Maximizing the chances of detecting pathogenic leptospires in mammals: the evaluation of field samples and a multi-sample-per-mammal, multi-test approach

S. M. TULSIANI^{*,†}, G. C. GRAHAM^{‡,§}, M. F. DOHNT[¶], M.-A. BURNS[¶] and S. B. CRAIG^{§,¶}

^{*}*School of Veterinary Science, The University of Queensland, Gatton, Queensland, 4343, Australia*

[†]*Australian Bio-security Cooperative Research Centre, The University of Queensland, St Lucia, Queensland, 4072, Australia*

[‡]*Organic Chemistry, Food Chemistry, Queensland Health Forensic and Scientific Services, P.O. Box 594, Archerfield, Queensland, 4108, Australia*

[§]*Faculty of Science, Health and Education, University of the Sunshine Coast, Sippy Downs Drive, Sippy Downs, Queensland, 4556, Australia*

[¶]*WHO/OIE/FAO Collaborating Centre for Reference and Research on Leptospirosis, Communicable Disease Unit, Queensland Health Forensic and Scientific Services, P.O. Box 594, Archerfield, Queensland, 4108, Australia*

Received 21 October 2010, Revised 19 November 2010,

Accepted 26 November 2010

Identification of wild animals that harbour the causative leptospires, and the identification of the most important of these 'wild reservoirs' (in terms of threat to human health), are key factors in the epidemiology of human leptospirosis. In an epidemiological investigation in the Australian state of Queensland, in 2007–2008, samples were collected from fruit bats (*Pteropus conspicillatus*) and rodents (to investigate the potential role of fruit bats in the maintenance and transmission of leptospires to ground-dwelling rodents) and checked for pathogenic leptospires. The results of these studies have now been carefully analysed in attempts to see which method of detection and type of test sample were best. The effects of pentobarbitone sodium used to euthanize wild mammals before collection of necropsy samples, on the survival and detection of leptospires *in vitro*, were also explored.

In the earlier field investigation, serum, renal tissue and urine were collected from wild mammals, for the detection of pathogenic leptospires by culture, the microscopic agglutination test (MAT), real-time PCR and silver impregnation of smears. Although 27.6% of the rodents investigated were found leptospire-positive, culture only yielded four isolates, probably because many cultures were contaminated. The main aims of the present study were to quantify the performance of the individual diagnostic tests and examine the reasons behind the high incidence of culture contamination.

The results of sensitivity and specificity analyses for the different diagnostic tests indicated that isolation by culture (the definitive diagnostic test for leptospiral shedding) had perfect (100%) sensitivity when compared with the results of the PCR but a low specificity (40%). The MAT performed poorly, with a sensitivity of 50% when compared against the results of culture. The prevalence of leptospiral carriage revealed by the PCR-based investigation of kidney and urine samples (59.2%) was higher than that revealed using any other method and far higher than the 2.0% revealed by culture. The results of the culture of renal tissue agreed fairly well with those of the PCR-based investigation of such tissue, with a Cohen's unweighted kappa coefficient (κ) of 0.5 ($P=0.04$). The levels of agreement between other pairs of tests were generally poor.

Reprint requests to: S. M. Tulsiani, School of Veterinary Science, The University of Queensland, Gatton, Queensland, 4343, Australia.
E-mail: s.tulsiani@uq.edu.au.

The presence of pentobarbitone sodium, at final concentrations of 27.8 or 167 mg/ml, did not affect the viability or the detection of leptospires in culture, and is therefore unlikely to reduce the chances of isolating leptospires from an animal that has been euthanized with the compound.

It appears that collecting multiple samples from each mammal being checked will improve the chances of detecting leptospires (and reduce the chances of reporting an inconclusive result for any of the mammals). For the identification of a leptospiral carrier, however, the use of just two detection methods (culture and PCR) and one type of sample (renal tissue) may give adequate sensitivity and specificity. Given the robustness of PCR to contamination and its high sensitivity (it can give a positive result when DNA from just two leptospiral cells is present in the sample), a PCR-based serotyping method, to allow the combined detection and characterisation of leptospires from field isolates, would be extremely useful.

A rodent or other wild mammal, acting as renal carrier, maintenance host or 'reservoir', usually forms the focal point in the epidemiology of human leptospirosis. Leptospires colonize the renal tubules of the carrier animals and are then shed intermittently with urine, human infection usually resulting from direct or indirect contact with such infectious urine or with environmental sources that have been contaminated with it (Faine *et al.*, 1999). In a carrier animal, leptospires may persist in the renal tubules for months, years or the animal's whole life. The epidemiology of the pathogens is almost always affected by serovar-wild-host specificity and varying levels of endemicity for each serovar.

Human leptospirosis is characterised by two phases of clinical disease following infection (Craig *et al.*, 2009b). In the acute phase, leptospires can be found in the blood, brain and mucosal and genital fluids (Lilenbaum *et al.*, 2008). Usually, after a period of about 7 days, leptospires are cleared from the bloodstream by phagocytosis, and the infection then enters its chronic or convalescent phase. The chronic phase is characterised by the presence of anti-leptospiral antibodies in the bloodstream and/or the colonization of leptospires in the kidney of the infected animal (Alston and Broom, 1958). An incidental host (such as a human) with a recent infection displays a short period of renal shedding, followed by a high antibody response that either results in rapid recovery or fails to prevent death. Although most renal carriers or maintenance hosts do not appear to demonstrate

any clinical malaise on leptospiral infection, some show a quick onset of illness and then a rapid recovery characterised by intermittent shedding and low antibody responses (Atxaerandio *et al.*, 2005). The level of histological damage that occurs in the kidneys of carrier animals shows considerable variation. As described by Faine *et al.* (1999), the kidneys of chronic carrier animals can appear normal or show the typical signs of chronic nephritis. The kidneys of cattle, pigs or sheep that are carriers may have microscopic, rough membrane protrusions beneath the capsule (Chappel *et al.*, 1992). Such hosts may shed leptospires in their urine continuously or intermittently, and the reasons behind this are poorly understood (Levett, 2001).

Success in the detection of leptospires in carrier animals, and the isolation and in-vitro growth of the leptospires, cannot be guaranteed. A definitive diagnosis in such carriers cannot be made without laboratory confirmation, and carrier status cannot be confirmed without the demonstration of renal carriage and shedding. In contrast, in incidental hosts, blood and body fluids can also be examined directly in the acute stage of infection (Craig *et al.*, 2009a) and diagnosis can be complete with the demonstration of antileptospiral antibodies only.

Culture, serology and molecular methods are typically employed to detect pathogenic leptospires in blood and tissue samples, and culture may also be used to isolate the pathogens. In a renal carrier, the direct culture of urine may not always provide an accurate result because the carrier may only

be shedding leptospires intermittently. The culture of renal tissue, specifically the cortical and sub-cortical region, provides a more definitive diagnosis in the event of renal colonization (Turner, 1970). Foetal tissue may also be tested, as there is some evidence of vertical transmission among carriers (Turner, 1970).

Serologically, the microscopic agglutination test (MAT) is employed by reference laboratories to detect antileptospiral immunoglobulins in serum and to demonstrate seroconversion in paired acute and convalescent samples of serum (Levett *et al.*, 2005). The MAT is specific for serovars that are antigenically related but cross-reactivity can occur. In carrier animals, high MAT titres are less representative of general exposure to a serovar and more indicative of a recent infection, such that new infections in a population can be monitored by routine MAT (Rivera, 2005).

