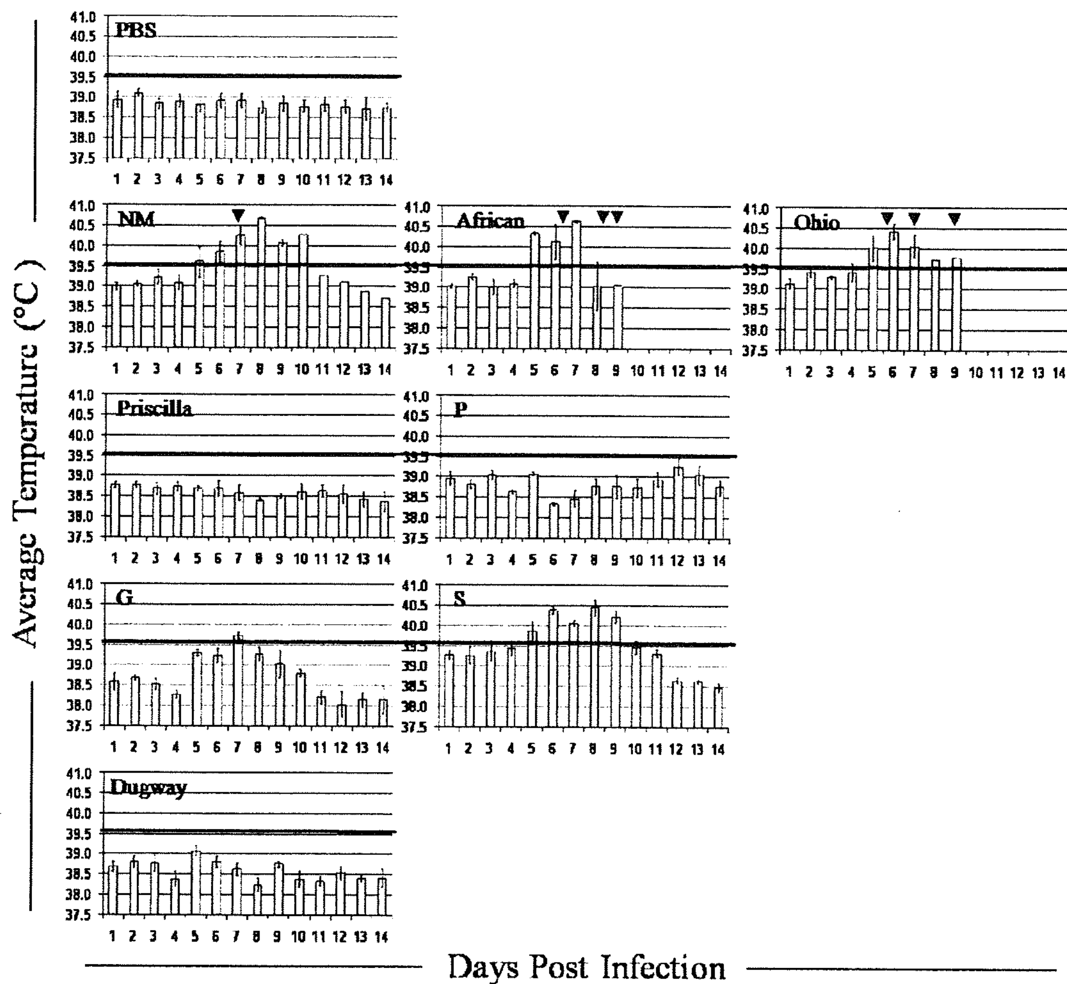

FIG. 4. Fever responses of guinea pigs to infection with high doses of *C. burnetii* isolates. The mean daily temperatures ± standard errors of the mean ($n = 3$) of animals infected with 2×10^6 bacteria of each *C. burnetii* isolate. Temperatures of ≥39.5°C (black lines) were considered fever. The arrows indicate days on which death occurred in NM-, African-, and Ohio-infected groups.

seen in guinea pigs infected with group I isolates. Subjectively, of all animals necropsied from each isolate group, hepatic granulomas from those infected with P were the greatest in size and number.

The livers of guinea pigs infected with the group V isolates G and S contained a few small granulomas and mild to moderate infiltration of lymphocytes along portal tracts. The hepatic changes observed in guinea pigs infected with group V isolates suggested that isolates from this group are less hepatovirulent than group IV isolates but more so than group I isolates.

No hepatic granulomas or other significant pathological changes were noted in guinea pigs infected with the group VI isolate Dugway. Liver weights did not vary significantly within or between genomic groups.

There were no significant differences in spleen weights at 28 days p.i. within or between genomic or dose groups. Animals infected with all isolates examined at 14 days p.i. (NM, P, G, and Dugway) had significantly larger spleens than PBS-injected control animals, and spleens from NM- and G-infected guinea pigs were significantly larger ($P < 0.01$ and $P < 0.05$, respectively) than those of P- and Dugway-infected animals (see Fig. S3 in the supplemental material). Pathological findings included multiple small granulomas in the spleens of group I-infected guinea pigs; fewer small granulomas were occasionally noted in animals infected with group IV and V isolates.

Heterologous protection of cross-vaccination and challenge in guinea pigs. The infection studies described here illustrate that there is pathotype diversity between *C. burnetii* isolates from different genogroups, and they are consistent with phylogenetic studies cataloging distinct gene contents (4). We therefore strove to determine whether this diversity was great enough to affect the ability of vaccines to protect against infection. Guinea pigs were given group I (NM) or group IV (S) vaccine and cross-challenged to evaluate potential heterologous protection against high-dose infection. Nonvaccinated guinea pigs developed a noticeable fever response by day 5 p.i.,

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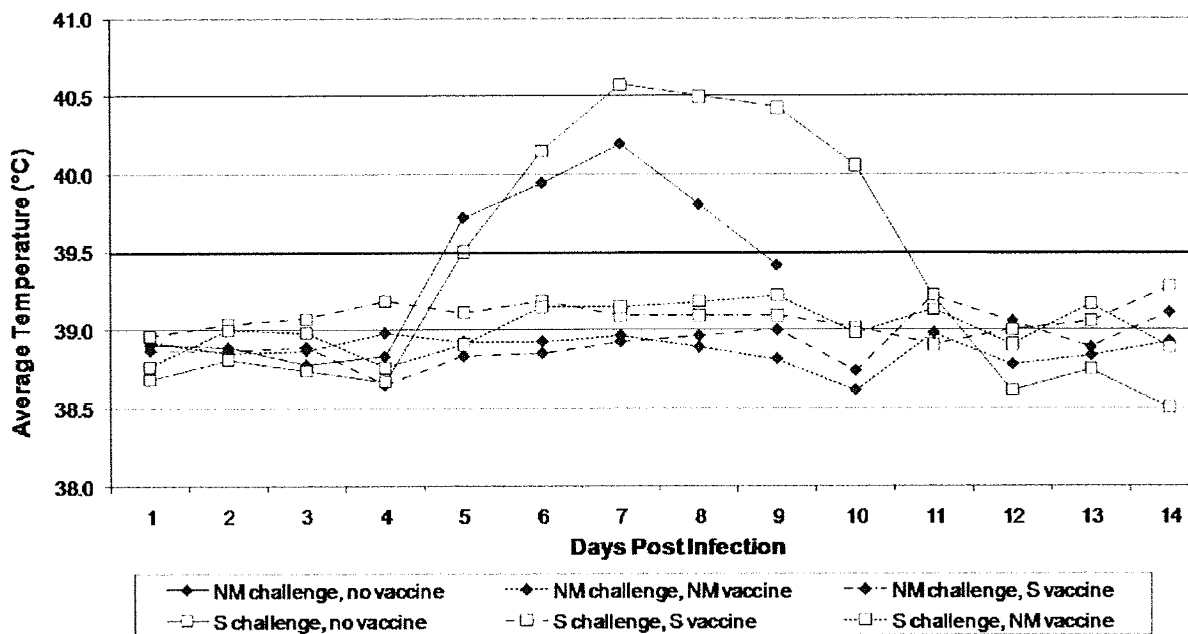

