INFECTION AND IMMUNITY, Aug. 1988, p. 1897–1900
0019-9567/88/081897-04$02.00/0

Vol. 56, No. 8

Two Distinct Forms of *Chlamydia psittaci* Associated with Disease and Infertility in *Phascolarctos cinereus* (Koala)

ADEEB A. GIRJES,[1] ANDREW F. HUGALL,[1] PETER TIMMS,[2] AND MARTIN F. LAVIN[1]*

Department of Biochemistry, University of Queensland, Brisbane,[1] and Animal Research Institute, Queensland Department of Primary Industries, Yeerongpilly,[2] Queensland, Australia

Received 4 November 1987/Accepted 7 April 1988

While several diseases associated with *Chlamydia psittaci* infection have been reported in *Phascolarctos cinereus* (koala), it is still unclear whether one or more chlamydial strains are responsible. In this study, we provide evidence, obtained by restriction enzyme and gene probe analysis, that two quite distinct strains of *C. psittaci* infect koalas; one strain was isolated from the conjunctivae, and the other was isolated from the urogenital tract and the rectum. A gene probe, pFEN207, containing the coding sequence for an enzyme involved in the biosynthesis of the chlamydial genus-specific lipopolysaccharide antigen, and a separate probe, pCPML-4N, prepared from a DNA fragment of a koala-infecting strain of *C. psittaci*, were used to determine the patterns of hybridization in the koala-infecting strains; these patterns were found to be quite distinct from those observed with *C. psittaci* isolates from other animals. We also demonstrated by hybridization analysis with an avian strain plasmid that all three koala urogenital isolates contain a plasmid and that there is no evidence for the presence of a homologous plasmid in any of the ocular isolates.

Concern exists for the survival of the marsupial *Phascolarctos cinereus* (koala). The threat to the existence of this animal is due to diminishing habitat as well as incidence of disease and infertility (24; A. S. Brown, Ph.D. thesis, University of Queensland, Brisbane, Queensland, Australia). Indeed, increased susceptibility to disease compared with that of other marsupials has been documented for the koala (6, 20). A 3.5-year study on Phillip Island, Victoria, Australia, demonstrated a decline in the koala population of approximately 50% (5). In another study, mean fertility in koala populations varied from 13% at Walkerville and 22% on Phillip Island to 63% on French Island (17). Pathological changes, in the form of ovarian cysts and vaginitis, were documented as early as 1919 (15) and subsequently in several reports (2, 3, 23, 28). Cockram and Jackson (4) were the first to report an association between infection with *Chlamydia psittaci* and keratoconjunctivitis in koalas. More recently, McColl et al. (19) isolated chlamydiae from the female koala reproductive tract and presented evidence that these organisms cause a severe reproductive tract disease leading to infertility.

Chlamydiae are divided into two major species: *Chlamydia trachomatis*, which is a pathogen of humans (12, 29), and *C. psittaci*, which causes a variety of diseases in a range of animals (30). A common, genus-specific antigen is shared by all *Chlamydia* species, but species can be differentiated by species-specific and serovar-specific antigens (1). Despite many biological similarities, *C. psittaci* and *C. trachomatis* have only about 10% homology in their DNA (11). Restriction endonuclease analysis has been used to differentiate between serovars of *C. trachomatis* (27). More limited studies with *C. psittaci* have differentiated avian and ovine isolates (7, 18). In recent studies, we have used restriction endonuclease analysis to differentiate ovine, bovine, avian, and feline *C. psittaci* isolates (13, 31). We have also used gene probe hybridization analysis, which proved to be a more sensitive method for differentiating these strains (31). This method of analysis allowed differentiation of a koala conjunctival isolate from all other *C. psittaci* isolates. In view of the variety of diseases caused by *C. psittaci* in the koala and the obvious association with infertility, it was of interest to determine whether more than one strain was involved.