With the advent of DNA–DNA hybridization techniques, such as real-time PCR, the detection of leptospiral DNA at high sensitivities (the equivalent of two or more leptospiral cells) in small samples of blood and tissue became possible. Over the last decade, several procedures for the diagnosis of leptospirosis based on real-time PCR have been described, including a method based on a TaqMan probe and the 16S ribosomal-RNA region (Smythe *et al.*, 2002b) and one based on SYBR chemistry and the LipL32 lipoprotein region, which represents an outer-membrane protein of leptospires (Levett *et al.*, 2005). Although the TaqMan-based assay of Smythe *et al.* (2002b) has not been used to characterise a serovar or ascertain the carrier or incidental status of a host, it has been employed to detect leptospiral DNA in renal tissue, confirming the presence of leptospires in the animal providing the sample (Cox *et al.*, 2005). Detection of leptospiral DNA in serum is indicative of a new infection rather than carrier status. As Faine *et al.* (1999) stated, the choice, timing and interpretation of the diagnostic test or tests used are

central to the accurate diagnosis of leptospiral status.

The wild animals that act as renal carriers of leptospires are generally assessed on field sites, with sample collection performed under relatively basic, field conditions. Sample contamination or degradation during collection, packaging and transportation may be a major problem. Ideally, necropsy samples need to be collected within 15 min of an animal's death, before post-mortem autolysis allows microorganisms to migrate between fluids and/or tissues (Galton, 1962). In the nutrient-rich, growth and enrichment media used to culture samples, fungal spores and non-target bacteria can compete with leptospires and over grow them (Faine *et al.*, 1999). Even when successfully cultured in isolation, leptospires are renowned for their fastidious requirements during culture. Regardless of the choice of growth medium, slight changes in temperature and pH, for example, can affect the survival and growth of leptospires *in vitro* (Goldstein and Charon, 1988; Faine *et al.*, 1999). These and other factors generally make the successful recovery of leptospires difficult.

In 2007–2008, Tulsiani *et al.* (2011) conducted field sampling in Far North Queensland (FNQ), Australia, to investigate the role of Australian fruit bats in the transmission of pathogenic leptospires to rodents. In this study, the leptospiral status of rodents caught in areas with and without fruit-bat (*Pteropus conspicillatus*) colonies (known as 'colony' and 'control' sites, respectively) was explored and attempts made to isolate leptospires from rodent samples. The prevalence of leptospiral carriage detected in the sampled rodents (27.6%) was relatively low and attempts to isolate leptospires by culture only yielded four isolates. The contamination of cultures, with fungi or non-leptospiral bacteria, was a major problem. The study differed from previous, related surveillance studies in the use of pentobarbitone sodium to euthanize the mammals that were investigated. Rivera (2005) euthanized field animals with a mixture of CO₂ and air, as toxicity of pentobarbitone to leptospires was

suspected. Barbiturates are also known to interfere with lipopolysaccharide structure, potentially leading to damage and dysfunction, of the liver, exocrine pancreas and kidney, that could directly affect studies on endotoxaemic models (Danhof *et al.*, 1984; Kazerani and Furman, 2006). *Mycobacterium avium* has, however, been successfully isolated from calves euthanized with pentobarbitone sodium (Waters *et al.*, 2003), and pentobarbitone has historically and routinely been used to euthanize animals without, apparently, compromising veterinary diagnostic processes, including the collection and culture of bacteriological specimens. In the absence of any clear effect of barbiturates on the isolation of bacterial pathogens from tissue, pentobarbitone sodium was chosen by Tulsiani *et al.* (2011) as a rapid and humane euthanasia agent (unpubl. obs.).

The present study is based entirely on data collected by Tulsiani *et al.* (2011), some of which have not been published previously. The main aim of the present study was to optimise the field-sampling techniques and diagnostic tests used for the detection of leptospires and to quantify the loss of samples attributable to contamination and/or poor handling. A second aim was to investigate the effect, if any, of pentobarbitone sodium on the survival and detection of leptospires *in vitro*.

MATERIALS AND METHODS

Samples Collected

BLOOD

Samples of whole blood were collected from 213 rodents and 57 spectacled fruit bats (*Pt. conspicillatus*). The volume collected from each mammal (100–2000 µl) depended on body weight (and represented about 5% of that weight). Each of the smaller samples was collected from a retro-orbital sinus, using a non-heparinized haematocrit tube (VetQuip, Castle Hill, Australia), whereas the larger samples were collected by cardiac puncture, using a 25-gauge needle affixed to a 3-ml syringe (BD, North Ryde, Australia). Each

sample was allowed to clot, in a 2-ml microfuge tube (Bacto Laboratories, Liverpool, Australia), so that serum could be separated off, within 3 h of blood collection, by centrifuging at 3000 × g for 1 min. Whenever possible, a 200-µl aliquot of each serum was placed in a clean microfuge tube, for use in real-time PCR, and the remainder, if any, was placed in another microfuge tube, for use in a microscopic agglutination test (MAT) (see below).

URINE

Overall, 137 rodents and 57 *Pt. conspicillatus* collected during the survey were dissected, with urine collected aseptically, either by aspiration from a full urinary bladder or by lavage of a less full bladder with 1 ml of semi-solid Ellinghausen–McCulloch–Johnson–Harris (EMJH) medium (using a 25-gauge needle and 3-ml syringe). Each urine sample was used to set up a culture in a 7-ml, screw-cap, glass Bijou bottle (Sarstedt, Ingle Farm, Australia) holding 3 ml semi-solid EMJH medium [1.5% (w/v) agar and 0.09% (w/v) sodium pyruvate]. Once sealed, the bottles were transported to the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, in Brisbane, for culture (see below).

Samples of RNA extracted from 265 samples of urine from the four most common species of Australian fruit bat (*Pt. conspicillatus*, *Pt. alecto*, *Pt. poliocephalus* and *Pt. scapulatus*) were obtained from the Queensland Department of Employment, Economic Development and Innovation (DEEDI), in Brisbane. These samples had been prepared, as part of the routine screening of fruit bats in Queensland and Northern Territory for *Hendra virus*, in 2008–2009 (see Table 1). Each represented the viral nucleic acids extracted, from a 560-µl sample of fruit-bat urine, using a QIAamp viral RNA mini kit (QIAGEN, Doncaster, Australia) according to the manufacturer's instructions. Real-time TaqMan PCR for the detection of leptospires (see below) was performed on each RNA

TABLE 1. Summary of the samples tested, diagnostic tests performed, mammals found positive and mammals with inconclusive results

