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AUTHORS: Mitchell, K E; Turton, J F; Lloyd, D H

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Isolation and identification of *Acinetobacter* spp. from healthy canine skin

Kayleigh E Mitchell*, Jane F Turton†, David H Lloyd‡

*Fernside Veterinary Centre, 205 Shenley Road, Borehamwood, Hertfordshire, WD6 1AT, UK.

† Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, Public Health England, 61 Colindale Avenue, London, NW9 5EQ, UK.

‡Department of Clinical Sciences and Services, Royal Veterinary College, University of London, Hawkshead Lane, North Mymms, Hertfordshire, AL9 7TA, UK.

Corresponding author: David H. Lloyd - david-lloyd@ntlworld.com
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Abstract
Background - *Acinetobacter* species can exhibit widespread resistance to antimicrobial agents. They are recognised as important nosocomial pathogens of humans, but are becoming increasingly recognised in opportunistic infections of animals.
Hypothesis/objectives - This study aimed to determine whether *Acinetobacter* spp. are carried on skin of healthy dogs and, if present, to identify the species.
Animals - Forty dogs were sampled at veterinary practices and rescue centres. They were free from skin disease and receiving no systemic or topical treatments.
Methods - Skin swab samples were collected from four sites on each dog and cultured. *Acinetobacter* spp. isolates were detected by biochemical tests and gas chromatography. The species was determined by sequencing the RNA polymerase β-subunit (*rpoB*) gene. Isolates were screened for OXA carbapenemase genes and class 1 integrons capable of carrying resistance genes, and subjected to antimicrobial susceptibility tests.
Results - For 25% dogs sampled (10/40), *Acinetobacter* spp. were isolated at one or more skin sites. Thirteen *Acinetobacter* spp. isolates were recovered from 160 samples. The most frequently cultured was *A. lwoffii* (7/13), followed by *A. baumannii* (2/13), *A. junii* (1/13), *A. calcoaceticus* (1/13), *A. pittii* (1/13) and one novel *Acinetobacter* species (1/13). Class 1 integrons and *bla*$_{OXA\text{-}23\text{-like}}$ were not detected. Isolates were susceptible to most antibiotics.
Conclusions and clinical importance - The study confirms *Acinetobacter* spp. can survive on canine skin, where they may be potential reservoirs for infection. This highlights the importance of good hygiene in veterinary practice, adhering to aseptic principles in surgery, and treatment based on culture and susceptibility testing where possible.

Introduction
Acinetobacter spp. are Gram-negative, non-motile, aerobic bacteria that have been recovered from diverse environmental sources and isolated from various infections, including septicaemia,[1] and equipment such as mechanical ventilators.[2] Some species, especially *A. baumannii*, but also *A. pittii*, *A. nosocomialis* and *A. ursingii*, amongst others, have a significant role in hospital-acquired infections in people and often exhibit widespread resistance to antimicrobials.[1,2] Although in comparison there is limited documentation of *Acinetobacter* infections in animals, there are reports of multi-resistant *Acinetobacter* infections acquired in veterinary hospitals[3,4] suggesting it is emerging as a significant nosocomial pathogen in dogs.

Acinetobacter spp. have been isolated from healthy companion animals at various sites such as the rectum and mouth,[5] and one study isolated *Acinetobacter* spp. from the skin of eight out of 10 dogs, although identification was only to genus level.[6] Species-level identification within the genus can be achieved by sequencing the RNA polymerase ß subunit gene (*rpoB*).[7]

