ウマバベシア病に対する国際標準診断法の開発

（研究課題番号13356007）

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4）研究成果による工業所有権の出願状況

1．五十嵐郁男、黄暁紅、玄学南、ウマバベシア感染用診断キット、
特願 2005-8062

研究成果の概要

ウマバベシア病は赤血球内に寄生する原虫 *Babesia equi* と *B.caballi* がダニによって馬に伝播され、発熱、貧血、黄疸などの症状を呈し、死亡率が10％～50％に達する家畜法定伝染病である。流行地域は南ヨーロッパ、アジア、中近東、アフリカ、中南米など全世界に及び、わずかに北欧、北米、豪州、日本が流行を免れている。ウマバベシア病が侵入すると、馬産業界に対する経済的打撃は甚大になるものと憂慮され、検疫監視体制の強化は各国の動物防疫の重要課題の一つとしてとらえられている。現在、日本を含む世界各国においてウマバベシア病の公式検査法として補体結合反応が採用されている。しかし、本法は感度が低く、アメリカではこの方法で検疫を通過した馬からバベシア原虫が発見された。したがって、人馬の国際交流が益々盛んになっている近年、バベシア病フリーの国に本病が侵入する可能性が危惧され、補体結合反応に替わる感度と特異性の高い診断法の開発が早急に必要である。

本研究はウマバベシア病に対する感度と特異性の高い国際標準診断法を開発・実用化することを目標として、*Babesia equi* と *B.caballi* の組換え原虫タンパク質の作製、免疫原性の解析、組換え原虫タンパク質を用いた ELISA、イムノクラマト法の確立、世界各国の血清試料を用いた血清診断法の評価を行い、下記のような成果を得た。

1. バベシア原虫の新規の抗原

感染ウマから得られた血清を用いたクローニングにより、新規の *B. equi* Be82、Be158 遺伝子 および *B. caballi* Bc134 遺伝子が得られた。大腸菌に効率良く高い濃度の蛋白質として産生させ、発現された原虫タンパク質を ELISA 用抗原として用い、その有用性を実験感染馬の血清を用いて検討した結果、高い特異性および感度が認められた。

2. ウマバベシア原虫組換え抗原産生の検討

B. equi のメロゾイト表面抗原（EMA-1）および *B. caballi* のロプトリー抗原の組み換抗原（BC48）を大腸菌の系を用いて産生し、発現された原虫タンパク質は分子量、抗原性など原虫由来のものと一致することが確認された。

3. 組換え抗原を用いたELISA

B. equi のメロゾイト表面抗原（EMA-2）および *B. caballi* のロプトリー 抗原の組み換え抗原（BC48）を用いた ELISA の有効性を検討するため、実験感染馬の血清を用いて検討したとろ、感度及び特異性に優れていることが示された。また、野外血清を用いた試験でも、同様の結果が得られている。

4. イムノクロマト法の基礎的検討

高濃度に発現された *B. equi* の EMA-2 および *B. caballi* の BC48 を用いて、ラテックス粒子に結合させる条件、粒子の大きさ、色、試料の希釈等反応系の条件設定について検討した。その結果、特異的反応が高く、短時間で判定可能なイムノクロマト法の開発に成功した。また、これら2種類の抗原を一つのスティックに固定して、同時に2種類のバベシア感染を診断できるキットも開発された。

以上のように本研究では、*B. equi* のメロゾイト表面抗原（EMA-2）および *B. caballi* の BC48 が最も診断用抗原として優れていることが判明した。また、両抗原を ELISA あるいはイムノクロマト法に用いた場合、特異性および感度に優れていることが示された。米国農務省ではこれらに類似した抗原を用いたｃELISA を開発し、公式検査法として導入したが、特異性が低いことが判明し、わずか1ヶ月半で補体結合反に戻した。従って、我々の開発した *B. equi* の EMA-2 および *B. caballi* の BC48 は、今後世界的な比較実験及び評価を行うことによって、国際的標準診断法の診断用抗原として使用される可能性が極めて高いと期待される。

Part II.

Babesiosis in domestic and wild animals in Asia

Chapter 1. Bovine babesiosis in Asia

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1. Introduction

Bovine babesiosis, a tick-borne disease caused by the intra-erythrocytic protozoan parasites from the genus *Babesia*, is characterized by fever, anemia, and hemoglobinuria (1). Various species of *Babesia* are known to infect cattle; however, the most important are those caused by *Babesia bovis* and *Babesia bigemina* (1, 2). This disease is found in tropical and subtropical regions of the world, including Asia, which corresponds to the distribution of the tick vectors (3). It has been estimated that half a billion cattle throughout the world may be endangered by bovine babesiosis (4). Since the disease has a major impact on the development of the livestock industries due to the losses of milk or meat production and sometimes the death of the infected animals, it is considered to be one of the most important infectious diseases of cattle from an economic viewpoint.

2. Pathogen and transmission

The *Babesia* parasites are members of the phylum Apicomplexa, suborder Piroplasmidea, and family Babesiidae (5). *B. bovis* and *B. bigemina* are the major causative agents of bovine babesiosis in Asia. Other species, such as *B. major* and *B. ovata*, have also been found in some countries; however, they are not highly pathogenic in cattle and are not considered to be such an important pathogen as *B. bovis* and *B. bigemina* (3, 6, 7). The disease caused by *B. bovis* is more severe and more difficult to control than that caused by *B. bigemina* (1). *B. bovis* is a small *Babesia* measuring approximately 2.4 _m long and 1.5 _m wide, whereas *B. bigemina* is a large *Babesia* measuring approximately 4 to 5 _m long and 2 to 3 _m wide (1). Single *B. bovis* organisms are round, oval, or irregular in shape, while paired forms are piriform or club-shaped. The angle between the paired organisms is often obtuse. Single forms of *B.*

bigemina are elongated or amoeboid in shape. Paired forms are typically piriform with an acute angle between the pair.

B. bovis and *B. bigemina* are transmitted by a variety of tick species, of which the most important species is *Boophilus microplus* (8). Both parasites follow similar life cycle patterns in adult *Boophilus* spp. Initial development takes place in basophilic epithelial cells of the gut wall, where multiple fission occurs with the formation of sporokinetes, and then the sporokinetes enter various organs of the tick and initiate cycles of multiple fission in the cells of tick organs, including the oocytes; thus, both *Babesia* pathogens are transmitted transovarially (8, 9). *B. bovis* is transmitted to cattle during the larval stage since the sporozoites that are infective for cattle occur in the salivary glands of the larval stage (10). On the other hand, the sporozoites of *B. bigemina* occur in the salivary glands in the nymphal and adult stages; thus, *B. bigemina* is transmitted during these stages (11). For transovarial transmission, in contrast to *B. bigemina*, ticks must be reinfected with *B. bovis* during the nymphal and adult stages since the larvae lose their infection after transmission occurs (12).

3. Clinical signs and pathogenesis

In general, the clinical signs that develop as a result of *B. bovis* and *B. bigemina* are similar. The first clinical signs of the disease occur at about the time the parasites reach parasitemia levels detectable in blood smears, usually between 8 and 16 days after they commence feeding (13). The most common signs of acute infection include fever, hemoglobinuria, and anemia (1). The rectal temperature of infected cattle rises as parasitemia increases and reaches 41-41.5 °C in 2-3 days (13). Infected animals lose their appetite, become dehydrated, and lose weight, and pregnant cattle may abort (14). The details of the clinical signs of acute infection have been observed and described by

Yonglin et al. (15). In animals infected with *B. bovis*, the central system may be involved, leading to hyperexicitability and often terminating with signs of cerebral damage, such as padding of the limbs, ataxia, mania, and coma (1, 13). The course of *B. bovis* infection is usually short, and the outcome is almost invariably fatal (14). Acutely affected cattle with *B. bigemina* infections exhibit less severe clinical signs than those with *B. bovis* infections (16). Non-fatal cases from *B. bovis* infections may take several weeks to recover completely, whereas recovery in non-fatal cases from *B. bigemina* infections is usually rapid (14, 16). In subacute infections, clinical signs are less pronounced and may be difficult to detect (14).

A major factor in the pathogenesis of bovine babesiosis is anemia (13). The parasites cause anemia by rupture of erythrocytes as they exit (17). As the parasites exit the infected bovine erythrocytes, parasite-associated proteolytic substances are released; these substances are believed to interact with blood components and to be responsible for several of the pathologic signs and symptoms associated with bovine babesiosis, including vasodilation, hypotensive shock, increased capillary permeability, edema, and vascular collapse (1, 18, 19). *B. bovis* infection leads to increased concentrations of fibrin (20). The fibrin coating of erythrocytes, which increases the adhesiveness of the red blood cells, causes adherence of erythrocytes to the inside of capillaries in the brain, which might block the blood supply and result in clinical signs with cerebral involvement (21). Parasitemias in brain capillaries could exceed 90%, although peripheral blood parasitemia is below 1% (1). In *B. bigemina* infection, the pathogenesis is almost entirely related to rapid, sometimes massive, intravascular hemolysis (14).

4. Epidemiology

Bovine babesiosis is present throughout Asia, although its distribution is not

completely known since no extensive epidemiological surveys have been conducted. However, it has been surveyed and reported in some Asian countries.

In Japan, bovine babesiosis, caused by *B. bigemina* and *B. ovata*, has been reported (7, 22). *B. bigemina* is found only in Okinawa prefecture, whereas *B. ovata* is widespread in Japan (3, 7, 22). *B. bigemina* has been now under control due to tick eradication by acaricides (23).

In China, the disease occurred sporadically and occasionally broke out in local areas (24, 25). Outbreaks usually occur when exotic animals are introduced into endemic areas or carrier animals are imported into *Babesia*-free areas (6). *B. bovis* and *B. bigemina* have been reported in 16 and 22 provinces, respectively (6). Statistical data in 19 provinces in 1979 indicates 33,387 cases of clinical bovine babesiosis and 4,946 cattle deaths as a result of this disease (6). In some endemic areas, the average infection rate and mortality rate of *B. bigemina* were 35.26% and 57.10%, while those of *B. bovis* were 39.35% and 49.61%, respectively (6). In most cases, the disease is caused by mixed infections of both *Babesia* parasites. As the active season of *B. microplus* is from March to October, cases of bovine babesiosis have been reported from April to November, with peak periods in May, June, and July (7, 25).

In India, only *B. bigemina* has been reported. In a five-year epidemiological study in and adjacent Ranthambhor National Park, India, a total of 307 serum samples and blood smears were collected from 60 wild ruminants, 100 cattle, and 147 buffaloes for serological and parasitological diagnoses (26). The serum samples subjected to enzyme-linked immunosorbent assay (ELISA) revealed anti-*B. bigemina* antibodies in 7.03% of wild and 11.97% of domestic ruminants. Stained blood smears showed *B. bigemina* infection in 5.7% in wild and 10.03% in domestic animals (26). A serological study of 214 serum samples collected from *B. microplus*-infested cattle from the state of

Orissa was conducted to detect anti-*B. bigemina* antibodies by ELISA (27). The test revealed 33.6% overall seroreactivity (27).

In Bangladesh, bovine babesiosis caused by *B. bigemina* is distributed all over the country and occurs throughout the year; however, high prevalence is noticed from March to June (28). *B. bigemina* is fairly common among indigenous and cross-bred animals in this country. The infections are mainly sub-clinical; however, clinical disease is sporadic (28). A seroprevalence study of bovine babesiosis indicated 14.5% of cases among native cattle and their exotic crosses (28).

In Sri Lanka, sera were collected from a minimum of 20 cattle at each of 14 localities in five climatic zones and tested for antibodies to *B. bovis* by the indirect fluorescent antibody test (IFAT) (29). Antibodies to *B. bovis* were detected in cattle from all the localities tested. Prevalence was consistently high among localities below 1,200 m and lower and more variable in the hill country (29).

In Malaysia, *B. bovis* and *B. bigemina* are a threat, particularly to susceptible imported cattle and their progeny (30). A serological survey conducted in cattle from 9 states showed that 72.6% were positive for both *Babesia* species (31). In an age-related study, high reactivity antibody to *B. bovis* and *B. bigemina* was detected in calves less than 1 month of age, and the reactivity decreased in calves between 1 to 3 months of age; then, the reactivity increased for both *Babesia* species in 6-month-old calves (31). These results indicated that *B. bovis* and *B. bigemina* are enzootic throughout peninsular Malaysia.

In Indonesia, a seroepidemiogical survey was conducted to determine the prevalence of antibodies to *B. bovis* in cattle by ELISA (32). A total of 1,448 serum samples were collected from Sumatra, Kalimantan, Sulawesi, Sumba, and Timor. The prevalence of positive samples to *B. bovis* was found in 96% (32). The results indicated

that the surveyed areas are endemic for *B. bovis*.

In Thailand, serum samples of 428 native cattle from 12 provinces and 532 buffaloes from 16 provinces were tested for antibodies of bovine babesiosis by IFAT (33). The prevalence of antibodies to *B. bovis* and *B. bigemina* was found to be 74.5% each in cattle and 28.2% and 41.1% in buffaloes, respectively (33). Clinical cases of babesiosis were mainly found from June to November (34).

In Mongolia, sera were collected from 283 cattle and tested for antibodies to *B. bovis* by ELISA (35). The mean prevalence of positive samples was found in 35.7%.

In Cambodia, bovine babesiosis caused by *B. bovis* and *B. bigemina* has been identified in several provinces by blood smear (36). Serological tests have been conducted in buffaloes in Kampong Cham and Ratanakiri provinces by ELISA. The prevalence of bovine babesiosis was 24% and 21.5%, respectively (36).

In the Philippines, bovine babesiosis caused by *B. bovis* and *B. bigemina* occurs occasionally in the field (37). In 1990, outbreaks in an imported herd of cattle from Australia were reported in Malaybalay and Bukidnon (37).

In Uzbekistan, Central Asia, *B. bovis* and *B. bigemina* have been reported with infection rates of 7.4% and 19.4%, respectively (38).

