NOTE *Public Health*

Epidemiological Survey for *Toxoplasma gondii, Chlamydia psittaci* var. *ovis*, *Mycobacterium paratuberculosis*, *Coxiella burnetii*, *Brucella* spp., Leptospirosis, and Orf Virus among Sheep from Northern Districts of Japan

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ABSTRACT. A serological survey was carried out in the northern prefectures of Hokkaido, Iwate and Aomori in Japan, for the presence of antibodies against *Toxoplasma gondii*, *Chlamydia psittaci* var. *ovis*, *Mycobacterium paratuberculosis*, *Coxiella burnetii*, *Brucella* spp., Leptospirosis, and Orf virus (ORFV). Out of 267 samples tested, highest overall prevalence (28.78%) was found for *T. gondii*. The 12.59% of tested sheep were positive for *C. psittaci* var. *ovis*. A total of 8.67% were found to be seropositive for *C. burnetii*. Levels of these infections were found in all three prefectures. Seroconversion to ORFV was detected in Hokkaido and Iwate Prefectures (2.57%). Animals were positive only for *L. ballum* (1.50%), in Hokkaido and Aomori Prefectures. No animals tested positive for *Brucella* spp. and *M. paratuberculosis*.
KEY WORDS: Japan, northern prefectures, sheep, zoonosis.

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The limited sheep population (11,000 heads, data from the Japan Livestock Industry Association, 2000) represents a niche sector in Japan. However, it is integrated into the epidemiology of zoonotic agents. Furthermore, sheep are also farmed with other domestic species, such as cattle, another susceptible species involved in diffusing pathogens with zoonotic potential. At present, in Japan, only *Caprine Arthritis Encephalitis* virus, Scrapie, *Mycoplasma ovipneumoniae* and Border disease have been examined in small ruminant populations [7–9, 14].

In order to explore the presence of pathogens with recognized zoonotic potential and to obtain a preliminary picture of their current epidemiology, a serological survey of antibodies against *Brucella* spp., *Toxoplasma gondii*, *Chlamydia psittaci* var. *ovis*, *Mycobacterium paratuberculosis*, *Coxiella burnetii* (Q fever), orf virus (ORFV - contagious pustular dermatitis or ecthyma), and Leptospirosis was carried out. This was to determine their prevalence in the northern prefectures in Japan, Hokkaido, Aomori and Iwate, where the majority of the Japanese sheep, a total of 4,775 sheep (43%), are bred. The sampling activities were supported also by veterinarians of the Agricultural Mutual Aid Association (NOSAI). Details of the sampling methodology and descriptions of the flocks were reported previously [7, 8].

Screening for anti-*Brucella* spp. antibodies was performed using a rapid serum agglutination test (Rose Bengal Plate Test, RBPT) using *B. abortus* biovar 1 strain 99 antigen covering *B. melitensis* and *B. abortus*, in accordance with the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organisation for Animal Health-*Office International des Épizooties* (OIE) [26]. Antigen and control sera were from Istituto Zooprofilattico Sperimentale Abruzzo and Molise (IZSA&M) in Teramo, Italy.

A complement fixation test (CFT) for the detection of antibodies against *C. burnetii* was used [2]. Q fever antigen was from Siemens (Germany), and positive and negative reference controls were from IZSA&M, Teramo, Italy.

The presence of antibodies against *Chlamydia psittaci* var. *ovis* was determined by using CFT as described by Donn *et al.* [4]. Antigen and positive and negative reference controls were from IZSA&M, Teramo, Italy.

CFT was performed as a confirmatory test for the screening for anti-*Brucella* spp. antibodies, using *B. abortus* biovar 1 strain 99 antigen covering *B. melitensis* and *B. abortus*, in accordance with the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the OIE [26]. Antigen and control sera were from IZSA&M, Teramo, Italy.

The testing for the presence of neutralizing antibodies against leptospirosis was performed using a microscopic agglutination test (MAT) as described by the Manual of Diag-

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nostic Tests and Vaccines for Terrestrial Animals of the OIE [27]. Antigens selected for use in the MAT included 8 strains (*L. ballum, L. bratislava, L. canicola, L. grippotyphosa, L. copenhageni, L. hardjo, L. pomona, L. tarassovi*) provided by the Istituto Zooprofilattico Sperimentale Lombardia and Emilia Romagna (IZSL&ER, Italy). Positive reference controls for each strain (IZSL&ER, Italy) were included in the test.

To detect antibodies against ORFV, an indirect enzyme-linked immunosorbent assay (ELISA) was used [3]. Antigen and control sera were from IZSA&M, Teramo, Italy. The whole organism antigen and a horseradish peroxidase-labelled secondary antibody were used to perform the test. Briefly, a 96-well microtiter plates were coated at 4°C overnight with 100 μl/well of positive and negative antigens diluted in 0.05 M carbonate buffer (pH 9.6). The plates were then washed 3 times with phosphate buffer solution containing 0.05% Tween 80 (PBST), pH 7.4. One hundred microliters of each sera diluted in PBST containing 1%, bovine serum albumin (BSA), pH 7.4, were added in duplicate to positive and negative antigen wells. The plates were then incubated for 1 hr at 37°C. After washing as described above, wells were filled with 100 μl of an appropriate dilution of horseradish peroxidase-labelled rabbit anti-sheep IgG (Sigma-Aldrich, St. Louis, MO, U.S.A.) in PBST containing 1% of BSA. Following incubation for 1 hr at 37°C and washing 3 times with PBST, wells were filled with 100 μl substrate solution containing 1 mM ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) and 4 mM H_2O_2 in 50 mM sodium citrate, pH 4.0, and the plates were shaken for 20 min at room temperature. The reaction was stopped by addition of 50 μl of sodium azide. The absorbance values of the samples were read at 405 nm. The cut-off value considered was 0.215. This was calculated by adding 3 times the standard deviation (SD) value to the mean optical density (OD) value obtained from 140 negative sera collected from genetically selected animals in an ORF-free flock [3].

Agar gel immunodiffusion (AGID) was used for the detection of antibodies against *M. paratuberculosis*. Briefly, 0.75% agarose was dissolved in 0.85% NaCl solution and buffered to pH 9.0 with 0.01 M tris hydroxymethyl aminomethane. Gel was poured in plates on a 4-mm-thick layer with wells of 5 mm in diameter in a hexagonal pattern of 6 peripheral wells for serum samples and the positive control (Allied Monitor Inc., Fayette, MO, U.S.A.) and a central well for the antigen (Allied Monitor Inc., Fayette, MO, U.S.A.). Plates were examined after 24 and 48 hr of incubation. The appearance of precipitation lines of identity with the control serum lines before or at 48 hr was considered positive. Absence of any precipitation line was recorded as a negative test result.

An indirect immunofluorescence (IIF) antibody assay was used for the detection of antibodies against *T. gondii*. Glass microscope slides with circular areas covered with the RH Sabin strain of *T. gondii* (Biomerieux, Marcy-Étoile, France) grown in mice were used. Four different dilutions of serum ranging from 1:40 to 1:320 were made in phosphate-buffered saline (0.01 M PBS, pH 7.6). Positive and negative controls sera (Fuller Laboratories, Fullerton, CA, U.S.A.) were included in each test. Twenty microliters of the diluted serum was incubated with the antigen preparation for 30 min at 37°C in a humid chamber. After incubation, the slides were washed with PBS and dried. Then 20 μl of the fluorescein isothiocyanate-conjugated rabbit anti-sheep IgG (Sigma-Aldrich, St. Louis, U.S.A.), diluted 1:40, was added onto the slides and incubated for 30 min at 37°C in a humid atmosphere. After a second washing with PBS, the slides were prepared with buffered glycerine (pH 9.5), mounted with coverslips and examined using a fluorescence microscope using 40× objective lenses. A reaction with a serum dilution 1:40 was considered reactive, and the final titer was the last dilution that still showed fluorescence in the periphery of the parasites.

Results of serological screening for antibodies to the considered pathogens are summarized in Table 1. The highest percentage of seropositive animals showed an immune reaction to *T. gondii* (28.78%) in 11 flocks from all the three considered prefectures, with prevalence at flock level being up to 72.72% and the titers ranging from 1:40 to 1:160 (Table 2). Positive sera originated from 69 ewes and 7 rams.

Table 1. Results of serological screening for antibodies to Leptospirosis, *Toxoplasma gondii*, *Chlamydia psittaci* var. *ovis*, *Brucella* spp., *Mycobacterium paratuberculosis*, Q fever, and orf virus in sheep from prefectures of northern Japan

	Positive	% Positive*	Negative	NE	Anticomplement	Total
T. gondii (IIF)	76	28.78	188	3	-	267
C. psittaci var. *ovis* (CFT)	33	12.59	229	3	2	267
Q Fever (CFT)	21	8.67	221	3	22	267
Orf virus (ELISA)	6	2.57	227	34	-	267
Leptospirosis (MAT) Tested for: *L. australis, L. ballum, L. canicola, L. grippotyphosa, L. icterohaemorragiae, L. pomona L. tarassovi, L. sejroe L. ballum*	4	1.50	262	1	0	267
Brucella spp. (RBPT, CFT)	0	0	267	0	0	267
M. paratuberculosis (AGID)	0	0	267	0	-	267

NE: Not executed due to insufficient aliquots for testing.
*Percentage computed excluding samples resulting anticomplement or not tested due to insufficient serum quantity.

