この通常の季節性インフルエンザに、遺伝変異型インフルエンザの出現で、百万人当たり何人の死亡者が上積みされたのかという超過死亡数を推定すると、①一九一八年～一九一九年のＨ１Ｎ１スペイン風邪（パンデミック）五千九百八十人（過剰推定死亡総数二千万人）、②一九二八年～一九二九年のＨ１Ｎ１（変異、ドリフト）九百六十七人（三百万人）、③一九三四年～一九三六年のＨ１Ｎ１（変異、ドリフト）五百二十人（百五十万人）、④一九四七年～一九四八年のＨ１Ｎ１　Ａダッシュ（亜型間の組み換え、リアソート）八十九人（三十万人）、⑤一九五一年～一九五三年のＨ１Ｎ１（亜型間の組み換え、リアソート）三百四十一人（百五十万人）、⑥一九五七年～一九五八年のＨ２Ｎ２（抗原シフト）アジア風邪（パンデミック）四百六人（二百万人）、⑦一九六八年～一九六九年のＨ３Ｎ２（抗原シフト）香港風邪（パンデミック）百六十九人（八十万人）、⑧一九七二年～一九七三年のＨ３Ｎ２　Ａ／ポート・チャーマー（変異、ドリフト）百十八人（五十万人）、⑨一九七五年～一九七七年のＨ３Ｎ２（変異、ドリフト）＋Ｈ１Ｎ１（ブタインフルエンザ流行）百二十四人（六十万人）、⑩一九七七年～一九七八年のＨ３Ｎ２（変異、ドリフト）＋Ｈ１Ｎ１（ロシア風邪）二百十人（百二十万人）、⑪一九九七年～一九九九年のＨ３Ｎ２　Ａ・シドニー（亜型間の組み換え、リアソート）＋Ｈ１Ｎ１（変異、ドリフト）四百九十五人（二百六十万人）、⑫二〇〇三年～二〇〇四年のＨ３Ｎ２　Ａ・福建（亜型間の組み換え、リアソート）＋Ｈ１Ｎ１（変異、ドリフト）百七十一人（八十万人）―などとなっています。

これに今回の二〇〇九年のＨ３Ｎ２＋Ｈ１Ｎ１（変異、ドリフト）＋ブタインフルエンザＨ１Ｎ１（パンデミック）が加わることになります。

このように、スペイン風邪で二千万人が死亡した後、同じ抗原変異で中間的な流行を起こした時に三百万人、それ以降は、時々大きな変異を遂げて百万人、百五十万人、二百万人程度の流行が繰り返されて起きています。

今回の場合は、一年間を振り返ってみても、最終的な超過死亡としては、せいぜい数十万人規模に過ぎないと予測されます。病原性の弱い株に、いつまでも最悪であったスペイン風邪をパンデミックのモデルとして、使用し続けることには疑問を抱かざるを得ません。

今回の新型インフルエンザが世界中を席巻しても、死亡者数はスペイン風邪の十分の一、もしくは二十分の一以下の規模に過ぎません。どのモデルを使うのが科学的に正当なのかは、今後、冷静に考えていかなければならないでしょう。

基本は自宅療養

日本の新型インフルエンザ行動計画（医療体制）は、最終責任は自分でとる姿になっています。考えてみれば当たり前のことでもあります。今回も海外での発症警告が流れて、徹底的な航空機内検疫を実施しましたが、結局はインフルエンザの侵入を許してしまいました。このように、潜伏期中にインフルエンザの侵入を阻止するのは、現実的には極めて難しいことです。

国内で発生しても、まだ一部地域に限られている時期には人数も少なく、感染者を第一種指定医療機関に入院勧告して隔離するなど、しっかりした対応も可能です。しかし、国内で広がり始めた段階になると、責任は国よりも各都道府県に移ってきます。隔離政策を取り続けていては、医療機関もパンク状態になってしまうので、入院勧告措置は解除せざるを得ません。入院は原則として重症患者、それ以外は在宅医療となり、医療機関も指定病院に止まらず、入院施設のある病院は対応していくことが求められてきます。

したがって、蔓延期から回復に徐々に向かうまでの間は、発熱相談センターがパンク状態になり、発熱外来も厳しい状態が予想されることから、一般の健康人が感染した場合には、基本的に一般外来で薬を処方してもらい、治るまで自宅療養せざるを得ません。もちろん、小児や高齢者、慢性疾病者などは、ハイリスクグループとして、その範疇には含まれません。

すでに、今回のインフルエンザの流行も、第二波が始まっており、第一波よりも大規模になるはずです。ワクチン投与も十月末くらいから始まりますが、全体としては、もう一、二回大きな流行を繰り返しながら終息していくと予想されます。

結局のところ、実際の流行が始まった段階では、国が何とかしてくれるとの淡い期待を抱くのではなく、自分で対処していくと気持ちを切りかえた方が、賢明なようです。

この新型インフルエンザ対応は、政権が変わっても継続されると思いますが、国は少なくともワクチンについて製造・輸入で三千二百万人分、さらに二千二百万人分を追加し、六千万人分近くを整備する方針です。治療薬としてのタミフル、リレンザに関しては、二千八百万人分をすでに備蓄しており、ワクチンと治療薬の両方で、総計一億三千万人分程度は確保されています。

理論通りにいけば、ほとんど大流行を見ないままで終わることにもなりますが、果たしてどこまでうまくいくのか、いまのところ定かではありません。

予防・治療については、ご存知のように、医療関係従事者やハイリスク者（小児、慢性疾患患者、高齢者）を中心に、優先順位がつけられています。

個人に関しては、月並みですが、マスクや手洗い、うがいを心がけるとともに、流行し始めたら、集会や人込みを避け、発症した場合には、重症度に応じて発熱外来、入院となりますが、多くの方々は自宅医療となります。

求められる危機管理対応

企業については、幾つかの指針がありますが、事業継続を前提としたシナリオ（危機管理対応）を作成しておくことが求められます。基本方針としては、職員の人命の安全を守るとともに、それとは矛盾するようですが、企業としての社会的責任も果たしていく、両立するプログラムが必要です。全ての機能を維持することは不可能ですので、予め業務の重要度による分類と具体的な人的資源の分配計画を立てておかなければなりません。

現実の危機管理として、インフルエンザ対策本部のような司令塔を構築、責任体制を明確化しておくことが重要です。しかも、一人のみの司令塔では、その責任者がインフルエンザに巻き込まれた瞬間に、全てが機能停止になってしまいます。代行者を確保しておかなくてはなりませんが、司令塔は企業内を含めた内外情報を収集、分析して職員に発信する役割も受け持ちます。

