was strongly positive but was only weakly positive for *T. canis* adult antigens. We abandoned TBLB because of his tendency to bleed from the bronchial mucosa when the fiberscope made contact. However, we suspected VLM because this patient had the same dietary history and similar symptoms as his son (Case 1). Therefore, we performed further serological tests. An Ouchterlony test against ES products of *T. canis* larvae was strongly positive (Figure 2B), whereas a Toxocara CHEK and plate ELISA of the serum sample and BALF were positive for larval ES antigens. He was started on albendazole treatment (600 mg/d for 33 days). By the end of this treatment, the cough and sputum production had disappeared, the serum eosinophil count had decreased, and the platelet count had increased. However, the non-specific IgE level remained high as in Case 1. Unfortunately, we stopped the drug treatment because of signs of impaired liver function in the form of elevated plasma alanine aminotransferase of up to 330 IU/L. Thereafter, this patient was lost to follow-up. No abnormality was found in the optic fundi or head on CT in either of these two cases.

DISCUSSION

Parasitic infections remain relatively common even in developed countries, caused in part by an increasing number of overseas travelers and pet owners. In Japan, the diversification of dietary habits is thought to be another reason for the persistence of food-borne parasitic diseases.[7] It had been believed that toxocariasis was a disease of children who accidentally ingested infective eggs of *T. canis*. In Japan, however, adult cases of toxocariasis have been reported after the ingestion of meat from quails, cows, and chickens,[8] suggesting that toxocariasis in Japan must be considered a food-borne parasitic disease of adulthood. After invasion of the host, these larvae hatch in the digestive track and migrate to the liver through the host portal system. Thereafter, some larvae continue to migrate to the lungs and systemic organs through the circulation, resulting in the symptoms characteristic of the clinical disease.[9,10] Recently, several cases of VLM by *A. suum* were reported in the southern part of Japan.[9,11] The origin of such infections involves swine husbandry and the inappropriate disposal of waste materials. In such cases, the antibodies against larval ES of *A. suum* should be detectable, although this was not possible with our patients.

Glickman and others[12] reported that hunting or living in a household with hunting dogs was a high risk factor for VLM in people, especially those with signs of allergies. In our cases, there was no history of allergies previously, although they have had such a risk factor. It is obvious that VLM symptoms were brought on by the ingestion of raw chicken livers because the event was the first onset. Therefore, we must consider the way of infection not only from hunting dogs directly but also from the raw meat of domestic fowl contaminated with *T. canis* eggs.[4,13] Incidentally, this is the first report that *Toxocara* sp. was proven to be recovered from raw materials of a meal directly.

While a definitive diagnosis in parasitic diseases is best made after the detection of worms or eggs from patients, the direct detection of *Toxocara* larvae is quite rare. Generally, food-borne toxocariasis can be diagnosed through a combination of a patient's admission with a history of ingesting raw meat, laboratory findings such as hypereosinophilic syndrome, and the presence of specific antibodies against larval ES antigens of *T. canis* in the serum. Our findings further suggest that specific antibodies in BALF should be measured in patients with respiratory symptoms.

We found that the ToxocaraCHEK, a rapid diagnostic test kit for toxocariasis, was inexpensive, easy to use, and accurate in detecting antibodies to larval ES products of *T. canis*.[14] This test kit was known to be useful for the detection of antibodies in vitreous fluid,[15] but we successfully applied it to the identification of antibodies in BALF in these cases. We believe that this is the first report to show the specific antibodies to the larval ES antigen of *T. canis* in BALF, although previous investigators showed an elevated eosinophil count in BALF.[3] These observations suggest that clinicians should consider food-borne toxocariasis in cases of severe respiratory disease with eosinophilia and a patient history of raw meat ingestion.

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Migration behaviour and pathogenesis of five ascarid nematode species in the Mongolian gerbil *Meriones unguiculatus*

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Abstract

To understand the characteristic features of the Mongolian gerbil, *Meriones unguiculatus*, as an animal model of ascarid infections, the migration behaviour and pathogenesis of larvae were investigated in experimentally infected gerbils. Embryonated eggs from each of *Toxocara canis*, *Baylisascaris procyonis*, *B. transfuga*, *Ascaris suum*, and *A. lumbricoides* were orally inoculated into gerbils and larvae were recovered from various organs at designated periods. In *T. canis*-infected gerbils, larvae were present in the liver 3 days after infection and in the skeletal muscle and brain via the heart and lungs at a similar rate. In *B. procyonis*- and *B. transfuga*-infected gerbils, larvae were present in the lungs within 24 h after infection, with some having reached the brain by that time. After 24 h, larvae of *B. procyonis* tended to accumulate in the brain, while those of *B. transfuga* accumulated in skeletal muscles. In *A. suum*- and *A. lumbricoides*-infected gerbils, larvae remained in the liver on day 5 post-infection and elicited pulmonary haemorrhagic lesions, which disappeared 7 days after initial infection. Thereafter, no larvae of any type were recovered. Ocular manifestations were frequently observed in *T. canis*- and *B. procyonis* infected gerbils, but were rare in *B. transfuga*-infected gerbils. In the cases of *A. suum* and *A. lumbricoides*, migration to the central nervous system and eyes was extremely rare, and larvae had disappeared by 2 weeks post-infection. Fatal neurological disturbances were observed in *B. procyonis*-infected gerbils, whereas irreversible non-fatal neurological symptoms were observed in the case of *B. transfuga*.

Introduction

Larval stages of ascarid nematodes elicit severe tissue damage when they invade hosts which are not normally the definitive host. The racoon roundworm, *Baylisascaris procyonis*, is particularly prone to cause a fatal neurological disturbance (Huff *et al.*, 1984; Kuchle *et al.*, 1993; Moertel *et al.*, 2001; Wise *et al.*, 2005). The dog roundworm, *Toxocara canis*, and the cat roundworm, *T. cati*, are also responsible for the visceral larva migrans syndrome (VLM) in humans (Glickman & Magnaval, 1993; Fisher, 2003). The VLM caused by these two roundworms is commonly known as toxocariasis, which is considered a disease of infants and children, although adults are also infected (Glickman *et al.*, 1987; Aragane *et al.*, 1999; Yoshida *et al.*, 1999). Moreover, outbreaks of VLM due to the pig roundworm, *Ascaris suum*, have been reported from the southern part of Japan (Maruyama *et al.*, 1996; Sakakibara *et al.*, 2002). Meningitis can occur when *Ascaris* larvae migrate into the central nervous system

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(Osoegawa *et al.*, 2001). However, a precise diagnosis of these animal-borne ascarid infections is not always possible and an adequate anthelminthic therapy against these infections was not available. The lack of a comprehensive study using animal models for ascarid infections has also prevented progress in this field.

The Mongolian gerbil *Meriones unguiculatus* is known to be susceptible to a variety of parasites including *Brugia pahangi* (Ash & Riley, 1970), *Strongyloides stercoralis* (Nolan *et al.*, 1993), *Nippostrongylus brasiliensis* (Horii *et al.*, 1993), and *Entamoeba histolytica* (Chadee & Meerovitch, 1984). Akao *et al.* (2000) and Takayanagi *et al.* (1999) demonstrated that gerbils could serve as an animal model for ocular toxocariasis due to both *T. canis* and *T. cati*. The occurrence of retinal haemorrhages including larval invasion into the retina was found to be quite high compared with that in mice after oral inoculation of infective eggs. However, no information is available on the migration route, the final site of infection, or on the pathogenesis of ascarid larvae in gerbils. Here, we present our findings on the characteristic features of ascarid infections in gerbils as they relate to the pathogenesis of VLM in humans.

Materials and methods

Mongolian gerbils *Meriones unguiculatus* ranging between 2 and 3 months of age, were raised in the Animal Centre of Nippon Medical University and were maintained under pathogen-free conditions. Only male gerbils with black hair were used and all experiments were carried in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Recovery of ascarid eggs

Eggs of *T. canis* were obtained from the uteri of adult worms collected from faeces following the administration of anthelmintics to naturally infected puppies. Worms of *B. procyonis* were recovered from the intestine of infected racoons (Sato *et al.*, 2002), and those of *B. transfuga* collected from the faeces of infected bears. Worms of *B. transfuga* were naturally expelled from bears during their fasting period just prior to hibernation. Adult worms of *A. suum* were obtained from slaughtered pigs and *A. lumbricoides* were collected after treatment with anthelmintics from naturally infected humans. Female worms of each species were isolated, and matured embryonated eggs were prepared following the method of Oshima (1961).

Fully embryonated eggs were treated with 50% hypochlorous acid for 10 min to remove their proteinous membranes. After repetitive washing with distilled water, 1000 eggs of each ascarid were inoculated into each gerbil through a gastric tube under light anaesthesia.

Recovery of larvae

After ophthalmological observations (Takayanagi *et al.*, 1999), four gerbils from each group were sacrificed using sodium pentobarbital at predetermined intervals. One gerbil from each group was examined histopathologically and the remainder were used for larval recovery from the gastrointestinal tract, liver, lungs with heart, and skeletal muscle including bone and genital organs except for the skin. The contents of the gastrointestinal tract were collected in a conical tube at 6 and 12 h after inoculation. Each organ was minced and digested with artificial gastric juice (0.5% 1:10,000 pepsin, 0.7% hydrochloric acid) for 2 h at 37°C along with vigorous agitation. After digestion, the fluids were sieved with a tea strainer, and centrifuged at $320 \times g$ for 5 min. The supernatant was discarded and a small amount of distilled water was added to the tube. The sediment was then spread out on glass, and larvae in the fluid were counted using a stereoscopic microscope. Examination of the brain tissue was performed as follows: each brain including the olfactory bulb, cerebrum, cerebellum, and pons was enucleated and minced into small pieces (approximately 2 mm^3) on a slide glass using forceps, and these were then covered with another slide glass. Migrating larvae were then counted using a microscope with eight or nine slide glasses being examined in each sample. Recovery rates were calculated from a mean of three gerbils at each period.