FIG. 5. Heterologous vaccination and challenge in guinea pigs. Shown are average daily temperatures of animals vaccinated with NM (dashed and dotted line), S (dashed line), or adjuvant alone (solid line) and challenged with high doses of NM (◆) or S (□). Temperatures of ≥39.5°C were considered fever.

and infection was lethal in three of three NM- and one of three S-challenged animals. Guinea pigs vaccinated with either formalin-killed NM or S were completely protected against fever development and death when challenged with either NM or S (Fig. 5).

DISCUSSION

The potential for genomic-group-specific pathogenicity of *C. burnetii* was evaluated using immunocompetent mice and guinea pigs and immunodeficient mice. The hypotheses that isolates belonging to the same genomic group would cause similar disease and that there would be distinctions in disease manifestations between isolate groups were supported by the findings presented here.

A detailed analysis of the Priscilla isolate dose-effect in SCID mice revealed differences in virulence of *C. burnetii* isolates. Disease development after Priscilla infection was progressive but slower than the development of the disease caused by NM previously reported in SCID mice (1); the survival time of SCID mice infected with Priscilla was longer with the same LD_{50}. This result supports the previous study by Moos and Hackstadt that evaluated the lesser ability of the Priscilla isolate to cause fever in i.p.-challenged guinea pigs (36). Interestingly, the mice infected with Priscilla did not exhibit cachexia until the terminal stages of infection, when they had extremely severe hepatosplenomegaly. Although the disease caused by Priscilla was milder than that associated with NM, all mice that developed clinical illness died. This result confirms the high infectivity and lethal potential of *C. burnetii*, which is not restricted to isolates that cause acute disease, and suggests that the SCID mouse model can be useful for evaluation of *C. burnetii* virulence.

The virulence of *C. burnetii* isolates tested in SCID mice was determined to be genomic group specific. Acute-Q fever-associated group I isolates caused the most rapidly progressing disease and the most severe pathological changes. Groups IV and V, isolates associated with chronic Q fever, caused a slower progression of disease. Overall, pathological changes in mice infected with group IV and V isolates were milder than those of group I-infected mice. The number of bacteria in the spleen at 28 days p.i. was greater in mice with severe disease from infection with group I isolates; however, the bacterial loads at the time of death were similar in all infected mice. This suggests that the rate of proliferation of *C. burnetii* in vivo may be virulence related. An in vitro comparison of infection in L929 cells using NM, Priscilla, and S isolates showed that all of the isolates could persistently infect, but Priscilla required a greater period of time to establish an infection (42), and it has been shown that inclusion-forming units produced by NM and Priscilla isolates were similar in Vero cells (36). However, because of developmental differences in clinical signs and pathological changes, the replication rate does not seem to be the only virulence factor involved, since clinical signs would then be similar with differences only in disease progression. At both time points, 28 days p.i. and the time of death due to infection, heart and lung lesions caused by group IV, V, and VI isolates were milder than those produced by infection with group I isolates. This observation seems to conflict with the hypothesis that isolates from chronic disease cause chronic Q fever, including heart disease. However, our observation is consistent with the report that isolates from heart lesions of

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chronic-Q fever patients have genetic characteristics similar to those of isolates from acute disease (46). The hypothesis that isolates from acute disease do not cause endocarditis has been supported by two other research groups (17, 24). The correlation between virulence and phylogeny has been controversial because of a lack of comprehensive studies. One study detected genes specific to isolates from acute disease in isolates from chronic Q fever patients and concluded that the isolates were not disease specific (46). The isolates used in the study were isolated by cell culture, and although the cell culture system is highly effective for isolation, isolates from acute disease are known to infect cultured cells more efficiently than isolates from chronic disease, so there remains a potential that the study collected only cell culture-adapted isolates. Several in vivo studies have reported isolate-specific virulence using guinea pig and mouse models (17, 24, 36); however, the number of isolates used in these studies was limited, making it difficult to conclude that there was genomic-group-specific virulence. The present study using eight isolates from four phylogenetic groups strongly supports the variation in virulence among *C. burnetii* isolate groups.

In the absence of functional T and B cells, cytokine profiles showed no group-specific differences. In immunocompetent mice, group I isolates caused a stronger immune response with high levels of multiple cytokines over a longer time than other groups. Interestingly, Dugway (group VI) induced the least change in CB-17 mice. The inflammatory-cytokine changes in immunocompetent mice in this study were similar to those in humans with acute Q fever (10): TNF-α and IL-6 were upregulated, but IL-1β was not. IFN-γ increased in CB-17 mice infected with group I isolates, and it is associated with the control of bacterial growth, stimulates phagosome-lysosome fusion, and may enable monocytes/macrophages to kill *C. burnetii* (13, 14). A difference in vacuole formation between isolates has also been shown, with NM and S developing within single large vacuoles while Priscilla occupied several smaller vacuoles per cell (18). This in vitro study suggested a difference in isolate ecology within host cells, which may be correlated with their virulence in vivo.

The ability to cause fever and respiratory illness was isolate and dose dependent in the guinea pig aerosol challenge model, with isolates from groups I and V causing disease consistent with human acute Q fever. Isolates within the same genomic group produced similar clinical illnesses, strongly supporting the mouse experiments demonstrating that genomic differences in the bacterial isolates do play a role in virulence. It was shown here that isolates associated with chronic disease, G and S, have the ability to cause acute disease in the guinea pig model. Our study confirmed and expanded the observations of Kazar et al. that the virulence of NM and S isolates was greater than that of Priscilla.

Lesny et al. compared the cross-immunity of whole-cell and soluble Q fever vaccines made from phase I NM, S, Priscilla, and Luga isolates. They found that vaccines from NM and Priscilla afforded a higher degree of protection than S and Luga vaccines and that whole-cell vaccines were more effective than soluble vaccines (28). In the guinea pig challenge study presented here, killed whole-cell vaccines made from isolates differing in LPS banding pattern (16), plasmid type (44), and genomic group (20), specifically isolates from groups I and V, conferred heterologous protection against virulent high-dose challenge in accordance with previous studies (28). This suggests that although the manifestations of disease and genomic contents differ among various isolate groups, the antigenic properties of whole-cell vaccines are shared enough that cross-protection is possible. Such information is valuable for the design of new vaccines and could be of the utmost importance in offering reliable protection in the event of an outbreak.