MATERIALS AND METHODS

Cell culture and *C. psittaci* DNA isolation. *C. psittaci* was grown on buffalo green monkey kidney (BGM) cells as described previously (31). Urogenital, rectal, and conjunctival swabs from koalas were collected into 1 ml of transport medium and stored at −70°C prior to use. Elementary bodies were purified by a modification of the method of McClenaghan et al. (18) by centrifugation through a Urografin-76 (Schering AG, Berlin, Federal Republic of Germany) gradient. DNA was extracted from purified elementary bodies by a modification of the method of Wenman and Lovett (32). Elementary bodies were suspended in lysis buffer (40 mM Tris hydrochloride, 0.1 M NaCl, 0.5% sodium dodecyl sulfate, 20 mM EDTA [pH 7.2]). Proteinase K was added to a concentration of 1.4 mg/ml, and the mixture was incubated at 55°C for 20 min and then further incubated at 37°C for 60 min. The resulting solution was extracted twice with equal volumes of phenol-$CHCl_3$ and then further extracted with $CHCl_3$ and ether. After precipitation with ethanol, DNA was suspended in Tris-EDTA buffer (pH 7.2).

Gene probes. The recombinant clone pCPML-4N was isolated from a gene library of a koala ocular strain of *C. psittaci* and prepared in the plasmid vector pUN121 (22), which was kindly provided by B. Nilsson (Stockholm, Sweden). Rabbit polyclonal antiserum raised against thiomersal-inactivated elementary bodies from a koala conjunctival strain of *C. psittaci* was used to screen the gene library. Anti-rabbit immunoglobulin G–alkaline phosphatase conjugate was used to detect antigen-producing clones according to the directions of the supplier (Promega Biotec, Madison, Wis.). pFEN207 was kindly provided by F. E. Nano, Rocky Mountain Laboratories, Mont. This recombinant plasmid consists of a 6.5-kilobase (kb) fragment of *C. trachomatis* LGV-434 DNA inserted into the *Bam*HI site of pUC8. This

* Corresponding author.

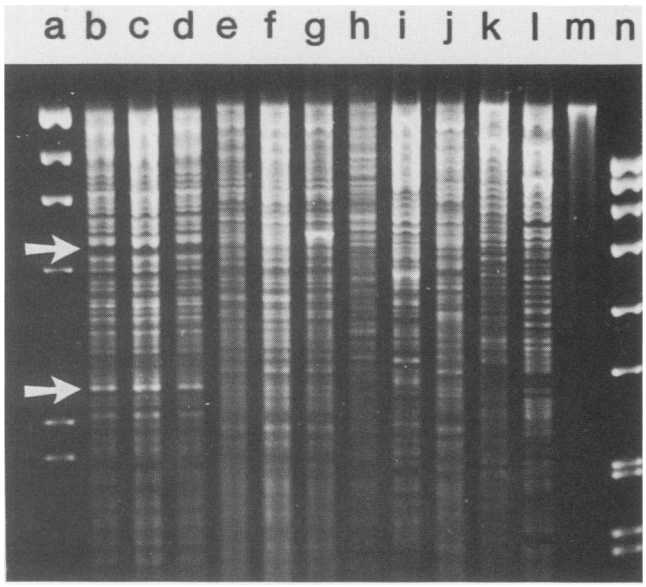

FIG. 1. Electrophoretic separation of DNA. Sources were λ DNA digested with *Hin*dIII (fragment sizes, 23, 9.4, 6.6, 4.4, 2.3 and 2 kb) (lane a); *Bam*HI-digested chlamydial DNA from koala conjunctivitis (lanes b to d), koala uterus (lane e), koala rectum (lane f), koala vagina (lane g), avian psittacosis (lane h), ovine abortion (lane i), SBE (lane j), feline conjunctivitis (lane k), *C. trachomatis* LGV 434 (lane l), and BGM (lane m); and SPP-1 DNA digested with *Eco*RI (fragment sizes, 7.8, 7, 5.9, 4.7, 3.4, 2.7, 1.9, 1.5, 1.3, and 1.1 kb) (lane n).