5. Resistance and immunity

The resistance of animals is influenced by several factors, such as age, breed, and inherent immunity (1). *Bos indicus* cattle, which are the majority in Asian countries, are less susceptible than *Bos Taurus* cattle, which are the European breeds (39, 40). Thus, the effect of the disease on Asian indigenous cattle is relatively small. In contrast, European breeds that are introduced into endemic areas are particularly susceptible to bovine babesiosis. Calves can receive *Babesia*-specific maternal antibodies via the

colostrum immediately after birth from immune mothers, which is considered to be a crucial factor in regulating the effect of *Babesia* challenge during the first month of life (41). Most cattle develop a durable immunity after recovering, and this immunity is not absolute but may last for life, even in the absence of reinfection (14).

6. Diagnosis

Definitive diagnosis of bovine babesiosis is the direct identification of the parasites in stained blood smears by microscopic examination (42). In animals showing clinical signs of acute infection, *Babesia* parasites are usually not difficult to identify. However, the diagnosis of subacute infections is often difficult, since parasitaemias are usually too low for the examination of stained blood smears (42). Nucleic acid probes and PCR techniques have been developed to detect small numbers of parasites in blood, but they are restricted to laboratories with facilities for molecular biology (43-46). An alternative for the detection of *Babesia* infections is the indirect identification of the parasites through the demonstration of antibodies by serological methods. Many serodiagnostic tests have been developed for the detection of antibodies to bovine *Babesia* species, such as indirect hemaglutination (IHA), the complement fixation test (CFT), the latex agglutination test (LAT), IFAT, and ELISA (47-56). However, only IFAT and ELISA are used routinely (57).

IFAT has been extensively applied to the diagnosis of bovine babesiosis. It is well suited to the low-technology environment (42). The cost per test is low, and most reagents required are readily available or can be produced on site (42). However, IFAT is unsuitable for use on large numbers of sera due to operator fatigue. Furthermore, the results of IFAT may be influenced by the subjective judgement of the operator (42, 58). In addition, some cross-reactions between the two parasites do occur when *B. bigemina*

antigen is used (59). ELISA has been described as a very sensitive and economic assay for the detection of specific antibodies (60). In contrast to IFAT, an objective reading is obtained, the data are amenable to computer evaluation, and a large number of samples can be handled (42). However, the quality of the antigens available is crucial to the development of a sensitive and specific test (42). Most antigens made from infected erythrocytes are contaminated with host erythrocytes and may lead to false-positive results due to anti-erythrocytic antibodies (42). Recently, ELISAs using recombinant antigens have been developed for the detection of bovine babesiosis (61-63). ELISAs using recombinant antigens produced from rhoptry-associated protein 1 (RAP-1) of *B. bovis* and a merozoite antigen of approximately 200-kDa (p200) of *B. bigemina* were shown to be highly specific for the detection of anti-*B. bovis* and *B. bigemina* antibodies, respectively (61-63).

7. Treatment

Treatment of bovine babesiosis usually involves the reduction of parasitemia and the moderation of the clinical signs of the infected animal (1). If specific and supportive treatment is given early on in the course of infection, before the onset of severe anemia or nervous system disorders, the infected animals always recover (11). A variety of chemical compounds have been successfully used for treatment, such as diamidine, quinoline, and acridine derivatives and imidocarb (64-71). Drug resistance to bovine *Babesia* spp. has not been confirmed (14). The available babesiacides in each country are different, depending on importation. Among available drugs in Asian countries, Berenil® (diminazene aceturate) is more commonly used (6, 26, 28, 34). For searching alternative and effective chemotherapeutic drugs, the anti-microbial drug triclosan [5-chloro-2-(2.4 dichlorophenoxy)-phenol] was evaluated for its growth-

inhibitory effects against *B. bovis* and *B. bigemina* in an *in vitro* culture (72). Although triclosan has an inhibitory effect on the *in vitro* growth of both parasites, further studies are necessary to examine the mode of drug action and the *in vivo* effect (72).

8. Control

In most Asian countries, the active control of bovine babesiosis is achieved mainly by chemotherapy, chemoprophylaxis, and vector control and less by immunization (6, 26, 28, 34, 73, 74). The latter is restricted to some countries, but it is not widely used. Chemotherapy is the most prominent method for controlling the disease. Chemoprophylaxis is also used for disease control, particularly in large outbreaks with high morbidity rates or when susceptible cattle are being transported through an endemic area (14). The eradication of tick vectors is the most effective way to control bovine babesiosis. It may be possible to achieve this aim under exceptional circumstances of geographical isolation, but, in most areas, tick eradication is impractical. The best way that can be achieved to prevent ticks feeding on cattle is by constantly treating cattle with acaricides (75). The method of application varies according to the facilities available and the resources of the farmer. The system most applicable to smallholder farmers is the use of a hand-pump spray to treat animals individually. Where larger numbers of cattle are involved, the use of a spray race or dipping tank is essential. Vaccines of different types have been developed and used to immunize cattle against bovine babesiosis. Live vaccines and inactivated vaccines have shown partial protection against challenge infection (76-80). However, vaccine failure sometimes occurs (81). For the control of babesiosis in endemic areas, either live or inactivated vaccines could be used. Inactivated vaccines are preferred in marginal areas, where bovine babesiosis is a seasonal event, and for the immunization of animals that

are to be moved from tick-free zones into endemic areas (82). In these situations, vaccination would prevent deaths during occasional unexpected exposure to *Babesia* and would not disseminate organisms that could be picked up and transmitted by tick vectors (82). Several recombinant vaccines have been developed to replace the current method of vaccination, but they are currently not available (83-86).

9. Conclusion

Bovine babesiosis is one of the greatest obstacles to the development of the livestock industry in Asia. The disease results in direct losses as a result of poor growth, poor milk production, and mortality in infected animals or indirect losses due to the high costs involved in controlling the disease. Eradication would at first appear to be the ideal approach to control bovine babesiosis; however, there have only been relatively few attempts with limited success over the years. Monitoring and surveillance can provide an epidemiological picture of the disease in terms of incidence/prevalence directed to the control of the disease. Research on bovine babesiosis, such as epidemiological studies and the development of diagnosis, vaccines, and integrated control methods, should be carried out. The information that will be gathered could help in designing more effective control strategies for bovine babesiosis in the future.

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Chapter 2. Equine Babesiosis in Asia

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1. Introduction

Babesiosis is an economically important disease of equids, and sporadic outbreaks are not uncommon. A significant segment of the equine population has carrier status, due to which the draugtability of these animals gets lowered, and poor farmers suffer economically (1). Equine babesiosis, also called piroplasmosis, is an acute, sub-acute, or chronic tick-borne disease of equidae caused by an intra-erythrocytic protozoa, *Babesia equi* or *Babesia caballi*. The disease is characterized by fever, sometimes of an intermittent nature, anemia, icterus and hepatomegaly, and splenomegaly. Billirubinuria and hemoglobinuria may be present, usually during the last phase of the disease condition. The disease caused by *B. equi* is more widespread and pathogenic than that by *B. caballi.*

2. Epidemiology

The *Babesia* genus falls in the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, suborder Piroplasmorina, and family Babesiidae. *Babesia equi* is a small piroplasm, measuring 2.0x1.0 μm, whereas *Babesia caballi* is a larger form that measures approximately 3.0x2.0 μm. Among erythrocytes, *Babesia equi* can be from spherical to ovoid to the characteristic 'Maltese-cross' form. *Babesia caballi* is mostly seen as two pear-shaped merozoites joined at their tips. The disease condition caused by these parasites is distributed throughout Asia, Europe, Africa, and South America, and its prevalence is synchronized with the existence of the tick vector.

Japan is apparently free of the disease. Recently, Ikadai et al. (2002) applied ELISA on 2,019 horse serum samples that had been collected between 1971-73 for detecting antibodies against *B. equi* and *B. caballi* (2). By ELISA, 5.4% and 2.2% of the

samples were observed to be positive for antibodies. These findings suggested the possibility of the existence of equine babesiosis in Japan, as the occurrence of the vector ticks, *Dermacentor reticulates* and *Rhipiceephalus sanguineus*, had already been determined in 1974 (3). However, recent study suggests that *Dermacento* sp. seemed to have been exterminated in Japan. Alternatively, *Haemaphysalis longicornis*, which is widely distributed in Japanese pastures, has raised questioned as real vector of equine *Babesia* (3). Therefore, in the light of these findings, it is important to investigate the actual status of equine babesiosis for future curtailment and control of infection.

Indian-bred horses and mules are considered to be preimmune carriers of the disease. Lingard (1904) recorded piroplasmosis in lower animals and man in India and assumed that mosquitoes were carriers of the disease (4). Stevenson (1944) recorded babesiosis in donkeys that had been imported to India from South Africa (5). These donkeys were carriers of the infection, and, due to voyage stress, the clinical form of the disease became apparent. Since then, sporadic cases of the disease have been recorded from time to time, but the disease rarely occurs in an enzootic form. In 1976, Gautam and Dwivedi observed a clinical form of *B. equi* infection in mares that had been imported from Poland. Seven mares succumbed to the disease, and the remainder were treated with Berenil® (6). Malhotra et al. (1978) recorded 50.1 percent prevalence of the disease among the horse population (7). Later Kumar et al. (1997, 2003) observed a 47.6 percent incidence by performing ELISA on horse and donkey samples (8, 9).

In Central Mongolia, a high prevalence of equine piroplasmosis has been reported (10); 88.2% and 84.5% of the horses tested were found to be sero-positive for *B. equi* and *B. caballi* antibodies by IFAT. Despite this high prevalence, very few clinical cases were

seen. Probably, the foals were infected at an early age and remained immune from further clinical infection. In China, equine babesiosis was recorded as early as 1943 in Heilonjian Province (11). A high rate of prevalence of equine babesiosis was observed from February to October, and April, May, and June were noted as months with a high incidence of disease. Horses were found to be more susceptible to the disease than mules and donkeys. Equine babesiosis is known to occur sporadically in local herds, and most local breeds have been found to be preimmune; however, outbreaks would be suspected in imported horses to an endemic area. Recently, seroepidmiological studies were conducted in Jilin Province of China by conducting ELISA using EMA-1 and P48 antigens of *B. equi* and *B. caballi*, respectively. Out of total samples tested 34% and 32% samples were found sero-poitive for *B. equi* and *B. caballi* infection, respectively (12).

3. Vector ticks

Ixodid ticks of the genera *Hyalomma*, *Dermacentor*, and *Rhipicephalus* have been identified as vectors for the transmission of either *B. equi* or *B. caballi* protozoa to a natural host. In tropical countries, including India, ticks of the *Hyalomma* species seem to have been a potential vector for the transmission of the disease to donkeys and horses. Ticks of the species *Hyalomma anatolicum anatolicum* (both sexes in the same ratio) were released on experimental donkeys, which showed a gradual rise in percent *B. equi* parasitemia. It was observed that the acini of the male ticks were more infected than those of the female ticks (13). The average percentage of infected acini per tick was 23.95% in males and 13.19% in females. These ticks were able to transmit infection to the natural host donkeys. In Mongolia, the tick vector for spreading equine babesiosis is unknown;

however, *Dermacentor nuttali Dermacentor salvarum*, and *Hyalomma dromedary*were observed to frequently infest horses and were reported to be the vector for *B. caballi*(14). Battasetseg et al. (2002) reported the amplification of specific equine *Babesia* parasite gene fragments in *D. nuttalli* ticks collected from different areas of Mongolia, suggesting their vectorial role in the transmission of the *Babesia* parasite (15). Bai et al. (1995) reported the transovarian transmission of *B. caballi* by *D. nuttali* in experimental horses of Chinese origin (16). *Dermacentor silvarum, Rhipiceephalus haemaphysaloides, D. niveu,* and *D. sinicus* have been reported as possible vector ticks for *B. equi* and *B. caballi* in China (11, 17).

4. Pathogenesis

The events in pathogenesis responsible for hemoglobinuria and subsequent anemia are obscure in equine babesiosis(18). Development of progressive anemia and hemoglobinuria in the last clinical phase of the disease is a pathognomonic sign in *B. equi* infection in horses and also in donkeys (19). The pathogenesis of anemia has not been fully elucidated. The adverse effects that *B. equi* and its metabolites inflict on donkey erythrocytes have been studied (20) in Indian donkeys. At high parasitemia, *B. equi* organisms were observed in neutrophils, indicating phagocytosis of the infected erythrocytes by the neutrophils. They reported a progressive increase in donkey erythrocyte membrane proteins, total phospholipids, and plasma malondialdehyde, thereby suggesting lipid peroxidation during the acute phase of the disease. Gradual decrease in hemoglobin and PCV values with the clinical progression of *B. equi* parasitemia in experimental donkeys was also observed. Further scanning electron

microscopy of *B. equi*-infected donkeys revealed spherocytes, sphero-echinocytes, sphero-stomatocyes, kinizocytes, and acanthocytes with pitting and fine granulation on their cell surface (21). This limited study concludes the occurrence of morphological changes and lipid peroxidation on the cell surface, leading to erythrocyte-phagocytosis and subsequent anemia during *B. equi* infection in donkeys.

5. Clinical manifestations

Infection caused by *B. equi* is more pathogenic and widespread than that by *B. caballi* protozoa. Mixed infections are not uncommon, and they make diagnosis difficult. The incubation period following an infective tick bite varies from five to 21 days (6, 22). The disease condition starts with intermittent fever up to 40°C followed by listlessness, depression, marked thirst, inappetence, watering eyes, and swelling of the eyelids. Affected donkeys are constipated, passing small, hard balls of feces covered with yellow mucus, and their overall health may deteriorate. Sometimes, donkeys will demonstrate colicky symptoms, such as, for example, looking at their flank, pawing, kicking at the belly region, and lying down and rolling, all of which are attributed to sluggish intestinal peristaltic movements and constipation. The most characteristic sign is the development of icterus, a mucous membrane that varies from pale pink to pale yellow to bright yellow in color. Petechiae or ecchymosed hemorrhages may be observed on the mucous membranes of the nasal passage, vagina, and third eyelids. Extreme enlargement of the spleen is a very common symptom in affected animals. Finally, color changes in the urine, from dark yellow to orange/brown, are observed, which indicates the presence of hemoglobin and bile pigments resulting from severe hemolysis of the infected

erythrocytes. Untreated or neglected cases will result in animals that are severely anemic and reluctant to move, suffer from malaise and inappetence, bend their necks down, as if disinterested in their surroundings, and exhibit signs of general weakness. Equines usually have a higher threshold for the escape of hemoglobin through urine from the circulation; hence, hemoglobinuria is observed as the last irreversible clinical sign signifying nephrosis and subsequent renal failure.