No. and (%) of animals examined by:																			
Species	Culture (urine and kidney)				MAT		PCR (serum)				PCR (urine and kidney)				Silver impregnation (kidney)		Any method		
	Source	Tested	Positive	Contaminated	Tested	Positive	With insufficient sample	Tested	Positive	Giving equivocal result	Tested	Positive	Giving equivocal result	Tested	Positive	With inadequate tissue on slide	Giving inconclusive result		
Any rodent	Field survey (control sites)	97	2 (2.0)	15 (15.4)	173	9 (5.2)	3 (1.7)	173	0 (0.0)	9 (5.2)	97	39 (40.2)	2 (2.0)	97	0 (0.0)	18 (18.5)	173	41 (23.7)	47 (27.1)
	Field survey (colony sites)	40	2 (5.0)	9 (22.5)	40	4 (10.0)	1 (2.5)	40	18 (45.0)	8 (20.0)	40	18 (45.0)	3 (7.5)	40	0 (0.0)	12 (30.0)	40	18 (45.0)	11 (27.5)
	Field survey (all sites)	137	4 (2.9)	24 (17.5)	213	13 (6.1)	4 (1.8)	213	0 (0.0)	17 (7.9)	137	57 (41.6)	5 (3.6)	137	0 (0.0)	30 (21.8)	213	59 (27.6)	58 (27.2)
	Tolga Bat Hospital	50	0 (0)	29 (58.0)	0	—	—	0	—	—	50	18 (36.0)	0 (0.0)	0	—	—	50	18 (36.0)	29 (58.0)
<i>Peropus conspicillatus</i>	Damage Mitigation Permit	7	0 (0)	1 (14.2)	7	1 (14.2)	0 (0.0)	7	1 (14.2)	0 (0.0)	7	2 (28.5)	0 (0.0)	0	—	—	7	3 (42.8)	1 (14.2)
	DEEDI (urine extracts)	0	—	—	0	—	—	0	—	—	265	207 (78.1)	1 (0.3)	0	—	—	265	207 (78.1)	1 (0.3)
<i>Pt. conspicillatus</i> , <i>Pt. alata</i> , <i>Pt. poliocephalus</i> and <i>Pt. scapulatus</i>	DEEDI (spleen extracts)	0	—	—	0	—	—	0	—	—	25	5 (20.0)	3 (12.0)	0	—	—	25	5 (20.0)	3 (12.0)
Any fruit bat		57	0 (0)	30 (52.6)	7	1 (14.2)	0 (0.0)	7	1 (14.2)	0 (0.0)	347	232 (66.8)	4 (1.1)	0	—	—	347	233 (67.1)	34 (9.7)
Any fruit bat or rodent		194	4 (2.1)	54 (27.8)	220	14 (6.3)	4 (1.8)	220	1 (0.4)	17 (7.7)	484	289 (59.7)	9 (1.8)	137	0 (0.0)	30 (21.8)	560	292 (52.1)	92 (16.4)

DEEDI, Queensland Department of Employment, Economic Development and Innovation.

eluate, on a positive leptospiral control (*Leptospira interrogans* serovar Pomona) and a positive *Hendra-virus* eluate, at a facility with level-3 biosafety.

KIDNEY

Kidney samples were collected from 137 rodents and 57 *Pt. conspicillatus* (see Table 1). Of the 57 fruit-bat specimens, 50 were acquired from the Tolga Bat Hospital (Herberton, Queensland, Australia), from bats that had been put down, with pento-barbitone sodium, because that had been severely ill as the result of toxin inoculated by paralysis ticks (*Ixodes holocyclus*) that had been feeding on the bats. The kidney samples from these bats had been collected at least 60 min after the bats had died. The seven other kidney samples from fruit bats that were investigated were each collected, under a Damage Mitigation Permit (DMP), in Queensland, within 10 min of each bat's death. Kidney tissue was collected, using standard aseptic dissection techniques, in the field. Each kidney was dissected out, dipped in 100% ethanol, flame-sterilized and then placed in a sterile Petri dish. A sterile scalpel and 23-gauge needle were used to dissect the kidney and cut a section across the cortex, medulla and renal pelvis. This section was placed in 3 ml semi-solid EMJH medium in a sterile, 7-ml Bijou bottle and transported to the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, in Brisbane, for culture (see below).

In addition, a 0.1-mm thick transverse section of each rodent kidney was placed on a glass slide and compression smeared (Frable, 1989) using a second slide held at right angles to the first. Each such smear was dipped in Diff-Quick fixative (Thermo Fisher Scientific, Scoresby, Australia) and allowed to air dry for 1 min before the slides were packed, one/mailler, in polypropylene slide mailers (Thermo Fisher Scientific) and transported to The University of Queensland's School of Veterinary Science, in Brisbane, for silver impregnation (see below).

A cross-section of the cortex of each kidney was also placed in 80% ethanol, for subsequent testing, in real-time PCR, for the DNA of pathogenic leptospires (see below).

SPLEEN

Samples of nucleic acids extracted from homogenates of the spleens of 25 fruit bats (*Pt. alecto*, *Pt. poliocephalus* or *Pt. scapulatus*) were obtained from DEEDI. The spleens had been collected from sick or injured bats around Brisbane, Queensland, in 1996–1997. Nucleic-acid eluates, created using QIAamp RNA mini kits, were tested for leptospiral DNA by real-time PCR (see below).

Tests Performed

CULTURE

At the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, in Brisbane, the samples received in semi-solid EMJH medium were sub-cultured into EMJH broth, incubated at 28–30°C and examined weekly for the presence of leptospires, by dark-field microscopy and/or by checking for sub-surface turbidity. The cultures were incubated for 13 weeks and then discarded if no growth had then been detected. Cultures that grew fungal colonies were also discarded. Cultures that showed growth of other bacteria (i.e. red spots, perhaps indicating *Staphylococcus aureus*, or other non-spirochaete, motile bacteria) were passed through sterile, 0.2-µm-pore membrane filters before being sub-cultured into fresh medium.

Cultures positive for leptospires were grown in 8-ml volumes of EMJH broth until there were at least 10⁸ cells/ml. The most probable leptospiral serogroup was determined by screening the unknown isolate against a known panel of antisera. The serogroup of the unknown culture was identified in serum agglutination tests (SAT; Faine *et al.*, 1999). Serogroup identity was accepted based on standard criteria for doubling-dilution titres

raised against a panel of 22 reference sera, with a threshold of 50% agglutination. Once an infecting serogroup was identified, an attempt was made to characterise the serovar with the 'gold-standard' cross absorption agglutinin test (CAAT). The CAAT uses heterologous antigens within the identified serogroup to agglutinate the unknown isolate until at least 10% of the heterologous titre is associated with one of the two antisera (Cerqueira and Picardeau, 2009).

For each successful isolation, a 1-ml aliquot of the culture in EMJH, with dimethyl sulphoxide added to give a final concentration of 2.5% (v/v), was cryopreserved in liquid nitrogen at -200°C (Palit *et al.*, 1986).

SEROLOGY (MAT)

MAT were used to check 220 sera (213 from rodents and seven from fruit bats) for *Leptospira*-specific antibodies. Each test or positive-control serum was diluted 1:25–1:6400 in phosphate-buffered saline at pH 7.4 (PBS), in microtitre plates (Millipore, North Ryde, Australia). A suspension of leptospires, representing one of a panel of known Australian or exotic serovars, was then added to each well. The contents of each well were mixed, by gently tapping the plates, before the plates were placed in an incubator at 30°C for 90 min. The level of agglutination in each well was then assessed by dark-field microscopy. The accepted end-point was the weakest dilution of serum that agglutinated at least 50% of the leptospires (when compared with the agglutination seen when the test serum was replaced with PBS). A mammal was considered seropositive if its serum gave an MAT titre of at least 1:50.

