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研究報告の概要

（背景）Plasmodium knowlesi（P. knowlesi）は東南アジアで次第にヒト・マラリアの原因と認められつつある。しかし、自然感染の詳細なプロスペクティブな臨床研究がない。

（測定法）急性P. knowlesi感染患者のpresentationと経過の系統的な研究において、臨床および検査データは2006年7月～2008年2月にKapit病院（サラワク、マレーシア）にPCRで確定された急性マラリアで入院した過去に治療経験のない非妊娠の成人から集められた。

（結果）152人の患者のうち、107人（70%）がP. knowlesiに、24人（16%）はP. falciparumに感染しており、そして、21人（14%）は三日熱マラリア原虫を持っていた。P. knowlesi感染者は、非特異的な発熱性の疾患を呈し、入院患者のベースライン中央値の寄生虫値は1387parasites/μL（四分位数間領域：6-222,570parasites/μL）を有し、そして全ての症例は入院又はその次の日に血小板減少を呈していた。
P. knowlesi感染患者のほとんど（93.5%）は、クロロキンとプリマキン治療に反応した合併症を伴わないマラリアであった。熱帯熱マラリアのWHO基準に基づくと、P. knowlesi感染の7人の患者（6.3%）は、入院時点で重症感染であった。最も頻度の高い合併症は呼吸困難であった。それは4人の患者では入院時にみられ、あとの3人の患者では入院後に発症した。入院時のP. knowlesi寄生虫血症は呼吸困難の独立した決定因子であり、入院時の血清クレアチニンレベル、血清ビリルビンそして血小板数と同様であった（各々P<0.002）。2人のP. knowlesiマラリア患者は死亡し、1.8%（95%信頼区間；0.2-6.6%）の致死率を示した。

（結論）Knowlesiマラリアは、広範囲の疾患を起こす。大部分の症例は合併症を伴わず、速やかに治療に反応する。しかし、約10人に1人の患者が潜在的に致命的な合併症を発病する。

使用上の注意記載状況・その他参考事項等

2. 重要な基本的注意
(1)本剤の原材料となる献血者の血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体、抗HTLV-I抗体陰性で、かつALT(GPT)値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査（NAT）を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した血漿を原料として、陽イオン交換体処理により人トロンビンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程においてリン酸トリ-n-ブチル(TNBP)/ポリソルベート80処理、ウイルス除去膜によるろ過処理、凍結乾燥の後、60℃、72時間の加熱処理を施しているが、使用に際しては、次の点に十分注意すること。

(30)

報告企業の意見	今後の対応
東南アジアでPlasmodium knowlesiが原因のマラリアはだんだん増えており、患者の多くはクロロキンとプリマキン治療に反応した合併症を伴わないマラリアであったが、約10人に1人の患者が致命的な合併症を発病する可能性があることについての報告である。 Plasmodium knowlesiは長い間、サルに感染するマラリア原虫とみなされていたが、ヒトに感染する5番目のPlasmodium属原虫と認められるようになってきている。 血漿分画製剤からのマラリア伝播の事例は報告はされていない。FDAが2000年6月に発行した"Guidance for Industry: Recommendations for Donor Questioning Regarding Possible Exposure to Malaria"においては、赤血球成分または血小板用の供血についてはマラリアに関連した供血停止条件を規定しているものの、血漿成分用の供血や血漿分画製剤の原料用の供血については規定していない。感染患者におけるマラリア原虫はメロゾイドの形で存在すると考えられ、このものは2-3μmの卵型であるとされている（最新医学大辞典第2版、医歯薬出版、1996）。万一、原料血漿にマラリア原虫が混入したとしても、除菌ろ過等の製造工程にて除去されるものと考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

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MAJOR ARTICLE

Clinical and Laboratory Features of Human *Plasmodium knowlesi* Infection

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Background. *Plasmodium knowlesi* is increasingly recognized as a cause of human malaria in Southeast Asia but there are no detailed prospective clinical studies of naturally acquired infections.

Methods. In a systematic study of the presentation and course of patients with acute *P. knowlesi* infection, clinical and laboratory data were collected from previously untreated, nonpregnant adults admitted to the hospital with polymerase chain reaction–confirmed acute malaria at Kapit Hospital (Sarawak, Malaysia) from July 2006 through February 2008.

Results. Of 152 patients recruited, 107 (70%) had *P. knowlesi* infection, 24 (16%) had *Plasmodium falciparum* infection, and 21 (14%) had *Plasmodium vivax*. Patients with *P. knowlesi* infection presented with a nonspecific febrile illness, had a baseline median parasitemia value at hospital admission of 1387 parasites/μL (interquartile range, 6–222,570 parasites/μL), and all were thrombocytopenic at hospital admission or on the following day. Most (93.5%) of the patients with *P. knowlesi* infection had uncomplicated malaria that responded to chloroquine and primaquine treatment. Based on World Health Organization criteria for falciparum malaria, 7 patients with *P. knowlesi* infection (6.5%) had severe infections at hospital admission. The most frequent complication was respiratory distress, which was present at hospital admission in 4 patients and developed after admission in an additional 3 patients. *P. knowlesi* parasitemia at hospital admission was an independent determinant of respiratory distress, as were serum creatinine level, serum bilirubin, and platelet count at admission ($P < .002$ for each). Two patients with knowlesi malaria died, representing a case fatality rate of 1.8% (95% confidence interval, 0.2%–6.6%).

Conclusions. Knowlesi malaria causes a wide spectrum of disease. Most cases are uncomplicated and respond promptly to treatment, but approximately 1 in 10 patients develop potentially fatal complications.

Five species of *Plasmodium* (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*) cause naturally acquired malaria in humans. The most recently identified species is *P. knowlesi*, which we previously reported to be the most common cause of hospitalization for malaria in the Kapit Division of Sarawak in Malaysian Borneo [1]. Further studies of blood samples from patients presenting with malaria in Sarawak, Sabah, and Peninsular states confirmed a much wider distribution within Malaysia [2]. There have also been reports of locally acquired *P. knowlesi* infections from Southern Thailand, the Myanmar-China border, the Philippines, and Singapore [3–7], indicating that transmission occurs in many Southeast Asian countries.

P. knowlesi is primarily a chronic infection of the long-tailed (*Macaca fascicularis*) and pig-tailed (*Macaca nemestrina*) macaques [8]. It is easily confused with *Plasmodium malariae* on blood film microscopy in cases of human infection, because the morphologic appearances are almost identical [9, 10]. However, *P. knowlesi* is unique amongst the primate and human malarias in that it has a 24-h erythrocytic cycle [10], which is a characteristic that is likely to accelerate the development of complications [2]. Information on the characteristics of knowlesi malaria in humans, however, is restricted to single case reports [3, 5, 7]; our previous retrospective study of 94 patients with uncomplicated cases, in

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which we described available data relating to clinical features at presentation only [1]; and our report of 4 fatal cases [2]. We have, therefore, undertaken a detailed, systematic, prospective study of the presentation and clinical course of patients with a diagnosis of confirmed acute knowlesi malaria.

PATIENTS AND METHODS

Study site. This prospective study was conducted in the Kapit Division, which has a total population of 109,000 people of mostly Iban ethnicity [1]. A single World Health Organization (WHO) level 2 hospital serves the Division, together with 3 polyclinics and 22 rural health clinics. Health policy mandates that all patients with malaria are hospitalized until negative blood smear results are obtained on 2 consecutive days. Treatment for malaria is provided free of charge.

Subjects. Recruitment was consecutive and took place during 2 periods totalling 17 months from July 2006 through February 2008. All nonpregnant patients aged ≥15 years who were admitted to Kapit Hospital with a blood film result positive for any *Plasmodium* species were eligible, provided that there was no significant comorbid disease and that they had taken no antimalarial treatment within the previous 14 days. Subsequent confirmation of malaria species was determined by nested polymerase chain reaction assays [1]. All patients provided witnessed informed consent to the study procedures, which were approved by the Medical Research Ethics Subcommittee of the Malaysian Ministry of Health. In an initial 2-month pilot study, most cases of *P. vivax* and *P. falciparum* infection were among logging camp workers returning from long periods in Oceania or Equatorial Africa, respectively. Because the demographic characteristics and background immunity of these patients were significantly different from those of patients with knowlesi malaria, their clinical and laboratory data are presented but are not compared directly with data for patients with *P. knowlesi* infection.