Chronic cases of equine babesiosis are more common in donkeys, and clinical signs are usually nonspecific and include mild appetence, poor work performance, or an inability to gain weight. Splenomegaly has been observed has been observed as an unusual finding in affected donkeys. Neonatal babesiosis has not been observed by the author and has been rarely reported. Severe hepatomegaly, splenomegaly, icterus, and internal hemorrhage have been reported (18).

6. Clinical pathology

Hemoglobin concentration, PCV level, and red blood cell counts are significantly lower in donkeys acutely infected with the *B. equi* parasite(20, 23). Acute infection is also characterized by severe leucocytosis, lymphopenia, and a high absolute neutrophil count (23, 24). Donkeys that died of *B. equi* infection showed a varying degree of emaciation, gross enlargement of the liver and spleen, and flabby kidneys (25). Small pinpoint petechial hemorrhages are also present in the liver, spleen, and cortical surfaces of the kidneys. The lungs are edematous and congested, and the lymph nodes are enlarged. Kupffer cells, observed microscopically, revealed a deposition of hemosiderin pigments(6, 25).

7. Diagnosis

7-1. Microscopic and serological examination

Microscopic demonstration of an intraerythrocytic parasite is still the best, most reliable, economical, and sustainable method for confirmative diagnosis of equine babesiosis. Thin and thick blood smears are suitable for this examination after staining with Giemsa stain, but they are unsuitable for the detection of carrier animals (26-28). Therefore, serological tests, such as the complement fixation (CF) test (29), indirect fluorescent antibody (IFA) test (30) [both are recommended official tests, according to OIE regulation], and enzyme-linked immunosorbent assay (ELISA) (8, 9, 31-36), are preferred. However, CFT has the inherent disadvantages of giving false-positive results and having low sensitivity for detecting latent infections. Due to the presence of some anti-complementary antibodies [IgG(T)] (37) and other factors in the equine serum, a CF test may give false-positive and -negative results (38). On the other hand, IFAT requires expertise and subjective variation for detecting positive nonspecific fluorescence. Different forms of ELISA have been standardized by many Asian researchers and have been validated on field samples (7-9, 12). Equine merozoite surface antigens (ema-1 and ema-2) have been used by many researchers for detecting specific antibodies against *B. equi* without any cross-reactivity with other *Babesia* spp. Knowles et al. (1991) confirmed that EMA-1 epitope is conserved in different isolates of *B. equi* from 19 countires, and they demonstrated the usefulness of monoclonal antibody-based competitive inhibition ELISA in the diagnosis of antibodies against *B. equi* in sera collected from different countries (38). Later, Tanaka et al. (1999) used the EMA-2-expressed antigen in ELISA

and demonstrated its efficacy in serodiagnosis and an epidemiological survey and, hence, on horses held for quarantine in Japan (39). To identify the usefulness of the new genes in the specific serodiagnosis of *B. equi* infection, Hirata et al. (2002) demonstrated the efficacy of the *B. equi* Be82 gene product in ELISA in detecting antibodies in field samples, and, furthermore, they constructed a series of clones with deletions in the Be82 gene product so as to improve the efficacy and reduce cross-reactivity with *B. caballi* (36). A clone with a deletion of the Be82 gene from positions 236 to 381 (Be82/236-381) was used to detect *B. equi*-infected horse sera without cross-reactivity with *B. caballi*-infected horse sera. Assays with clones from which other gene products were deleted showed decreased sensitivities or remained nonspecific for the detection of B. equi-infected horse sera. These results suggest that the Be82/236-381 gene product is a novel antigen for the diagnosis of *B. equi* infection in horses. Recently, Kumar et al. (2003) reported the use of single-dilution ELISA for the sero-diagnosis of *B. equi* antibodies (9). In conventional serial-dilution ELISA, the end titer of a sample is calculated by serial two-fold dilution of the sample until the optical density (OD) becomes negative. However, in a single-dilution ELISA, only one particular dilution of the serum sample is tested (for this reason, it is called a single-dilution ELISA). The OD value of the one-dilution tested is used in the regression equation for predicting the end antibody titer. Therefore, single-dilution ELISA is less cumbersome, more economical in terms of consumption of antigen/conjugate/reagents, and suitable for field studies. Kumar et al. (2003) demonstrated that a single-dilution ELISA is as sensitive and specific as a serial-dilution ELISA in determining the *B. equi* antibody's end titer of a sample (9).

7-2. Parasitic DNA detection

During the early phase of infection, the antibody titer and, hence, the parasitemia are very low, which makes it difficult to accurately diagnose the disease by conventional serological tests. In these cases, the detection of parasitic DNA is preferred, and many tests have been standardized on this parameter, that is, for example, with Southern hybridization with specific DNA probes, PCR, nested PCR, and PCR-based restriction fragment polymorphism analysis (RELP). Among these methods for the detections of DNA, PCR has been more popular and widely used test. The PCR reaction can amplify the target *Babesia* spp. DNA many times and is able to detect a very low quantity of DNA and, hence, of parasitemia. As little as 1 pg of the parasitic DNA can be successfully amplified and detected with a routine PCR test. Bashiruddin et al. (1999) designed PCR primers from the 16S rRNA gene of *B. equi* and *B. caballi* (40). They demonstrated that PCR could detect an approximated parasitemia of 0.000083% for *B. equi* compared with the reported limits of 0.001% for the microscopic examination of stained blood smears and up to 0.00025% for DNA probes. Furthermore, they reported that PCR detected more samples as positive, which had been found negative by the standard CF test. They concluded that PCR systems may be useful in the diagnosis of equine babesiosis adjuncts to serological, microscopic, and cultural methods, especially for the import and export testing of horses.

Rampersad et al. (2003) compared the efficacy of PCR and nested-PCR with routine blood-smear examinations (41). They concluded that the Wright-Giemsa-stain could be used to identify *Babesia* in mostly clinically ill animals and that nested PCR could be used to detect the organism in a large number of apparently healthy animals and

would be more sensitive than normal PCR. The results of this study suggested that nested PCR is superior to both the Wright-Giemsa-stain and primary PCR methods and should be considered for the routine detection of *B. equi* in both healthy and clinically ill horses.

8. Chemotherapy

The *B. equi* infection is more resistant to chemotherapy than *B. caballi* infection, and, usually, frequently repeated doses are required. A variety of drugs have been used for the treatment of *B. equi* infection in donkeys of Indian origin. Most of the drugs improve the clinical signs but do not completely eliminate the infection from the body. At the same time, drug treament is seldom mandatory in endemic areas but results in a serious relapse of the disease in the event of physiologically and physically stressful conditions. Tetracycline-like chlorotetracycline hydrochloride (Aureomycin®, Ledera Laboratories) and oxytetracycline hydrochloride (Terramycin®, Pfizer) are effective only against *B. equi* when given intravenously daily for two or more days at a dosage of at least 5.5 mg/kg body weight (42). The time interval between doses seems to be important. Four intramuscular doses of imidocarb dipropionate (Imizol® Burroughs Wellcome and Co., Gr. Britain) 72 h apart at 4 mg/kg body weight cleared *B. equi* from horses but not from infected donkeys, which died even after treatment (43, 44). Two-dose therapies with imidocarb at a 48h interval at 5 mg/ml were found to be quite effective in bringing about the clinical recovery in infected donkeys (24).

Dennig (1965) reported that diminazene diaceturate (Berenil® Hoechst Pharmaceuticals, Ltd.) was the only drug that was successful against mild-to-moderate *B. equi* infection in horses and donkeys but not effective against acute infection (45).

Further diminazene diaceturate was effective in eliminating *B. caballi* infection but not the *B. equi* parasite (46, 47). Singh et al. (1980) applied diminazene diaceturate to donkeys infected with the *B. equi* parasite at a dose of 12 mg/kg body weight (24). Two intramuscular injections were given at 24h apart. These researchers observed that parasitemia only declined two to three days after treatment. It has been considered that some time is required for the initiation of drug action against a multiplying parasite, which should preclude its field use. Copper glycinate has been proved successful in treating clinical cases due to *Babesia bigemina* infection in cattle (48). However, similar trials in splenectomized and non-splenectomized donkeys infected with *B. equi* parasite with an intravenous injection of copper glycinate at 1.5 mg/kg and two injections with a 24h interval proved unsuccessful in reducing fulminating parasitemia (49).

Encouraged by the high therapeutic efficacy of artemisinin (qinghaosu) derivatives, namely, artesunate, arteether, and artemeter, against multiple cases of falciparum malaria (50-52) and buparvaquone against tropical bovine theileriosis by *Theileria annulata* (53), we tested these drugs for their therapeutic efficacy (alone or in drug combinations) against *B. equi*-infected splenectomized donkeys (9). Individually, arteether (5 mg/kg daily for 3 days, intramuscularly) and buparvaquone (5 mg/kg daily for 4 days, intravenously) were found to have no parasite clearing efficacy, and the treated donkeys died within 5-6 days after showing high parasitemia and clinical symptoms of the disease. However, the parasite multiplication in animals treated with artesunate (2.5 mg/kg daily for 4 days, intramuscularly) was restricted, but only during the treatment period; after that, all the treated donkeys died. The parasite was cleared from the blood of all the animals treated with imidicarb and the arteether+buparvaquone

combination within 2-5 days post-treatment (PT), but, in both of these groups, recrudescence of the *B. equi* parasite was observed within 55-58 days PT, and the mean survival period post-treatment was 66-69 days. The hemato-biochemical parameters on these animals had shown that imidocarb had a deleterious effect on the liver function, while the combination of arteether+buparvaquone was found to be safe. This limited study indicates that the combination of arteether+buparvaquone could be a better choice than imidocarb for treating *B. equi* infection in donkeys (9).

9. Immunological control

Little effort has been made to develop a suitable and potent vaccine for the control of equine babesiosis in horses and donkeys. After diagnosis of the infection in equids, the second step would be to curtail and control the disease. Some efforts toward vaccinological control have been made using crude *B. equi* immunogen in donkeys. Singh et al. (1980) immunized donkeys with a *B. equi*-infected erythrocyte lysate followed by boosted inoculation (24). Immunized donkeys survived after the challenge of infection but became carriers of the *B. equi* parasite. Salem et al. (1990) also tried crude vaccine on donkeys and reported protection upon the challenge (54). Recently, Kumar et al. (2002) immunized the donkeys with immunogen so that each dose of the immunogen would contain the lysate of 2×10^{10} parasitized erythrocytes (23). The immunogen was mixed with adjuvant Quil A and injected into the animal, and one boosted inoculation was repeated. The immunized donkeys survived the challenge infection (1×10^{11} parasitized erythrocytes), and, simultaneously, a very high humoral and cell-mediated immune response was mounted by the immunogen in the immunized donkeys, as monitored by

ELISA and the lymphocyte stimulation assay, respectively, during the experimental period. The most astonishing part of the experiment was the absence of any parasitemia after splenectomy of the immunized donkeys, which survived after the challenge infection. This experiment had proved that a *B. equi* crude immunogen can elicit a strong immune response against *B. equi* infection and, also, that there is a possibility for the vaccinological control of the infection. Further 112, 45, 33, and 18 kDa polypeptides were identified as immunodominant in *B. equi* merozoite antigen and reacted strongly with the serum collected from immunized challenge-survived donkeys (23). More future vaccinological experiments will be required to develop a potent subunit vaccine.

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Chapter 3. Canine babesiosis

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1. Introduction

Canine babesiosis is tick-borne disease caused by two intraerythrocytic protozoan parasites, *Babesia canis* and *B. gibsoni*. Both *B. canis* and *B. gibsoni* infections are prevalent in Asia, although the former is less common. Traditionally, canine piroplasms were mainly identified based on their morphological properties: all large piroplasms were thought to be *B. canis*, whereas all small piroplasms were thought to be *B. gibsoni*. In contrast to *B. gibsoni*, three different subspecies of *B. canis* have been proposed based on their biological properties: *B. canis rossi*, which is transmitted by *Haemaphysalis leachi* in Southern Africa and causes a usually fatal infection in domestic dogs even after treatment; *B. canis vogeli*, which is transmitted by *Rhipicephalus sanguineus* (distributed throughout various tropical and subtropical regions, including many Asian countries) and leads to a moderate, often clinically inapparent infection; *B. canis canis*, which is transmitted by *Dermacentor reticulates* in Europe and shows a more variable pathogenicity, usually lying between the aforementioned extremes (1, 2, 3, 4). These three biologically different subspecies of large piroplasms were recently confirmed as true subspecies based on genetic characterizations (3, 5). On the other hand, recent knowledge, based on genetic analyses of genes from canine piroplasms, also shows that there are at least three distinct subtypes or subspecies for small piroplasms: a classic Asian type *B. gibsoni*; a small organism recently identified in northern Spain and called *Theileria annae*; and a small organism identified in California that remains unnamed (4, 6, 7, 8, 9).

2. Morphology

B. canis belongs to large *Babesia* (Fig. 1), is 3-5 μm in length, is piriform, and often occurs in pairs (10, 11). Pleomorphism of shape may be seen, organisms varying from amoeboid to ring forms. Multiple infection of erythrocytes is possible, up to and sometimes more than 16 organisms occurring in a single red blood cell (10, 11).

Organisms have also been found in endothelial cells of lungs and liver and in macrophages due to erythrophagocytosis (10).

B. gibsoni belongs to small *Babesia* (Fig. 2), is 0.5-2.5 μm in length, appears in pleomorphism in shapes that are frequently annular or oval, often appears alone, and may display a ring form (11). Occasionally, a maltase cross-form is observed (12).

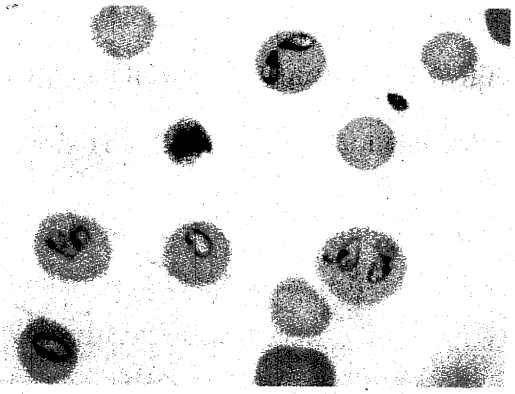

Fig. 1. *B. canis* usually appears as paired piriform organisms in canine red blood cells.