PCR

Nucleic acids were extracted from 220 sera (213 rodent and seven fruit-bat) and 194 kidney samples (137 rodent and 57 fruit-bat) in ethanol, using the High Pure PCR template preparation kit (Roche Scientific, Penzberg, Germany) according to the manufacturer's

instructions. With each serum, 200 μl were incubated at 72°C with 40 μl proteinase K (from the kit) and 200 μl lysis buffer (also from the kit), to lyse any cells. With each kidney sample in ethanol, the kidney tissue was ground to a coarse powder in a sterile, porcelain, mortar and pestle, with approximately 5 ml liquid nitrogen. Approximately 30 mg of the ground tissue were then transferred to a microcentrifuge tube, mixed with 40 μl proteinase K and 200 μl lysis buffer, and incubated at 55°C for 1–3 h or until the tissue was digested completely.

Nucleic acids were then extracted, as described in the kit protocol, using an inhibitor-removal buffer and wash buffer, the extracted DNA being eluted in a final volume of 200 μl .

The concentration of DNA in the eluate was checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific), with a concentration of ≥ 50 ng/ μl considered acceptable.

Each 200- μl eluate was then divided into four equal aliquots, which were stored at -20°C until needed.

Real-time TaqMan PCR was performed on each eluate, using a previously described assay (Slack *et al.*, 2007). Positive, negative and 'equivocal' samples were defined as having cycle-to-threshold (Ct) values of 5–40, >45 and 41–45 cycles, respectively. Any equivocal samples were re-run once (Smythe *et al.*, 2002b). The Ct values were also used to categorize leptospiral levels as 'heavy' (Ct <27), 'moderate' (Ct=27–35) or 'light' (Ct >35), as previously described by Cox *et al.* (2005). The Ct values for the positive controls all indicated 'heavy' leptospiral levels.

SILVER IMPREGNATION

The Warthin–Starry method of silver staining (Young, 1969) was used to reveal leptospires in the smears of rodent kidney. Positive control slides were prepared from histological sections of a (paraffin-embedded) whole kidney that was known to positive for

leptospire (the kidney coming from a laboratory mouse that had been experimentally infected at the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, in Brisbane). To minimize subjective error, all the stained smears were examined by one person and then cross-checked by another, at The University of Queensland's School of Veterinary Science.

Effects of Pentobarbitone Sodium

A commercial solution of pentobarbitone sodium (Lethabarb; Virbac, Miperra, Australia) was diluted with an equal volume of sterile water and then used to euthanize all the rodents and 50 of the 57 sampled fruit bats. An experiment was carried out to explore the effect of this euthanasia agent on the growth and survival of pathogenic leptospire in in-vitro cultures. A suspended culture of *L. interrogans* serovar Australis strain Ballico was serially decimally diluted with PBS, in a microtitre plate, so that each well contained 50 µl of a suspension containing between 10 and 10⁸ cells/ml. To each well was then added 50 µl pentobarbitone-sodium solution (the commercial solution diluted, with sterile water, to give 334.0 or 55.6 mg pentobarbitone sodium/ml) or, as a control, the same volume of PBS. After sealing the plates with paraffin film and mixing the well contents, by gently tapping the plates, the plates were incubated for 90 min at 30°C. Cultures were examined and concentrations of leptospire in them were evaluated (in a NanoDrop spectrophotometer) after 1.5, 5 and 24 h of incubation. The test was replicated on two more occasions, once with fresh culture and once with a culture that had been growing in the same EMJH medium for over 6 days.

Ethics

All animal experiments were conducted in accordance with the guidelines of the University of Queensland Animal Ethics Committee (SAS/388/06/CRC) and Queensland Parks and Wildlife Services (WISP04118306).

Data Analysis

Contamination of samples was defined by the presence of fungal spores or by the presence of bacteria (other than leptospire) that could not be cleared by filtration. For the present study, each test mammal was simply considered positive or negative in each type of test and in any type of test (with the species of leptospire ignored). As the results of the PCR-based examination of urine showed perfect concordance with the results of the PCR-based examination of kidney tissue, these two sets of results were analysed and presented as those of one test. A test mammal was considered 'contaminated for culture' if all three samples (two of kidney tissue and one of urine) taken from that animal, for culture, produced contaminated cultures.

Each diagnostic test was further evaluated for performance based on the inconclusive reporting of a result because of contamination (in the case of culture), the insufficiency or absence of a sample (MAT and silver staining) or an 'equivocal' reading in both replicates (PCR).

Sensitivity, specificity and prevalence estimates were generated for each of the tests, once by taking the results of culture as the 'gold standard' (i.e. as an accurate representation of true positive and negative status) and once by taking the results of the PCR-based assays (of urine and kidney) as the 'gold standard'. Unweighted Cohen's kappa tests were used to evaluate the agreement between the results of two methods, with κ -values of <0, >0 and 1 indicating agreements that were 'below-chance', 'above-chance' and perfect, respectively.

A Pearson's two sample *t*-test with unequal variance was used to compare leptospiral numbers in cultures, before and 1.5, 5 and 24 h after exposure to pentobarbitone sodium.

RESULTS

Culture

Overall, just four (2.0%) of the cultured samples, of renal tissue or urine from 137

FIG. 1. The percentages of the samples collected from rodents (■) and fruit bats (□) for which test results were inconclusive because of contamination that could not be cleared (culture), serum samples that were too small (microscopic agglutination test), equivocal readings (PCR) or inadequate tissue on slides (silver staining).

rodents and 57 fruit bats, yielded leptospiral isolates. All four positive samples were of rodent samples. Twenty-four (17.5%) of the rodents and 30 (52.6%) of the fruit bats were 'contaminated for culture', the 30 fruit bats comprising just one (14.3%) of the seven from which samples were collected within 10 min of death but 29 (58.0%) of the 50 from which samples were collected at least 60 min post-mortem (see Table 1 and Figure 1).

Although cultures of urine collected by flushing were more likely to be contaminated than cultures of kidney tissue or

cultures of urine collected by aspiration, two of the four strains of *Leptospira* that were successfully isolated came from cultures of urine collected by flushing the urinary bladder. Of the seven cultures of urine collected from fruit bats by flushing the bats' bladders, four (57%) were contaminated (see Figure 1 and Table 2).

MAT

No serum samples tested for the presence of leptospiral antibodies by MAT were apparently

compromised by contamination. Four (1.87%) of the serum samples collected from rodents (but none of those collected from fruit bats) were, however, too small to perform accurate MAT (Table 1).

Overall, 13 (6.1%) of the 213 rodents and one (14.2%) of the seven fruit bats checked by MAT were found seropositive (see Table 1 and Figure 1). Although sera from two *Rattus sordidus* were found positive for *L. interrogans* (serovars Australis and Zanoni) by MAT, samples from these two rodents were found negative by PCR and culture. These two 'discrepant' samples were included in the analyses of test performance and as indicators of exposure but excluded from the estimates of the prevalences of leptospiral carriage and infectious-carrier status.

PCR

Although no leptospiral DNA was detected in the sera of 213 rodents, it was found in the serum of one (14.2%) of the seven fruit

bats investigated. The fruit bat with PCR-positive serum was found negative by culture and MAT but to have PCR-positive kidney tissue and urine. This 'discrepant' sample was included in the analyses of test performance and as an indicator of exposure but excluded from the estimates of the prevalences of leptospiral carriage and infectious-carrier status. No PCR-based investigation of rodent or fruit-bat serum was apparently compromised by contamination but each of 17 (7.9%) of the 213 rodent sera that were tested by PCR gave equivocal results (i.e. Ct values of 41–45) in two PCR-based tests.