Histopathology

Tissue samples of liver, lungs, femoral muscle, brain, and gastrointestinal tract including the stomach, duodenum, ileum, caecum and rectum were fixed in 10% neutral formalin solution. Serial sections were prepared and stained with haematoxylin and eosin or periodic acid Schiff haematoxylin.

Results

Changes in the recovery rates of larvae from various organs after oral inoculation of the five ascarid species were recorded (fig. 1) and recovery rates arranged in the order of the migration route shown in fig. 2. In *Toxocara canis*-infected gerbils, almost all larvae were recovered from the intestinal wall up to 24 h after infection. Thereafter, larvae began to appear in the liver and lungs (by day 3), and then in the skeletal muscle and brain. The number of larvae in the muscle and brain were approximately equal. Macroscopically, haemorrhagic lesions which were observed in the lung 3, 5, 7 and 14 days after infection gradually disappeared. Ophthalmoscopically, a motile larva was observed in the retina 14 days after infection.

In *B. procyonis*- and *B. transfuga*-infected gerbils, the recovery rates of larvae were significantly lower than that in the case of *T. canis*. Both species of larvae had migrated into the lungs through the liver within 24 h after infection, and some had already arrived in the brain by this time, resulting in small haemorrhagic foci in the brain (fig. 3). With *B. procyonis*, ocular invasion by the larvae was observed and neurological disorders such as rotational and involuntary movement or paraplegia emerged, resulting in the mortality of gerbils between days 15 and 20 post-infection. Macroscopically, the gerbils exhibited fresh petechial haemorrhages of the lung 1 day after infection, but no new haemorrhagic lesions were evident beyond that time. Ophthalmic examination

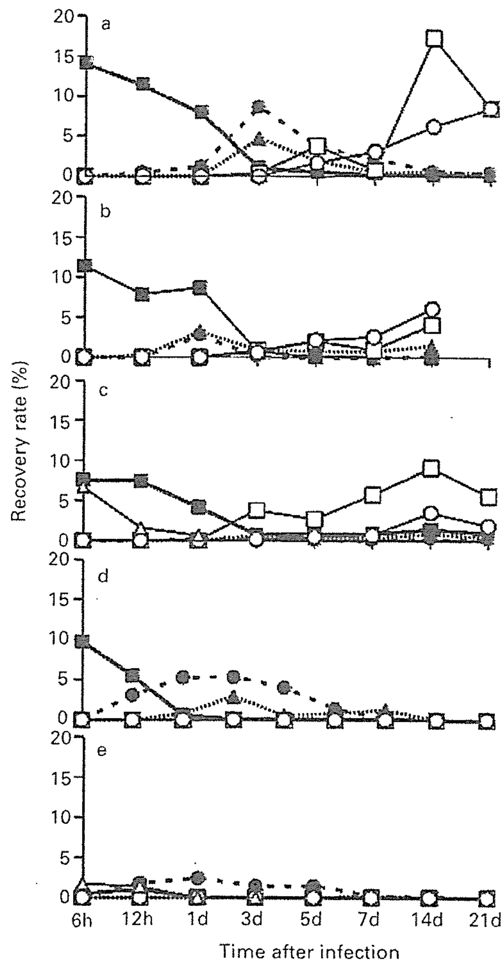

Fig. 1. Recovery rates (%) of larvae from various organs in gerbils after oral inoculation with five ascarid species up to day 21 post-infection. a, *Toxocara canis*; b, *Baylisascaris procyonis*; c, *B. transfuga*; d, *Ascaris suum*; e, *A. lumbricoides*; ■, gastrointestinal tract; ●, liver; ▲, lung and heart; □, muscles; ○, brain; △, intestinal contents.

demonstrated motile larvae in the retina and severe chorioretinitis 7 days after infection. The number of migrating larvae of *B. procyonis* in the brain was higher than that of *B. transfuga*-infected gerbils at all times after the infection. By day 3 post-infection an average of 6.3 larvae (range 6–7) in *B. procyonis*-infected gerbils and 1.7 larvae (range 1–2) in *B. transfuga*-infected gerbils were found. By day 7, an average of 26.0 larvae (range 23–31) in *B. procyonis*-infected gerbils and 6.7 larvae (range 5–8) in *B. transfuga*-infected gerbils were recovered. Ophthalmic and neurological abnormalities were less severe than those observed with *B. procyonis*, although gait difficulty and circulatory movements in the same direction were presented by day 10 post-infection. The number of *B. transfuga* larvae in the skeletal muscle was higher than

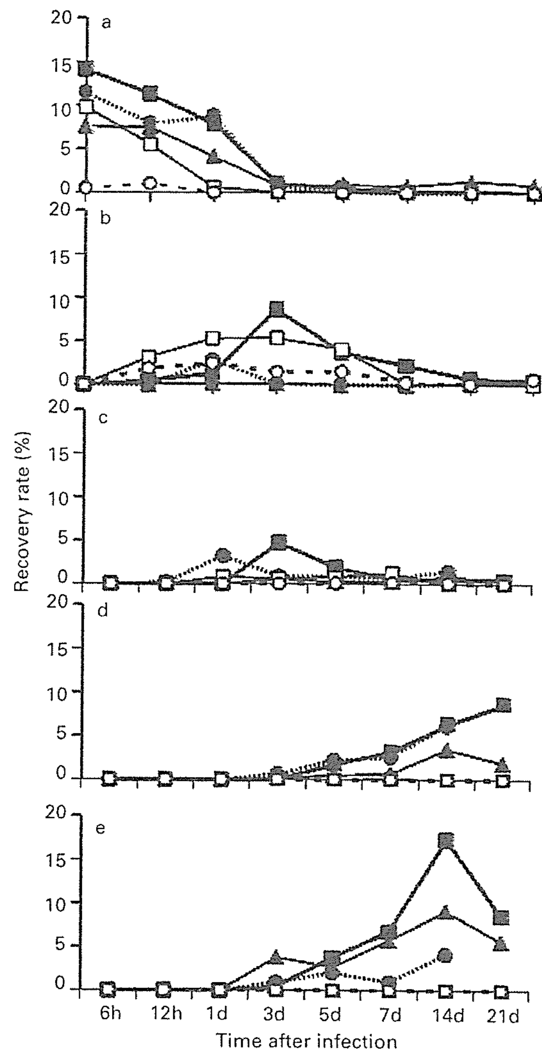

Fig. 2. Recovery rates (%) of larvae in gerbils after oral inoculation with five ascarid species to show the route of migration. ■, *Toxocara canis*; ●, *Baylisascaris procyonis*; ▲, *B. transfuga*; □, *Ascaris suum*; ○, *A. lumbricoides*; a, gastrointestinal tract; b, liver; c, lung and heart; d, brain; e, muscles.

that in the brain; i.e. an average number of larvae recovered were 37.7 (range 21–67) in muscle and 1.7 (range 1–2) in brain at the day 3 post-infection, and 91.0 (range 95–100) in muscle and 34.7 (range 24–49) in brain at the day 14 post-infection.

With *A. lumbricoides*, the average recovery rate of larvae after 6 h of infection was 2.3% (range 1.7–2.6%) compared with about 10% or higher in other ascarid parasites (14.1% in *T. canis*, 11.4% in *B. procyonis*, 16.3% in *B. transfuga* and 9.7% in *A. suum*; fig. 1a). Migrating larvae were present in the liver 12 h after infection, and remained there for up to 5 days after infection in the case of *A. lumbricoides* and

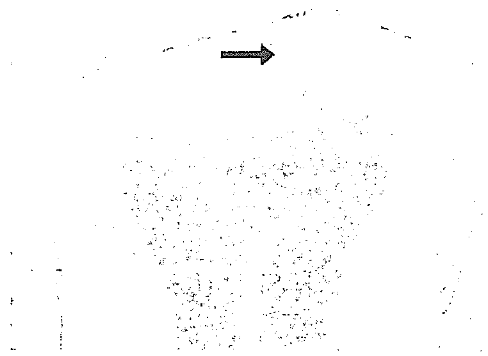

Fig. 3. A fresh haemorrhagic lesion in the granular layer of the cerebellum in gerbils 24 h after infection with *Baylisascaris procyonis*; larva (arrowed) in the molecular layer of cerebellum immediately beneath the pia mater.

7 days in the case of *A. suum*. On day 7, haemorrhagic lesions in the lungs were the most prominent feature in gerbils infected with both species, although these lesions gradually disappeared and no larvae were recovered thereafter from any organ.

As far as the migration route is concerned, the highest recovery rate in the gastrointestinal wall occurred in *T. canis*-infected gerbils followed by *B. procyonis*-, *A. suum*-, and *B. transfuga*-infected gerbils 6 h after infection. Larvae of both species of *Ascaris* immediately migrated away from the intestinal wall to the liver within 24 h after infection, with *Toxocara* and *Baylisascaris* larvae remaining there until the end of experiment. *Ascaris lumbricoides* larvae were minimally recovered from not only the gastrointestinal tract but also from other organs throughout the experiment. The recovery rate of ascarid larvae from the brain was high in the case of *T. canis* (3.1%) and *B. procyonis* (2.6%) at day 7 post-infection as compared with *B. transfuga* (0.7%), even though *B. procyonis*-infected gerbils did not survive until the end of the experiment. On the other hand, no *A. lumbricoides* larvae were found and only one *A. suum* larva was observed on day 7 post-infection. The recovery rate from skeletal muscles was high in the case of *T. canis* and *B. transfuga*, although the number of muscle stage larvae of *B. transfuga* was always higher than that in *B. procyonis*.

