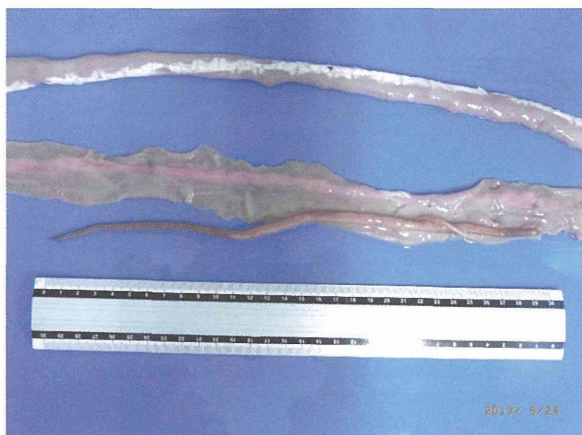
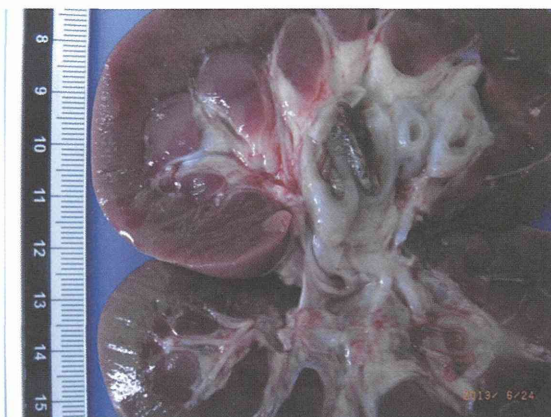
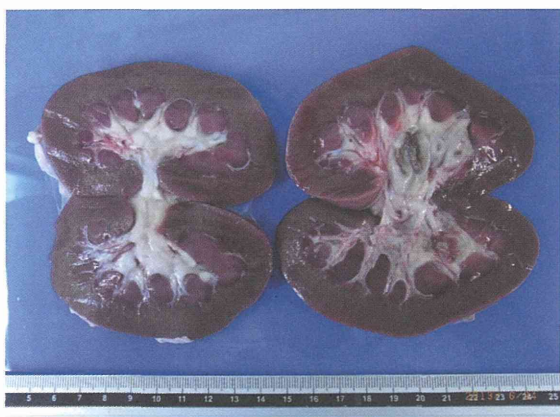


回虫(雌雄成虫)  
イノシシ・小腸



豚腎虫  
イノシシ・腎臓腎盂

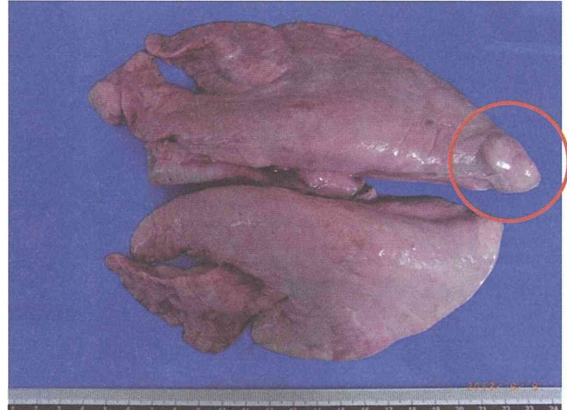


# イノシシ

鞭虫(大腸内容)

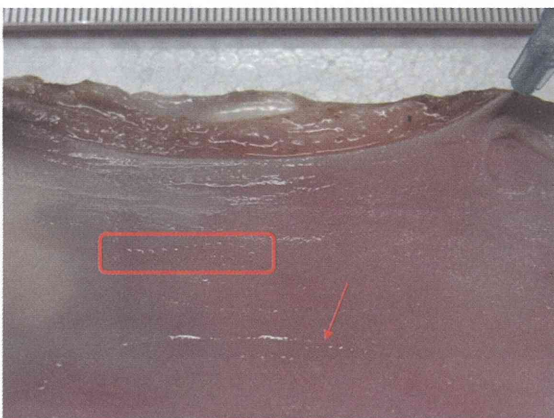


寄生虫性気腫(肺)



# イノシシ

食道虫



有齒豚胃虫





# 肝蛭 シカ・肝臓



# シカ肉処理の注意点 1 —ウイルス・細菌—

前田 健

## 要 約

シカに限らず野生動物が健康であるとは限らないし、健康に見えても多くのウイルス・細菌などの病原体を保有している。外見に異常が認められるものは何らかの病気を持っている可能性が高いので食用にすべきではない。狩猟の際の血液の混入が激しい個体は、血液には病原体が存在する可能性が高いので食用にすべきではない。また、細菌の増殖を防ぐために、捕獲後は速やかに解体し、冷蔵保存すべきである。解体の際には、腸内には多くの細菌が存在するため、腸の内容物の混入に注意する。さらに、臓器の異常にも注意をする必要がある。臓器に結節、白斑、出血痕などが認められれば、何らかの異常が生じていることを意味する。調理する際には、充分加熱するとともに、他の食材を同時に扱わないことが重要である。最後に、最も重要なことは“生食は厳禁”である。本稿では、シカ由来の人獣共通感染症として問題となる E 型肝炎、腸管出血性大腸菌感染症、サルモネラ感染症、結核に焦点を絞り、これまでの事例を紹介し、問題点を解説した。

## はじめに

シカにおける感染症については未だ不明な点が多い。しかし、シカから人に感染する感染症は幾つか知られており、

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多くの研究者の御協力を得て野生動物から伴侶動物まで広く浅くウイルス感染症の調査研究を行っております。One World, One Healthのもと、獣医学の役割が大きくなっています。何らかの形で貢献していきたいと考えています。

注目を浴びた例も幾つか報告されている。本項ではシカ由来で問題となっている E 型肝炎、腸管出血性大腸菌感染症、サルモネラ感染症、結核に焦点を絞り紹介する。また、対策が重要であるが、ウイルス・細菌に限ったことではなく、毛づやが悪い個体、死亡してから時間がたった個体、病変らしきものが認められる個体は食用しない、生食は避ける、血液や腸内容物の汚染を避ける、など基本的な対策が最も効果的である。また、喫食する際には、十分に火を通すことが重要である。基本的に、牛や豚の生肉や生レバーから発生する食中毒があるように、シカも同様に汚染源となり得ると考えるのが重要である。

## E 型肝炎

### 1) シカ肉由来の E 型肝炎

2003 年 4 月に兵庫県で冷凍シカ肉を喫食した 2 家族 7 名中 4 名が発症し、急性期の血清から E 型肝炎ウイルス (HEV)-IgM 抗体および HEV 遺伝子が検出された。冷凍シカ肉残品から検出された HEV の遺伝子配列が患者から検出された HEV 遺伝子のものとほぼ一致した。これは食品の摂食と E 型肝炎の発症との直接的な関係が確認された世界初の事例である<sup>1)</sup>。しかし、わが国の野生シカにおいては、抗体をもつ個体は極めて少数であり、HEV の保有動物とは考えにくい。しかし、シカ肉から人への直接伝播が報告されているので注意が必要である。HEV の主な保有動物としては豚であると考えられているが、野生動物ではイノシシ肉からの感染が多く報告されている。

### 2) E 型肝炎とは？

HEV 感染によって引き起こされる急性肝炎（稀に劇症肝炎）である。慢性化することはない。HEV は主として食肉や飲水を介して経口感染する。開発途上国に常在し散



表1 E型肝炎患者数

年	患者数
平成11年	0
平成12年	3
平成13年	0
平成14年	16
平成15年	31
平成16年	41
平成17年	43
平成18年	71
平成19年	56
平成20年	44
平成21年	56
平成22年	66

感染症発生動向調査より

発的に発生している疾患であるが、時として汚染された飲料水などを介し大規模な流行を引き起こす。一方、先進国においては、食肉を介した人獣共通感染症である。

### 3) 症 状

不顕性感染（感染しても症状がでない）が多い。発症した場合は、高率に黄疸を伴う。平均6週間の潜伏期（感染してから発症までの間）の後に、発熱、悪心・腹痛等の消化器症状、肝腫大、肝機能の悪化が現れ、多くは安静を保つことにより治癒する。しかし、まれに劇症化する。E型肝炎の特徴として、妊婦が妊娠晩期に感染すると劇症化しやすいといわれており、高齢者ほど重症化しやすい。