Leptospiral DNA was detected in nucleic-acid extracts of the kidneys of 57 (41.6%) of the 137 rodents and 232 (66.8%) of the 347 fruit bats investigated. Each mammal found to have a PCR-positive kidney sample (and no others) was found to have PCR-positive urine. Twenty (35.0%) of the 57 *Pt. conspicillatus* investigated had PCR-positive kidney and urine samples. Most (78.1%) of the 265 DEEDI-supplied extracts from fruit-bat urine and five (20.0%) of the 25 extracts of splenic homogenates that were investigated were also found PCR-positive for leptospirosis (Table 1). No nucleic-acid extracts of kidney tissue, urine or spleen tissue were apparently compromised by contamination but all of the kidney and urine extracts from five (3.6%) of 137 rodents and the kidney, spleen and/or urine extracts from four (1.1%) of 347 fruit bats — none (0%) of the 57 *Pt. conspicillatus* investigated, one (0.3%) of the 265 DEEDI-supplied extracts from fruit-bat urine and three (12.0%) of the 25 homogenized spleen extracts — gave irresolvable equivocal results in PCR-based tests (see Table 1 and Figure 1).

Silver Impregnation

No leptospire were seen in any of the smears of rodent kidney that were stained using the Warthin–Starry method of silver impregnation (no fruit-bat samples were tested for leptospirosis by this method).

TABLE 2. The numbers of urine samples collected by bladder flushing and the numbers of those samples giving positive or contaminated cultures

Species	No. of samples		
	Tested	Positive	Contaminated
RODENTS			
<i>Isodon macrorus</i> *	2	0	0
<i>Melomys burtoni</i>	18	0	4
<i>Melomys cervinipes</i>	24	0	3
<i>Mus musculus</i>	11	1	2
<i>Perameles nasuta</i> *	2	0	0
<i>Rattus fuscipes</i>	41	1	6
<i>Rattus leucopus</i>	0	—	—
<i>Rattus lutreolus</i>	2	0	0
<i>Rattus rattus</i>	18	0	5
<i>Rattus sordidus</i>	17	0	3
<i>Rattus tunneyi</i>	0	—	—
<i>Uromys caudimaculatus</i>	2	0	0
Any	137	2	23
<i>Pteropus conspicillatus</i>	7	0	4

*Bandicoots that were treated as rodents in the data analyses.

Although none of the smears was obviously affected by contamination, the smears produced from the kidneys of 30 (21.8%) of the 137 rodents investigated in this way were considered to hold inadequate tissue. These 30 mis-processed or damaged smears were categorized as 'no tissue detected' in the data analyses (see Figure 1 and Table 1).

Pentobarbitone Sodium

After incubation for 1.5, 5 or 24 h, concentrations of leptospires in the test wells were not significantly affected by the presence of pentobarbitone sodium. At the end of the 24-h incubation, for example, the two tested dilutions of the drug gave very similar results ($P=0.89$), the leptospire densities in the test wells then correlating with the numbers of leptospires inoculated into each well, whether pentobarbitone sodium was present — even at the higher concentration tested ($R=0.82$; $P<0.001$) — or not (Fig. 2). Some agglutination of leptospires was observed in the cultures set up using leptospires that had been in the same culture for at least 6 days immediately before their use. This agglutination was, however, similar whether or not pentobarbitone sodium was present.

Comparison of Culture with the Other Tests

When the results of culture were treated as the gold standard, the performances of the PCR-based testing of tissue/urine appeared to be quite good, with a sensitivity of 100% and a specificity of 40%. Although silver impregnation gave the highest specificity (100%), its sensitivity was zero. The MAT also performed reasonably well, with a sensitivity of 50% and a high specificity of 98%. Although the results of culture indicated an overall prevalence of leptospiral carriage of 2.0%, the corresponding prevalences indicated by MAT (6.1%), silver impregnation (0.0%) and, particularly, the PCR-based investigation of tissue/urine (59.2%) were very different (Table 3).

Five tested mammals gave 'discrepant' results. Two of these were rodents that were found MAT-positive but negative for leptospires by culture or PCR; data for these two animals were excluded when reporting renal shedding but included in the analyses of exposure and the comparisons of the diagnostic tests. In addition, a *Melomys burtoni* from a control site (found infected with *L. interrogans* serovar Australis), a specimen of the same species from a colony site (*L. interrogans* serovar Zanoni) and a *Mus musculus* from a colony site (*L. interrogans* serovar Australis) were all found MAT-negative but positive for leptospires by culture of kidney or urine and in PCR-based tests of kidney or urine; the data for these three rodents were included in the estimates of exposure and prevalence as well as the comparisons of the diagnostic tests.

Comparison of PCR-based Investigation (of Urine or Tissue) with the Other Tests

If the results of the PCR-based investigation of urine/tissue were treated as the gold standard, culture and silver impregnation both appeared to offer perfect (100%) specificity but very poor sensitivities, of 3% and zero, respectively (Table 3). The MAT also had poor sensitivity (10%) but high specificity (97%).

Overall Result per Specimen, Using Multiple Tests

The number of positive outcomes, as reflected in the prevalences of leptospiral carriage indicated by the results of each type of test, was greatest with the PCR-based investigation of urine and tissue extracts, followed by MAT, then culture and finally by silver impregnation. For the rodents investigated, the level of agreement between the results of culture and MAT ($\kappa=0.45$; $P=0.05$) was poorer than that between culture and PCR of urine/tissue ($\kappa=0.50$; $P=0.04$). For the fruit bats, Cohen's kappa could not be estimated for a comparison

FIG. 2. Leptospire concentrations seen in cultures, after incubation for 24 h, in the absence (■) or presence (□) of pentobarbitone sodium at 167 mg/ml (a) or 27.8 mg/ml (b).

between the results of culture and any of the other detection methods (because the observed concordance was zero in each

case) and the levels of agreement between the results of MAT and PCR of urine/tissue ($\kappa=0.48$; $P=0.04$), MAT and PCR of

TABLE 3. The performance of each diagnostic test when the results of culture or the results of the PCR-based investigation of kidney were used as the 'gold standard' (showing the pooled results for the rodent and fruit-bat samples)

Gold standard	Variable	Value for:				
		Microscopic agglutination test	PCR (serum)	PCR (kidney)	Silver impregnation	Culture
Results of culture	Sensitivity (%)	50	0	100	0	–
	Specificity (%)	90	90	40	100	–
	Indicated prevalence (%)	6.1	0.5	59.2	0	–
Results of PCR	Sensitivity (%)	10	0	–	0	3
	Specificity (%)	97	98	–	100	100
	Indicated prevalence (%)	6.1	0.5	–	0	2.0

serum ($\kappa=0.05$; $P=0.03$), and PCR of urine/tissue and PCR of serum ($\kappa=0.02$; $P<0.001$) were also poor.

DISCUSSION

Concept and Hypothesis: Justification

In the observational study described by Tulsiani *et al.* (2011), which had a cohort design, the role of Australian fruit bats in the transmission of pathogenic leptospires to rodents was explored. Ground-dwelling rodents associated with fruit-bat (*Pt. conspicillatus*) colonies were compared, in terms of leptospiral carriage, with similar rodents that lived in areas without fruit-bat colonies. The demonstration of renal shedding of leptospires by fruit bats, via the isolation of leptospires from fruit-bat kidney or urine samples, was a key aim, as this is the definitive means of demonstrating active carriage and would provide a culture collection of isolates that could be characterised and compared (with, for example, the serovars present in the local rodents).