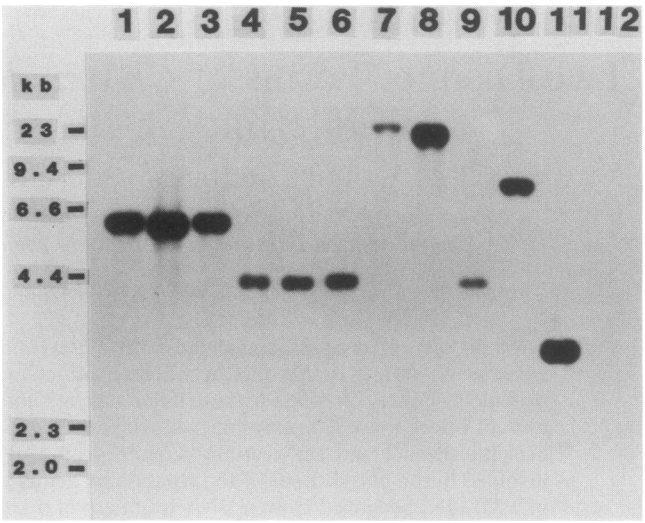

FIG. 2. Southern blot hybridization with a koala ocular strain probe of *Eco*RI-digested *C. psittaci* DNA from various sources. The probe used was a ^{32}P-labeled, 5.3-kb insert of pCPML-4N cloned in this laboratory from a koala strain of *C. psittaci*. Lanes: 1 to 3, koala conjunctivitis; 4 to 6, koala urogenital isolates; 7, avian psittacosis; 8, ovine abortion; 9, SBE; 10, feline conjunctivitis; 11, *C. trachomatis* LGV 434; 12, BGM.

fragment contains a gene which directs the expression of the chlamydial genus-specific lipopolysaccharide antigen in *Escherichia coli* (21). A 6.2-kb plasmid from an avian psittacosis isolate, which had been cloned into the *Eco*RI site of pUC13 (unpublished results), was also used as a probe.

Restriction enzyme digestions and hybridization analysis. *C. psittaci* DNA samples were digested to completion with an excess of the appropriate restriction enzyme according to instructions supplied by the manufacturer (Boehringer GmbH, Mannheim, Federal Republic of Germany, or Amersham Corp., Arlington Heights, Ill.). Electrophoretic separation was carried out on 0.8% agarose gels, and DNA was transferred to Hybond-N (Amersham). Hybridization was carried out with ^{32}P-labeled gene probes (described in the figure legends) by the method of Maniatis et al. (16). After hybridization, filters were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 20 min and for 20 min in 2× SSC–0.1% sodium dodecyl sulfate at 65°C. Filters were then exposed to Kodak XAR-5 X-ray film at −70°C for various times.

RESULTS

C. psittaci was isolated from five koalas: two had ocular infections, two had urogenital infections, and one had both. DNA extracted from these isolates was compared by restriction enzyme analysis with *Bam*HI (Fig. 1). It is evident that the three ocular strains show similar patterns of fragmentation (Fig. 1, lanes b, c, and d). Comparison with uterine (lane e), rectal (lane f), and vaginal (lane g) isolates (subsequently referred to as urogenital isolates) revealed a marked difference in digestion patterns (arrows). The three urogenital isolates appear to have the same fragmentation pattern, which is also similar to that in the sporadic bovine encephalomyelitis (SBE) strain (Fig. 1, lane j). The rather prominent band in the vaginal isolate at approximately 5 kb (Fig. 1, lane g) has been observed in other isolates. We have cloned this fragment and shown it to be simian virus 40. It appears to be induced from the BGM cells used to culture *C. psittaci* under certain conditions of infection.