Acinetobacter baumannii is the species associated most frequently with nosocomial infections and outbreaks in humans;[1,2] it is also recognised as a nosocomial pathogen in animals.[3,4] However, other species have pathogenic significance.[1] *Acinetobacter baumannii* has an intrinsic, naturally-occurring carbapenemase gene ($bla_{\text{OXA-51-like}}$), present in all isolates*;* PCR detection of this gene provides a convenient identification method for this species. $Bla_{\text{OXA-23-like}}$ is intrinsic to *A. radioresistens*, but is an acquired carbapenemase gene in other species of *Acinetobacter*, especially *A. baumannii*, in which it is consistently associated with resistance. Another useful marker is the class 1 integron, involved in acquisition of resistance, frequently present in 'outbreak' strains of *A. baumannii*.[8] A previous study found that companion animals in veterinary clinics shared the same clonal lineages of *A. baumannii* and carried the same carbapenem resistance determinants as those from humans.[9]

This study was undertaken to establish whether *Acinetobacter* species were present on skin of healthy dogs and to determine the species and their distribution. This may give an indication of potential reservoirs for infection of susceptible animals.

Materials and methods

The sample population comprised 40 dogs, free from skin disease and not receiving any systemic or topical antimicrobial or corticosteroid treatment. Four establishments were visited for a diverse sample population and to reduce the possibility of carriage frequency being influenced by transmission: a companion animal hospital and rescue centre in central London (nine dogs), a dog rescue centre in west London (10 dogs), a veterinary practice in Hampshire (11 dogs) and a veterinary teaching centre in Hertfordshire (10 dogs). Sampling was carried out over a four week period in 2008. The procedure was approved by the teaching centre ethics committee.

Swabs were taken from four skin sites on each dog: muzzle, axilla, inguinal region and the interdigital space between digits two and three on the left hind foot, and inoculated onto both nutrient agar and Leeds *Acinetobacter* Medium (LAM). These media were prepared in-house. LAM was prepared as previously described.[10] Based on a preliminary study by the first author, nutrient agar was the superior medium for yielding *Acinetobacter* species and LAM was a selective medium to increase the likelihood of finding *Acinetobacter baumannii*.

Acinetobacter spp. colonies were initially selected based on morphology, a negative oxidase reaction and the appearance of a white colony on chromogenic agar (Brilliance UTI Agar, Thermo Scientific Oxoid Microbiology Products, Basingstoke, UK); see (http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0949; accessed 27/12/2017).
This was followed by generic confirmation by PCR as described below. Isolates were stored on cryobeads at -70°C.

Antibiotic susceptibility testing was by agar dilution and was carried out in 2017 on stored isolates and interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations and breakpoints (http://www.eucast.org/clinical_breakpoints/).

Polymerase Chain Reaction (PCR)

Amplification of the *rpoB* gene followed by sequencing of the amplicon was carried out as previously described*.*[1] A multiplex PCR was also performed, to detect class 1 integrase, $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23-like}}$ genes.[1] *Acinetobacter baumannii* NCTC 13421 was used as a positive control. *rpoB* sequences were clustered on a BioNumerics database (Applied Maths, Sint-Martens-Latem, Belgium) which included the sequences of the type strains of all the currently described species. The gene sequence was also uploaded to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), which compared and aligned the sequence to all publicly available *Acinetobacter rpoB* sequences.

Testing for international clones I, II and III of *A. baumannii* was by multiplex PCR.[11] Variable Number Tandem Repeat (VNTR) analysis at four highly discriminatory loci (VNTR-1, VNTR-10, Abaum_845 and Abaum_3468) was carried out in a single reaction using previously described fluorescently labelled primers and PCR products sized on a capillary sequencer (Applied Biosystems, Foster City, CA, USA).[12]

Statistical analysis
Using a statistical software package (SPSS Statistics 17.0; SPSS Inc, Chicago, IL, USA), a Chi-square test (χ^2) was performed, to test significance for the distribution of *Acinetobacter* species among the skin sites and the frequencies of isolation of the different species. Fisher's exact tests were performed for those variables where there were less than five observations per cell. A *P* value of <0.05 was used as a cut off value to assess the evidence of significance of the relationship between the considered variables.

