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SHORT COMMUNICATIONS

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Microflora of the Pouch of the Koala (*Phascolarctos cinereus*)

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ABSTRACT: Microflora of the pouch epithelium of 17 female koalas (*Phascolarctos cinereus*) were examined in relation to their current reproductive status and recent reproductive history. No microbial growth was observed in pouch swabs from 13 of 17 (76%) koalas, including four females without young, seven with pouch young and two with back young (i.e. permanently emerged from the pouch). Growth of bacteria or yeasts was observed in pouch swabs from four koalas, each of which had experienced mortality of its pouch young during the current breeding season. Seven species of microorganisms were isolated, including *Pseudomonas aeruginosa*, *Serratia marcescens* and *Enterococcus faecalis*. Based on the absence of microflora in the majority of females examined, we propose that the pouch epithelium normally provides a hostile environment for microbial colonization.

Key words: Koala, pouch epithelium microflora, pouch young mortality, *Pseudomonas aeruginosa, Serratia marcescens, Enterococcus faecalis.*

Diseases resulting from microbial infection have been described in both captive and free-ranging koala (*Phascolarctos cinereus*) populations (Backhouse and Bolliger, 1961; Dickens, 1975, 1976; Butler, 1978; Wood, 1978; Brown et al., 1984, 1987). In a review of the diseases of captive koalas, Wood (1978) reported mortality of 3- to 5-month-old pouch young in "epidemic proportions" at Lone Pine Koala Sanctuary. *Pseudomonas pyocyanea*, now known as *P. aeruginosa* (Cowan and Steel, 1965), was identified as the infective agent. In other isolated cases, bacteria including *Klebsiella* sp. and *Proteus* sp. were implicated as pathogens (Wood, 1978). However, little information is available on the normal microflora of the pouch epithelium of the koala or of other marsupials. In the quokka (*Setonix brachyurus*), pouches of apparently healthy lactating and non-lactating females harbored a variety of bacteria (Yadav et al., 1972), but no pouch flora were present in one female quokka expecting a birth; Yadav et al. (1972) suggested that the secretions of the pouch skin may contain antibacterial substances in expectant animals. We report the results of a clinical investigation of the pouch microflora of female koalas, in relation to the animals' current reproductive status and recent reproductive history.

Seventeen captive female koalas held at Lone Pine Koala Sanctuary in southeast Queensland (Australia; 27°32′S, 152°58′E) were examined. The animals were provided with fresh browse *ad libitum*, from two to five *Eucalyptus* spp. daily and received no other supplementary food of any kind. The female koalas ranged in age from 20 mo to 12 yr. Eight carried pouch young and two carried back young (i.e. permanently emerged from the pouch) at the time of examination. General descriptions of the individual females including their age, reproductive status (presence or absence of young) at the time of pouch swabbing, and previous reproductive history, are presented in Table 1.

Routine husbandry procedures at Lone Pine included regular weighing of each koala, and visual inspection of the pouch interior in females due to give birth. As a result, the animals were accustomed to being handled and examined. For each koala tested in the present study, one person held

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TABLE 1. Identification, age and reproductive status at time of swabbing of 17 female koalas. Reproductive success (weaning or mortality of young) over the preceding 2.5 years is also shown.

Koala identification	Age (yr)	Current reproductive status or age of any dependent young (days)	Reproductive success over preceding 2.5 yr	
			Number weaned	Number lost
K-1	2	never mated	0	0
K-2	2	young lost (≤61)[a]	0	1
K-3	3	pouch young (84)	1	0
K-4	4	pouch young (19)	1	1
K-5	4	pouch young (112)	0	2
K-6	4	pouch young (173)	0	2
K-7	5	mated 84 days earlier[b]	3	0
K-8	5	young lost (≤15)[a]	0	4
K-9	6	pouch young (118)	2	0
K-10	6	back young (225)[c]	2	0
K-11	7	pouch young (50)	0	2
K-12	8	mated 33 days earlier[d]	3	0
K-13	8	pouch young (62)	2	0
K-14	9	mated 49 days earlier[b]	2	0
K-15	10	pouch young (40)	3	0
K-16	10	back young (278)	2	0
K-17	12	young lost (24–40 hr)[e]	2	2