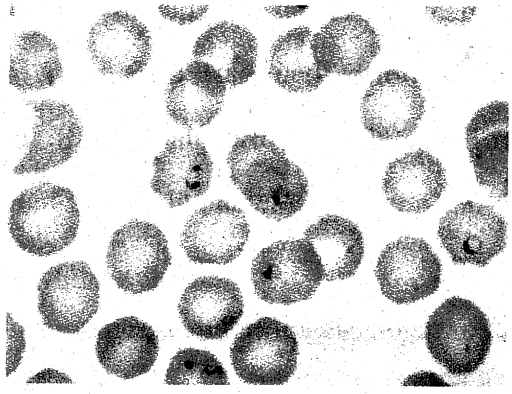

Fig. 2. *B. gibsoni* usually appears as ring form organisms in canine red blood cells.

3. Life cycle and transmission

H. leachi, *R. sanguineus*, and *D. reticulates* are known to be transmitting vectors of *B. canis* (10, 11, 12). On the other hand, *H. bispinosa*, *H. longicornis*, and *R. sanguineus* are considered as transmitting vectors of *B. gibsoni* (10, 11, 13). The development cycles of both *Babesia* are considered to be similar. Following attachment of infested ticks, sporozoites are released into the blood circulation, infecting erythrocytes. Within the erythrocytes, the parasites propagate by binary fission, an asexual form of schizogony, resulting in merozoites. Naïve ticks attach to the infected dogs and become infected with *Babesia* organisms when they ingest a blood meal. In addition to ticks, transmission of canine babesiosis may also occur through blood transfusion, oral abrasion bites, and contaminated needles and instruments. Certain biting insects may also serve as mechanical vectors.

Transplacental transmission may also occur.

4. Distribution

In Asia, *B. gibsoni* infection is much more common and serious than *B. canis* infection. Only *B. canis vogeli* infection is occasionally reported from Asian countries, whereas *B. gibsoni* is frequently and widely reported from many Asian countries (13, 14). To date, there have been many reports of *B. gibsoni* infection in India (15, 16), Sri-Lanka (17, 18), Bangladesh (18), China (19), Japan (13, 20, 21, 22, 23), Malaysia (24), Korea (25), Vietnam (19), and Iran (13).

5. Pathogenesis and clinical signs

The pathogenesis of canine babesiosis varies considerably with the species and strains. Both young and old dogs are susceptible to babesiosis; however, young dogs are more susceptible and frequently have severer infections (26). This is different from the case of bovine babesiosis, in which calves are more resistant than older animals (26). Infested ticks need to complete the transmission with feeding for 2 to 3 days (12). The incubation period of *B. canis* is 10-21 days, and, for *B. gibsoni*, it is 7-21 days. The most common clinical signs of acute cases are fever, marked anemia with icterus, inappetence, marked thirst, weakness, prostration, and often death (11). Hemoglobinuria is usually associated with peracute cases in which the loss of red blood cells is marked (10). In chronic cases, there may be irregular temperature, a capricious appetite, and marked loss of conditions. Anemia is severe, and the dogs are listless and become very weak and emaciated. In some severe and fatal infections, there is no direct relationship between the clinical condition and the degree of parasitemia (10). Erythropoiesis is active even in profound cases of anemia, and reticulocytes appear from the early to the late stages. Central nervous system involvement, which is less common than the other manifestations, causes locomotor disturbances, paresis,

epileptiform fits, and other CNS signs. The spleen is enlarged, with dark red, soft pulp and prominent splenic corpuscles. The liver is enlarged and yellow with pathologic changes ranging from congestion to centrilobular necrosis. Small hemorrhages are sometimes present on the heart, pleura, bronchi, and intestines.

6. Diagnosis

It is important to determine the species, subspecies, and genotype that causes canine babesiosis, since the virulence, prognosis, and response to antibabesial drugs may be different for each organism. The diagnosis of canine babesiosis is mainly based on the detection of intraerythrocytic *Babesia* organisms by the microscopic examination of Giemsa-stained thin blood smear films. *B. canis* generally appears as a paired piriform measuring 3-5 μm (Fig. 1), and *B. gibsoni* usually appears as a singular, signet ring shape measuring 0.5-2.5 μm (Fig. 2). A microscopic examination is simple and suitable for the acute stage of canine babesiosis. However, the detection of the intraerythrocytic *Babesia* organisms is sometimes very difficult and not suitable for practical use in inapparent or chronic infection because of low levels of parasitemia. Hence, alternatively, the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) with infected erythrocytes as an antigen have been used for the serological diagnosis of *B. gibsoni* infection (13, 27). These tests are particularly useful for the identification of chronically infected dogs with significantly low parasitemia. In general, IFAT and ELISA for babesial parasites are highly sensitive but only moderately specific because of antigenic cross-reactions with other closely related *Babesia* species (27). In these tests, antigenic cross-reactivity between the two canine *Babesia* species has been reported (27). Furthermore, dogs that were experimentally infected with *B. gibsoni* developed cross-reactive antibody to canine protozoan parasites, such as *B. canis*, *Toxoplasma gondii*, and *Neospora caninum* (27). In addition, when whole parasites are used as antigens, their quantities can vary from batch to batch. In addition, the antigen production for these tests requires experimentally infected dogs, making production time-consuming and expensive. Therefore, the development of a high-quality serodiagnostic system using parasite-specific antigens is required for the diagnosis of canine babesiosis.

The molecular search for diagnostic antigens for canine babesiosis infection has focused on the identification of immunodominant antigens that are recognized by sera from animals infected with geographically distant isolates and from both acutely and chronically infected animals. Recently, Fukumoto et al. have identified two genes both encoding immunodominat antigens, P29 and P50 of *B. gibsoni*, and evaluated their diagnostic potential in ELISA (28, 29). The ELISAs with recombinant P29 or P50 were able to differentiate clearly between *B. gibsoni*-infected dogs and *B. canis*-infected dogs or non-infected dogs (28, 29, 30, 31). A positive result is dependent on an antibody response by the host, which may take up to 8 days to develop (30).

Recently, the introduction of a polymerase chain reaction (PCR) has allowed the development of simple, specific, and sensitive diagnostic methods for the detection of canine piroplasms (32, 33). In addition, molecular analysis based on PCR offered a better understanding of the phylogenetic relationships and classification of canine piroplasms (3-9).

Severe combined immune deficiency (SCID) mice, whose erythrocytes had been replaced by canine erythrocytes (Ca-RBC-SCID), were established for the propagation of canine piroplasms (34, 35). Inoculation of Ca-RBC-SCID with blood samples (xenodiagnosis) suspected of being infected with *B. canis vogeli* or *B. gibsoni*, for example, is also useful, especially when the level of parasitemia is significantly low.

In vitro cultivation of canine piroplasms has also been established (18, 36, 37). The methods can be used as a means of amplifying piroplasms in dogs in which parasites might have otherwise been missed.

7. Treatment

Chemotherapy against canine babesiosis at the early phase of infection is very important to reduce the severity of disease and mortality, although it cannot completely eliminate the parasites. Diminazene acceturate, phenamicine isethionate, and pentamicine isethionate have been demonstrated to be effective against canine babesiosis (10). Trypan blue and quinoline derivatives, such as acapron, are effective against *B. canis* infection but not against *B. gibsoni* infection (11). Only diminazene acceturate (Ganazeg) has been utilized for the treatment of *B. gibsoni* infection in Japan. However, it sometimes induces side effects, such as weakness, irritability, paralysis,

non-responsiveness to stimuli, and fatal central nervous system hemorrhage. Furthermore, the production of Ganazeg was recently stopped because of these severe side effects. Since then, the treatment and control of canine *B. gibsoni* infection have become very difficult in Japan. Supportive therapy, such as intravenous fluids and transfusions, is recommended for canine babesiosis, particularly for dogs with severe anemia.

8. Prevention and control

For the control of canine babesiosis, vaccination is generally considered to be the most effective means. It is known that the inactivated whole parasite antigen or soluble parasite antigen that is derived from a supernatant of an *in vitro* culture of *Babesia* parasites is a useful antigen for vaccination and induces partial protection against babesiosis (37, 38). However, the quantities and quality of the antigens frequently vary from one batch to another. This sentence is a little too long and not quite clear. Do you mean, "Furthermore, the production of whole parasites requires that dogs be experimentally infected, and the production of soluble parasite antigen requires a significantly large volume of canine erythrocytes and a large scale of *in vitro* cultured parasites. All these requirements make this method expensive, time-consuming, and impractical as a vaccine development method against canine babesiosis. Recombinant vaccines, corresponding to immunodominant antigens of *B. gibsoni*, could be used to overcome the problems outlined above (39).

Currently, an effective vaccine is not commercially available in Asian countries. Therefore, tick control is considered the most important means for the prevention of canine babesiosis, since treatment is not always successful. The environment should be treated to decrease the number of ticks; dogs should be treated to control tick infestations; and ticks should be removed from parasitized dogs as quickly as they are detected. In addition, owners should be aware that dogs that have survived from babesiosis, particularly from *B. gibsoni* infection, remain subclinically infected. These dogs may suffer a relapse of the disease or serve as point sources for the further spread of disease in a given area. Dogs that have recovered from babesiosis should never be used as donors for blood transfusions.

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Part III.

Basic studies for babesiosis by Asian scientists

Chapter 1. Molecular approaches for the development of babesial vaccines

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1. Introduction

Babesia parasites are tick-transmitted intra-erythrocytic protozoa in the phylum Apicomplexa. They infect a wide variety of wild and domestic animals and are responsible for enormous economic losses to the livestock industry worldwide (1,2). Moreover, some are major etiologic agents of human babesiosis (3). During the asexual growth cycle in a natural host, the merozoites internalize the host erythrocytes via multiple adhesive interactions of several protozoan molecules with the host cell components. Thus, the parasites destroy the infected erythrocytes, which results in severe clinical symptoms, such as high fever, anemia, hematuria, and hemoglobinuria, in the infected hosts (1,2). Therefore, a better understanding of basic molecular mechanism(s) during the asexual growth cycle may accelerate the development of effective vaccines against babesiosis.

Babesia microti has been used as a useful experimental model to study the immune mechanisms of babesiosis in many other animals. Mice infected with *B. microti* produce a transiently high parasitemia but subsequently recover from the acute infection (4). The recovered mice are resistant against re-infection with *B. microti* (5,6). $CD4^+$ T cells and gamma interferon (IFN-γ) produced by the $CD4^+$ T cells are considered to play vital roles in the resolution of *B. microti* infection (5-7).

In this review, we discuss four recent developments that have made a remarkable contribution to understanding the molecular mechanism of *Babesia* parasites and, hence, the identification of vaccine targets.

2. Heparin covers the surface of babesial merozoites and inhibits the subsequent invasion into the host erythrocytes (4)

Heparin is a highly sulfated form of heparan sulfate (HS) and is well known as an inhibitor of the blood coagulation system (8). The HS and heparin are complex entities

composed by anionic, linear mucopolysaccharides with alternating uronic acid and hexosamine residues in which a limited set of monosaccharide units gives rise to a number of complex sequences by variable substitution with the *O*-sulfate, *N*-sulfate, and *N*-acetyl groups (9). While the HS is produced in most cell types, heparin is a biosynthetically derived component of mast cells and basophils (10) and has a molecular weight (MW) of approximately 3,000 to 37,500 Da with an average MW of 13,000 Da (11). Due to the sulphate and carboxylate residues, heparin is highly negatively charged and has a selectively high affinity for various molecules (12). Moreover, heparin has been used as a model glycosaminoglycan (GAG) to study the HS interaction with its binding partners (13).

In this study, the inhibitory efficacy of heparin was principally demonstrated on the growth of *Babesia* parasites. The multiplications of *Babesia bovis*, *B. bigemina*, *B. equi,* and *B. caballi* in *in vitro* cultures and of *B. microti in vivo* (mice) were significantly inhibited in the presence of or treatment with heparin. Treatment with heparin restricted the invasion of babesial merozoites to new erythrocytes, as evidenced by the increased number of extra-erythrocytic (free) merozoites, and finally led to a complete clearance of the intra-erythrocytic parasites. Furthermore, fluorescein isothiocyanate-labeled heparin (heparin-FITC) was preferentially found on the surface of extracellular merozoites in confocal laser scanning microscopy (Fig. 1).

The heparin-FITC results indicated the presence of heparin-binding molecule(s) on the surface of free merozoites. The interaction between the heparin-binding molecule(s) and exogenous heparin appears to play a critical role in the interruption of the attachment/invasion process of merozoites on the erythrocytic surface. Previous studies with heparin-FITC-labeled *Toxoplasma gondii* reported that the fluorescence was localized near the subapical region of free tachyzoites (14). The *T. gondii* surface antigen, SAG3, is known to show a heparin-binding property and mediates the attachment of tachyzoites to the cellular HS proteoglycan of

host cells (15). In *P. falciparum*, heparin interacts with the circumsporozoite (CS) protein expressed on the surface of sporozoites (16). The binding of the CS protein to cellular HS proteoglycans is required for the attachment of sporozoites to hepatocytes. It is noteworthy that the HS proteoglycans are utilized in the host recognition by some parasitic microbes (17). Together with the fact that the HS-like GAG is located on the surface of erythrocytes (18,19), free babesial merozoite can be considered to recognize the HS-like GAG of host erythrocytes via the parasitic molecule(s) with an affinity to heparin.

The observations suggested that heparin wraps the surface of free babesial merozoites and inhibits subsequent invasion into the host erythrocytes. This enforces the identification of the heparin-binding molecule(s) for understanding the process of merozoite attachment and invasion. Furthermore, such a merozoite surface molecule might become an effective babesial vaccine, which might induce antibodies inactivating the process of merozoite attachment.

3. Cellular localization of *Babesia bovis* merozoite rhoptry-associated protein 1 and its erythrocyte-binding activity (20)

Apicomplexans utilize several rhoptry proteins in their invasion and further development within the host cell (21,22). In *Plasmodium* parasites, extracellular merozoites attach to the host erythrocytes and reorient to bring the apical organelles close to the attachment interface, and, through the interaction of protozoan ligands with several surface receptors, the rhoptry products are released at the point of membrane invagination. In *Babesia bovis*, which is pathogenic in bovines (1,2), a rhoptry protein, designated as rhoptry-associated protein-1 (RAP-1), has previously been identified (22). The RAP-1 has an apical location on the surface of *B. bovis* merozoites (23-26) and is detectable as a rhoptry component by immuno-electron microscopy (27,28). The RAP-1 of *B. bovis* has substantial sequence homology with the RAP-1 of other *Babesia* parasites (23,29) and contains several epitopes immunogenic to host B cells (27).