Clinical procedures. Detailed demographic characteristics, history, and examination findings were recorded on a standard form. A baseline blood sample was obtained for routine biochemical and hematological testing, and regular monitoring of temperature, blood pressure, and pulse rate was started. Treatment was administered promptly according to the Malaysian Ministry of Health Guidelines. Because there are no current guidelines for *P. knowlesi* malaria, the guidelines for *P. malariae* were used. Patients with uncomplicated knowlesi malaria received oral chloroquine (25 mg base/kg over a 3-day period) followed by primaquine (15 mg daily for 2 days) given as a gametocidal agent. Oral and/or intravenous hydration was administered at the discretion of the treating physician. Patients presenting with or developing features of severe malaria were treated in accordance with WHO guidelines [11] except that the thresholds for hyperparasitemia and anemia were changed to >100,000 asexual forms/μL of whole blood and <7.1 g of hemoglobin/dL, respectively, to allow for the low immunity levels of the local population. If indicated clinically, patients were transferred to Sibu Hospital for intensive care.

All patients were assessed clinically and by microscopic examination of blood films on each inpatient day. Additional laboratory tests were performed as indicated by the clinical state of the patient. Parasite clearance time and fever clearance time were taken as the number of days to the first of at least 2 follow-up assessments at which the patient had negative blood film results and was afebrile, respectively. When the patient was afebrile and had negative blood film results for 2 consecutive days, additional blood samples were obtained for routine biochemical and hematological tests before discharge. Patients returned on the 28th day after hospital admission for clinical review and blood tests.

Laboratory procedures. All blood films were examined by 2 experienced microscopists. The parasite density was first determined at Kapit Hospital on the basis of the number of parasites per 500 white blood cells and the total white blood cell count for each patient. Microscopic examination was repeated in Kuching, with the second microscopist blinded to the initial result. The mean of the 2 parasite densities was used in data analysis. Parasite DNA was extracted from blood spots that had been collected on filter paper, and the *Plasmodium* species was determined by nested polymerase chain reaction for *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*, as described elsewhere [1, 12].

Hematological profiles were determined on site using semiautomated methods (Sysmex model KX-21N). Serum sodium, potassium, glucose, creatinine, bilirubin, alanine aminotransferase (ALT), and albumin levels were either assayed on site (AVL 9180 and Hitachi 902; Roche/Hitachi, Roche Diagnostics) or serum samples were stored at −80°C before transfer on dry ice to the Biochemistry Department, Fremantle Hospital (Freemantle, Australia), for analysis (Cobas Integra 800; Roche Diagnostics). An additional uncuffed blood sample was collected into a chilled fluoride-oxalate tube, centrifuged immediately and separated plasma stored at −80°C before transfer on dry ice to Fremantle Hospital for plasma lactate assay (COBAS INTEGRA 800). Other laboratory investigations, including blood cultures, urine dipstick testing, microscopic examination, and chest radiography were performed as indicated clinically.

Statistical analysis. Data were analyzed using SPSS software, version 15.0 (SPSS). Normally distributed variables were compared using the Student's *t* test or analysis of variance and the Scheffé post hoc test. All other data were analyzed using nonparametric methods (the Wilcoxon rank-sum test or Friedman test). Proportions were compared with use of Fisher's exact test. Multiple logistic or linear regression analysis using forward conditional modeling was performed to determine baseline associates of complications or markers of severity, respectively. Plausible predictive variables with a statistically significant ($P < .05$) univariate association with the specific severity outcome were selected for inclusion in the model. These variables were log-transformed prior to model entry if they were nonnormally distributed and a stepwise forward selection procedure was then performed to identify the significant independent associates in each case.

RESULTS

Baseline characteristics. The number of patients who participated in the study in relation to all malaria admissions to Kapit Hospital during the recruitment period is shown in figure 1. Their baseline demographic and clinical features are summarized in table 1. *P. knowlesi* infections were acquired locally by both sexes and across all age groups, with 93 (87%) of patients reporting recent activities in the jungle or forest-fringe in the Kapit Division. All regions along the Rejang River and its associated tributaries were represented, and there was no significant clustering of cases. Confirming our pilot study findings, most of the cases of vivax and falciparum malaria (31 cases; 69%) were imported, and the numbers were relatively small.

The overall median duration of symptoms prior to hospitalization was 5 days (interquartile range, 3–5 days), but 2 patients were unwell for >10 days before hospitalization. Symptoms were typically nonspecific. Fever and chills were present in almost all cases, and other frequent symptoms included abdominal pain, breathlessness, and productive cough. Tachypnea, pyrexia, and tachycardia were common clinical signs (table 1).

The results of baseline laboratory investigations are summarized in table 2. The level of parasitemia at hospital admission was relatively low in the *P. knowlesi* group, but there was a wide range that included 3 patients (2.8%) with parasite densities >100,000 parasites/μL and 33 patients (30.8%) with densities <500 parasites/μL. The most common abnormal laboratory finding was thrombocytopenia (<150,000 platelets/μL), which was present in 104 patients (98%), with 31 (29%) of 107 patients having a platelet count <50,000 platelets/μL. The 3 patients who did not have thrombocytopenia (155,000, 152,000, and 167,000 platelets/μL) had low parasitemias (5, 126, and 170 asexual forms/μL, respectively), and all became thrombocytopenic within 24 h (with nadir values of 90,000, 131,000, and 112,00 platelets/μL, respectively). Lymphopenia was found in 7 (6.5%) of patients at presentation, but all patients had normal values by the time of hospital discharge. Anemia was uncommon at hospital admission. Only 5 (4.6%) of the patients had a hemoglobin concentration <10 g/dL, whereas none of the patients met the criteria for severe anemia. Mild hepatic dysfunction, usually comprising an elevated serum ALT level and a low serum albumin level, was relatively common. Mild-to-moderate hyponatremia (range, 122–135 mmol/L) was evident in 29% of cases, all of which responded to rehydration and antimalarial therapy.

On the basis of WHO criteria for severe falciparum malaria [11], 8 (7.5%) of the patients with *P. knowlesi* infection had severe infections at presentation (table 3). The most frequent clinical presentations of severe infection were respiratory distress (diagnosed in 4 patients on the basis of a respiratory rate >30 breaths/min, oxygen saturation <94% by pulse oximetry, auscultatory findings, and radiographic changes), hyperparasitemia (3 patients), and jaundice (serum total bilirubin >43 μmol/L in 3 patients). There were 3 cases of renal failure (serum

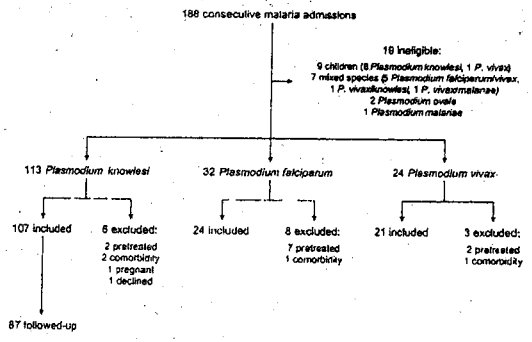

Figure 1. Flow chart showing patient recruitment, exclusion, and follow-up in a study of human *Plasmodium knowlesi* infection in Malaysia.

Table 1. Demographic and Clinical Characteristics of Patients Admitted to Kapit Hospital (Sarawak, Malaysia) with Untreated Malaria Categorized by *Plasmodium* Species