感染防止策としては、リスクに応じた管理措置や教育・啓蒙が求められますが、感染者が出た場合には、早期の自宅待機や集会・会議の延期措置などで、物理的封じ込めが大切です。

具体的な感染防止策としては、①手洗い・うがい励行、咳エチケット徹底などインフルエンザの基本的知識の周

知、②入口の外での手洗い所（消毒液使用場所）設置による手洗い励行、③発熱中の従業員・訪問者の出勤・入場禁止、④ラッシュ時の公共交通機関利用を防ぐ時差出勤、自家用車・自転車・徒歩などによる出勤―などが挙げられますが、最近では病院以外でも入口に消毒薬などを置いて手洗いを励行する施設も多くなってきています。

対人距離維持の観点からは、①出張や会議の抑制（電話会議やビデオ会議の利用）、②職場・食堂などの配置替え、時差利用による接触距離の維持、③対面販売に際する顧客へのマスク配布、④不要不急業務、感染リスクの高い業務の一時停止、⑤在宅勤務、職場内での宿直実施、⑥従業員や訪問者同士の不用意な接近を避けるための通路の一方通行、⑦フレックスタイム制導入で同時期における職場内従業員数の減少、⑧ガラスなどの仕切り設置による訪問者からの飛沫遮断―などが求められてきます。

また、立入制限の観点からは、①従業員や訪問者の職場に入る前の発熱や咳の有無に関する質問や検温、②職場での入口や立入場所の制限、③一度の訪問人数制限―などが考えられます。

その他、①職場の清掃・消毒の一日数回実施、②社内感染者発生や感染疑いのある場合の発熱センターへの連絡、③感染疑いのある顧客への責任者対応―なども挙げられます。

混同された感染力と病原性

本日のまとめですが、今回、ＷＨＯも含め世界中が多少混乱した要因として挙げられるのが、感染力と病原性の混同です。両者は全く別のもので、そもそもインフルエンザウイルスの感染性とは上部気道（気管、気管支）で増えるウイルスほど広がりやすく、一方、下部気道（肺）で増えるウイルスは重症化（肺炎）しやすいものの、感染力は低くなります。

しかし、上部、下部の双方で増えるウイルスは、当然、感染性も高く重症化しやすい怖いウイルスです。さらにこれが呼吸器以外でも増える、全身感染を起こすウイルスでは病原性も極めて高く、感染は致命的です。高病原性トリインフルエンザに感染したトリは、本当に次々と死んでいきます。スペイン風邪の一部は、呼吸器以外でも増える高病原性を有していたかもしれませんが、これ

までヒトには、まだこのような事例は出現していません。

また、感染性には種の壁があります。ヒトへのウイルス感染を考えた場合、ヒトからの感染力が最も強く、その次がブタで、トリからヒトに直接感染するウイルスは極めて稀です。

しかし、ひとたび種の壁を越えてヒトに感染した場合には、新しい宿主に適応するまでウイルスが変異を遂げ続けていくため、その過程で病原性が上がったり、伝播力が強くなったりします。専門の関係者はそのことを最も心配しています。

しかし、一般的には、その新型インフルエンザウイルスも、何年か経過するうちに、徐々に抗原変異を遂げていき、最終的に季節性インフルエンザとしてヒトに定着します。

最後にひと言、危機管理と持続社会について触れさせていただきます。今回のインフルエンザもそうですが、目に見えて増大するリスクに対応して、どんどん管理措置レベルを強化していくことは、容易に合意が得られます。税金はかかるものの、リスクを評価する科学者、管理する行政機関、巻き込まれる消費者のいずれにとっても責任が減少するからです。

問題は、流行がおさまって減少するリスクに対して、管理措置を徐々に緩和することの難しさです。リスク措置の緩和には強化以上に責任が伴います。誰も責任をとらないと、リスクがなくなっても、施策はそのまま残ることにもなります。

しかし、この緩和措置が成功してこそ持続社会が維持できることになります。危機管理は持続社会をつくるために行うもので、管理体制を強化したままにして、次から次へと新しい感染症に税金を費やしていては、社会は崩壊してしまいます。危機管理のために作られた仕組みが社会を滅ぼしてしまっては本末転倒です。危機管理の本質は強化するだけでなく、いかにソフトランディングして持続社会を構築していけるかです。

日本は世界でも類のない、ひたすら危機管理水準を上げ続け、一度上げたらその水準から下げないことを善とする傾向が強い社会です。このままではどこかで疲弊して潰れてしまうのではないかと、私などはこれまでの感染症対応から不安感を抱いています。

今回のブタインフルエンザについても、一、二回の大

流行を経て、いずれ収束していくと思われますが、その時には、行政だけでなく、企業も国民も冷静な対応ができることを期待しています。ご清聴ありがとうございました。

【質疑応答】

問　新型インフルエンザのワクチン接種に関して、どのように考えれば良いでしょうか。

吉川　私は有効性が高いと思います。今回の新型インフルエンザウイルスは、二〇〇九年四月にカリフォルニアで最初に採取されましたが、それから大きく変異していません。したがって、いま世界中で流行しているブタインフルエンザに関しては、いま製造されている不活化ワクチンで十分免疫を誘導できると存じます。

季節性インフルエンザの場合、ウイルスが時間をかけて変異して効かなくなったり、流行する型を読み違えたりして、ワクチンが効かないこともありますが、今回のワクチンは十分に効くはずです。

問　大流行が来る前に、ワクチンが接種できないのではと心配していますが。

吉川　いますでに流行の第二波が来ています。運よくまだ感染していない方と、すでに感染してしまった方がおられると思いますが、物は考えようです。

ハンディを持っている方は大変ですが、普通の健康状態の方であれば、今回のブタインフルエンザは病原性が強くないので、それほど心配はないように思われます。ワクチンを接種しても、不活化ワクチンですと、一年から二年で免疫がなくなってしまいます。しかし、病原性が低い、いまのうちに感染すれば生涯免疫力が残ります。

現に、四月から五月にかけて、アメリカでは病原性が低いうちに感染して免疫をと、インフルエンザパーティが開催されているという驚くべき報道がありました。もちろん、ワクチンが間に合えばそれに越したことはありませんが、普通の健康な方であれば、季節性のインフルエンザとそれほど重篤性は変わらないため、早目に病院で抗ウイルス薬を処方して貰えば十分に完治します。「ワクチンを接種していない」と言って思い悩む必要はありません。いずれにしても、私どものワクチン接種は最後になると思います。

