APPLIED MICROBIOLOGY, June 1973, p. 981-990

Vol. 25, No. 6
Printed in U.S.A.

Identification of the *Prototheca* Species by Immunofluorescence

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Received for publication 16 February 1973

Studies were carried out to develop fluorescent antibody reagents for the identification of the *Prototheca* species and for their differentiation from morphologically similar fungi of various genera in formalin-fixed tissues. Antisera against representative isolates of *P. filamenta*, *P. moriformis*, *P. stagnora*, *P. wickerhamii*, and *P. zopfii* were produced in rabbits. Antiglobulins, labeled with fluorescein-isothiocyanate that intensely stained most cells of the homologous species, were selected for use as potential diagnostic reagents. By adsorbing the conjugates with selected heterologous cross-staining protothecae, reagents that were both sensitive and specific were obtained. Evaluation of the adsorbed conjugates with sections of tissue infected with protothecae, sections of tissue infected with morphologically similar fungi, and cultures of protothecae showed that these reagents are useful for the rapid and reliable identification of the *Prototheca* species.

Prototheca species are microscopic, achlorophyllous, single-celled organisms which occur ubiquitously in nature. They have been isolated from such diverse sites as slime flux of trees, feces and fingernails of man, potato skin, acid stream water, sludge in waste stabilization ponds, and other habitats.

Protothecae cells are hyaline and globose to oval in form; they range in size from 1.3 to 13.4 by 1.3 to 16.1 μm and have thick walls that give a cellulotic reaction with chlorozinc iodine (2).

Reproduction in the genus *Prototheca* is asexual. As the cells mature their cytoplasm undergoes a process of cleavage to form endospores. The endospores increase in size upon release from the mother cell and go through an assimilative stage. When a cell is one-half to two-thirds the size of its parent cell, cleavage lines appear and two to twenty spores develop, which are initially irregular in shape (Fig. 1). The mother cell apparently breaks by pressure from the enlarging spores; release of the spores is passive (4).

Prototheca species were first implicated as agents of human disease in 1964. The first reported case caused by *Prototheca zopfii* (*segbwema*), occurred on the foot of a rice worker in Sierra Leone (5, 6). The second case, in Durham, N.C., was described in a woman who had diabetes mellitus and metastatic carcinoma of the breast; the lower portion of one leg was involved, and the etiologic agent was found to be *P. wickerhamii* (11). A third infection involved the scalp and forehead of a man in Johannesburg, South Africa; the etiologic agent was also *P. wickerhamii* (14). A fourth report describes three alleged cases of cutaneous protothecosis that were diagnosed on the basis of histopathology alone (19). Regrettably, the photomicrographs in this last report were of too low a magnification to enable recognition of protothecae; the reader was thereby left with some doubt as to the accuracy of the diagnosis.

Arnold and Ahearn (2) isolated *P. filamenta* from a case of athlete's foot. This organism was thought to be a skin saprophyte. Sonck and Koch (18) recovered *P. wickerhamii* from five patients with dermatologic disease but suggested that the isolates were present as skin saprophytes.

Protothecosis has been reported frequently in lower animals. As early as 1952, a *Prototheca* sp. was implicated as the cause of bovine mastitis (13); additional cases of bovine protothecal mastitis have subsequently been re-