Discussion

Takayanagi *et al.* (1999) demonstrated that the Mongolian gerbil is a suitable animal model for ocular toxocariasis because of the high incidence of ocular invasion by the larvae. However, little is known about the migratory behaviour or pathogenesis of ascarid larvae in gerbils. In the present study, *T. canis* larvae migrated to the liver within 3 days after infection, and were thereafter distributed equally in skeletal muscles and the brain. These results are similar to those of Olson (1962) and Sprent (1952), suggesting that the migration route and final site of infection have little influence on the development of ocular toxocariasis in gerbils.

In the present study, *B. procyonis* larvae more so than *B. transfuga* were likely to accumulate in the brain and all gerbils infected with *B. procyonis* died from severe neurological disturbances within 2 weeks after infection. On the other hand, gerbils infected with *B. trasfuga* survived throughout the duration of the experiment, despite exhibiting neurological disorders. The number of *B. transfuga* muscle stage larvae was always higher than in *B. procyonis*-infected gerbils. Sato *et al.* (2004) reported that the *B. procyonis* and *B. transfuga* larvae that had migrated into the brain of gerbils were larger than those of *T. canis*; however, no significant differences in larval size were observed between *B. procyonis* and *B. transfuga*. These results suggest that severe neurological disorders caused by *B. procyonis* could be attributed to the total amount of larvae in the brain. Additionally, these findings suggest that *B. procyonis* larvae may have a neurotropism, whereas *B. transfuga* larvae may have an affinity for muscular tissue. Further studies are needed to better understand the pathogenetic differences between *B. procyonis* and *B. transfuga* larvae in the brain of infected gerbils. Ophthalmologically, the lesions elicited by both species closely resembled each other although the incidence was extremely low in *B. transfuga*-infected gerbils. These results indicate that *B. transfuga* should not be used as an alternative parasite for studying diffuse unilateral subacute neuroretinitis induced by *B. procyonis* in gerbils (Akao *et al.*, 2003).

In the present study, the infectivity of *A. suum* and *A. lumbricoides* in gerbils was very low, with migration to the central nervous system being minimal and no ophthalmological changes were found. Therefore, *A. suum* and *A. lumbricoides* are considered inappropriate parasites for studying ophthalmological and neurological disorders in gerbils. Severe to mild pulmonary haemorrhagic lesions were common in infected gerbils, although a complete healing of these lesions occurred in the case of *A. suum* and *A. lumbricoides*. Interestingly, no larvae were recovered from any organs of these gerbils beyond 14 days post-infection. Mouse models have shown a similar pattern (Slotved *et al.*, 1997, 1998). To further document the migratory behaviour of *A. suum* and *A. lumbricoides* larvae in gerbils after 7 days of infection, the contents of the gastrointestinal tract were examined daily between days 8 and 13 post-infection because we assumed that the larvae might return to the intestine via the larynx and pharynx. However, no larvae were detected (data not shown), suggesting their rapid expulsion from infected gerbils.

Further studies are needed to more fully elucidate the migration behaviour and pathogenesis of *T. cati* so that we may potentially improve the therapy against this important zoonotic parasite of human VLM (Akao *et al.*, 2000; Fisher, 2003).

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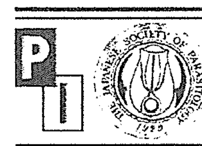

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Review

Toxocariasis in Japan

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Abstract

Toxocariasis has long been considered a parasitic disease affecting pet owners and children who often play in sandboxes at public parks. Recent cases of this animal-borne infection, however, indicate that its clinical manifestations and etiologies are changing. In this article, we will describe the critical characteristic features of toxocariasis alongside the contributions of Japanese researchers to a better understanding of the disease.
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Keywords: *Toxocara canis*; *Toxocara cati*; Toxocariasis; Visceral larva migrans

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1. Introduction

Among animal-borne diseases, toxocariasis is one of the most popular parasitic infections in the world, caused by the larval stage of *Toxocara* spp. Humans are infected mainly by the tiny developmental stage of the parasite, which belong to the family Ascaridoidea, through their pet dogs and cats. Other natural hosts include wild Canidae for *Toxocara canis* and wild felines for *Toxocara cati*. Symptoms depend on organs affected and the magnitude of infection. It is usually a non-fatal disease, but the larvae migrate through the eyes and can cause severe vision disability or even blindness.

In 1950, Dr. Wilder, an American ophthalmologist, histopathologically identified a nematode of unknown etiology in the retinas of 26 out of 46 enucleated eyes with retinoblastoma [1].

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Two years later, Beaver et al. [2] recognized the same parasite in the liver of three young children. Shortly afterwards, the parasite was correctly identified as an infectious stage larva of *T. canis* [3–5]. Since then, many clinicians and biologists have been accumulating knowledge of *Toxocara* and toxocariasis.

In this review article, we describe the lesser-known contributions of Japanese researchers to the understanding of *Toxocara* and toxocariasis. This articles builds on the work of Kondo [6], focusing on the topics that he did not cover in his review and on new findings since his publication.

2. Toxocariasis in humans

2.1. Clinical cases

Toxocariasis is clinically classified into four types: visceral, ocular, neurologic, and covert [7,8]. In 1963, the first report on toxocariasis in Japan was presented orally at the 32nd Annual Meeting of the Japanese Society of Parasitology by Fushimi et al. [9]. A 14 year-old boy was admitted to a university hospital because of fever, hepatomegaly and persistent eosinophilia. The patient died from severe anemia six months later. Though no autopsy or serological examinations were performed, the patient was strongly suspected to have suffered from visceral toxocariasis. In the early 1960s, immunological tests for parasitic infections, especially for helminthiasis, had only just begun, and antigen for the diagnosis of toxocariasis was not yet known.

Just as in other parasitic infections, direct demonstration is the only way to make definite diagnosis of toxocariasis. However, it is difficult to find the larva in either tissue biopsies or autopsies due to its very small size. So far in Japan, one morphologically and two pathologically confirmed cases have been reported [10–12]. Two additional reports, both of ocular toxocariasis, were doubtful because of the lack of characteristic features of the parasite; the authors nevertheless reproduced the microscopic findings of the purported larva in their papers [13,14]. One of these two cases showed increased antibody production in vitreous fluid against *Toxocara* antigen prepared from larval excretory–secretory product (LES), suggesting that the case might be attributable to ocular toxocariasis.

Serology is an alternative method for the diagnosis of toxocariasis. A method has been established for *in vitro* cultivation of the larvae, with LES prepared from the culture medium serving as an antigen. Detection of specific antibodies against LES provides evidence of *Toxocara* infection in individual patients and useful tool for understanding the epidemiological characteristics of this disease. The first serological survey in Japan was reported by Matsumura and Endo [15] using sera of 83 clinically healthy children. In their sample, 3.6% tested were positive for LES. In another study, Matsumura and Endo [16] demonstrated that 20 of 530 adults possessed the IgG antibody to LES. The positive individuals were thought to have a latent or past infection. In a large-scale seroepidemiolgical survey, Kondo et al. [17] collected 3277 sera from 14 prefectures in Japan and tested for LES antibodies. Antibodies were confirmed in 52 individuals (1.6%), but geographical patterns were notable: the highest prevalence rate was observed in Miyagi Prefecture (6.1%), and the lowest was in Ibaragi Prefecture (0.5%). The researchers concluded that the overall seroprevalence rate was in good agreement with those reported from other countries [17–19].

Based on improvements in the field of serology, diagnosis of toxocariasis is usually made by detection of the specific antibody to LES, along with clinical manifestations such as eosinophilia, eosinophilic pneumonia, or ophthalmoscopic findings.

2.2. Characteristic features of toxocariasis

2.2.1. Toxocariasis as a food-borne infectious disease

Using serological methods, there were nearly 200 reports of toxocariasis in the database of Japana Centra Revuo Medicina, and almost 300 cases have been diagnosed in Japan in the past two decades. Among these cases, some significant reports have provided a new perspective on the pathogenic mechanisms of toxocariasis.

Since Beaver et al. [2] introduced the concept of visceral larva migrans, characterized by chronic eosinophilia with granulomatous lesions in the liver, toxocariasis was regarded as a disease in children who were infected by soil contaminated with embryonated eggs [20]. In 1983, Sakai et al. [21] reported a case toxocariasis after ingestion of raw chicken liver. The 57-year-old man was admitted to a hospital due to cough, fever and weight loss. Complete blood count revealed a marked increase in eosinophils in peripheral blood with leukocytosis, and serum antibody against *T. canis* was strongly positive. Before onset, he and his friends had eaten raw chicken livers derived from his poultry and boar farm. Soon after the meal, they experienced abdominal pain, vomiting and diarrhea, but the symptoms improved within two days after ingestion. One month later, his chief complaints emerged. Two similar cases were subsequently reported by the same group [22].

These cases clearly indicates that the disease should be considered a food-borne parasitic infection. Four additional papers describing six patients were published in Japan in the 1980s [22–25]. These patients, all male and between 22 and 51 years of age, had a history of eating raw meat or liver of fowl and/or cattle before onset of symptoms. The possibility that raw liver of domestic animals can transmit the pathogens of human visceral larva migrans was substantiated by Lee et al. [26] of Yonsei University College of Medicine in Korea. They found that a dietary habit of raw liver was much more frequently seen in males than in females, especially in the 31–40 age group. Experimental studies revealed that chicken, cattle and swine were able to act as paratenic hosts for *T. canis* [27–29]. Most of the adult cases reported in recent years in Japan are categorized as this type of infection [30].