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Epizootology and experimental infection of Yokose virus in bats

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Abstract

To reveal whether bats serve as an amplifying host for Yokose virus (YOKV), we conducted a serological survey and experimentally infected fruit bats with YOKV isolated from microbats in Japan. YOKV belongs to the Entebbe bat virus group of vector unknown group within the genus *Flavivirus* and family *Flaviviridae*. To detect antibodies against YOKV, we developed an enzyme-linked immunosorbent assay (ELISA) using biotinylated anti-bat IgG rabbit sera. Serological surveillance was conducted with samples collected in the Philippines and the sera supplied from Malaysia. One of the 36 samples from the Philippines (2.7%) and 5 of the 26 samples from Malaysia (19%) had detectable ELISA antibodies. In the experimental infections, no clinical signs of disease were observed. Moreover, no significant viral genome amplification was detected. These findings

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revealed that YOKV replicates poorly in the fruit bat, suggesting that fruit bats do not seem to serve as an amplifying host for YOKV.
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Keywords: ELISA; Yokose virus; Bat; Experimental infection; Anti-bat IgG; Chiroptera

Résumé

Afin de vérifier si les chauves-souris servent comme hôte amplificateur pour le virus Yokose (YOKV), nous avons mené des études sérologiques sur des chauves-souris frugivores expérimentalement infectées par YOKV. Le virus Yokose appartient à la famille des virus Entebbe, dont le vecteur est inconnu et appartenant au genre Flavivirus, de la famille des Flaviviridae. Pour détecter les anticorps contre YOKV, nous avons développé une technique « ELISA » utilisant des sérums de lapin anti-IgG de chauves-souris. Notre surveillance sérologique s'est effectuée sur des échantillons en provenance des Philippines et des sérums récoltés en Malaisie. Un prélèvement sur 36 provenant des Philippines (2,7%) et cinq sur 26 provenant de Malaisie (19%) ont développé des anticorps détectés par le test ELISA. En revanche, aucun signe clinique de maladie n'a été développé sur les animaux infectés expérimentalement. En outre, aucune amplification du génome viral n'a été détectée. Il en résulte que YOKV se propage mal chez les chauves-souris frugivores, ce qui suggère que les chauves-souris frugivores ne servent pas comme hôte amplificateur du virus Yokose.
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Mots clés : ELISA ; Yokose virus ; Chauve-souris ; Infection expérimentale ; IgG anti-chauve-souris ; Chiroptères

1. Introduction

Bats, the only mammals capable of flight, display large amounts of diversity and account for 20% of the 4800 mammalian species recorded in the world. During the past decade, bats have been associated with several emerging zoonotic agents including Hendra, Nipah, Lyssa, Ebola, and severe acute respiratory syndrome coronavirus-like viruses [1–5]. Therefore, bats are thought to be an important reservoir for many mammalian viruses. Specifically, numerous viruses have been isolated from the genus *Flavivirus*, family *Flaviviridae* [6–9]. While the epizootology of these viruses remains unknown, nucleotide sequences for some flaviviruses have been reported [8–10]. Yokose virus (YOKV) is a flavivirus that has been isolated from a bat in Japan in 1971. YOKV belongs to the Entebbe bat virus group within the genus *Flavivirus* and family *Flaviviridae*.

To investigate the possibility that bats serve as a reservoir for Japanese encephalitis virus (JEV) during the winter, Oya et al. attempted to isolate arthropod-borne viruses from bats in Oita Prefecture, Japan [11]. During this 1971 investigation, YOKV was isolated from the long-fingered bat *Miniopterus fuliginosus*. Recently, Tajima et al. [11] reported the complete nucleotide sequence of YOKV. Kuno and Chang [9] compared the complete nucleotides sequences with those of other flaviviruses. They concluded that YOKV is genetically closer to yellow fever virus or Sepik virus than JEV, and it is most closely related to Entebbe bat virus.

Previous phylogenetic analyses of the genus *Flavivirus* have revealed that flaviviruses can be divided into three groups: mosquito-borne, tick-borne, and unknown vector groups [12,13]. Although YOKV is classified into the Entebbe bat virus group of vector unknown group, conserved sequence element 1 (CS1) in the 3′-untranslated region of YOKV is similar to that of mosquito-borne viruses, which are known to display highly conserved CS regions [11,14]. Therefore, Tajima et al. [11] suggested that YOKV belongs to the mosquito-borne virus group. Moreover, previous reports indicated that Entebbe bat virus can replicate in mosquito cells in vitro [15], and experimental infection of Entebbe bat virus using frugivorous and insectivorous bats showed no viral growth in bats [16]. Since the initial isolation with YOKV in 1971, there have been no additional reports on the isolation or antibody detection of YOKV from bats or mosquitoes. Therefore, to determine whether bats serve as a natural or amplifying host for YOKV, we conducted a serological survey and experimental infection studies in bats with YOKV.

To detect antibodies against YOKV, we developed an ELISA using biotinylated anti-bat IgG rabbit sera. In this system, polyclonal anti-bat IgG rabbit sera were used as described in a previous paper [17]. The developed anti-bat IgG reacts only with bat IgG but not with IgG of other mammalian species. Therefore, this ELISA detects bat-specific IgG antibodies. Using the conventional ELISA, a serological survey was performed on bat serum samples collected from the Philippines and Malaysia.

2. Materials and methods

2.1. Cell culture and virus growth

Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), penicillin, and streptomycin. The Oita-36 strain of YOKV was kindly provided by Dr. T. Takasaki (National Institute of Infectious Diseases). The virus was grown in Vero cells on 700-cm^2 roller bottles. Infection was performed at a multiplicity of 0.005 $TCID_{50}$/cell with an inoculum containing 5 ml of serum-free maintenance medium (-SMM) in DMEM, 2.95 g triptose phosphate broth, 5 g L-glutamine–Na, 1 g glucose, 0.5 g yeast extract, 0.292 g L-glutamine/L, penicillin, and streptomycin. Following virus adsorption at 37 °C for 1 h, cells were washed with -SMM and 100 ml of -SMM was added per bottle. Four days after infection, ten 100-ml bottles of infectious fluid were harvested and cellular debris was removed by low-speed centrifugation (2000 × *g*, 15 min, 4 °C). The resulting supernatant was collected, and half was used for virus purification while the remainder was used for virus inactivation.