Taken together, the results of the study as published by Tulsiani *et al.* (2011) and the present results (based on data collected in the same study) indicate lower-than-expected prevalences of leptospiral carriage among rodents at the colony (22.5%) and control (42.5%) sites and among fruit bats (35.1% if the DEEDI-supplied extracts are excluded, otherwise 78.1%). Attempts at

isolation by culture only yielded four isolates (all from rodents and none for fruit bats), perhaps because of the contamination of many samples. In the present study, an analytical review was performed to assess the degree and reasons for sample compromise, check the levels of agreement between the results of the various diagnostic tests employed, investigate the merits of a multi-sample approach (to increase the chance of detecting leptospires) and provide recommendations for the optimal methodological approaches to be used in similar field trials in the future. In addition, an in-vitro experiment was conducted to investigate whether the pentobarbitone sodium used as an euthanasia agent was toxic to leptospires and may therefore have contributed to the general lack of success in the isolation of leptospires by culture.

Reliability of Each Test

The testing of tissue and/or urine samples in assays based on real-time PCR indicated the highest prevalence of leptospiral carriage (59.2% overall, using samples from 484 mammals). Fifty-seven such samples (of 137) from rodents, 20 (of 57) from the fruit bats collected at the colony sites in FNQ, and 232 (of 347) from other fruit bats collected in Australia (including the DEEDI-supplied extracts) were found PCR-positive. At the same time, the PCR-based testing of tissue and urine gave the lowest percentage of inconclusive results, with just 4.3% of the

484 mammals tested giving two equivocal Ct values each. These results indicate that the PCR-based testing of tissue/urine is a relatively robust method, perhaps because it is very specific and so largely unaffected by sample contamination. The real-time PCR used in the present study is capable of detecting just 10 leptospiral cells in a urine sample and just two such cells in serum (Smythe *et al.*, 2002b), the assumption being that urine contains PCR-inhibitory compounds that are not found (or are found at lower concentrations) in serum. In the present study, encouragingly, the results of the PCR-based testing of kidney tissue showed perfect concordance with the results of the PCR-based testing of urine, partly, perhaps, because leptospiral loads were at least 'moderate' in almost all (86.2%) of the samples involved (data not shown). The DEEDI-supplied samples of nucleic acids from fruit-bat urine and spleen were extracted using the QIAamp Viral Mini RNA kit, which has not yet been validated for leptospirosis diagnosis via real-time PCR. Therefore, although the results of the PCR-based testing of the DEEDI-supplied extracts of fruit-bat urine indicated a high prevalence of leptospiral carriage amongst fruit bats (93.2%), which is in concordance with previous findings (Smythe *et al.*, 2002a; Cox *et al.*, 2005), they were excluded from the analyses of test robustness. Furthermore, the urine extracts provided by DEEDI were prepared using urine samples collected underneath fruit-bat diurnal camps. As each such sample could contain the urine of several bats, the prevalence of PCR-positivity among the DEEDI-supplied urine extracts is likely to be an over-estimate of the true prevalence within the sampled fruit-bat population. Despite these limitations, the detection of leptospiral DNA in the urine and splenic tissues of all four of the common species of fruit bat from around Queensland and the Northern Territory and the high prevalence indicated by the PCR-based testing of (probably pooled) urine samples from such bats are a significant indicator of

renal shedding and the leptospiral burdens in the bat colonies, which could pose a significant transmission risk to in-contact rodents.

Although the detection of *Leptospira*-specific antibodies by the MAT indicated the second highest prevalence of leptospiral carriage (6.1%), this was far lower than that revealed by PCR. The presence of antibodies can be used as an indicator of infection but *Leptospira*-specific IgG antibodies are only detectable in serum collected in the convalescent phase of infection (Cerqueira and Picardeau, 2009), making the period during which the MAT would give a positive result quite narrow. The MAT is consequently an unreliable test to detect active renal shedding in a renal carrier. When a human patient or other animal is in the acute phase, antileptospiral antibodies will not be detected in its serum but leptospires and/or leptospiral DNA may be detected in its bloodstream. In the present study, the mammals that were found either positive by culture or PCR of tissue but MAT-negative or to have leptospiral DNA but no MAT-detectable antibodies in their serum may simply have been sampled when they were in the acute phase. During this phase of the infection, it should be possible to isolate leptospires by the culture of blood samples. For the maintenance hosts (renal carriers) of leptospires, however, it is extremely difficult to identify animals in the acute phase of infection unless mark-recapture studies are performed over several generations.

Leptospires were only isolated from four (2.0%) of the 194 mammals checked by culture. Isolation by culture also gave the highest percentage of inconclusive results (27.8% of samples), all attributable to unwanted fungal or bacterial contamination. When, for the rodents, the results of culture of tissue or urine were compared with the results of PCR-based testing, 53 PCR-positive specimens were found culture-negative, highlighting the limitations of using culture as the exclusive method of leptospiral detection (Bolin, 1996). When

the results of PCR were used as the gold standard, culture appeared to have perfect specificity but a poor sensitivity of just 3%. If the culture results were used as the gold standard, the performance of MAT also appeared poor, with a sensitivity of 20%. The silver impregnation of tissue smears performed the poorest of all the tests, with no sample found smear-positive and a sensitivity of zero (whether culture or PCR was assumed to be the accurate test).

The level of agreement between the results of any two tests was generally poor, as indicated by low κ -values. Relatively few mammals were found positive by any one test, however, and only one was found positive for leptospires by all three main tests (i.e. culture, MAT and PCR). The poor between-test agreement and the successful isolation, by culture, of leptospires from one kidney sample from a rodent specimen, whilst cultures of the other kidney sample and the urine sample from the same mammal had to be discarded because of contamination, indicates the benefit, in carrier-host studies, of collecting multiple samples from each test animal and performing multiple tests on each animal.

Problems with Individual Tests

Most negative cultures were found to be heavily contaminated. Although the isolation of leptospires by culture is, by definition, a deterministic test, it is not often the most reliable because of problems with contamination and the viability of leptospires (Webster *et al.*, 1995). Furthermore, samples for culture, if collected at necropsy, need to be collected immediately or soon after death, to avoid post-mortem bacterial invasion (Galton, 1962; Bolin, 1996). The importance of speed in the collection of necropsy samples is reflected in the present study, where cultures from 29 of 50 fruit-bat samples collected at least 60 min post-mortem were contaminated. Cultures of kidney or urine samples from 24 rodents and 30 fruit bats were found contaminated

and it appeared particularly hard to collect urine samples by flushing bladders without contaminating the samples (Table 2), probably with air-borne bacteria and fungi. The culture of urine samples might be improved by diluting the samples with culture medium and passing the mixture through a sterile 0.2- μ m-pore filter **before** incubating it and/or by wiping the urinary tract, pre-dissection, with sterile swabs. Although the probability of contamination was high when urine samples were collected by flushing, this method did yield two *Leptospira* isolates, indicating that the optimisation of this technique may be worthwhile.

The compression smearing of kidney tissues, followed by silver impregnation and microscopical examination, was ineffective in detecting leptospires, perhaps because of non-specific background staining, precipitation of silver ions and/or the rupture of cells during smearing (Ellis *et al.*, 1983). Although the results of silver staining of tissue smears were also found to be disappointing in some earlier studies (Galton, 1962; De Koning *et al.*, 1987; Webster *et al.*, 1995), the similar staining of tissue that had been fixed in formalin, embedded in paraffin wax and sectioned gave better results (Chappel *et al.*, 1992; Faine *et al.*, 1999), perhaps because such processing helps to keep cells intact (Smith *et al.*, 1994). Paraffin embedding would also avoid the problem of the loss of smears (which adversely affected the investigation of 30 samples in the present study), although tissue sections can also fall off slides.

