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Culture of the Causative Organism of Donovanosis (*Calymmatobacterium granulomatis*) in HEp-2 Cells

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We report successful culture of *Calymmatobacterium granulomatis* by standard cell culture methods. Swabs were obtained from lesions in three patients with a clinical diagnosis of donovanosis. For two patients, there was histological confirmation of the disease (i.e., the presence of Donovan bodies in Giemsa-stained smears). Specimens were inoculated onto cycloheximide-treated HEp-2 cell monolayers in RPMI 1640 medium (supplemented with fetal calf serum, $NaHCO_3$, vancomycin hydrochloride, and benzylpenicillin). At 48 h, organisms resembling Donovan bodies were identified in monolayer cultures from all three specimens. The organisms appeared as pleomorphic bacilli with characteristic bipolar staining and "safety pin" appearance. Using a PCR designed to differentiate *C. granulomatis* from the *Klebsiella* species (which have a high degree of molecular homology), we were able to demonstrate that the cultured organisms produced a PCR product identical to that obtained from the original swab specimens. It is now possible to test in vitro susceptibility of *C. granulomatis* to antibiotics and to provide a ready source of DNA and antigenic material to enable the development of serological tests and, possibly in the future, a vaccine.

Donovanosis is a chronic, genital ulcerative disease caused by infection with *Calymmatobacterium granulomatis*. It is an important infection in Southern Africa, parts of Southeast Asia, Papua New Guinea, the Caribbean, and in northern and central Australia (11, 13). *C. granulomatis* is an intracellular, gram-negative, pleomorphic, encapsulated bacterium which shares many morphological and serological characteristics with *Klebsiella* species (10). Recently, by sequencing a region of the *phoE* (phosphate porin) gene, we have shown that *C. granulomatis* has a high degree of molecular homology with other *Klebsiella* species (3). We have since sequenced a number of unrelated genes and have consistently shown that there is greater than 99% homology at the nucleotide level between *C. granulomatis* and members of the *Klebsiella* genus that are pathogenic to humans (5). However, we have also shown that primers targeting genes of the sucrose regulon of the klebsiellae (2, 15) did not give a PCR product with *C. granulomatis*-derived DNA, allowing us to distinguish between the organisms (6).

C. granulomatis cannot be routinely cultured on conventional media, and earlier attempts have relied upon the inoculation of egg yolk sacs (1). The last successful culture using this method was reported in 1962 (7). Recently, Kharsany et al. (8) cultured the organism in human monocytes. We now report successful culture of the organism from three patients with typical clinical features of donovanosis in human epithelial (HEp-2) cells by a technique identical to that used for culture of *Chlamydia.*

MATERIALS AND METHODS

Specimens. Specimens were obtained from three women with clinical signs of donovanosis. Three cotton-tipped swabs were obtained from each lesion after the area was washed with normal saline. One swab was used to prepare an air-dried smear for histopathology. In addition, an impression smear was obtained by pressing a glass slide directly onto the lesion. Smears were stained with 10% Giemsa stain overnight and examined for the presence of typical histopathological features of donovanosis, particularly the presence of Donovan bodies (14). A second swab was transported to the laboratory in 1 to 10 ml of normal saline for PCR, while a third swab was placed in 1 ml of transport medium (glucose phosphate buffer [pH 7.2], 10% fetal calf serum, 20 mg of vancomycin hydrochloride per liter, 2.5 mg of amphotericin B per liter) for cell culture. Specimens in transport medium were stored at −70°C for a period ranging from 2 to 14 days and were thawed at 37°C immediately prior to testing.

Klebsiella pneumoniae (S1650 RDH) and *Klebsiella rhinoscleromatis* (NCTC 5046) were obtained from the Royal Darwin Hospital culture collection and processed as control specimens.

Cell culture. HEp-2 monolayer culture was established on four coverslips in 5-ml shell vials containing 1-ml portions of RPMI 1640 medium supplemented with 10% fetal calf serum, 0.2% $NaHCO_3$, vancomycin hydrochloride (20 mg/l), and benzylpenicillin (100 U/ml). The monolayer was inoculated (25 to 50 μl) with the specimen in transport medium, and the vial was then centrifuged at 33°C for 45 min at 1,000 × *g*. Just prior to inoculation, cycloheximide (0.7 mg/l) was added to inhibit further growth of HEp-2 cells.

After incubation at 35°C in 5% CO_2 for 24 to 72 h, one coverslip was fixed with methanol, stained with 10% Giemsa stain for 20 min, and observed by light microscopy. At the same time, the culture medium was centrifuged and the pellet was plated on horse blood agar (HBA; Oxoid) and on MacConkey agar plus crystal violet (Oxoid) and incubated at 37°C for 48 h. The second coverslip from each set was wet mounted onto a glass slide and examined by interference microscopy. The third vial was used for PCR analysis. The fourth vial from each set was stored at −70°C for subsequent repassaging. Duplicate sets of cultures were also set up, to which gentamicin (20 μg/ml) was added 1 h postinfection.