Importantly, the purified recombinant RAP-1 has proved effective in inducing a protective immunity to some extent in the vaccinated cattle (30). Furthermore, Mosqueda et al. (31) reported that the RAP-1 is also expressed in sporozoites and that the specific antibodies can inhibit the attachment of sporozoites to host erythrocytes. In order to understand the biological role of RAP-1 expressed in *B. bovis* merozoites, the cellular localization of *B. bovis* merozoite RAP-1 and its erythrocyte-binding activity were investigated in this study.

In an indirect immunofluorescent antibody test, the RAP-1 was detectable in all developmental stages of merozoites (ring and the subsequent pear-shaped forms) and extracellular merozoites. In the early stage of merozoite development, the RAP-1 appears in the parasite cytoplasm, which later thins out and blankets the host cell cytoplasm but remains in the cytoplasm of newly formed parasites (Fig. 2). The preferential accumulations of RAP-1 on the surface of the erythrocytic membrane and near the outer surface of parasites were demonstrated by immuno-electron microscopy in the later developmental stages. An erythrocyte-binding assay with the lysate of merozoites proved the binding ability of RAP-1 to bovine erythrocytes. An anti-RAP-1-specific antibody prevented the interaction of RAP-1 with bovine erythrocytes and significantly inhibited the proliferation of *B. bovis* in the *in vitro* culture.

The cellular localization of RAP-1 in the later developmental stages suggests that the RAP-1 interacts with several components, which are contained in the cytoskeleton and membrane of infected erythrocytes. The RAP-1 family might function as proteases (32), which can exhibit a proteolytic activity to the erythrocytic components, associating with the rupture of host cells and subsequent release of merozoites. In contrast, the erythrocyte-binding activity of RAP-1 suggests that the RAP-1 also has an essential and functional participation in the invasion of merozoites into the host erythrocytes. The growth inhibition of *B. bovis* parasites in the presence of anti-RAP-1-specific antibody suggested that the antibody neutralized the erythrocyte-binding activity of RAP-1 during the extracellular phase of the parasite, thus

disrupting the parasite invasion to new erythrocytes. However, another possibility, the blockage of mature merozoite release by covering the RAP-1 located on or near the membranes of infected erythrocytes, could not be excluded.

It is very important to study the multiple functions of RAP-1 not only during the invasion of merozoites into erythrocytes but also during the egression of matured merozoites from host cells in more detail. Additionally, the identification of the erythrocyte surface receptor recognized by RAP-1 is also necessary. Studies on rhoptry proteins, such as *B. bovis* RAP-1, will certainly boost the successful development of recombinant vaccines against babesiosis.

4. Stage-specific localizations of *Babesia equi* EMA-1 and EMA-2 in the erythrocytic phase and EMA-2-specific interaction with the erythrocytic skeleton (33)

Equine babesiosis caused by *Babesia equi* is more pathogenic and widespread in equid than that by *Babesia caballi* (34,35). In *B. equi,* two kinds of the most immunodominant antigens, equi merozoite antigen (EMA) -1 (34 kDa) and -2 (30 kDa), have been identified (36-38). EMA-1 is geographically conserved among all *B. equi* isolates (37) and shares significantly high homologies in the amino acid sequence with the counterpart proteins of many *Theileria* parasites (36,39). EMA-1 and -2 genes have 52 % amino acid identity with each other (39). Additionally, EMA-1 and -2 have glycosyl-phosphatidylinositol (GPI) anchor-specific motifs in their sequences, suggesting that these proteins are expressed on the outer surface of merozoites with the GPI anchor (39). In this study, the cellular localizations of the EMA-1 and -2 during the asexual development of merozoites and their binding activities to the erythrocytic skeleton were examined by using the anti-EMA-1 or -2 mono-specific antibodies.

The indirect fluorescent antibody test (IFAT) demonstrated that the EMA-1 and -2 were mutually co-expressed during the early stage but not in all developmental stages of *B. equi* merozoites. The co-expression of EMA-1 and -2 was observed in the pre-invasive and

subsequently internalized merozoites in the invaded erythrocyte. Then, IFAT using the pre-incubated *B. equi* culture with these antibodies showed that both of the EMA-1 and -2 are exposed on the surface of extracellular merozoites. During the multiplicative phase after invasion, which leads to a classical 'Maltese-cross' formation (40,41), it was observed that the EMA-1 and -2 expressions were dominant only during the initial multiplying phase. When the merozoites fully developed and detached themselves from the bunch of 'Maltese-cross' formations, the expressions of EMA-1 and -2 completely disappeared. These findings indicated that the expressions of EMA-1 and -2 on the merozoite surface are highly stage-specific.

Furthermore, the intra-erythrocytic merozoite was shown to shed only EMA-2 in the cytoplasm or inside the membrane of infected erythrocytes before the 'Maltese-cross' formation (Fig. 3). The shedding behavior of EMA-2 was a very unique finding, and that of EMA-1 was not detectable. In *Plasmodium* parasites, secondary processing of merozoite surface protein-1 (MSP-1) by serine proteases leads to its cleavage into MSP-1_{19} and MSP-1_{33}, whereas the MSP-1_{33} is shed quantitatively in the cytoplasm of infected erythrocytes and the MSP-1_{19} remains on the surface of invading parasite (42,43). The processing is associated with the successful invasion and intra-erythrocytic survival of merozoites because the inhibition of secondary processing aborts the merozoite invasion. Secondary processing of the surface protein has not been identified in *Babesia* parasites, but this feature may be possible by the EMA-2 in *B. equi* in the light of present observation and needs more investigation. Shedding of EMA-2 inside the membrane of infected erythrocytes might have some important functions after the internalization of merozoites.

In the erythrocyte-binding assay, the recombinant EMA-2 gene product showed a specific interaction with the Triton X-100-insoluble fraction of equine erythrocytes (erythrocytic skeleton), but the EMA-2 did not. The specific binding of EMA-2 reinforces the observation of EMA-2-shedding behavior. In our preliminary study, the addition of anti-EMA-2-specific

antibodies significantly prevented the *in vitro* growth of *B. equi*, suggesting that the development of EMA-2-based vaccine might be possible (Kumar et al., in preparation). Further experiments are required to understand the biological roles of merozoite surface antigens of *B. equi*.

5. Maltese cross form-related antigen of *Babesia microti* induces a protective immunity against challenge infection in mice

Babesia microti is a rodent form of *Babesia* and also known as a major etiologic agent of human babesiosis (3). Hundreds of cases of human babesiosis by *B. microti* have been reported in the northeastern and upper mid-western areas of the United States (44-46). Recently, *B. microti* infection has also been reported in Taiwan (47) and Japan (48). Under a light microscope (Fig. 4), ring- and pear-shaped forms of *B. microti* are often observed in the infected erythrocytes. The 'Maltese cross' form, however, which consists of four masses in an erythrocyte, is rarely seen, but it is often described to be characteristic of *B. microti* (3,49,50) as well as that of *B. equi* (40,33). We had earlier reported that the monoclonal antibody (mAb) 2-1E specifically recognizes the 'Maltese cross' form of *B. microti* (50). In this study, the role of the Maltese cross form in the infectious course of *B. microti* and the immunogenic potential of the Maltese cross form-related antigen (MRA), which is recognized by the mAb 2-1E, were examined in mice.

First, the Maltese cross form was revealed to increase rapidly in the peripheral blood of infected mice prior to the peak of parasitemia. In an indirect immunofluorescent antibody test (IFAT), the mAb 2-1E was found to be reactive with the ring form, with parasites undergoing the transformation to the 'Maltese cross' form and subsequent division and also with extracellular merozoites, although the reactive parasites were remarkably rare (only 1% of all parasites). The results of IFAT and the correlation between the appearance of the Maltese cross

form and the rapid development of parasitemia suggest that a small population of the ring form transforms into the Maltese cross form and produces four progenies, thus contributing to the rapid development of parsitemia.

Next, the MRA gene was isolated from a *B. microti*-cDNA expression phage library by immunoscreening with the mAb 2-1E, and the nucleotide sequence was determined. The deduced amino acid sequence of MRA showed a partial similarity to the BMN 1-15 of *B. microti* (51). The recombinant MRA was synthesized by a baculoviral expression system. The immunization of mice with the recombinant MRA in combination with Freund's adjuvants induced a significant protection against the challenge infection with *B. microti.* Freund's adjuvants are known to induce a strong cell-mediated immunity as well as a humoral response in rodent malaria infection (52,53). In *B. microti* infection, cell-mediated immunities, especially by $CD4^+$ T cells and IFN-γ, are known to play important roles in the protection (5-6). Because passive immunization with the mAb 2-1E did not influence the development of parasitemia in the *B. microti*-infected mice (50), the cell-mediated immunity induced by a combination of recombinant MRA and adjuvants might inhibit the reproductive expansion of *B. microti* merozoites in mice. It would be interesting to determine why such a rarely detectable antigen can induce protective immunity, and further studies toward the resolution will throw a light on new preventive strategies for protozoan infections.

B. microti not only is of zoonotic importance but also has been studied as a model for many other babesiosis. Besides *B. microti*, *B. equi* is also known to have the 'Maltese cross' forms in the erythrocytic phase. From the nucleotide sequence or antigenicity of *B. microti* MRA, it might be possible to isolate the homologous MRA genes from the *B. equi* cDNA library (54). Because the severity of *B. equi* infection is also associated with the erythrocytic phase, as in *B. microti* infection, and responsible for enormous losses to the horse industry, this report will also be valuable for the development of protective measures against equine babesiosis in the future.

6. Concluding remarks

The severity of babesiosis is associated with the asexual multiplication of merozoites in erythrocytes, leading to high parasitemia and subsequently erythrolisis, anaemia, and malaise in the infected animals. Many studies have been carried out, with limited success, for the development of an effective babesial vaccine that can restrict the growth of the parasite and completely eliminate the parasite from the host (23,30,55-59). For the development of such an ideal vaccine, it is highly desirable to understand the basic molecular mechanism(s) during the asexual growth cycle of *Babesia* merozoites, particularly, the processes of merozoite invasion into, maturation in, and escape from the infected erythrocytes.

In the erythrocytic phase of *Babesia* parasites, there are several strategies that might help in the development of preventive measures. The first could be based on the pre-invaded phase of merozoites. In this phase, extracellular merozoites are directly exposed to the humoral immune components of the host. By the blockage of merozoite attachment to and invasion into the host erythrocytes, the replication of merozoites might be controlled in the vaccinated animals. The identification of heparin-binding molecules located on the surface of free merozoites or rhoptry proteins secreted from the apical organelle of merozoites will provide important information for the development of a babesial vaccine. Furthermore, the search for the identification of ligands/receptors on the erythrocyte, which facilitate merozoite invasion, is of paramount importance. A second candidate may be targeted at the maturation step of merozoites, in which the vaccine can prevent the internalization of intracellular merozoites. Humoral immunity induced by the vaccine ideally recognizes the surface of free merozoites, and, after the merozoite has invaded, these antibodies will restrain the subsequent maturation process of merozoites within the infected erythrocytes, making it its own graveyard. Although *B. equi* EMA-2 might be targeted for the development of the vaccine, further investigations will be

required. A third target is the escape step, when the merozoites rupture the infected erythrocytes. Intracellular merozoites must secrete protease-related enzymes into the cytoplasm of infected erythrocytes and digest the erythrocytic components for their escape. The blockage of these proteases in the mature merozoites would abort the escape process. Finally, a search for the protozoan molecules/epitopes responsible for inducing cell-mediated immune responses would also help in mounting the cell-mediated immune responses against the reproduction of merozoites by way of stimulating $CD4^+$ T cells and IFN-γ. Consequently, further efforts to identify potential protozoan components necessary for the development of ideal vaccines for babesiosis are warranted for the containment of the infection.

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[Figure legends]

Figure 1. Demonstration of heparin binding on the surface of free merozoites. *B. bovis* (A)- or *B. caballi* (B)-infected erythrocytes were pre-incubated with heparin-FITC followed by fixing with acetone/methanol and then examined under a confocal laser scanning microscope. The heparin-antigen reaction (green) and nucleus (red) were visualized with FITC and propidium iodide (PI) staining, respectively. Note: Diffused fluorescence reaction was detectable only around free merozoites. Bar = 5 μm.

Figure 2. Methanol-acetone-fixed smears of *B. bovis*-infected erythrocytes incubated with anti-RAP-1-specific mAb 1C1 and then observed with confocal laser microscopy. MAb-antigen reaction (green) and nucleus (red) were visualized with the FITC-conjugated secondary antibody and PI staining, respectively. Note: In the early stage of merozoite development, the RAP-1 appears in the parasite cytoplasm (A), which later thins out and blankets the host cell cytoplasm but remains in the cytoplasm of a newly formed parasite (B and C). Bar = 5μm.

Figure 3. Methanol-fixed smears of *B. equi*-infected erythrocytes incubated with anti-EMA-1 (A) or EMA-2 (B) mono-specific antibodies and then observed with confocal laser microscopy. MAb-antigen reaction (green) and nucleus (red) were visualized with the FITC-conjugated secondary antibody and PI staining, respectively. Note: The intra-erythrocytic merozoite sheds only EMA-2 in the cytoplasm or inside the membrane of infected erythrocytes before the 'Maltese-cross' formation. Bar = 5μm.

Figure 4. Light micrograph of intra-erythrocytic *B. microti* in Giemsa-stained blood smears. An arrow indicates the Maltese cross forms, which were rarely observed in the *B. microti*-infected murine erythrocytes. Bar = 5μm.