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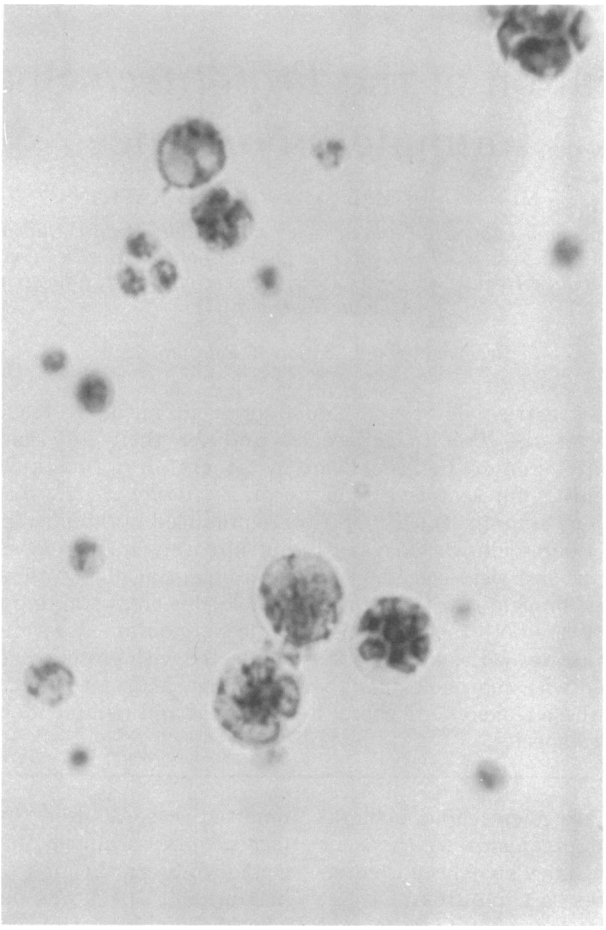

FIG. 1. *P. wickerhamii cells in lactophenol cotton blue. Note cells at various stages of development and mother-cells containing daughter cells. Magnification at ×1400.*

ported (1, 7, 15). In 1968, *P. zopfii* was found in skin lesions, adjacent bone, and regional lymph nodes of a deer (8). *Prototheca* species have also been implicated in disseminated disease in dogs, involving such organs as the liver, brain, kidneys, and eyes (16, 20, 21).

The microscope morphology of the *Prototheca* species in tissue is the same as that observed in culture. An organism can easily be recognized as a *Prototheca* species if the characteristic endosporulating cells are visible. If these diagnostic structures are not visible, protothecae may resemble nonsporulating cells of *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, and some stages of *Coccidioides immitis*, *Pneumocystis carinii*, and *Rhinosporidium seeberi;* thus, diagnosis of a *Prototheca* infection by histopathologic techniques might be difficult if not impossible.

Diagnosis of protothecosis depends upon the demonstration of the cells of a *Prototheca* species in tissue and, ideally, the isolation and identification of the organism in culture. Although differentiation of the species by carbohydrate and alcohol assimilation (2) is straightforward, the methods involved are time consuming. Moreover, identification of the species in fixed tissue or other clinical material cannot be made with physiological tests. Therefore, a more rapid procedure for the differential identification of the *Prototheca* species was considered desirable. Since the fluorescent antibody (FA) technique has been widely applied to the identification of microorganisms, we believed it worthwhile to determine its usefulness for the detection and identification of the *Prototheca* species.

The purpose of this study was to develop specific FA reagents for the rapid and reliable detection and differentiation of the *Prototheca* species in culture and in fixed tissue.

(This investigation is a portion of a dissertation submitted by the senior author to the University of North Carolina in partial fulfillment of the requirements for the degree of Doctor of Public Health in the School of Public Health.)

MATERIALS AND METHODS

Production of antisera. Antisera were produced in rabbits against a representative isolate of each of the five recognized *Prototheca* species (2): *P. filamenta*, *P. moriformis*, *P. stagnora*, *P. wickerhamii*, and *P. zopfii*. The organisms were grown in Sabouraud dextrose broth (SDB) for 7 days at 25 C on a rotary shaker (160 rpm), then killed with 1.0% buffered neutral formalin for 24 h, and washed and suspended in 0.5% buffered neutral formalin. The cell-antigen concentration was adjusted to a density of a no. 5 McFarland nephelometric standard in 0.5% buffered neutral formalin.

The immunization schedules and dosages used for the production of antisera were as follows. The rabbits received 1, 2, and 2 ml of the respective antigen intravenously (i.v.) on 3 successive days for 3 consecutive weeks. Four weeks after the initial course of inoculations, the rabbits received the first of three series of booster injections; 8 weeks later the second booster series was given and 12 weeks later the third one was given. Each series of booster injections consisted of the i.v. administration of the respective antigens in 1-, 2-, and 2-ml doses on 3 consecutive days of 1 week.