2.2.2. Respiratory illness and toxocariasis

In animal models in rodents, hatched larvae migrate into the lungs through the liver after ingestion, resulting in liver dysfunction and pneumonia [31–33]. In humans, similar manifestations are well documented in the literature [30,34–36]. Pulmonary lesions appear on computed tomography as multifocal subpleural nodules with halos or ground-glass

opacities and ill-defined margins. Additionally, transient pulmonary infiltrates are a characteristic finding. Morimatsu et al. [30] recently reported a familial case of visceral toxocariasis after consumption of raw chicken livers. In this case, the patients, a father (71 years old) and his son (45 years old), ate raw chicken livers three weeks before onset and then developed mild fever, general fatigue, headache and respiratory disorder. The specific antibody to LES was identified both in their serum samples and in bronchoalveolar lavage fluid (BALF). *T. canis* larvae were recovered from chicken liver from the same source as that ingested by the patients. These cases showed that BALF is a reliable specimen to demonstrate LES antibodies when the patient shows respiratory illness.

2.2.3. Urticaria-like skin lesions and toxocariasis

Parasitic infection is often said to be associated with chronic urticaria [37]. This is still a controversial issue, but acute urticaria is certainly associated with infection with larva from the marine fish parasite, *Anisakis simplex* [38]. Japanese have long tradition of eating raw fish, sashimi and sushi, and anisakidosis is a common parasitic infection in Japan. It is well documented that urticaria is closely related to the infestation of *Anisakis* larva [38,39]. As with anisakidosis, an allergic reaction could be elicited by the invasion of *Toxocara* larvae and result in skin rash that looks like hives. These skin manifestations might occur as a result of immunological response to larval metabolites [40,41].

In 1999, the first confirmed case of toxocariasis with larva in subcutaneous tissue was reported [11]. A 26-year-old female with fever, headache, and dry cough was admitted to a university hospital. Her peripheral blood smear showed an eosinophilia (61%) and her chest radiograph revealed multiple nodules. A diagnosis of visceral toxocariasis was made after detection of LES antibodies. During her hospitalization, several brown itchy nodules, which were thought to be prurigo, developed on her legs. Histological examination showed *Toxocara* larva in the center of an eosinophilic and lymphocytic abscess. The patient admitted frequently eating raw beef liver almost one year before her hospitalization for its purported health benefits. We can learn from this case that larvae migrating into subcutaneous tissue directly elicit pruriginous skin lesions.

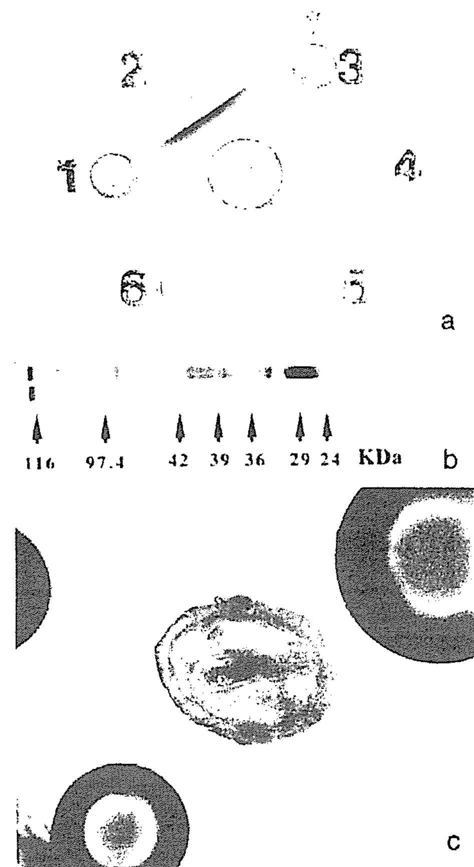

Fig. 1. The results of double gel diffusion (a) and western blot (b) tests of a patient of visceral toxocariasis. Strong precipitin bands were obviously observed between larval excretory–secretory products (LES) of *Toxocara canis* and patient's serum by means of double gel diffusion test. Antigens used in this test were adult worm extract (AEX) of *T. canis* (1), LES of *T. canis* (2), AEX of *Dirofilaria immitis* (3), AEX of *Ascaris suum* (4), LES of *Anisakis simplex* (5) and AEX of *Ascaris lumbricoides* (6). Western blot test shows a whole range of LES molecules were reacted with the patient's serum (upper strip) but not with a normal control serum (bottom strip). An embryonated egg recovered from the soil in the park where the patient often played (c). A fully developed and live *Toxocara* larva was found in the egg.

2.2.4. Toxocariasis is a disease that affects adults rather than children

Many reviews from western countries indicated that children under 12 years old, who often play outside, are the most affected age group for toxocariasis [42,43]. They are accidentally infected with *T. canis*/*T. cati* eggs, which expelled in feces puppies and fully develop in the surrounding environment within two to four weeks. Therefore, contaminated soil is the most important etiological source for toxocariasis [44,45]. Hori et al. [46] reported a case of visceral toxocariasis in a 1.5-year-old girl with fever, hepatomegaly, and eosinophilia (73%). The patient had a history of pica, particularly eating soil from a nearby park where she frequently played with her brother. Serological examination strongly suggested that she was suffering from *Toxocara* infection (Fig. 1a, b). They also found many embryonated eggs from the soil in the park that contained a live larva closely resembling *T. canis* eggs (Fig. 1c). Fortunately, her brother showed a negative result in serological tests.

In a review article of Barriga [47], the average age of visceral toxocariasis was 9.5 years, and only 18% of patients were adults. However, in recent investigations, adults rather than young children were more frequently affected by this parasite. This tendency is particularly true for ocular toxocariasis. Yoshida et al. [48] described that, among 38 Japanese cases of ocular toxocariasis, 34 (89%) were older than 20 years of age, and suggested that clinical features observed in these patients were somewhat different from those of previously reported cases [49]. Therefore, ocular toxocariasis is no longer merely a disease of young children, but affects any age group having a risk factor such as consumption of raw meat or close contact with contaminated soil.

As of the end of 2006, 584 clinically suspected cases of toxocariasis (112 of visceral type and 472 of ocular type) have been referred to our laboratory for detection of the anti-*Toxocara* antibody. We omitted 109 cases from this study due to a lack of description of the patient's age and sex. In visceral toxocariasis, the male-to-female ratio in the remaining sample was 2.04 (male: 53, female 26). The average age was 39.2±21.7 (range, 0–83 years old) in male and 31.3±23.9 8range, 0.5–82 years old) in female. On the other hand, the male-to-female ratio in ocular toxocariasis group was 1.16 (male: 213, female: 183). The average age was 39.3±18.5 among males (range, 2–83 years old) and 37.6±18.2 among females (range, 2–74 years old). There were no significant differences in age distribution between males and females (Fig. 2). A similar result was obtained by Fujino et al. in 1998 [50].

2.2.5. Myelitis and toxocariasis

According to the case-control study by Magnaval et al. [51], migration of *T. canis* larvae in the human brain does not frequently induce recognizable neurological signs, but is possibly responsible for repeated low-dose infections. These light parasitic burdens usually do not appear to elicit a special clinical symptom, but in some cases, sever neurological disorders such as encephalitis, myelitis and meningitis are manifested [52]. In Japan, Ota et al. [53] reported a case of eosinophilic meningo-encephalo-myelitis due to *Toxocara* infection. The patient, a 21-year-old woman, showed frontal headache, low-grade fever and convulsion. She had a long history of close contact with her pet dog. Immunological tests were strongly positive for LES antigen in both her serum and cerebrospinal fluid. Based on clinical evidence and characteristic features in similar patients, Kira and his colleagues proposed a new disease entity: "atopic myelitis" or "parasitic myelitis." They assumed that allergic reaction to LES might be involved in this neurologic disorder [54]. Interestingly, most of the patients lived in Kyushu District, in the south of Japan, suggesting that myelitis due to *Toxocara* infection might be a regional clustering disease.

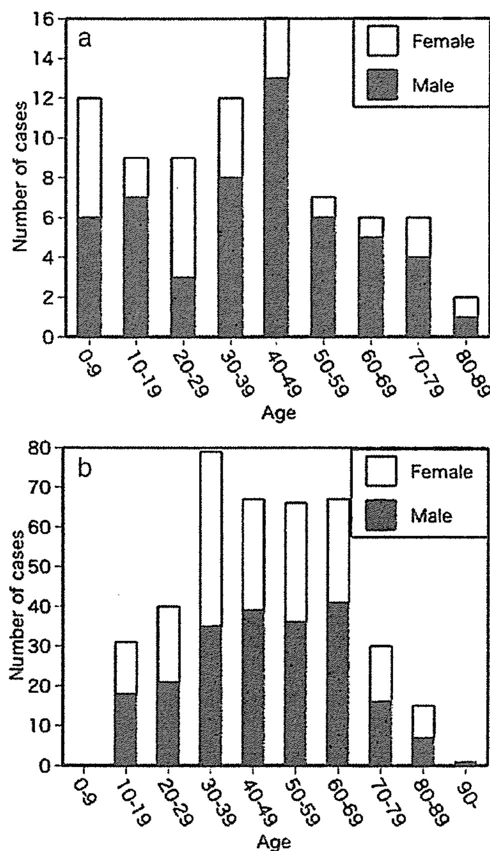

Fig. 2. Age distribution of suspected cases of visceral (n=79)(a) and ocular toxocariasis (n=396)(b) referred to our laboratory from August 1994 to December 2006.