PCR. Template DNA for PCR was prepared from original swabs in saline and infected HEp-2 cells. After the cells were released from the monolayer (by freezing at −70°C, thawing at 37°C, sonicating, and mixing) or from the swabs (by vortexing), the cells were collected by centrifugation in microcentrifuge tubes (12,000 × *g* for 10 to 15 min). The pellets were resuspended in 100 μl of 50 mM NaOH. Samples were then overlayed with paraffin oil and heated at 95°C for at least 15 min prior to neutralization with 16-μl portions of 1 M Tris-HCl, pH 8.0.

Two sets of primers that amplify a segment of the *phoE* (phosphate porin) gene (5′ CTATGACAGCAAGGATGGCGA 3′ and 5′ CTGGTACTGCAG GGTCAGA 3′) and a segment of the *scrA* (sucrose transport) gene (15) (5′ TGCTGTCCAACATCTTCGTG 3′ and 5′ ATAATCACCGTCAGGAACGG 3′) were used. We have shown this method to be highly specific for donovanosis (6).

The final reaction mixture contained 1.0 μl of template DNA, 0.5 μM each primer, 0.2 mM deoxynucleotide triphosphates, 1× PCR buffer (10 mM Tris-HCl [pH 8.0], 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin), and 0.5 U of *Taq* polymerase in a final volume of 25 μl. Samples were subjected to 30 cycles of PCR, with 1 cycle consisting of denaturation (94°C, 40 s), annealing (55°C, 40 s), and extension (72°C, 60 s) in a thermal cycler (Corbett Research, Sydney, New South

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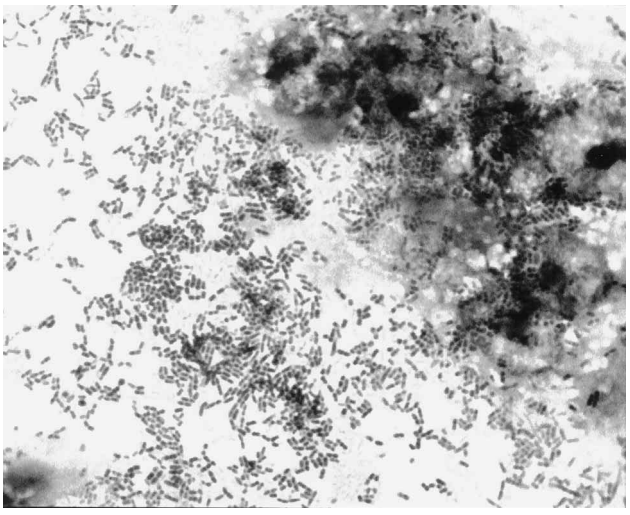

FIG. 1. Giemsa-stained culture in Hep-2 cells after 48 h of incubation.

Wales, Australia). Positive (*K. pneumoniae* and *K. rhinoscleromatis*) and negative (no DNA) controls for the DNA extraction and PCR procedures were included. PCR products were analyzed by agarose gel electrophoresis.

DNA sequencing. The PCR product amplified from bacteria in their third passage through HEp-2 cells was purified (Microcon; Amicon) and sequenced by the dye-terminator method (Applied Biosystems).

RESULTS

Microscopy. Organisms resembling Donovan bodies were identified in monolayer cultures from all three specimens. The organisms appeared as pleomorphic bacilli with a halo, suggesting the presence of a capsule. The characteristic bipolar staining and "safety pin" appearance was apparent in elongated forms of the organism, some of which appeared to be dividing (Fig. 1). Both extracellular and cell-associated forms of the organism were observed. The addition of gentamicin did not completely inhibit the growth of the bacteria, suggesting that they were multiplying intracellularly. We have successfully carried out three serial passages of *C. granulomatis* from one sample.

Interference microscopy. Interference microscopy of wet-mounted monolayer cultures confirmed the observations of light microscopy and showed that the cultured organisms were nonmotile bacilli.

Other flora from the specimens. After passage through culture, the only organism cultured on HBA and MacConkey agar from one patient was *Oligella urethralis* while *Pseudomonas aeruginosa* was the only organism to grow on these media from another patient. No growth on either HBA or MacConkey agar was observed from the third patient after 48 h of incubation. As expected, *K. pneumoniae* and *K. rhinoscleromatis* were the only organisms to grow on these media from the respective control cultures.