It was not possible to use the above immunization procedures for the production of antiserum to *P. filamenta* because the rabbits invariably died from anaphylactoid shock during the second week of the initial course of i.v. injections. A modified procedure was used which consisted of the i.v. injection of 1, 2, and 2 ml of antigen on 3 successive days for the first week and 1 ml of antigen intramuscularly on 3 consecutive days for weeks 2 and 3. The animals received three series of booster injections at the same time as that used for the production of antisera to the other *Prototheca* species. Each booster series consisted of the intramuscular injection of 1 ml of antigen on 3 consecutive days for 1 week.

One week after the third booster series, the rabbits were bled. Both preimmunization sera and antisera were stored at −20 C until fractionated.

Conjugation of globulins. Preimmunization globulins and antiglobulins were obtained by two precipitations of the sera with half-saturated ammonium sulfate. The precipitates were dissolved in distilled water and dialyzed at 4 C against 0.85% saline until free of ammonium sulfate. The globulins were conjugated to fluorescein isothiocyanate by the procedure of Kaplan and Ivens (9). Uncombined fluorescein was removed by dialysis against phosphate-buffered saline (PBS), pH 7.2, at 4 C. The labeled globulins were adjusted to 1.0 g of protein per 100 ml, merthiolated (1:10,000; Lilly), and stored at 4 C.

Adsorption of conjugates. Nonspecific tissue staining factors were removed by adsorbing conjugates two times with acetone-dried spleen powder (Nutritional Biochemicals Corp., Cleveland, Ohio, No. 2977) for one hour at 4 C. Cross-reacting factors for heterologous *Prototheca* species and morphologically similar fungi were eliminated by adsorbing two volumes of conjugate with one volume of packed formalin-killed cells of the adsorbing organism for 2 h in a 37-C water bath. The isolates of the respective *Prototheca* species used for immunization were also used for adsorption. *Prototheca* species used for adsorption were grown in SDB at 25 C for 7 days on a rotary shaker; cells of the heterologous fungi used for adsorption were grown in Brain Heart Infusion broth at 37 C for 7 days on a rotary shaker. The respective growth was harvested, formalinized (1.0%), washed twice with PBS (pH 7.2), and packed by centrifugation.

Preparation of material for testing. For testing of cultures (Table 1), heat-fixed smears of aqueous suspensions of 7-day-old growth were used. The staining procedure used has been described in a previous report (9). Tissues (Table 2) were processed and stained according to the method of Kaplan and Kraft (10).

Production of experimental infections. Only two *Prototheca* species, *P. wickerhamii* and *P. zopfii*, have been known to cause disease, and tissues naturally infected with these two organisms were available. In the case of the other *Prototheca* species, tissues from experimentally infected animals had to be used. Experimental infections were produced in guinea pigs by intradermally injecting 0.1 ml of a 1.0% (vol/vol) saline suspension containing a 7-day-old growth of the respective organism. Seven days later the animals were sacrificed and the infected tissue was excised and fixed in 10% buffered neutral formalin.

Visualization of staining reactions. Stained preparations were examined with a Reichert Biozet microscope fitted with a cardioid dark-field condenser. A Reichert Fluorex unit fitted with an Osram HBO-200 high-pressure mercury vapor lamp was used as the light source. A 442-1 American Optical interference filter (17) was used in combination with a GG-9 ocular filter.

The intensity of the staining was rated according to the criteria of Cherry et al. (3) as follows: 4+, 3+, 2+, 1+, positive reactions; and +/−, 0, no reactions.

RESULTS

Staining properties of unadsorbed conjugates. Three series of booster injections were required to obtain antiglobulins which, when labeled with fluorescein, would stain isolates of the homologous species 2–4+. The staining properties of these conjugates for the homologous and heterologous *Prototheca* species are presented in Table 3. The least cross-reactive reagent was the one prepared from antiglobulins to *P. filamenta*, which cross-stained only cells of *P. stagnora*. By contrast, the fluorescein-labeled *P. stagnora* antiglobulins cross-reacted strongly with all of the heterologous *Prototheca* species. The fluorescein-labeled antiglobulins to *P. moriformis* and

