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Isolation of *Chlamydia psittaci* from Naturally Infected African Clawed Frogs (*Xenopus laevis*)

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An inclusion-forming agent was isolated from the livers of commercially raised African clawed frogs (*Xenopus laevis*) involved in an epizootic of high morbidity and mortality. Original isolation was made in McCoy cells. This agent was identified as *Chlamydia psittaci* based on the formation of typical intracytoplasmic inclusions which developed within 48 h, were not stained by iodine, and were resistant to sulfadiazine. The isolate from one particular frog (designated as strain 178) was further studied and found to be lethal for 7-day-old embryonated chicken eggs after intra-yolk sac inoculation. This strain was demonstrated not to be pathogenic for mice when inoculated intraperitoneally. The cell culture isolate of *C. psittaci* was transmitted to uninfected *X. laevis*, causing disease and death.

The genus *Chlamydia* consists of two species, *Chlamydia trachomatis* and *Chlamydia psittaci* (6). *C. trachomatis* is well known as a human pathogen and has been associated with a variety of ocular, urogenital, and respiratory infections in humans. Among these are trachoma, inclusion conjunctivitis, lymphogranuloma venereum, urethritis, cervicitis, and pneumonia of infants (7, 8). Various strains of *C. psittaci* have been isolated from and implicated as pathogens in many different species of wild and domestic birds and mammals. The disease spectrum of *C. psittaci* in animals is large and includes conjunctivitis, polyarthritis, enteritis, endometritis, placentitis, encephalitis, and pneumonitis (12). *C. psittaci* is capable of causing severe pneumonia or systemic nonrespiratory infections in humans (8).

The extent to which *C. psittaci* is associated with disease in animals other than birds and mammals has not been determined. *C. psittaci* has been isolated from various naturally and experimentally infected ectoparasites, but the role of the ectoparasites, if any, in the transmission of disease has not been well established (7, 12). Although *C. psittaci* has been successfully grown in cell cultures from cold-blooded animals such as the tortoise (9, 10), there have been no reports of *C. psittaci* isolated as the causative agent of a naturally occurring disease in cold-blooded animals.

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MATERIALS AND METHODS

Frogs. The original six clinically ill African clawed frogs (*Xenopus laevis*) were obtained from a commercial supplier whose colony of frogs was experiencing an epizootic. Healthy *X. laevis*, putatively *Chlamydia* free, were obtained from the University of Michigan Amphibian Facility for subsequent *Chlamydia* transmission experiments. During experimental infections, the frogs were housed in polycarbonate cages (47.5 by 26.3 by 20.0 cm) in ca. 10 cm of dechlorinated water. The water was changed three times weekly. Animal housing conformed to the Centers for Disease Control and National Institutes of Health guidelines for handling animals infected with *C. psittaci* (1).

Necropsy and histopathology. The frogs were grossly and microscopically examined after spontaneous death or after being killed with Metofane (Pitman-Moore, Washington Crossing, N.J.). Necropsy was performed shortly after death for those frogs that died spontaneously or immediately after death for those frogs that were sacrificed. Livers, kidneys, hearts, lungs, and spleens were collected for histopathological examination. Tissues were fixed in 10% buffered neutral Formalin, and 5-μm paraffin sections were made.

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TABLE 1. Necropsy of original six index cases of chlamydiosis in African clawed frogs (*X. laevis*)

Frog no.	Age	Sex	Mode of death	Gross appearance of liver	Isolation of *C. psittaci* from liver
173	Adult	ND[a]	Spontanoeus	Brown, mottled, friable	Yes
174	Adult	F	Spontaneous	Gray-black, mottled, enlarged	Yes
175	Adult	F	Spontaneous	Not remarkable	Yes
176	Adult	F	Sacrifice	Not remarkable	No
177	Juvenile	ND	Sacrifice	Dark brown, congested	Yes
178	Adult	ND	Sacrifice	Gray-green, mottled	Yes

[a] ND, Not determined.

Staining was performed with hematoxylin and eosin, Brown and Brenn (tissue Gram stain), periodic acid-Schiff, or Giemsa stains.

Electron microscopy. Material was fixed in neutral buffered Formalin, minced into 1-mm cubes, and placed in phosphate-buffered 2% glutaraldehyde at 4°C for 1 h. The tissue was postfixed in 1% osmium, stained with uranyl acetate and lead citrate, and embedded in Epon for thin sectioning.