### 4) 感染経路

感染経路は経口感染であり、HEVに汚染された食物、水等の摂取により感染することが多い。人から人への感染は、稀である。

### 5) 予 防

手洗い、飲食物の加熱が重要である。動物の内臓、特に豚レバーを食べる際には、中心部まで火が通るよう十分に加熱することが重要である。

### 6) 患者の発生状況

患者の発生は平成14年以降徐々に増えている。男性が女性と比較して圧倒的に多い（表1）。

## 7) E型肝炎ウイルスについての見解

シカ肉によるE型肝炎の発生は少ない。しかし、実際に、感染例もあることから注意を要する。野生動物に関してはイノシシ肉が汚染源となることが多い。解体する際には血液の混入に注意する。また、生食は絶対してはならない。我々の調査でも5%前後のイノシシの血液からウイルスが検出されている。

## 腸管出血性大腸菌感染症

### 1) 国内シカ肉由来腸管出血性大腸菌感染症例

平成21年6月に群馬県で捕獲された野生のシカ肉を生そのまま喫食した茨城県の10名のうち1名が、血便、腹痛、嘔吐等の食中毒症状を呈して、入院した。患者の便およびシカ肉の残りから腸管出血性大腸菌O157が検出された。そのため、シカ肉による腸管出血性大腸菌感染症と断定された。

平成13年、大分県内で購入したシカ肉（生食用）を持ち帰り、福岡県内の自宅で喫食した5歳の女の子が発症した。その後、同様に喫食した家族からも腸管出血性大腸菌O157が検出された。大分県においてシカ肉を検査した結果、12検体中5検体から腸管出血性大腸菌O157が検出された。最初に発症した女の子、自宅にあったシカ肉の食べ残し、大分県で検査されたシカ肉から分離された菌株は全く同じDNAパターンを示した。このことから、腸管出血性大腸菌O157に汚染されたシカ肉（生食用）が原因であると断定された。

### 2) 米国の自家製シカ肉ジャーキーによる感染例

自家製シカ肉ジャーキーを食べた6名が腸管出血性大腸菌感染症と確定診断され、5名が腸管出血性大腸菌感染症と疑われる食中毒事例が発生した。患者、ジャーキーの残り、同じシカの調理前の肉、シカの死体を解体するためののこぎりから腸管出血性大腸菌O157:H7が分離された。近隣の森のシカの糞からは32検体中3検体の9%からO157:H7が分離されている。また、実験室で様々な温度と時間で乾燥させたO157を接種したシカ肉からも菌が分離され、62.8℃10時間で乾燥させても大腸菌が生きていることが証明された。結論としては、腸管出血性大腸菌

表2 腸管出血性大腸菌感染症患者の発生状況

年	患者数
平成 11 年	3,115
平成 12 年	3,652
平成 13 年	4,436
平成 14 年	3,186
平成 15 年	2,998
平成 16 年	3,760
平成 17 年	3,594
平成 18 年	3,922
平成 19 年	4,617
平成 20 年	4,329
平成 21 年	3,879
平成 22 年	4,135
平成 23 年	3,900

感染症発生動向調査より

O157:H7 はシカに常在しており、人の感染源となることが証明された。野生動物の肉を処理する際には、牛などの食肉処理と同様の注意が必要である<sup>2)</sup>。

### 3) 腸管出血性大腸菌感染症とは？

赤痢菌の毒素と類似の毒素を産生する大腸菌による食中毒である。腹痛、出血を伴う水様性の下痢などを発症する。重症化し、死に至ることもある。本菌の特徴は少数で発症するため、二次感染が起きやすい。また、この菌は強い酸抵抗性を示し、胃酸の中でも生残する。

腸管出血性大腸菌は血清型によりさらにいくつかに分類され、本食中毒の原因となっているものは、血清型 O157 が主であるが、この他に O26, O111, O128 および O145 などがある。

### 4) 症 状

O157 感染症では、全く症状がないものから軽い腹痛や下痢のみで終わるもの、さらには頻回の水様便、激しい腹痛、著しい血便とともに重篤な合併症を起こし、時には死に至るものまで様々である。多くの場合、3～5日の潜伏期をおいて頻回の水様便で発病する。さらに、激しい腹痛を伴い、まもなく著しい血便となることがある（出血性大腸炎）。有症者の6～7%では、症状がでたのち約5～7日後に、溶血性尿毒症症候群（HUS）または脳症などの重症合併症が発症する。なお、激しい腹痛と血便を認めた場合は、その数日後にこれらの重症合併症を起こすことがあ

り、特に注意が必要である。

### 5) 感染経路

感染経路は経口感染である。動物の腸管内に生息し、糞尿を介して食品、飲料水を汚染する。少量でも発病する。加熱や消毒処理には弱い。

#### <過去の原因食品>

井戸水、牛肉、牛レバー刺し、ハンバーグ、牛角切りステーキ、牛タタキ、ローストビーフ、シカ肉、サラダ、貝割れ大根、キャベツ、メロン、白菜漬け、日本そば、シーフードソース、ハンバーガー、ミートパイ、アルファルファ、レタス、ハウレンソウ、アップルジュースなど。

### 6) 予 防

食肉は中心部までよく加熱する（75℃、1分以上）。野菜類はよく洗浄。と畜場の衛生管理、食肉店での二次汚染対策を十分に行う。低温保存の徹底。

### 7) 腸管出血性大腸菌感染症についての見解

本細菌の特徴は少数でも食中毒を引き起こす点である。肉は加熱すれば大丈夫であるが、生で食べる野菜などに細菌がついた場合は問題となる。生肉を処理した際の包丁・まな板・箸などはよく洗ってから、あるいは消毒薬で消毒してから使用すべきである。牛肉での生食が制限され、生レバーが規制されようとしている。これは腸管出血性大腸菌感染症がその発端となっている。シカと牛での感染率の比較はできないが、ほぼ同様だと考えている。シカ肉に限らず野生動物肉の生食は避けるべきである。また、ジャーキーでの発生例は、本菌は熱に弱い、中途半端な温度だと死滅しないことを意味している。加熱に関しては75℃1分以上が必要である。

## サルモネラ感染症

### 1) サルモネラ感染によるニホンジカの死亡例

国内の公園で飼育されていたニホンジカ 30 頭のうち 7 頭が死亡した。3 頭を検査した結果、臓器および腸内容から *Salmonella* Typhimurium が分離され、病理所見ではカタル性腸炎と肝臓の巣状壊死が認められた。免疫組織学検査で小腸病変部に *Salmonella* O4 抗原が検出され、本症例



はニホンジカのサルモネラ感染症と診断された。公園での発生は公衆衛生学的に重要なことから継続検査を実施したところ、治療後の糞便および徹底消毒後の土壌からは本菌は分離されなかった<sup>3)</sup>。

## 2) 野生のシカにおけるサルモネラの保有率と血清型

米国のネブラスカ州でハンターにより回収されたシカの糞便におけるサルモネラの保有率を調べた。その結果、1%のシカの糞便からサルモネラが分離され、その血清型はLitchfield, Dessau, Infantis, Enteritidisであった。サルモネラ菌の保有率は低かったが、人や動物に病気を引き起こす病原性サルモネラの血清型が存在していた<sup>4)</sup>。

## 3) シカにおけるサルモネラ症の概要

本症は、サルモネラ菌の感染により引き起こされる、急性あるいは慢性の腸炎である。腸炎に加えて流死産や乳房炎を主徴とする。届出伝染病に指定されており、人獣共通感染症である。

生後1か月以内の若齢動物は感受性が高く、急性例では1～7日で敗血症により死亡する。感染は保菌動物の糞便中に排泄された菌の経口感染が主体である。菌は腸管内で増殖し、腸炎を起こす。菌は体内で長く生存する。乳汁や膾分泌物にも菌が排泄され、排泄された菌は数か月間生存し、感染源となる。糞便に汚染された敷きわら、飼料、飲料水なども感染源となる。さらに、ネズミ、鳥類なども汚染・感染源となり、牧場などにおける菌の清浄化は多くの場合は困難である。

急性・慢性の下痢、重篤な例では菌血症や敗血症を呈する。急性例では発熱、食欲減退、悪臭のある黄色下痢便・粘血便、やせ、脱水などがみられる。成熟動物では症状を示さない例が多い。妊娠動物では流死産がみられる。

予防には発症動物や保菌動物の隔離、感染動物の淘汰などが行われる。

## 4) 人のサルモネラ症

サルモネラの臨床症状は多岐にわたるが、最も普通に見られるのは急性胃腸炎である。通常8～48時間の潜伏期を経て発病するが、Enteritidis感染では3～4日後の発病も珍しくない。症状はまず悪心および嘔吐で始まり、数時間後に腹痛および下痢を起こす。下痢は1日数回から十

