU	UU					UU		—	—	3
BDV-f.1																																						
BDV 91-F-6732	.			UG				GC	AU			GC	GC	UA	GG	GG	—	—	—	.				AU			.	.	UA				AU	UG	U	—	—	1
BDV 91-F-6731	.			UG					GU			GC	GC	UA	GG	GU	—	—	—	.	CG	UA		AU			.	.	UA				AU	UG	U	—	—	3
BDV-f.2																																						
BDV 37A	AU			UG					AU			GC	GC	UA	AG	AU	—	—	—	.		AC		AU			.	.	UA				AC	UG	C	—	—	4
BDV 33S	AU			UG					AU		UA	GC	GC	UA	AG	AU	—	—	—	.		AU		AU			.	.	UA				CC	UG	C	—	—	4
BDV-g.1																																						
BDV Gifhorn	.		GC	UG				GC	GU		GC	GU	UA	CC	AG	UC	—	—	—	.				UA			.	.			GC	UG		CU	UG	A	—	0
BDV-g.2																																						
BDV 06-F-0083	.		GC	CG				GC	AU		GC	AU	CG	CU	AG		—	—	—	.	CG	UG		UA			.	.		UA	GC			UU	UC	—	—	1
BDV 85-F-588	.		GC	UG				GC	AU		GC	AC	CG	CU	AG		—	—	—	.	CG	UG		UA			.			UA	GC			UU	UC	—	—	1
BDV-h																																						
BDV/Burdur/05-TR	.		GC	UG					GC		CG	GC	GC	UC	AC	AA	—	—	—	.	CG		UA	GC		GU	.	.	AC					CG	CU	UU	UU	1
BDV/Aydin/04-TR	.			UG					GC			GC	GC	UC	AC	AA	—	—	—	.	CG		UA	GC		AU	.	.	GC	UG				CG	AU	UU	CU	2

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Table II. Continued.

Variable locus	V1																			V2								V3											
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10		(*)
BDV-2	**AU**	**UA**	**AU**		**A**	**UA**	**CG**	**GC**		**CG**	**GC**					**AU**		—	—	**GU**	**UG**	**CG**	**UA**		**GC**	**GC**	**GC**	**AU**	**GU**	**CG**	**AU**	**CG**	**CG**		**GU**	**A**	—		
BDV-2 712/02	.			UG					AU			CA	GC	CG	AC		G	—	—	.			UA	GC			.	.	GU					UU	GG	A	—		2
BDV-2 TO/121/04	.			UG					AU			UA	GC	CG	GC		G	—	—	.			UA	GC			.	.	GU					UA	GU	A	—		2
Variable locus	V1																			V2								V3											
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10		(*)
CSFV	**GU**	**UA**	**AU**	**UG**	**A**	**UA**	**CG**	**GC**	**GC**	**UA**	**GC**	**AC**	**GC**	**CG**	**AA**			—	—	**AU**	**CG**	**CG**	**CG**	**UA**		**GC**	**GC**	**AU**	**UA**	**CG**	**AU**	CG				—	—		
CSFV-a.1																																							
CSFV Alfort	.															CU	—	—	—	.					GU		.	.					AU	UA	U	—	—		2
CSFV Alfort 187	.			UU												GA	—	—	—	.					AU		.	.					AC	CA	C	—	—		3
CSFV Brescia	.															GA	—	—	—	.	AG				AU		.	.					AU	CA	C	—	—		3
CSFV-a.2																																							
CSFV Switzerland 1/93	.															CC	—	—	—	.					GU		.	.					AU	UA	U	—	—		2
CSFV Pader	.															GG	—	—	—	.					GC		.	.					AU	CA	C	—	—		2
CSFV-a.3																																							
CSFV Saitama/81	.															GA	—	—	—	AC					GU		.	.					AC	CA	A	—	—		2
CSFV-a.4																																							
CSFV Fukuoka/72	.											GC			GA	GA	—	—	—	.					AU		.	.					AC	CA	C	—	—		2
CSFV Honduras	.									UG	AU	GC				UG	—	—	—	.					AU		AC	.					AC	CA	C	—	—		2
CSFV-b																																							
CSFV 5440/99	.											GC				AG	UA	—	—	.				AU	GC	AC	.	.					UC	UA	A	—	—		2
CSFV-c																																							
CSFV Okinawa/86	.										AC	AU			GA	GA	—	—	—	.					GC		AC	AG					AU	CA	U	—	—		4
CSFV Kanagawa/74	.							AU		UG	AU	AU			GA	GA	—	—	—	.					GC		.	AG	AU					CA	U	—	—		3
PRONGHORN	AU	GC	GC	UG	A	UA	CG	UA	UA	CG	CG	UA	CG	AU	GU	—	—	—	—	GU	CG	CG	GC	GC	GU	GU	GU	AU	GU	UA	AU	GA	CA	—	—	—	—		4
GIRAFFE	AU	CG	AU	UU	A	UA	CG	AU	AU	CG	GC	CG	AU	NU	AU	AG	UG	A	—	AC	CG	CG	UA	AU	UA	CG	GC	AU	GC	UA	CG	CG	AU	GU	UA	—	—		7
BUNGOWANNA	CG	AU	GC	UG	A	GC	CG	UA	UA	GC	GC	AC	AA	AC	GU	GA	—	—	—	UA	CG	AU	GC	UA	AU	GU	GC	AU	GU	CG	UA	GU	CG	CG	CG	AU	GA	A	7

Table III. Example of sequence secondary structure alignment applied for the determination of the degree of genetic homogeneity among sequences clustered in a single group (PNS method numerical taxonomy qualitative verification-first step). Highly conserved base pair positions are excluded. Species identification divergence limit value: 13. Y: G or U. HV: highly variable.