Fifty-five Suffolk and Suffolk crossbreeds, 6 crossbreeds Southdown x Poll Dorset, 9 Corriedale, 3 Black, 2 Cheviot and 1 Romanov, were affected.

Seroconversion against *C. psittaci* var. *ovis* (12.59%) was detected in 10 flocks in the 3 prefectures. Positive sera originated from 31 ewes and 2 rams: 12 Suffolk, 17 crossbreeds (mainly Suffolk x Cheviot, and Suffolk x Corriedale), 1 Corriedale, 1 Cheviot, and 2 Black. The average incidence of seropositive animals in individual herds ranged from 5.0% to 33.33% in the ten sampling groups from seropositive flocks. Titers ranged from 1:16 to 1:256 (Table 3).

Using CFT, sera from 21 animals (8.67%), out of 242 sera examined, were observed to be positive for anti-*C. burnetii* immunoglobulins (Table 1). Positive sera originated from 8 flocks from Hokkaido and 1 flock each from both Iwate and Aomori Prefectures. The average incidence of seropositive animals in individual herds ranged from 5% to 29% in the 10 sampling groups from seropositive flocks. The obtained titer with CFT was 1:8 in all tested positive sera. The seropositive animals included all ewes and 1 ram, but of different breeds, including Suffolk, Cheviot, Corriedale and crossbreed animals.

Antibodies against ORFV were reported in 3 flocks from Hokkaido and Iwate. The 6 positive animals were all Suffolk

Table 2. Indirect immunofluorescence (IIF) test. Results of serological screening for antibodies to *Toxoplasma gondii* in sheep from prefectures of northern Japan

Flock No.	Prefecture	IIF titer				NE	Total /flock
		Negative	40	80	160		
1	Hokkaido	11	5	3		1	20
2	Hokkaido	10	3	7			20
3	Hokkaido	16	3	1			20
4	Hokkaido	19		1			20
5	Hokkaido	17	1	2			20
6	Hokkaido	14	1	4	1		20
7	Hokkaido	10	5	5			20
8	Hokkaido	6	7	7			20
9	Hokkaido	20					20
10	Hokkaido	3	3	5			11
11	Iwate	20					20
12	Iwate	9	1	8	1	1	20
13	Aomori	20					20
14	Aomori	13		2		1	16
Total		188	29	45	2	3	267

NE: Not executed due to insufficient aliquots for testing.

Table 3. Complement fixation test (CFT). Results of serological screening for antibodies to *Chlamydia psittaci* var. *ovis* in sheep from prefectures of northern Japan

Flock No.	Prefecture	CFT titer						Anticomplement	NE	Total /flock
		Negative	16	32	64	160	256			
1	Hokkaido	14	4	1					1	20
2	Hokkaido	17	3							20
3	Hokkaido	19	1							20
4	Hokkaido	20								20
5	Hokkaido	15	4	1						20
6	Hokkaido	18	2							20
7	Hokkaido	20								20
8	Hokkaido	17	2	1						20
9	Hokkaido	18	2							20
10	Hokkaido	10							1	11
11	Iwate	15	5							20
12	Iwate	12	3	2			1	2		20
13	Aomori	20								20
14	Aomori	14	1						1	16
Total		229	27	5			1	2	3	267

NE: Not executed due to insufficient aliquots for testing.

ewes of 5 to 7 years of age. Antibodies against *L. ballum* were reported in 4 Suffolk ewes (from 4 to 8 years of age) from 2 flocks from Hokkaido and Aomori Prefectures, respectively.

The prevalence of the different infections was found to vary between prefectures (Table 4). Prevalence of antibodies to *T. gondii*, the most diffuse pathogen in this study, was relatively high in Hokkaido and Iwate Prefectures (33.16% and 25.64%, respectively) when compared with the prevalence detected in animals from Aomori Prefecture (5.71%). The highest percentage of positive sheep for *C. psittaci* var. *ovis* was reported in Iwate Prefecture (28.94%). The highest prevalence for *C. burnetii* was found in Aomori Prefecture (17.24%). The prevalences of ORFV and *L. ballum* were low (not exceeding 3.18% and 2.77%, respectively) and differently distributed, with both being absent from Aomori and Iwate Prefectures, respectively.

Comparison between different age categories and the percentage of seropositive sheep showed that age ranged from 1 to 10 years only in the case of sheep infected with *C. burnetii* (Table 5). For most of the pathogens, no seroconversions were present in animals at 1 year of age, and seroconversion was infrequent after 8 years of age. Seroprevalence usually raises with age, although this was not reported in this study. This might be related to the low number of samples from animals more than 8 years of age (three 9-year-old animals, six 10-year-old animals and one 12-year-old animal), and culling of old animals.

All the 267 collected samples were tested for *Brucella* spp. antibodies. Three samples (H8/17, H9/1 and H9/9) were positive in the RBPT, which was used as qualitative screening test. Sera originated from two different flocks from Hokkaido. Complement fixation was used as a confirmation test. None of the sera were confirmed to be positive for *B. melitensis* and *B. abortus*, showing titers <20 UI. Similarly, none of the tested animals were positive for antibodies against *M. paratuberculosis*.

This survey demonstrated positiveness for antibodies to *T. gondii*, *C. psittaci* var. *ovis*, *C. burnetii*, *L. ballum* and ORFV in sheep flocks in the northern prefectures of Japan.

Concerning brucellosis, to date, Japan is officially free from the disease in domestic animals. Only rare cases of *B. abortus* have been reported in cattle in 1992, 2002 and 2008 [28]. *B. suis* has never been reported in Japan. In 2006, the first serological survey in wild animals revealed 7.8% positiveness for antibodies against *Brucella* spp. in the Japanese wild boar (*Sus scrofa leucomystax*) from Shikoku region, in southern Japan [25]. With reference to small ruminants,

Table 4. Comparison between different prefectures of northern Japan for the percentage of sheep positive for antibodies to some pathogens with zoonotic potential. No animals reacted to *Brucella* spp. and *Mycobacterium paratuberculosis* antigens

Prefecture	% Positive				
	Toxoplasma	*Chlamydia*	Q fever	Orf	Leptospirosis *L. ballum*
Hokkaido	33.16	11.11	8.47	3.18	1.57
Iwate	25.64	28.94	2.77	2.50	0
Aomori	5.71	2.85	17.24	0	2.77
Total	28.78	12.59	8.67	2.57	1.50

Table 5. Comparison between different age categories for the percentage of sheep positive for antibodies to *T. gondii*, *C. psittaci* var. *ovis*, *C. burnetii*, *L. ballum*, and orf virus. Percentage computed excluding samples resulting anticomplement or not tested due to insufficient serum quantity

Age category (years)	*T. gondii*	*C. psittaci* var. *ovis*	*C. burnetii*	ORFV	*L. ballum*
1	0	0	21.42	0	0
2	11.76	11.76	12.5	0	0
3	39.02	23.07	0	0	0
4	36.36	2.27	4.54	0	2.22
5	11.11	4.44	7.31	7.69	0
6	44.44	14.81	13.63	7.69	3.7
7	42.42	24.24	0	3.12	0
8	10	20	20	0	10
9	0	0	0	0	0
10	33.33	0	40	0	0
12	0	0	0	0	0
Unknown	38.09	23.80	20	0	0

the last occurrence of *B. melitensis* in Japan was reported in 1949. *B. abortus* has never been reported in sheep and goats. Five zookeepers in the City of Kawasaki developed brucellosis in 2001 after attending the delivery of a baby moose (*Alces alces*). Subsequent investigations confirmed the infection in a goat at the zoo, but no information on isolation or characterization has been provided [6]. In this study, none of the sera tested were serologically positive for *Brucella* spp. This confirms the absence of the disease in sheep in the studied area in 2007 and corroborates the official free status of the country for brucellosis.

None of the sera tested were serologically positive to *M. paratuberculosis*. However, further epidemiological studies in small ruminant populations are needed, taking into consideration the presence of the pathogen in cattle in the country [17] and the importance in public health due to evidence of the potential role in the etiopathogenesis of Crohn's disease in humans, which is not strong but should not be ignored [24].