数回で、3～4日持続するが、1週間以上に及ぶこともある。小児では意識障害、痙攣および菌血症、高齢者では急性脱水症および菌血症を起こすなど重症化しやすく、回復も遅れる傾向がある。

予防には原因食品、特に食肉および鶏卵の低温保存管理が重要である。また、調理時および調理後の汚染防止が基本である。

## 5) サルモネラ感染症に関する見解

シカの腸内にも病原性の強いサルモネラ菌が存在しており、シカでの死亡例もある。腸内容物が混入することのないように解体時の注意が必要である。また、腸管出血性大腸菌感染症に対する対応で、サルモネラ感染症も予防することができる。注意すべきは、捕獲したシカおよび処理したシカ肉をできるだけ速やかに低温保存することである。万が一、菌が肉に付着しても増殖を避けることができる。

## 結 核

### 1) シカの結核感染例

2009年10月と1月に大阪府内の動物園で6頭および13頭の結核に感染したシカが見つかった。そのため、動物園では全てのシカを家畜伝染病予防法に基づき安楽死を実施した。シカが感染したのは牛型結核菌であった。

1984年秋田県内の動物園で飼育されていたニホンシカが結核で死亡し、このシカから牛型結核菌 *Mycobacterium bovis* が分離された。同居群16頭についてツベルクリン・テストによる *M. bovis* の浸潤調査を実施した。このうち7頭がツベルクリンに反応し、これらのツベルクリン反応例すべてに結核病巣が確認された。*M. bovis* は、石灰化病変の強かった1例を除く6頭の病変部から分離された<sup>5)</sup>。

1988～1990年までの3年間に、青森県内の養鹿場で東南アジア産のキバノロおよびニホンジカ計288頭のうち、47%にあたる136頭が、また自家産の同居していたシカ146頭中66%にあたる96頭が結核病により死亡した。

### 2) シカにおける結核病の概要

本病は、牛型結核菌 *M. bovis* の呼吸器感染による慢性感染症である。結核病巣が主に肺および頸部・胸腔内リン

パ節に形成され、重症例は発咳、呼吸困難などを呈し、法定伝染病に指定されている。シカは本菌に対して特に高い感受性を示し、全身結核により高い死亡率を示す。集団発生例がある。米国では結核病に罹患したシカから食肉性野生動物への伝播が問題となっている。

感染動物の大部分は不顕性感染の状態になり、外見上はほとんど異常ない。しかし、発咳、あくびの多発などの呼吸器症状とやせ、被毛失沢を呈した進行症例では、数週間で死亡する。

新規の動物導入にあたってはツベルクリン反応検査を実施すべきである。患畜発生群については、全頭を対象に年数回のツベルクリン検査を実施し、陽性個体の殺処分とともに疑陽性畜の自主淘汰を行う。感染動物舎はオールアウトし、石灰による徹底的な消毒後に清浄群からの新規導入を図り、汚染動物舎の再使用は避けます。抗菌剤による化学療法は実施しない。

### 3) シカの結核に関する見解

シカの結核に関しては、野生のシカに関する疫学情報は得ていないが、野生のシカにも結核が存在する可能性は高い。肺や肝臓、リンパ節に白い顆粒（結節）が認められる場合は食用すべきではない。また、解体時にも、動物の毛づやなどに注意して、解体する。さらには、臓器の病変の有無の確認は重要である。

### その他の細菌

国内で市販されているシカ肉から検出される人獣共通感染症を引き起こす細菌について以下の報告がある。

1993年12月から1994年4月にかけて13の小売店から30頭のシカ肉について、豚丹毒属菌、エルシニア属菌、リステリア属菌、サルモネラ属菌、カンピロバクター属菌の存在について調べた。豚丹毒属菌は、15サンプルの50%のシカ肉から分離された。腸炎エルシニア菌は10

頭のシカ肉から分離された。しかし、リステリア属菌、サルモネラ属菌、カンピロバクター属菌はシカ肉からは分離されなかった<sup>6)</sup>。

### 最後に

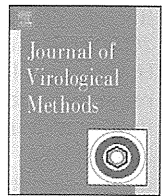
近年、家畜の生肉や生レバーに関する規制が厳しくなっている。シカは牛と同様の病原体を保有しており、イノシシは豚と同様の病原体を保有していると考えることが重要である。ウイルス・細菌だけでなく、多くの病原体が潜んでいる。狩猟者・解体者・消費者は安全に食用することができるように、以下の最低限の注意事項は守るべきである。

- ①毛づやなど、全身症状が悪そうなシカは食用しない。
- ②血液・腸の内容物には筋肉以上に病原体が存在しているので、筋肉への汚染に注意する
- ③捕獲した死体や解体後の肉はできるだけ速やかに冷蔵保存する（冷蔵でも増える病原菌は存在する）。できれば冷凍する（冷凍により死滅する病原体も存在する）。
- ④生食は厳禁。十分（内部まで）加熱する。
- ⑤生肉を処理した調理器具で、他の材料を調理しない。十分な洗浄や消毒を行う。

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## Development and application of an indirect enzyme-linked immunosorbent assay for serological survey of Japanese encephalitis virus infection in dogs

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### A B S T R A C T

#### Article history:

Received 16 February 2012

Received in revised form 6 September 2012

Accepted 10 September 2012

Available online 6 October 2012

#### Keywords:

Japanese encephalitis virus (JEV)

Enzyme-linked immunosorbent assay

(ELISA)

Serological survey

Dog

Japanese encephalitis virus (JEV) causes serious acute encephalitis in humans and horses. Although dogs are good sentinels for assessing the risk of JEV infection to humans, a virus neutralization test has been the only method available for measuring the levels of JEV antibody in dogs. In this study, an indirect enzyme-linked immunosorbent assay (ELISA) using purified viral particles as an antigen, was developed for serological survey of JEV infection in dogs. In dogs inoculated experimentally with JEV, the ELISA detected anti-JEV IgM 3 days after infection, with IgM levels peaking 7 days after infection. Anti-JEV IgG was detected 14 days after infection and peaked on 21–28 days after infection. Virus neutralization titers correlated with anti-JEV immunoglobulins measured by the ELISA. To test the utility of the new assay, the seroprevalence of JEV infection among 102 dogs in Kyushu, Japan, was examined by IgG ELISA and by virus neutralization. The correlation coefficient between the IgG ELISA and virus neutralization was 0.813 ( $p < 0.001$ ); comparison of the IgG ELISA and virus neutralization showed a sensitivity and specificity of 82% and 98%, respectively. The IgG ELISA was used to survey dogs in Bangkok, Thailand and 51% of these dogs were found seropositive for JEV. These data suggest that in the capital city of Thailand, the risk of infection with JEV remains high.

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### 1. Introduction

Japanese encephalitis virus (JEV) is a member of genus *Flavivirus*, family *Flaviviridae*. JEV is transmitted primarily by *Culex tritaeniorhynchus* vector mosquitoes, and pigs play a main role as amplifiers. The virus is widely endemic in nations of Southeast Asia and the Western Pacific, including Japan and Thailand (Mackenzie et al., 2001), with an annual incidence of approximately 50,000 cases and 10,000 deaths in humans (Erlanger et al., 2009).

In Thailand, 1500–2500 JE cases had been reported annually throughout the 1970s and 1980s (Olsen et al., 2010). In contrast, between 2005 and 2010, the annual number of encephalitis cases in Thailand ranged from 322 to 431, including 36–78 confirmed JE cases per year (Thailand Ministry of Public Health). This dramatic reduction in the number of JE cases over the past 20 years has been the result of an effective human vaccination program by the Thailand Ministry of Public Health. However, a recent (2009)

serological survey showed that 39% of pigs are seropositive for JEV in Thailand; the seroprevalence of JEV was especially high (67%) in pigs in Chiang Mai province, which includes the second biggest city of Thailand (Prompiram et al., 2011). In addition, the number of JE cases in Thailand still exceeds those in other countries where a JEV vaccine is used, such as Japan and Korea (Erlanger et al., 2009). However, recent human risk of JEV infection in the capital city, Bangkok, remains unknown.

In Japan, serological surveys for JEV infection among pigs have been performed annually. However, it is unclear whether serological surveys among pigs accurately reflect the risk of JEV infection to humans, since pig farms typically are separated from urban areas in Japan. Therefore, another method for monitoring JEV infection risk in humans in urban areas is necessary. In the previous serological survey of JEV, one fifth of dogs in urban and residential areas in Japan were infected with JEV (Shimoda et al., 2010). In addition, experimental infection of JEV in dogs showed that dogs infected with JEV do not exhibit any symptoms or detectable viremia, although dogs do develop high JEV antibody titers (Shimoda et al., 2011). These data indicate that dogs are good sentinels to assess the risk of human infection with JEV.