Variable locus	V1																			V2								V3									
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10 (*)
Type 47																																					
BVDV1	**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		**—**	**—**		**CG**	**CG**	**UA**	**GC**			**GC**	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**
BVDV1 1/B/01	.								AU		CG		CA	AG	GA	AA	A	—	—	AU					GC	AC	.	.	GU		AU			CA	UC	UC	. 0
BVDV1 Europa	.								AU		CG		CA	GG	GA	AA	—	—	—	AU					GC	AC	.	.	GU		AU				UU	GA	. 0
BVDV1 Lamspringe 735	.								AU		CG		CA	GG	GA	AA	—	—	—	AU					GC	AC	.	.	GU		AU				UU	AC	. 0
BVDV1 SE5726	.								AU		CG		CA	GG	GA	AA	—	—	—	AU					GC	AC	.	.	GU		AU				UC	AC	. 0
BVDV1 Vkl	.								AU		CG		CA	GG	GA	AA	—	—	—	AU					GC	AC	.	.	GU		AU				UC	UC	. 0
BVDV1 16484/93	.								AU		CG		CA	AG	AA	A	—	—	—	AU					GC	AC	.	.	GU		AU			CG	UC	UC	. 0
BVDV1 SE5572	.								AU		CG		CA	AG	AA	AA	—	—	—	AU					GC	AC	.	.	GU		AU				UA	AC	. 0
BVDV1 SH9/11	.								AU		CG		CA	GU	GA	AA	—	—	—	AU					GC	AC	.	.	GU		AU				UC	AC	. 0
BVDV1 9466/91	.								AU		CG		UG	AG	GA	AA	A	—	—	AU					GC	AC	.	.	GU		AU		UG		UU	UC	. 0
BVDV1 16-111	.								AU		CG		AA	GG	AA	AA	—	—	—	AU					GC	AC	.	.	GU		AU				UC	UC	. 0
BVDV1 3479-97-I	.		AU						AU		CG		AU		GG	AA	A	—	—	AU					GC	AC	.	.	GU		AU				UU	AC	. 1
BVDV1 Europa	5																																				
BVDV1 Lamspringe 735	5			1																																	
BVDV1 SE5726	4			2				1																													
BVDV1 Vkl	3			2				2				1																									
BVDV1 16484/93	4			5				6				5			4																						
BVDV1 SE5572	5			4				2				3			4			4																			
BVDV1 SH9/11	4			3				2				1			2			5		3																	
BVDV1 9466/91	4			5				5				6			5			7		6			6														
BVDV1 3596/86	6			3				2				3			4			8		5			4			7											
BVDV1 16-111	5			4				4				3			2			4		4			4			6		6									
BVDV1 3479-97-I	7			6				5				6			7			9		6			6			6		7			7						
	1/B/01			Europa				Lamspringe 735				SE5726			Vkl			16484/93		SE5572			SH9/11			9466/91		3596/86			16-111						

Values from 1 to 9, Subtype relative divergence % 0, Result: Homogeneous.

Table IV. Example of sequence secondary structure alignment for the determination of genetic distance between genogroups (PNS method numerical taxonomy qualitative verification-second step). Highly conserved base pair positions are excluded. Species identification divergence limit value: 13. Y: G or U. HV: highly variable.

Variable locus	V1																			V2								V3										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)
Type 99x106																																						
Type 99																																						
BVDV2	**AU**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**AU**	**CG**	**UA**	**CG**	**CG**				**AU**				**CG**	**CG**		**AU**	**UA**	**GC**	**GU**	**AU**	**GU**	**CG**		**CG**	**CG**	**AU**	**UG**	**UC**	**—**	
BVDV2 VS-123.4	.								GC				UA	GA	GA	CG	GU	G	—	AU			UA		UG		.	.			AU					CU	U	2
BVDV2 VS-63	.								GC				UA	AC	GG	CG		A	—	AU			UA		UG		.	.			AU					UU	U	2
Type 106																																						
BDV	**GC**	**UA**	**AU**		**A**	**UA**	**CG**	**AU**		**CG**	**AU**					**UG**		—	—	**AU**	**UG**	**CG**	**CG**		**GC**	**GC**	**GC**	**AU**	**AU**	**CG**	**AU**	**CG**	**CG**		**AA**	—	—	
BDV JH2816	.			UG					GC			GC	GU	CU	AG		—	—	—	.				CG			.	.						UC		—	—	2
BDV ChlEs	.			UG					GC			GC	AU	CU	AG		—	—	—	.	UA			CG			.	.						UC	AC	—	—	3
BVDV2 VS-123.4	19	19																																				
BVDV2 VS-63	19	19																																				
	BDV JH2816	ChlEs																																				
Values 19, Subtype relative divergence % 100, Result: Divergent																																						
Type 99 × 108																																						
Type 99																																						
BVDV2	**AU**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**AU**	**CG**	**UA**	**CG**	**CG**				**AU**				**CG**	**CG**		**AU**	**UA**	**GC**	**GU**	**AU**	**GU**	**CG**		**CG**	**CG**	**AU**	**UG**	**UC**	**—**	
BVDV2 VS-123.4	.								GC				UA	GA	GA	CG	GU	G	—	AU			UA		UG		.	.			AU					CU	U	2
BVDV2 VS-63	.								GC				UA	AC	GG	CG		A	—	AU			UA		UG		.	.			AU					UU	U	2
Type 108																																						
BVDV2	**AU**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**AU**	**CG**	**UA**	**CG**	**CG**				**AU**				**CG**	**CG**		**AU**	**UA**	**GC**	**GU**	**AU**	**GU**	**CG**		**CG**	**CG**	**AU**	**UG**	UC	—	
BVDV2 354	.							AU					UA	GA	GA	CA	AA	G	—	AC			UA		CG		.	.	AU		AU				UA		U	3
Values 8 11, Subtype relative divergence % 0, Result: Related.																																						

for research work through identification of peculiar characteristics in strategic genomic regions.