2.3. T. cati

Because morphological differences between *T. canis* and *T. cati* in the adult stage are apparent [55], *T. cati* is easy to identify when patients expel adult worms. It has been suggested that *T. cati* could develop in children through the ingestion of the immature worm of *T. cati* [56]. More than 26 cases were reported so far [56,57], but there was only one case was reported from Japan. A 5-year-old male boy was admitted to a hospital due to a complaint of vomiting 3 worm-like foreign bodies. These worms were morphologically identified as two female and one male immature worms [58].

On the contrary, there are few reports of human intestinal infection with adult worms of *T. canis* [59], and many of these are believed to be erroneous observations [60]. Serological discrimination between toxocariasis canis and toxocariasis cati, however, is not so apparent, because of complete cross-reactivity between the two LESs, although *T. cati*-specific LES has been identified [61]. Therefore, distinguishing between *T. canis* and *T. cati* is even more difficult if somatic antigens are used in the serological diagnosis [62–64]. For the precise serodiagnosis of toxocariasis, a great deal of additional research effort is needed to obtain *T. cati*-specific LES antigens.

3. Advances in serological diagnosis

3.1. Antigens

As mentioned above, the most reliable and suitable antigen for the diagnosis of toxocariasis is LES from *T. canis*. Once the larvae are cultivated *in vitro*, they are viable for up to two years. During this period, no morphological changes have been observed, but chemosusceptibility to some compounds were found to have changed [65], suggesting that the physiological natures of the larva do change over this time period. The nature of LES was extensively studied by Maizels and colleagues [61,66–68]. Around the same time, Sugane and Oshima demonstrated that LES had an ability to induce not only IgG and IgM antibodies, but also IgE antibody in mice. Allergenic activity was lost when LES was treated with guanidine hydrochloride and 2-mercaptoethanol. LES also showed a cross-reaction with serum from *Ascaris suum*-infected mice

[69]. In addition, studies have identified numerous lectin-specific glycoconjugates on the surface of the larvae [61,66–68,70–73], and these have been found to dynamically change during the course of infection in murine [74] and rabbit models [75].

Although the antigenicity and specificity of LES is fairly high, cross-reaction to other parasites, especially nematode parasites, have been observed [76]. To overcome this problem, Yamasaki et al. [77] produced a recombinant antigen that reacted with serum from patients with toxocariasis but not from those with roundworm or hookworm infections.

3.2. Rapid diagnostic test for toxocariasis

For many years, numerous diagnostic measures, such as the double gel diffusion test, immunoelectrophoresis, indirect hemagglutination test, latex agglutination test, plate-based ELISA, membrane-based dot-ELISA, etc., have been employed to detect specific antibodies against LES. However, these tests require 1.5 hours or more to obtain an accurate result. In 1997, a new rapid diagnostic test kit for the detection of anti-LES antibody was introduced by us [78]. The test is based on the antigen-sensitized nitrocellulose membrane-based assay. It is easy to perform, does not require any sophisticated apparatus or expertise and the results can be obtained within 3 min. This test kit can even detect the antibody in intraoccular fluid.

4. Conclusion

In this review article, we present an overview of human toxocariasis in Japan. Due to space limitations, we do not describe in detail the aspects of experimental investigations concerning biology, immunology and molecular biology using animal models. However, we briefly pay special attention to Japanese investigators who contributed to advance the understanding of toxocariasis. In early studies, Oshima established a standard method for the oral inoculation of eggs, in which the albuminoid coat of the egg is first removed in order to prevent the adhesion of eggs onto glassware [79]. Sugane is a longtime co-worker of Oshima, and his colleagues are actively engaged in the field of immunology [80–88]. They demonstrated many examples of cellular immunity to *Toxocara* infection in mice. The late Dr. Tsuji made pioneering efforts to develop immunodiagnostic techniques for toxocariasis [50,89,90]. Recently, Mongolian gerbils, *Meriones unguiculatus* have been established as a suitable animal model for experimental ocular and neurologic toxocariasis [91–94].

Human toxocariasis is a public health hazard not only in children but also in adults, both in developing and developed countries. There are still questions to which we have no answers: How does ocular toxocariasis develop? Why do nearly half of ocular toxocariasis patients not produce detectable antibody to LES? What is the pathogenesis of neurologic toxocariasis? What mechanisms are involved in the reemergence of *Toxocara* larvae during pregnancy both in definitive and undefinitive hosts? In addition, we have not yet established an effective anthelmintic against *Toxocara* parasites in the tissue stage, especially for the ocular toxocariasis. Continuous efforts should be made to address these issues. Finally, toxocariasis is a disease that afflicts two of the very best and oldest friends of humans: dogs and cats. Therefore, we must continue to study this puzzling disease both for the sake of humans, and for that of our animal friends.

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猫ひっかき病

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日本大学農獣医学部卒、東京大学大学院修了（農学修士）。（財）競走馬理化学研究所、日本大学の助手を経て 2005 年から現職。2005 年「猫ひっかき病の疫学に関する研究」で日本獣医学会賞を受賞。細菌性人獣共通感染症（とくにバルトネラなど細胞内寄生細菌）および細菌性食中毒の疫学、病原性発現機序解明がおもな研究テーマ。趣味は釣り（フライ）、カヌー、スキー、バイク等のアウトドア系。「樹を見て森を見ず」にならないよう、研究に関しても常にフィールドに目を向け、その成果を社会に還元できるように心がけている。

現在、わが国の猫の飼育頭数は 1,100 万頭ともいわれている。最近では猫は単にペットというより、むしろコンパニオン・アニマルあるいは家族の一員として飼育されているため、ヒトと濃密に接触する機会も多くなっている。そのため、猫を介して感染する人と動物の共通感染症（以下、共通感染症）の 1 つである猫ひっかき病（Cat-scratch disease; CSD）の症例が多数報告され、医学領域でも注目されるようになってきた。CSD は 1950 年にフランスで報告された当初から、猫が関与する原因不明の疾病として今日まで至ったが、1990 年代の初頭に米国において、新種の細菌 *Bartonella henselae* が、猫ひっかき病の主要な病原体であることが明らかとなった。これが契機となって、猫ひっかき病に関する種々の事実が明らかとなってきた。

病原体

B. henselae が、主要な病原体である。自然病原巣である猫体内では、赤血球のなかに存在している。*B. henselae* は小型（2 × 0.5 ～ 0.6 μm）のグラム陰性、多形性単桿菌で、運動性（twitching）を示すが、鞭毛は確認されていない。発育にはヘミン等の赤血球成分を必要とする。血液寒天培地に塗沫し 35 ～ 37℃，5%CO_2 の気相で 2 ～ 4 週間培養すると、灰白色、表面が隆起したカリフラワー状、非溶血性、直径約 0.5 ～ 1mm 程度の微小なコロニーを形成する（写真 1）。

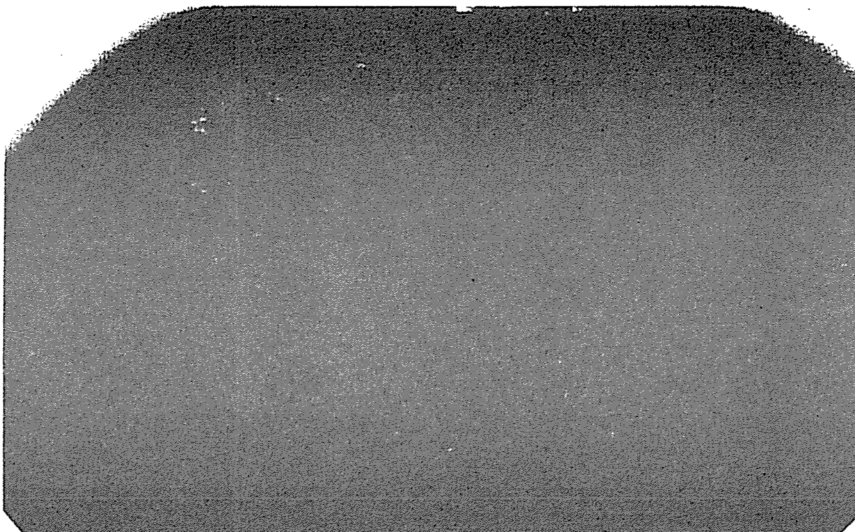

写真 1　血液寒天培地上の *Bartonella henselae* のコロニー

猫ひっかき病の臨床症状

定型的な CSD では、猫から受傷後、3 ～ 10 日目に受傷部、すなわち菌の侵入部位（通常，手指や前腕）に虫さされに似た病変が形成され、丘疹（写真 2）から水疱に、また、一部では化膿や潰瘍に発展する場合もある。これらの初期病変から 1、2 週間後にリンパ節の腫脹が現れる。リンパ節炎は、一般に一側性で、鼠径部，腋窩（写真 3）あるいは頸部リンパ節に多く現れる [19, 25]。わが国の 130 名の CSD 患者のうち、リンパ節の腫脹を呈した患者は 84.6% で、そのうち 33% は頸部、27% が腋窩部、18% が鼠径部のリンパ節であった [29]。通常、リ

写真 2　猫から受傷後 2 週間目にできた丘疹（左前胸部）

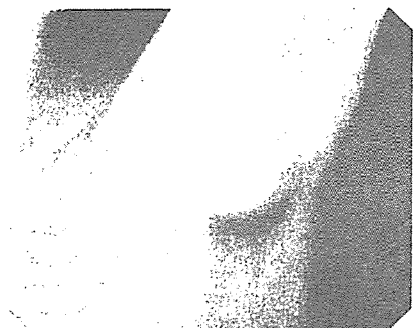

写真 3　腋窩リンパ節が鶏卵大に腫脹した例（6 歳男児）（写真提供：公立八女総合病院院長，吉田博先生）

ンパ節の腫脹は疼痛を伴い、数週〜数カ月間持続する。多くの症例で、全身感染の徴候、すなわち、発熱、悪寒、倦怠、食欲不振、頭痛等を示すが、一般に良性で自然に治癒する。