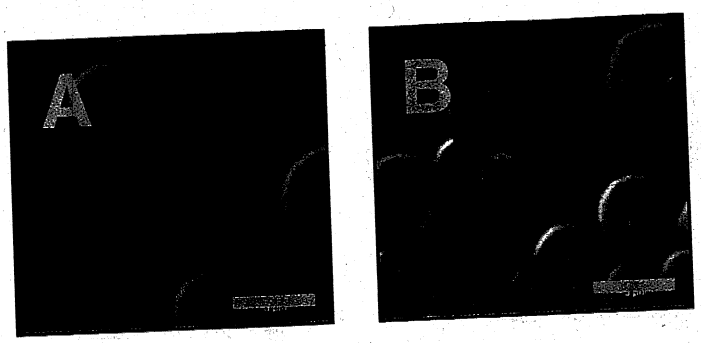

Fig. 1

Fig. 2

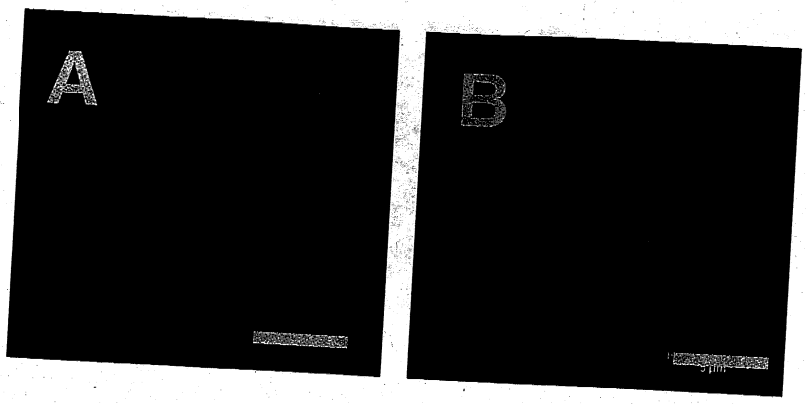

Fig. 3

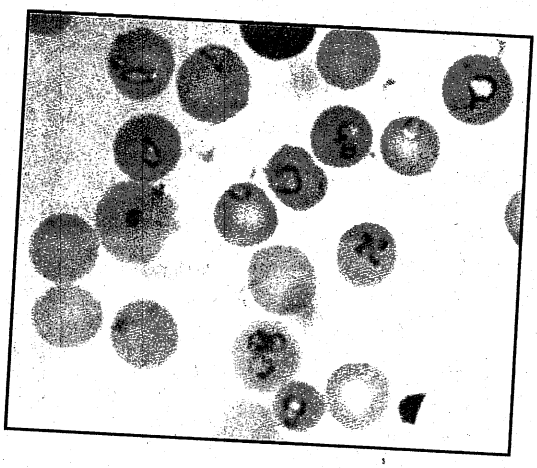

Fig. 4

Chapter 2. Host immune responses against Babesia infection

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1. Introduction

Babesiosis caused by *Babesia* spp., the hemoprotozoan parasites, has been responsible for major economic losses in the cattle industry and momentous concern on the international trade of horses throughout the world. Several studies have resulted in rapid, effective diagnostic tools, but the development of control strategies including chemotherapy and, especially, vaccines has been retarded due to limited information about the host immune responses against the protozoa. Cattle that recovered from an acute infection of *B. bigemina* or *B. bovis* experience the latent infection and become resistant to challenge infection with the homologous strain (1, 2). In addition, immunization with the dead parasite or parasite extract can give protective immunity against the homologous or heterologous parasites (3, 4). These findings indicate the possible use of a non-living parasite as a vaccine. However, it is difficult to use cattle to demonstrate the mechanism by which protective immunity is elicited against babesiosis. In this respect, the mouse model is very useful to examine the host immune responses to *Babesia* parasites. Macrophages, IFN-γ, and its producer, $CD4^+$ T cells, are considered pivotal in resolving the primary infection and preventing the subsequent infection in the

Babesia infections in mice. Both innate and acquired immunity, especially, a T-cell-mediated response rather than a B-cell-mediated one, contribute to defense against *Babesia*. However, based on the studies of malaria, the robust production of nitric oxide by activated macrophages may induce severe pathology, such as cerebral babesiosis and renal disfunction. We hereafter describe 1) the importance of innate and cell-mediated immunity and associated cytokines in murine babesiosis in view of our recent findings, 2) the immunopathology in babesiosis, and 3) the current status of and future perspective toward the effective vaccine development against babesiosis.

2. Immune response against the experimental *Babesia* infection in mice

B. rodhaini and *B. microti* are often used in the mouse model of babesiosis. *B. rodhaini* is known to be highly lethal, since mice injected with even only one parasite are killed. On the other hand, *B. microti*, which also infects humans, causes non-lethal, self-limited infection in mice. The *B. microti*-infected BALB/c mice initially show parasites in the peripheral blood at 4 days (acute stage). Parasitemia reaches a peak at around 10 days and gradually decreases, and then no parasites are present at 3 weeks after infection (resolution stage). The host spleen has been implicated in the elimination

of *Babesia* parasites and parasitized erythrocytes in several studies that showed that splenomegaly is often observed in the infected animals (5-8) and that the splenectomized or inherently asplenic mice are highly susceptible to *Babesia* infection (9, 10). T and B lymphocytes, natural killer (NK) cells, and macrophages in the spleen are clearly responsible for protective immunity against babesiosis. Both innate and acquired immunity contribute to the resolution of the primary infection and the protection against the subsequent challenge infection.

2-1. Innate immunity

Although innate immunity is actually a nonspecific response, it is essential in antibabesial activity, since activation of nonspecific immune responses upon unrelated stimuli can provide resistance against babesiosis (11-15). The major factor that contributes to host innate immunity against *Babesia* parasites is macrophages, since macrophages phagocytize *Babesia* parasites and parasitized erythrocytes (5, 7, 16). Macrophage depletion with silica eliminates protection against *B. microti* (17). In addition, macrophage inhibition (18) or depletion (19) totally abolished the protection

of mice immunized against *B. rodhaini*, causing high mortality. This is also explained by the better protection of naïve mice against *B. microti* by the adoptive transfer of immune macrophages rather than that of immune T cells (20). Although the importance of NK cells, which also contribute to host innate responses, in the protection against *Babesia* parasites was also proposed (21-23), this is still controversial (24). Based on studies on malaria (25-27), NK cells as well as T cells may be activated by IL-12 and IL-18 produced by macrophages that sense the infecting parasites or parasitized erythrocytes and then, in turn, produce IFN-γ to activate macrophages with the help of macrophage-derived TNF-α. The effective killing of parasites is most likely accomplished by nitric oxide (NO) and reactive oxygen species (ROS) that are produced by activated macrophages (16, 28, 29). Infection of the inducible NO synthase (iNOS)-KO mice with *B. microti* resulted in high parasitemia in the early stage of infection compared to the normal mice (Igarashi et al., in press), indicating that macrophage-mediated innate mechanism is responsible for the resolution of *Babesia* infection in the early stages. Besides, TNF-α, which was described to play a role in the late stage of *Babesia* infection in the past (30), requires further work for the elucidation of its role in

the early stages. Simultaneously, such innate mechanism triggers the antigen presentation by macrophages and/or dendritic cells to T and B cells, leading to the initiation of acquired immunity against babesiosis.

2-2. Acquired immunity

Acquired immunity, also termed specific immunity, is elicited not only in the resolution stage of the primary infection but also in the early stage of the secondary infection. Acquired responses consist of cell-mediated immunity and humoral immunity, which are chiefly promoted by T and B cells, respectively.

Cell-mediated immunity: Involvement of cell-mediated responses was also first proposed by the importance of the host spleen in defense against babesiosis. The adoptive transfer of immune splenocytes, including not only macrophages but also lymphocytes, can protect mice from babesiosis (20, 31-33). Among the lymphocyte populations, T cells involved in cell-mediated immunity play a significant role in the effective protection in the resolution stage of *Babesia* infection, since infection of the nude mice, which congenitally lack thymes, with *B. microti* resulted in persistent, high

parasitemia, which reached 70% at around 10 days post-infection and thereafter remained at about 40% (34, 35). The adoptive transfer of immune thymocytes to immunodeficient mice (36) or of immune T cells to naïve mice (37) confers immunity to *B. microti*. In addition, an increase in the delayed-type hypersensitivity, which is an indicator of cell-mediated responses, particularly by $CD4^+$ T helper type 1 (Th1) cells, was observed in *B. microti* infection (38). Further, $CD4^+$ cell depletion made the naïve or recovered mice more susceptible to *B. microti* infection than $CD8^+$ cell depletion as well as the normal condition (30, 35). The study by Shimada et al. (39) demonstrated that the depletion of $CD4^+$ cells at 1 week after infection led to the complete elimination of the parasites, similarly to the case of control mice. They also showed an increased rate of $CD4^+$cells/$CD8^+$cells in the early infection. The adoptive transfer of splenocytes, which was obtained from the $CD8^+$-cell-depleted or normal mice that had recovered from the primary infection, to naïve mice provided protective immunity against the infection, whereas the transfer of $CD4^+$- cell-depleted splenocytes did not (30). In the intraerythrocytic stage, $CD8^+$ T cells, termed cytotoxic T cells, may not be involved in the clearance of parasitized erythrocytes, since erythrocytes are not capable of antigen

presentation due to lack of the major histocompatibility complex (MHC) class I molecules. Therefore, $CD4^+$ T cells are responsible for the resolution of the primary infection and prevention of the subsequent infection.

The $CD4^+$ cells are known to function as helper T cells, which consist of the T helper type 1 (Th1) subset, which secretes IL-2 and IFN-γ and activates macrophages, and the T helper type 2 (Th2) subset, which elicits the proliferation of B cells by secretion of IL-4, IL-5, and IL-6 (40). Specific IFN-γ production by Th1 cells generally occurs in the resolution phase of *B. microti* infection. Igarashi et al. (30) demonstrated a prolonged infection with persistent parasitemia of 15-25% for more than 3 weeks in the IFN-γ KO mice infected with *B. microti*. Challenge infection of the drug-cured IFN-γ KO mice with *B. microti* also resulted in high parasitemia, whereas the recovered control BALB/c mice showed strong protection against challenge infection. When the spleen cells from the infected normal and $CD4^+$- or $CD8^+$-cell-depleted mice were stimulated with a babesial antigen *in vitro*, an increased level of IFN-γ was detected in the normal and $CD8^+$-cell-depleted spleens at 4 days post-infection, but not in the $CD4^+$-cell-depleted spleen. Furthermore, depletion of IFN-γ by anti-IFN-γ mAb after

infection resulted in an increase in parasitemia. In addition, IL-4 was secreted in neither the normal nor mAb-pretreated mice. The IL-4-depleted mice did not develop parasitemia. Correspondingly, Chen et al. (41) showed a protective role of the macrophages activated by the T-cell-derived IFN-γ in *B. microti* infection in the early stage of infection. Therefore, IFN-γ released by $CD4^+$ Th1 cells is of significant importance in the clearance of parasites in both the resolution stage and early stage of infection, probably with regard to macrophage activation, as described above, which may be needed for the persistent activity of the innate mechanism.

Humoral immunity: Although humoral immunity mediated by B cells and antibodies has also been reported as essential to the protection of mice and hamsters against *B. microti* (9, 36, 42), humoral responses are considered less important than T-cell-mediated responses. The severe combined immune deficiency (SCID) mice that lack both the T and B cells showed a prolonged infection with a persistently high parasitemia to the same extent as nude mice, when infected with *B. microti* (34, 35). Cavacini et al. (43) demonstrated that the B-cell-depleted mice successfully controlled the primary infection with *B. microti*. Likewise, suppression of antibody production by irradiation

did not affect the protection of mice immune to *B. rodhaini* (33). However, indeed, serum obtained from mice in the recovery phase of infection with *B. microti* could inhibit the growth of the parasites *in vitro*, suggesting that antibabesial antibodies were elicited in the resolution stage (41). Antibodies in immune serum have been demonstrated to neutralize babesial sporozoites and merozoites at the extracellular stage (44). Considering that *Babesia* species are the intracellular parasites, the protective role for antibody-mediated immunity in babesiosis seems to be restricted to the extracellular parasites in the blood stream.

3. Immunopathology in the *Babesia* infection

The pathogenesis of babesiosis is mainly anemia due to the rupture of infected erythrocytes. In addition, other minor, but severe, pathologies also exacerbate symptoms facilitating host death, especially in the lethal babesiosis caused by *B. rodhaini* and *B. bovis*.

3-1. Cerebral babesiosis

Despite the low parasitemia observed in the peripheral blood, sequestration of the parasitized erythrocytes in the capillary bed of brain results in cerebral babesiosis in cows. Such a blockage of the cerebrovascular microcirculation is similarly observed in cerebral malaria, which is caused by the human malarial parasite *Plasmodium farciparum*. Humans suffering from cerebral malaria, a disease that causes around 1 million deaths per year, show paralysis and coma. Therefore, there seems to be a similar mechanism(s) by which cerebral babesiosis and cerebral malaria occur (45). Interestingly, NO has been implicated in an exacerbation of cerebral malaria, despite its protective roles. The pathogenesis of cerebral malaria is clearly due to the rosette formation of the parasitized erythrocytes and intact erythrocytes and the sequestration of parasitized erythrocytes in the cerebral vasculature, where they may cause microvascular obstruction and/or local induction of proinflammatory cytokines (25, 46, 47). It has been suggested that iNOS-derived NO induced in the vascular endothelium by proinflammatory cytokines may traverse the blood-brain barrier and affect local neuronal function, by mimicking and exaggerating the physiological effects of endogenous nNOS-derived NO (46). On the other hand, the levels of NO in the

cerebrospinal fluid, which may be more reflective of the local cerebral production of NO and is less affected by diet and other confounders, increased in cerebral malaria (48). While constitutive nNOS-derived NO gives normal neurological functions, iNOS-derived NO may be toxic to the brain (49). This indicates that normal brain functions can become affected by the continuous production of large amounts of NO (iNOS origin) in an area that is usually exposed to low and intermittent levels of NO (nNOS origin) (50). Therefore, if this mechanism can be applied to cerebral babesiosis, the inhibition of the local induction of proinflammatory cytokines and iNOS-derived NO or RNI in the brain may reduce a severe pathology in babesiosis.