2.2. Virus purification

Virus purification was performed according to the procedures outlined for JEV [18,19]. Briefly, YOKV was precipitated from the supernatant by incubation with PEG6000 (10 mM) and NaCl (400 mM). After an overnight incubation at 4 °C, the mixture was centrifuged (5000 × *g*, 60 min, 4 °C) and the pellet containing the virus was resuspended in 1 ml Tris–saline–EDTA buffer (TEN buffer). Virions were further purified on 12 ml of a

continuous 10–60% sucrose gradient. The gradients were centrifuged in an RPS40T rotor (Hitachi, Tokyo, Japan) at 100,000 × *g*, for 2 h at 4 °C. Fifteen fractions were collected from the bottom of each tube. Each fraction was assayed by using both ELISA and titration method. Peak fractions that demonstrated the highest ELISA reactivity were collected and used as ELISA antigens.

2.3. Virus inactivation and purification

Virus inactivation was performed using 37% formalin at a final concentration of 0.1%, according to the procedures for JEV [18,19]. The inactivated virus was purified as described above. The peak fractions were collected and dialyzed with PBS. The inactivated virus was filtered through a 0.22 μm low protein-binding (GV-type) filter unit, and stored at 4 °C until it was used as a bat immunogen.

2.4. Immunizations and blood sampling

Two Leschenault's rousette bats (*Rousettus leschenaulti*) were immunized with inactivated and purified virus. One milliliter of the inactivated virus was inoculated intraperitoneally twice at 4-week intervals to obtain positive sera containing anti-YOKV antibodies. Serum samples were collected at days 14 and 28 postinoculation. Briefly, a small vein on the patagium was cut with a scalpel for blood sampling, and filter papers were soaked with approximately 50 μl of blood. The filter papers were transferred into Eppendorf tubes and eluted in 200 μl PBS by centrifugation (3000 rpm, 10 min, 4 °C). After discarding the filter papers, eluates were centrifuged again and the supernatants were stored as serum samples. These samples were estimated to be equal to a 1:5 sera dilution. On day 28 after collecting sera, two bats were inoculated with a secondary injection. On day 35 after the first inoculation, the two bats were anesthetized using a 1.5 mg intraperitoneal injection of ketamine hydrochloride and were euthanized via intracardiac exsanguination.

2.5. Sample collection for the serological survey

Bat serum samples were collected in the Philippines and Malaysia. Insectivorous bats were collected from two sites in the Philippines (Fig. 1). Sites were chosen on the basis of a previous survey [20]. All captured bats were anesthetized by administering a 15 mg/kg intraperitoneal injection of ketamine hydrochloride. We measured the size of each bat (weight, forearm length, head and body length, ear length, and tail length) and examined additional morphological features (uropatagium, tragus, and nose-leaf). Species were identified based on gross morphology according to the classification criteria for chiropterans [21] (Table 1).

Blood was obtained by cardiac puncture and stored at 4 °C until centrifugation. Sera were frozen at −20 °C during transport and later stored in a −80 °C freezer upon arrival. Fruit bat sera collected in Malaysia were kindly supplied by Dr. A. Rayari (University of Malaysia, Sarawak) and Dr. T. Imada (JICA project leader at the Veterinary Research Institute).

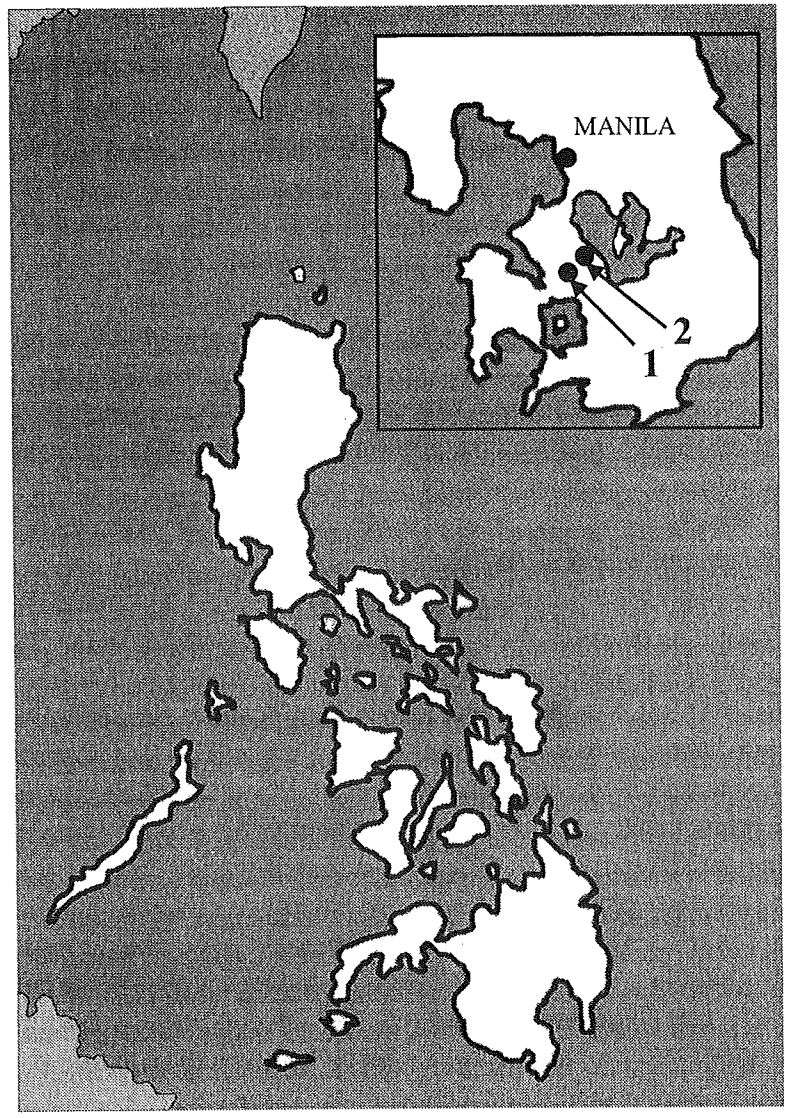

Fig. 1. Map of the Philippines outlining bat collection sites from two locations. 1 = Calbang, Laguna; 2 = Santa Rosa, Laguna.

Table 1
Bat species and collecting sites

Bat species	The Philippines (Laguna)		Malaysia	Total
	Calubang	Santa Rosa		
Collecting cite				
Taphozous melanopogon	1/31			1/31
Scotophilus kuhlii		0/5		0/5
Unidentified species (fruit bats)			5/26	5/26
Total	1/31	0/5		6/62

*The number of antibody positive sera/the number of sera tested.