While observations of restriction endonuclease digestion patterns are useful for detecting gross differences between different chlamydial strains, the method is lacking in sensitivity. Accordingly, we have used cloned chlamydial genes as probes to compare the ocular and urogenital strains. The results obtained by using the 5.3-kb insert of pCPML-4N cloned from a koala ocular strain of *C. psittaci* in this laboratory demonstrate an obvious difference in hybridization patterns between the ocular and urogenital strains (Fig. 2). A single band at 5.3 kb was observed for all three ocular isolates (Fig. 2, lanes 1 to 3), while a fragment hybridizing at 4.4 kb was found for the urogenital isolates (Fig. 2, lanes 4 to 6) after digestion with *Eco*RI. The samples in Fig. 2, lane 3 (ocular) and lane 4 (uterine), were isolated from the same animal. The pattern of hybridization was different for other *C. psittaci* strains (Fig. 2, lanes 7 to 10), with the exception of the SBE strain, which showed a band of approximately the same size as the urogenital isolates (Fig. 2, lane 9). Figure 2, lanes 11 and 12, contain DNA from *C. trachomatis* lymphogranuloma venereum (LGV) and BGM, respectively. It was also possible to differentiate between the two koala strains with a genus-specific antigen-gene probe, pFEN207, isolated from *C. trachomatis*. DNA digested with *Bam*HI gave a different pattern of hybridization in ocular (Fig. 3, lanes 1 to 3) and urogenital (Fig. 3, lanes 4 to 6) isolates. It is interesting to note that, with this probe also, the size of the fragment hybridized in the urogenital isolates was similar in size to that hybridized in the SBE strain (Fig. 3, lane 9).

In view of the ubiquitous presence of plasmids in *C. trachomatis* strains, it has been suggested that they may play a role in the growth cycle of the organism (25). Since the presence or absence of plasmids could provide information

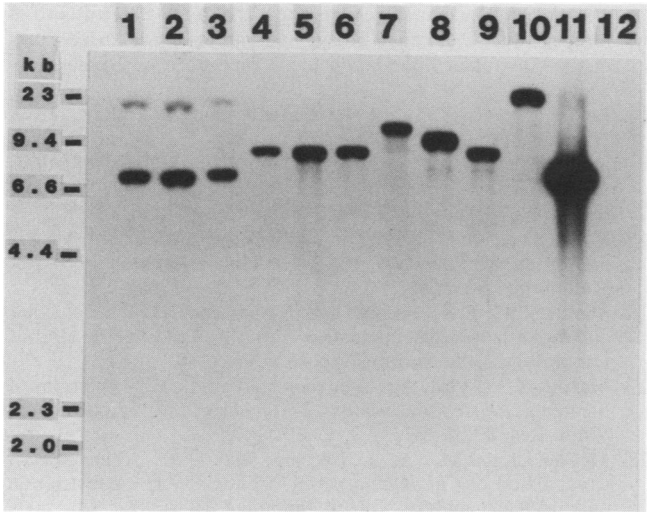

FIG. 3. Southern blot hybridization with a chlamydial genus-specific probe (pFEN207) of *Bam*HI-digested chlamydial DNA from various sources. Lanes: 1 to 3, koala conjunctivitis; 4 to 6, koala urogenital strains; 7, avian psittacosis; 8, ovine abortion; 9, SBE; 10, feline conjunctivitis; 11, *C. trachomatis* LGV 434; 12, BGM.

on the infectivity of different *C. psittaci* strains or be a means of differentiating between strains, we screened our isolates with a ^{32}P-labeled psittacosis plasmid (cloned by P.T.) as a probe. The results presented in Fig. 4 show a single hybridization band in all three koala urogenital isolates (lanes 5 to 7). No hybridization was observed with DNA from any of the ocular strains (Fig. 4, lanes 8 to 10). The koala plasmid is approximately 7.4 kb in size, which corresponds well with the *C. trachomatis* plasmid (Fig. 4, lane 2), and is significantly larger than the psittacosis plasmid, which is 6.2 kb in size (Fig. 4, lane 3). A plasmid present in the SBE isolate is approximately the same size as that in the urogenital isolates (Fig. 4, lane 4).

DISCUSSION

This report has provided the first evidence for infection of koalas by more than one strain of *C. psittaci*. On the basis of gene probe analysis, it seems likely that there is one ocular strain and another strain with a propensity to infect the urinary tract and rectum. Even with the same koala, it is possible to distinguish site-specific infection by *C. psittaci*.