Serological tests such as virus neutralization and hemagglutination inhibition (HI) have been the primary assays used to detect

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JEV-specific antibodies in serum (Anderson et al., 2011; Chiou et al., 2007; Mall et al., 1995; Ting et al., 2004; Watanabe et al., 2008). The HI requires a large volume of serum, while the virus neutralization requires a special facility (e.g., biosafety level-2 or -3) and a high level of technical skill. In contrast, enzyme-linked immunosorbent assay (ELISA) does not require the use of live JEV, and the procedure is simple and requires only a small amount of sample. Other laboratories have reported the use of an indirect IgG ELISA for JEV serological surveys among pigs and bats (Cui et al., 2008; Hamano et al., 2007; Nidaira et al., 2007; Xinglin et al., 2005; Yang et al., 2006).

In the present study, an indirect ELISA to detect JEV antibodies in dogs was developed using sera of dogs infected experimentally with JEV and domestic dogs in Kyushu and Hokkaido, Japan. The test was then applied for a serological survey of dogs in Thailand to assess the risk of human infection with JEV.

## 2. Materials and methods

### 2.1. Cells

Vero cells (JCRB number JCRB9013), which are derived from African green monkey, were purchased from the Health Science Research Resources Bank (HSRRB, Tokyo, Japan). Vero cells were cultured at 37 °C and 5% CO<sub>2</sub> in Eagle's minimum essential medium (EMEM; GIBCO, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal calf serum (FCS; JR Scientific, Woodland, CA, USA), 1 mM sodium pyruvate, 100 U/ml of penicillin, and 100 µg/ml of streptomycin (GIBCO). Mosquito-derived C6/36 cells (JCRB number IFO 50010) were cultured at 28 °C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% heat-inactivated FCS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin.

### 2.2. Virus

JEV/sw/Chiba/88/2002 was originally isolated from the serum of a healthy pig in 2002, and has been genetically classified into genotype I (Nerome et al., 2007). The virus was propagated in C6/36 cells grown at 28 °C in DMEM supplemented with 2% FCS, and was stored at –80 °C until use.

### 2.3. Dog serum samples

Sera of dogs infected experimentally with JEV were collected sequentially and stored at –80 °C in a previous study (Shimoda et al., 2011). Briefly, three female dogs were inoculated intraperitoneally and subcutaneously with  $5 \times 10^6$  plaque-forming units (PFU) per dog of JaOH0566, a viral strain (kindly provided by Dr. Ishikawa (Biken, Kagawa, Japan)) that was originally isolated from a JE patient in 1966. Serum samples, which had been collected at a series of time points, were used for ELISA and virus neutralization. The animal experiments were approved by Animal Research Ethics Board of Faculty of Agriculture, Yamaguchi University.

For the serological survey, a total of 183 family dog sera were collected from domestic dogs in Kyushu and Hokkaido districts, Japan, and Bangkok, Thailand. These samples were collected from veterinary hospitals in Japan, or by visiting the owners of the examined dogs in Thailand. All owners were informed of the research objectives before sampling and data collection. To classify the dogs examined in this study, the owners also were asked to provide data about the sex, age, breed, and housing of their dogs. The definitions of outdoor (group A) and indoor dogs (group B) in this study are always (100%) staying outside of the house and, staying inside of the house more than 80% of the time, respectively. Note that all sera used for the virus neutralization were inactivated by incubation at

56 °C for 30 min to prevent the inactivation of JEV by complement in serum samples, and then stored at –20 °C until use.

### 2.4. Virus neutralization

To determine the presence in sera of virus neutralizing antibodies against JEV, an 80% plaque reduction virus neutralization was carried out as described previously (Ohno et al., 2009; Shimoda et al., 2010, 2011). Briefly, sera were diluted 1:5 and then were serially two-fold diluted in EMEM containing 2% FCS. The diluted sera or medium alone were mixed with equal volumes of virus solution containing 100 PFU of JEV/sw/Chiba/88/2002, and the mixtures were incubated at 37 °C for 90 min. After incubation, the mixtures were inoculated to subconfluent Vero cells and incubated at 37 °C for 90 min. After two washes with EMEM, the infected cells were overlaid with 0.8% agarose (Lonza, Rockland, ME, USA) in EMEM containing 5% FCS. Following 4 days incubation at 37 °C in 5% CO<sub>2</sub>, the cells were fixed with 10% buffered formaldehyde for 1 h, and the agarose layer was removed. After staining with crystal violet, plaques were counted. Sera that reduced the number of plaques by more than 80% in comparison with the mean number of plaques in control wells were considered to be JEV antibody positive.

### 2.5. ELISA

For ELISA, inactivated JEV originating from the Beijing 01 strain was selected as the antigen. This strain had been propagated in Vero cells, inactivated with formaldehyde, and purified by ultracentrifugation, and the product was intended for use as a vaccine antigen in humans. The inactivated JEV was diluted to 5 µg/ml with adsorption buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6), and distributed at 100 µl per well into 96-well microplates (Maxisorp; Nunc, Roskilde, Denmark). Control wells received an equivalent volume of adsorption buffer without antigen. After incubation at 37 °C for 2 h, plates were placed at 4 °C overnight. The wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween20 (PBS-T) and then incubated with 100 µl per well of 0.1% bovine serum albumin (BSA) (Fraction V; Sigma, St. Louis, MO, USA) in PBS at 37 °C for 30 min. Test sera were diluted with PBS-T containing 10% FCS. Wells were washed three times with PBS-T, and then diluted sera were added to duplicate wells, and plates were incubated at 37 °C for 30 min. Next, the wells were washed three times with PBS-T and incubated with 100 µl per well of diluted peroxidase-conjugated sheep anti-dog IgG or goat anti-dog IgM antibody (Bethyl Laboratories, Montgomery, TX, USA) at 37 °C for 30 min. Following washing three times with PBS-T, 100 µl of horseradish peroxidase substrate kit (Bio-Rad, Hercules, CA, USA) was added to each well. After incubation at room temperature for 30 min, the enzymatic reaction was stopped by adding 100 µl of 2% oxalic acid to each well. The absorbance was measured by a spectrophotometer (Bio-Rad) with a 405 nm filter. All results were corrected by the value of the non-antigen control.

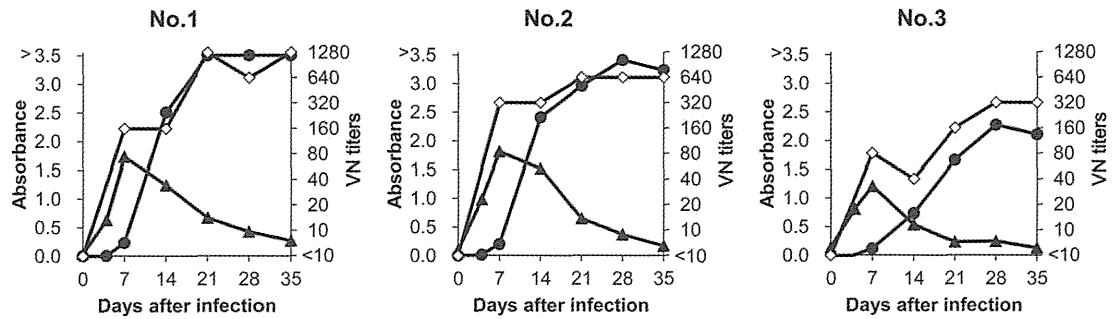
### 2.6. Optimization of concentration of ELISA antigen

The optimal concentration of the antigen was determined by cross-titration using the serially diluted antigen and the serially diluted serum from one mouse infected with JEV as the primary antibody. Based on the results of this preliminary study, an optimal antigen concentration of 5 µg/ml was selected (data not shown).

### 2.7. Statistical analysis

Chi-square test was performed to assess statistically the seroprevalence of JEV among dogs using IgG ELISA, and Pearson's correlation coefficient was calculated to determine the correlation





**Fig. 1.** Enzyme-linked immunosorbent assay (ELISA) values using 1:500 diluted sera and virus neutralization titers in dogs infected experimentally with Japanese encephalitis virus (JEV). Absorbances in IgG (closed circle) and IgM (closed triangle) ELISA are compared with virus neutralization (VN) titers (open diamond) (Shimoda et al., 2011).

between virus neutralization and IgG ELISA. *p* values of <0.05 were considered statistically significant.