The present study showed positiveness for antibodies in sheep to well-known zoonotic diseases, i.e., toxoplasmosis, chlamydiosis and leptospirosis. In humans, the major concern for toxoplasmosis is congenital infection, causing severe sequelae in the infant (e.g., mental retardation, blindness and epilepsy). In Japan, a recent study reported 34.9% of pregnant women, from Hokkaido and Hyogo Prefectures, tested positive for *T. gondii* antibodies. It was likely that ingestion of raw or undercooked meat was the main risk factor for acute *T. gondii* infection in Japanese pregnant women. The incidence of congenital toxoplasmosis in Japan was estimated to be at least 0.0126% (1.26 per 10,000 births) [29]. Concerning ovine chlamydiosis, the risk of infection for humans is considered low, but pregnant women in particular should prevent contact with potentially infected small ruminants. Antibodies against *C. psittaci* var. *ovis* were reported in the majority of the tested sheep flocks. The observation of high titers up to 1:256 (Table 3) indicates the specificity of the seroconversion. However, further investigations will be necessary to clarify the epidemiology of the pathogen, taking into account that positive reactions with titers between 1:10 and 1:40 may relate to an infection with *C. pecorum* [20].

Concerning "*akiyami*" (autumn disease) (the Japanese name for leptospirosis), the disease was responsible for more than 200 reported human deaths annually in Japan until the 1960s, mostly in rice-field farmers. Since then, this number has decreased, and currently the annual incidence of leptospirosis in the country is low (<1 per 100,000) [23]. By 2003, leptospirosis had become a notifiable disease of the National Epidemiological Surveillance of Infectious Diseases (NESID), and fewer than 20 cases have been reported annually [18]. Serovars *Icterohaemorrhagiae*, *Copenhageni*, *Autumnalis*, *Hebdomadis* and *Australis* have been recognized as major causes of human leptospirosis [23], out of the 13 serovars of the causative *Leptospira* estimated by MAT in Japan [18]. In our survey, antibodies against a different serovar of *Leptospira* were detected, *L. Ballum*, which is almost exclusively associated with exposure to infected rats and mice [16].

Detection of antibodies against *C. burnetii* confirms the previous studies on the presence of Q fever in sheep in Japan, which was first reported by Htwe *et al.* [10]. The positive rate (8.64%) in the present study showed a low pathogenic pressure in 2007 and was lower than the previously reported level (17.6%) of infection [10]. The reported lower prevalence was probably due to the use of the CFT instead of the more sensible IIF test previously used by Htwe *et al.* [10].

Other investigations on prevalence of coxiellosis in animal populations have been focused on cattle, particularly in dairy cattle with reproductive disorders [21], and have shown relatively high prevalences (58.9% and 60.4%). Reports indicated also serological evidence in wild animals [5]. Furthermore, cats are considered a significant source of *C. burnetii* responsible for human outbreaks [19].

In Japan, Q fever has been reportable since 1999. Every year, 7 to 46 clinical cases are reported in humans [19]. Atypical pneumonia due to *C. burnetii* among children has been reported, and the positive rate was 39.7% of positiveness among patients suffering from respiratory diseases [22]. The prevalence of *C. burnetii* antibodies in samples from adult humans showed overall seroconversion rates ranging from 8.6% to 16.5%, suggesting an occupational risk to humans living in close contact with animals [11].

Contagious pustular dermatitis infection in sheep in Japan was first reported in the 1940s [1]. Serological surveys demonstrated that 88.5% of sheep in Hokkaido were seropositive (AGID test) based on 52 tested samples [15]. An extensive outbreak of disease occurred in sheep in Hokkaido [13], and ORFV was isolated also in Iwate Prefecture [12]. Our study revealed a sensibly lower prevalence (2.57%) when compared with that reported by Kuroda *et al.* [15]. No human case has been reported in Japan so far.

The demonstration of circulation of some bacterial and viral zoonotic agents in sheep flocks in the northern prefectures of Japan, based on serological analysis, advanced the knowledge on pathogens affecting domestic sheep in Japan. Considering the zoonotic potential of these pathogens, further studies are needed. Awareness of professionals in the appropriate fields and consumers should be enhanced to ensure a preventive approach in public health. Furthermore, according to the OIE, brucellosis, chlamydiosis, paratuberculosis and Q fever are included in the list of notifiable diseases of importance to international trade [28].

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NOTE *Bacteriology*

Detection of Hemotropic Mycoplasmas in Free-Living Brown Sewer Rats (*Rattus norvegicus*)

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ABSTRACT. The prevalence of hemotropic mycoplasmas in wild rodents is largely unknown. Here, we report the presence of hemoplasmas in blood samples collected from brown sewer rats (*Rattus norvegicus*) trapped during rodent control around an animal hospital in Morioka, Japan. We examined nine rats using real-time PCR and end-point PCR, and found one rat (11.1%) that was positive for a hemoplasma infection. The 16S rRNA gene and 16S to 23S rRNA intergenic spacer region of the hemoplasma detected in a wild-caught rat were amplified using PCR. The nucleotide sequences of the PCR products were further determined and compared to those of other hemoplasmas. Our examinations revealed the presence of a hemoplasma that has not previously been described in rodents. The pathogenic traits of this hemoplasma remain unexplored.

KEY WORDS: hemoplasma, mycoplasma, wild-caught rat.

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Hemotropic mycoplasma, also called hemoplasma, is a newly defined group of uncultivable pathogens, which have been classified solely using nucleotide sequences of the 16S rRNA or RNase P RNA genes. Hemotropic mycoplasma has been classified in this manner, because of a lack of an appropriate means to examine its biological or serological properties [9]. This group is composed of formerly *Eperythrozoon* and *Hemobartonella* (previously *Bartonella*) species and newly identified hemotropic mycoplasmas. Hemoplasma infection, which is accompanied by erythrocyte hemolysis, has been reported in a variety of mammalian species [9]. Hemoplasmas have been detected in rodents, including mice, rats and hamsters, as an etiological agent of infectious anemia or splenomegaly, and these hemoplasmas have collectively been identified as *Bartonella muris* based only upon microscopic observation [13]. However, the prevalence of hemoplasma infections in wild rodents has remained largely undocumented. In this report, we demonstrate a hemoplasma that has not been described previously; the hemoplasma was found in brown sewer rats (*Rattus norvegicus*) wild-caught near an animal hospital.

Nine wild-caught rats were trapped in July 2012 during sanitation measures at the Iwate University veterinary hospital (latitude 39.7N and longitude 141.1E) in Japan. Anti-coagulated blood samples were collected under ether anesthesia from seven of the rats, and blood was also taken from two of the rats that were found dead; blood samples were stored at −80°C prior to examination. No clinical sign was apparent in the rats. The protocol used in the present study was approved by the Animal Care and Use Committee of Iwate University, and all animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals established by the Committee. Total DNA was extracted from 200 μl blood samples collected from rats using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Negative controls consisting of 200 μl phosphate-buffered saline solution were prepared with each batch. Extracted DNA samples were stored at −80°C prior to examination.

For preliminary screening of hemoplasma infections, universal PCR primers for the hemoplasmas' 16S rRNA gene were used, as described by Tasker *et al.* [16]. Real-time PCR was performed using a SmartCycler instrument (Cepheid, Sunnyvale, CA, U.S.A.) with SYBR Premix Ex *Taq* (Code #RR041A, TaKaRa Bio., Otsu, Japan). The reaction mixture contained 1 μl of each primer (10 pmol/μl), 12.5 μl of 2X premix reaction buffer and water to a volume of 23 μl. Finally, 2 μl (200 *pg*) of a DNA sample was added to this mixture as a template. Amplification was achieved with 40 cycles of denaturation at 95°C for 5 sec, renaturation at 57°C for 20 sec and elongation at 72°C for 15 sec, after the initial denaturation at 94°C for 30 sec. Fluorescence readings in a channel for SYBR Green I were taken throughout the experiments.

After real-time PCR, the melting experiment was performed from 60°C to 95°C at 0.2°C/sec with a smooth curve setting averaging one point. Melting peaks were visualized by plotting the first derivative against the melting temperature (*Tm*) as described previously [7]. The *Tm* was defined

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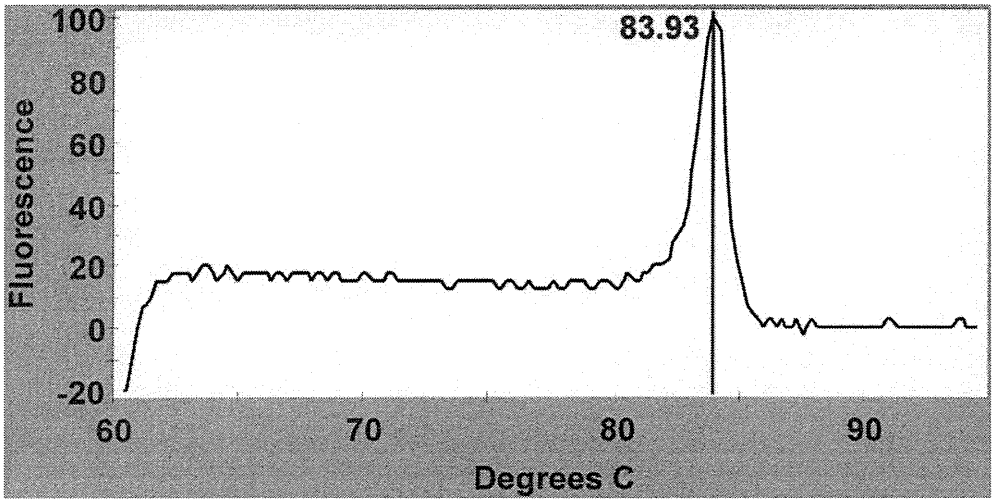

Fig. 1. Melting curve analysis for the real-time PCR product from the 16S rRNA gene of the hemoplasma strain detected in the peripheral blood of a wild-caught rat. Melting temperature was depicted as a single peak at 83.93°C.