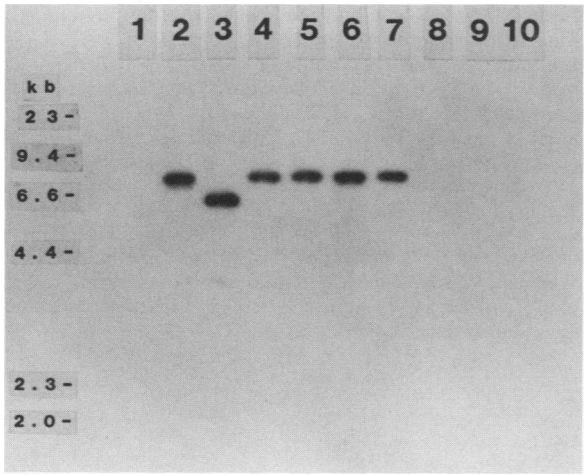

FIG. 4. Hybridization of ^{32}P-labeled psittacosis plasmid to koala and other chlamydial DNAs. Lanes: 1, BGM; 2, *C. trachomatis* LGV 434; 3, psittacosis plasmid; 4, SBE; 5 to 7, koala urogenital isolates; 8 to 10, koala ocular isolates.

Chlamydiae can be differentiated antigenically by species-specific, subspecies-specific, and serovar-specific epitopes and on the basis of pathogenicity and other biological properties (1). The molecular basis for these antigenic differences has remained largely unknown. More recently, chlamydial species and *C. trachomatis* biovars have been differentiated by analysis of DNA fragments generated by restriction enzyme digestion (27). As has been pointed out previously, this approach is useful for detecting gross differences between DNAs of different strains but is lacking in sensitivity (Fig. 1). For *C. trachomatis*, more than one restriction enzyme was required to discriminate between strains or serovars (27). While gene probes have been used to detect chlamydial DNA in spot (10), sandwich (26), and in situ (8) hybridizations, only one recent report, from this laboratory, has differentiated chlamydial strains by DNA polymorphisms (31). In this study, we have succeeded in distinguishing between chlamydial strains in the koala at a molecular level with different gene probes. Unlike *C. trachomatis*, in which plasmids are ubiquitously observed (9, 14), only some strains of *C. psittaci* contain plasmids (7). Furthermore, the *C. psittaci* plasmid was found to vary in size. It is interesting to note that plasmids were present in conjunctival strains in guinea pig (strain GPIC) and sheep but were absent from all ovine abortion strains (7). In this study the reverse was true for koalas, with plasmids being found in urogenital and rectal strains but not in ocular strains. The size of the urogenital strain plasmid is about 7.4 kb, similar to that reported for the plasmid in a GPIC strain but considerably greater than the 6.2-kb estimate for the plasmid in a cloned psittacosis sample used in this study. We have cloned the koala urogenital strain plasmid and are at present characterizing it. Presence of plasmids may prove to be a useful diagnostic probe for distinguishing between the two chlamydial strains in the koala.

We have noted previously the similarity of the koala urogenital strain and the SBE strain when restriction enzyme patterns and gene probe analyses were used. A further similarity is the presence of plasmids of the same size in the two isolates (Fig. 4). These results raise the possibility of a close link between the bovine strain and the urogenital koala strain and may be important in future epidemiological studies on disease in the koala. However, since only one bovine strain was available, it is somewhat premature to suggest that a single or closely related strain is involved in both cases.

Samples for this study were collected largely in southeast Queensland, where both urogenital and ocular infections have been observed. However, on Phillip Island, Western-port Bay, Victoria, there is a high prevalence of genital tract pathology and infection, with little evidence of ocular infection (Brown, Ph.D. thesis). The fertility of that population is low because of reproductive failure in females over 3 years of age (5), and *C. psittaci* is strongly implicated in that failure (Brown, Ph.D. thesis; K. A. Handasyde, Ph.D. thesis, Monash University, Melbourne, Victoria, Australia). The evidence presented in this report of organ specificity of infection by *C. psittaci* together with the poor survival of chlamydiae outside the host organism supports both ocular and venereal transmission of disease in koalas. We have initiated a project with R. W. Martin, A. K. Lee, and K. A. Handasyde (Monash University, Melbourne, Victoria, Aus-

tralia) to determine whether the organism implicated in disease and infertility on Phillip Island is the same as that isolated from the urogenital tract of animals 2,000 km away in southeast Queensland.