3-2. Renal failure

The lethal *Babesia*-infected animals generally show hematurea. This is pathologically confirmed due to tubulorrhexis and glomerular nephritis, but the causes are not completely elucidated yet. The possible involvement of the immune complex in glomerulonephropathy induced by *Babesia* infection as well as malaria has been identified (51). The mice infected with *B. rodhaini* underwent the acute course, showed

anemia and hematurea at 5 days post-infection, and died in 7 days. In the kidneys of infected mice, electron microscopy showed an increase in the mesangial cells and matrix as well as electron-dense deposits in the mesangial matrix and along the glomerular basement membrane, which contained electron-dense deposits of antibodies, complement and *Babesia* antigens, confirmed by immunohistochemistry, indicating that these pathological changes were triggered by the immune complex. In addition, tubular necrosis was sometimes observed. On the other hand, pathological changes in the kidney of non-lethal *B. microti* infection appeared to be less severe than *B. rodhaini* infection. Interestingly, to date, iNOS as well as the inflammatory cytokines has been implicated in such renal insufficiency, as well as in the brain dysfunction seen in falciparum, but not in vivax malaria (50). Therefore, further investigation of the mechanism responsible for renal dysfunction in babesiosis is also needed.

4. Current vaccine development against babesiosis in animals

An extensive effort has so far been directed to the development of an effective vaccine against malaria, but it has proved very difficult to design. Chemically produced or

recombinant antigens, which had been evaluated as a vaccine in experimental models in laboratories, were demonstrated to be not very effective in field examinations in Southeast Asia and Africa. Since then, the construction of novel strategies for vaccine development has been receiving attention. One of the factors involved in such difficulty is the antigenic variation of malarial parasites (52). In babesiosis, a vast majority of work done to develop vaccines has focused on two bovine parasites, *B. bovis* and *B. bigemina*. Live avirulent strains were obtained by rapid passage of the parasite in the splenectomized calves or infection with the parasites derived from infected cows or *in vitro* cultures followed by treatment with antibabesial chemotherapeutics (53-57). Further, soluble antigens called "exoantigens" obtained from *in vitro* cultures have also been developed (58, 59). However, the development of more effective and safer vaccines is needed because there are potential hazards, such as reversion to virulence and contamination with unwanted host proteins. To overcome this drawback, several attempts to develop potential subunit vaccines against bovine babesiosis are currently being made (60, 61). Antigens from the apical complex proteins, which are of special interest because of their putative role in host erythrocyte invasion, have been the focus,

since immune responses sufficient for protection could be expected. For instance, a high molecular antigen, 11C5 (70-200 kDa), and a cysteine-rich protein, 12D3 (38 kDa), which are not very immunodominant, successfully provided protection against the infection (60). SBP1 (77-80 kDa), located on the spherical body, and RAP-1 (60 kDa), located on the rhoptry of the parasite, elicited protective immunity (60, 62-65). However, MSA-1 (42 kDa) and MSA-2 (44 kDa), the major surface antigens of merozoites, triggered the proliferation of *B. bovis*-specific T cells without induction of protective immunity, which is due to their antigenic variations for evasion of host immunity (64). On the other hand, the RAP-1 antigen was also identified in *B. bigemina* (66), and immunization with *B. bigemina* RAP-1 protected cows from the challenge infection with an increased induction of IFN-γ (67-69). Actually, although RAP-1 is also polymorphic in both *Babesia* spp., the variants of homologous parasites share a highly conserved epitope (70-72). Therefore, the recombinant RAP-1 can be used to elicit sufficient immune responses against the homologous *Babesia* parasites. Whereas the RAP-1 antigen is highly immunogenic for T and B cells, the presence of the anti-RAP-1 antibody is irrelevant regarding the degree of protection (73-75), supporting the

significant importance of T-cell- and macrophage-mediated mechanisms for the host defense against *Babesia* parasites, as described above. Overall, further elucidation of the host immune mechanism and immune-mediated pathology is required to identify or select the appropriate antigens for the development of subunit vaccines against *Babesia* infection.

5. Conclusion

Knowledge of the host response, the resistance process, and the mediators committed against *Babesia* infection is essential to progress toward a better means of prophylaxis and eradication. In the mouse model, macrophages, IFN-γ, and its producer, $CD4^+$ T cells, are considered pivotal in resolving the primary infection and preventing the subsequent infection. Both innate and acquired immunity, especially, a T-cell-mediated response rather than a B-cell-mediated one, contribute to defense against *Babesia*. However, based on the studies of malaria, the robust production of nitric oxide by activated macrophages may induce severe pathology, such as cerebral babesiosis and renal disfunction. This review paper discusses the mechanisms of antibabesial immunity and immune-mediated pathology and introduces the current advances in the development of vaccines against babesiosis, principally in cows.

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Chapter 3. Recent advances in the chemotherapy of babesiosis by Asian scientists

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1. Introduction

Babesiosis is a tick-borne disease caused by intraerythocytic parasites of the genus *Babesia* in the phylum Apicomplexa (1). In 1888, the first vast outbreak of "redwater" occurred in Romania, presumably caused by *Babesia bigemina*, leading to the death of more than 40,000 cattle (2). Since then, babesiosis has attracted increasing attention, not only in the livestock industry but also as an emerging zoonosis in humans (3).

Some drugs available for treating animal babesiosis, including diminazene diaceturate (Berenil® and Ganaseg®), amicarbalide diisethionate (Diampron®), oxytetracycline, phenamidine, and imidocarb dipropionate (Imidocarb® and Imizol®), have been proved to be highly effective (4-7). In the case of human babesiosis, drug combinations of either clindamycin and quinine or atovaquone and azithromycin have been considered as a standard treatment regimen (8,9). Nevertheless, many compounds have proved to exert severe side effects as well as toxicity.

Drug resistance in *Babesia* parasites has not yet become a major menace to the treatment measures (10). However, the experimental production of imidocarb-resistant parasites and the existence of amicarbalide isethionate-resistance have been reported in *B. bigemina* and *B. bovis*, respectively (11,12). In this regard, the search for new drugs

and new drug strategies is becoming increasingly more important, in particular, the design of compounds that specifically target the parasite's life cycle without any side effects in the host.

This review provides an overview of some of the present approved and clinically used drugs against domestic animal and human babesiosis, as well as insights into several current topics on basic drug research on the Asian continent for the past years.

2. Clindamycin

The semi-synthetic antibiotic clindamycin in combination with quinine was first applied in 1982 in a newborn infant suffering from babesiosis (13). Subsequently, this combination has become the treatment of choice for human babesiosis (14,15). Despite its high efficacy in clearing infections, untoward reactions, such as vertigo, tinnitus, and gastrointestinal symptoms, as well as treatment failures in immunocompromised patients, have been reported (16).

Recently, a research group reported the usefulness of clindamycin alone and in combination with tetracycline as a treatment measure against the rodent *Babesia rodhaini* (17,18). It was found that double therapies consisting of two treatments of

100-mg clindamycin and 100-mg clindamycin or 100-mg clindamycin and 100-mg tetracycline and a single therapy of 200-mg clindamycin or 100-mg tetracycline given orally completely cured the parasitemia of the mice. Concentrations of 50 mg and 100 mg of clindamycin were able to suppress the parasitic growth and prolong the life span of the infected mice for one day, although they could not clear the parasites from the blood (17). Furthermore, a significant increase in the weight of the spleen, the number of spleen mononuclear cells, and their phagozytic activity in the early stage of clindamycin-treatment in BALB/c mice inoculated with *B. rodhaini*, as well as a subsequent decrease in the final stage, have been observed. These findings point to the effectiveness of clindamycin therapy for murine babesiosis (18). Similar results have been obtained in dogs (Beagles) infected with *Babesia gibsoni*, where oral treatments with 25 mg/kg of clindamycin per kilogram of body weight for a period of two weeks gradually reduced parasitemia (19).

In both studies, the treatments with clindamycin induced morphological changes in the parasites, indicating their degeneration (17,19). Additionally, in dogs treated with clindamycin, the number of $CD4^+$ cells, which are involved in antibody production, significantly increased, especially after treatment, and a rapid humoral antibody

response occurred (20). Although clindamycin could not completely clear the parasitemia, it efficiently stimulated both humoral and cellular immunity and ameliorated the clinical conditions of the infected dogs (20). Clindamycin is suggested to act by inhibiting peptide bond formation through binding to the 50S ribosomal units of susceptible microorganisms (21), but its mode of action in *Babesia* parasites remains unknown.

3. Azithromycin and quinine

In human babesiosis, the combination of atovaquone and azithromycin was found to show similar efficacies to the clindamycin/quinine combination with the advantage of fewer adverse drug reactions. The macrolide antibiotic azithromycin (9-deoxo-9a-methyl-9a-aza-homoerythromycin A), an azalide analog of erythromycin, is formed by a chemical modification (*i.e.*, the inclusion of a methyl-substituted nitrogen at position C9) (22), which renders the compound several fold more active against parasites than erythromycin (23). Macrolide antibiotics are known to inhibit protein synthesis while reversibly binding to the 50S subunit and inhibiting the transpeptidation/translocation process, causing premature detachment of incomplete peptide chains and subsequent

cell death (24). The efficacy of azithromycin has been proven against various protozoal infections (25,26). Recently, a Chinese research team published a case report of a Taiwanese woman infected with *B. microti*, who was initially treated with the standard drugs clindamycin and quinine (9). In order to eradicate the parasite, a subsequent treatment measure consisting of a combination of 500 mg of azithromycin twice per day and 650 mg of quinine three times a day followed for a period of 10 days. Subsequently, blood smears and hamster inoculation of the patient's blood proved the complete clearance of the parasite. Although the efficacy of azithromycin in combination with quinine to treat human *Babesia* infections remains to be defined, this study demonstrated the ability of this drug combination to eliminate a persistent *Babesia* infection irretrievably from the patient's blood following antibiotic treatment.

4. Curdlan sulfate (CRDS)

Curdlan sulfate (CRDS) is a sulfated polysaccharide with 1-3b-d-glucan as its main chain that inhibits the T-cell tropic strains of the human immunodeficiency virus (HIV) (27,28). Analysis of the effect of CRDS on binding of HIV-1-neutralizing antibodies to gp 120 demonstrated that both the continuous epitopes on the V3 loop and the

discontinuous CD4 binding site of gp 120 represent targets for CDRS, suggesting that CRDS interferes with the membrane fusion process during HIV-1 infection (29). In HIV, the 50% inhibitory concentration (IC_{50}) values *in vitro* range from 1,000 to 5,000 μg/ml (30,31). Curdlan sulfate inhibited the growth of *P. falciparum in vitro* (32), although the mode of inhibition is still unknown. In *B. bigemina*, CRDS irreversibly inhibited the growth *in vitro* at concentrations of 50 and 100 μg/ml (33). Moreover, 20 mg/ml of CRDS was injected intravascularly twice a day into splenectomized and infected SCID mice, leading to a sharp decrease of parasitemia after a four-time drug treatment and to the complete clearance of the parasites by day 11 post-inoculation. The authors speculated that CRDS might affect the invasion of the *Babesia* merozoite into erythrocytes by blocking the merozoite attachment to specific receptors, such as sialic acid on the erythrocytes, and subsequently interrupt the growth cycle of the parasite. Alternatively, it is possible that CRDS directly or indirectly affects the growth and multiplication of the intraerythrocytic parasites. Interestingly, in contrast to *B. bigemina*, the growth of *P. falciparum* was incomplete, even with much higher doses of CRDS (32). This suggests that the mode of action of CRDS is different within parasite groups, presumably indicating that CRDS acts on several targets. Although the

inhibitory effect of CRDS on the growth of *Babesia* parasites has been clearly proved, the need to determine the mechanism of action of the CRDS on *Babesia* parasites still remains, so that its effectiveness can be fully developed.

5. Artesunate, pyrimethamine, and pamaquine

Among the few alternative drugs for the treatment of malaria, artemisinin derivatives have been promising as special candidates for the fight against multiple drug-resistant parasites in humans (34). This group, including artesunate, arteether, and artemether, is derived from the Chinese herb *Artemisia annua* (qinghao) (35) and has been proved to reduce the malarial parasitemia more rapidly than other known anti-malarial drugs (36).

A combination of the 2,4-pyrimidinediamine (pyrimethamine) and the *p*-aminobenzoic acid analogue sulfadoxine was also found to act synergistically in the treatment of human malaria caused by chloroquine-resistant *P. falciparum* (37).

Pamaquine, an 8-aminoquinoline, is the first synthetic anti-malarial drug (38) and, subsequently, has also been evaluated as an anti-theilerial and anti-coccidial agent (39,40).

Artemisinin, pyrimethamine, and pamaquine significantly and irretrievably inhibited the *in vitro* growths of *B. equi* and *B. caballi*, although artesunate was unable to exert a destructive effect on *B. caballi* (41). Interestingly, in donkeys experimentally infected with *B. equi* and subsequently treated with either 2.5 mg/kg of artesunate intramuscularly (i.m.) or the combination of 5 mg/kg of arteether (i.m.) and 5 mg/kg of the antitheilerial agent buparvaquone intravenously, parasite multiplication was restricted and cleared, respectively, but only for the duration of the treatment period (42). In addition, the same authors reported that, 55 to 58 days post-treatment, the recrudescence of the parasites was observed, finally leading to the death of the experimental animals.

The inhibitory mechanisms of artemisinin are not fully understood. A previous study (36) proposed a mechanism in which several malaria proteins specifically undergo selective alkylation in the presence of artemisinin (43). Recent studies reported that artemisinin decreases the deformability of infected erythrocytes, leading to a disruption of the infected erythrocytes by the host immune system (44). On the other hand, pyrimethamine inhibits the parasitic dihyrofolate reductase, necessary for the folate metabolism, therefore, interrupting the parasitic life cycle (45). Additionally, pamaquine is assumed to act on receptor recycling

due to interference with the protozoan calmodulin function at the endosome (46,47). Therefore, the action modes of the three compounds in *Babesia* parasites are highly interesting and might provide effective information that could be used to synthesize the parasite-specific drugs.

6. Triclosan

The synthetic 2-hydroxydiphenyl ether triclosan has been used for more than 30 years as a widely accepted anti-bacterial and anti-fungal agent (48) in health care products, such as soaps, toothpastes, and mouthwashes, and it is commonly used in household fabrics and plastics (49-51). Due to the difficulty of creating triclosan-resistant microbes (52), this compound is very promising for its effectiveness and safety when applied.