[a] Loss discovered at time of swabbing.
[b] No birth resulted from mating.
[c] Young permanently emerged from pouch.
[d] Birth expected within 48 hr (ultimately no birth resulted).
[e] Young lost within 24–40 hr of birth (swab taken 17 days later).

the animal in a sitting position with its hindlegs slightly abducted. The pouch opening was parted by a second person, enabling a third person to avoid contamination from the animal's fur while introducing a cotton-tipped swab moistened with sterile water. The swab was rotated and rubbed against the epithelial lining of the pouch. If a small pouch young was present, the swab was directed away from the baby's head and swabbing confined to the vacant side of the pouch.

Four different culture media were used for initial isolation of microorganisms: brain heart infusion agar (BHIA) (Oxoid Ltd, Basingstoke, Hampshire, United Kingdom) supplemented with 5% defibrinated horse blood and 0.5% yeast extract (Oxoid); Columbia blood agar base (COBA) (Oxoid) supplemented with 5% defibrinated horse blood and *Streptococcus* selective supplement containing colistin sulfate and oxolinic acid (Oxoid); desoxycholate lactose agar (DHLA) (Difco Laboratories, Detroit, Michigan, USA); and de Man, Rogosa, Sharpe agar (MRSA) (Oxoid). BHIA was a nonselective medium, while COBA, DHLA and MRSA were used for selective isolation of streptococci, enterococci and lactobacilli, respectively.

Immediately after sampling, each pouch swab was streaked directly onto each medium. The nonselective medium (BHIA) was always streaked first. Duplicate inoculated BHIA and COBA plates were prepared, to allow anaerobic as well as aerobic incubation, at 37.5 C for 72 hr. Anaerobic incubation was conducted in Bio-Bag (Type A) Multi-Plate environmental chambers (Becton Dickinson and Co., Cockeysville, Maryland, USA), which provide an atmosphere of 8 to 12% carbon dioxide, 10 to 20% hydrogen, and 70 to 80% nitrogen. DHLA plates were incubated aerobically only, at 37.5 C for 48 hr. MRSA plates were incubated microaero-

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TABLE 2. Microorganisms isolated and their relative abundance in pouch epithelial swabs from four female koalas.

Microorganism isolated and relative abundance[a]	Koala identification			
	K-2	K-6	K-8	K-17
Bacteria				
Acinetobacter lwoffi	−	++	−	−
Enterococcus faecalis	−	++	+	−
Klebsiella pneumoniae	−	+	−	−
Pseudomonas aeruginosa	−	−	+++	+++
Serratia marcescens	−	+++	++	−
Streptococcus bovis	+	−	−	−
Yeast				
unidentified species	+++	−	−	−

[a] +++ = heavy growth, ++ = moderate growth, + = a few colonies, − = absent.

philically at 37.5 C for 72 hr in Bio-Bag Type Cfj environmental chambers (Becton Dickinson and Co.) with an atmosphere of 5 to 12% oxygen, 5 to 10% carbon dioxide, and 80 to 90% nitrogen.

After incubation, the plates were examined for microbial growth. Colonies on the anaerobic BHIA and COBA plates were subcultured in duplicate on BHIA to allow further aerobic and anaerobic incubation. Isolates appearing only on the anaerobic subcultured BHIA plates were considered obligate anaerobes.