ACKNOWLEDGMENTS

We thank S. Brown, F. Carrick, and B. Weigler for the koala *C. psittaci* isolates.

This work was supported in part by grants from American Express and the Australian National Parks and Wildlife Service. A.A.G. is supported by a grant from the Iraqi Government.

LITERATURE CITED

1. **Allan, I.** 1986. Chlamydial antigenic structure and genetics, p. 73–80. *In* D. Oriel, G. Ridgway, J. Schachter, D. Taylor-Robinson, and M. Ward (ed.), Proceedings of the Sixth International Symposium on Human Chlamydial Infections. Cambridge University Press, Cambridge.
2. **Backhouse, T. C., and A. Bolliger.** 1961. Morbidity and mortality in the koala (*Phascolarctos cinereus*) Aust. J. Zool. **9**:24–37.
3. **Butler, R.** 1978. The koala, p. 174–175. *In* T. J. Bergin (ed.), Zoological Parks Board of New South Wales, Sidney, Australia.
4. **Cockram, F. A., and A. R. B. Jackson.** 1974. Isolation of a chlamydia from cases of keratoconjunctivitis in koalas. Aust. Vet. J. **50**:82–83.
5. **Every, K. R.** 1986. Evaluation of a decline in population of the koala, *Phascolarctos cinereus* (Goldfuss), in Ventnor Res., Phillip I., Vic., by means of a triple-count technique. Aust. Wildl. Res. **13**:517–525.
6. **Gordon, G., and D. G. McGreevy.** 1978. The koala, p. 125–131. *In* T. J. Bergin (ed.), Zoological Parks Board of New South Wales, Sydney, Australia.
7. **Herring, A. J., M. McClenaghan, I. D. Aitken, and J. Honeycombe.** 1986. Nucleic acid techniques for strain differentiation and detection of *Chlamydia psittaci*, p. 578–580. *In* D. Oriel, G. Ridgway, J. Schachter, D. Taylor-Robinson, and M. Ward (ed.), Proceedings of the Sixth International Symposium on Human Chlamydial Infections. Cambridge University Press, Cambridge.
8. **Horn, J. E., M. L. Hammer, S. Falkow, and T. C. Quinn.** 1986. Detection of chlamydia trachomatis in tissue culture and cervical scrapings by *in situ* hybridization. J. Infect. Dis. **153**:1155–1159.
9. **Joseph, T., F. E. Nano, C. F. Garon, and H. D. Caldwell.** 1986. Molecular characterization of *Chlamydia trachomatis* and *Chlamydia psittaci* plasmids. Infect. Immun. **51**:699–703.
10. **Kahane, S., and I. Sarov.** 1986. Detection of chlamydia by DNA hybridization with a native chlamydial plasmid probe, p. 574–577. *In* D. Oriel, G. Ridgway, J. Schachter, D. Taylor-Robinson, and M. Ward (ed.), Proceedings of the Sixth International Symposium on Human Chlamydial Infections. Cambridge University Press, Cambridge.
11. **Kingsbury, D. T., and E. Weiss.** 1968. Lack of deoxyribonucleic acid homology between species of the genus *Chlamydia*. J. Bacteriol. **96**:1421–1423.
12. **Kuo, C. C., H. H. Chen, S. P. Wang, and J. T. Grayston.** 1986. Characteristics of TWAR strains, a new group of chlamydia, p. 321–324. *In* D. Oriel, G. Ridgway, J. Schachter, D. Taylor-Robinson, and M. Ward (ed.), Proceedings of the Sixth International Symposium on Human Chlamydial Infections. Cambridge University Press, Cambridge.
13. **Lavin, M. F., P. Timms, and A. A. Girjes.** 1986. Chlamydial infection in koalas: a molecular approach, p. 81–88. *In* Board of Management, Australian Koala Foundation (ed.), Proceedings of the Australian Foundation Inc. Conference on Koala Management.