With about 80 nucleotides, the palindromic loci represented a very limited portion of the virus genome. Within these short sequences, only 21 nucleotides were sufficient for the evaluation to obtain, with certitude, the characterization of the genus. BVDV-1 species was characterized through the evaluation of only 7 nucleotides. Similarly, genotypes were defined with only 6 to 10 nucleotides. These peculiar aspects sum up the high specificity of the PNS method and the reliability of the provided results. Accuracy for typing could be obtained also at sub-genotype level of genotype BVDV-1b. The two sub-genotypes could be identified with changes in only one base- pairing at the level of V2 locus in position 7: BVDV-1b1–AU; BVDV-1b2–G-C or G*U. The PNS method can provide a clear picture of species and genotype boundaries, due to the exclusive consideration of strategic and highly conserved regions, and consequently helps to avoid unclear classification, since PNS in base-pairings corresponds to radical evolutionary changes, which can generate new species and genotypes. The results of the PNS method are essentially qualitative, through the identification of base-pair markers for species and genotype definition. However, the quantitative verification of relatedness between species, and segregation into genotypes supported the rationale for the PNS procedure and confirmed the reliability of the keys for identification of genotypes and the results provided by the method. Values of divergence, according to changes in nucleotide base-pairs, through their mean values, provided clear indications on relatedness among species genotypes and divergence in the species. At genotype level, values expressed the heterogeneity among strains in genotypes. Homology among genotypes changed with low values indicating strong correlation and higher values indicated a range of divergence among genotypes reaching marked genetic distance. The identification of homogeneous groups within genetically heterogeneous species was important to define the theoretical phylogenetic origin of the virus strains. For example, in the BVDV-1 species, the two main evolutionary branches could be identified. Both were related to genotypes BVDV-1a and BVDV-1b, core of the species, and possible prototypes in the species. The location of base-pair changes in the three variable loci could be identified for each evolutionary step generating the different genotypes. The relatedness among genotypes was characterized by shared base-pairs and occurrence of further stable mutations. Mean values of divergent base-pairs, indicated and quantified the genetic relatedness among genotypes in the species. These values reflected nucleotide characteristic in the specific base-pairs, which were either shared by related genotypes or divergent thereby indicating genetic distance among them. This aspect was fundamental, since characteristic and un-shared base-pairs were very limited only partially allowing the discrimination of genotypes in the species and providing only qualitative indications of segregated genotypes not sufficiently explanatory of genetic relatedness. In order to define a rationale for identification, genotypes were also identified by specific combination of base- pairings in the sequence. These base-pairings were non-specific when considered separately and expressed phylogenetic markers. Stable mutation characteristic for each genotype could be identified at the level of the different variable loci, giving rationale to relatedness among genotypes in the species.

Due to the world-wide distribution and economic importance of the *Pestivirus* species, with respect to difficulties in the control of the diseases, it is important to understand the genetic differences among virus isolates, their evolutionary history and the impact of the genetic peculiarities on virulence and epidemiology. The identification of viral types or subtypes based on genetic changes should improve our understanding of virus epizootiology and will provide markers for biological difference flanked by the development of in vitro criteria for virulence. Genetic markers for taxonomy could be applied for accurate phylogenetic analysis and potential indication of the virus evolutionary history. Knowledge of different phenotypic features is in particular necessary for the genus *Pestivirus*, which constitutes a group that seems to get more complicated as new isolates are described, despite the fact that, in the majority of the cases, the genomic portion under study is the most conserved, the 5'-UTR. This is in agreement with the wide range of clinical signs associated with *Pestivirus* infections and the problems, faced in controlling these infections. Sequence evaluation based on the secondary structure analysis of the palindromic structures in the 5'-UTR, applied for numerical analysis of genetic markers of virus biological activity, such as tissue affinity, virulence, and related pathological disorders, host range and capacity to cross species barrier, could be used as indicators of the impact of virus infection to greater advantage, an improvement in contrast to available software programs, limited to phylogenetic analyses. The above mentioned aspects will contribute to the improvement of diagnostic and characterization methods in defining appropriate control and prophylactic measures.

4. CONCLUSION

The useful application of the PNS method appeared an appropriate approach for differential diagnostic in order to solve cross-infections which may obscure the rationale for the definition of the *Pestivirus* species according to their animal host. Nomenclature for *Pestivirus* species is predominantly dependent on the animal host species from which they were isolated. There is extensive antigenic cross-reactivity among species, and, they can cross the host species barrier and infect various animal species, even

in case of CSFV, long believed an exception and apparently restricted to swine. Furthermore, PNS is based on the analysis of secondary structure at the level of palindromic variable loci in the 5'-UTR genomic RNA, well conserved and critical region in pestiviruses, thus providing numerical taxonomy accuracy of the genus *Pestivirus* and identifying nucleotide variations that at this level assume high importance in terms of virus evolutionary history.

The preparation of the software for the PNS method removed the main drawback due to manual searching of relevant base-pairings and direct observation of the sequence, simplified the genotyping procedure for users' easy access and rapid testing with reliable results, allowing the consideration of secondary structures predicted at the three variable regions in the 5'-UTR for the classification of *Pestivirus*. The PNS software version 2.0 improved the presentation of the secondary structure sequences, providing variable loci alignment useful for the evaluation of relatedness among strains at the level of strategic genomic sequences. This aspect could be important also for possible adaptation of the methodology to other positive polarity RNA virus species, such as Poliovirus or Hepatitis C virus.