The advantages of real-time PCR include relatively short turn-around times, elimination of the need to produce reference hyperimmune antisera for convalescent responses, the reduction of sample contamination (using closed-tube techniques), the need for no post-PCR manipulation (in the form of gel electrophoresis and ethidium-bromide staining), high sensitivity, and high specificity (Smythe *et al.*, 2002b; Levett *et al.*, 2005; Slack *et al.*, 2007). In the

present study, the PCR-based testing of either kidney samples or urine gave the highest levels of detection, with 57 (41.6%) of 137 rodents and 20 (35.1%) of 57 fruit bats from FNQ found positive (even though some of the corresponding cultures were contaminated). Some obvious disadvantages of real-time PCR, at least in this context, are its inability to characterise the serogroup or genotype that is contributing any leptospiral DNA that is detected, and its inability to prove the presence of viable leptospires. The cycle-to-threshold value (Ct) is indicative of the amount of target DNA in a sample (Cox *et al.*, 2005) and may therefore be generally representative of the infectiousness of a sample. Several molecular assays have been proposed to overcome the limitations outlined above, including restriction-enzyme analysis of PCR products (REA; Perolat, 1990), the sequencing of amplicons (Wangroongsarb *et al.*, 2007), the random amplification of polymorphic DNA (RAPD; Corney *et al.*, 1993), multilocus variable number tandem repeat analysis (MLVA; Slack *et al.*, 2007) and multi locus sequence typing (MLST; Thaipadungpanit *et al.*, 2007).

Reactions with Pentobarbitone Sodium

Leptospires can survive low concentrations of sodium and, in fact, most culture media contain 10–40 mM sodium (ions), for salinity. When Toon and Rowland (1983) investigated the pharmacokinetics of barbiturates in rats treated with the drugs, they found that barbiturates have greater tissue affinity than plasma affinity. Although this characteristic should reduce the exposure of leptospires to barbiturates in their host, the possibility that even low plasma concentrations of such compounds may be toxic to the pathogens remains. In the present study, however, there was no evidence that pentobarbitone sodium had any effect on the survival or detection of leptospires (Fig. 2), even when present at concentrations far higher than those that occur in the plasma of a

mammal that has been euthanized with the drug. It therefore seems unlikely that the low level of successful isolation of leptospires reported here can be attributed to the use of pentobarbitone sodium as the euthanasia agent, although it remains possible that the solution of pentobarbitone sodium injected into the mammals was contaminated, leading to the contamination of the mammal's tissues (Hansen, 2000) and the subsequent contamination of cultures of some of those tissues.

Multi-sample Approach: Conclusions

In the accurate diagnosis of leptospirosis, the context in which the result will be interpreted is a crucial factor in the selection of samples to be collected and the diagnostic test or tests to be performed. In addition, the timing of the tests (Tulsiani *et al.*, 2010) and the constraints of the study, such as animal ethics and productivity considerations, influence access to desirable samples. In a clinical-disease scenario, the MAT-based detection of leptospiral antibodies or the PCR-based detection of leptospiral DNA in serum samples indicates past or new infection (Craig *et al.*, 2009a). In the context of experimental or field-based studies on renal carriage or transmission, however, the main aim is likely to be the demonstration of active infection and transmission potential. This may be best achieved by direct culture of infectious tissue or fluid, to confirm the presence of leptospires. In such cases, the detection of leptospiral DNA in the bloodstream is indicative of a new or second infection and should not be used as evidence of carrier status. The detection of such DNA in renal tissue or fluid would, however, be indicative of shedding and therefore of infectiousness (Cox *et al.*, 2005).

If the purpose of the study is simply to detect the presence or absence of clinical leptospirosis, then the PCR-based investigation of kidney tissue and serology by MAT may be the most expedient and effective

combination of tests. Such an approach would be unsuitable, however, if the researcher wanted to compare isolates and the characteristics of infecting serovars (e.g. across host species and study sites), when direct culture would be the recommended test.

The results of the present study indicated weak agreement between the results of any two test methods. For the purposes of this study, however, high sensitivity was more important than high specificity, and a combination of three methods — the PCR-based testing of tissue/urine, direct culture and MAT — appeared to offer both high sensitivity and reliability. The use of a single diagnostic test with low sensitivity and robustness, such as culture, would have given a poor estimate of the prevalence of leptospiral infection/exposure, especially if only one test sample had been collected from each mammal. Ideally, multiple samples should be collected from each test mammal and several methods should be used to investigate those samples (with the choice of methods dependent on the main aims of the study). The present results demonstrate the importance of adopting aseptic sampling techniques in the field and indicate that the contamination of samples collected for culture may be avoided or reduced by immediate necropsy and the filtering of flushed-urine samples on site. The results also reveal the robustness of 'molecular-based' diagnostics to contamination and low sample yield. Although gel-based genotyping, using amplicons produced by PCR, has fallen from favour because of problems with reproducibility (Slack *et al.*, 2007), future priority should be given to the development of non-gel-based molecular methods (rather than serological techniques) for the detection and characterisation of leptospires from reservoir and incidental hosts.

ACKNOWLEDGEMENTS. The authors thank Dr R. Cobbold and L. Smythe for their comments and assistance in the preparation

of this manuscript. Funding was provided by the Australian Biosecurity Co-operative Research Centre.

REFERENCES

- Alston, J. M. & Broom, J. C. (1958). *Leptospirosis in Man and Animals*. Edinburgh: E. & S. Livingstone.
- Atxaerandio, R., Aduriz, G., Ziluaga, I., Esteban, J. I., Maranda, L. & Mainar-Jaime, R. C. (2005). Serological evidence of *Leptospira interrogans* serovar Bratislava infection and its association with abortions in cattle in northern Spain. *Veterinary Record*, **156**, 376–380.
- Bolin, C. A. (1996). Diagnosis of leptospirosis: a re-emerging disease of companion animals. *Seminars in Veterinary Medicine and Surgery (Small Animal)*, **11**, 166–171.
- Cerqueira, G. M. & Picardeau, M. (2009). A century of *Leptospira* strain typing. *Infection, Genetics and Evolution*, **9**, 760–768.
- Chappel, R. J., Prime, R. W., Miller, B. D., Mead, L. J., Jones, R. T. & Adler, B. (1992). Comparison of diagnostic procedures for porcine leptospirosis. *Veterinary Microbiology*, **30**, 151–163.
- Corney, B. G., Colley, J., Djordjevic, S. P., Whittington, R. & Graham, G. C. (1993). Rapid identification of some *Leptospira* isolates from cattle by random amplified polymorphic DNA fingerprinting. *Journal of Clinical Microbiology*, **31**, 2927–2932.
- Cox, T. E., Smythe, L. D. & Leung, L. K.-P. (2005). Flying foxes as carriers of pathogenic *Leptospira* species. *Journal of Wildlife Diseases*, **41**, 253–257.
- Craig, S. B., Graham, G. C., Burns, M.-A., Dohnt, M. F., Smythe, L. D. & McKay, D. B. (2009a). Lymphopenia in leptospirosis. *Annals of Tropical Medicine and Parasitology*, **103**, 279–282.
- Craig, S. B., Graham, G. C., Burns, M.-A., Dohnt, M. F., Smythe, L. D. & McKay, D. B. (2009b). Haematological and clinical-chemistry markers in patients presenting with leptospirosis: a comparison of the findings from uncomplicated cases with those seen in the severe disease. *Annals of Tropical Medicine and Parasitology*, **103**, 333–341.
- Danhof, M., Hisaoka, M. & Levy, G. (1984). Kinetics of drug action in disease states. II. Effect of experimental renal dysfunction on phenobarbital concentrations in rats at onset of loss of righting reflex. *Journal of Pharmacology and Experimental Therapeutics*, **230**, 627–631.
- De Koning, J., Bosma, R. B. & Hoogkamp-Korstanje, J. A. A. (1987). Demonstration of spirochaetes in patients with Lyme disease with a modified silver stain. *Journal of Medical Microbiology*, **23**, 261–267.
- Ellis, T. M., Robertson, G. M., Hustas, L. & Kirby, M. (1983). Detection of leptospires in tissue using an immunoperoxidase staining procedure. *Australian Veterinary Journal*, **60**, 364–367.