***C. psittaci* isolation from index cases.** Livers or kidneys or both were collected for *C. psittaci* isolation. Tissues were collected aseptically and inoculated within 2 to 4 h or stored frozen at −70°C until inoculation. Tissues were transported and held in a sucrose-phosphate buffer, 2 SP (2). The tissue specimens were ground with a sterile mortar and pestle to achieve an approximate 10% (wt/vol) tissue suspension in 2 SP. The suspensions were centrifuged briefly at 800 rpm (170 × *g*) to remove large tissue fragments and debris. The supernatant was further diluted 1:10 in 2 SP. The inocula (0.2 ml) from the 1:10 dilutions were overlaid onto cover slip monolayers of McCoy cells. The McCoy cells had been pretreated for 72 h with 5-iodo-2-deoxyuridine by the method of Wentworth and Alexander (13). After centrifugation for 1 h at 3,200 rpm (2,500 × *g*), the cover slips were rinsed briefly with calcium- and magnesium-free phosphate-buffered saline (pH 7.5), and freshly made tissue culture medium (Earle minimal essential medium [pH 7.2 to 7.4] with 2% fetal bovine serum, gentamicin [10 μg/ml], and amphotericin B [2 μg/ml]) was added. The inoculated monolayers were incubated for 48 to 72 h at 35 to 37°C. After incubation, the cover slips were stained with Jones iodine or Giemsa stain. Quantitation of inclusion bodies was made by averaging the number of inclusions in 20 representative fields and multiplying by the number of high-power fields per cover slip, i.e., 620. Quantitation was based on triplicate or quadruplicate cover slips.

Characterization of isolates. The isolates from *X. laevis* were characterized by staining reactions (Jones iodine versus Giemsa stain), the morphology of the inclusions, the susceptibility to 25 μg of sulfadiazine per ml, (Lederle Laboratories, Pearl River, N.Y.), and the effect of centrifugation on inclusion body formation.

Experimental infection in frogs. Liver suspensions from two index cases of chlamydiosis were inoculated into the dorsal lymph sac of four healthy *X. laevis*, and the presence of inclusion bodies was determined by histological examination. Liver homogenate from normal healthy *X. laevis* was inoculated as a control.

Further studies were performed with one particular isolate of *C. psittaci* from index case number 178. This isolate (strain 178) was first obtained in the McCoy cell isolation system and passed in the same system. A suspension of strain 178 passage material was serially diluted in 10-fold dilutions to 10^{-6}, and 0.2 ml of each dilution was inoculated into the dorsal lymph sac of healthy frogs (six frogs per dilution). As a control, an undiluted suspension of McCoy cells was inoculated into six healthy frogs (0.2 ml per frog). All frogs were monitored for 3 weeks. Necropsies were performed on frogs that died spontaneously, and liver and kidney specimens were aseptically removed for reisolation attempts. At 3 weeks, all frogs still living were killed, and necropsies were performed as described above. Reisolation attempts for *C. psittaci* were made in McCoy cells. Serum specimens from infected and control frogs were collected for subsequent testing.

Mouse inoculation studies. Mice used were of an outbred stock of white mice designated Mdh (Carworth Farms Webster) and were obtained from R. Myers of the Animal Care Section, Michigan Department of Public Health. The colony was predetermined to be *Chlamydia* free by serological testing of sentinel mice by immune adherence hemagglutination (4). Serial 10-fold dilutions of *C. psittaci* 178 were made (undiluted through 10^{-6}, and 0.5 ml of each dilution was inoculated intraperitoneally into six mice. Undiluted McCoy cells and a known *C. trachomatis* strain (strain G29 [E/UW-17/Cx]; originally obtained from E. R. Alexander, University of Washington) were used as controls.

After 12 days of observation, the mice were anesthetized with Metofane, exsanguinated, and necropsied. The sera obtained from these mice were tested by immune adherence hemagglutination against a *Chlamydia* group antigen (obtained from the Centers for Disease Control). Sections of livers, spleens, and kidneys were collected and pooled for *C. psittaci* isolation attempts with the McCoy cell system as described above.

Embryonated chicken egg inoculation. The yolk sacs of 7-day-old embryonated chicken eggs were inoculated (0.2 ml per egg) with ca. 10^2 to 10^3 inclusion-forming units (IFU) of strain 178, and 0.2 ml of 2 SP was inoculated into control eggs. The yolk was collected after the embryos had died, and titration of *C. psittaci* was carried out in the McCoy cell system.