Results
Ten out of 40 dogs carried *Acinetobacter* spp. at one or more skin sites (prevalence of 25%). *Acinetobacter*-positive dogs were detected from dogs at three of four centres visited; four at the rescue centre, three at the veterinary practice and three at the teaching centre. Three of the 10 dogs carried *Acinetobacter* spp. at two skin sites and the remainder at one site.

The frequencies of isolation of *Acinetobacter* species from the different skin sites were similar (Table 1). Of the 13 isolates, four were from the muzzle, three from the axilla, three from the inguinal region and three from the interdigital space.

Of the 160 skin swabs taken, 13 yielded *Acinetobacter* species. Two of the isolates possessed the $bla_{\text{OXA-51-like}}$ gene, confirming their identity as *Acinetobacter baumannii.* One isolate was from a muzzle; the other from an inguinal region of the same dog sampled at the teaching centre; neither possessed a class 1 integron. Both shared identical VNTR profiles (12,20,2,9), did not belong to international clones I, II or III (which predominate among human clinical isolates) and were susceptible to most antibiotics, including the carbapenems (imipenem and meropenem), aminoglycosides, ceftazidime, ciprofloxacin and colistin. No isolates possessed a $bla_{\text{OXA-23-like}}$ gene, the most common acquired carbapenemase gene found in *Acinetobacter* species in the UK.

The most common *Acinetobacter* species isolated was *A. lwoffii* (seven isolates) (Table 1). The distribution was relatively even from skin sites. One isolate was *A. junii,* (muzzle), one *A. pittii,* (interdigital region) and one *A. calcoaceticus* (interdigital region). One isolate from an axilla, confirmed as belonging to the genus *Acinetobacter* by fatty acid analysis, did not cluster closely enough with any of the currently described *Acinetobacter* species by *rpoB* sequencing, suggesting it represents a novel *Acinetobacter* species. The *rpoB* sequence was submitted to GenBank under accession number FJ157977.1

In this study, the rates of isolations of *Acinetobacter* species from the four skin sites were not significantly different and statistically there was no difference between the overall isolation rates of each species (cut-off value $p < 0.05$).

Discussion
Although small, this study confirms that *Acinetobacter* species can be carried on healthy canine skin. The most significant organism in terms of its pathogenicity, *A. baumannii,* was isolated twice, from one dog. This species is rarely found on human skin or outside hospital environments and our findings suggest that a similar situation may exist in dogs.[13] Its recovery from a patient at the teaching hospital could be due to transfer, directly or indirectly, from the clinical environment or other dogs including animals that are immunocompromised or on long-term antibiotic therapy.

The frequencies of isolation of *Acinetobacter* species from the four skin sites indicate that there is no predilection for carriage at the sites sampled. No carriers were found at the companion animal hospital and rescue centre in central London. This is unlikely to be due to sampling error as both nutrient agar and LAM culturing was done from this clinic. These results from the only clinic in a city location could perhaps reflect the influence of environment on carriage of *Acinetobacter* spp. None of the isolates in this study carried $bla_{\text{OXA-23-like}}$ acquired carbapenemase gene or class 1 integrons, however a subsequent study has found $bla_{\text{OXA-23-like}}$ in isolates from dogs.[9]

The antibiotic susceptibility of the isolates is interesting and suggests that dogs do not serve as a reservoir for multidrug-resistant *Acinetobacter s*pp. but larger studies would be required to confirm this. Nevertheless, the fact that potentially pathogenic *Acinetobacter s*pp. isolates can survive on skin highlights the importance of maintaining rigorous hygiene, disinfection and antisepsis, as proposed for veterinary clinics and hospitals.[3]

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Table 1 *Acinetobacter* species cultured from each skin site from 10 dogs providing positive cultures.

Species	Muzzle	Axilla	Inguinal	Interdigital	Total
A. lwoffii	2	2	2	1	7
A. junii	1	-	-	-	1
A. pittii	-	-	-	1	1
A. baumannii	1	-	1		2
A. calcoaceticus	-	-	-	1	1
Novel species	-	1	-	-	1
Total	4	3	3	3	13