Variable	*Plasmodium knowlesi* (*n* = 107)	*Plasmodium falciparum* (*n* = 24)	*Plasmodium vivax* (*n* = 21)	*P*
Age, years				
Mean value (± SD)	44.9 ± 14.94[a]	38.7 ± 9.64	35.5 ± 10.61	.006
Range	16–79	15–53	15–51	
Male sex	56.1[b,c]	95.8	100	<.001
Iban ethnicity	91.6	95.8	76.2	.073
Occupation				<.001
Farmer	49.5	4.2	9.5	
Logging/plantation worker	27.1[b,c]	91.7	71.4	
Other	23.4	4.2	19	
Self-reported previous malaria	26.2[b,c]	75	57.1	<.001
Previous foreign travel	19.6[b,c]	91.7	71.4	<.001
Foreign travel within previous 4 weeks	0.9[b,c]	83.3	52.4	<.001
Duration of illness, median days (IQR)	5 (3–7)	2.5 (1–4.75)	3 (1–5)	<.001
Symptom				
Fever/chills	100	91.7	95.1	NA
Headache	94.4	87.5	52.4	NA
Rigors	89.7	79.2	85.7	NA
Malaise	89.7	91.7	66.7	NA
Anorexia	83.2	70.8	52.4	NA
Myalgia	87.9	79.2	90.2	NA
Cough	56.1	54.7	47.6	NA
Nausea	56.1	87.5	28.5	NA
Vomiting	33.6	41.7	19.0	NA
Abdominal pain	52.3	37.5	23.8	NA
Diarrhea	29.0	47.5	33.3	NA
Clinical findings				
Axillary temperature, median °C (IQR)	37.6 (37.0–38.5)	37.8 (37.0–38.5)	37.0 (36.8)	NA
Respiratory rate, median breaths/min (IQR)	26 (22–31)	25.5 (22.3–28.5)	27 (24.5–29.0)	NA
Pulse rate, mean beats/min (± SD)	95 ± 16	99 ± 17	97 ± 18	NA
Arterial blood pressure, mean mmHg (± SD)	89 ± 11	85 ± 9	89 ± 9	NA
Capillary refill time, median secs (IQR)	2 (2–3)	2 (2–3)	2 (2–3)	NA
Palpable liver	24.3	29.2	16.7	NA
Palpable spleen	15.0	20.8	23.8	NA

NOTE. Data are percentage of patients, unless otherwise indicated. IQR, interquartile range; NA, not assessed; SD, standard deviation.

[a] $P < .05$ vs *P. vivax*.
[b] $P < .01$ vs *P. falciparum*.
[c] $P < .01$ vs *P. vivax*.

creatinine level ≥265 μmol/L despite fluid resuscitation), 2 cases of hypotension (systolic blood pressure ≤80 mmHg despite fluid resuscitation), and 1 case of hypoglycemia (venous plasma glucose level <2.2 mmol/L). There were no cases of unrousable coma. A combination of features was present at hospital admission in 3 patients.

Clinical course. Clinical and parasitological outcomes together with changes in key hematological and biochemical variables during hospitalization and at day 28 for patients with knowlesi malaria are summarized in tables 4 and 5. There was no clinical, laboratory, or radiological evidence of other infections or conditions at study entry, during hospitalization, or at follow-up that would have influenced outcome. When patients with knowlesi malaria were discharged from the hospital, platelet counts had increased, and all patients had values that were within the normal range by day 28. Most of the remaining hematological and biochemical parameters had improved by hospital discharge. Abnormal laboratory values had resolved in all 87 patients with knowlesi malaria who attended for day 28 review.

Three patients, including 2 patients without complications at hospital admission, developed respiratory distress (table 3). A total of 7 (6.5%) of the 107 patients in the knowlesi group, all of whom were female, presented with or developed respiratory distress. Of those patients with evidence of severe knowlesi malaria either at presentation or during treatment, 2 died (table 3). Patient 1 had parasitemia at presentation (parasite density, 222,570 parasites/μL), evidence of multiorgan failure,

Table 2. Laboratory Results for Patients Admitted to Kapit Hospital with Untreated Malaria Categorized by *Plasmodium* Species

Variable	Normal range	*Plasmodium knowlesi* (*n* = 107)	*Plasmodium falciparum* (*n* = 24)	*Plasmodium vivax* (*n* = 21)
Parasite count, parasites/μL	NA	1387 (6–222,570)	26,781 (1840–271,760)	4258 (324–32,132)
Hemoglobin level, g/dL	11.3–15.7	13.3 (12.0–14.3)	12.9 (12.3–13.6)	13.5 (12.6–13.8)
White blood cell count, × 10^3 cells/μL	3.1–10.3	5.6 (4.7–7.0)	6.3 (5.3–8.6)	6.1 (4.9–7.8)
Neutrophil count, mean neutrophils × 10^3/μL (± SD)	.2–5.3	3.7 ± 1.8	4.6 ± 2.4	4.6 ± 2.2
Lymphocyte count, × 10^3 cells/μL	0.8–2.7	1.5 (1.1–2.0)	1.0 (0.8–1.4)	1.0 (0.6–1.7)
Platelet count, mean value × 10^3 platelets/μL (± SD)	150–450	71 ± 35	108 ± 59	118 ± 51
Prothrombin time, secs	NA	13 (12–15)	15 (13–16)	12 (12–14)
Blood group O, % of patients	NA	28.0	12.5	9.5
Serum creatinine level, μmol/L	<133	86 (73–100)	89 (80–97)	89 (76–98)
Serum sodium level, mmol/L	136–152	137 (135–140)	138 (135–140)	138 (135.5–141)
Serum total bilirubin, μmol/L	<21	13 (9–18)	17 (12–22)	16 (10–21)
Serum alanine aminotransferase level, IU/L	<40	36 (25–54)	26 (20–40)	27 (13–55)
Serum albumin level, g/dL	>36	36 (33–39)	38 (35–41)	41 (39–46)
Serum glucose level, mmol/L	4–8	6.2 (5.3–6.7)	6.4 (5.7–7.2)	6.2 (5.5–7.0)
Plasma lactate level, mmol/L	<2	1.6 (1.2–2.0)	1.5 (1.2–2.0)	1.5 (1.1–2.0)

NOTE. Unless otherwise indicated, data are median value (interquartile range). NA, not applicable.

hypoglycemia, and lactic acidosis. This patient died within 6 h after hospital admission despite intensive treatment with intravenous quinine, broad spectrum antibiotics, and ionotropic and ventilatory support. Patient 8 presented with symptoms and signs of a right hemiparesis and sensory inattention and had a history of uncontrolled hypertension. The patient's parasite density at hospital admission was 214,000 parasites/μL. She was treated with intravenous quinine but developed respiratory distress that required mechanical ventilation. After showing signs of improvement, she experienced neurological deterioration on the seventh day of hospitalization and died 24 h later. No neuroimaging studies were possible.

Baseline* P. knowlesi *parasitemia, complications, and markers of severity. Patients reporting breathlessness or vomiting had greater geometric mean parasite counts than did those who did not report these symptoms ($P = .025$ and $P = .038$, respectively). In a logistic regression model, presentation with or development of respiratory distress was positively and independently associated with the admission ln(parasitemia) and inversely associated with the admission hemoglobin level ($P = .004$ and $P = .015$, respectively). In multiple linear regression, (1) ln(parasitemia) and age were independent positive associates of ln(admission serum creatinine) ($P < .001$ and $P = .007$, respectively), (2) ln(parasitemia) and ln(plasma glucose) were independent associates of ln(admission serum total serum bilirubin) ($P = .003$ and $P = .008$, respectively), and (3) ln(parasitemia) was an independent associate of the ln(admission platelet count) and absolute differences between day 28 and hospital admission platelet counts ($P = .002$ and $P = .004$, respectively). In other multivariate models, ln(parasitemia) was not an independent associate of the admission hemoglobin level ($P = .49$) or serum ALT level ($P = .70$). In receiver operating characteristic curve analysis, parasitemia was a good predictor of complications after excluding hyperparasitemia (area under the receiver operating characteristic curve, 0.90 [95% confidence interval, 0.82–0.98]; $P < .001$). The prespecified 100,000/μL threshold was highly specific (specificity, 100%) but had a sensitivity of 30%.

DISCUSSION

The present study provides the first detailed, prospective evaluation of *P. knowlesi* infection in an area of Malaysian Borneo in which it is the most common locally acquired human malaria. Although there were demographic differences between the 3 groups of patients with malaria, there were no presenting symptoms or signs that distinguished knowlesi malaria from either falciparum or vivax malaria. Consistent with available—albeit, incomplete—retrospective data [1, 2], most cases of knowlesi malaria were uncomplicated and responded promptly to treatment with chloroquine and primaquine, but complications developed in nearly 1 in 10 patients. Because the number of cases of severe knowlesi malaria was small, an accurate case fatality rate is difficult to ascertain, but the case fatality rate was 1.8% (95% confidence interval, 0.2%–6.6%) in our sample. Malaria may have been a contributory factor rather than the sole cause in our patient who presented with a stroke. Nevertheless, *P. knowlesi* infections occur in older as well as younger adult patients in the Kapit Division, and the vital organ dysfunction caused by this parasite may unmask underlying significant comorbidities.