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Prevalence of antibodies against Parainfluenza virus type 3, Respiratory syncitial virus and bovine Herpesvirus type 1 in sheep from Northern Prefectures of Japan

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Summary
Ovine sera collected in the Prefectures of Hokkaido, Aomori and Iwate in the Northern Japan were examined for the presence of antibodies against Respiratory syncytial virus (RSV), bovine Herpesvirus type 1 (infectious bovine rhinotracheitis: IBR) and Parainfluenza virus type 3 (PIV3) using serum neutralisation (SN) and enzyme-linked immunosorbent assay (ELISA) tests. Twenty-three animals (11.73%) out of the 196 tested were sero-positive to PIV3. Sixteen animals (8.69%) out of the 184 tested reacted to RSV. No animals were positive to IBR antigen. Sero-conversions to PIV3 were detected in Hokkaido and Iwate (14.92% and 8.82%, respectively). Antibodies against RSV were detected in Hokkaido (9.23%) and Aomori (14.28%). Although no diagnostic measures were in place, the infections did not appear to be related to any reduction in sheep productivity.

Prevalenza di anticorpi contro il virus Parainfluenzale di tipo 3, il virus Respiratorio sinciziale e l'Herpesvirus bovino di tipo 1 in pecore nelle Prefetture settentrionali del Giappone

Parole chiave
Giappone,
Pecore,
Rinotracheite infettiva bovina,
Virus Parainfluenzale di tipo 3,
Virus Respiratorio sinciziale.

Riassunto
Sieri ovini, raccolti nelle prefetture settentrionali del Giappone (Hokkaido, Aomori e Iwate), sono stati esaminati per la presenza di anticorpi contro il virus Respiratorio sinciziale (RSV), l'Herpesvirus bovino di tipo 1 (rinotracheite infettiva bovina: IBR) e il virus Parainfluenzale di tipo 3 (PIV3) applicando i test di siero neutralizzazione (SN) ed enzyme-linked immunosorbent assay (ELISA). Ventitré animali (11,73%) su 196 campioni testati sono risultati sieropositivi per PIV3. Sedici animali (8,69%) su 184 campioni testati hanno reagito per RSV. Nessun animale è risultato positivo per antigene IBR. Seroconversioni verso PIV3 sono state identificate nelle prefetture di Hokkaido e Iwate (rispettivamente 14,92% e 8,82%). Anticorpi contro RSV sono stati riscontrati nella prefettura di Hokkaido (9,23%) e in quella di Aomori (14,28%). Sebbene non siano state applicate misure diagnostiche, le infezioni non hanno mostrato nessuna relazione con una riduzione di produttività nelle pecore.

Respiratory disorders are among the most important problems associated with small ruminant health, causing morbidity and mortality. Respiratory syncytial virus (RSV) and Parainfluenza virus type 3 (PIV3) are among the most well known diseases that affect the respiratory system of sheep and goats (1, 2). Sheep are susceptible to bovine Herpesvirus type 1 (BoHV1), agent of infectious bovine rhinotracheitis (IBR). This is a pathogen of worldwide importance, which primarily affects cattle. So far, the studies conducted on respiratory viral infections in Japan have been mainly focused on cattle (7, 8, 9), hence only scarce information is available on epidemiology of virus pathogens in sheep. No previous epidemiological surveys on RSV, PIV3 or IBR have been undertaken on small ruminants in Japan. Furthermore, no clinical cases due to these infections have been reported among sheep flocks.

To explore the presence of the RSV, PIV3 and IBR and to obtain a preliminary picture of their epidemiology, a serological survey was carried out from September 2007 to January 2008 in the Prefectures of Hokkaido, Aomori and Iwate in the Northern Japan, where the majority of the Japanese sheep, a total of 4,775 sheep (43%), are bred. Details of the sampling methodology and descriptions of the flocks have been reported (5).

The presence of antibodies against PIV3 and RSV was determined by using serum neutralisation (SN) test. In a 96-well plate, inactivated serum samples were diluted from an initial dilution of 1:2 by doubling and placed in contact with $100TCID_{50}$ of previously titrated PIV3 SF-4 or RSV RB-94 strains. After incubation for 1h at 37°C with 5% CO_2 to enable viral neutralisation, 5×10^5/ml of Madin-Darby bovine kidney (MDBK) cells - suspended in minimum essentials medium (MEM) (Eurobio, Cortaboeuf, France) and containing penicillin 100IU/ml, streptomycin 100µg/ml, gentamicin 5µg/ml, nystatin 50 IU/ml and 10% foetal calf serum (FCS) (Sigma, Hamburg, Germany) - were added to each well. After 5 days, the cytopathic effect (CPE) in the wells was evaluated and the antibody titre was defined as the highest serum dilution able to inhibit at least 75% of the virus' CPE. Positive and negative reference sera, cell and virus controls (Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Teramo, Italy) were included in each plate.

Serological testing for antibodies against BoHV1 glycoprotein B was performed by enzyme linked immunosorbent assay (ELISA), using a commercial kit (IDEXX IBR gB, IDEXX, Westbrook, Maine, USA), following the manufacturers' instructions.

As for the flock production, the annual lambing rate was calculated as number of lambs born per ewes exposed to the ram and it was based on a lambing season occurring from February to April, with an exception being made for 1 farm where the reproductive cycle was related to 3 breeding seasons. The proportions of screened pathogens infection rate of the sampled animals were compared using the Pearson's correlation coefficients statistics in order to calculate the possibility of a relationship between the prevalence of infection and production parameters such as annual lambing rate, annual lamb mortality rate and annual adult mortality rate. Differences were considered to be significant at $P<0.05$.

Results of serological screening for antibodies to RSV, PIV3 and IBR in sheep from each Prefecture of the Northern Japan are summarized in Table I. All the 267 sera were submitted to IBR testing. Not all the samples were applicable to serological tests for RSV and PIV3 antigens (Table I). Some sera showed cytotoxicity (indicated by cell death, probably caused by the sub-optimal condition of the samples) or they were not tested due insufficient serum quantity. All such samples (n = 83 for RSV and n = 71 for PIV3) were then excluded.