Direct fluorescent antibody detection of *Chlamydia*. The identity of strain 178 as *Chlamydia* was further confirmed by using a fluorescein-labeled conjugate obtained from Bartels Immunodiagnostics, Bellevue, Wash. The conjugate used does not distinguish between *C. trachomatis* and *C. psittaci*.

RESULTS

Isolation from index cases. The necropsies on the original six index cases are summarized in Table 1. The gross pathological observations were unremarkable, with the exceptions noted pertaining to the appearance of the liver in five of the six cases. The histopathological description of chlamydial lesions in these African clawed frogs has recently been published by Newcomer et al. (5). Case number 176 showed no remarkable gross pathological findings, and the isolation attempts for *C. psittaci* were negative in this particular frog. The other five frogs all had *C. psittaci* isolated from the liver tissue specimens. *Chlamydia*-like inclusions were detected in the McCoy cell system. In each case the inclusions were intracytoplasmic, non-iodine staining, diffuse in character, and readily visible at 48 h post-inoculation. All of these characteristics are typically those of *C. psittaci* inclusions. The average number of inclusions counted in the McCoy cell monolayers indicated that the original liver specimens contained ca. 3.5×10^5 to 7.0×10^5 IFU/g of liver tissue.

Characterization of strain 178. (i) Growth characteristics. *C. psittaci* 178 was isolated and passaged in McCoy cells. It was determined that pretreatment with 5-iodo-2-deoxyuridine was not necessary for the growth of this agent. In addition, centrifugation did not appear to be necessary for infecting cell culture. Strain 178 produces non-glycogen-containing inclusions, as demonstrated by the fact that the inclusions stained only with Giesma stain and not iodine. Typical Giemsa-stained inclusions in McCoy cells are shown in Fig. 1. Treatment of strain 178 with 25 μg of sulfadiazine per ml had no effect on the size or number of inclusions formed. In contrast, a known strain of *C. trachomatis* was completely inhibited by the same concentration of sulfadiazine.

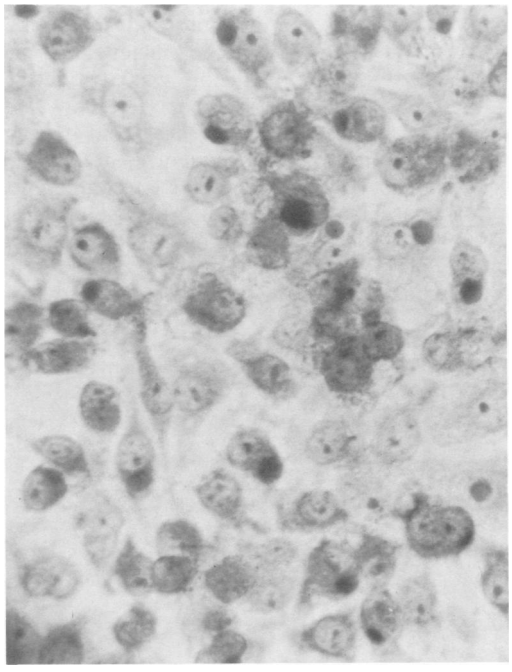

FIG. 1. Inclusions of strain 178 in McCoy cells (Giemsa stained). ×250.

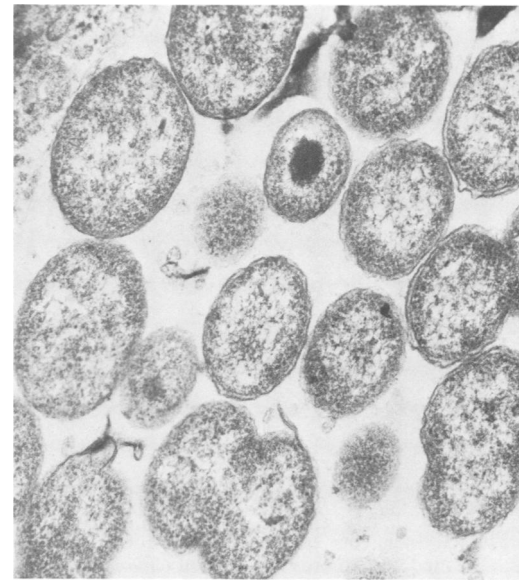

FIG. 2. Electron micrograph of strain 178 in Kupffer cell of *X. laevis* demonstrating initial bodies and an intermediate body. ×35,000.