Despite the significantly lower peripheral blood parasitemia, the patients with knowlesi malaria had clinical and laboratory profiles that were largely similar to those for patients with *P.*

Table 3. Details of Knowlesi Patients Presenting with (Patients 1–8) or Developing (Patients 9 and 10) Severe Malaria

Patient	Age, years	Sex	Hyperparasitemia	Hypotension	Acute renal impairment	Jaundice	Hypoglycemia	Lactic acidosis	Severe anemia	Acute pulmonary edema or respiratory distress syndrome	Outcome
1	68	F	Yes (parasite count, 222,570 parasites/μL)	Yes (systolic blood pressure, 80 mm Hg)	Yes (serum creatinine level, 320 μmol/L)	Yes (total serum bilirubin, 45 μmol/L)	Yes (plasma glucose level, <1.1 mmol/L)	Yes (plasma lactate level, 17.4 mmol/L)	No	Yes	Died
2	36	M	Yes (parasite count, 178,000 parasites/μL)	No	Yes (serum creatinine level, 385 μmol/L)	No	No	No	No	No	Discharged
3	50	F	No	No	No	Yes (total serum bilirubin, 87 μmol/L)	No	No	No	Yes	Discharged
4	71	M	No	Yes (systolic blood pressure, 79 mm Hg)	No	No	No	No	No	No	Discharged
5	66	M	No	No	No	Yes (total serum bilirubin, 66 μmol/L)	No	No	No	No	Discharged
6	61	F	No	No	No	No	No	No	No	Yes	Discharged
7	69	F	No	No	Yes (serum creatinine level, 418 μmol/L)	No	No	No	No	Yes	Discharged
8	36	F	Yes (parasite count, 214,000 parasites/μL)	No	No	Yes (total serum bilirubin, 178 μmol/L)	No	No	No	Yes	Died
9	73	F	No	No	No	No	No	No	No	Yes	Discharged
10	54	F	No	No	No	No	No	No	No	Yes	Discharged

NOTE. Severe malaria was defined on the basis of World Health Organization criteria for severe falciparum malaria [11]: hyperparasitemia was defined as >100,000 parasites/μL. Severe anemia was defined as hemoglobin concentration <7.1 g/dL. Hypotension was defined as systolic blood pressure ≤80 mm Hg; Acute renal impairment was defined as a serum creatinine level >265 μmol/L despite rehydration. Jaundice was defined as serum bilirubin level >43 μmol/L. Hypoglycemia was defined as a serum glucose level <2.2 mmol/L. Hyperlactatemia was defined as a lactate level >6.0 mmol/L. Acute pulmonary edema or respiratory distress was defined as a respiratory rate >30 breaths/min plus oxygen saturation <94% on room air and/or pulmonary infiltrates visible on a chest radiograph.

Table 4. Measures of Outcome in Patients Categorized by *Plasmodium* Species

Variable	*Plasmodium knowlesi* (n = 107)	*Plasmodium falciparum* (n = 24)	*Plasmodium vivax* (n = 21)
Fever clearance time, h	20 (12–31)	20 (11–37)	16 (4–28)
Parasite clearance time, days	1 (1–2)	3 (2–3.75)	3 (2–3)
Duration of hospitalization, days[a]	3 (3–4)	4 (4–5)	4 (3–4)

NOTE. Data are median value (interquartile range).

[a] Excludes 2 patients who died and 2 patients with hospital admission and day 1 data only.

falciparum and *P. vivax* infection, with a wide spectrum of illness. The most frequent complication in our cohort was respiratory distress, which affected 1 in 15 patients. It is also a relatively common sequelum of severe falciparum malaria [13]. Respiratory distress can reflect pulmonary edema, acute respiratory distress syndrome, or metabolic acidosis. In our group, a pulmonary, rather than metabolic, etiology was the main cause, because we measured blood lactate concentrations and had access to chest radiographs and pulse oximetry. The strong association between parasitemia at hospital admission and the development of respiratory distress in our patients suggests that parasite-specific effects that increase pulmonary capillary permeability rather than iatrogenic fluid overload or the syndrome of inappropriate anti-diuretic hormone secretion are responsible, as in falciparum malaria [14]. Patients with falciparum malaria who develop respiratory distress have a relatively poor prognosis [13], and both of our patients who died developed this complication. Respiratory distress has also been reported as a rare complication of vivax [15–17] and ovale [18, 19] malaria. We cannot explain the disproportionate number of female patients with this complication in the *P. knowlesi* group.

Although the women in our cohort, compared with the men, had lower serum albumin concentrations at presentation (34.5 g/L vs 38.0 g/L; $P < .001$), sex association has not been reported in the case of the other human malarias and is likely to be attributable to the play of chance in the present study.

The *P. knowlesi* parasitemia at hospital admission was also strongly and independently associated with renal dysfunction, and 3 patients developed renal failure despite resuscitation and rehydration. As with respiratory distress, this is another complication of falciparum malaria that could be mediated by the parasite [20], although the microvascular sequestration that may contribute to *P. falciparum*–associated renal dysfunction [21] is not known to occur in *P. knowlesi* infection. The presence of *P. knowlesi* parasitemia at hospital admission was also independently associated with the total serum bilirubin but not serum ALT level. This could reflect relatively brisk hemolysis associated with the short (24-h) erythrocytic cycle rather than abnormal liver function, but the median parasitemia was low, and there was no inverse association with hemoglobin level at hospital admission. It is still possible that hepatic dysfunction is a relatively late vital organ complication of *P. knowlesi* malaria

Table 5. Changes in Laboratory Test Results between Hospital Admission and Discharge and Hospital Admission and Day 28 in Patients with *Plasmodium knowlesi* Infections

Variable	Change from hospital admission to discharge (n = 103)	Change from hospital admission to day 28 (n = 87)
Hemoglobin level, g/dL	−1.3 ± 1.0	0 ± 1.3[a]
White blood cell count, ×10³ cells/μL)	0.1 ± 1.8	1.2 ± 2.1[a]
Neutrophil count, median value × 10³ cells/μL (IQR)	−0.6 (−1.5 to 0.6)[a]	0.5 (−0.5 to 1.45)
Lymphocyte count, median value × 10³ cells/μL (IQR)	0.7 (0.40–1.3)[a]	0.9 (0.4–1.5)[a]
Platelet count, median value × 10³ platelets/μL (IQR)	65 (31–113)[a]	184 (144–222)[a]
Serum creatinine level, median μmol/L (IQR)	−8.5 (−19 to 1)[a]	−12 (−21 to 0)[b]
Serum sodium level, median mmol/L (IQR)	2 (0.1–5)[a]	3 (0–8)[a]
Serum total bilirubin, median μmol/L (IQR)	−6 (−15 to −3)[a]	−7 (−12.9 to −4.3)[a]
Serum alanine aminotransferase level, median IU/L (IQR)	−1 (−10 to 11)	−18 (−32.9 to −4)[a]
Serum albumin level, g/dL	−1.0 ± 2.8	4.8 ± 4.1[a]
Serum glucose, median mmol/L (IQR) (n = 56)	−0.4 (−1.1 to 0.8)	−0.6 (−1.21 to 0.37)[b]
Plasma lactate level, median mmol/L (IQR) (n = 56)	0.1 (−0.5 to 0.4)	−0.1 (−0.5 to 0.5)

NOTE. Unless otherwise indicated, data are mean value ± standard deviation. IQR, interquartile range.

[a] $P < .01$.

[b] $P \leq .05$.

but—as evidenced by patient 1, who presented with jaundice, hypoglycemia, and lactic acidosis—it is one with potentially devastating metabolic consequences.

Consistent with the nonsequestering nature of *P. knowlesi*, we did not observe significant neurologic sequelae except in patient 8, who had evidence of a stroke in the context of preexisting cerebrovascular risk. In addition, in contrast with the group of patients with *P. falciparum* infection, the group of patients with *P. knowlesi* included no patients with severe anemia. Both severe anemia and neurologic disturbance have been reported recently as common manifestations of severe vivax malaria [22, 23], but these complications were observed in patients who were younger than those in the present study and in areas of much greater malaria transmission of multiple *Plasmodium* species.

Despite the very high prevalence of thrombocytopenia among our patients with *P. knowlesi* infection (100%, compared with <80% in other human malarias [24–26]), none had a clinically evident coagulopathy. This is consistent with the relative infrequency of bleeding episodes complicating severe falciparum malaria [11], but it is possible that a low platelet count (52,000 platelets/μL) and prolonged prothrombin time (17 sec) contributed to an intracerebral hemorrhage in the patient with knowlesi malaria who died of a probable stroke. The almost invariable presence of thrombocytopenia could facilitate diagnosis of knowlesi malaria. In addition, the significant association between platelet count and *P. knowlesi* parasite density and, in turn, the relationship between parasitemia and markers of severity, could imply that very low platelet counts are of prognostic significance. Such a relationship has been found among African children with falciparum malaria [27].