The SN test revealed 23 samples out of the 196 sera examined positive for anti-PIV3 immunoglobulins (Table I); this corresponds to a prevalence of 11.73%. At flock level positiveness ranged between 5.55% and 88.23%, whereas titres ranged from 1:8 to 1:256. PIV3 infection was detected in 5 out of the 14 sampled flocks. Levels of infection were found in flocks from Hokkaido and Iwate Prefectures, but not in the Aomori Prefecture. Four Suffolk, 1 Cheviot, 1 Corriedale, and 17 cross-breeds, mainly Suffolk × Cheviot, 1 rams and all the other ewes, were affected.

RSV infection was detected in 3 flocks, with an overall prevalence of 8.69%; 16 animals, out of 184 sera

Table I. *Results of serological screening for antibodies to IBR, RSV and PIV3 in sheep from the Prefectures of Hokkaido, Aomori and Iwate in the Northern Japan.*

	Positive	% Positive*	Negative	NE	Toxic	Total
IBR (ELISA)	0	0	267	–	–	267
RSV (SN)	16	8.69	168	64	19	267
PIV3 (SN)	23	11.73	173	69	2	267

NE = not executed due to insufficient aliquots for testing; * Percentage computed excluding samples resulting toxic or not tested for insufficient serum quantity.

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examined, were observed to be positive for anti-RSV immunoglobulins (Table I). Positive sera originated from 2 flocks from the Hokkaido Prefecture and 1 flock from the Aomori Prefecture. None of the sera collected from the Iwate Prefecture were found to be positive. The percentage of positive sheep was 9.23% and 14.28% in Hokkaido and Aomori Prefectures, respectively. The average incidence of seropositive animals in individual herds was 13.33%, 26.66% and 55.55% respectively for the 3 sampling groups from sero-positive flocks. The obtained titres with SN were 1:256 in all tested positive sera. The seropositive sheep were all females and except for 1 ram. The sheep were of different breeds, 4 Suffolk, 10 Suffolk x Cheviot cross breeds, and 2 Romanov x Poll Dorset x Suffolk cross breeds.

The variation of prevalence of the different infections among the 3 Prefectures is reported in Table II. The analysis of the percentage of sheep positive for antibodies to RSV and PIV3 per age categories showed that for both the pathogens no seroconversions were present in animals of 1 and 2 years of age and in animals older than 7 years. Seroconversion was related mainly to single infections. However, antibodies against RSV and PIV3 were simultaneously identified in 10 animals from the same flock (sample 5 from Hokkaido Prefecture).

Table II. *Comparison of the 3 Prefectures in Northern Japan for the percentage of sheep positive for antibodies to the different respiratory viruses.* No animals reacted to infectious bovine rhinotracheitis antigen.

	Positive (%)	
	PIV3	**RSV**
Hokkaido	14.92	9.23
Iwate	8.82	0
Aomori	0	14.28
Total	11.73	8.69

PIV3 = Parainfluenza virus type 3; RSV = Respiratory syncytial virus.

The assessment of the possible impact of RSV and PIV3 infections on the production levels in the sampled flocks did not reveal a clear correlation with the reported levels of seropositive animals (Table III). However, with concern to the annual lamb mortality rate, it is noteworthy that in 4 flocks losses of 20% or more have been reported. In 3 out of 4 of these flocks animals were found seropositive to RSV. Although no diagnostic measures were in place and the observation was not statistically significant (p = 0.05539), this may suggest a relation of RSV infection with lamb mortality.

All the 267 samples collected were tested for IBR antibodies. None of the tested animals resulted serologically positive.

This survey demonstrates positiveness for antibodies to PIV3 and RSV in sheep flocks in the Northern Prefectures of Japan, where the majority of the Japanese sheep are bred. The survey also provides the first serological evidence of the occurrence of these diseases in sheep in the country. Interviews with farmers revealed that no previous investigations on these pathogens have been carried out in all of the randomly selected sampling units for this study,

Table III. *Comparison of different production parameters for the percentage of sheep positive for antibodies to Respiratory syncytial virus and Parainfluenza virus type 3.*

Flock No.	Prefecture	RSV	PIV3	Annual lambing rate	Annual lamb mortality rate	Annual culling rate	Mortality rate among adults
1	Hokkaido	0	21.42	NR	NR	NR	5
2	Hokkaido	0	0	0.72	1.29	0	4.76
3	Hokkaido	0	0	1.62	3.46	14.77	9.2
4	Hokkaido	NE	NE	1.1	12.78	2.97	0
5	Hokkaido	55.55	88.23	1.61	20	11.73	8.33
6	Hokkaido	0	0	1.48	17.09	10.33	9.09
7	Hokkaido	0	0	1.58	16.92	6.66	2.22
8	Hokkaido	13.33	0	2.44	20.53	NR	NR
9	Hokkaido	0	5.55	1.23	0	0	0
10	Hokkaido	0	50	NR	NR	NR	10
11	Iwate	0	0	1.61	6.89	0	11.76
12	Iwate	0	17.64	1.38	9.83	24.03	4.8
13	Aomori	0	0	1.54	25.35	17.64	2.94
14	Aomori	26.66	0	1.14	21.87	0	9.09

NE = not executed due to insufficient aliquots for testing; NR = not recorded.

which then should be regarded as the first source of preliminary information on the epidemiology and distribution of such pathogens for the years 2007 and 2008.

The demonstration of respiratory virus circulation in sheep flocks in the Northern Prefectures of Japan, based on serological analysis, advanced the knowledge on pathogens affecting domestic sheep in Japan. These interesting findings deserve further evaluations in order to examine the full extent of the problem in small ruminant populations, taking into account that infections with PIV3 and RSV are characterized by a potential negative impact on animal health (1, 2), also indirectly, predisposing lambs to a severe pneumonia caused by several serotypes of *Pasteurella haemolitica* (enzootic pneumonia) (3, 10, 11, 12).