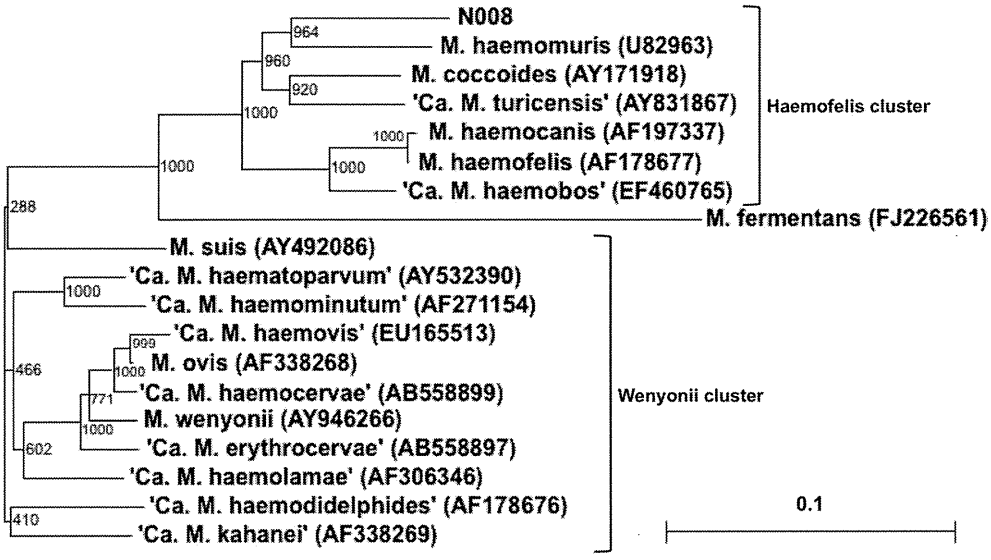

Fig. 2. Comprehensive neighbor-joining phylogenetic tree generated by the nucleotide sequences of 16S rRNA genes showing the evolutionary relationship among hemoplasmas (accession numbers are given in parentheses) and a newly identified rat hemoplasma strain, N008. *Mycoplasma fermentans* PG18 was included as an out-group. The bootstrap values are indicated at the branch points. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances that were used to infer the phylogenetic tree (scale bar, 0.1 nucleotide substitutions per site).

as a peak of the curve; if the highest point was a plateau, the mid-point was identified as the *Tm*. Of the nine blood samples tested using real-time PCR, one rat was found to be positive for a hemplasma infection. The *Tm* of the positive sample was 83.93°C (Fig. 1). Our previous experiments indicated that the input amount of DNA, the copy number of the target and the presence of co-infections with several targets did not influence the *Tm* [12].

The positive sample from the real-time PCR experiment was further subjected to end-point PCR to amplify the entire region of the 16S rRNA gene. End-point PCR was carried out with 50-μl reaction mixtures each containing 1 μl of DNA solution, 0.5 μl of TaKaRa LA *Taq*™ (5 units/μl), 5 μl of 10X LA PCR™ Buffer II (TaKaRa Bio.), 8 μl of 25

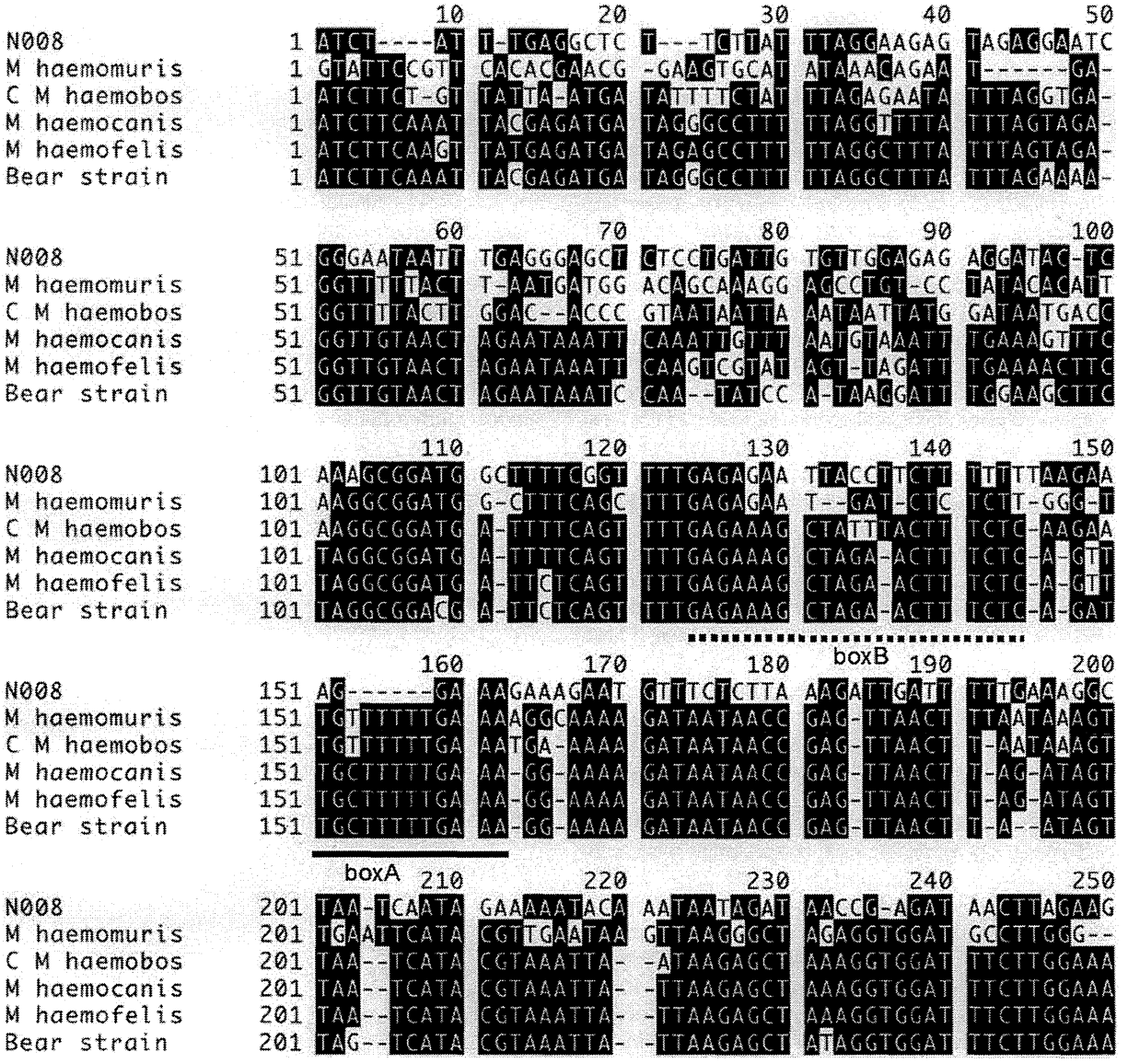

Fig. 3. Nucleotide sequence alignment of the ITS region from the six hemoplasma sequences, *M. haemofelis*, *M. haemocanis*, '*Candidatus* M. haemobos', *M. haemomuris*, bear strain and rat strain N008. Accession numbers for the nucleotide sequences of *M. haemomuris*, *M. haemofelis*, *M. haemocanis*, '*Ca.* M. hamobos' and bear strain are AB080799, AB638408, AF197337, AB638407 and AB725596, respectively. Nucleotide sequence numbers are given from a consensus sequence. Homologous nucleotides are shown as inverted characters. Dashes indicate nucleotide gaps between adjacent nucleotides introduced for the alignment. BoxA is underlined, and boxB is shown by a dotted line. Notably, the boxA motif was missing in the rat hemoplasma strain, N008.