Although our study included relatively few patients with severe knowlesi malaria, we provide preliminary data relating to the incidence of severe disease. A larger study on the main complications and pathophysiology of knowlesi malaria is in progress, with the aim of establishing specific criteria for severity. It is likely that those for severe falciparum malaria, including neurologic sequelae, severe anemia, and hyperparasitemia [11], may not adequately address the unique biologic properties of *P. knowlesi*. In the case of falciparum malaria, $\geq$250,000 parasites/μL (or 5% parasitized erythrocytes) is conventionally used [11], but thresholds as low as 100,000/μL have been associated with increased mortality and have been used for nonimmune patients [28, 29]. It is therefore important to determine knowlesi-specific markers of disease severity, especially an accurate risk-associated threshold parasitemia.

Our study shows that knowlesi malaria is a significant cause of morbidity in the Kapit Division, extends available data to characterize the spectrum of illness and its clinical course, and confirms our previous observation that life-threatening complications can supervene [2]. Knowlesi malaria is widely distributed in Southeast Asia; it affects mainly people who enter forests or the forest fringe, but the transmission ecology of this potentially serious disease may be changing [30]. Recently, European travellers to Malaysia have received a diagnosis of knowlesi malaria following their return home [31, 32]. The increase in tourism in Southeast Asia may mean that more cases are detected in the future, including in Western countries. Clinicians assessing a patient who has visited an area with known or possible *P. knowlesi* transmission should be aware of the diagnosis, its clinical manifestations, and its course.

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No. 28

医薬品 研究報告 調査報告書

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研究報告の概要

○英国の匿名扁桃検体中の疾患関連プリオンタンパク質の陽性率:横断的便乗検査

目的:英国の一般集団における疾患関連プリオンタンパク質(PrP(CJD))のより正確な陽性率を確認し、健康への脅威となる変異型クロイツフェルトヤコブ病(vCJD)伝播を低減するための適切な公衆衛生対策に役立てること。

デザイン、調査対象:イングランドおよびスコットランド全域で選択的扁桃摘出術で摘出された匿名扁桃腺検体を対象に、横断的便乗調査を実施した。

主要評価項目:分析原理の異なる2つの酵素免疫法を用いて調べたPrP(CJD)の有無(いずれかの方法で陽性となった場合は、免疫組織化学法または免疫ブロット法による更なる検査を行う)。

結果:2008年9月末までに63,007検体の検査が終了した。このうち12,753例は、vCJDのほとんどが発生した1961～85年生まれの集団であり、19,908例は感染牛肉および加工品によりBSEに曝露した可能性がある1986～95年生まれであった。検査検体のうち、両方の酵素免疫法で明らかな陽性となったものはなかった。1つの検査法で陽性となり、もう一方があいまい(擬陽性)となったのは2検体のみで、残り9検体は両方とも擬陽性結果となった。276検体は、どちらか一方の検査法で初回陽性であった。繰り返し陽性率は15%以下であり、検査法とカットオフの定義により左右された。免疫組織化学法または免疫ブロット法を実施した検体(初回陽性となった276検体すべてを含む)のうちPrP(CJD)陽性となった検体はなかった。

結論:観察された扁桃検体中のPrP(CJD)有病率は、1961～95年生まれの集団では0/32,661(95%信頼区間0～113/100万)であった。1961～85年生まれの有病率は0(95%信頼区間0～289/100万)で、過去の虫垂組織の調査(292/100万、95%信頼区間60～853/100万)より低かったが統計的に矛盾はなかった。引き続き扁桃検体を集めて検索することで、特に年長の集団、あるいは他の補完的な大規模な匿名の組織調査(特に剖検組織)の検索により、PrP(CJD)の陽性率の算出精度は更に高まるであろう。

報告企業の意見	今後の対応
英国の匿名扁桃検体中の疾患関連プリオンタンパク質の陽性率調査において、PrP(CJD)陽性が確認された検体はなかったとの報告である。	これまでの疫学研究等では、血液製剤を介して古典的CJD(弧発性、遺伝性および医原性CJD)が伝播するという証拠はない。またCJDの病原因子とされる異常プリオンがアルブミン製剤の製造工程で効果的に除去されるとの報告もあるが、輸血あるいは第Ⅷ因子製剤によりvCJDに感染する可能性が示唆されたことから、今後も引き続き情報の収集に努める。なお、日本赤十字社は、CJD、vCJDの血液を介する感染防止の目的から、献血時に過去の海外渡航歴(旅行及び居住)、CJDの既往歴(本人、血縁者)、hGH製剤投与の有無を確認し、該当するドナーを無期限に献血延期としている。

使用上の注意記載状況・その他参考事項等
赤十字アルブミン20 赤十字アルブミン25 赤十字アルブミン20%静注4g/20mL 赤十字アルブミン20%静注10g/50mL 赤十字アルブミン25%静注12.5g/50mL 血液を原料とすることに由来する感染症伝播等

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RESEARCH

Prevalence of disease related prion protein in anonymous tonsil specimens in Britain: cross sectional opportunistic survey

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ABSTRACT

Objective To establish with improved accuracy the prevalence of disease related prion protein (PrP^{dcp}) in the population of Britain and thereby guide a proportionate public health response to limit the threat of healthcare associated transmission of variant Creutzfeldt-Jakob disease (vCJD).

Design Cross sectional opportunistic survey.

Study samples Anonymised tonsil pairs removed at elective tonsillectomy throughout England and Scotland.

Setting National anonymous tissue archive for England and Scotland.

Main outcome measure Presence of PrP^{dcp} determined by using two enzyme immunoassays based on different analytical principles, with further investigation by immunohistochemistry or immunoblotting of any samples reactive in either assay.

Results Testing of 63 007 samples was completed by the end of September 2008. Of these, 12 753 were from the birth cohort in which most vCJD cases have arisen (1961-85) and 19 908 were from the 1986-95 cohort that would have been also exposed to bovine spongiform encephalopathy through infected meat or meat products. None of the samples tested was unequivocally reactive in both enzyme immunoassays. Only two samples were reactive in one or other enzyme immunoassay and equivocal in the other, and nine samples were equivocally reactive in both enzyme immunoassays. Two hundred and seventy six samples were initially reactive in one or other enzyme immunoassay; the repeat reactivity rate was 15% or less, depending on the enzyme immunoassay and cut-off definition. None of the samples (including all the 276 initially reactive in enzyme immunoassay) that were investigated by immunohistochemistry or immunoblotting was positive for the presence of PrP^{dcp}.

Conclusions The observed prevalence of PrP^{dcp} in tonsils from the 1961-95 combined birth cohort was 0/32 661 with a 95% confidence interval of 0 to 113 per million. In the 1961-85 cohort, the prevalence of zero with a 95% confidence interval of 0 to 289 per million was lower than, but still consistent with, a previous survey of appendix tissue that showed a prevalence of 292 per million with 95% confidence interval of 60 to 853 per million. Continuing to archive and test tonsil specimens, especially in older birth cohorts, and other complementary large scale anonymous tissue surveys, particularly of post-mortem tissues, will further refine the calculated prevalence of PrP^{dcp}.

INTRODUCTION

Although the risk to the population of Britain of dietar exposure to the bovine spongiform encephalopath agent that causes variant Creutzfeldt-Jakob disease (vCJD) has been virtually eliminated, the occurrenc to date of four cases of vCJD infection resulting fror blood transfusion has made real the threat of a second ary epidemic through healthcare associated human t human transmission.[1-4] These cases from blood trans fusion have also established the existence of an infec tive asymptomatic stage in human vCJD. Estimatin the prevalence of this asymptomatic infective stage although technically challenging, is essential to guid a proportionate public health response to reduce th risk of healthcare associated transmission.