**3. Results**

**3.1. Optimization of dilution of dog sera**

The optimal serum dilution was determined using sera collected from three dogs infected experimentally with JEV. The IgG ELISA was carried out using sera diluted to 1:100 and 1:500. The observed increases of JEV antibody after JEV challenge were consistent with the pattern seen by the virus neutralization. Using 1:100 diluted serum, the OD value of the IgG ELISA reached over 3.500 (the limit of detection) after day 14 in dog No. 1 and dog No. 2 (data not shown). On the other hand, absorbance exceeded 3.500 only after day 21 in dog No. 1 and on day 28 in dog No. 2 using 1:500 diluted sera (Fig. 1). In addition, in dog No. 3, the highest absorbance in 1:500 diluted sera was 2.270 on day 28. Therefore, to avoid false-positives and to examine the correlation between the absorbance of IgG ELISA and virus neutralization titers, a dilution of dog sera of 1:500 was selected for subsequent phases of this study.

**3.2. Determination of cut-off value**

For any serological surveys, determination of the cut-off value is important. To determine the cut-off value, IgG ELISA was performed using 5 µg/ml of the JEV antigen and 1:500 diluted dog sera as the primary antibody. Eleven dog sera collected in Hokkaido, Japan, where there was no evidence of the prevalence of JEV in dogs (Shimoda et al., 2010), were examined using IgG ELISA. The absorbance values obtained from these 11 specimens ranged from 0.013 to 0.200. The mean IgG ELISA value was 0.052 with a standard deviation (S.D.) of 0.053 (data not shown). The mean value plus 3 S.D. (a value of 0.211) was selected as the cut-off value between positive and negative results in this study.

**3.3. Comparison of antibody response in dogs infected experimentally**

In dogs infected experimentally with JEV, anti-JEV IgM was detected on day 3, peaked on day 7, and then gradually decreased. On the other hand, anti-JEV IgG increased on day 7 and peaked on days 21–28 (Fig. 1). For comparison, virus neutralization titers reached a first peak on day 7, and then started to increase again on day 14. Therefore, it is inferred that the titer detected by virus neutralization corresponds to the sum of IgG and IgM concentrations.

**3.4. Specificity and sensitivity of IgG ELISA**

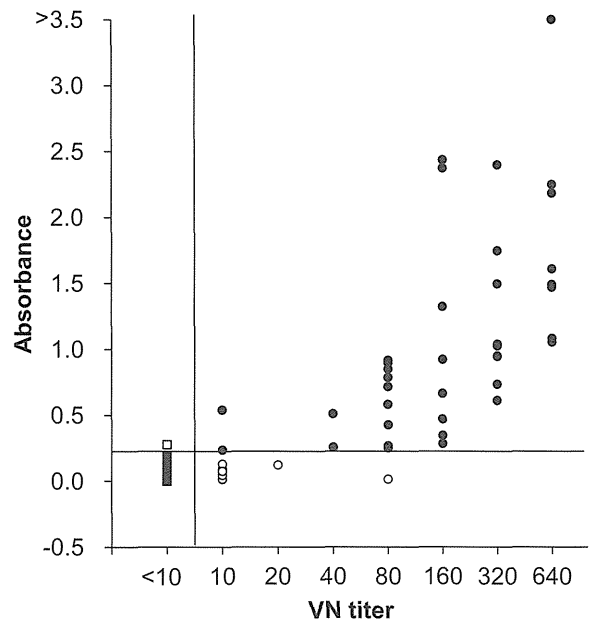
To investigate the specificity and sensitivity of IgG ELISA, 102 serum samples collected from dogs in Kyushu, Japan, were

**Table 1**

Comparison of the results between virus neutralization and IgG ELISA among dogs in Kyushu, Japan.

IgG ELISA	Virus neutralization		Total
	Positive	Negative	
Positive	40	1	41
Negative	9	52	61
Total	49	53	102

examined by both IgG ELISA and virus neutralization. Of 49 serum samples judged positive by virus neutralization, 40 and 9 were positive and negative in the IgG ELISA, respectively; of 53 serum samples judged negative by virus neutralization, only one was positive in the IgG ELISA (Table 1, Fig. 2). Thus, in comparison with the results in virus neutralization, the sensitivity and specificity of IgG ELISA were 82% and 98%, respectively. The correlation coefficient between IgG ELISA and virus neutralization was 0.813 (*n* = 102, *p* < 0.001). These results indicated that the result of IgG ELISA showed significant correlation with that of virus neutralization.



**Fig. 2.** Comparison of IgG ELISA values using 1:500 diluted sera and virus neutralization titers among dogs in Kyushu, Japan. Absorbances in IgG ELISA and virus neutralization titers are plotted. Closed circles, positive by both IgG ELISA and virus neutralization; closed squares, negative by both IgG ELISA and virus neutralization; open circles, positive only by virus neutralization; open squares, positive only by IgG ELISA. The cut-off value for IgG ELISA was set at 0.211 (see main text).

**Table 2**  
Seroprevalence of JEV by IgG ELISA among dog population in Bangkok, Thailand.

	Housing			Age (year)			Total
	Group A <sup>a</sup>	Group B <sup>b</sup>	Both	0–3	4–6	7–	
No. of examined dogs	44	9	17	13	33	24	70
No. of JEV-positive dogs	29	0	7	5	17	14	36
% of JEV-positive dogs	66	0	41	38	52	58	51

<sup>a</sup> Dogs are always (100%) staying outside of the house.

<sup>b</sup> Dogs are kept inside of the house more than 80% of the time.

### 3.5. Seroprevalence of JEV among dogs in Thailand

To survey JEV infection among dogs in Bangkok, Thailand, 70 serum samples were examined by IgG ELISA, revealing that 36 out of 70 dogs (51%) were seropositive for JEV. The seroprevalence among older dogs (>3 years) tended to be higher than that among young dogs (3 years and under), but any significant difference was not observed. In addition, 29 out of 44 (66%) dogs which were kept outside houses (group A) were seropositive for JEV; in contrast, all dogs which were kept inside houses (group B) were seronegative (Table 2). The serum samples from dogs in Thailand were also screened using IgM ELISA, but the resulting absorbances were less than 0.3 (data not shown).

## 4. Discussion

Previous studies showed that dogs are good sentinels to assess the risk of human infection with JEV, given that dogs share their life space with humans, are not vaccinated against JEV, do not display symptoms when infected, do not serve as amplifiers for JEV, and maintain virus neutralization titers for long periods after infection (Shimoda et al., 2010, 2011). These previous studies indicated that the virus neutralization was a suitable method for serological surveys of JEV infection in dogs, but some sera, especially sera from wild animals, inhibited nonspecifically JEV infection in the virus neutralization. Therefore, although surveys in humans typically employed a 50% plaque reduction assay, serological surveys in wild and companion animals were performed using an 80% plaque reduction assay (Ohno et al., 2009; Shimoda et al., 2010). In addition, the virus neutralization is time-consuming and requires a special facility. Therefore, other methods for detection of JEV antibodies are desirable. In this study, an indirect ELISA was developed to detect immunoglobulin for JEV in dogs, and the assay was applied in a serological survey on JEV infection to assess the risk of JEV infection to humans.

A preliminary ELISA study (using sera from dogs infected experimentally with JEV) tested the use of an unpurified extract from Vero cells infected with JEV/sw/Chiba/88/2002 as the ELISA antigen. This extract yielded lower absorbances than those seen with inactivated JEV antigen (data not shown). Therefore, inactivated JEV was selected as the ELISA antigen for the present study. This inactivated JEV antigen corresponds to the JEV vaccine that has been used in Japan since 2009, and is derived from the Beijing 01 strain (JEV genotype III) that has been propagated in Vero cells, inactivated with formaldehyde, and purified by ultracentrifugation. Thus, in the present study, different viruses, Beijing 01 and JEV/sw/Chiba/88/2002, were used in ELISA and virus neutralization, respectively. To avoid non-specific reaction in ELISA, the vaccine for human use originated from Beijing 01 was selected, because the vaccine is, to our knowledge, the most purified JEV reagents available. For virus neutralization, JEV/sw/Chiba/88/2002 routinely used in our laboratory (Ohno et al., 2009; Shimoda et al., 2010, 2011) was selected, because the plaques formed by the virus are large and clear. Virus neutralization using this strain could detect JEV

antibodies in various animal species, including dogs, raccoons, wild boars, and raccoon dogs (Ohno et al., 2009; Shimoda et al., 2010). In addition, some reports described that antibodies to JEV are cross-reactive among various JEV strains by virus neutralization (Kitano, 1989; Liu et al., 2011). Therefore, it seems that the ELISA and virus neutralization developed in this study can detect a broad range of JEV antibody.