The differences in perceived infectious doses noted when ODs, particle counts, and genome copy enumerations were compared underline the importance of using multiple quantitation methods to compare studies with earlier observations. Some of the differences in disease manifestations seen in guinea pigs in this study could be due to slight differences in the infectious doses delivered. For instance, Priscilla and P both induced hepatic changes, although guinea pigs infected with P appeared to develop more severe lesions than those infected with Priscilla, which had a lower infectious dose by OD and qPCR. The difference in infectious dose as determined by the genome copy number could account for this variation. However, G and S both caused fever, and although guinea pigs infected with G did not attain the same degree of febrile response as S-infected animals, quantitation by particle count and real-time PCR showed infectious doses of S to be over a log unit lower than those of G. It could be argued that Priscilla-infected guinea pigs did not develop fever because fewer bacteria were present in the aerosol challenge; however, the group IV isolates did not induce fever at any of the challenge doses while group I isolates induced fever even at the lowest dose. We believe that, despite the variation in the infectious dose depending on the enumeration technique, the significant differences noted among genotypic groups are valid.

Phase variation is the only well-characterized phenotypic difference that is related to virulence in *C. burnetii* (50). Although LPS may be a major virulence determinant, and isolate LPS banding patterns have been correlated with acute or chronic disease (16), other components alone or in association with LPS may be responsible for differences in mortality in SCID mice and fever development in aerosol-challenged guinea pigs. It has been hypothesized that differences in the lipid A component are responsible for the variations in virulence, but lipid structural information indicates they are similar. The combination of a variety of factors expressed by phase I bacteria likely governs the ability of *C. burnetii* to infect cells and to maintain continuous growth within the phagolysosome. Indeed, the combination of pathotype variation of disease in infected guinea pigs and cross-protection of different isolates suggests conserved predominant antigenic components with virulence determinant specificity.

A recent report compared all open reading frames of NM phase I to those of African, Ohio, P, G, S, and Dugway, among others (4), and a majority of the open reading frames deleted from NM in the other isolates were either hypothetical or nonfunctional; however, a few were associated with assorted cellular functions. Beare et al. compared the complete genome sequences of NM, K, G, and Dugway and found distinct collections of pseudogenes and unique gene contents that may contribute to pathotype-specific virulence, including type II and type IV secreted effector molecules (5). Integrating our in vivo data with these molecular details, as well as with other in

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vitro studies, may reveal the critical virulence determinants of *C. burnetii* and ultimately identify targets for vaccine and therapeutic intervention.

Isolates of phase I *C. burnetii* have the potential to cause a range of clinical signs, including fever, pneumonia, hepatitis, and splenomegaly. Isolates from one human chronic-disease group induced mild to moderate acute disease in the physiologically relevant guinea pig aerosol challenge model, while a separate isolate group representing several chronic-disease isolates caused no acute disease. All isolates examined were capable of producing disease in the immunocompromised SCID mouse model, and genogroup-consistent trends were noted in cytokine production in response to infection in the immunocompetent-mouse model. In these studies, isolates within the same genomic group caused similar pathological responses, with a distinction in strain virulence between established genogroups, sustaining the theory that genetic differences in the bacterial isolates affect their virulence.

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Human leptospirosis cases and the prevalence of rats harbouring *Leptospira interrogans* in urban areas of Tokyo, Japan

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Thirteen patients with leptospirosis were identified, as confirmed by laboratory analysis during the last 5 years in our laboratory, who came from urban areas of Tokyo, Japan. All of the patients came into contact with rats before the onset of illness. Seventeen per cent of Norway rats captured in the inner cities of Tokyo carried leptospires in their kidneys. Most of these rat isolates were *Leptospira interrogans* serovar Copenhageni/Icterohaemorrhagiae. Antibodies against these serovars and their DNA were detected in the patients. This suggests that rats are important reservoirs of leptospirosis, and that rat-borne leptospires occur in urban areas of Tokyo.

INTRODUCTION

Leptospirosis is caused by infection with pathogenic *Leptospira*. It is a globally important zoonotic disease that affects humans in rural and urban settings, in both industrialized and developing countries (Bharti *et al.*, 2003; Levett, 2001; McBride *et al.*, 2005). Transmission of *Leptospira* pathogens to humans occurs mainly through indirect contact with water or soil contaminated by the urine of infected animals (Faine *et al.*, 1999). Leptospirosis has become an important public health problem in Asia and Latin America. In these tropical areas, large outbreaks of leptospirosis are most likely to occur after floods, hurricanes or other disasters. Leptospirosis has also become an urban problem in developing countries. Outbreaks occur in poor urban slum communities during seasonal periods of heavy rainfall (Johnson *et al.*, 2004; Ko *et al.*, 1999; LaRocque *et al.*, 2005). The risk of infection in urban inhabitants is not limited to developing countries because the importance of urban leptospirosis has already been recognized in inner-city populations of the USA (Vinetz *et al.*, 1996). In the present study, we report the presence of leptospirosis and rat reservoirs of leptospires in urban areas of Tokyo, Japan.

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Abbreviation: MAT, microscopic agglutination test.

The GenBank/EMBL/DDBJ accession numbers for the *flaB* sequences of rat isolates and patient samples are AB454100–AB454125.

A table of detection test data and a figure of PFGE results are available as supplementary data with the online version of this paper.

METHODS

Serodiagnosis of patients with clinically suspected leptospirosis. The microscopic agglutination test (MAT) for detection of anti-*Leptospira* antibodies in patient serum samples was performed (Faine *et al.*, 1999) using a battery of reference strains described previously (Koizumi *et al.*, 2008). These reference strains were cultivated in liquid modified Korthof's medium with 10 % rabbit serum at 30 °C (Faine *et al.*, 1999). Detection of IgM was also carried out by IgM dot enzyme-linked immunoassay (*Dip-S-Ticks*; PanBio) for cases 6 and 8 (Supplementary Table S1 available with the online journal).

Isolation of leptospires from rats. Norway rats (*Rattus norvegicus*) were captured using live traps at 14 locations in urban areas of Tokyo from 2002 to 2007. For the isolation of leptospires, rat kidneys were inoculated into medium and cultivated as described above.

PCR. DNA was extracted from *Leptospira* isolates, and the blood and urine samples of patients, using a DNeasy tissue kit (Qiagen). Extracted DNAs were subjected to PCR to detect the *Leptospira flaB* gene (*flaB*-PCR; Kawabata *et al.*, 2001; Koizumi *et al.*, 2003). Sequencing of amplicons was performed by the dideoxynucleotide chain-termination method using a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems).

Identification of serogroups of rat isolates. The serogroups of the isolates were identified by MAT using a panel of anti-*Leptospira* rabbit sera for serovars Australis, Autumnalis, Canicola, Copenhageni, Hebdomadis and Icterohaemorrhagiae, which are present in the main island of Japan.

PFGE. PFGE of rat isolates was carried out as described previously (Koizumi *et al.*, 2009).