Measurement of prevalence in the 1961-85 birt cohort is a priority, given that 138 of the 167 cases c vCJD to date in Britain have been in this group (with 3 cases in the 1961-9 and 99 in the 1970-85 birt cohorts). Data are available from previous analyses c appendix and tonsil specimens for the presence of dis ease related prion protein (designated PrP^{dcp}) b immunohistochemistry and immunoblotting.[5 6] Th first study screened 11 947 appendix specimens an 1427 tonsil specimens by immunohistochemistry an found three positives in the appendixes from the 1961 85 birth cohort, giving a prevalence of 292 (95% cor fidence interval 60 to 853) per million.[5] A second stud found no positives in 2000 tonsil specimens screene by both immunohistochemistry and immunoblotting half of these tonsils were from patients aged ove

9 years and hence in the birth cohort likely to have had dietary exposure to bovine spongiform encephalopathy. Uncertainty about the true prevalence was increased when back calculation using plausible assumptions from the observed clinical vCJD cases suggested a much lower prevalence of sub-clinical vCJD infection than would be predicted from the finding of PrP^{CJD} in three appendixes.[5,7]

The absence of a suitable blood test for PrP^{CJD}, and doubt about the clinical interpretation for a patient of a positive test result from testing any tissue, created major organisational and technical challenges for our large scale prevalence survey of PrP^{CJD}. To facilitate semi-automated enzyme immunoassay screening, we chose anonymised surgically removed tonsil pairs collected prospectively for the study reported here, rather than appendix tissue already archived in paraffin blocks that would have needed more labour intensive and slower immunohistochemical screening. PrP^{CJD} is known to accumulate to relatively high levels in the tonsils of people with vCJD, although, because of the difficulty of identifying such cases, it has not yet been shown to be present pre-clinically.[8,9]

Commercially available enzyme immunoassay kits are routinely used for testing for bovine spongiform encephalopathy, scrapie, and other animal prion diseases; however, when our survey began no validated kits were available for testing human samples for PrP^{CJD}. We therefore issued a formal tender calling for manufacturers to take part in an enzyme immunoassay selection study and to supply suitable kits. The companies that responded were each sent two blinded panels of samples. Two assays, from Microsens and Bio-Rad, were able to detect brain from vCJD cases diluted 10^{-3} and spleen diluted 10^{-2} into tonsil homogenate (Jillian Cooper, personal communication), and we selected these for use in this study. We now report the results of testing of the first 63 007 specimens from the intended collection of 100 000 in a national anonymous tissue archive.

METHODS

Test validation

We obtained unfixed palatine tonsil samples from 32 sheep with scrapie and 10 that were uninfected, as well as aliquots of unfixed frozen tonsil tissue taken at autopsy from six patients who died of vCJD. We prepared 12% homogenates from these and tested them by both enzyme immunoassays after making a dilution series from 10^{-1} to 10^{-5} with negative human tonsil homogenate. We used a panel of 250 human tonsils that had been previously tested and found to be negative by immunoblotting and immunohistochemistry as examples of "true" negative controls.[6]

Survey tissue samples

Paired tonsil samples from people of all ages, and from operations done between January 2004 and September 2008, were collected from hospitals throughout England and Scotland. One tonsil of the pair was collected as fresh tissue chilled to 4C, and the other tonsil was collected in formalin. Tonsils arrived at the study centre an average of 65 (mode 50, median 113) hours after operation. Once transferred to suitable containers, samples were stored either at −80C (fresh tissue) or at room temperature (fixed tissue).

Patients or their carers were given a leaflet explaining the aims of the study and that any result from testing their tonsil could not be traced back to them. An explicit paragraph and tick box to exercise a right to opt out of inclusion in the survey was included in the pre-tonsillectomy consent forms.

Investigatory algorithm

We homogenised a specimen of each tonsil pair and screened it with both enzyme immunoassays. We defined samples as "reactive," "high negative," or "negative" by a calculation based on the optical density readings from enzyme immunoassay for each microtitre plate. A reactive sample was within three standard deviations of the cut-off, and a high negative was within four standard deviations. We further investigated all samples that were initially reactive in either enzyme immunoassay or gave a high negative result in both enzyme immunoassays by immunoblotting and immunohistochemistry. We re-tested any sample that was high negative in one or other enzyme immunoassay by both enzyme immunoassays, and if it gave a reactive or high negative result in either we investigated it further by immunoblotting and immunohistochemistry. On occasion, we repeated immunoblotting tests with the same and with alternative antibodies.

Definition of a positive result

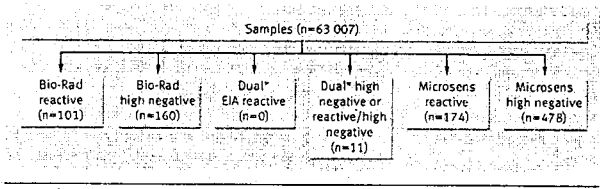
We defined a tonsil positive for PrP^{CJD} as one identified by enzyme immunoassay that was immunohistochemistry positive, had the expected specific protein band pattern in immunoblotting, or both.

RESULTS

Test performance

At a dilution of 10^{-3}, 31 of 32 scrapie sheep samples were reactive in both enzyme immunoassays, and at a 10^{-4} dilution 21were reactive in the Microsens enzyme immunoassay and 16 were reactive in the Bio-Rad enzyme immunoassay. One positive sample was detectable only at a dilution of 10^{-1}. Dilutions of 10^{-2} and 10^{-3} could be detected by immunoblotting.

The six tonsil aliquots from human vCJD cases varied in the amount of lymphoid germinal centre tissue that was present, as judged by visual inspection. Depending on the quality of the tissue, PrP^{CJD} was detectable down to a dilution of 10^{-3} in the Microsens enzyme immunoassay and 10^{-2} in the Bio-Rad enzyme immunoassay (table 1). The amount of PrP^{CJD} detected varied, as judged by the optical density values. This variation may have been due to biological differences in some cases, but an important contributory factor will have been the quality of the available tissue. Immunoblotting of aliquots of the vCJD samples showed that the expected specific band patterns of PrP^{CJD} were



Enzyme immunoassay screening of human tonsil tissue homogenates for PrP^{CJD} *Dual enzyme immunoassay (EIA) reactive samples gave optical density readings above the cut-off classified as "reactive" in both Bio-Rad and Microsens tests; dual high negative or reactive/high negative samples gave optical density readings above the cut-off classified as "high negative" in both Bio-Rad and Microsens tests or was reactive in one and high negative in the other. All EIA reactive samples and most high negative samples were subject to both immunoblotting and immunohistochemistry testing (see text)

detectable. The sensitivities of the enzyme immunoassays were comparable to the immunoblotting results.

Survey specimens collected

Between January 2004 and October 2008, a total of 67 696 tonsil pairs had been archived after collection from 134 hospital trusts throughout England and Scotland. We received forms without tonsil tissue for 1426 patients who objected and 762 in whom clinical pathology examination had been requested. All regions of England contributed samples, and 5651 came from Scotland between January 2006 and September 2008.

We also tested another 2015 anonymous specimens, from tonsillectomies done in the southeast of England between July 2000 and August 2002, of which half were from patients aged over 9 years at operation, and that were untested as part of an earlier survey.[6]

Table 1| Enzyme immunoassay results on available tonsil tissue from six variant Creutzfeldt-Jakob disease (vCJD) cases (including sample of brain from one case)*: highest dilutions for reported result

	Bio-Rad		Microsens	
Dilution†	Optical density	Interpretation	Optical density	Interpretation
Specimen 1:				
Tonsil 10^{-2}	0.06	High negative	0.12	Reactive
Brain 10^{-3}	0.39	Reactive	0.08	Reactive
Specimen 2:				
Tonsil 10^{-2}	0.04	Negative	0.11	Reactive
Specimen 3:				
Tonsil 10^{-2}	0.06	High negative	0.20	Reactive
Specimen 4:				
Tonsil 10^{-1}	0.04	Negative	0.10	Reactive
Specimen 5:				
Tonsil 10^{-3}	0.04	Negative	0.09	Reactive
Specimen 6:				
Tonsil 10^{-1}	0.13	Reactive	0.21	Reactive

*Three specimens supplied by National CJD Surveillance Unit (including paired tonsil and brain) and three by MRC Prion Unit.
†Dilution from 12% homogenate (10^0); 10^{-1} dilution is therefore equivalent to 0.012 g/ml vCJD tonsil tissue homogenate; as dilution is in negative homogenate, total tissue concentration was 0.12 g/ml for all samples tested.

Enzyme immunoassay screening results

By the end of September 2008, we had screened 63 00 samples with both enzyme immunoassays and, wher indicated, completed investigatory testing (figure).