mM $MgCl_2$ (final 4.0 mM), 8 μl of dNTP mixture (2.5 mM each), 0.2 μl each of the forward primer (5'-AGAGTTTGATCCTGGCTCAG-3', equivalent to nucleotide numbers 11 to 30 of *M. wenyonii*(AY946266) or 5'- ATATTCCTACGGGAAGCAGC-3', which is equivalent to nucleotide numbers 328 to 347 of *M. wenyonii*) and the reverse primer (5'- ACCGCAGCTGCTGGCACATA-3', equivalent to nucleotide numbers 503 to 522 of *M. wenyonii* or 5'-TACCTTGTTACGACTTAACT-3', equivalent to nucleotide numbers 1,446 to 1,465 of *M. wenyonii*) (50 pmol/μl each) and water to a final volume of 50 μl. After the mixture was overlaid with 20 μl of mineral oil, the reaction cycle was carried out 35 times with denaturation at 94°C for 30 sec, annealing at 58°C for 120 sec and extension at 72°C for 60 sec in a thermal cycler. The end-point PCR product from the 16S rRNA gene was fractionated on horizontal, submerged 1.0% SeaKem ME agarose gels (FMC Bioproducts, Rockland, ME, U.S.A.) in TAE buffer (40 mM Tris, pH8.0, 5 mM sodium acetate, 1 mM disodium ethylenediaminetetracetate) at 50 volts for 60 min. After electrophoresis, the gels were

stained in ethidium bromide solution (0.4 μg/m*l*) for 15 min and visualized under a UV transilluminator. DNA was extracted using a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and was subjected to direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The nucleotide sequence of this rat hemoplasma 16S rRNA gene was most similar to the sequence of *M. haemomuris* (formerly *Bartonella muris* or *Haemobartonella muris*) Shizuoka strain that was isolated from a small field mouse (*Apodemus argenteus*) in Japan [14]. Sequence homology between them was 87%, which was lower than the critical cut-off level of a same species [1].

The 16S rRNA gene sequence obtained from a rat hemoplasma was similar to other hemoplasma sequences from the DNA database using Clustal W [17]. A phylogenetic tree was generated using the neighbor-joining method [15] from a distance matrix corrected for nucleotide substitutions using the Kimura two-parameter model [8]. The data were re-sampled 1,000 times to generate bootstrap values (Fig. 2). The 16S rRNA gene nucleotide sequence of a hemoplasma strain detected in a sewer rat was distinct from those of other hemoplasmas. The 16S rRNA gene sequences are widely used in microbiology for identifying uncultivable microorganisms as new species; 16S rRNA gene sequences have also been the basis for the reclassification of hemotropic *Mycoplasma* species [10, 11]. In our previous examinations, hemoplasmas were divided into two phylogenetic groups, Haemofelis and Wenyonii clusters [18]. The hemoplasma strain detected in a wild-caught rat belonged in the Haemofelis cluster in the present study.

Next, we amplified the 16S-23S rRNA intergenic transcribed spacer (ITS) region of this specimen using end-point PCR with the forward primer Hemo16-23S-F and the reverse primer, as described previously [2]. The ITS region nucleotide sequence was determined as described above and compared to those of other hemoplasmas (Fig. 3). The ITS region of the genus *Mycoplasma* is well conserved within a species and has been used for a genetic marker for identification and classification of mycoplasmas [3]. Nucleotide sequence homology at the ITS region between the rat strain N008 and *M. haemomuris* Shizuoka strain was 37%, and this suggests that they are genetically distinct. No spacer tRNA gene was identified within the ITS region of the rat hemoplasma strain, which is a common feature that is consistent with the other species of the genus *Mycoplasma* [4, 5]. The rat hemoplasma strain lacked the boxA motif that is common to other mycoplasma species examined so far [6], despite the presence of the boxB motif.

In the present study, we used 16S rRNA phylogenetic analysis to demonstrate, in a wild-caught rat, a hemoplasma strain that was most closely related to *M. haemomuris* Shizuoka strain [14]. Although *M. haemomuris,* previously called *Bartonella muris* or *Haemobartonella muris,* was a species name given to an anemic pathogen isolated from an albino rat [10, 11], the alleged 16S rRNA gene nucleotide sequence of *M. haemomuris* was determined using a morphologically identified hemoplasma strain isolated from a small field mouse [14]. Similarly, hemoplasmas detected in mice, rats or hamsters have been identified on the basis of morphology. This may pose a question whether all the hemoplasma strains detected in these rodents were identical or a same species. It is not certain that there is a genetic relationship among these hemotropic pathogens isolated from mice, rats and hamsters. Thus, our findings may address this problem by demonstrating differences in nucleotide sequences of the 16S rRNA gene and the ITS region of the hemoplasmas detected in mice and rats.

In conclusion, we used 16S rRNA phylogenetic analysis to demonstrate the presence of a hemoplasma, in a wild-caught brown rat, which was similar but not identical to *M. haemomuris*. Both murine hemoplasmas were distinct from each other in the primary and secondary structures of the ITS region. Our data indicate that there is genetic variation among murine hemoplasmas. The nucleotide sequence of the 16S rRNA gene combined with the ITS region has been deposited to the DNA database under the accession number AB752303.

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SHORT COMMUNICATION

The bacterial cell division protein FtsZ forms rings in swarmer cells of *Proteus mirabilis*

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Abstract Bacterial FtsZ assembles and constricts after chromosomal segregation in the course of cell division. Here we examined the localization of FtsZ in multinucleated swarmer cells of *Proteus mirabilis* by immunostaining. FtsZ was found to localize to the point of karyomitosis in swarmer cells of *P. mirabilis*, which is equivalent to filamentous mutants of *Escherichia coli* defective in the *ftsI* or *ftsQ* genes that are involved in later steps of cell division. Thus our findings suggest that the appearance of swarmer cells results from cellular functions immediately after FtsZ assembly.

Keywords FtsZ assembly · Immunostaining · *Proteus mirabilis* · Swarmer cell

Introduction

Proteus mirabilis—a urinary pathogen—is known to form a bull's-eye pattern on solid medium as it swarms over the entire surface of agar plates. These swarmer cells, characterized by a 10- to 40-fold increase in cell length, a drastic increase in the number of flagella, and production of higher levels of specific virulence factors, are related closely to establishment of infection (Allison et al. 1992, 1994). *P. mirabilis* has long been considered to initiate cell differentiation into swarmer cells only upon contact with a solid surface by inhibition of flagellar rotation or by cell–cell signaling (Alavi and Belas 2001; Fraser and Hughes 1999; Rauprich et al. 1996; Sturgill and Rather 2004). However, appearance of swarmer cells is reported in artificial urine at pH 6.5 (Jones et al. 2007) as well as in acidic broth containing urea (Fujihara et al. 2011). Despite many reports on swarmer cell differentiation of *Proteus*, the exact mechanisms of cell elongation remain largely unknown. In *Escherichia coli*, of ten proteins known to be essential for cell division—FtsA, B, I, K, L, N, Q, W, Z and ZipA—FtsZ, the tubulin homologue that forms the cytokinetic Z ring (Löwe and Amos 1998), plays a key role in cytokinesis. Although FtsA and ZipA both help to tether FtsZ to the inner-membrane, either one is capable of supporting formation of the Z ring. Following Z ring formation, FtsK, Q, B, L, W, I and N are required in that order for further maturation of the Z ring to a septal ring. Additionally, FtsA and ZipA are also required for this maturation (de Boer 2010; Pichoff and Lutkenhaus 2002). In the present study, we examined the localization of FtsZ by immunostaining to help understand the cell division process of swarmer *Proteus*.

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Materials and methods

Fluorescent microscopic observation

FtsZ ring organization was examined as described by Den Blaauwen et al. (1999) with a minor modification. Briefly, swarmer cells of *P. mirabilis* NBRC3849 was harvested from the edge of colonies incubated overnight at 37 °C on

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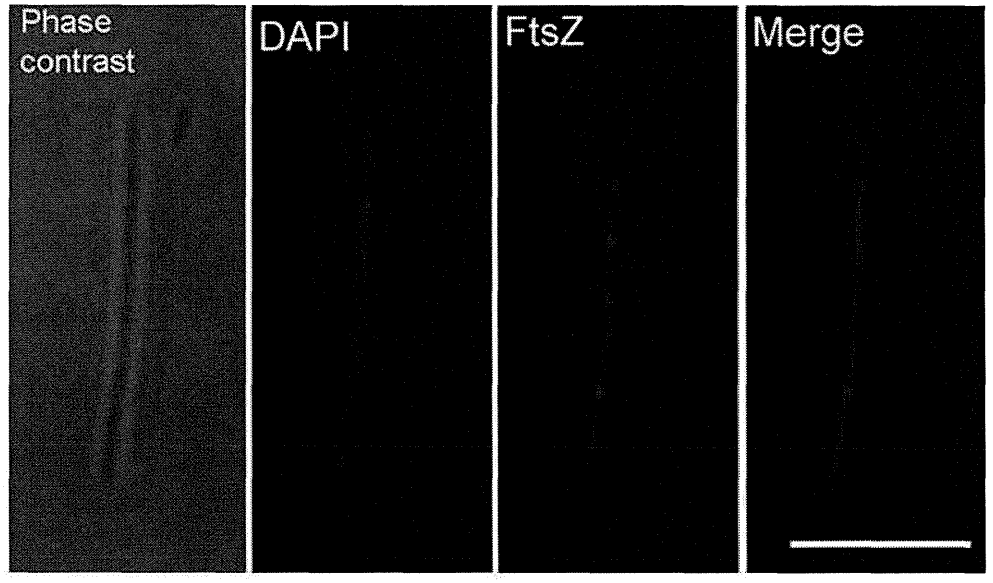