Sensitivity and specificity of the IgG ELISA, as well as correlation with virus neutralization, were determined using domestic dogs in Kyushu, Japan, where there is no evidence of the prevalence of other flaviviruses. Absorbances in the IgG ELISA significantly correlated with the virus neutralization titer (0.813;  $n = 102$ ,  $p < 0.001$ ), suggesting that the IgG ELISA is suitable for detection of JEV antibodies in dogs. Sensitivity (82%) of the IgG ELISA in this study was not high, as nine dogs that were seropositive by virus neutralization were seronegative by the IgG ELISA. Since sensitivity may have been decreased due to the use of highly diluted sera (1:500), these nine serum samples with inconsistent results were examined at higher serum concentrations (1:10, 1:20, 1:40, and 1:80). The results showed that significant increases of the IgG ELISA values were obtained in 5 out of 9 samples (data not shown), suggesting these 5 dogs might be seropositive for JEV. In the development of this IgG ELISA, the specificity was the first priority for avoiding false-positive results. Therefore, 1:500 dilutions of sera were used as the primary antibody, providing reduced background (compared to 1:100 dilutions) and increased specificity (Figs. 1 and 2).

In Thailand, only a few surveys for JEV infection have been reported recently (Olsen et al., 2010; Prompiram et al., 2011). In particular, the risk of human infection with JEV in the capital city, Bangkok, remains unknown. In this study, a serological survey of JEV among dogs in Bangkok showed that 66% of dogs belonging to group A possessed antibodies to JEV, while JEV antibodies were not detected in any dogs belonging to group B. In Japan, the seroprevalence of dogs belonging to group A (45%) was 5.5-fold higher than that of dogs belonging to group B (Shimoda et al., 2010). These data suggest that the risk of human infection with JEV in Bangkok may be similar to or even higher than that in Japan.

Some inconsistent results between the IgG ELISA and virus neutralization were also observed in dogs in Thailand, as one of the putatively seronegative dogs by virus neutralization yielded a high absorbance (of 2.084) by the IgG ELISA (data not shown). It is hypothesized that this dog was infected with another flavivirus, such as dengue virus (DENV), which is endemic in Thailand (Hemungkorn et al., 2007). Upon testing by indirect IgG ELISA (using DENGUE IgG INDIRECT ELISA (Panbio, Queensland, Australia) only as the DENV antigen), this serum sample displayed a high ELISA titer against DENV (data not shown). Thus, this dog may have been infected with DENV.

ELISA of sera from dogs infected experimentally with JEV showed that anti-JEV IgM appeared earlier than IgG and then disappeared rapidly (Fig. 1), which is consistent with the results of a previous study on experimental infection of JEV against pigs (Ohkubo et al., 1984). These data suggest that the detection of anti-JEV IgM in this assay indicates recent infection with JEV. In the survey of Thai dogs, no dog possessed significant levels of anti-JEV



IgM, suggesting that infection with JEV had occurred more than 2–3 weeks before sampling. However, the values of the indirect ELISA for detection of anti-JEV IgM are not quantitative, since the binding strength of IgM is weaker than that of IgG, and the absorbance of anti-JEV IgM also may depend on the amount of anti-JEV IgG. The development of an IgM-capture ELISA, which has been used previously in serological surveys of humans and pigs (Hamano et al., 2007; Jacobson et al., 2007), might be required for the assessment of recent JEV infection.

In conclusion, an indirect ELISA was described for the detection of JEV antibodies in dogs; this assay allowed us to examine a large number of samples at once and to assess the levels of anti-JEV immunoglobulins in canine specimens quantitatively. This method is expected to facilitate the assessment of the human risk of JEV infection.

## Acknowledgments

JEV JaOH0566 was kindly provided by Dr. Ishikawa (Biken, Kanonji, Kagawa, Japan). Porcine serum samples used to isolate JEV/sw/Chiba/88/2002 were provided by Chiba Prefectural Institute of Public Health. This work was supported by grants from the Ministry of Health, Labour, and Welfare of Japan (H20-Shinko-Ippan-003), the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Japan Society for the Promotion of Science.

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## Production and Characterization of Monoclonal Antibodies to Japanese Encephalitis Virus

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(Received 26 December 2012/Accepted 8 March 2013/Published online in J-STAGE 22 March 2013)

**ABSTRACT.** In this study, eighteen monoclonal antibodies (MAbs) to recent Japanese encephalitis virus (JEV) genotype I were produced and characterized by plaque reduction neutralization test, western blot analysis, indirect immunofluorescence assay and enzyme-linked immunosorbent assay. All MAbs recognized only envelope (E) protein or conformational epitope of E and precursor membrane proteins. Two MAbs (7E5 and 3-3H8) possessed virus-neutralization activity, and their escape mutants possessed a change of glutamine to histidine at the position of 52 of E protein, suggesting that these neutralizing MAbs recognize the domain I-II hinge region of E protein. Five MAbs recognized all examined flaviviruses, and two were specific to JEV. These MAbs may be useful for differentiation and diagnosis of flaviviruses.

**KEY WORDS:** flavivirus, Japanese encephalitis virus, monoclonal antibody.

doi: 10.1292/jvms.12-0558; *J. Vet. Med. Sci.* 75(8): 1077-1080, 2013

Japanese encephalitis virus (JEV) is a member of genus *Flavivirus*, family *Flaviviridae*. JEV is transmitted primarily by *Culex tritaeniorhynchus* vector mosquitoes, and pigs play a main role as amplifiers. The virus is widely endemic in nations of Southeast Asia and the Western Pacific, including Japan [10], with an annual incidence of approximately 50,000 cases and 10,000 deaths in humans [3].

Many methods have been used for serological survey of JEV in various animals, such as plaque reduction neutralization test (PRNT), hemagglutination inhibition (HI) assay and enzyme-linked immunosorbent assay (ELISA). Among them, PRNT is currently "gold standard" for the serological differentiation of flavivirus infections [11]. However, to perform PRNT using live JEV, a special facility, biosafety level 2 and high level of technical skill are required. Also, a large volume of serum samples is required to perform either PRNT or HI assay. In contrast, ELISA does not require the use of live virus and the procedure is simple, and requires only a small amount of serum samples. We and other laboratories have reported the usability of indirect IgG ELISA for serological survey of JEV [2, 4, 14, 21-23]. Although indirect ELISA allows us to examine a large number of samples at once, the cross-reactivity of the antibody with other flaviviruses obstructs us to perform the JEV-specific diagnosis. Since dengue virus (DENV) and JEV have overlapped geographic distributions in several countries in Southeast Asia [10], diagnostic tools to differentiate JEV from other flaviviruses are required.

In Asia including Japan, a main genotype of JEV has changed from genotype III to I since 1990's [1, 9, 13, 17]. Although there is a report that antigenicity between genotypes I and III is different [18], the difference of antigenicity among JEV genotypes has been obscure. Furthermore, many monoclonal antibodies (MAbs) to JEV were reported and have been used as diagnostic and research tools, but there is no MAb to JEV genotype I. In this study, MAbs to recent JEV genotype I were produced, and the specificities were compared among various flaviviruses.

BALB/c mice (5 weeks old, male) were intraperitoneally immunized with JEV/sw/Chiba/88/2002 3 or 4 times at intervals of three weeks. The mice were euthanized 3 to 7 days after the final immunization, and the splenocytes were fused with P3U1 myeloma cells by using 50% polyethylene glycol solution (Hybri-Max<sup>TM</sup>; Sigma-Aldrich, St. Louis, MO, U.S.A.). The hybridoma cells were diluted in selection media, GIT media (Wako, Osaka, Japan) containing 2% hypoxanthine-aminopterin-thymidine (HAT) supplement (GIBCO, Grand Island, NY, U.S.A.), 10% BM-condimed H1 hybridoma cloning supplement (Roche Diagnostics, Mannheim, Germany) and 10% FCS, and incubated for 7 to 10 days at 37°C and 5% CO<sub>2</sub>. The screening of hybridomas was performed by either PRNT or indirect immunofluorescence assay (IFA) using JEV/sw/Chiba/88/2002. PRNT was carried out as described previously [16, 19-21]. For IFA, fluorescein-conjugated goat anti-mouse IgM+IgG+IgA (Southern Biotech, Birmingham, AL, U.S.A.) was used as a secondary antibody.