14. **Lovett, M., C. C. Kuo, K. Holmes, and S. Falkow.** 1980. Plasmids of the genus *Chlamydia*, p. 1250–1252. *In* J. D. Nelson and C. Grassi (ed.), Current chemotherapy and infectious disease, vol. 2. American Society for Microbiology, Washington, D.C.
15. **MacKenzie, W. C.** 1919. The comparative anatomy of Australian mammals, part iv, p. 33–40. Jenkin, Buxton and Co., Melbourne, Australia.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 149–186. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Martin, R. W.** 1981. Age-specific fertility in three populations of the koala, *Phascolarctos cinereus* (Goldfuss), in Victoria. Aust. Wildl. Res. **8**:275–283.
18. **McClenaghan, M., A. J. Herring, and I. D. Aitken.** 1984. Comparison of *Chlamydia psittaci* isolates by DNA restriction endonuclease analysis. Infect. Immun. **45**:384–389.
19. **McColl, K. A., R. W. Martin, L. J. Gleeson, K. A. Handasyde, and A. K. Lee.** 1984. Chlamydia infection and infertility in the female koala (*Phascolarctos cinereus*). Vet. Rec. **115**:655.
20. **McKenzie, R. A.** 1981. Observations on diseases of free living and captive koalas. Aust. Vet. J. **57**:243–246.
21. **Nano, F. E., and H. D. Caldwell.** 1985. Expression of the chlamydial genus-specific lipopolysaccharide epitope in *Escherichia coli*. Science **228**:742–744.
22. **Nilsson, B., M. Uhlen, S. Josephson, S. Gatenbeck, and L. Philipson.** 1983. An improved positive selection plasmid vector constructed by oligonucleotide mediated mutagenesis. Nucleic Acids Res. **11**:8019–8030.
23. **Obendorf, D. L.** 1981. Pathology of the female reproductive tract in the koala, *Phascolarctos cinereus* (Goldfuss), in Victoria, Australia. J. Wildl. Dis. **17**:587–592.
24. **Obendorf, D. L.** 1983. Causes of mortality and morbidity of wild koala, *Phascolarctos cinereus* (Goldfuss), in Victoria, Australia. J. Wildl. Dis. **19**:123–131.
25. **Palmer, L., and S. Falkow.** 1986. A common plasmid of *Chlamydia trachomatis*. Plasmid **16**:52–62.
26. **Palva, A., H. Jousiemies-Somer, P. Saikku, P. Vaanamen, H. Soderlund, and M. Ranki.** 1984. Detection of *Chlamydia trachomatis* by nucleic acid sandwich hybridization. FEMS Microbiol. Lett. **23**:83–89.
27. **Peterson, E. M., and L. M. de la Maza.** 1983. Characterization of *Chlamydia* DNA by restriction endonuclease cleavage. Infect. Immun. **41**:604–608.
28. **Pratt, A.** 1937. The call of the koala, p. 96–99. Robertson and Mullens, Melbourne, Australia.
29. **Schachter, J., and H. D. Caldwell.** 1980. Chlamydial characterization of TWAR strains, a new group of *Chlamydia psittaci*. Annu. Rev. Microbiol. **34**:285–309.
30. **Storz, J., and P. Spears.** 1977. Chlamydiales: properties, cycle of development and effect on eukaryotic host cells. Curr. Top. Microbiol. Immunol. **76**:167–214.
31. **Timms, P., F. W. Eaves, A. A. Girjes, and M. F. Lavin.** 1988. Comparison of *Chlamydia psittaci* isolates by restriction endonuclease and DNA probe analysis. Infect. Immun. **56**:287–293.
32. **Wenman, W. M., and M. A. Lovett.** 1982. Expression in *E. coli* of *Chlamydia trachomatis* antigen recognized during human infection. Nature (London) **296**:68–70.