- Faine, S., Adler, B., Bolin, C. & Perolat, P. (1999). *Leptospira and Leptospirosis*, 2nd Edn. Melbourne, Australia: MediSci.
- Frable, W. J. (1989). Needle aspiration biology: past, present, and future. *Human Pathology*, **20**, 504–517.
- Galton, M. M. (1962). Methods in the laboratory diagnosis of leptospirosis. *Annals of the New York Academy of Sciences*, **98**, 675–685.
- Goldstein, F. S. & Charon, N. W. (1988). Motility of the spirochete *Leptospira*. *Cell Motility and Cytoskeleton*, **9**, 101–110.
- Hansen, A. K. (2000). *Handbook of Laboratory Animal Bacteriology*. Boca Raton, FL: CRC Press.
- Kazerani, H. R. & Furman, B. L. (2006). Comparison of urethane/chloralose and pentobarbitone anaesthesia for examining effects of bacterial lipopolysaccharide in mice. *Fundamental and Clinical Pharmacology*, **20**, 379–384.
- Levett, P. N. (2001). Leptospirosis. *Clinical Microbiological Reviews*, **14**, 296–326.
- Levett, P. N., Morey, R. E., Galloway, R., Turner, D. E., Steigerwalt, A. & Mayer, L. W. (2005). Detection of pathogenic leptospires by real-time quantitative PCR. *Journal of Medical Microbiology*, **54**, 45–49.
- Lilenbaum, W., Varges, R., Brandao, F. Z., Cortez, A., De Souza, S. O., Brandao, P. E., Richtzenhain, L. J. & Vasconcelos, S. A. (2008). Detection of *Leptospira* spp. in semen and vaginal fluids of goats and sheep by polymerase chain reaction. *Theriogenology*, **69**, 837–842.
- Palit, A., Haylock, L. M. & Cox, J. C. (1986). Storage of pathogenic leptospires in liquid nitrogen. *Journal of Applied Bacteriology*, **61**, 407–411.
- Perolat, P. (1990). rRNA gene restriction patterns of *Leptospira*: a molecular typing system. *Research in Microbiology*, **141**, 159–171.
- Rivera, D. (2005). *Identifying ecological factors affecting populations of reservoir hosts of leptospirosis: implications for management*. M. Phil. thesis, School of Animal Studies, The University of Queensland, Gatton, Australia.
- Slack, A. T., Symonds, M., Dohnt, M. F., Harris, C., Brookes, D. & Smythe, L. D. (2007). Evaluation of a modified TaqMan assay detecting pathogenic *Leptospira* species against culture and *Leptospira*-specific IgM ELISA in a clinical environment. *Diagnostic Microbiology and Infectious Disease*, **57**, 361–366.
- Smith, C. R., Ketterer, P. J., McGowan, M. R. & Corney, B. G. (1994). A review of laboratory techniques and their use in the diagnosis of *Leptospira interrogans* serovar Hardjo infection in cattle. *Australian Veterinary Journal*, **71**, 290–294.
- Smythe, L. D., Field, H. E., Barnett, L., Smith, C. S., Dohnt, M. F., Symonds, M., Moore, M. R. & Rolf, P. F. (2002a). Leptospiral antibodies in flying foxes in Australia. *Journal of Wildlife Diseases*, **38**, 182–186.
- Smythe, L. D., Smith, I. L., Smith, G. A., Dohnt, M. F., Symonds, M., Barnett, L. & McKay, D. B. (2002b). A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. *BMC Infectious Diseases*, **2**, 13.
- Thaipadungpanit, J., Wuthiekanun, V., Chierakul, W., Smythe, L. D., Petkanchanapong, W., Limpaboon, R., Apiwatanaporn, A., Slack, A. T., Suputtamongkol, Y., White, N. J., Feil, E., Day, N. & Peacock, S. (2007). A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. *PLoS Neglected Tropical Diseases*, **1**, 1–6.
- Toon, S. & Rowland, M. (1983). Structure–pharmacokinetic relationships among the barbiturates in rat. *Journal of Pharmacology and Experimental Therapeutics*, **225**, 752–763.
- Tulsiani, S. T., Lau, C. L., Graham, G. C., van den Hurk, A. F., Jansen, C. C., Smythe, L. D., McKay, D. B. & Craig, S. B. (2010). Emerging tropical diseases in Australia. Part I. Leptospirosis. *Annals of Tropical Medicine and Parasitology*, **104**, 543–556.
- Tulsiani, S. T., Cobbold, R. C., Graham, G. C., Dohnt, M. F., Burns, M.-A., Leung, L. K.-P., Field, H. E., Smythe, L. D. & Craig, S. B. (2011). The role of fruit bats in the transmission of pathogenic leptospires in Australia. *Annals of Tropical Medicine and Parasitology*, **105**, 000–000.
- Turner, L. H. (1970). Leptospirosis. III. Maintenance, isolation and demonstration of leptospires. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **64**, 623–646.
- Wangroongsarb, P., Chanket, T., Gunlabun, K., Hoang Long, D., Satheanmethakul, P., Jetanadee, S., Thaipadungpanit, J., Wuthiekanun, V., Peacock, S. J., Blacksell, S. D., Smythe, L. D., Bulach, D. M. & Kalambaheti, T. (2007). Molecular typing of *Leptospira* spp. based on putative O-antigen polymerase gene (*wzy*), the benefit over 16S rRNA gene sequence. *FEMS Microbiology Letters*, **271**, 170–179.
- Waters, W. R., Miller, J. M., Palmer, M. V., Stabel, J. R., Jones, D. E., Koistinen, K. A., Steadham, E. M., Hamilton, M. J., Davis, W. C. & Bannantine, J. P. (2003). Early induction of humoral and cellular immune responses during experimental *Mycobacterium avis* subsp. *paratuberculosis* infection of calves. *Infection and Immunity*, **71**, 5130–5138.
- Webster, J. P., Ellis, W. A. & MacDonald, D. W. (1995). Prevalence of *Leptospira* spp. in wild brown rats (*Rattus norvegicus*) on UK farms. *Epidemiology and Infection*, **114**, 195–201.
- Young, B. J. (1969). A reliable method for demonstrating spirochaetes in tissue sections. *Journal of Medical Laboratory Technology*, **26**, 248–252.