RESULTS AND DISCUSSION

Human leptospirosis cases in urban areas of Tokyo

In the last 5 years (from the first case on 4 September 2003 to the last on 18 September 2008), we carried out laboratory examinations for leptospirosis for 55 cases. According to their physicians in Tokyo, the symptoms in these patients matched those of leptospirosis. A total of 16 cases were revealed to be positive for leptospirosis during the period of the study; 13 were from Tokyo (Table 1, Supplementary Table S1 available with the online journal) and the other 3 cases were from Bali (Indonesia), Borneo (Malaysia) and Fiji (data not shown). Among the 13 cases in Tokyo, 12 patients were definitively diagnosed by MAT (fourfold increase in antibody titre between acute and convalescent serum samples or reciprocal MAT titre of at least 400 in a single serum sample), including 4 patients who were also positive for the leptospiral *flaB* gene by PCR using urine or blood specimens. One probable case was demonstrated by anti-leptospiral IgM and a MAT titre of 1 : 160. All patients were hospitalized with severe manifestations, such as acute renal failure and jaundice, indicating that they had contracted Weil's disease (a severe type of leptospirosis). All patients declared that they had come into contact with rats (Table 1). Two patients (nos 1 and 2) worked at a place where they came in contact with rats, and rats were frequently found in the houses or stores of other patients (nos 3–13). The patients neither performed agricultural work nor undertook recreational activity in an endemic area, nor were they exposed to possible reservoir animals other than rats, which are generally considered as high risk behaviours. Among the 39 leptospirosis-negative cases, only 4 patients came in contact with rats (1 patient was a construction worker, and rats were seen at the houses of the other 3 patients). This indicated that the ratio of contact with rats among leptospirosis-positive cases was significantly higher than that among leptospirosis-negative cases in urban areas of Tokyo (Fisher's exact test, $P<0.01$). Since leptospirosis became a reportable disease in Japan (from November 2003), another laboratory-confirmed case other than the 13 cases mentioned above was reported from a regional medical centre in Tokyo in September 2006. The patient saw rats at his restaurant (T. Iida, personal communication).

Isolation and characterization of *Leptospira interrogans* from Norway rats captured in urban areas of Tokyo

We captured 127 Norway rats (*R. norvegicus*) at 14 locations in urban areas of Tokyo from 2002 to 2007. Leptospires were isolated from 22 of the 127 rats from 6 of

Table 1. Human leptospirosis in urban areas of Tokyo diagnosed in our laboratory from 2003 to 2008

Patient no.	Year	Sex	Age (years)	Occupation	Association with rats
1	2003	M	66	Construction worker	Probable environmental contamination with rat urine
2	2004	M	35	Sewer worker	Probable environmental contamination with rat urine
3	2005	F	53	Housewife	Rats appeared frequently in patient's house
4	2005	M	65	Butcher	Patient cleaned the urine and faeces of rats in his store without wearing gloves
5	2006	M	51	Fish dealer	Rats appeared frequently in patient's store
6	2006	M	62	Unknown	Rats appeared frequently in patient's house
7	2006	M	54	Fish dealer	Patient cleaned the urine and faeces of rats in his store without wearing gloves
8	2007	M	51	Day worker	Rats appeared frequently in patient's house (he had been bitten by a rat at his house)
9	2007	F	57	Restaurant worker	Rats appeared frequently in patient's restaurant
10	2008	M	57	Supermarket salesman	Patient was involved in killing rats captured at his store
11	2008	M	56	Fish market worker	Rats appeared frequently in the fish market
12	2008	M	58	Unknown	Patient had contact with rat urine in his house
13	2008	M	68	Restaurant chef	Patient had been bitten by a rat in his restaurant

F, Female; M, male.

the 14 places (Table 2). Nucleotide sequences of the partial *flaB* gene from 18 rats captured at locations F, G, H, K and M were identical (GenBank accession numbers AB454100–AB454117) and those from 4 rats at location A (the *flaB* sequences were identical among the four; GenBank accession numbers AB454118–AB454121) were different in six bases from those described above. The sequences from the 18 rats were identical to those of the reference strains of *L. interrogans* serovar Copenhageni and Icterohaemorrhagiae, suggesting that all the isolates were *L. interrogans*. These isolates reacted equally with anti-Copenhageni and anti-Icterohaemorrhagiae sera, but not with the other sera (data not shown). The *Not*I restriction patterns of the genomes of the isolates from the 18 rats were identical on PFGE not only to each other, but also to the reference strains of serovars Copenhageni and Icterohaemorrhagiae (Supplementary Fig. S1 available with the online journal). It has been determined by PFGE that the genomes of leptospiral serovars have been remarkably conserved over time and across a wide geographical distribution (Herrmann *et al.*, 1991, 1992). Most (but not all) serovars give unique patterns on PFGE carried out using the restriction enzyme *Not*I, although the *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae are indistinguishable. These two serovars are also very difficult to distinguish by serological methods (Kobayashi *et al.*, 1984). These results indicate that isolates from the 18 rats belonged to *L. interrogans* serovar Copenhageni or Icterohaemorrhagiae, which are known to frequently cause Weil's disease in Japan and other countries. We could not carry out MAT for serogroup identification and PFGE on the four isolates at location A due to poor growth.

Table 2. Isolation of leptospires from rats captured in urban areas of Tokyo

Location*	No. of rats captured	No. of rats from which *Leptospira* was isolated (%)
A – park	15	4 (27)
B – park	2	0
C – street	4	0
D – street	8	0
E – building	12	0
F – street	29	12 (41)
G – street	1	1 (100)
H – garden (house)	4	3 (75)
I – park	1	0
J – street	12	0
K – street	13	1 (8)
L – street	5	0
M – store	2	1 (50)
N – street	19	0
Total	127	22 (17)

*Location M was a store in which patient 7 used to work; other locations are not related to the putative exposure sites of the patients.

Conclusion

In 5 of the 13 human leptospirosis cases (cases 1–3, 6 and 7), the patients were infected with serovar Copenhageni or Icterohaemorrhagiae as shown by serological and PCR-based evidence (Supplementary Table S1 available with the online journal). Cross-agglutination and even paradoxical reactions are observed in MAT, but the existence of antibodies against serovars Copenhageni and Icterohaemorrhagiae in all other serum samples suggests the possibility of infection with these serovars (Supplementary Table S1 available with the online journal). Nucleotide sequences of the partial *flaB* gene from urine and blood (patients 1, 2, 3 and 7; GenBank accession numbers AB454122–AB454125) were identical to those from the rat isolates. In particular, *Leptospira* was isolated from a rat captured at the store where patient 7 previously worked. Although there is a possibility of recall bias, all the patients said they had rat contact (Table 1). Dogs and cats may also serve as reservoir hosts in urban settings. We attempted to isolate leptospires from the kidney tissues of stray or abandoned dogs and cats in Tokyo (304 dogs and 77 cats), but *Leptospira* was not obtained. These results strongly suggest that the patients contracted leptospirosis (Weil's disease) from rats in urban areas of Tokyo, though the possibility of involvement of other reservoir animals cannot be excluded.

Outbreaks of leptospirosis are recognized through occupational exposure, such as rice farming and other agricultural activities in rural areas of the tropics (Tangkanakul *et al.*, 2000). Leptospirosis has also become a health problem in urban slums in developing countries (Johnson *et al.*, 2004; Ko *et al.*, 1999; LaRocque *et al.*, 2005). In 'developed countries', recreational activities have recently been identified as a significant risk factor for leptospirosis (Bharti *et al.*, 2003; Levett, 2001; McBride *et al.*, 2005). The present study suggests that humans could contract leptospirosis through occupational exposure or exposure during activities of daily life in environments contaminated with rat urine containing leptospires in urban areas in Tokyo. Leptospirosis constitutes one of the neglected diseases in Japan except for Okinawa Prefecture (Nakamura *et al.*, 2006; Narita *et al.*, 2005). This is one of the reasons why few cases have been identified over the 5 year period despite the high carriage of leptospires in rats in Tokyo. A high prevalence of *Leptospira* spp. in Norway rats from urban settings has also been reported from temperate and tropical endemic countries, but human leptospirosis in urban areas is underreported even in an endemic country (Ariyapruchya *et al.*, 2003; Demers *et al.*, 1985; Doungchawee *et al.*, 2005; Easterbrook *et al.*, 2007; Krøjgaard *et al.*, 2009). Physicians and public health authorities should, therefore, be aware of the severe risk of contracting leptospirosis associated with rats in urban areas.