CSDの非定型的な症状は、5〜10%の割合で発生する。症状としては、パリノー症候群（耳周囲のリンパ節炎、眼球運動障害等）、脳炎、骨溶解性の病変、心内膜炎、肉芽腫性肝炎、あるいは血小板減少性の紫斑等が報告されている[4, 21, 22]。*B. henselae* の心内膜炎は、猫ひっかき病の非定型的な症状として認められ、とくに猫との接触がある心臓弁膜症患者に多くみられる[8, 12, 20]。脳炎はCSDのもっとも重篤な合併症の1つで、リンパ節炎を発症してから2〜6週後に発症する[4, 7]。多くは、後遺症なしに完全に治癒する。

免疫不全状態の人が *B. henselae* に感染した場合、細菌性血管腫（bacillary angiomatosis: BA）を起こす[1, 2, 31,36]。BAは上皮様血管腫症（epitheloid angiomatosis）とも言われ、血液の充満した嚢腫を特徴とした皮膚の血管増殖性疾患で、臨床的にはカポジ肉腫のような紫色や無色の小胞あるいは嚢胞性皮膚病変である[17]。実質臓器に嚢腫が波及した場合、細菌性肝臓紫斑病（bacillary peliosis hepatic: BP）、脾臓性紫斑病（splenic peliosis）ともよばれる。

わが国の猫ひっかき病発生状況

わが国では、全国的なCSD患者数に関する統計はない。神戸市と福岡市の医師に行ったアンケート調査において、医師が経験した共通感染症のうちCSDは外科系医師では1位、内科系医師では2位にランクされている[54]。猫の飼育頭数を考慮すると、わが国でも相当数のCSD患者が発生しているものと考えられる。

わが国の63名のCSD患者について調べたところ、小児から老人まで全年齢層に発生しているが、とくに、10歳未満の男児に多発している。この理由として、男児は猫の取り扱いが乱暴なため、不用意にひっかかれる機会が多いためと考えられる。また、CSD患者の60%以上は女性で、10代と40代の女性に多発する傾向がみられる（表1）。わが国では、この年代の女性は猫と接したり、猫の飼育や世話をすることが多いため、ひっかかれる機会も多いと考えられる。血清学的な調査でも、わが国のCSD患者の *B. henselae* 抗体陽性率は、男性に比べ女性で有意に高く、また健常な獣医系の学生でも女性に高い傾向が見られている[16]。

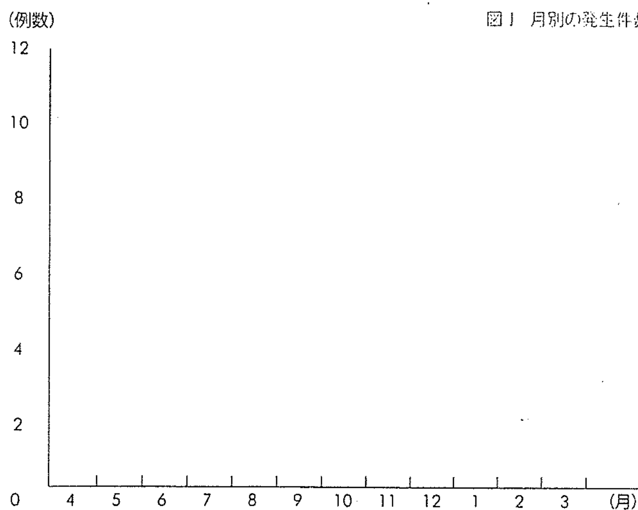

図1 月別の発生件数

（公立八女総合病院院長　吉田 博 先生データ）

CSDは1年の後半、秋から冬にかけて多発している（図1）。この理由として、夏のネコノミの繁殖期に *B. henselae* に感染する猫が増加し、その後、寒い時期になると猫は室内にいることが多くなるため、飼い主が猫から受傷する機会が増えるのではないかと考えられている。

猫の飼育・受傷歴のない健常者グループの *B. henselae* 抗体陽性率は2.3%（4／173）であるのに対し、猫の飼育歴・受傷歴のあるグループでは12.5%（10／80）、CSD患者の同居家族では21.4%（3／14）であることが示されている[32, 38]。これらの事実は、猫が本症の重要な感染源であることを示している。その一方で発症に猫が関与していない事例[39, 40]、猫と接触しただけでも発症した事例、あるいは犬が関与した事例なども報告されている（表2）[15, 33, 37]。

表1 猫ひっかき病患者の年齢・性別

年齢	男	女	計
0〜9	6	1	7
10〜19	4	8	12
20〜29	4	5	9
30〜39	0	5	5
40〜49	6	10	16
50〜59	4	3	7
60〜69	1	2	3
70〜79	0	3	3
80〜89	0	1	1
計(%)	25(39.7)	38(60.3)	63

（公立八女総合病院院長　吉田 博 先生データ）

表2 猫ひっかき病の発症原因

関与した動物	発症原因	例数	%
猫	ひっかき傷	26	41.3
	接触のみ	24	38.1
	咬傷	5	7.9
犬		4	6.3
ネコノミ		3	4.8
不明		1	1.6
計		63	100

（公立八女総合病院院長　吉田 博 先生データ）

ベクターと感染経路

CSD患者は、ネコノミ（*Ctenocephalides felis*）が多く寄生した子猫を飼育している人で多発している[17]。また、*B. henselae* 保菌猫に寄生していたネコノミからも本菌が分離されたこと[11]や猫から採取したノミの33.3%（12／36）から *B. henselae* のDNAが検出されていること[13]から、ネコノミが *B. henselae* のベクターである可能性が示唆された。Chomelら[6]は、SPF猫を用いたノミの感染実験から、猫間における *B. henselae* の伝播にネコノミが関与していることを明らかにした。また、感染猫を吸血したネコノミの糞をほかの猫に接種することで、感染が成立することも報告されている[30]。日本の猫でもノミが本菌の重要なベクターであることが、血清疫学的に示されている[27]。猫では、ノミの糞便中に排泄され猫の体表に付着した菌をグルーミングの際に歯牙や爪に付着・汚染させることにより、猫間あるいは猫から人へ創傷感染するものと思われる（図2）。

現在のところ、ノミから人への *B. henselae* 感染は明らかにされていないが、猫から受傷していないにもかかわらず、ノミから感染したと思われる例が、日本[39]やオーストラリア[9]で報告されている（表2）。また日本では、飼い猫に寄生していた多数のノミに吸血された後にCSDを発症し、そのネコノミからも *B. henselae* のDNAが検出されるとともに患者の鼠径リ

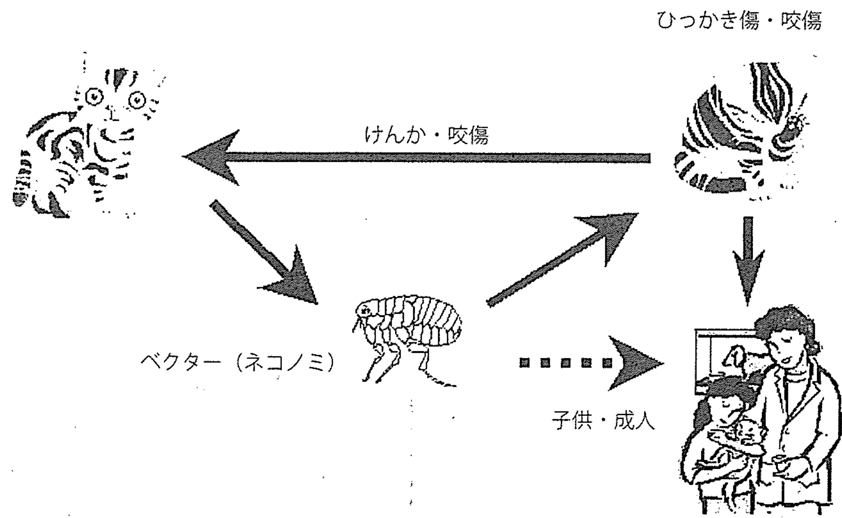

図2　猫ひっかき病の感染経路

ンパ節から本菌が分離された例が報告されている[28]。ネコノミは広い宿主域を有することから、感染猫の血液を吸血したノミが人へ本菌を伝播する可能性も否定できない（図2）。

猫の臨床症状

B. henselae に感染した猫は、通常臨床症状を示さない。猫を実験的に *B. henselae* に感染させた場合、1～2週間で菌血症（菌量：3～10^6CFU/mL）に達し、2～3カ月間持続する[5, 10]。自然感染した猫では1～2年もの間、菌血症が持続した例も報告されている[14, 18]。

実験的に感染させた猫では、発熱、一過性の神経機能障害、傾眠、食欲不振などの臨床症状が報告されている[3, 30]。

猫の *B. henselae* 感染状況

わが国では、1995年に初めて猫から *B. henselae* が分離されている[23]。全国の690頭の飼育猫を対象とした調査では、その7.2%（50／690）が *Bartonella* 属菌を保菌していたこと、保菌率は北海道，宮城県の0%から沖縄県（島尻郡）の20%で、南の地方や都市部の猫で高いことが明らかになっている（図3）[26]。また、3歳齢以下の若い猫では老齢の猫に比べ保菌率が有意に高いことも明らかとなっている（図4）。