Previously, the growth-inhibitory efficacy of triclosan had been demonstrated in *P. falciparum* and *T. gondii in vitro* and rodent *P. berghei in vivo* (52,53). Triclosan is considered to target the parasitic *trans*-2-enoyl-acyl-carrier-protein-(ACP)-reductase (also known as inhA or FabI), which is an important enzyme in the final, regulatory step of parasitic type II fatty acid synthesis (55). Concentrations of 50 to 200 μg/ml of

triclosan were also found to completely inhibit the *in vitro* growth of equine *B. caballi* and *B. equi* and bovine *B. bovis* and *B. bigemina*, representing 50% inhibitory concentrations (IC_{50}s) of 4.8 to 60 μg/ml (56). Moreover, the treated parasites lost their typical shapes, became pycnotic, and showed the occurrence of large vacuoles in their cytoplasm (56). However, the mode of action of triclosan in *Babesia* parasites remains unknown. Pharmacokinetically, triclosan has no acute oral toxicity and is rapidly absorbed, metabolized in the liver, and excreted by the kidneys without accumulating over time (57, 58). In conclusion, drugs based on the biochemical triclosan structure are promising for their potency and safety in *in vivo* applications.

7. Clotrimazole, ketoconazole, and clodinafop-propargyl

The imidazole derivatives and anti-fungal agents, clotrimazole (CLT) (1-[(2-chlorophenyl) diphenylmethyl]-1*H*-imidazole) and ketoconazole (KC) (cis-1-acetyl-4-[4-[[2- (2,4-dichlorophenyl) -2- (1*H*-imidazole-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl] piperazine), had been reported to inhibit the *in vitro* growth of *P. falciparum* (30-32) and the *in vivo* replication of *P. berghei* and *T. cruzi* (62). On the other hand, the herbicide clodinafop-propargyl (CP) ((2*R*)-2-[4-[(5-Chloro-3-fluoro-2-

pyridinyl) oxy]phenoxy] propanoic acid-2-propynyl ester) is known to successfully inhibit the growth of *T. gondii* (63).

The potent inhibitory efficacies of these compounds were evaluated against the *in vitro* growths of *B. equi*, *B. caballi*, *B. bovis*, and *B. bigemina* (64,65). CLT was found to be effective in a dose range of 15 to 60 μM (IC_{50}: 2 to 23.5 μM), followed by KC (50 to 100 μM; IC_{50}: 6 to 50 μM) and CP (500 to 750 μM; IC_{50}: 265 and 450 μM). In transmission electron microscopy, extensive damage was observed in the cytoplasm of drug-treated parasites. In bovine parasites, the combinations of CLT/KC, CLT/CP, and CLT/KC/CP acted synergistically, while the combination of KC/CP was exclusively effective in *B. bovis*, but not in *B. bigemina*. In sharp contrast, in both equine parasites, the combination of KC/CP alone exerted significantly enhanced growth-inhibitory (Bork *et al.*, in preparation).

Although the anti-malarial effects of imidazole compounds have been studied since the early 1980s (66), their modes of action have not been fully understood. In fungi, KC inhibits sterol-14-a-dimethylase, a microsomal cytochrome P450-dependent enzyme system (67) that is also detectable in *P. yoelii* (68) and *P. berghei* (69). Moreover, in *P. falciparum*, CLT binds to the erythrocytic heme to disturb the parasitic

hemoglobin catabolism (70). Herbicides such as CP are known to be toxic for apicomplexan parasites due to their inhibition of the fatty acid and isoprenyl pyrophosphate biosynthesis (71) by targeting the essential enzyme, acetyl-CoA-carboxylase (ACC) (63). Because these compounds successfully inhibited the growth of *Babesia* parasites, the clarification of their action modes is strongly required.

8. Heparin

More than 65 years ago, in 1938, heparin was discovered (72). Due to its anticoagulative activity (73), it has been applied as an essential drug for the diagnosis and treatment of heart and blood vessel diseases (74). Heparin is a mixture of sulfated polysaccharides, in which a limited number of monosaccharide units give rise to several different sets of complex sequences by variable substitution with *O*-sulfate, *N*-sulfate, and *N*-acetyl groups (75). Heparin is a biosynthetically derived component of mast cells and basophils (76), and its molecular weight (MW) ranges between 3,000 and 37,500 Da with an average MW of 13,000 Da (77). Besides its application as an anticoagulant, heparin has been reported to be effective for the treatment of human cerebral malaria (78,79). The inhibitory capacity of heparin was also proved *in vitro*

studies with *P. falciparum* (80,81) and *T. sergenti* (82) and in experimental infection of *Rhesus* monkeys with *P. knowlesi* (83). However, the precise inhibitory mechanisms against these hemoprotozoa have not been fully understood.

The inhibitory effects of heparin against the growths of *B. bovis, B. bigemina, B. equi,* and *B. caballi in vitro* and of the rodent *B. microti in vivo* were examined (84). It was found that heparin significantly inhibited the growth of these parasites in a dose- and time-related manner. The IC_{50} values of heparin ranged from 30 to 410 μg/ml in the *in vitro* experiments, while, *in vivo*, it was calculated as 81 μg per 20 g body weight. Additionally, free merozoites exclusively reacted with a fluorescein isothiocyanate-labeled heparin on their surfaces, indicating that heparin-binding molecules are present on the surface of extraerythrocytic parasites. Because the merozoite invasion was completely inhibited by heparin, these authors concluded that the interaction between the heparin-binding molecule(s) and exogenous heparin must play a critical role in the disruption of the invasion process. Further investigation on the growth inhibition by heparin will be helpful for the design of new drugs against babesiosis.

9. Concluding remarks

In addition to the already established drugs, such as clindamycin, azithromycin, and quinine, several new compounds, which are known to act on the essential targets of different protozoan parasites, have been tested in these topics. Although the operation modes of these drugs are unknown, the results are meaningful for the development of babesial drugs.

In general, three different strategies are summarized to identify new drugs. The first strategy is designed to target directly the essential enzymes in the parasitic pathways, while the second strategy is based on the indirect intervention of the drug with the parasite's metabolism. Finally, the third strategy consists of the disturbance of the parasite's invasion into host erythrocytes, therefore, disrupting the asexual replication. With regard to the first strategy, triclosan, CP, KC, and pyrimethamine target essential enzymes in the parasitic fatty acid synthesis or in the parasitic microsomal cytochrome P450-dependent system. Concerning the second strategy, artesunate and CLT act indirectly, while disturbing the heme catabolism of the parasite. In contrast, following the latter strategy, heparin hampers the erythrocyte invasion by covering the surface of extracellular merozoites with heparin, therefore, interrupting the parasite's life cycle. Effective drugs for the treatment of various infectious diseases commonly work by

targeting the specific pathway or activity of the pathogens. Examples of the efficacy/reliability are many antibiotics that can inhibit protein synthesis only in bacteria but not in mammals due to the difference in the bacterial and mammalian pathways. In conclusion, the goal of sustained drug research should be based on the finding and exploitation of the differences between the parasites and their hosts in view of their lifecycle and metabolism. To prevent side effects and possible drug-resistance phenomena, it will be necessary to evaluate parasite-specific targets and, subsequently, to develop target-directed compounds.

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先端研究最前線

基礎研究に基づく検疫体制の強化

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病原性大腸菌O157、口蹄疫、BSEなどの発生により、今までになく国民の大きな関心が食物の安全性に向けられている。食物の自給率が約四〇%と低く、また家畜の飼料もその多くを外国からの輸入に依存しているわが国では、食物の安全性確保のため、輸入される食物、生産方法、衛星管理、人畜共通感染症の発生などに関する情報を世界的規模で収集することが重要である。また、病原微生物をテロの道具として用いるバイオテロリズムの危険性は、湾岸戦争、米国での炭疽事件で現実のものとなってきた。病原微生物は発見や予防対策が非常に困難であり、人はもちろん家畜、植物にも被害を及ぼし、社会、経済活動に対する影響は甚大なものとなる。すでにアメリカではバイオテロリズム対策を国家の安全保障に及ぼす重要な政策として位置づけている。今後さらに自由貿易が促進され、これまで以上に諸外国からの食料輸入が増加することが予想されることから、新興感染症、食物の安全性、食物の安全管理とバイオテロリズムに対する対策が必要であり、技術的能力を高め、より良い制圧・診断法の開発が必要となる。そしてこれらの食の安全に関する科学データに基づいた疾病制圧政策や新しい基準を設定することが重要である。さらに、食の安全を脅かす感染症について外国の研究機関、研究者との情報交換、診断・治療方法の開発など国際的な協力・基準の確立も重要な検討課題である。昨年十月、原虫病研究センターを中核施設として申請した、「動物性蛋白質資源の生産向上と食の安全確保―特に原虫病研究を中心として―」が「21世紀COEプログラム」生命科学部門のプログラムとして選定された。国際化次代における食の安全性の確保、特に海外からの原虫病の侵入阻止に関する研究は本研究センターの大きな柱となっており、本稿では、監視対策の一例としてウマバベシア症の診断を例にわれわれの取り組みについて紹介する。

ウマバベシア病は赤血球内に寄生する二種類のバベシア原虫*Babesia equi*と*B.caballi*が病因で、馬に発熱、貧血、黄疸などの症状を引き起こし、死亡率が一〇%～四〇%に達する家畜法定伝染病である。流行地域は南ヨーロッパ、アジア、中近東、アフリカ、中南米など全世界に及び、わずかに北欧、北米、豪州、日本が流行を免れている。本病はダニによって伝播されるが、日本国内でもこれらの病原体を媒介可能なダニが存在する。わが国では、国際的要請によって軽種馬の輸出入の自由化や人馬の文化的国際交流がますます盛んになっており、年間二、四〇〇～三、二〇〇頭の馬が輸入されている。現在、日本を含む世界各国においてウマバベシア病の公式検査法として補体結合反応が採用されている。しかし、本法は感度が低く、アメリカではこの方法で検疫を通過した馬からバベシア原虫が発見された。したがって、現在の検疫体制ではバベシア病フリーの国に本病が侵入する可能性が危惧され、補体結合反応に替わる感度と特異性の高い診断法の開発が早急に必要であり、その実用化による検疫体制の強化が世界各

国から切望されている。

本病は法定家畜伝染病に指定されているため、P3研究棟以外での馬感染実験は不可能である。したがって、原虫抗原の確保や精製が困難であり、新しい診断法の開発が世界的に遅れている。本学原虫病研究センターは、わが国で初めて*B.equi*及び*B.caballi*の連続培養法の確立に成功し、常時研究材料や血清診断法の抗原の確保を容易にした。本病の診断は、顕微鏡を用いて原虫を直接検出することが一番確実な方法である。しかし、感染初期や慢性感染の場合、絶対的な原虫数が少ないため、原虫の検出は極めて困難である。われわれは二種類のバベシア原虫を一種類の培養液で増殖させる方法など培養法の改良を重ねて、診断に応用する可能性について種々検討した。その結果をモンゴル国において、実証な診断への応用を試みた。抗体が陽性で血液塗抹標本では原虫が認められない材料を用いて二週間培養法で原虫の検出を試みたところ、高い割合で原虫が認められ、確定診断の方法として試験管内培養法が応用可能であることが判明した。

また、バベシア原虫のゲノムプロジェクトも開始されている。ゲノム解読により、原虫の増殖、病原性、免疫に関与する遺伝子の検索が可能になり、①原虫を殺滅する薬剤、病気の発症を抑制する薬剤、原虫の免疫回避を無効にする薬剤などの新しい治療薬の開発、②感度と特異性の高い診断法や原虫病を予防できるワクチンの開発等に発展する成果が期待されている。現在、遺伝子増幅法（PCR）による遺伝子診断法が検討され、原虫感染の初期診断に有効であることが明らかにされつつある。

ウマバベシア病の確定診断には、血液塗抹標本や培養法による原虫の検出や遺伝子診断が理想的である。しかし、原虫の形態や培養に熟練した技術者、培養設備や研究者がいる特定の研究機関や大学においてのみ培養法による確定診断は実施可能である。そこで、間接的に原虫感染を証明するために、抗体を検出する血清診断法が用いられているが、上記のように感度や特異性に問題があり、感度と特異性の高い診断法の開発が緊急課題となっている。われわれはモノクローナル抗体の作製およびDNAライブラリーの作製等による血清診断法に有効な原虫抗原の遺伝子の検索を進めている。すでに、大腸菌並びにバキュロウィルス－昆虫細胞系で組換え原虫タンパク質を作製した。これを抗原として用いて、これまでの血清診断法と同等かそれ以上の感度および精度を有するELISA法が開発されている。この方法は多数のサンプルを一度に検査するのに適している。しかし、特別の検査機器および結果を得るまでに数時間を要する。したがって、一般の研究室や野外での使用には適さない。そこで、われわれは検索感度が優れ、かつ短時間で判定でき、特別の機器を必要としない簡易診断法、イムノクロマト法の開発に着手し、その実用化も間近である。この方法は、特別な試薬や機器も必要でなく、検索試料の保存も室温で良く、かつ五～十分で診断可能である。今後の実用化により、感度・精度の高い国際標準法として普及させることを計画中である。

以上のように、ウマバベシア病を例にして、検疫体制の強化のため、基礎研究に基づく応用診断技術の開発について述べた。われわれはさらにこれらの方法を世界各国の研究機関や大学との連携による国際的比較試験を実施し、国際獣疫事務局（OIE）により世界的な認証を受けることを目指している。また、研究者の人材育成や研究者の国際的ネットワークの形成により地球規模での汚染状況の疫学調査研究を推進することも極めて重要であることを認識している。BSEやSARS等で経験しているごとく、感染症の発生の情報公開や対策が科学的見地よりも政治的かつ経済的側面が優先され、結果的に経済的あるいは社会的に大きな被害を受け、さらに国際的問題に発展したと理解している。したがって、本学原虫病研究センターは、あらかじめ国際標準診断法の採用や世界各国の大学や研究機関を中心とした国際的研究ネットワークを形成し、常時最新の技術・情報を交換することにより、利害関係に関わらない中立的学術拠点を維持することに努めている。そのことによって、各国の政府や産業界に対して適切な対処方法を提言し、人畜共通感染症等を基盤とした第二、第三のBSEやSARS騒動に対しても事前に対処することができ、人間および動物の健康福祉、そして食の安全と安心に貢献できるものと考えられる。