Furthermore, while ovine farming is a relatively minor sector in Japan - the population is constituted by 11,000 heads (according to the Japan Livestock Industry Association 2000) – it is worth considering that in some farms other domestic animals, i.e. cows, were housed close to sheep pens or had access to common pastures. A sheep flock (sample 7 from Hokkaido Prefecture) originated from a farm mainly focused on dairy cattle breeding, thus being in close contact with a herd of 700 black Japanese cows. Preventive measures should be carefully considered to avoid diffusion and impact on valuable breeding cattle farming. This is evident if one were to consider the potential adverse effects, both direct or indirect, on production of these pathogens detected in sheep and to take into account that in cattle PIV3 and RSV are among the main causes of respiratory disorders (6) along with bovine diarrhoea virus (BVDV). The same goes for IBR, which is known to cause major welfare and economic problems in cattle, the potential for infection in sheep remains consistent when considering that the infection is present in cattle (8, 9) and in particular it is most frequent in Hokkaido, as indicated by reports from 2005 to 2011, which described up to 42 outbreaks in 2009 (14). The importance of sheep in the epidemiology of IBR remains limited, considering the lower capacity of spreading the virus (4). However, according to the World Animal Health Organisation (*Office International des Épizooties*: OIE), IBR is included in the list of reportable diseases of importance to international trade (13).

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Note

Salivary Nitrate and Nitrite May Have Antimicrobial Effects on *Desulfovibrio* Species

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The antibacterial effects of salivary nitrate/nitrite on the growth of three *Desulfovibrio* species were examined. The bacteria did not grow on plates with ≥0.2 mM nitrate or ≥1.0 mM nitrite. They were also incubated in filter-sterilized saliva. *D. desulfuricans* was reduced on the order of $>10^2$ compared with the control solution (phosphate-buffered saline) in nine out of the 10 participants.

Key words: nitrate; nitrite; saliva; *Desulfovibrio*

Dietary nitrates, mainly from vegetables, fruits, and water, are absorbed into the bloodstream from the stomach and small intestine and concentrated by a factor of 10 to 20 by the salivary glands.[1)] Both diet and indigenous nitrate due to the oxidation of nitric oxide synthesized from L-arginine are significant sources of plasma nitrate.[2)] Salivary nitrate is then reduced to nitrite by oral microorganisms are usually found in saliva at concentrations of 0.3 to 2.6 mM[3)] and 0.2 to 2.0 mM.[4)] Salivary nitrate/nitrite might have antimicrobial effects against food and water-borne pathogens.[5)] We hypothesize that the addition of a physiological level of nitrite to gastric acid renders it bactericidal, possibly because of acidified nitrite and other active nitrogen species.[5)] Acidified nitrite is also antibacterial against oral pathogens, such as the cariogenic bacteria, *Streptococcus mutans*,[6)] and periodontal bacteria, including *Fusobacterium nucleatum*, *Eikenella corrodens*, and *Porphyromonas gingivalis*.[4)]

The addition of nitrate is effective in preventing the production of highly toxic hydrogen sulfide, a metabolite of anaerobic sulfate-reducing bacteria (SRB), under environmental conditions, as in waste-water and oil reservoirs.[7,8)] Adding nitrate causes an increase in the redox potential level and can result in bacterial growth inhibition.[7)] The genus *Desulfovibrio*, a common SRB in the human intestine,[9)] includes opportunistic pathogens[10)] and is considered to be an etiologic agent associated with chronic periodontitis[11)] and ulcerative colitis.[12)] To our knowledge, no information is available on the effect of salivary nitrate/nitrite on *Desulfovibrio* spp. growth. Hence, the purpose of this study was to investigate the effect of salivary nitrate/nitrite on the growth of *Desulfovibrio* species before and after the ingestion of vegetables that contain high levels of nitrates.

D. desulfuricans (NRBC 13699) and *D. vulgraris* (NRBC 104121) were purchased from the Biological Resource Center (National Institute of Technology and Evaluation, Chiba, Japan). *D. piger* (GAI 05137) and *D. fairfieldensis* (GAI 05146) were provided by the Division of Anaerobe Research, Life Science Research Center, Gifu University. To date, the four *Desulfovibrio* species described above have been isolated from humans.[13)] Each species was inoculated onto Wilkins-Chalgren Anaerobe Agar Medium (Oxoid, Basingstoke, UK) and checked for purity, then cultured on nitrate- or nitrite-containing medium. The composition of the Wilkins-Chalgren Anaerobe Medium (pH 7.1 ± 0.2) was 10.0 g/L of tryptone, 10.0 g/L of gelatin peptone, 5.0 g/L of yeast extract, 1.0 g/L of glucose, 5.0 g/L of sodium chloride, 1.0 g/L of L-arginine, 1.0 g/L of sodium pyruvate, 0.005 g/L of hemin, and 0.0005 g/L of menadione. All bacterial species were incubated at 37 °C in a container with Anaeropack-Anaero (Mitsubishi Gas Chemical, Tokyo, Japan).

Each bacterial species was inoculated onto nitrate- or nitrite-containing Chalgren-Anaerobe agar plates in duplicate with an inoculation loop. The final medium concentrations were adjusted to 0.2, 0.5, 1.0, 5.0, 10.0, and 20.0 mM by adding a solution of 200 mM sodium nitrate or of 200 mM sodium nitrite (Kanto Chemical, Tokyo, Japan). All the plates were incubated at 37 °C in a container with Anaeropack-Anaero (Mitsubishi Gas Chemical, Tokyo, Japan). The experiment enrolled 10 healthy university students and staff volunteers (nine male and one female, aged 22 to 42 years). Written informed consent was obtained from all participants, and the study protocol was approved by the Human Ethics Committee of Iwate University. The participants were asked to refrain from eating vegetables at breakfast. Before and 30 to 40 min after eating 150 g of lettuce, which is tantamount to 159 mg of nitrate,[14)] 8 to 9 mL of whole saliva was collected. The nitrate/nitrite concentration in saliva usually rises within 30 min after nitrate loading, and remains at a high level for 4 h.[1)] Approximately 6.5 mL of each saliva sample was centrifuged at 3,500 rpm (8,000 *g*) for 5 min. The supernatant liquid was filtered with a filter (Puradisk™ 25 mm, GE