2.6. ELISA

ELISAs were performed according to the procedures described for JEV [22]. To standardize the reagents used for the labeled avidin–biotin enzyme-linked immunosorbent assay (LAB-ELISA), antigen and antibody concentrations were determined by checkerboard titration. Briefly, YOKV antigen was diluted with coating buffer at a concentration of 15 μg/ml and ELISA plates were coated with 100 μl/well of the antigen solution. After washing the plates, 50 μl of serum from each sample was appropriately diluted with blocking buffer (PBS, pH 7.4, containing 0.5% Tween 20 and 5% chicken serum) and delivered to each well. The plates were then kept at 37 °C for 1 h. After washing the plates, biotin-labeled anti-bat IgG rabbit serum was diluted in blocking buffer at 1:3200 and 50 μl of the diluted solution was added to each well. Anti-bat IgG rabbit serum [17] was biotin-labeled by the methods described by Chang et al. [23]. The plates were incubated at 37 °C for 30 min. After washing the plates, horseradish peroxidase-labeled avidin (Sigma, St. Louis, MO, USA) was diluted in blocking buffer at 1:1000 and 50 μl of the diluted solution was added to each well. After incubation at 37 °C for 30 min, the plates were washed and 100 μl of prepared TMB substrate solution (TMB Microwell Peroxidase Substrate; Kappel, USA) was added to each well. Following incubation for 5 min at room temperature, 100 μl of stop solution (1 M H_2SO_4) was added to each well and OD values were determined.

Minor nonspecific reactions were observed in the control wells without serum and antigen. In the control wells without antigen, some of the OD values were slightly higher than those with the antigen; however, the values were all lower than 0.06 and neared 0. The cutoff point was estimated as the sum of the average OD values of the control wells without serum and antigen and an additional factor of 0.1 in accordance with the procedures for JEV [22]. According to the above criteria, when antiserum was used at the highest dilution in a checkerboard titration (1:100), the end point titer of antigen was determined at 1:800 (a putative 1 unit). We examined 10 negative control samples at a dilution of 1:25–1:1600 using 1:100 diluted antigen (as a putative 8 units). A cutoff value of 0.23 was set according to the following calculation: mean each wells plus (S.D.) × 3. According to these criteria, when the antiserum was used at a dilution of 1:100 in the checkerboard titration, the end point titer of antigen was determined at 1:800. Therefore, the YOKV antigen was used at 1:100 (8 units) for the LAB-ELISA assay.

2.7. Neutralization test

Neutralization tests were performed using the microtiter method. One hundred $TCID_{50}$ of virus was incubated with twofold serially diluted serum samples and added into Vero cells seeded in a 96-well plate. The titer of neutralization antibody was determined based on the highest serum dilution, which completely suppressed a cytopathic effect.

2.8. Experimental infection

Leschenault's rousette bats were obtained from zoos in Japan. These fruit bats were housed in separate cages in an air-conditioned room. The animals were fed several kinds of

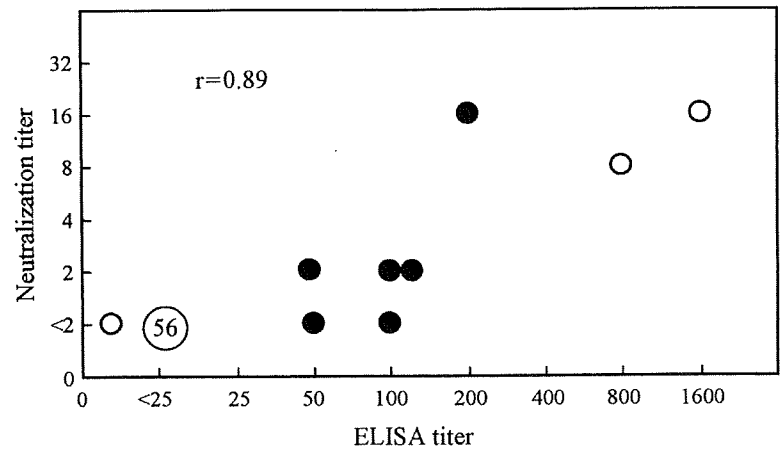

Fig. 2. Comparison of YOKV antibody titers in bat serum samples collected from the Philippines and Malaysia (close circle), and immunized bats. Titers were determined using ELISAs and NT tests; two positive and one negative serum samples were collected from immunized and control bats (open circle). The number in the circle indicates the number of serum samples. The correlation coefficient was calculated using the field sample titers.

fruit with free access to water. Serum samples were collected from the orbital sinus under anesthesia by diethylether.

Nine of the seronegative bats were randomly selected for experimental infection and placed in a negative-pressure isolator. The bats were inoculated intraperitoneally with 1 ml of solution containing 10^7 $TCID_{50}$/ml of YOKV. The bats were separated into three groups, each containing three bats. One group each was sacrificed by cardiac puncture on days 2, 4, and 7 postinoculation following anesthesia by a 1.5 mg intraperitoneal injection of ketamine hydrochloride. The experiment was conducted according to the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agriculture and Life Sciences, the University of Tokyo. Serum samples were obtained via whole blood by centrifugation at 3000 rpm for 10 min at 4 °C. Organs (liver, kidney, spleen, lung, and brain) were also collected. During the experimental infection, bats were examined daily for clinical symptoms of infections. Urine and fecal specimens were collected using a clean translucent plastic sheet spread along the bottom of the cage. Virus isolation was attempted from these samples (i.e., organs, serum, urine, and feces). Each sample was homogenized in DMEM as 10% suspensions and assayed for viral titers using $TCID_{50}$ on Vero cells. Each sample was also tested using RT-PCR to detect YOKV RNA. Finally, serum samples were tested using NT and ELISA.

2.9. Detection of viral genome by RT-PCR

Viral RNA extraction was performed on samples obtained from infected bats using an SV Total RNA Isolation System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. SuperScript™ One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA, USA) was used for RT-PCR. The primer set (5′-ATAAGACAGCCAACCATTGC-3′ and 5′-TATCCGGCAAATCCAATCAC-3′) was targeted to a 320-bp fragment of the envelope gene and designed to be specific to YOKV according to a prior report [24].

3. Results

3.1. Immunization

To obtain sera positive for anti-YOKV antibodies, two bats were immunized with inactivated and purified virus. Antibodies were first detected in Bat 2 with a titer of 1:25 on day 14 postinoculation. Anti-YOKV antibodies reached the peak titer (1:800 in Bat 2 and 1:1600 in Bat 1) a week after the second inoculation on day 35 (Fig. 2). On day 35, the bats were killed by intracardiac exsanguination under anesthesia. Neutralizing antibodies were also tested on days 0 and 35. We could not obtain sufficient serum volumes to perform the neutralizing test for days 14 and 28. Neutralizing titers were found to be low on day 35 (1:8 in Bat 2 and 1:16 in Bat 1).