In one or other of the enzyme immunoassays, 27 samples gave an optical density defined as reactive an 638 were classed as high negative (figure). To defin the repeat reactivity rate by enzyme immunoassay we retested 487 reactive and high negative sample by enzyme immunoassay at the beginning of the pro ject, before immunohistochemistry and immunoblot ting confirmatory testing. The repeat reactivity rat was 15% (7/48) for the initially reactive samples an 3.5% (4/116) for the initially high negative samples ir the Bio-Rad enzyme immunoassay. The equivalent fig ures for the Microsens enzyme immunoassay were 12% (7/60) and 10% (26/263). All initially reactive samples and any initially high negative samples tha gave a repeat reactive or high negative result enzyme immunoassay were subject to immunohisto chemistry and immunoblotting confirmatory testing Any samples that were initially reactive or high nega tive but which were not repeat tested by enzyme immu noassay went directly for immunohistochemistry and immunoblotting (figure).

No samples were clearly reactive in both enzyme immunoassays. One was reactive by Microsens and high negative by Bio-Rad, and another was reactive by Bio-Rad and high negative by Microsens. Nine were high negative by both the Microsens and Bio-Rad enzyme immunoassays. Seven of these 11 samples were methionine homozygote at codon 129 of the prion protein gene (*PRNP*) and four were heterozygote; only four (three homozygote and one heterozygote) were from people born before 1996 and therefore likely to have had dietary exposure to bovine spongiform encephalopathy.

Immunoblotting results

We demonstrated satisfactory immunoblotting perfor mance, using two different protocols in two separate laboratories, by testing the tonsil tissue taken at autopsy from vCJD patients, as well as by spiking experiments using scrapie sheep tonsil tissue, scrapie infected hamster brain, and human vCJD brain tissue.

None of the survey sub-sample investigated by immunoblotting gave a protein banding pattern consistent with the presence of PrP^{CJD}. Some samples that showed a single band, which was not consistent with any expected pattern, were re-tested by immunoblotting either with the same antibody or with different antibodies, including 3F4 and a secondary antibody designed to reveal non-specific antibody interactions. Only one sample still showed a single immunoblotting band; it was methionine homozygote at codon 129 and from a patient in the 1986-90 birth cohort, and it was negative by immunohistochemistry.

Immunohistochemistry results

More than 800 tonsils, selected on the basis of the enzyme immunoassay results, have been investigated

by immunohistochemistry in one or other of two experienced laboratories, and none was scored positive for PrP^{CJD}.

Prevalence estimates

Overall, 32 661 (52%) of the 63 007 samples tested came from people born in 1995 or earlier who were alive at the time when bovine spongiform encephalopathy contaminated meat was being consumed (table 2). The observed prevalence of PrP^{CJD} in this group was zero (95% confidence interval 0 to 113 per million). Combining the 1986-90 and 1991-5 cohorts gave a prevalence of zero with an upper 95% confidence limit of 185 per million. The prevalence in the combined 1996-2000 and 2001-7 unexposed cohorts was also zero with an upper 95% confidence limit of 122 per million.

Although the zero per million prevalence seen in the 1961-85 cohort (upper 95% confidence limit 289 per million) was different from the 292 per million (95% confidence interval 60 to 853 per million) found in the earlier survey of appendix tissue,[5] the 95% confidence intervals for both surveys overlapped (a formal comparison of the prevalence estimates gives a P value of 0.09).

DISCUSSION

Initial results from testing the tonsil specimens in a national anonymous tissue archive have shown the prevalence of PrP^{CJD} to be zero in 63 007 overall and zero in 12 753 in the birth cohort in Britain in which most cases of vCJD have occurred. Interpretation of this finding, and of the difference between it and the earlier survey of appendix tissue, depends critically on three factors: the sensitivity of the test system chosen to screen the tonsil specimens, the representativeness of the sample specimens of the people most vulnerable to vCJD disease, and the natural history of the infectivity of bovine spongiform encephalopathy in individual patients, particularly the time when PrP^{CJD} first appears pre-clinically in tonsil compared with appendix tissue and how long it persists.

Table 2 | Prevalence of disease related prion protein (PrP^{CJD}) in Britain by birth cohort (positive/total; rate per million with 95% confidence intervals*)

Birth cohort	Current (2004-September 2008) national tissue survey: tonsils	Earlier† (1995-9) national tissue survey	
		Appendices	Tonsils
1940 and before	NA	NA	0/225
1941-60	NA	0/573	0/266
1961-85	0/12 753; 0 (0 to 289)	3/10 278; 292 (60 to 853)	0/694
1986-90	0/9 564; 0 (0 to 386)	0/396	0/119
1991-5	0/10 344; 0 (0 to 357)	NA	0/106
1996-2000	0/15 708; 0 (0 to 253)	NA	0/17
2001-7	0/14 638; 0 (0 to 252)	NA	NA
Total	0/63 007; 0 (0 to 59)	3/11 247; 267 (55 to 779)	0/1 427; 0 (0 to 2 582)

NA=not available.
*95% confidence interval calculated only when denominator exceeds 1000.
†Data from separate tissue survey of 2000 tonsils (July 2000-August 2002) in southeast England (including London)[6] not included.

Test sensitivity

Three experiments investigated the sensitivity of the enzyme immunoassays. The first was the enzyme immunoassay selection study, the second was the interrogation of the enzyme immunoassays with tonsil tissue from sheep with scrapie, and the third was the use of tonsil tissue from patients who died from vCJD. Overall, these indicated that the Microsens enzyme immunoassay was more sensitive than the Bio-Rad enzyme immunoassay for detection of PrP^{CJD} in lymphatic tissue. The most sensitive detection was by the Microsens enzyme immunoassay with a sample containing 12 µg vCJD tonsil tissue; the equivalent for the Bio-Rad enzyme immunoassay was 480 µg vCJD tonsil tissue (table 1). When used for screening, 12 000 µg tonsil tissue was applied to the Microsens enzyme immunoassay and 48 000 µg to the Bio-Rad enzyme immunoassay. Therefore, the two enzyme immunoassays should have been sufficiently sensitive to detect PrP^{CJD} in tonsils from asymptomatic people incubating vCJD if levels of PrP^{CJD} were a 10th to a 1000th of those in patients with symptoms.

The dual enzyme immunoassay tonsil screening protocol may be at least as sensitive as any other large scale testing for abnormal prion protein that could have been used. The enzyme immunoassays use different test principles and antibodies, perhaps reinforcing the sensitivity of each. Reading of the results was automated, and we used a range of controls on each 96 well plate of tests. We deemed the use of a single enzyme immunoassay cut-off value as commonly applied to screen a population with many positives to be inappropriate, as this particular set of samples was expected (and found) to be overwhelmingly negative. Therefore, we calculated the cut-off value for each plate individually, and this method almost doubled the number of specimens that were selected for further investigation by immunoblotting and immunohistochemistry.

Several reasons exist why a specimen could have given a false high (reactive or high negative) optical density reading in either or both enzyme immunoassays: inadequate proteinase K digestion of PrP^{C} (the normal cellular form of PrP) for the Bio-Rad enzyme immunoassay, inadequate removal of PrP^{C} bound to the capture polyanion for the Microsens enzyme immunoassay, non-specific antibody interactions owing to the high antibody concentration in tonsil tissue, and poor sample quality or technical failures. Therefore, applying more specific immunoblotting and immunohistochemistry tests to confirm whether PrP^{CJD} was present was essential.

In comparison with immunohistochemistry, the volume of tonsil tissue screened by enzyme immunoassay was relatively large. Immunohistochemistry on appendix tissue may also be less specific than immunoblotting, so that prevalence estimated by immunohistochemistry screening may tend to overestimate the true situation.[9] However, to tackle the lingering uncertainty that screening immunohistochemistry might be more sensitive than dual enzyme immunoassay screening, a further study to re-test 10 000 of the archived tonsils by immunohistochemistry has been commissioned. These 10 000 samples comprise those from patients in the 1961-85 birth cohort, as well as any samples that gave optical density readings above the cut-offs in either of the two enzyme immunoassays. The results from this major undertaking should be available some time during 2009.