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Healthcare UK, Buckinghamshire, UK). After incubation, all bacterial cells were harvested and suspended in 1 mL of phosphate-buffered saline (PBS) solution at pH 7.4 yielding a final concentration of approximately 10^9 cells/mL (turbidity, McFarland's no. 6). Ten μL of each bacterial suspension was inoculated into 1 mL of filter-sterilized saliva or PBS solution as negative control. The culture was incubated at 37 °C for 4 h. After 4 h, 100 μL of bacterial slurry containing the *Desulfovibrio* species was serially diluted to 10^{-2}, 10^{-3}, and 10^{-4} in PBS solution for viable bacterial counting. The bacterial suspension and the control were also serially diluted, to 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. One hundred μL of each dilution was inoculated onto Chalgren-Anaerobe plates and incubated at 37 °C in a container with Anaeropack-Anaero (Mitsubishi Gas Chemical, Tokyo, Japan). Each whole saliva sample was diluted 100-fold with deionized water and filtered (Whatman Grade no. 2). The nitrite concentrations were measured by a standard colorimetric method involving diazotization with sulphanilamide and coupling with *N*-(1-naphthyl)ethylendiamine to form an azo-dye, which was measured spectroscopically at 540 nm. Nitrate was measured after reduction to nitrite by passing it though a cadmium-copper column. A rate of more than 95% reduction was obtained with 10 μM of standard solution.

D. desulfuricans colonies were observed after 48 h of incubation, while *D. vulgraris* and *D. fairfieldensis* colonies were not observed until 5 to 7 d of incubation. *D. piger* colony was not observed after 10 d of incubation. Hence, *D. desulfuricans*, *D. vulgraris*, and *D. fairfieldensis* were used in subsequent experiments. The results of the nitrate/nitrite plate test are described in Table 1. No *Desulfovibrio* species grew on any of the nitrate plates. *D. desulfuricans* and *D. vulgraris* grew on the plates with a nitrite concentration ≤0.5 mM, while *D. fairfieldensis* did not grow on any nitrite plates. The concentrations of nitrite increased after ingestion of lettuce, from 0.35 ± 0.10 to 1.24 ± 1.76 mM. The nitrate concentration also increased, from 0.99 ± 0.69 to 2.26 ± 1.24 mM (Table 2). The number of *D. desulfuricans* (cfu/mL) was reduced in all the participants. A 10^2 order of reduction was seen in nine out of 10 participants after incubation of pre- or post-prandial saliva. The difference between pre- and post-prandial saliva was not clear, because an increased number of *D. desulfuricans* (cfu/mL) was seen in three participants. *D. vulgraris* grew in the control solution, and

Table 1. Growth of *Desulfovibrio* Species on Nitrate and on Nitrite Plates

		0.2	0.5	1	5	10	20 mM
D. desulfuricans	NO^{2-}	+	+	−	−	−	−
	NO^{3-}	−	−	−	−	−	−
D. vulgraris	NO^{2-}	+	+	−	−	−	−
	NO^{3-}	−	−	−	−	−	−
D. fairfieldensis	NO^{2-}	−	−	−	−	−	−
	NO^{3-}	−	−	−	−	−	−

NO^{2-}, nitrite; NO^{3-}, nitrate
+, grew; −, did not grow

Table 2. Effects of Pre- and Post-Prandial Salivary Nitrate/Nitrite Concentrations on *Desulfovibrio* Species

Participants		Nitrite (mM)	Nitrate (mM)		*D. desulfuricans* cfu/mL	*D. vulgraris* cfu/mL	*D. fairfieldensis* cfu/mL
				Bacterial solution	4.0 × 10^7	2.3 × 10^6	ND
				Control	4.9 × 10^7	6.1 × 10^6	ND
1	Preprandial	0.31	0.34		ND	ND	ND
	Postprandial	1.40	0.97		9.0 × 10^5	ND	ND
2	Preprandial	0.41	1.90		1.47 × 10^5	ND	ND
	Postprandial	5.90	2.36		1 × 10^3	3 × 10^3	ND
3	Preprandial	0.23	0.11		6.9 × 10^4	ND	ND
	Postprandial	0.52	4.10		8.7 × 10^5	ND	ND
4	Preprandial	0.31	1.61		ND	ND	ND
	Postprandial	0.24	2.20		ND	ND	ND
5	Preprandial	0.32	1.20		7.2 × 10^4	3.0 × 10^4	ND
	Postprandial	1.97	3.42		1.98 × 10^6	8.9 × 10^4	ND
6	Preprandial	0.52	1.95		4.3 × 10^6	ND	ND
	Postprandial	1.62	0.16		5.1 × 10^5	ND	ND
7	Preprandial	0.26	1.25		ND	ND	ND
	Postprandial	1.26	2.90		ND	ND	ND
8	Preprandial	0.32	0.75		1.08 × 10^5	ND	ND
	Postprandial	1.30	3.56		ND	ND	ND
9	Preprandial	0.32	0.67		1 × 10^3	ND	ND
	Postprandial	0.12	1.10		ND	7 × 10^3	ND
10	Preprandial	0.49	0.11		6.2 × 10^6	1.0 × 10^4	ND
	Postprandial	0.63	0.36		3.8 × 10^6	2.0 × 10^4	ND
mean ± SD	Preprandial	0.35 ± 0.09	1.50 ± 1.67				
	Postprandial	0.99 ± 0.70	2.11 ± 1.40				

ND, not detected

colonies were detected on 6 out of 20 plates after incubation with the saliva solution, and the number of cfu/mL was reduced on the order of $>10^2$ as compared to control. *D. fairfieldensis* did not grow on any plates in this experiment (Table 2).