(ii) Morphological characteristics. Electron micrographs demonstrated that the morphology of isolate 178 in livers from experimentally infected frogs was characteristic of the *Chlamydia* genus. Two stages in the developmental cycle of the *Chlamydia* genus, dividing initial bodies and intermediate bodies, are shown in Fig. 2.

Isolate 178 was stained after passage in McCoy cells by using a commercial fluorescein-labeled *Chlamydia* antibody prepared in goats (Bartels Immunodiagnostics). Both the frog isolate (Fig. 3) and a control strain of *C. trachomatis* stained equally well with this conjugate. The inclusions were easily detected within 48 h and showed typical intracytoplasmic morphology.

(iii) Pathogenicity of strain 178. The cell culture isolate from frogs was inoculated into normal healthy frogs (*X. laevis*). The selection of a 3-week cutoff period for this experiment was based on the previously observed morbidity and death of frogs inoculated with liver homogenate from *C. psittaci*-infected frogs. The results of

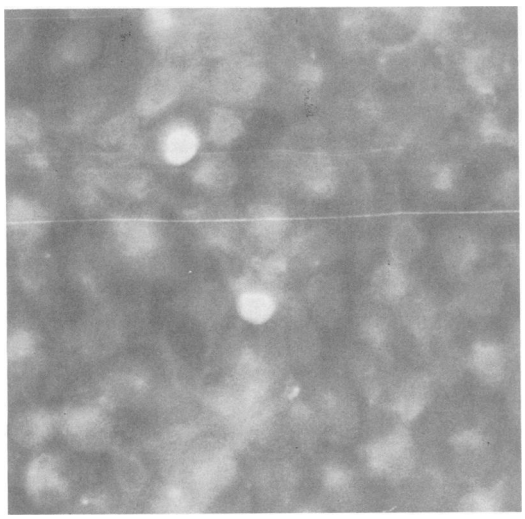

FIG. 3. Inclusions of strain 178 in McCoy cells at 48 h (stained with fluorescein-labeled *Chlamydia* antibody). ×400.

this experiment indicated that the 50% lethal dose appears to be <10 IFU when inoculated by the dorsal lymph sac route (Table 2). Histologically, all groups of frogs inoculated with strain 178 demonstrated the presence of inclusions in liver, kidney, and/or spleen sections. Reisolation of *C. psittaci* was made from representative frogs in the undiluted, 10^{-3}, and 10^{-5} groups. One of the six frogs in the control group had *C. psittaci* isolated from a pool of tissue collected at necropsy. This frog was not clinically ill, and it was assumed that, despite precautions taken, cross contamination from an infected frog must have occurred late in the experimental time frame. Attempts to demonstrate a serological conversion to the *Chlamydia* group antigen in the experimentally infected frogs were unsuccessful because of the anticomplementary activity of the frog sera.

Strain 178 was not pathogenic for mice via the intraperitoneal route when mice were inoculated with as many as 10^6 IFU. None of the mice inoculated with strain 178 at this dosage level developed any overt sign of infection. After 12 days, the mice were sacrificed and exsanguinated, and necropsy specimens were taken. Attempts to isolate *C. psittaci* from livers, spleens, and kidneys were all negative. Control mice inoculated with either *C. trachomatis* or a suspension of McCoy cells were similarly negative. Evidence of infection was seen, however, by using the immune adherence hemagglutination test to compare preinoculation sera with sera taken at death. The mice which received 10^4 to 10^6 IFU of strain 178 demonstrated a rise in titer to the *Chlamydia* group antigen (Table 3). The mice inoculated with *C. trachomatis* did not show such a rise.

Approximately 10^2 to 10^3 IFU of strain 178 was inoculated into the yolk sacs of 7-day-old embryonated chicken eggs (six eggs per group). One embryo from each group died within 3 days as a result of trauma. The remainder died at 7 or 8 days post-inoculation. All yolk sacs were harvested at 8 days. All control eggs inoculated with 2 SP survived and yielded no *C. psittaci*. The infected eggs yielded $\geq 10^6$ IFU of strain 178 per ml of yolk when titrated in McCoy cells, indicating proliferation of the organisms in yolk sacs.