Two of the three positive samples in the retrospective immunohistochemistry study of appendix tissue were valine homozygous at codon 129 of *PRNP*.[5 10] Therefore, we can be confident that the antibodies used in our immunohistochemistry analysis would have showed PrP^{CJD} in a valine homozygote if it was present. The antibodies used in the enzyme immunoassay and immunoblotting would similarly be likely to detect PrP^{CJD} in a valine homozygote and, by extension, PrP^{CJD} in a heterozygote. Although the immunoblotting profiles of valine homozygote and heterozygote vCJD are unknown, they may be expected to consist of three or four glycoforms.[11] The immunoblotting profile of the spleen in a case of asymptomatic vCJD infection in a heterozygote patient showed similarities to that in clinical vCJD spleen samples in methionine homozygote patients, with a predominance of the diglycosylated band.[2] We did not observe by immunoblotting any pattern similar to any recognised profiles in sporadic CJD or vCJD.[12-16] The only repeatedly anomalous immunoblotting pattern seen was of a single immunoblotting band in an immunohistochemistry negative sample, which was methionine homozygote at codon 129 of *PRNP*.

Representativeness of sample

The age and sex characteristics of the samples in our study reflected the current age and sex distribution of people having tonsillectomy: 72% of those born in 1995 or earlier in our survey were female, compared with 48% of those born since 1995. Although only 44% of vCJD cases to date have been in women, we do not think that the predominance of females in our older sample of tonsils could have biased our findings with respect to prevalence of PrP^{CJD}.

Given the very strong association between PrP^{CJD} and people who are homozygous for methionine at *PRNP* codon 129,[5] it is important to note that our sample was likely to have been representative of this genetic susceptibility: an analysis of 466 of the tonsils in our survey showed 47% to be methionine homozygotes at codon 129, consistent with what was expected.[10 17-20] Therefore, of the 32 661 tonsils tested from people born before 1996, approximately 15 351 (47%) would have been from methionine homozygotes.

Several differences must be considered when comparing results between surveys. First and foremost is that previously appendix tissues were screened by immunohistochemistry, whereas we screened tonsil tissue by enzyme immunoassay. Secondly, an average of 10 years elapsed between when the previous large sample from the 1961-85 birth cohort had their appendixes removed (during 1995-9) until our sample had their tonsils removed (mostly in 2006-7)—10 years during which abnormal prion protein levels might be expected to have increased rather than diminished. Within this birth cohort, however, the average age of appendicectomy was estimated to be four years older than the average age of tonsillectomy, so the average duration of the opportunity for PrP^{CJD} to increase between the appendicectomy samples and the tonsillectomy samples would have been about six years. On the other hand, the relatively older appendix sample that was collected earlier may conceivably have contained a wave of infectivity in the 1961-85 cohort of the British population that was not present in the younger tonsil group that was sampled later.

Detailed information on previous operative history was sought on every vCJD case diagnosed in Britain. Seventeen of 167 patients were reported to have had tonsillectomy; 14 of these were in the 1961-85 birth cohort, and the remaining three were in the pre-1961 birth cohort. None was likely to have had specimens included in this or the earlier tonsil survey (Hester Ward, personal communication).[6]

Natural history

While PrP^{CJD} has been found consistently by immunoblotting and immunohistochemistry in tonsil tissue from patients with vCJD,[8 9 21-24] PrP^{CJD} in a tonsil from an asymptomatic person has yet to be reported. Given, however, that tonsillar tissue has been shown to accumulate PrP^{Sc} before the onset of clinical disease in non-human primates and well before the onset of clinical disease in sheep experimentally infected orally with bovine spongiform encephalopathy,[25 26] we considered tonsil tissue to be a reliable substrate for a survey of prevalence in humans. Also, the use of fresh tonsil tissue allowed more comprehensive laboratory testing, if necessary, after the initial screening assays.

PrP^{CJD} has been observed to accumulate in appendix tissue in vCJD (19/20 positive/tested)[9 27 28] and, in two cases, before symptoms developed.[29 30] However, data on the timing of the appearance of PrP^{CJD} in different peripheral lymphoreticular tissues during the prolonged incubation period of vCJD are sparse. The rate of accumulation of PrP^{CJD} in tonsil and appendix tissue could differ such that the findings of surveys of appendix and tonsil tissues would also differ. The positive samples found in the appendix survey presumably came from people who were infected a relatively short time earlier, during the peak of the bovine spongiform encephalopathy epidemic.[5] Moreover, should the incubation period for prion disease be considerably longer in people with different genotypes, uncertainty about the timing of the appearance of detectable PrP^{CJD} in these will increase, with concomitant implications for the interpretation of results of PrP^{CJD} prevalence surveys.

Animal experiments have shown that high infectivity, and indeed disease, can be present in the absence of detectable proteinase K resistant PrP^{Sc}.[31] The extent to

WHAT IS ALREADY KNOWN ON THIS TOPIC

Statistical back calculation based on cases of vCJD to 2004 has given estimates of between 10 and 190 further clinical cases over the next few decades

A study of archived appendix and tonsil tissues found a prevalence of lymphoreticular accumulation of pathogenic prion protein consistent with the existence of between 520 and 13 000 sub-clinical cases

Therefore, a discrepancy exists between estimates, which needs to be resolved to ensure that proportionate public health measures are implemented

WHAT THIS STUDY ADDS

Testing of tissue from more than 63 000 tonsils, of which 12 763 were from the 1961-85 birth cohort, has not shown evidence for the presence of the pathogenic form of the prion protein

The prevalence of sub-clinical vCJD infection in Britain may be lower than that given by previous estimates, with an upper limit of 289 per million in the 1961-85 birth cohort

which this observation can be generalised is, however, unclear, as PrP^{CJD} has been shown to be present in the lymphoid tissues of all vCJD patients tested.[9 27] If other, more reliable, indicators of vCJD become available, screening the existing samples with tests for these markers, and thereby determining whether any vCJD positives have been missed by looking only for PrP^{CJD}, may be possible.

Data from animal experiments also show "clearance" of abnormal prion protein after inoculation.[31 32] Therefore, the abnormal prion protein found in the earlier survey of appendix tissue may conceivably have been transient and eventually cleared without leading to disease, so that the appendix survey result would not have been replicated by the later tonsil survey.

Conclusion

We tested more than 32 000 tonsils from people in the age range most exposed to meat contaminated with bovine spongiform encephalopathy, and believed to be asymptomatic when sampled, for disease related prion protein. Using two sensitive enzyme immunoassays, with selective application of specific immunoblotting and immunohistochemistry techniques, we found no samples positive for PrP^{CJD}, a prevalence of 0 per million (with an upper 95% confidence limit of 113 per million). For the 1961-85 birth cohort, the prevalence of zero with a 95% confidence interval of 0 to 289 per million was lower than, but still consistent with, the earlier study of appendix tissue (60 to 853 per million). A P value of 0.09 applies to the comparison of the two prevalence estimates. These two surveys may not, however, be directly comparable owing to differences in testing methods, tissues sampled, and the time the tissues were removed (typically about 10 years earlier in the previous study). More data are needed through continuing the testing of tonsils from people born before 1996, despite the low frequency of tonsillectomy in older birth cohorts. In addition, creation and testing of other anonymous tissue archives, such as one based on coronial autopsies, or a repeat of the appendix survey on an even larger scale, should provide a larger sample set of the people most exposed to the bovine spongiform encephalopathy agent.[33]

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This study was originally proposed by a Medical Research Council and Department of Health committee. It was overseen by an Expert Advisory Group on the Laboratory Testing Strategy for Large Scale Abnormal Prion Prevalence Studies with a membership of N Andrews, D W G Brown, J P Clewley, J Cooper, R Eglin, E Gadd, O N-Gill, M Head, D A Hilton, J W Ironside, G Jackson, C M Kelly, G Mallinson, D Matthews, P Minor (chair), P P Mortimer, N Raven, J R Stephenson, and J D F Wadsworth. Data from this anonymous tonsil survey were discussed in public at meetings of the Spongiform Encephalopathy Advisory Committee (www.seac.gov.uk) in December 2007 and April 2008; and see a statement issued in August 2008 (www.seac.gov.uk/statements/state-cjd-infections.pdf).
Contributors: JPC designed and analysed the laboratory studies and wrote the paper with ONG, who initiated the study and did clinical and epidemiological analyses. CMK recruited hospitals to the study and did epidemiological analyses. NA did statistical and epidemiological analyses. KV organised the National Anonymous Tissue Archive laboratory, tonsil processing, and enzyme immunoassay testing. GM, MK, and RD did the immunoblotting. DAH, PE, JWI, LMcC, and DLR did the immunohistochemistry. JWI provided some of the vCJD clinical tissue used in the work. HEA did the codon 129 genotyping. JPC and ONG are the guarantors.
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