Tentative identification of isolates to the level of family was performed from their colonial and cellular morphology, Gram staining, spore formation and aerobic or anaerobic growth. Further identification of bacterial isolates to the level of genus and species was conducted using commercially available identification kits API 20 E and API 20 Strep (API System, Montalieu, Vercieu, France), and oxidase, catalase and nitrate reduction tests (Buchanan and Gibbons, 1974). Yeast isolates were subcultured from BHIA onto Sabouraud Dextrose Agar (Oxoid), and incubated aerobically at 30 C for up to 10 days. Yeast identification was attempted using commercially available kits ATB 32 C (API System) and Yeast Biological Card (Vitek Systems, Port Melbourne, Victoria, Australia), and a germ tube test (Reynolds and Braude, 1956). Based on the number of colonies of each microorganism identified on the plate media, the composition and relative abundance of the microflora of the pouch was estimated.

We initially considered that the humidity and warmth of the pouch would offer an ideal environment for microbial growth. We also speculated that the pouch flora of females with back young would be more numerous and diverse than that of females with small pouch young, since any microorganisms occurring outside the pouch could be mechanically transferred as the baby was suckled through the pouch opening. Contrary to these hypotheses, no microbial growth was obtained from the pouches of 13 of 17 (76%) koalas examined. These included four females without dependent young (K-1, K-7, K-12 and K-14), seven females with pouch young ranging in age from 19 to 118 days (K-3, K-4, K-5, K-9, K-11, K-13 and K-15), and two females with back young (K-10 and K-16) (Table 1).

Microbial growth was observed from the pouch swabs of only four koalas (K-2, K-6, K-8 and K-17). No obligate anaerobic bacteria were identified from any samples. Seven species of microorganisms were isolated; their relative abundance in the pouches of these four females is summarized in Table 2. *Pseudomonas aeruginosa* was present in relatively large numbers in two animals (K-8 and K-17), and was the

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only isolate from K-17. Both *Serratia marcescens* and *Enterococcus faecalis* were isolated from two koalas (K-6 and K-8). The *E. faecalis* isolated from K-6 was β-hemolytic, while that from K-8 was an α-hemolytic strain. *Acinetobacter lwoffi*, *Klebsiella pneumoniae* and *Streptococcus bovis* each were recovered in relatively low numbers from single animals (K-6, K-6, and K-2, respectively). The pouch flora of K-2 was dominated by a yeast which could not be confidently identified. The isolate gave a negative germ tube test; thus, it was not *Candida albicans* (Reynolds and Braude, 1956).

In the current study, three of the four females from which microbial growth was obtained (K-2, K-8 and K-17) did not have pouch young at the time of sampling. K-8 lost her young within the 15 days between its birth and the time of pouch swabbing, when the pouch was discovered to be empty. K-17's young had disappeared when a second pouch check was performed 24 to 40 hours after birth. Loss of pouch young was discovered at the time of swabbing in K-2, who had given birth 61 days earlier. At 61 days after birth, the pouch of K-2 contained an amorphous mass of thick brown paste, which may have been the highly decomposed body of the young. In the case of K-2, we suggest that pouch infection may have developed after the death of the young, not before it. In the case of K-17, however, where the neonate disappeared from the pouch within hours of birth, it seems likely that infection was the cause of death, rather than the result of it.

A 173-day-old pouch young was removed from K-6 for clinical examination immediately prior to swabbing the pouch epithelium. The young's head was protruding through the pouch opening, but it was unresponsive to touch. On removal from the pouch the baby was found to be very small for its age and extremely thin. There was evidence of mastitis in the developed (left) mammary gland of the pouch. Milk expressed after disinfection of the teat yielded a β-hemolytic *Streptococcus* sp. and *Serratia marcescens*. The young died 48 hr after hand-raising was commenced; on post-mortem examination there was evidence of typhlitis and septicemia. Culture of the young's heart blood yielded a pure growth of *S. marcescens*, while that of the cecal contents revealed *S. marcescens*, *E. faecalis* (β-hemolytic strain) and *K. pneumoniae*, corresponding to the flora of the mother's pouch and mammary gland.

Based on the evidence in this study, we propose that the epithelial surface of the koala pouch normally provides a hostile environment for microbial colonization. In the majority of females examined, irrespective of their reproductive status, no viable microflora were isolated from pouch swabs. Further work to evaluate the antimicrobial potential of the pouch environment of koalas is underway.

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