Fig. 1 Fluorescence micrograph of a swarmer cell. Cells were immunostained with polyclonal anti-FtsZ rabbit antibody followed by Cy3-conjugated goat anti-rabbit secondary antibody. Nucleoids were visualized by treating the cells with 4′,6-diamidino-2-phenylindole (DAPI). The Z rings and nucleoids are shown in *red* and *blue*, respectively. *Bar* 10 μm

Luria agar. After fixation by 2.8 % neutral-buffered formalin and 0.04 % glutaraldehyde, swarmer cells were treated by 0.1 % Triton X-100 followed by 100 μg/mL lysozyme solution. FtsZ was stained with a polyclonal anti-FtsZ rabbit antibody (1:200, Agrisera, Vännas, Sweden) followed by Cy3-conjugated goat anti-rabbit secondary antibody (1:1,000, Rockland Immunochemicals Gilbertsville, PA) for fluorescent microscopic observation. All antibodies used were diluted in phosphate-buffered saline containing 1 % bovine serum albumin. Nucleoids were stained using 2.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Dojindo, Kumamoto, Japan) and visualized under an Eclipse TE2000-U fluorescence microscope (Nikon, Tokyo, Japan).

Transmission electron microscopic observation

Swarmer cells treated with lysozyme as described above were incubated in methanol containing 0.3 % H_2O_2 to eliminate endogenous peroxidase. FtsZ was stained with a polyclonal anti-FtsZ rabbit antibody (1:200, Agrisera) followed by biotinylated donkey anti-rabbit secondary antibody (1:1,000, Jackson ImmunoResearch Laboratories, West Grove, PA). Cells reacted with avidine-biotin complex reagent (Vector Laboratories, Burlingame, CA) were colorized by 3-3′-diaminobenzidine tetrahydrochloride (Kondoh et al. 2011) and then observed in an H-800 transmission electron microscope (Hitachi, Tokyo, Japan).

Results and discussion

Immunofluorescent microscopy revealed that FtsZ is localized between separated chromosomes in most swarmer cells (Fig. 1). In another study, filamentous cells treated with FtsZ inhibitor (Beuria et al. 2005; Jaiswal et al. 2007) or FtsZ mutants (Pogliano et al. 1997) lacked FtsZ ring formation and segregated chromosomes. On the other hand, FtsZ assembly was observed in filamentous mutants of *ftsA*, *ftsI* or *ftsQ* genes in *E. coli* (Addinall et al. 1996; Pogliano et al. 1997). Propidium iodide staining revealed chromosomal segregation in filamentous mutants of *ftsI*; however, between one and eight FtsZ rings formed in cells having 16 nucleoids (Pogliano et al. 1997). Considering the increase in the number of FtsZ rings upon prolonged incubation hours (Addinall et al. 1996), these filamentous mutants of the *ftsA*, *ftsI* or *ftsQ* genes essential to the cell division after FtsZ assembly might be identical to our swarmer cells depicted by immunofluorescent staining. On the other hand, the *ftsA* mutant showed an indented or sausage-like appearance, despite smooth filaments in the *ftsI* or *ftsQ* mutants (Begg and Donachie 1985; Taschner et al. 1988). In swarmer cells

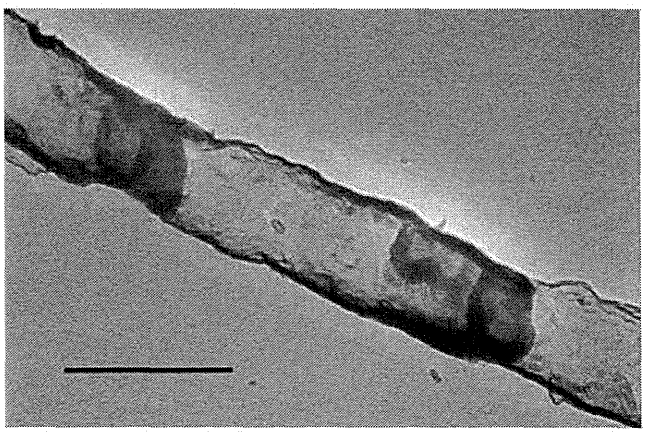

Fig. 2 Transmission electron micrograph of swarmer cells. Cells were immunostained with polyclonal anti-FtsZ rabbit antibody followed by biotin-conjugated anti-rabbit antibody prepared in donkeys. *Bar* 1 μm

Author's personal copy

of *P. mirabilis*, the FtsZ ring was found in smooth filamentous cells by electronmicroscopic observation (Fig. 2). This suggested some proteins, like FtsI or FtsQ which function immediately after the FtsZ assembly, were inhibited in the swarmer cells. To our knowledge, no electron microscopic study has been reported on filamentous cell of *ftsB*, *K*, *L*, *N*, *W* and *zipA* mutants. Thus, considering the order of these functions, dysfunction of FtsK, B, L and W may bring the same results with dysfunction of FtsI or EtsQ and swarmer *P. mirabilis*.

In conclusion, we demonstrated for the first time FtsZ localization in swarmer cells of *P. mirabilis* between separated nucleoids by immunofluorescent microscopy. Although further extensive studies on the functions of cell division proteins are necessary, our results provide a new insight into cell division and swarmer cell elongation in *Proteus*.

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Full Length Research Paper

Palindromic nucleotide substitutions: A new software for *pestivirus* genotyping

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The genus *Pestivirus* of the family *Flaviviridae* is represented by four established species; Bovine viral diarrhea virus 1 (BVDV-1), Bovine viral diarrhea virus 2 (BVDV-2), Border disease virus (BDV) and Classical swine fever virus (CSFV) and a tentative "Giraffe" species. The palindromic nucleotide substitutions (PNS) in the 5' untranslated region (UTR) of *Pestivirus* RNA have been described as a new, simple and practical method for genotyping. The preparation of specific software was considered for an easy access of the users to the method. In the present study, the new software, named PNS, was prepared and the application on the genotyping procedures with the keys for *Pestivirus* identification as specific PNS at genus, species and genotype level, respectively, was evaluated on five hundred-tirty-four sequences. The software is freely available at www.pns-software.com

Keywords: Genotypes, palindromic nucleotide substitutions, *Pestivirus*, software.

INTRODUCTION

The genus *Pestivirus* of the family *Flaviviridae* is represented by four established species; *Bovine viral diarrhea virus 1* (BVDV-1), *Bovine viral diarrhea virus 2* (BVDV-2), *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV) and a tentative "Giraffe" species (King *et al.*, 2012). The *Pestivirus* genome, single-stranded, positive polarity RNA, is composed by a sequence of about 12,500 nucleotides. It can be divided into three regions: a 5'-untranslated region (UTR), a single large open reading frame encoding region, and a 3'-UTR. The 5'-UTR is highly conserved among all members within the genus *Pestivirus*, thus being useful for the characterization of species or genotypes. The primary structure analysis, by sequence alignment and construction of phylogenetic trees, is the most common method for the classification of *Pestivirus* strains. Genetyx-Mac, DNASIS, Clustal X(Thompson *et al.*, 1997) are among the software used for typing virus strains based on sequence alignment. In reality, the genomic sequence is tri-dimensional. The reproduction of the third structure is highly problematic. However, it is relatively easy to predict the secondary structure, according to the most probable nucleotide binding, with lowest folding energies. The secondary structure of the 5'-UTR can be divided into four domains, A-D, with domain D encompassing the 3' two thirds of the UTR predicted to fold into a complex palindromic stem-loop structure, including an internal ribosome entry site (IRES), as observed in poliovirus (Deng and Brock, 1993; Harasawa, 1994), thus representing critical regions of the 5´-UTR, responsible for translational, transcriptional and replicational events in pestiviruses.

The *Pestivirus* genome has a relatively long 5'-UTR upstream of the polyprotein open reading frame. Although the nucleotide sequence of the 5'-UTR is well conserved among the members of the *Pestivirus* genus, the 5'-UTR has been shown to contain at least three variable loci. The nucleotide substitutions in these variable loci are particularly important because the 5'-UTR of positive-sense RNA viruses generally includes regulatory motifs, which are indispensable to viral survival. Therefore, random mutations at the 5'-UTR have a high probability of incompatibility with viral survival. Thus stable nucleotide variations at this level assume high importance in terms of virus evolutionary history.

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Table 1. Summary of *Pestivirus* strains (n 533) evaluated according to the Palindromic nucleotide substitution (PNS) method at the 5' untranslated region of RNA.