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COINFECTION OF *LEPTOSPIRA* SPP AND *TOXOPLASMA GONDII* AMONG STRAY DOGS IN BANGKOK, THAILAND

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Abstract. Leptospirosis and toxoplasmosis are zoonotic diseases with global importance. Asymptomatic animals harboring these pathogens may act as carriers to other animals including humans. The objective of this study was to investigate the seroprevalence of *Leptospira* and *Toxoplasma* infections in stray dogs in Bangkok. A total of 230 stray dogs from monasteries in a Bangkok district were examined for specific antibodies to *T. gondii* and *Leptospira*. The seroprevalence of *T. gondii* was determined by a modified latex agglutination test (cut off 1≥64). A microscopic agglutination test was performed to detect antibodies to *Leptospira* (cut off, 1:100). The seroprevalences of *T. gondii* and *Leptospira* were 10.9% (25/ 230) and 83.5% (192/230), respectively. *Leptospira* serovar *bataviae* was the most predominant (20.3%) serovar. Co-infection with *Leptospira* and *Toxoplasma* was found in 22 dogs (9.6%). The prevalence of *Toxoplasma* in females was significantly higher than in males ($p<0.05$), but no significant differences was observed for *Leptospira*. The high seroprevalence of these two diseases in dogs is of public health concern because close contact between dogs and humans may provide a link between a reservoir in the environment and susceptible humans.

INTRODUCTION

Leptospirosis and toxoplasmosis are important zoonoses affecting both animals and human beings. The incidence of human leptospirosis is higher in the tropics than in temperate regions but transmission occurs in both industrialized and developing countries (WHO, 2003). The prevalence of different leptospiral serovars in a human population depends on the reservoir animals, as well as local environmental conditions, occupation, agronomical, and agricultural practices. Southeast Asia is endemic for leptospirosis. It has been reported that dogs play an important role in the transmission of leptospirosis to humans in India (Venkataraman and Nedunchelliyan, 1992). In Thailand, stray dogs have become of public health concern since the numbers increase annually, especially in Bangkok metropolitan areas (Jittapalapong *et al*, 2003). This increases the risk for spreading of leptospirosis to humans since the infection results from

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exposure to urine of carrier mammals, either directly or via contaminated soil or water.

Toxoplasma gondii is an obligate, intracellular, protozoan parasite with a worldwide distribution. Infection with *T. gondii* is wide spread in many species of animals, including humans, and it is estimated to infect 30% of the human population worldwide (Aspinall *et al*, 2002). Although the infection usually does not cause a significant problem for healthy individuals, it can be life-threatening for congenitally-infected and immunosuppressed individuals (Chintana *et al*, 1998).

T. gondii infects human beings either by direct contact with infected animals, ingesting oocysts or by eating raw, undercooked meat or unpasteurised milk containing infective stages of the parasite (Riemann *et al*, 1975; Sacks *et al*, 1982). Dogs play a role in transmission of *T.gondii* to humans without having clinical signs of *Toxoplasma* infection similar to cats (Lindsay *et al*, 1997). Dogs are also capable of mechanically shedding oocysts, contaminating the environment (Dubay, 1996).

These facts indicate the importance to public health of investigating the prevalence of both *Leptospira* and *Toxoplasma* infections in stray dogs. The objective of this study was to determine the seroprevalence of *Leptospira* and *Toxoplasma* infections in stray dogs in Bangkok, Thailand.

MATERIALS AND METHODS

Study areas and animals

The dog samples were collected in a district located in the center of Bangkok. More than 20 Buddhist monasteries exist in this area and many stray dogs inhabit in this area. A total of 230 stray dogs were obtained randomly from monasteries in this district. One hundred fifteen male and 115 female dogs were collected. Dogs were examined for sex, general condition and status of ectoparasites. Blood samples were collected and the sera were separated by centrifugation at 500*g* for 30 minutes at the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University and stored at -20°C until analysis.

Detection of antibodies to *Leptospira* spp

Serum samples were tested for the infection due to leptospirosis using the following 21 serovars as antigens: *L. bratislava, L. autumnalis, L. ballum, L. bataviae, L. canicola, L. cellidoni, L. cynopteri, L. djasiman, L. grippotyphosa, L. hebdomadis, L. icterohaemorrhagiae, L. javanica, L. louisiana, L. manhao, L. pomona, L. ranarum, L. sarmin, L. sejroe, L. shermani, L. tarassovi,* and *L. patoc.* A microscopic agglutination test was performed as previously described (Cole *et al*, 1973). In brief, the dog sera were inactivated by heating at 56°C for 30 minutes and diluted by two fold dilution beginning with a 1:25 solution on a U-shaped micro plate. A volume of 50 μl of each dilution was made for each serum sample with an approximate concentration of 10^8*Leptospira* organisms per sample and incubated at 37°C for 2-4 hours. Only antigens and an equal volume of PBS were added to the plate as negative antigen controls. Rabbit serum positive for homologous strain antigens were used as a positive control. Fifty percent agglutination at a 1:100 dilution was considered to be positive (WHO, 2003).

Detection of antibodies to *Toxoplasma gondii*

Antibody to *T. gondii* was analyzed using a latex agglutination test kit (TOXOCHECK-MT 'Eiken' Chemical, Tokyo, Japan). Positive and negative controls were used for each test. The procedure was carried out as previously described (Jittapalapong *et al*, 2005). The cut-off titer for a positive test was 1:64, as set by the manufacturer of the

Table 1
Results of *Leptospira* and *Toxoplasma* in stray dogs in Bangkok, Thailand.

Seropositive	Overall		Sex			
			Males		Females	
	Number	%	Number	%	Number	%
Leptospira only	183	79.6	101	87.8	82	71.3
Toxoplasma only	25	10.9	7	6.1	18	15.7
Co-infection	22	9.6	7	6.1	15	13
Leptospira overall	205	89.1	108	93.9	97	84.3
Toxoplasma overall	47	20.4	14	12.2	33	28.7
Total	230		115		115	

test kit (Tsubota *et al*, 1977a).

Statistical analysis

A chi-square test (χ^2) was used to examine for significant differences between findings. A probability less than 0.05 were considered to be statistically significant.

RESULTS

Seroprevalence of *Leptospira* in stray dogs

Of 230 dog sera samples, 205 (89.1%) were seropositive for *Leptospira* infection, 183 were infected with *Leptospira* only (Table 1).

The positive rates in males and females were 87.8% and 71.3%, respectively, the difference was not significant. Fifteen different serovars of leptospira had titers ranging from 1:100 to 1:800. *Leptospira* serovar *bataviae* was the most predominant serovars (20%) (Table 2). There were co-infections with more than 1 serovar of *Leptospira* in some dogs. There were no significant differences showed in *Leptospira* infections between the sexes.