TABLE 1. *Cultures of Prototheca species used*

Organism	CDC identification no.	Source[b]
P. chlorelloides	B-1420	Pore, W. Va. 842; Poyton, Cornell, U., 69-19A
P. filamenta	B-1273[a]	Ahearn, Georgia State, ATCC 22432
P. moriformis	B-1266[a]	NRRLY-6864; SEC PR-5; Starr, Ind. 328; Cambridge 263/5
P. moriformis	B-1414	NRRL Y-6867; Cooke, SEC PR-28
P. moriformis	B-1444	NRRL Y-6865; vanNeil, Hopkins IV 7.3.2.1; Cooke, SEC PR-9
P. moriformis	B-1445	NRRL Y-6869; Cooke, SEC PR-31
P. moriformis	B-1446	NRRL Y-6871; Cooke, SEC PR-53
P. pastoriensis	B-1421	Pore, W. Va. 843; Poyton, 68-20A
Prototheca sp.	B-1415	Pore 763
Prototheca sp.	B-1416	Weitzman, N.Y. 655-72
Prototheca sp.	B-1417	Pore, 838
Prototheca sp.	B-1418	Phaff, Calif. C387
Prototheca sp.	B-1447	Agricul. Dept., U. Penn.
P. stagnora	B-1277[a]	NRRL Y-6872; Cooke, Type sp. SEC L-1690
P. trispora	B-1413	Feo, 179-A, Venezuela
P. ubrizsyi	B-1412	Zsolt, Hungary
P. wickerhamii	B-1269	NRRL YB-5163; Capriotti, 61P
P. wickerhamii	B-1274	CDC 45-1029-70
P. wickerhamii	B-1275	CDC 45-1068-70
P. wickerhamii	B-1278	NRRL YB-4330
P. wickerhamii	B-1279	NRRL Y-6870; SEC L-2-14
P. wickerhamii	B-1280[a]	NRRL Y-7021
P. wickerhamii	B-1281	NRRL Y-7029; Ahearn, 964
P. wickerhamii	B-1282	NRRL Y-7045; Nielson, Duke U.
P. zopfii (*ciferri*)	B-1265	NRRL YB-4825; Nagao Inst. 7660; Ciferri, HMS-1154; Phaff, 60-49
P. zopfii (*portoricensis*)	B-1267	NRRL YB-4826; Nagao Inst. 7669; Ciferri, HMS-1154; Phaff, 60-48
P. zopfii	B-1268	NRRL YB-2462
P. zopfii	B-1270[a]	NRRL Y-7054; Frank, Wooster, Ohio
P. zopfii	B-1271	NRRL Y-7056; Frank, Wooster, Ohio
P. zopfii	B-1272	NRRL Y-7057; Frank, Wooster, Ohio
P. zopfii (*segbwema*)	B-1276	NRRL YB-7032
P. zopfii	B-1283	NRRL YB-990;
P. zopfii	B-1284	NRRL YB-4121;
P. zopfii	B-1285	NRRL Y-6868; SEC PR-30; NRRL YB-833
Chlorella sp. (achloric)	B-1419	Pore, 841; Poyton, 69-7A

[a] Immunization strains.

[b] CDC, Center for Disease Control; ATCC, American Type Culture Collection; NRRL, Northern Regional Research Laboratory, U.S. Dept. Agr.

P. zopfii were identical in their extent of cross-reactivity.

The cross-reactivity of each of the unadsorbed conjugates with organisms of other genera is presented in Table 4. Each reagent cross-stained one or more of these fungi but, with some exceptions, the intensities of the reactions were of low order.

The labeled preimmunization globulins did not stain any of the *Prototheca* species or any of the fungi tested.

Staining properties of the adsorbed conjugates. Since specific reagents of adequate sensitivity could not be produced by simple dilution, the possibility of preparing them by adsorption was explored. Each of the labeled antiglobulins was adsorbed with cells of each of the heterologous *Prototheca* species. The staining properties of each adsorbed conjugate for the homologous as well as the heterologous *Prototheca* species are summarized in Table 5. With the exception of reagents for the differentiation of *P. zopfii* from *P. moriformis*, it was possible to prepare conjugates for the specific identification of the *Prototheca* species by selective adsorption of the labeled antiglobulins. A

conjugate specific for *P. filamenta* was prepared by adsorption of the labeled antiglobulins to *P. filamenta* with cells of any of the other *Prototheca* species. FA reagents for the specific identification of *P. stagnora* and *P. wickerhamii* were prepared by adsorption of the labeled antiglobulins to each of these species with cells of *P. zopfii*. It was possible to prepare an FA reagent for the differentiation of *P. zopfii* and *P. moriformis* from the other *Prototheca* species by