3.2. Serological survey

Of the 60 bat serum samples collected in the Philippines, 36 had sufficient volumes and quality for analysis. Twenty-six additional samples were supplied by Dr. A. Rayari (University of Malaysia, Sarawak) and Dr. T. Imada (JICA project leader at the Veterinary Research Institute). Sera were screened at a dilution of 1:25 and each sample was tested three times by ELISA. Of the 62 serum samples tested, 6 (9.6%) were determined to be positive in the ELISA assay; ELISA titers of each serum sample were determined. Neutralization tests (NTs) were also conducted using collected bat sera at a concentration of 1:2. Of the 62 samples, five were determined to be positive. Although the titers of these five samples were also determined, they were low.

Yokose antibody titers obtained by ELISA were compared with those obtained by NT (Fig. 2). Close correlations existed between ELISA and NT titers ($r = 0.89$).

3.3. Cross-reactivity of Japanese encephalitis virus antigen

Serological tests used for flaviviruses, such as ELISAs or fluorescent antibody techniques, have been reported to show high cross-reactivity with other flaviviruses [25,26]. Therefore, to check the specificity of the YOKV ELISA, an ELISA with the JEV antigen substituted for YOKV antigen was conducted. ELISA titers of the JEV antigen were determined using positive sera from Bat 1. The heterologous titer using the JEV antigen was 1:50 whereas the homologous titer (using the YOKV antigen) was 1:1600. Therefore, we found that the homologous titer was 32 times higher than the heterologous titer. ELISA with the JEV antigen was also conducted using the bat serum samples described in Section 3.2. Titers of all the samples were less than 1:25. Furthermore, the serum samples collected from bats which were obtained from Japan Zoos were also screened by ELISA using the JEV antigen. Titers of all samples were less than 1:25.

3.4. Experimental infection

An ELISA was used to exclude bats that were positive for antibodies against YOKV. Fourteen percent of the fruit bats (Leschenault's rousette bats) collected from several zoos

Table 2
Prevalence of ELISA antibodies in fruit bats obtained from zoos

ELISA titer	No. of sera
100	3 (4.6%)
50	3 (4.6%)
25	8 (12%)
<25 (=neg.)	50 (78%)

Table 3
Results of RT-PCR and virus isolation from sera, tissue samples, urine, and feces

Days after inoculation	No. bats tested	RT-PCR for YOKV (real time PCR)							Virus isolation
		Sera	Liver	Kidney	Lung	Brain	Spleen	Urine and feces	
2	2	–	–	–	–	–	–	–	–
	1	–	+	–	–	–	–	–	–
4	3	–	–	–	–	–	–	–	–
7	3	–	–	–	–	–	–	–	–

(+) Positive; (–) negative.

in Japan had antibodies against YOKV (Table 2). All of the bats seropositive against YOKV were obtained from zoos in the western portion of Japan. Nine of the seronegative bats were selected and experimentally infected with YOKV. During experimental infection, none of the bats inoculated with YOKV showed clinical signs of infection. Viral particles were not recovered from any of the collected samples. Viral genome amplification was not detected in any of the samples including sera, organs (brain, heart, kidney, liver, lung, and spleen), feces, and urine; however, viral RNA was detected in the liver of one bat that was killed 2 days after inoculation (Table 3).

Sera collected from the infected bats were tested using a NT (Table 4). NT titers of six preinoculated serum samples were less than 1:4. For the remaining three samples, NT was

Table 4
Neutralizing titers of the bats experimentally infected with YOKV

Bat no.	Days after inoculation			
	Pre	2	4	7
1	<4	<4		
2	<4	<4		
3	–	<4		
4	<4	–	8	
5	<4	–	<4	
6	–	–	4	
7	<4	–	–	4
8	<4	–	–	8
9	–	–	–	4

(–) Not tested.

not performed due to insufficient volumes of serum. No NT antibodies were detected in sera obtained 2 days after inoculation. On days 4 and 7 postinoculation, NT antibodies were detected in all samples except for one bat killed on day 4, and the NT titers were less than 8.

4. Discussion

We developed an ELISA system using biotin-labeled anti-bat-IgG rabbit serum to detect antibodies against YOKV in bat sera and conducted serological surveys using this system. One of the 36 samples collected from the Philippines and five of the 26 samples from Malaysia had detectable antibodies against YOKV. These results suggest that YOKV is distributed not only in Japan, but also in other Asian countries. The possibility exists that antibodies detected in this survey were against other flaviviruses, since antibodies against flaviviruses have been reported to show high cross-reactivity with other flaviviral antigens [26]. Thus, we conducted an ELISA in which we substituted the JEV antigen to confirm assay specificity. The ELISA using the JEV antigen was found to react with the positive serum against YOKV from the immunized bat; however, the titer against JEV was much lower than the homologous titer using YOKV antigen. We also tested field samples using an ELISA with the JEV antigen, and all samples were negative (data not shown). Moreover, ELISA and NT antibody titers from both field samples and positive sera from immunized bats showed a close correlation. These data suggest that the antibody detected in this survey was specific to YOKV.

Serological surveys of several viruses have been conducted using bat sera collected in the field; however, most of the surveys were performed using a NT or fluorescent antibody tests [2–5,27]. Obtaining sufficient volumes of blood necessary for these serological tests is difficult, particularly in smaller species including microbats. Moreover, these assays are not suitable for testing large numbers of samples at one time. Therefore, an ELISA is a powerful tool that can be helpful in serological surveys of infected bats. However, no known conventional ELISAs are available, except assays using protein G or competitive techniques with monoclonal antibodies [1,28]. In this study, we demonstrated that a conventional ELISA using biotin-labeled anti-bat IgG rabbit sera was able to detect antibodies in bat sera obtained from field studies.