Seroprevalence of *T. gondii* infections

The overall seropositive rate of *T. gondii* was 20.4% (47/230); 25 dogs (10.9%) were infected with only this parasite. The *T. gondii* seropositive rate in males (15.7%) was significantly higher than that in females (6.1%) ($p<0.05$). The highest titer was 1:512.

Table 2
Leptospira serovars found in stray dogs, Bangkok, Thailand.

Serovars	% Positive	Titer
bratislava	1	1:100
autumnalis	3	1:100-1:200
bataviae	20	1:100-1:800
cynopteri	1	1:100
grippotyphosa	2	1:100
hebdomadis	2	1:100
louisiana	1	1:100
manhao	1	1:200
pomona	1	1:100
ranarum	3	1:100-1:200
sarmin	3	1:100-1:200
sejroe	6	1:100-1:200
shermani	4	1:100-1:200
tarassovi	7	1:100-1:200
patoc	8	1:100-1:200

Co-infection with *Leptospira* and *Toxoplasma*

Co-infection with *Leptospira* and *T. gondii* was found in 22 dogs (9.6%). The posi-

tive rate of co-infection in males was significantly higher than in females ($p<0.05$).

DISCUSSION

The importance of stray dogs as a source of human infection is well recognized (Thorten *et al*, 1971; Aslantas *et al*, 2005). The large number of stray dogs in the monastery increases the possibility of acquiring an infection. In many tropical countries, dogs are significant reservoir of zoonoses and may be an important source for outbreaks (Levett, 2001).

Stray dogs are easily infected by *Leptospira* via rodents living in monasteries in Bangkok. *Leptospira* serovars *canicola* and *icterohaemorrhagiae* are common serogroups infecting dogs, but the relative importance of these two serogroups varies from country to country (Rentko *et al*, 1992; Faine, 1994; Weekes *et al*, 1997). In Thailand, a proposed shift in predominant infecting serovars is thought to be responsible for the spread of leptospirosis in the northeastern provinces of Thailand between 1996 and 2003 (Tangkanakul *et al*, 2005). The changing epidemiology of canine leptospirosis in Thailand reflects the variable nature of leptospirosis within animal populations.

In our study, *Leptospira* serovar *bataviae* was found to be the predominant serovar in stray dogs. In Thailand, leptospirosis in pet dogs is normally controlled by a bivalent vaccine using the serovars, *canicola* and *icterohaemorrhagiae*. Humoral immune responses induced by the bivalent vaccine appear to be serovar-specific (Faine *et al*, 1999). These results indicate animals that receive the bivalent vaccine may not be protected from leptospirosis due to other serovars, such as *bataviae, tarassovi, patoc,* and *sejroe.* Urines of infected dogs are a risk to the dog owner and their family.

Climate and ecological conditions influence some endemic diseases in Thailand and may be factors that trigger outbreaks (Tangkanakul *et al*, 2005). Flooding during the rainy season favors the transmission of disease (Fuortes and Nettleman, 1994; Levett, 2001). Ecological and epidemiological variations in domestic and wild animals are also factors that may influence disease transmission. The role of rats has been reported to play in increasing role in the transmission of leptospirosis (Hartskeerl and Tepstra, 1996; Levett, 2001; Bhati *et al*, 2003). Analysis of contributing factors, such as animal reservoirs, methods of prevention and control and animal vaccination and treatment, should be in included in the evaluation and control of disease in these areas (Levett, 2001; Tangkanakul *et al*, 2005).

The seroprevalence of *Toxoplasma* infection in stray dogs in Bangkok was 20.4%. Our previous study found a prevalence of 9.4% in stray dogs from 38 districts in 2003 (Jittapalapong *et al*, 2007). The data suggest the prevalence of *Toxoplasma* in dogs differs by location. The rate in this district is considerably higher than in previous studies in Bangkok. The route of transmission to stray dogs is thought to be by ingestion of rodents that have infective cysts. Another route suggested is the ingestion of food or water contaminated with oocysts shed by stray cats in the environment. *Toxoplasma* infection is prevalent among rodents (Holliman, 1997). Rats with *T. gondii* infection appear to be less neophobic than uninfected rats (Webster *et al*, 1994). Consequently, increased activity and movement in *Toxoplasma*-infected rodents may increase the chance of predation by cats or dogs. Dogs have been reported to be a mechanical vector of toxoplasmosis (Lindsay *et al*, 1997).

Our findings regarding *Leptospira* and *T. gondii* infections in stray dogs in Bangkok, Thailand signify a significant potential public health problem.

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原　著

国産狂犬病ワクチンを用いたWHO方式による狂犬病曝露前免疫の検討

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Key words：rabies, pre-exposure immunization, vaccine

要　旨

本邦での狂犬病曝露前免疫は，組織培養不活化ワクチン1回量1.0mLを4週間隔で2回，その後6～12カ月後に1回皮下注射する方式が標準である．この方式では，多くの渡航者にとって，時間的な制約から3回の基礎免疫を完了することは極めて困難である．WHOでは曝露前免疫を行う方法として，初回接種日を0日として，0，7，28日に狂犬病ワクチンを接種することを推奨している（WHO方式）．WHO方式を用いれば，1カ月で基礎免疫を完了することが可能であるが，国産の組織培養不活化狂犬病ワクチンを用いて，WHO方式による曝露前免疫の有効性や安全性は，これまで検討されていない．

同意を得た健康成人26名を対象に，国産の狂犬病ワクチンをWHO方式に従って0，7，28日に接種し，血中抗狂犬病抗体価の測定を2回目接種直前（7日目），2回目接種3週間後（28日目），および3回目接種後2週間（42日目）に施行した．28日目の抗体価は，0.5EU/mL以下であった3例を除いて，0.7～3.5EU/mLであった．42日目には，全員の抗体価が1.6EU/mL以上であり，十分な抗体上昇が見られた．WHO方式による曝露前免疫法は，本邦の標準法に劣らず効果的であった．また，発赤や疼痛など局所副反応は認めたが，全身副反応など重篤な有害事象は認めなかった．国産の組織培養不活化ワクチンを使用したWHO方式による狂犬病曝露前免疫の有効性と安全性は，共に高いと考えられる．

〔感染症誌　82：441～444，2008〕

序　文

世界保健機関（WHO）は，狂犬病流行地において動物による咬傷を受けた場合，抗狂犬病免疫グロブリン（RIG）の投与と組織培養不活化ワクチン接種による曝露後発症予防を勧告している[1]．一方，曝露前免疫を受けていれば，RIGの投与は不要となる．RIGは世界的に不足しており，入手が容易でないうえ，日本では市販されていない．曝露後免疫の効果を確実にするうえで，狂犬病曝露前免疫を行っておくことは重要である．

本邦での狂犬病曝露前免疫は，組織培養不活化ワクチン1回量1.0mLを4週間隔で2回，その後6～12カ月後に1回皮下注射する方式が標準である[2]（標準法）．しかし，多くの渡航者は海外渡航が決定してから，出国までの時間が1～2カ月程度であるため，本邦の標準法では3回の基礎免疫を完了することは困難である．このため，狂犬病常在国への渡航者や長期滞在者の多くは，狂犬病ワクチン接種を2回だけ済ませ，出国している[3]．