わが国の猫の8.8～15.1%が *B. henselae* 抗体陽性であることが示されている[24, 27, 35]。Maruyamaら[27]は、1～3歳齢の若い猫、室外飼育の猫やノミの寄生のあった猫で抗体陽性率が有意に高かったこと（図5）、保菌率と同様に南の地方や都市部の猫で高いことを明らかにしている。これにより、わが国の猫の *Bartonella* 感染率は、飼育環境、ノミの分布・寄生状況あるいは地域の猫の密度に関係しているものと思われる。

猫の *B. henselae* 感染率は、国、地域、あるいは調査対象とした猫等によってさまざまである。

診断

CSDを臨床診断する場合、鼠径リンパ肉芽腫、化膿性炎、非定型抗酸菌症、結核、ブルセラ症、野兎病、伝染性単核症、コクシジオマイコーシス、ヒストプラズマ症、ホジキン病、サルコイドーシス等のリンパ節が腫脹するほかの疾病との類症鑑別が必要である。

血清診断には、*B. henselae* 抗原を用いた間接蛍光免疫抗体法（IFA）が用いられる。このIFAでは、IgM抗体が1:16希釈以上、IgG抗体が1:64希釈以上で特異的な蛍光

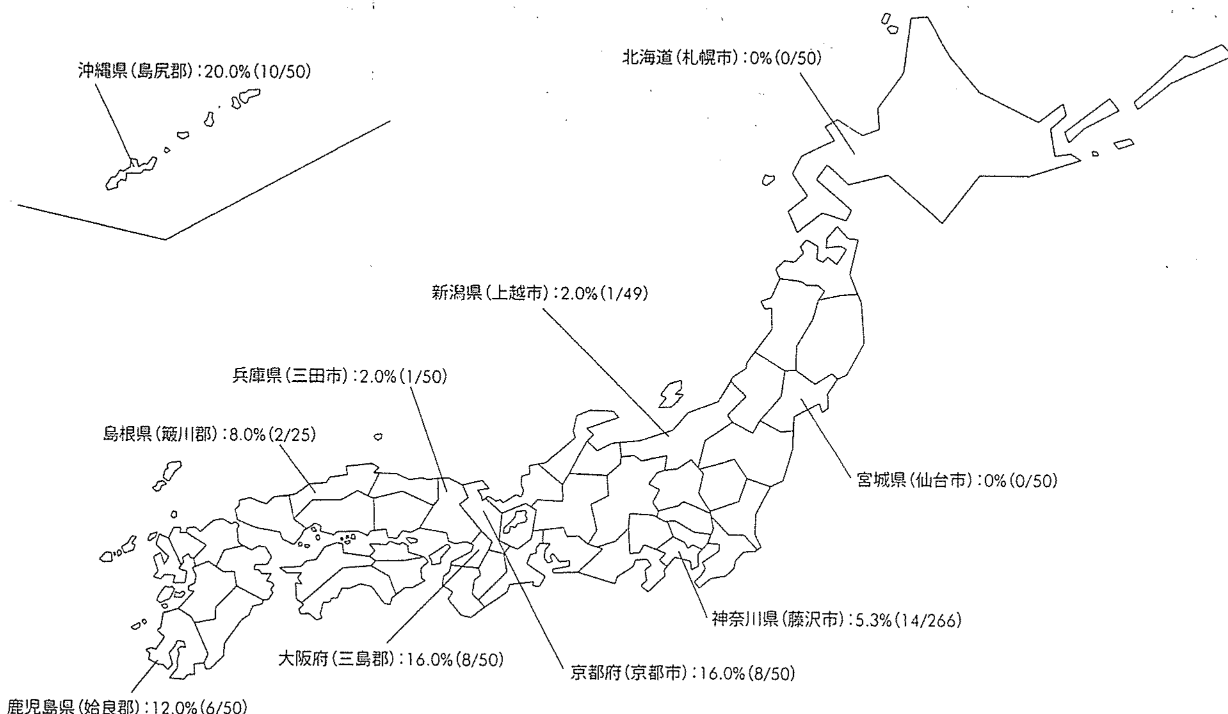

図5　地域別にみた猫の *Bartonella* 属菌保菌率
（Maruyama S ら, J Vet Med Sci, 62, 273～279, 2000. より改変）

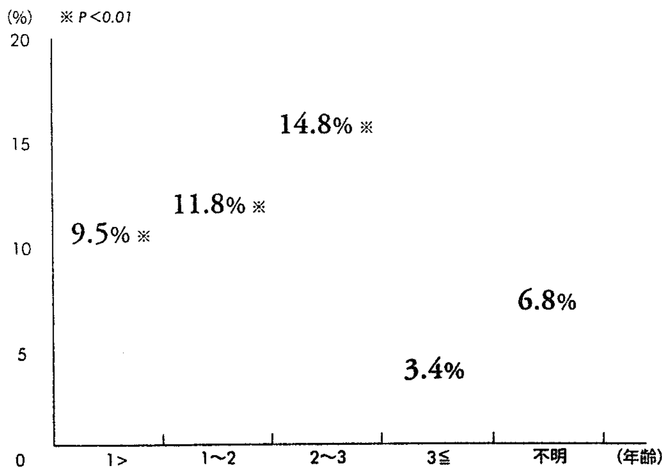

図4 年齢別にみた猫の保菌率（Maruyama S. ら, Microbiol. Immunol. 47:147～153, 2002, より改変）

がみられたものを陽性とする。ペア血清でIgM抗体が検出されなかった場合、IgG抗体価に4倍以上の差がみられたものを陽性とする。数カ月以内に*B. henselae*の感染があった場合、通常患者のIgG抗体価は1:256以上を示す。

患者血液、リンパ節生検材料から本菌を分離することは非常に難しく、また培養から同定までに時間がかかるため、PCR法により臨床材料中の*B. henselae*の遺伝子を検出する方法も診断上有用である[25)]。

治療

定型的CSDに対して各種の抗菌性物質による治療が試みられているが、多くの症例でその効果は認められていない。通常、特別な治療をしなくとも2～3週間で自然に治癒する。

一方、BAやBPには、エリスロマイシン、リファンピシン、ゲンタマイシン、ドキシサイクリン、シプロフロキサシン等が有効である。

猫ではドキシサイクリン、リンコマイシン、アモキシシリンの連続経口投与で、ある程度菌血症のレベルを抑制できるが、完全には除菌できない[10)]。

予防

CSDの発症には猫が深く関与しているものの、猫と接したり猫から受傷することでただちに発症することはない。性格のおとなしい猫を飼う、定期的な爪切り、猫（とくに子猫）との接触後の手指の洗浄、猫による外傷の消毒、ならびにネコノミの駆除等の一般的な衛生対策で対応する。子供のいる家庭内で猫を飼育する場合、ノミ対策を施された猫や*B. henselae*菌血症が陰性であることを確認された猫を飼育することも考慮する。また、免疫不全状態にある人は、CSD以外の感染症の可能性も考慮して、猫との接触は避けるべきである。

※*B. henselae*菌の分離・同定と抗体測定（人・猫）に関しては、日本大学生物資源科学部獣医公衆衛生学研究室・丸山総一（TEL & FAX：0466-84-3386）までお問い合わせください。

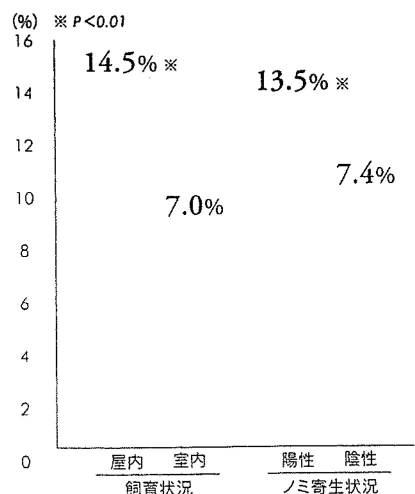

図5 猫の飼育状況・ノミ発生状況による*B.henselae*抗体陽性率
（Maruyama S. ら, Microbiol. Immunol. 47:147～153, 2003, より改変）

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Multispacer Typing To Study the Genotypic Distribution of *Bartonella henselae* Populations

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Bartonella henselae*, a worldwide fastidious bacterium, has a feline reservoir and is pathogenic for humans. However, the relationship between human and cat isolates of *B. henselae*, as well as its population dynamics and geographic heterogeneity, is not fully understood, in part because of the absence of appropriate typing methods. Multilocus sequence typing (MLST), the most discriminatory genotyping method for *B. henselae*, identified seven genotypes and suggested that human isolates arose from a limited number of cat isolates. Herein, we estimated the discriminatory power of multispacer typing (MST) by studying 126 *B. henselae* cat isolates from various areas of Europe, Asia, and the United States. We identified the nine most variable intergenic spacers conserved by both *B. henselae* and *Bartonella quintana* genomes. By comparing the sequences obtained from these nine spacers for each studied isolate, we identified 39 MST genotypes. The distribution of isolates into MST genotypes matched their phylogenetic organization into four clusters. MST showed that European and Asian isolates were different, in contrast with American isolates, but failed to identify pandemic strains. Our study demonstrated that MST is a powerful method for genotyping *B. henselae* at the strain level and may serve in studying the population dynamics of this bacterium and understanding the relationships between cat and human isolates. Finally, we provide a free-access MST-Rick online software program (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst) that investigators may use to compare their own MST sequences to our database.

Bartonella henselae is a gram-negative, fastidious bacterium associated with cats. Its transmission among cats is mediated by the cat flea, *Ctenocephalides felis* (7). Infected cats may remain bacteremic for long periods, thus playing a major role as a reservoir for the bacterium (6, 24). Human infection occurs through cat scratches or bites (22) and presents as cat scratch disease (2), bacillary angiomatosis (23), peliosis hepatis (32), endocarditis (18), or a variety of other, less frequent manifestations (14).