From complete nucleotide sequence analyses of YOKV, Tajima et al. [11] suggested that YOKV belongs to the mosquito-borne group. Thus, to reveal whether bats serve as an amplifying host for YOKV, we conducted an experimental infection study of YOKV in bats and examined viral growth and pathogenicity. Since YOKV was originally isolated from a species of microbat, *M. fuliginosus*, it would be preferable to conduct experiments on viral characteristics of YOKV not only with fruit bats but also with *M. fuliginosus* or other microbats. However, maintaining and feeding microbats, especially insectivorous bats, is difficult. Therefore, we conducted our experimental infection studies on the fruit bat *R. leschenaultia*. Prior to experimental infection, we performed an ELISA to exclude fruit bats that had been previously exposed to (i.e., were positive for) antibodies against YOKV. Fourteen percent of the fruit bats collected from several zoos in the western portion of Japan were positive for YOKV antibodies. These positive sera against YOKV were also tested using an ELISA with the JEV antigen. All samples were negative. Given

that Leschenault's rousette bats have been bred in each zoo, and reared separately in open-air cages, it seems likely that these antibody positive bats had been exposed to YOKV at the zoos themselves. Although no accounts of viral isolation or antibody detection of YOKV have been reported since the initial one in 1971, YOKV appears to be present in Japan.

No clinical signs of disease were observed in fruit bats following viral infection. Moreover, significant viral genome amplification was not detected in any of the samples, except for one liver sample obtained from a virus-inoculated bat killed at day 2 postinoculation. No viral particles were isolated from any of the samples and antibody responses were low. These results reveal that YOKV replicates poorly in Leschenault's rousette bats, and might suggest that fruit bats do not serve as an amplifying host for YOKV. Our results from the serological field survey demonstrate a low prevalence of YOKV in bats from the Philippines and Malaysia, further supporting this suggestion. YOKV may have additional amplifying hosts besides bats, such as mosquitoes. To confirm the viral pathogenicity in microbats and also the relationship between YOKV and mosquitoes, further studies are needed. Although no cases of YOKV infection have been reported in other animals, a single human case of febrile illness, possibly caused by Sepik virus, has been published [29]. Interestingly, Sepik virus exhibits high nucleotide sequence similarities with YOKV. Further studies are necessary to more fully elucidate the pathogenicity of YOKV.

Acknowledgments

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NOTE *Immunology*

Molecular Cloning and Expression Analysis of Bat Toll-Like Receptors 3, 7 and 9

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ABSTRACT. In this study, cDNA of Toll-like receptors (TLR) 3, 7 and 9 were synthesized and completely sequenced. The coding regions of cDNA for bat TLR3, TLR7 and TLR9 were 2,718, 3,150 and 3,090 bp in length, respectively. The open reading frames encoded 905, 1,049 and 1,029 amino acids for TLR3, TLR7 and TLR9, respectively. The nucleotide sequences, predicted amino acid sequences and predicted domain structures of the three bat TLRs had high homology with those of other mammals. In addition, the expression profiles of each TLR in main organs were analyzed. Expression of TLR3 was highest in the liver, whereas the expressions of TLR7 and TLR9 were highest in the spleen.

KEY WORDS: chiroptera, expression analysis, molecular cloning, sequencing, toll-like receptor.

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Infectious diseases transmitted by bats have been increasing recently. These diseases include rabies, Ebola hemorrhagic fever, Nipah virus infection and probably SARS-corona virus infection [4, 10, 16, 17]. In addition, some virus inoculation experiments in bats suggest that many of the bat-associated pathogens cause no clinicopathology in the bats themselves or cause less damage than is seen in other animals [6, 8, 13, 15]. Although many studies on viruses carried in bats are under way, little is known about the emergence of these viruses and their interactions with the host.

The current study is the first analysis of the cDNA sequences of bat Toll-like receptors (TLR) 3, 7 and 9 and their mRNA expressions in various bat organs. TLRs recognize structurally conserved molecules derived from microbes that have breached physical barriers and play a key role in the innate immune system [1]. Among the TLR classes, TLRs 3, 7, 8 and 9 are involved in virus detection. For example, TLR3 detects double-stranded RNA synthesized during viral genome replication of a single-stranded RNA virus. TLR7 and TLR8 bind single-stranded RNA, and TLR9 recognizes unmethylated cytosine-phosphate-guanine motifs common to both bacterial and viral genomic DNA [14]. In addition, only TLR3, TLR7 and TLR9 induce type 1 interferon during viral infection [14].

Leschenault's Rousette bats (*Rousettus leschenaulti*) were housed under controlled conditions using an air conditioner and a moisture chamber. The animals were kept in steel cages and fed fruit and water at the same time every day. All experiments were performed in accordance with the Animal Experimentation Guidelines of the University of Tokyo and approved by the Institution Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, University of Tokyo.

Fresh spleen samples were collected from bats anesthetized with diethyl ether. The spleens were quickly preserved in RNAlater (Ambion, Austin, TX, U.S.A.) and frozen at −80°C until use. Total RNA was isolated from the bat spleen with TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. The primary cDNA was synthesized by reverse transcription with Superscript®III reverse transcriptase (Invitrogen) using an Oligo $(dT)_{12-18}$ primer (Invitrogen), followed by PCR with Thermo-Start® Taq DNA Polymerase (ABgene). In order to clone cDNA from the coding region of bat TLR mRNA, RT-PCR was performed using primer sets designed from the ortholog sequence data of the equine, swine and feline, which are available in the GenBank nucleotide database. The primers were as follows: TLR3 f1, 5'-AACTCCATCCAAAGCTGGAGCCAGAA; TLR3 r1, 5'-GCTATGTTGTTGTTGCTTAGATCCAGAATG; TLR3 f2, 5'-CTGGATCTAAGC AACAACAACATAGCCAAC; TLR3 r2, 5'-ACCGAGAA CTCGATGCACTGAAACATTCCA; TLR7 f1, 5'-CTAGATGGTTTCCTAAAACTCTGCCCTGTG; TLR7 r1, 5'-CATTATAACAACGAGGGCAATTTCCACT; TLR7 f2, 5'-AACTTCTTGGCCAAAGAAATTGGGGATGC; TLR7 r2, 5'-TGAGTAATTCCTTCTGATTGAAAATA; TLR7 f3, 5'-ACTGTCCCTGAGAGATTATCCAACTGTTCC; TLR7 r3, 5'-TTGCTAAGCTGTATGCTCTGGGAAAG; TLR9 f1, 5'-CTGACGCCTGAGGACCTGG CCAATCTGAC; TLR9 r1, 5'-CAGGTGGGCAAAGGACACCTTCTTGTGGTA; TLR9 f2, 5'-AGCTGGACATGCATGGCATCTTCTTCCGCTC; TLR9 r2, 5'-GCCATTGCTTAGGGCCTTCAGCTGGTTTCC; TLR9 f3, 5'-ACCTGCGCCTCTGCCTGGATGAGGCCCTCT; and TLR9 r3, 5'-GGTTATAGAAGTGGCGGTTGTCCCTGGTCA. The amplified products were cloned into plasmids by using a TOPO TA Cloning® Kit (Invitrogen),