Total of 18 hybridomas were finally isolated and intraperitoneally injected into pristane-treated BALB/c mice (5 weeks old, male) to produce ascites. The immunoglobulin subclass of MAbs was determined using an IsoStrip mouse monoclonal antibody isotyping kit (Roche Applied Science,

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Table 1. Characterization of MAbs by IFA, ELISA, western blot and PRNT

MAbs	Isotype	IFA <sup>a)</sup>				ELISA <sup>a)</sup> (G III)	Western blot <sup>a)</sup> (G I)	PRNT	
		prM	E	prME	prM+E			Chiba (G I)	JaOH0566 (G III)
3-1A1	IgG2a	-	+	+	+	>3.50	-	<1:100	NT <sup>b)</sup>
3-1A5	IgG2a	-	+	+	+	>3.50	+	<1:100	NT
3-2A1	IgG2a	-	+	+	+	>3.50	-	<1:100	NT
3-2D6	IgG2a	-	+	+	+	>3.50	+	<1:100	NT
3-4B3	IgG2a	-	+	+	+	>3.50	-	<1:100	NT
3-3B10	IgG2b	-	+	+	+	>3.50	+	<1:100	NT
1-1A7	IgM	-	-	+	+	0.05	-	<1:100	NT
3-1D7	IgG3	-	-	+	+	2.40	-	<1:100	NT
3-3D1	IgM	-	-	+	+	2.05	-	<1:100	NT
4-4E9	IgG2a	-	-	+	+	1.82	-	<1:100	NT
3-2D1	IgM	-	-	+	+	2.35	-	<1:100	NT
3-1A2	IgG2a	-	-	+	+	2.38	-	<1:100	NT
4-4A8	IgM	-	-	+	+	3.30	-	<1:100	NT
3-1C2	IgG2a	-	-	+	+	0.09	-	<1:100	NT
3-1B5	IgG2a	-	-	+	+	2.53	-	<1:100	NT
7E5	IgM	-	-	+	+	1.53	-	1:102400	1:102400
3-3H8	IgM	-	-	+	+	1.65	-	1:102400	1:204800
4-3E8	IgG2a	-	+	+	+	0.28	-	<1:100	NT

a) 100-fold diluted mouse ascites was used for IFA, ELISA and western blot analysis. b) NT: Not tested.

Mannheim, Germany) according to the manufacturer's instructions. The heavy chain subclasses of MAbs are shown in Table 1, and their light chains were all kappa isotype.

To determine the protein recognized by MAbs, plasmids expressing precursor membrane (prM) and/or envelope (E) proteins were constructed. RT-PCR was carried out from JEV/sw/Chiba/88/2002 by RNA LA PCR<sup>TM</sup> Kit (AMV) Ver.1.1 (TAKARA, Otsu, Japan) using random 9 mer and primer pairs EcoprMF (5'-ACA GAA TTC ACC ATG GGA GGA AAT GAA AGC TCG-3') and BglMR (5'-ACA AGA TCT TCA ACT GTA AGC CGG AGC GAC C-3') for prM, EcoprMF and BglER (5'-ACA AGA TCT TCA GGC ATG CAC ATT GGT CGC-3') for prME and EcoEF (5'-ACA GAA TTC ACC ATG CAA CGT GTG GTG TTT ACT ATT C-3') and BglER for E. Amplified cDNAs were cloned in the *EcoRI*-*BglII* site of pCAGGS [15]. The resultants were named as pCAG-prM, pCAG-E and pCAG-prME, respectively. Then, 293T cells were transfected with expression plasmids using Lipofectamine<sup>TM</sup> LTX and Plus<sup>TM</sup> Regent (Invitrogen, Carlsbad, CA, U.S.A.). As the result, all MAbs recognized prME protein and co-expressed prM and E, and seven recognized E protein alone and none recognized prM protein alone (Table 1). The previous study indicates that the prM protein assists in the proper folding of E protein [7]. Therefore, the other eleven MAbs may recognize the conformational epitope in the presence of prM protein. Furthermore, western blot analysis using lysate of JEV/sw/Chiba/88/2002 infected Vero cells was carried out, resulting that three MAbs (3-1A5, 3-2D6 and 3-3B10) recognized a molecular mass of 50 kilodalton by western blot analysis under non-reducing condition (Table 1). This result also supported that all of our established MAbs did not react with linear epitope and recognized conformational epitope of E protein.

To determine the binding ability to purified vaccine

antigen, Beijing 01 (genotype III), ELISA was carried out according to our previous report [21]. Fifteen MAbs showed significant reactivity (Absorbance >1.0), while three MAbs (1-1A7, 3-1C2 and 4-3E8) showed no or less reactivity (Absorbance <1.0) (Table 1). These results indicated that most of the MAbs may be applicable for development of tools for JEV antibodies detection, such as competitive ELISA using purified vaccine antigen.

PRNT was performed to determine the virus-neutralization (VN) activity of ascites of each MAb to JEV. As the result, two MAbs (7E5 and 3-3H8) showed significant VN activity to both JEV/sw/Chiba/88/2002 and JaOH0566. Furthermore, to identify the epitope recognized by these MAbs, escape mutants, 7E5 mar Chiba, 3-3H8 mar Chiba and 7E5 mar JaOH0566 were selected by harvesting JEV in the presence of MAb 7E5 or 3-3H8, and amino acid sequences of capsid, prM and E were compared between parent strains and escape mutants. As the result, only one amino acid change was identified in the E proteins of 7E5 mar Chiba and 3-3H8 mar Chiba at E52 (Gln→His), and two amino acid changes were identified on the E protein of 7E5 mar JaOH0566 at E52 (Gln→His) and E276 (Ser→Arg) (Table 2). Based on these sequence analyses of escape mutants, MAbs 7E5 and 3-3H8 recognized the domain I-II hinge region of E protein, which has been previously described as an important neutralizing epitope for JEV [5, 6, 8, 12]. These results confirmed that the domain I-II hinge region of E protein recognized by these MAbs is a major target of VN antibody.

To determine the cross-reactivity of the MAbs with various flaviviruses including genotypes I (JEV/sw/Chiba/88/2002, JEV/eq/Tottori/2003 and Jakagawa2000), III (JaOH0566 and JaGAR-01) and V (Muar) of JEV, Murray Valley encephalitis virus (MVEV) (unknown strain), West Nile virus (WNV) (NY99), serotypes 1 (Hawaii), 2 (NC), 3 (H87)

Table 2. Amino acid changes in neutralization-resistant escape mutants

	Viruses	Q52	S276
		(Domain II)	(Domain II)
Parents	Chiba	Q	S
	JaOH0566	Q	S
Escape mutants	7E5 mar Chiba	H	S
	3-3H8 mar Chiba	H	S
	7E5 mar JaOH0566	H	R

and 4 (H241) of DENV, yellow fever virus (17D) and tick borne encephalitis virus (Sofjin), each MAb was examined by IFA using Vero cells infected with these viruses. As the result, five MAbs (3-1A1, 3-1A5, 3-2A1, 3-2D6 and 3-4B3) cross-reacted with all flaviviruses examined in this study, and two MAbs (3-1A2 and 4-4A8) specifically recognized all examined genotypes of JEV (Table 3). One MAb (3-1D7) recognized all of examined JEV, MVEV and WNV, suggesting that 3-1D7 might be specific for JEV serocomplex (Table 3). These JEV-specific MAbs and cross-reactive MAbs may be useful for detection of flaviviruses and may be applicable for differentiation of JEV infection from other flavivirus infection by immunohistochemistry and competitive ELISA.

In conclusion, these MAbs must be useful for further development of diagnostic methods of flavivirus infection.

**ACKNOWLEDGMENTS.** JEV JaOH0566 was kindly provided by Dr. Ishikawa (Biken, Kanonji, Japan). This work was supported by grants from the Ministry of Health, Labour and Welfare of Japan (H23-Shinko-Ippan-003) and by Grant-in-Aid for Japan Society for the Promotion of Science Fellows.

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Table 3. Cross-reactivity of MAbs against various flaviviruses by IFA<sup>a)</sup>

MAbs	JEV GI			JEV GIII		JEV GV	MVEV	WNV	DENV				YFV	TBEV
	Chiba	Tottori	Kagawa	JaOH	JaGAR				1	2	3	4		
3-1A1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-1A5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-2A1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-2D6	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-4B3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-3B10	+	+	+	+	+	+	+	+	+	+	+	+	+	–
1-1A7	+	+	+	+	+	+	–	+	+	+	+	+	–	–
3-1D7	+	+	+	+	+	+	+	+	–	–	–	–	–	–
3-3D1	+	+	+	+	+	+	–	+	–	–	–	–	–	–
4-4E9	+	+	+	+	+	–	+	+	–	–	–	–	–	–
3-2D1	+	+	+	+	+	+	+	–	–	–	–	–	–	–
3-1A2	+	+	+	+	+	+	–	–	–	–	–	–	–	–
4-4A8	+	+	+	+	+	+	–	–	–	–	–	–	–	–
3-1C2	+	+	+	–	+	+	–	–	–	–	–	–	–	–
3-1B5	+	+	+	+	+	–	–	–	–	–	–	–	–	–
7E5	+	+	–	+	–	–	–	–	–	–	–	–	–	–
3-3H8	+	+	–	+	–	–	–	–	–	–	–	–	–	–
4-3E8	+	+	–	+	–	–	–	–	–	–	–	–	–	–

a) IFA was performed using 100-fold diluted mouse ascites.