SRB are strict anaerobes, and it might have been expected that they would be found primarily at sites at which the redox potential is low, as in subgingival plaque. However, SRB is commonly found at higher redox sites as the posterior tongue, anterior tongue, and vestibular mucosa in the oral cavity of healthy adults.[15,16] Willis *et al.*[15] have suggested that oxygen-tolerant SRB strains may be expected to grow in areas of unfavorable redox potential, such as the oral cavity. Many studies have indicated that nitrate also eliminates odors caused by sulfate-reducing bacteria. Our results, which indicate that microorganism growth is inhibited even at 0.2 mM nitrate, are consistent with previous studies. Myhr *et al.*[8] have reported that injection of 0.5 mM nitrate over 2.5 to 3.5 months led to complete elimination of hydrogen sulfide in an oil reservoir model column. In fecal batch culture, the addition of nitrate to a final concentration of 1.0 mM resulted in a 68% reduction in hydrogen sulfide after a 24 h of incubation.[17]

After incubation in saliva, the number of *Desulfovibrio* colonies was significantly reduced as compared to the control, indicating that saliva has antibacterial effects on bacteria. Although the levels of nitrate and nitrite are increased, the effect of eating vegetables on the growth of the bacteria is not clear. Higher numbers (cfu/mL) were seen after incubation of postprandial saliva in some participants. The antibacterial effect of nitrate/nitrite on the bacteria was difficult to observe, probably due to rich ions, carbohydrates, and proteins in the saliva. In addition, fasting saliva usually contains approximately 0.2 mM nitrate, which might be enough for an antibacterial effect on *Desulfovibrio*. In conclusion, the results of this study suggest that human saliva significantly inhibits the growth of *Desulfovibrio* species, probably due to nitrate and nitrite.

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Supplementary Materials
www.sciencemag.org/content/344/6182/376/suppl/DC1
Materials and Methods
Figs. S1 to S8
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Movies S1 to S3

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Genome Sequence of the Tsetse Fly (*Glossina morsitans*): Vector of African Trypanosomiasis

International *Glossina* Genome Initiative*†

Tsetse flies are the sole vectors of human African trypanosomiasis throughout sub-Saharan Africa. Both sexes of adult tsetse feed exclusively on blood and contribute to disease transmission. Notable differences between tsetse and other disease vectors include obligate microbial symbioses, viviparous reproduction, and lactation. Here, we describe the sequence and annotation of the 366-megabase *Glossina morsitans morsitans* genome. Analysis of the genome and the 12,308 predicted protein–encoding genes led to multiple discoveries, including chromosomal integrations of bacterial (*Wolbachia*) genome sequences, a family of lactation-specific proteins, reduced complement of host pathogen recognition proteins, and reduced olfaction/chemosensory associated genes. These genome data provide a foundation for research into trypanosomiasis prevention and yield important insights with broad implications for multiple aspects of tsetse biology.

African trypanosomiasis is transmitted by the tsetse fly to humans (sleeping sickness) and livestock (nagana) throughout sub-Saharan Africa, with an estimated 70 million people at risk of infection. Rearing livestock in endemic areas is difficult to impossible and results in an economic loss in agricultural output of several billion U.S. dollars per year. Human infections are fatal if untreated, but tools for disease control are limited because it has not been possible to develop vaccines and current trypanocidal drug treatments result in undesirable side effects with growing reports of drug resistance. The reduction or elimination of tsetse populations is an effective method for disease control that could be improved with greater knowledge of their biology and genetics (*1*).

Tsetse flies are key representatives of the dipteran clade Calyptratae, which represents 12% of the known diversity within the dipteran order. Many of the calyptrate species are blood feeders of biomedical importance (*2*). In addition, members of the calyptrate family of Glossinidae and superfamily Hippoboscoidea, to which tsetse belong (fig. S1) (*3*), are defined by the ability to nourish intrauterine offspring from glandular secretions and give birth to fully developed larvae (obligate adenotrophic viviparity). Tsetse flies live considerably longer than other vector insects, which somewhat compensates for their slow rate of reproduction. Trypanosome infections in tsetse are acquired by blood feeding from an infected vertebrate host, and trypanosomes have to overcome multiple immune barriers to establish an infection within the fly. As a result, trypanosome infection prevalence is low in field populations and in experimentally infected tsetse (*4*). Tsetse have symbionts that compensate for their nutritionally restricted diet by the production of specific metabolites and influence multiple other aspects of the fly's immune and reproductive physiology (*5*).

In 2004, the International *Glossina* Genome Initiative (IGGI) was formed (*6*) to expand research capacity for *Glossina*, particularly in sub-Saharan Africa, through the generation and distribution of molecular resources, including bioinformatics training. An outcome of the effort undertaken by IGGI is the annotated *Glossina morsitans* genome presented here and further developed in satellite papers on genomic and functional biology findings that reflect the unique physiology of this disease vector (*7–14*).

Characteristics of the *Glossina* Genome

A combination of sequencing methods were used to obtain the *Glossina morsitans morsitans* (*Gmm*) genome, including Sanger sequencing of bacterial artificial chromosomes (BACs), small-insert plasmid and large-insert fosmid libraries, and 454 and Illumina sequencing (tables S1 and S2). The sequences were assembled into 13,807 scaffolds of up to 25.4 Mb, with a mean size of 27 kb and half the genome present in scaffolds of at least 120 kb. The 366-Mb genome is more than twice the size of the *Drosophila melanogaster* genome (fig. S2A and table S3). Clear conservation of synteny was detected between *Glossina* and *Drosophila*, but with the blocks of synteny tending to be twice as large in *Glossina* due to larger introns and an increase in the size of intergenic sequences, possibly as a result of transposon activity and/or repetitive sequence expansions. Sequences from most of the major groups of retrotransposons and DNA transposons are found in the *Glossina* genome (table S4). These sequences comprise ~14% of the assembled genome, in contrast to 3.8% of the *Drosophila*

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