DISCUSSION

We report here on the isolation of *C. psittaci* from a naturally occurring infection of African clawed frogs (*X. laevis*). To our knowledge this is the first report of *C. psittaci* as a pathogen in frogs. Although *Chlamydia*-like inclusions have been histologically seen in mollusks (3) and other ectotherms (9, 10), we are unaware of isolations being made from cold-blooded animals.

The *C. psittaci* isolate from *X. laevis* was typical in its inclusion morphology, staining characteristics, and resistance to sulfadiazine. Detection of inclusion bodies in cell cultures was best carried out with Giemsa stain or a fluorescein-labeled anti-*Chlamydia* conjugate. It was possible, however, to see the inclusions of the isolate from frogs even with Jones iodine stain. The inclusions did not stain any darker with iodine than did the cell nucleus, but the morphology of the inclusion was distinctive. Although originally isolated in a 5-iodo-2-deoxyuridine-treated McCoy cell system by centrifugation, it was subsequently determined that neither 5-iodo-2-deoxyuridine treatment nor centrifugation was necessary for isolation in McCoy cells. The electron microscopic examination of this organism further confirmed its identity as a typical *Chlamydia* strain which

TABLE 2. Experimental infection of healthy frogs (*X. laevis*) with strain 178 of *C. psittaci*

Dilution inoculated	Inoculum size (IFU)	No. of frogs dead/ total no. of frogs inoculated
Undiluted	10^4	6/6
10^{-1}	10^3	6/6
10^{-2}	10^2	6/6
10^{-3}	10	6/6
10^{-4}	1	2/6
10^{-5}	<1	1/6
Control[a]		0/6

[a] McCoy cell suspension.

goes through a normal developmental cycle involving initial body and intermediate body formation.

Evidence to establish this isolate of *C. psittaci* as the causative agent for this epizootic was obtained by transmitting the disease to healthy uninfected *X. laevis* and reisolating the agent after death. Unfortunately, a serological conversion in frogs could not be demonstrated because of the anticomplementary activity of frog sera.

It was possible, however, to demonstrate a serological conversion in mice. Although there was no clinical evidence of infection or positive cultures, mice which had received 10^4 IFU or greater responded with a rise in antibody to the *Chlamydia* group antigen. This lack of virulence for mice is unlike that of the *C. psittaci* strains observed from psittacine birds and humans, which normally can cause death in mice within 10 days (7).

Similar to most strains of *C. psittaci*, this isolate from frogs was highly virulent for embryonated eggs. The titers obtained in eggs were quite high in comparison to cell culture. In this respect strain 178 is quite different from *C. trachomatis*, which is relatively less virulent in eggs than is *C. psittaci*.

Serotyping of *C. psittaci* strains has lagged behind the serotyping of *C. trachomatis*, and at this point there is no recognized system for the serotyping of *C. psittaci* (7). There have been, however, attempts to biotype *C. psittaci* based on inclusion morphology and response to DEAE-dextran and cycloheximide (11). Further studies of strain 178 will involve such biotyping.

The source of the infection in this epizootic has not been determined. It was revealed that the colony of *X. laevis* was being fed condemned beef liver at the time of the outbreak, and *C. psittaci* is known to be the cause of several different types of bovine infections. Another possible source could have been wild animal contact. There was a period of time before the epizootic during which the frogs were released into a pond in which they would have had contact with many animals in nature. The frogs had been moved back indoors, however, over 2 months before the onset of the epizootic.

Further studies are needed to characterize this strain of *C. psittaci*. Since we have demonstrated that *C. psittaci* can be a pathogen for *X. laevis*, it remains to be determined whether other genera of frogs can be infected. The whole area of *C. psittaci* as a pathogen in cold-blooded animals must be studied if the ecology and epidemiology of this organism are to be understood.

TABLE 3. Serological response to *C. psittaci* 178 in mice after intraperitoneal injection[a]

Inoculum	Inoculum size (IFU)	IAHA titer to CGA
C. psittaci 178	10^6	1:8
		1:64
		1:32
	10^5	1:32
		1:32
		1:16
	10^4	1:32
		<1:8
		<1:8
	10^3	<1:8
		<1:8
		<1:8
C. trachomatis	10^5	<1:8
		<1:8
		<1:8
McCoy cell suspension		<1:8
		<1:8
		<1:8

[a] Three mice per group. IAHA, immune adherence hemagglutination; CGA, *Chlamydia* group antigen. Results at 12 days post-inoculation are given.

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