TABLE 2. *Prototheca-infected tissues used*

Species	CDC[a] identification no.	Tissue	Source
P. filamenta	T-1345	Skin, guinea pig	Sudman, experimental infection
P. moriformis	T-1346	Skin, guinea pig	Sudman, experimental infection
Prototheca sp.	T-1049	Intestine, dog	Van Kruiningen, Storrs, Conn.
Prototheca sp.	T-1173	Lymph node, cow	Migaki, Armed Forces Institute of Pathology (AFIP)
Prototheca sp.	T-1187	Intestine, dog	Povey, Bristol, England
Prototheca sp.	T-1139	Lymph node, cow	Frank, Wooster, Ohio
Prototheca sp.	T-1349	Kidney, mouse	Pore, W. Va.
Prototheca sp.	T-1353	Lymph node, cow	Migaki, AFIP
Prototheca sp.	T-1354	Lymph node, cow	Migaki, AFIP
P. stagnora	T-1335	Skin, guinea pig	Sudman, experimental infection
P. wickerhamii	T-1337	Skin, guinea pig	Sudman, experimental infection
P. wickerhamii	T-1261	Scalp, human	Mars, Johannesburg, S. Africa
P. zopfii	T-1334	Skin, guinea pig	Sudman, experimental infection
P. zopfii	T-1343	Hindquarter, cow	Hanischen, Munich, Germany
P. zopfii	T-1344	Hindquarter, cow	Hanischen
P. zopfii	T-1341	Lymph node, deer	Frese, Germany

[a] CDC, Center for Disease Control.

TABLE 3. *Staining properties of unadsorbed fluorescein-labeled Prototheca species antiglobulins for the homologous and heterologous Prototheca species in culture*

Organism	CDC[a] identification no.	Staining reactions with fluorescein-labeled globulins against				
		P. filamenta	*P. moriformis*	*P. stagnora*	*P. wickerhamii*	*P. zopfii*
P. filamenta	B-1273[b]	4+	0-1+	0-4+	0-+/-	0-1+
P. moriformis	B-1266[b]	0	2-4+	3+	0-+/-	3-4+
P. stagnora	B-1277[b]	1-2+	2+	4+	0-+/-	2+
P. wickerhamii	B-1280[b]	0	0-4+	0-4+	4+	0-3+
P. wickerhamii	B-1269	0	0-2+	0-4+	4+	0-3+
P. wickerhamii	B-1274	0	0-2+	0-3+	4+	0-2+
P. wickerhamii	B-1275	0	0-2+	0-2+	4+	0-3+
P. wickerhamii	B-1278	0	0-4+	0-4+	4+	0-4+
P. wickerhamii	B-1279	0	0-2+	0-4+	4+	0-3+
P. wickerhamii	B-1281	0	0-3+	0-4+	4+	0-3+
P. wickerhamii	B-1282	0	0-3+	0-4+	4+	0-3+
P. zopfii	B-1270[b]	0-+/-	2+	4+	0-2+	3-4+
P. zopfii	B-1265	0	2-3+	4+	1-2+	3-4+
P. zopfii	B-1267	0-+/-	3-4+	3-4+	3+	3-4+
P. zopfii	B-1268	0	3-4+	4+	1-3+	3-4+
P. zopfii	B-1271	0	2-3+	3-4+	2+	3-4+
P. zopfii	B-1272	0	3+	4+	2-3+	3-4+
P. zopfii	B-1276	0	4+	4+	2+	3-4+
P. zopfii	B-1283	0	2-4+	3-4+	1-2+	3-4+
P. zopfii	B-1284	0	2+	3+	1-2+	3-4+
P. zopfii	B-1285	0	2-3+	3-4+	2-3+	3-4+

[a] CDC, Center for Disease Control.
[b] Immunization strains.