狂犬病曝露前免疫を行う方法として，WHOでは初回接種日を0日として，0，7，28日に力価が2.5IU/mL以上の組織培養不活化狂犬病ワクチンを三角筋上部へ筋注することとしている[2]（WHO方式）．この方法では，1カ月間で基礎免疫を完了することができる．世界的に標準的な方法であるが，国産の組織培養不活化ワクチンを用いて，その有効性や安全性を検討した報告はこれまでにない．

今回我々は，同意を得た健康成人に対して，国産の組織培養不活化ワクチンを用いて，WHO方式による狂犬病曝露前免疫の有効性と安全性を調査した．

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対象と方法

1. 対象

これまで狂犬病ワクチンの接種歴がなく，接種を希望された海外派遣予定者26人を対象とした．本調査の目的，調査項目，接種ワクチンと予想される副反応について文書，および口頭で説明し，WHO方式による狂犬病曝露前免疫を行うことの同意を得た．尚，本研究に先立って，当院の倫理委員会に研究計画書を提出し，承認を得た．

2. 接種ワクチン及び接種方法

化学及血清療法研究所（化血研）製組織培養不活化狂犬病ワクチンのロットRB02（1，2回目接種の全て，3回目の18例）とRB03（3回目の8例のみ）を用いた．狂犬病ワクチンは溶解液1.0mLで溶解した後，利き腕とは反対側の上腕に全量を皮下注射した．狂犬病ワクチンはWHO方式に従って，初回接種日を0日として，0，7，28日の計3回接種した．

3. 局所および全身副反応

被接種者には発赤，腫脹，疼痛，掻痒感などの自覚症状の有無について観察して，次回接種時および採血時に報告するように依頼した．

4. 抗体検査

血中抗狂犬病抗体価は，2回目接種直前（7日目），2回目接種3週間後（28日目），および3回目接種後2週間（42日目）に測定した．1，2回目の抗体価測定日は，2回目，3回目のワクチン接種日に合わせた．3回目の抗体価測定日に関しては，過去の報告ではワクチン接種2〜3週間後に測定されており[4][5]，本研究でもそれに倣い3回目接種後2週間とした．抗体価は，化血研臨床検査センターに依頼して，Platelia™ rabies kit（BIO-RAD Laboratories）を用いて，ELISA法で測定した．

結　果

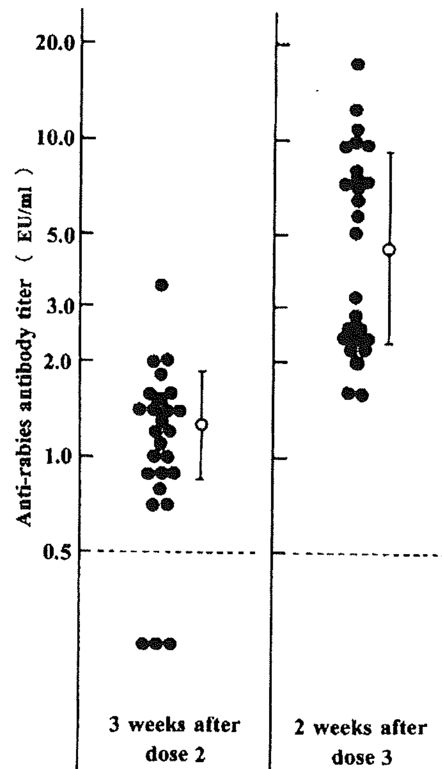
1. 対象者の年齢分布

対象者は健康な男性26例であった．年齢分布は25歳から55歳で，20歳代が12例，30歳代が12例，40歳代が1例，50歳代が1例であり，平均年齢は31.1±6.5歳であった．

2. 血中抗狂犬病抗体価

2回目接種直前（7日目）の血中抗狂犬病抗体価は，全例が0.5EU/mL未満で陰性であった．2回目接種3週間後（28日目）の抗体価は，0.5EU/mL以下であった3例を除いて，0.7〜3.5EU/mLであり，抗体陽性者の幾何平均値は1.3EU/mLであった．3回目接種後2週間（42日目）には，28日目の検査で0.5EU/mL以下であった3例も含めて，全例の抗体価が陽性となった．抗体価は1.6〜17.2EU/mLで，幾何平均値は4.6EU/mLであった．42日目の抗体価は上昇が大きい群（A群）と，比較的小さい群（B群）に分かれていた（Fig. 1）．A群は14例からなり，幾何平均抗体価は8.4EU/mLであった．B群は12例からなり，幾何平均抗体価は2.3EU/mLであった．

Fig. 1 Anti-rabies antibody titers examined 3 weeks after dose 2 and 2 weeks after dose 3.
Japanese rabies vaccine was injected subcutaneously on days 0, 7, and 28 and blood samples were taken on days 7, 28, and 42. Solid circles : anti-rabies antibody titers for each subject examined (left column - titers examined 3 weeks after dose 2; right column - titers examined 2 weeks after dose 3). Open circles : geometric mean titers among subjects with positive titers, with solid vertical lines indicating ±SD. Dotted horizontal line : protective level against overt rabies reported by the World Health Organization.



3. 接種後の局所および全身症状

3回のワクチン接種期間中に，ワクチン接種部位に発赤を認めた例は2例，腫脹を認めた例は4例，疼痛を認めた例は2例，掻痒感を認めた例は1例であった．発熱，頭痛，全身倦怠感などの全身症状を報告した例はなかった．

考　察

本邦での標準法を用いて，海外渡航前に3回のワクチン接種を行い，基礎免疫を完了することは多くの渡航者にとって困難である．しかしWHO方式による狂犬病曝露前免疫では，1カ月で基礎免疫を完了することが可能である．狂犬病は発症すれば，ほぼ100％死に至る疾患であり，ワクチン接種が唯一の狂犬病予防死を免れる手段であることを考えれば，出国までの時間が限られた渡航予定者にとって，1カ月で基礎免疫が完了できることの利点は極めて大きい．

本調査において，ワクチン2回目接種3週間後（28日目）に測定した抗体価は，3例の陰性例を除いて，良好な抗体上昇が見られた．3回目のワクチン接種終了後2週間（42日目）で測定した血中抗狂犬病抗体価は，全例1.6EU/mL以上となり，幾何平均値は4.6 EU/mLであった．これはWHOが定める発症防御レベル（0.5IU/mL）を大きく超えるものである．2回目接種後の抗体価が陰性であった3例も，ワクチン3回接種2週間後（42日目）の抗体価は，それぞれ2.2 EU/mL，2.4EU/mL，5.1EU/mLと上昇が認められ，本法の有効性は十分高いと考えられた．基礎免疫完了後の抗体価が上下2群に分かれ，免疫応答が良い群とそれほどではない群が存在することが示唆された．3回目ワクチン接種の際に，ロットRB03を8例（31％）に使用したが，それぞれ上下の2群に分散しており，抗体価がロットの違いによるものではないと考えられた．抗体価が2群に分かれた明らかな理由は不明であり，今後の検討が必要である．接種部位の発赤や腫脹は見られたものの，全身副反応など重大な有害事象は認められなかった．