Species	Number of strains	Host	Geographical origin
BVDV-1	274	Cattle, Sheep, Pig, Deer, Roe deer, Human, Contaminant	Argentina, Austria, Belgium, Brazil, Canada, China, Belgium, France, Germany, India, Ireland, Italy, Japan, New Zealand, Slovakia, South Africa, Spain, Sweden, Switzerland, UK, USA
BVDV-2	77	Cattle Sheep Contaminant	Argentina, Austria, Belgium, Brazil, Canada, France, Germany, Italy, Japan, Netherland, NewZealand, Slovakia, Tunisia, UK, USA
BVDV-3*	3	Cattle	Brazil, Thailand
BDV	131	Sheep, Pyrenean chamois, Cattle, Pig, Reindeer, Wisent	Australia, France, Germany, Japan, New Zealand, Spain, Switzerland, Tunisia, Turkey, UK, USA
BDV-2*	3	Sheep, Goat	Italy
CSFV	43	Pig, Sheep	China, France, Garmany, Honduras, Italy, Japan, Malaysia, Netherlands, Poland, Russia, Spain, Switzerland, USA
Pronghorn*	1	Pronghorn	USA
Giraffe*	1	Giraffe	Kenya
Bungowannah*	1	Pig	Australia

* Tentative species

Nucleotide sequences at the three variable loci, V1, V2 and V3, in the 5´-UTR of pestiviruses have been shown to be palindromic and capable of forming a stable stem-loop structure peculiar to each *Pestivirus* species. Nucleotide substitutions in the stem regions always occur to maintain the palindromic sequence and thereby form a stable stem-loop structure. Thus, this type of mutation was referred to palindromic nucleotide substitutions (PNS). Based on the above mentioned considerations, the observation of nucleotide variations among virus strains at the level of the three specific palindromes in the 5'-UTR has been conceived as method for genotyping (Harasawa and Giangaspero, 1998). The method named palindromic nucleotide substitutions (PNS) appeared to be simple and practical, showing comparable results with other procedures based on the primary structure comparison.

According to palindromic nucleotide substitutions, 534 sequences (Table 1) have been segregated into nine species within the genus *Pestivirus* (Giangaspero and Harasawa, 2011). In addition to the four established species, Harasawa *et al.* (2000) characterized the taxonomic status of a giraffe strain, based on the 5' untranslated region, as a new cluster among *Pestivirus* species. Furthermore, other four tentative species (BVDV-3, BDV-2, Pronghorn and Bungowannah) have been recently proposed (Vilček *et al.*, 2005, Kirkland *et al.*, 2007, Giangaspero and Harasawa, 2011). Genotypes have been identified in species showing heterogeneity: *Bovine viral diarrhea virus 1* (Giangaspero *et al.*, 2001), *Bovine viral diarrhea virus 2* (Giangaspero *et al.*, 2008, Giangaspero and Harasawa, 2011), *Border Disease virus* (Giangaspero, 2011) and *Classical Swine Fever virus* (Giangaspero and Harasawa, 2008). The observation made on the nucleotide sequences of the three variable loci at the level of the 5'-UTR genomic region of *Pestivirus* strains allowed to the identification of consensus motifs shared by all the *Pestivirus* species. The characteristic palindromic nucleotide substitutions have been identified at genus, species and genotype level, respectively (Tables 2 and 3). The palindromic loci represented, with about 80 nucleotides, a very limited portion of the virus genome. Within these short sequences, it was sufficient the evaluation of only 21 nucleotides to obtain with certitude the characterization of the genus. Species were characterized through the evaluation of only 6 to 19 nucleotides. Similarly, the genotype was defined with only 6 to 10 nucleotides. These peculiar aspects resumed the high specificity of the PNS method and the reliability of the provided results.

With the aim of improving the PNS method in a full computerization of the procedure and avoid the main limitation due to the manual searching of relevant base pairings and direct observation of the sequence, a specific software was conceived for an easy access of the users and a rapid testing with reliable results applying the patterns for *Pestivirus* identification.

MATERIALS AND METHODS

The program was realized using the "C++" programming language (Ellis and Stroustrup, 1990) and adapted to run under the Windows operative system. The software was constructed in order to evaluate virus nucleotide sequences up to 15,000 bases. In general, RNA virus sequences from 5'-UTR genomic region, deposited in international databases, include approximately 250-350

Table 2. Palindromic nucleotide substitutions (PNS) characteristic to the genus *Pestivirus*. The position of base Pairings is defined by numbering from the bottom of the variable locus

Genus	Locus	Characteristic PNS markers
	V1	Absence in position 22 - size of V1 21 bp (exception U); C C bulge in position 11; A-U in position 10; C-G in position 8 (exceptions U*G, U-A and G G bulge); U-A in position 7 (exception G-C and A A bulge); A in position 6 (exception G); U*G in position 5; U in position 5 right nucleotide; G-C in position 4.
	V2	GGGGU loop (exception GGGGC); C-G in position 8 (exception U*G).
Species	**Locus**	**Characteristic PNS markers**
BVDV-1	V1	U-A in position 15 (exception U*G or C-G);
	V2	G-C in position 5 (exception A-U);
	V3	G-C in position 5; A in position 10 (exceptions A-U, G-C or A C, A A or G A bulges or absence).
BVDV-2	V1	A-U or A C bulge in position 20 (exceptions G*U, C C or A A bulges); A,G or U in position 21 (exception G G);
	V2	U-A or U*G in position 6 (exception C A bulge);
	V3	A-U or A C bulge in position 7 (exception G-C).
BVDV-3 tentative species (HoBi group)	V1	U-A in position 15;
	V3	G-C or G*U in position 3; A-U or G-C in position 7; A in position 10.
BDV	V1	G-C or A-U in position 15 (exceptions C U and A C bulges);
	V3	U C and U U bulges or U*G in position 7 (exceptions A-U, U-A and C C, A C, C U and C A bulges).
BDV-2 tentative species (Italian ovine isolates)	V1	U-A or C A bulge in position 15;
	V3	G*U or G G bulge in position 8.
CSFV	V1	U-A in position 13 (exception U*G);
	V3	U-A in position 2; U or C in position 8 (exception A).
Pronghorn tentative species	V1	G-C in position 2; U-A in position 9; U-A in position 12; U-A in position 15;
	V2	G-C in position 4;
	V3	G A bulge in position 5.
Giraffe tentative species	V1	C-G in position 2; U*G in position 20;
	V2	C-G in position 7;
	V3	C-G in position 4; G*U in position 7.
Bungowannah tentative species	V1	A-U in position 2; G-C in position 7; U-A in position 9; U-A in position 12; G-C in position 13;
	V2	A-U in position 3; G-C in position 4;
	V3	U-A in position 4; G A bulge in position 10; A in position 11.

nucleotides. However, the size of the sequence may change up to a virus genome sequencing performed on the entire RNA. Therefore the program was conceived to allow the analysis of large size sequences. Primary objective was to identify in the test sequence the three variable loci V1, V2 and V3 characteristic for genotyping procedure. The sequences to be tested were prepared in text file type (.txt) as input for the program. No other characters then bases were allowed in the file.

Once verified the compatibility of the input file, trough a first step, the program loaded in memory the sequence. In a second step the three variable sequences were

Table 3. Palindromic nucleotide substitutions (PNS) characteristic to the *Pestivirus* species genotypes. The position of base pairings is defined by numbering from the bottom of the variable locus.

BVDV-1 genotypes	Locus	Characteristic PNS markers
BVDV-1a	V1	U*G or C-G in position 14.
	V2	G*U or G-C in position 7.
	V3	A-U in position 4.
BVDV-1b1	V1	U-A in position 14 (exception G A bulge).
	V2	A-U in position 7 (exception A C bulge).
	V3	G-C in position 4 (exception A-U).
BVDV-1b2	V1	U-A in position 14 (exception A A bulge).
	V2	G*U or G-C in position 7.
	V3	G-C in position 4.
BVDV-1c	V1	C-G in position 14 (exception C A bulge).
	V2	A C bulge in position 7.
	V3	A-U in position 4.
BVDV-1d	V1	C-G in position 14.
	V2	A-U in position 7.
	V3	G-C in position 4.
BVDV-1e	V1	C-G in position 14.
	V2	G-C in position 7.
	V3	A C bulge in position 2; G-C in position 4.
BVDV-1f	V1	C-G in position 14.
	V2	A-U in position 7.
	V3	A-U in position 4.
BVDV-1g	V1	C-G in position 14; U*G in position 17.
	V2	G-C in position 7.
	V3	G*U in position 4.
BVDV-1h	V1	C-G in position 14.
	V2	C U bulge in position 6; G-C in position 7.
	V3	G-C in position 4.
BVDV-1i	V1	C-G in position 14.
	V2	G*U in position 1; G-C in position 7.
	V3	G-C in position 4; A A or G A bulges in position 8.
BVDV-1j	V1	C-G in position 14.
	V2	A-U in position 1; G- C in position 7; A C bulge in position 9.
	V3	G-C in position 4; G-C or G A bulge in position 6.
BVDV-1k	V1	C- G in position 14; C-G or C A bulge in position 16; A A bulge in position 19.
	V2	A-U in position 1; G-C in position 7.
	V3	G-C in position 4.
BVDV-1l	V1	CG in position 14.
	V2	UA in position 1; AC in position 7.
	V3	GC in position 4.
BVDV-1m	V1	C-G in position 14.
	V2	G-C in position 7; A C bulge in position 9.
	V3	G-C in position 4; A A bulge in position 6.
BVDV-1n	V1	C-G in position 14; C-G in position 15.
	V2	G-C in position 7; A-U in position 5.
	V3	G-C in position 4.
BVDV-1o	V1	C-G in position 14.
	V2	G-C in position 7; U-A in position 5.
	V3	G-C in position 4.
BVDV-2 genotypes	**Locus**	**Characteristic PNS markers**
BVDV-2a	V1	C-G in position 16; U*G, C-G or U-A in position 18.
BVDV-2b	V1	G-C in position 12; U-A in position 16; G A, G G or A C bulges in position 17; G G or G A bulges in position 18.
	V3	higher V3 loop, U in position 10.
BVDV-2c	V1	G-C in position 12; C-G in position 14; C-G in position 16; G A, G G or A C bulges in position 17; G A bulge in position 18.
	V3	higher V3 loop, C in position 10;