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## Pseudorabies Virus Infection in Wild Boars in Japan

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(Received 21 April 2011/Accepted 2 July 2011/Published online in J-STAGE 15 July 2011)

**ABSTRACT.** In Japan, most pig populations are now free from pseudorabies virus (PRV) due to the recent success of an extensive eradication program. However, PRV infection persists in Japanese wild boars (*Sus scrofa leucomystax*), representing another potential reservoir for the virus in Japan. In this study, the seroprevalence of PRV in wild boars captured in three different prefectures was ascertained. A virus neutralization (VN) test showed that 6 of 173 serum samples (3%) were positive for VN antibody; glycoprotein E-ELISA revealed infection with the wild-type, but not the available vaccine strain, PRV. These results indicate that PRV has continued to spread among wild boars in Japan.

**KEY WORDS:** Aujeszky's disease, pseudorabies virus, wild boar.

*J. Vet. Med. Sci.* 73(11): 1535-1537, 2011

Pseudorabies virus (PRV), a member of the genus *Variellovirus* in the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* [9], is the causative agent of a contagious and epidemic disease, Aujeszky's disease, which affects swine at various production phases, causing high mortality in naive and newborn piglets and abortion in pregnant sows. This disease results in significant economic losses for the swine industry [3].

In Japan, a PRV eradication program in domestic pigs has been successfully implemented; consequently, most domestic pig populations are now free from wild-type PRV and therefore are not vaccinated with a live attenuated vaccine. As this vaccine virus has a deletion in the glycoprotein E (gE) gene, vaccinated pigs can be serologically differentiated from those infected with wild-type PRV by the gE-ELISA. In 1997, a PRV epidemic in Nara Prefecture, Japan, resulted in the deaths of 24 hunting dogs due to PRV infection acquired after eating raw wild boar (*Sus scrofa leucomystax*) meat (Kouda, T. *et al.*, In the 129th Annual Meeting of the Japanese Society of Veterinary Science, Tsukuba, 2000). This event suggested that wild boars may represent a potential source of PRV infection for other animals. However, there is currently little information about the present situation of PRV prevalence among wild boars in Japan. Here, we present the results of a seroepidemiological study of PRV in wild boars in Japan in order to clarify the seroprevalence of PRV in this population.

A total of 173 serum samples were collected from wild boars in three different prefectures A, B and C, of Japan (Table 1). These three prefectures were located in the western part of Japan, were not next to each other and were free from PRV in their pig populations. Most animals were

hunted with government permission during the winter season. Sera were inactivated by incubation at 56°C for 30 min and then kept at -20°C until use.

Cloned porcine kidney (CPK) cells were kindly provided by the National Institute of Animal Health in Japan; these were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, NY, U.S.A.) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Subsequently, the PRV Indiana strain was propagated in the cultured CPK cells. To assess the presence of virus neutralization (VN) antibody to PRV, sera were serially twofold diluted in DMEM containing 2% FCS. Diluted sera and medium (control) samples were mixed with equal volumes of a solution containing 50 plaque-forming units (PFU) / 100 µl of PRV, and then incubated at 37°C for 1 hr. CPK cells were then inoculated with these samples. After adsorption for 60 min at 37°C in 5% CO<sub>2</sub>, treated cells were washed twice with DMEM and then overlaid with 0.8% agarose (SeaPlaque GTG Agarose, Lonza, ME, U.S.A.) in DMEM containing 10% FCS. The plates were then incubated at 37°C in 5% CO<sub>2</sub> for 3 days. The cells were fixed with 5% buffered formaldehyde for 1 hr, and the agarose layers were removed. After staining with crystal violet, plaques were counted. Sera that reduced the number of plaques by more than 80%, as compared with the mean number of plaques in control wells, were considered positive.

The results of VN assays revealed that at least 6 sera (3%) had VN antibodies against PRV (Table 1). PRV seropositivity was found in 2 of 50 wild boars (4%) in Prefecture A, 4 of 71 (6%) in Prefecture B, and none of the 52 in Prefecture C. The VN titers in the 6 positive sera ranged from 1:40 to 1:160 (Table 2). PRV-positive boars were over three years old and relatively heavy (49-81 kg), indicating that infection may have occurred a few years before sampling took place and may not have occurred recently.

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Table 1. Seroprevalence of PRV in wild boars

Prefecture	Period	Number of examined sera	Number of PRV-positive sera (% of PRV-positive sera)
A	Sep. 2009 –Nov. 2010	50	2 (4%)
B	Nov. 2007 –Mar. 2010	71	4 (6%)
C	Jan. 2010 –Dec. 2010	52	0 (0%)
Total		173	6 (3%)

Table 2. VN titers and antibodies to PRV gE in PRV-positive sera

Prefecture	Date	Sex	Age <sup>a)</sup> (Body weight)	gE ELISA S/N ratio <sup>b)</sup>	VN titer
A	Nov. 26, 2010	F	Over 3 (65 kg)	0.04	1:160
A	Oct. 4, 2010	F	Over 3 (73 kg)	0.19	1:80
B	Feb. 15, 2008	M	N.A. (52 kg)	0.05	1:160
B	Feb. 19, 2008	M	N.A. (53 kg)	0.19	1:80
B	Mar. 3, 2010	M	N.A. (81 kg)	0.10	1:40
B	Feb. 16, 2010	M	N.A. (49 kg)	0.15	1:80

a) Age was presumed from their teeth.

b) S/N ratio was calculated as a ratio of the absorbance of a well with serum to the absorbance of a control well without serum. Serum with an S/N ratio of  $\leq 0.60$  was judged as positive.

N.A.) Data is not available.

To confirm PRV seropositivity, PRV gE-ELISA was carried out using HerdChek PRV g1 (gE) Antibody ELISA (IDEXX Laboratories, Westbrook, ME, U.S.A.). This assay detects the antibody to wild-type PRV gE by competition with a monoclonal antibody, thereby distinguishing the antibody induced by wild-type PRV from that induced by the vaccine strain [11]. The results showed that all 6 VN-positive sera contained antibodies to wild-type, virulent PRV gE, and not the vaccine strain.

The present results were consistent with the PRV epidemic in hunting dogs in 1997, because prefecture B is next to Nara Prefecture, suggesting that virulent PRV continues to spread among wild boars in Japan. However, the reported seroprevalences of PRV in wild boars in western (10%) and eastern (9%) Germany [10, 12], France (6%) [1], south-central Spain (56%) [5], Croatia (55%) [16] and the southern U.S.A. (34–61%) [2, 6, 7, 14] seem to be higher than that in Japan (3%). In addition, only one previous report indicated that wild boars in Shikoku, in southern Japan, were free from PRV [8]. These results suggest that the low seroprevalence of PRV in wild boars in Japan might be indicative of the success of the PRV eradication program in domestic pigs.

PRV-seropositive wild boars can act as a source of infection for other wildlife species, including wild canids and hunting dogs [13]. Mortality associated with PRV infection has also been documented in endangered species such as the Florida panther (*Felis concolor*) in the U.S.A. [4], and PRV has been suggested as a possible factor contributing to the declining numbers of the Eurasian lynx (*Lynx lynx*) in Slovenia [15]. Taken together with our results, these trends indi-

cate that PRV infection in wild boars should become a target for eradication programs like the one implemented in the domestic pig population in Japan.

In conclusion, since PRV continues to infect wild boars in Japan, even in the prefectures without PRV in pigs, it is necessary to take precautions concerning possible transmission of PRV from wild boars to pigs.

**ACKNOWLEDGMENTS.** Cloned porcine kidney (CPK) cells were kindly provided by the National Institute of Animal Health in Japan. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and from the Ministry of Health, Labour and Welfare of Japan (H23-Shokuhin-Ippan-008). H.Y.A.M. received a financial support from the Egyptian government (Faculty of Veterinary Medicine, South Valley University) as a part of a scholarship for his PhD degree.

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