標準法を用いての狂犬病曝露前免疫の効果に関しては，皮内・皮下併用群と標準法を用いた群のワクチン投与後の抗体価の推移について検討した髙山らの報告に見られる[5]．標準法を用いた群において，2回目接種6カ月後の幾何平均中和抗体価は0.4IU/mL以下であったが，ワクチン接種4週間後で測定した幾何平均中和抗体価は2.7IU/mLであった．今回の接種試験では採血時期をはじめ，その対象群の年齢，性別，人数や抗体測定法などに違いがあるため，厳密な比較は不可能である．また，抗体価測定は42日目で終了とし，抗体価の持続性に関しても検討は行っていない．しかし，上述の報告から明らかであるように，時間とともに狂犬病抗体価は減衰するものの，ワクチン接種後にすみやかに良好な抗体上昇がみられることから，1カ月間で2回のワクチン接種のみでも十分プライミング効果があると考えられる．WHO方式は1カ月間でワクチン接種を3回行うため，本方式による曝露前免疫は標準法と比較しても，少なくとも同等以上の効果が期待できると考えられる．

2006年11月，国内で36年ぶりに相次いで輸入狂犬病患者が発生した[6)7)]．交通手段の発達により，日本から数時間で狂犬病常在地に渡航できることを考えると，今後も輸入狂犬病患者が発生する可能性は十分認識しておく必要がある．今後新たな狂犬病患者を出さないためにも，海外渡航者への情報提供，注意喚起を行うとともに，ワクチンが接種できる医療側の体制を早急に整備する必要がある．また，本邦でRIGの入手が困難である以上，曝露後免疫をより確実にし，狂犬病犠牲者をなくすためには，狂犬病曝露前免疫を行うことが重要である．WHO方式による狂犬病曝露前免疫は，短期間で基礎免疫を完了することができる有用な接種方式であり，今回の調査により国産の狂犬病ワクチンを用いても，その効果や安全性は高いことが明らかになった．出国までに時間が限られた狂犬病常在地への渡航者には，WHO方式による曝露前免疫を勧めてよいと考える．

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平成20年9月20日

WHO Recommended Pre-exposure Prophylaxis for Rabies Using Japanese Rabies Vaccine

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After severe exposure to suspected rabid animal, WHO recommends a complete vaccine series using a potent effective vaccine that meets WHO criteria, and administration of rabies immunoglobulin (RIG). RIG is not available globally, and is not marketed in Japan. If pre-exposure prophylaxis for rabies is given, RIG is unnecessary even after severe exposure. It is thus important to give pre-exposure prophylaxis for rabies to people who plan to go to rabies-endemic areas.

In Japan, pre-exposure prophylaxis for rabies consists of 3 doses of cell-culture rabies vaccine. The first two doses are given 4 weeks apart, and the third dose is given 6-12 months after the first dose, all of which are injected subcutaneously (standard regimen). People who plan to travel abroad to rabies-endemic areas may know of their destinations only 1 or 2 months in advance at best. Therefore, it is virtually impossible to complete the 3 dose regimen for rabies in Japan.

Pre-exposure prophylaxis recommended by WHO consists of 3 doses given intramuscularly on days 0, 7, and 28, making it possible to complete pre-exposure prophylaxis in one month. This WHO recommended pre-exposure prophylaxis using Japanese cell-cultured rabies vaccine (PCEC-K) has not been studied, so we elected to fill the gap using PCEC-K, administered based on the WHO recommendation and examined its efficacy and safety.

Subjects were 26 healthy volunteers with no previous rabies vaccination giving oral and written consent. Vaccine was administered on days 0, 7, and 28, and rabies antibody levels were tested on days 7, 28, and 42. On day 7, every antibody level was negative. On day 28, antibody levels were between 0.7-3.5EU/mL, with the exception of 3 cases still negative. On day 42, all cases, including the 3 negative cases, exceeded 1.6EU/mL, providing sufficient protection against rabies. This result was not inferior compared to the standard regimen. Local adverse effects such as erythema and pain were noted, but none were serious.

In conclusion, WHO recommended pre-exposure prophylaxis for rabies using PCEC-K is considered effective and safe.

中国からのコンテナに潜んでいたネコに咬まれて狂犬病曝露後発病予防を受けた１例

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はじめに

日本国内での狂犬病はヒトでも動物でも、３例の輸入ヒト狂犬病を除いて、50年以上発生の報告がない。しかし、近隣のアジア諸国では、発生件数の相違はあれ、今なお狂犬病の発生が続いている。交通手段が発達し、輸出入に伴う運搬も頻繁となっている現状では、狂犬病常在地にて感染した日本人渡航者が帰国後に発症する輸入狂犬病および狂犬病ウイルス感染動物が日本に侵入する可能性は常に考えておく必要がある。最近、狂犬病常在病予防を行った症例を経験したので、経過と問題点を報告する。

症例と経過

症例は埼玉県内で事業を営む男性。中国から搬入されたコンテナ内にネコが潜んでいることに気づき捕獲しようとして右手第１指を咬まれた。出血あり。傷を水で洗った後、医療機関を受診して、消毒と抗生剤の処方を受けた。同日、保健所から紹介されて当院を受診し、狂犬病ワクチン１回目接種と破傷風トキソイドの注射を受けた。捕獲したネコは保健所に通報し、動物愛護センターに一時保護された。

男性は、曝露後発病予防スケジュールに従って、３日後に２回目、７日後に３回目の狂犬病ワクチン接種を受けたが、14日後の４回目接種の予約日には来院しなかった。すでに、加害ネコが10日間元気であれば、狂犬病の可能性が否定され、狂犬病ワクチン接種を継続する必要はないとの説明をしていたので、当局から被害者の男性に加害ネコが元気であるとの連絡が行ったために受診しなかったものと判断していた。

男性の初診日に、輸入動物を介する狂犬病輸入の可能性もあったため、主治医の判断で厚生労働省に通報した。数日後、男性から加害ネコの検査結果が届かないとの連絡があったため、厚生労働省に問い合わせたが、確定的な回答は得られなかった。

初診日から33日後に男性が２回目の破傷風トキソイド接種のため来院した。このとき加害ネコの観察結果が未着であることを知った。このため、動物検疫所にに問い合わせて、加害ネコの観察に関しては、男性の狂犬病ワクチン４回目接種予定日に、観察が終了し、翌日に異常がないことを農林水産省動物衛生課衛生課に書面で報告したこと、農林水産省から厚生労働省へはその日のうちにメールで結果が送付されたことが判明した。また、動物検疫所では、通常捕獲した動物はすぐに安楽死させて脳組織の検査を行っているが、今回は厚生労働省の要請により加害ネコの観察を行ったことも伝えられた。その後、厚生労働省から加害ネコの観察および脳組織検査結果が被害者の男性と主治医に届いた。

考　察

各種商品の流通が盛んになり、運送の方法もコンテナ輸送が主流になっている。外国からコンテナで商品が輸入される場合、財務省が示す一定の条件を満たした場合、港湾部でコンテナは開かれずに注文主がいる地域の保税蔵置場に配送されるので、コンテナ内に小動物が潜んでいても税関で発見されることはない。

財務省によれば、現在国内には約500カ所の保税蔵置場が、沿岸部ではなく内陸部にある。欧米などの遠方から長時間かけて運搬される場合や、高温や低温など過酷な環境にさらされる場合はコ