Table 3. Continues

BVDV-2d	V1	A-U in position 9; A-U in position 12; U-A in position 16; G A, G G or A C bulges in position 17; G A bulge in position 18.
	V3	higher V3 loop, U in position 10.
BVDV-2e	V1	U-A in position 8; C C bulge in position 20.
	V3	G-C in position 7.
BDV genotypes	**Locus**	**Characteristic PNS markers**
BDV-a	V1	A-U or C U bulge in position 9; A A or A G bulges in position 18 (exception G G bulge).
	V2	A-U in position 1.
	V3	A A, G A or A C bulges in position 8.
BDV-b	V1	G-C in position 9; G-C or G G bulge in position 18; G*U or G G bulge in position 20.
	V2	G*U in position 1.
	V3	U-A or C A bulge in position 8.
BDV-c	V1	G-C or U C bulge in position 20; U or U U bulge in position 21.
	V2	A C bulge in position 1.
	V3	C C bulge in position 7.
BDV-d	V1	G-C or A-U in position 9; U*G, G-C, G*U or G G bulge in position 18 (exception A G bulge).
	V2	G*U, G-C in position 1 (exceptions A-U and C U bulge).
	V3	U-A, C-G, U*G, A A or C A bulges in position 8.
BDV-e	V1	U-A, C-G or U*G in position 16.
	V3	C U bulge in position 1; G*U or U U bulge in position 2.
BDV-f	V3	U-A in position 2; U*G in position 7; U or C in position 8.
BDV-g	V1	G-C in position 3; U-A or C-G in position 16.
	V3	G-C in position 4.
BDV-h	V2	G-C in position 5.
	V3	G-C or A C bulge in position 2; C-G in position 7; U U bulge in position 9; U Uor C U bulge in position 10.
CSFV genotypes	**Locus**	**Characteristic PNS markers**
CSFV-1	V1	A C bulge in position 15 (exception G-C).
	V2	U-A in position 5; G:Y in position 7.
	V3	A-U in position 1.
CSFV-2	V1	G-C in position 15; A G bulge in position 19; U-A in position 20.
	V2	A-U in position 5; A C bulge in position 7.
	V3	A-U in position 1; U C bulge in position 6.
CSFV-3	V1	A-U in position 15.
	V2	U-A in position 5; G-C in position 7.
	V3	A_G bulge in position 1.

identified and further the secondary structures were determined according to the Watson-Crick base pairings, with strong bindings as adenine uracil or cytosine guanine, tolerated pairings in secondary structure as guanine uracil and pairings without binding forming bulges as cytosine cytosine. Since timine is equivalent to uracil, the program was adapted to translate as timine in case test sequences were constructed with uracile. In addition, the nucleotides were considered indifferently when expressed in small or capital letters in the sequence file.

PNS class

The most important class of the software was named PNS and, in addition to a series of data structure definitions, it contains two fundamental methods which are respectively: the *SearchPNS,* for loading in memory the test sequence nucleotides and to determine correct V1, V2 and V3 structures; the *CreateHTML*, in charge of V1, V2 and V3 structure analysis by known parameters in order to recognize PNS and create an Html file containing the results of the analysis.

Loading in memory of key data structures

The PNS software uses four data structures to memorize and keep available information during the analytical process.

The memorization of all possible nucleotide pairings and the related bindings was done by a character matrix.

The nucleotides read in the input file were stored in an array (Lodi and Pacini, 1998) for a maximum dimension of 15,000.

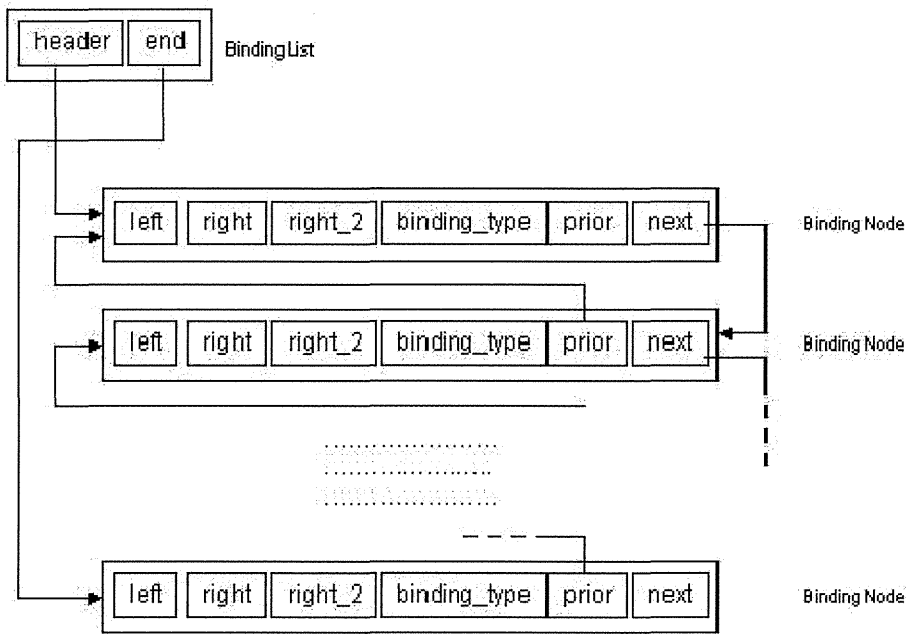

Figure 1. Schematic presentation of the pointer list management in the PNS program memory

The analysis parameters for typing genus, species and genotypes were stored in three arrays of a structure (*struct*) named *Records*, which contains respectively the following fields: *name, structure, position, left, right, binding*.

The V1, V2 and V3 structures were stored using three pointers list (Lodi and Pacini, 1998) defined by two different type of *struct*. The first defined the single node of the list and the second defined the structure of list type. Nodes were linked among them in a sequential order by virtual bindings represented by pointers, addresses of memory, so that a single node contains the pointer at a following node. The list node contains the following fields: *left, right, right_2, binding_type, prior, next*. A schematic presentation of the list management in the program memory is reported in Figure 1.

PSet abstract structure

On the base of the model proposed by Apicella (1999), an abstract parametric class or template, called *PSet,* was realized in order to obtain a dynamic and flexible structure allowing the manage of a double linked pointer list of generic type T objects.

The use of *PSet* was necessary for the program, expecially during the phase of V1, V2 and V3 structure determination where the selection of the correct structure was realized on the base of defined criteria among a large number of possible structures. A *PSet* class opportunely instanced facilitate the entire list recording, the evaluation of the possible structures and the identification of the appropriate one.

Construction of the V1, V2 and V3 palindromic structures

The appropriate structure of type V1, V2 or V3 of a tested sequence was selected among a large number of possible structures identified according to well defined criteria, according to specific program procedures. The three variable loci in the test sequence were identified starting by the V2 locus due to the invariable number of nucleotide composing the palindrome (23 bases) and the characteristic conserved 4 guanine sequence of the loop (5´-GGGG-3´). The calculation of the secondary structure was based on the search of palindromes with the higher number of Watson-Crick base pairings with strong bindings (C-G; G-C; A-U; U-A), which are most probable, with priority for the stability of the structure in confront to tolerated pairings (G*U; U*G) and pairings forming bulges (CC; AA; AC; AG; CA; GG; GA; UU; CU; UC). The construction of the palindrome structures respected the principle of the correct curbing of the sequence at the loop level, thus avoiding formation of base pairing generating strong or weak, tolerated bindings. In other terms, if at loop were present 3 nucleotides n_1, n_2, n_3 the potential binding occurring between n_1 and n_3, for example G-C, was not considered. Similarly, in case of 4 nucleotides (n_1, n_2, n_3, n_4) the potential binding between