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and the inserts were confirmed by DNA sequencing (Applied Biosystems). To determine the remaining 5'- and 3'-terminal gene sequences, both 5' and 3' methods of rapid amplification of cDNA ends (RACE) were performed using a 5'-Full RACE Core Set (Takara, Tokyo, Japan). Additional specific primers were designed based on the first decoded sequences to confirm the bat TLR nucleotide sequences. The TLR gene specific primers were as follows: TLR3 F1, 5'-CTCCACTTACAAGACGAGGAAACT; TLR3 R1, 5'-GTTCTGTATAGCTGGGTGTTGCT; TLR3 F2, 5'-CCCAGTCTCA TAGAGAAGCTTTG; TLR3 R2, 5'-ATAAGTCCTTGAAGACCTCTGCTG; TLR3 F3, 5'-TCTTTCTCATCTCCATGTCCTTAAC; TLR3 R3, 5'-GAGAC GTATTTCCATAGAAGAGAGAG; TLR7 F1, 5'-ATGC TCTGTTCTCTTCAACCTGAC; TLR7 R1, 5'-TTGGGAAAGATCTAGTTCCCTAAG; TLR7 F2, 5'-TTACGT CTACATAGCAACTCCCTTC; TLR7 R2, 5'-CCCAATTAAAAGACTTGAGCCC; TLR7 F3, 5'-GGTATGCCTCCAAATCTAAAGACTC; TLR7 R3, 5'-GAACATTCTTG GTGAGACATCTTTG; TLR9 F1, 5'-ACTCCCTGTCATGGGCCCTTGCCATGGTGC; TLR9 R1, 5'AGTTC ATCTGCAGACTCAGGTTG; TLR9 F2, 5'-CAAACTCA ACCTGTCCTTCAATTAC; TLR9 R2, 5'-ATTGAGCTGCCGCAGATTCCT; TLR9 F3, 5'-CTGCTGAAGGCC CTGACCAAC; and TLR9 R3, 5'-GGCTGTCCGCCGTGCTATTC. The lengths of the PCR products were 891, 1,054, 1,276, 1,021, 1,198, 1,222, 1,214, 1,177 and 1,049 bp, respectively. Sequence analysis was conducted using an ABI 3130/3130xl Genetic Analyzer (ABI). The cDNA sequences of bat TLR3, TLR7 and TLR9 were deposited in the GenBank database (Accession numbers: AB_472355, AB_472356 and AB_472357). The coding regions of cDNA for TLR3, TLR7 and TLR9 were 2,718, 3,150 and 3,090 bp in length, with open reading frames encoding 905, 1,049 and 1,029 amino acids, respectively. The sequences of the nucleotides and their predicted amino acids share very high homology to the TLRs of other mammals. Bat TLR3, TLR7 and TLR9 had 85.5 to 89.7%, 88.2 to 90.5% and 80.6 to 82.4% sequence homology, respectively, with other mammals. The predicted amino acid sequences of TLR3, TLR7 and TLR9 were 82.8 to 88.5%, 85.7 to 88.0% and 74.3 to 7.2% homologous with those of other mammals, respectively (Table 1). Like other mammalian TLRs [5, 11, 18, 19], bat TLR3, TLR7 and TLR9 mRNA nucleotide and predicted amino acid sequences showed high similarities to those of other mammalian orthologs. The phylogenic tree generated by comparing bat TLR amino acid sequences and those of other animals showed that bat TLRs are closely related to equine and carnivore TLRs (data not shown), which supports the findings of a previous study [9]. Nucleotide and predicted amino acid sequences were analyzed by GENETYX-WIN (Ver 4.0, Software Development Co., Ltd., Tokyo, Japan). The accession numbers of the nucleotide data used for homology analysis were as follows: TLR3, NM_001081798, XM_540020, NM_001008664, NM_003265; TLR7, NM_001081771, NM_001048124, NM_001033761, NM_016562; TLR9, NM_001081790, NM_001002998, NM_183081, NM_017442. Bat TLR3, TLR7 and TLR9 domain structures were compared with those of other mammalian species orthologs using the SMART [12] and NCBI BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results showed that bat TLR3, TLR7 and TLR9 have highly conserved leucine-rich repeat (LRR) and Toll/Interleukin-1 receptor (TIR) domains that play critical roles in innate and acquired immunity [2, 3] (data not shown).

Table 1. CDS length of the TLR cDNA and sequence similarities

	CDS lengths (bp)	Nucleotides (%)	Amino acids (%)
(A) TLR3			
Chiroptera	2,718	–	–
Equine	2,715	89.7	88.5
Canine	2,718	88.6	85.9
Bovine	2,715	85.5	82.8
Human	2,715	86.6	84
(B) TLR4			
Chiroptera	3,150	–	–
Equine	3,153	90.5	87.9
Canine	3,153	88.2	88
Bovine	3,177	88.7	87
Human	3,150	88.2	85.7
(C) TLR9			
Chiroptera	3,090	–	–
Equine	3,096	82.3	77.2
Canine	3,093	82.4	76.6
Bovine	3,090	81.6	75.6
Human	3,099	80.6	74.3

In addition, semi-quantitative RT-PCR was performed to assess the mRNA expression ratios of TLR3, TLR7 and TLR9 in various organs. Eight tissues or organs (kidney, liver, spleen, brain, ovary, small intestine, lung and heart) were obtained from young female bats (n=5). The respective procedures described above were used to process each organ and synthesize the template cDNA used in the semi-quantitative RT-PCR. The expression of beta-actin mRNA was used as an internal control. The primers used in semi-quantitative RT-PCR were as follows: TLR3 quantitate F, 5'-AGTTGACTCAAATACCTGACGACC; TLR3 quantitate R, 5'-CAAGTTCTTCACGGCTTAGTACG; TLR7 quantitate F, 5'-CAAAGAGACTCCTTCTTCCTTGAG; TLR7 quantitate R, 5'-CTAAGAGATTCACTCTCCATGGTCC; TLR9 quantitate F, 5'-CTACTACAAGAACCCGTGCGAC; TLR9 quantitate R, 5'-GTACAGAAAGTTCTCACTCAGGTCC; beta-actin F, 5'-ATTGTGCGTGACAT CAAGGAGAAG; and beta-actin R, 5'-ATTCCTGCTTGCTGATCCACATCT. PCR cycles were conditioned in which growth curves of all organs PCR products run on mid-logarithmic phase. The PCR products were analyzed in 2% agarose gel, and DNA band intensities were quantified using the Scion Image software (Scion Cor-