Although criteria exist for classifying *Bartonella* isolates as new species (27), there is a need for a method able to reliably identify *B. henselae* at the strain level. Such a method would allow investigation of the relationships between cat and human isolates, the question of whether epidemic strains occur in cats, and the geographic heterogeneity of *B. henselae* isolates. Various methods have been proposed for typing *Bartonella* isolates (10, 12, 19, 20, 26, 29, 34). Of these, sequence-based methods have the advantages of being applicable to clinical or environmental specimens and producing reproducible and comparable results. On the basis of comparison of 16S rRNA gene sequences, *B. henselae* isolates were classified into two main genotypes, i.e., types I and II. This gene was considered a useful delineation among isolates because the two genotypes also exhibited different serotypes and possessed consistently distinguishable protein profiles (26). Sequences from the *ftsZ* (12), *gltA* (10), 35-kDa protein-encoding (26), *groEL* and *pap31* (34) genes, and from the 16S-23S intergenic spacer (20), later permitted the identification of three, two, two, four, and six genotypes, respectively, that did not exactly match 16S rRNA gene types. To date, the most discriminatory typing method for *B. henselae* isolates is multilocus sequence typing (MLST) incorporating nine genes (21). This method distinguished seven genotypes among 37 human and cat isolates and suggested that lateral gene transfer occurs among *B. henselae* isolates (21). Although these investigators and others suggested that human infection is caused by a limited number of specific *B. henselae* genotypes (4, 10, 21), the discriminatory power of the genotyping methods that they used and the small number of *B. henselae* isolates that they studied were insufficient to allow any statistically significant conclusions to be drawn. Therefore, a genotyping tool with greater discriminatory power for genotyping *B. henselae* at the strain level is needed to investigate the diversity and population structure of this bacterium.

Recently, we applied a new genotyping method to *Bartonella quintana*, i.e., multispacer typing (MST) (13). This method allows genotyping of bacteria at the strain level. MST, initially

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developed for *Yersinia pestis* (11), was also applied with success to strains of other human pathogens, including *Rickettsia conorii* (15), *Rickettsia prowazekii* (35) and *Coxiella burnetii* (16). MST was developed with the assumption that intergenic spacers are more variable than genes for genotyping bacteria at the strain level. In this study, to estimate the usefulness of MST for studying the population genetics of *B. henselae*, we applied it to a large collection of cat isolates.

MATERIALS AND METHODS

Study design. One hundred twenty-six *B. henselae* cat isolates of various geographic origins were incorporated in this study (Table 1). All 38 European isolates were grown in our laboratory. For the other 88 isolates, from the United States and Asia, we studied DNA extracted by two of the authors (B.B.C. and L.G.) from their isolates.

***Bartonella henselae* culture and DNA extraction.** *B. henselae* isolates were cultivated on Columbia agar with 5% sheep blood (BioMerieux, Marcy l'Etoile, France) at 37°C in 5% CO_2 (Genbag CO_2 system; BioMerieux). Genomic DNA of *B. henselae* strains was extracted by using the Chelex procedure as previously described (9) or the QIAmp Tissue kit following the manufacturer's recommendations (QIAGEN, Hilden, Germany).

Selection of target sequences. We aligned the genomic sequences of *B. henselae* (GenBank accession number BX897699) and *B. quintana* (BX897700) by using the BLASTn (1) and GenomeComp (33) software programs to identify conserved pairs of consecutive genes. Then, intergenic sequences were aligned using the CLUSTAL W program (31). We classified intergenic spacers conserved by both genomes, with sizes ranging from 150 to 600 bp, by degree of similarity and then selected the 20 most variable spacers (detailed in Table 2).

PCR amplification and sequencing. Primers were designed to amplify the 20 most variable spacers fulfilling the above criteria using the Primer 3.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers for amplifying the 20 most variable spacers were selected within genes flanking the selected spacers and are listed in Table 2. All primers were obtained from Eurogentec (Seraing, Belgium). Their specificity was predicted by comparison with GenBank using the BLASTn software (1). PCRs were carried out in a PTC-200 automated thermal cycler (MJ Research, Waltham, Mass.). One nanomolar concentration of each DNA preparation was amplified in a 25-μl reaction mixture containing 50 pM of each primer; 200 μM (each) dATP, dCTP, dGTP, and dTTP (Invitrogen, Gaithersburg, Md.); 1 U of eLONGase polymerase (Invitrogen); 1 μl of eLONGase buffer A; and 4 μl of eLONGase buffer B. The following conditions were used for amplification: an initial 3 min of denaturation at 94°C was followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 52°C, and extension for 1 min at 68°C. Amplification was completed by holding the reaction mixture for 10 min at 68°C to allow complete extension of the PCR products. PCR products were purified by using the MultiScreen PCR filter plate (Millipore, Saint-Quentin en Yvelines, France) as recommended by the manufacturer. PCR products were sequenced in both directions by using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit with Amplitaq polymerase FS (Perkin-Elmer, Coignieres, France) as described by the manufacturer. Sequencing products were resolved using an ABI 3100 automated sequencer (Perkin-Elmer). Sterile water was used as a negative control in each PCR assay. Sequences from each genotype were checked twice in both directions to ensure the reliability of the MST method.

Sequence analysis and phylogenetic analysis. Nucleotide sequences were edited using the Autoassembler package (Perkin-Elmer). For each intergenic spacer, a genotype was defined as a sequence exhibiting unique mutations. MST genotypes were defined as unique combinations of spacer genotypes. Multiple alignment of sequences was carried out using the CLUSTAL W software (31). Phylogenetic analysis of the studied isolates was obtained using the neighbor-joining and maximum parsimony methods within the MEGA 3 software (25). For this purpose, sequences of the selected spacers were concatenated. To facilitate sequence comparison with our MST sequences, we developed an online site named MST-Rick. This site contains a local BLAST to help scientists compare their sequences to our database.

Statistical tests. The genotypic variability of *B. henselae* isolates according to their geographic origin was estimated using Fisher's exact test. A difference was considered significant when P was <0.05.

Nucleotide sequence accession numbers. The different genotypes for the discriminatory spacers have been deposited in the GenBank database under accession numbers DQ383226 to DQ383270.

RESULTS

MST genotyping. One thousand four hundred thirteen intergenic spacers were found conserved by *B. henselae* and *B. quintana* genomes. Among them, 293 had a size ranging from 150 to 600 bp. We tested the 20 most variable (S1 to S20) of these 293 spacers among the 126 *B. henselae* cat isolates available. Nine of the spacers (S1 to S9) were found highly variable among these isolates (Table 2). The tRNA-Ala/GCA-tRNA-Ile/AUC spacer (S1), flanked by two tRNA genes, was found to be the most variable spacer among the nine tested, with five variable nucleotide positions and a 15-bp sequence fragment presenting either as a single copy or repeated up to five times, depending on the isolate (Fig. 1; Table 3). Sequences from the S1 spacer classified the 126 isolates into nine genotypes. The BH2865724-*dut* spacer (S2), with 14 variable nucleotide positions, was the second most variable spacer and allowed the 126 tested isolates to be classified into seven genotypes (Table 3). The *dnaJ*-related protein–*cobS* spacer (S3) held eight variable nucleotide positions and classified the 126 isolates into six genotypes (Table 3). The *pssA*-oxidoreductase (S4) and *carB*-cold shock protein (S5) spacers had nine and five variable nucleotide positions, respectively, and classified the 126 isolates into five genotypes each (Table 3). The *alr-gcvP* (S6) and *ftsK*-oxidoreductase spacers (S7) contained eight variable nucleotide positions each and classified the 126 isolates into four genotypes each (Table 3). The BH2864883-BH2864884 (S8) and *acpP2*-malate oxidoreductase (S9) spacers harbored eight and four variable nucleotide positions, respectively, and classified the 126 isolates into three genotypes each (Table 3). In total, 69 variable nucleotide positions were found within the nine intergenic spacers (Table 3). Each variable nucleotide was checked three times to ensure the reliability of MST. Only two alleles at each variable position were found, with the exception of position 256 within the *alr-gcvP* spacer. At this position, 117 isolates had a thymine (types 2 and 3), compared to a cytosine in five European isolates (type 1) and a guanine in four American isolates (type 4) (Table 3). By combining the genotypes obtained from each variable spacer, the 126 tested isolates could be classified into 39 MST genotypes (Table 1). Each of the 39 genotypes was identified based on sequence specificities from either a single spacer or a combination of a maximum of seven spacers (Fig. 2). Sequences from each genotype from the nine spacers were added to the MST-Rick database (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst).

Among the 39 MST types, 24 MST types (types 1, 3, 4, 6, 8 to 12, 14, 16, 19 to 21, 23, 26, 28 to 31, 34, 36, 37, and 39) contained only one isolate each and five MST genotypes (types 17, 24, 27, 32, and 33) contained only two isolates each (Table 1). The 19 Asian isolates were distributed into 12 MST genotypes, compared to 6 ($P < 0.01$) and 24 ($P = 0.03$) MST types for the 38 European and 69 American isolates, respectively (Fig. 3). Among the 39 MST genotypes, 10 (types 6, 10, 20, 21, 23, 32, and 36 to 39), 21 (types 1, 3, 4, 7 to 9, 11, 14 to 19, 24 to 30, and 34), and 5 (types 4, 9, 12, 22, and 31) genotypes were specific to Asian, American, and European isolates, respectively.

A significant difference in genotypic diversity was found between *B. quintana* (4 MST types out of 71 isolates) (13), and