PCR. Monolayer cultures and original swab samples were processed for PCR as outlined above. All three clinical isolates gave PCR products for *phoE* but did not give a product for *scrA*. The same pattern was obtained with the DNA templates from bacteria passaged through HEp-2 cells (Fig. 2). However, as expected, *K. pneumoniae* and *K. rhinoscleromatis* gave PCR products for *phoE* and *scrA*.

DNA sequencing. The DNA sequence of the *phoE* PCR product amplified from bacteria in their third passage through HEp-2 cells was identical to that previously determined for *C. granulomatis* (3, 5).

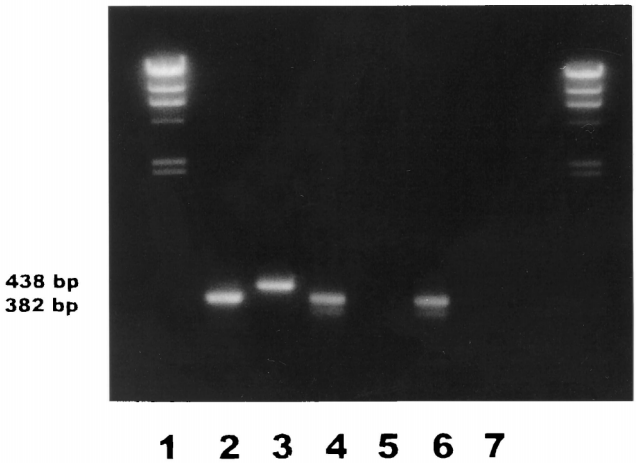

FIG. 2. PCR products derived from DNA templates from control organisms, original swab specimens, and culture specimens. Lane 1, molecular size markers; lane 2, 382-bp fragment (*phoE*) from *K. pneumoniae*; lane 3, 438-bp fragment (*scrA*) from *K. pneumoniae*; lane 4, *phoE* from original swab; lane 5, *scrA* from original swab; lane 6, *phoE* from culture; lane 7, *scrA* from culture. The rightmost lane (not labelled) contains no DNA (negative control).

DISCUSSION

Donovanosis has not attracted the same attention in the research community as have other more common sexually transmitted diseases, and as a result, diagnostic methods for the condition remain underdeveloped. Nevertheless, the disease is a major cause of morbidity and, more recently, of mortality, as infection is likely to increase the risk of transmission of human immunodeficiency virus (12).

Donovanosis is an uncommon but important sexually transmitted disease in northern and central Australia, and we have developed a research strategy to improve surveillance (11), diagnostic approaches (3), and treatment (4). Our major diagnostic focus has been on the development of a sensitive and specific PCR for the detection of the disease. In the process we have found that the causative organism has a high degree of homology with other *Klebsiella* species in many genes (5) but that *C. granulomatis* lacks the sucrose regulon (6). Therefore, differentiation of the organisms is now possible by using a combination of primers that target the *phoE* gene (which is common to both *C. granulomatis* and the *Klebsiellae*) and primers for the *Klebsiella scrA* gene (15) (which is lacking in *C. granulomatis*).

There is considerable evidence that the organisms cultured from our patients are *C. granulomatis*. The patients from whom the samples were obtained had the typical clinical features of donovanosis, and the original swab was positive by PCR (i.e., *phoE* positive, *scrA* negative) in each case. The impression and swab smears Giemsa stained overnight were negative for Donovan bodies for one patient but positive for the other two patients. The cultured organisms morphologically resemble Donovan bodies at various stages of maturity, including the characteristic safety pin appearance. Giemsa staining revealed the typical features seen in histological specimens. Furthermore, the *phoE* gene amplified from cultured organisms in their third passage was of identical sequence to that previously determined for *C. granulomatis* (3, 5). Contaminating organisms were found in two of the three isolates, but each was readily identified.

Gentamicin has previously been shown to rapidly kill extracellular members of the family *Enterobacteriaceae* while being

incapable of eliminating intracellular *Enterobacteriaceae* (9). Growth of *C. granulomatis* in the presence of gentamicin therefore suggests that the organisms are multiplying intracellularly. Moreover, *C. granulomatis*, like *Chlamydia*, does not appear to require host-cell protein synthesis as the culture was performed in the presence of cycloheximide. This property will enable the specific labelling of *C. granulomatis* proteins in future biochemical studies.

Kharsany et al. (8) reported successful culture of *C. granulomatis* with a human monocyte coculture system. This method is dependent upon the availability of fresh monocytes from healthy donors and is time-consuming and technically demanding. Our technique utilizes a standard culture technique which can be used in any laboratory routinely culturing *Chlamydia.*

The development of this simple, routine culture system for donovanosis will allow the testing of in vitro susceptibility to antibiotics and will provide a ready source of DNA and antigenic material to enable the development of serological tests and, possibly in the future, a vaccine.

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