TABLE 4. *Staining properties of unadsorbed fluorescein-labeled Prototheca species antiglobulins for morphologically similar fungi in culture*

Organism	CDC[a] identification no.	Staining reactions with fluorescein-labeled globulins against				
		P. filamenta	*P. moriformis*	*P. stagnora*	*P. wickerhamii*	*P. zopfii*
B. dermatitidis	B-414	0-1+	0-1+	0-4+	0-2+	0-+/-
B. dermatitidis	45-730-71	0-1+	0-+/-	0-3+	0-1+	0-+/-
B. dermatitidis	76-62	0-+/-	0-+/-	0-3+	0-+/-	0
C. neoformans	B-551	0-2+	0-+/-	0-3+	0-+/-	0
C. neoformans	56-295-72	0-2+	0	0-3+	0	0-2+
C. neoformans	56-322-72	0	0	0-1+	0	0
H. capsulatum	B-923	0-+/-	0-+/-	0-+/-	0-+/-	0-+/-
H. capsulatum	K-643	0-4+	0-+/-	0-2+	0-+/-	0-+/-
H. capsulatum	A-827	0-4+	0-2+	0-3+	0-2+	0-+/-
P. brasiliensis	137	0-+/-	0-+/-	0-2+	0-2+	0-1+

[a] CDC, Center for Disease Control.

TABLE 5. *Staining properties of the fluorescein-labeled Prototheca species antiglobulins adsorbed with heterologous Prototheca species for the Prototheca species in culture*

Fluorescein-labeled antiglobulins	Adsorbing antigen[a]	Staining reactions of organisms				
		P. filamenta (1)[b]	*P. moriformis* (1)	*P. stagnora* (1)	*P. wickerhamii* (8)	*P. zopfii* (10)
P. filamenta	None	4+	0-1+	0-3+	0	0-1+
	P. moriformis	3-4+	0	0	0	0
	P. stagnora	3-4+	0	0	0	0
	P. wickerhamii	3-4+	0	0	0	0
	P. zopfii	3-4+	0	0	0	0
P. moriformis	None	0-1+	2-4+	0-2+	0-3+	2-4+
	P. filamenta	0	0-3+	0-4+	0-2+	0-3+
	P. stagnora	0	0-2+	0	0	0-2+
	P. wickerhamii	0	1-3+	0	0	1-3+
	P. zopfii	0	0	0	0	0
P. stagnora	None	2-4+	3-4+	4+	4+	3-4+
	P. filamenta	0	3-4+	4+	3-4+	3-4+
	P. moriformis	0	0	3-4+	0	0
	P. wickerhamii	1-3+	0-2+	1-4+	0	1-3+
	P. zopfii	0	0	3-4+	0	0
P. wickerhamii	None	0	0-2+	0-+/-	4+	1-3+
	P. filamenta	0	2-3+	0-3+	3-4+	2-3+
	P. moriformis	0	0	0-1+	2-4+	0
	P. stagnora	0	0-1+	0	2-4+	0-1+
	P. zopfii	0	0	0-1+	2-4+	0
P. zopfii	None	0-1+	3-4+	0-2+	0-4+	3-4+
	P. filamenta	0	3-4+	3-4+	1-3+	3-4+
	P. moriformis	0	0	0	0	0
	P. stagnora	0	1-2+	0	0	1-2+
	P. wickerhamii	0-1+	1-2+	0-1+	0	1-2+

[a] Immunization strains.
[b] No. of strains used.

adsorbing the antiglobulins to each of these species with cells of either *P. stagnora* or *P. wickerhamii*. However, it was not possible to prepare conjugates for the differentiation of *P. moriformis* from *P. zopfii*. Adsorption of labeled antiglobulins to either of these two organisms with cells of the other species eliminated the staining capacity of the adsorbed reagent for the homologous as well as the heterologous species.

The specific conjugates for the individual *Prototheca* species were tested for cross-reactivity with morphologically similar fungi. None of the reagents cross-stained these organisms except the *P. stagnora* conjugate, which stained *B. brasiliensis*, *C. neoformans*, and *P. brasiliensis*, but only at low levels of intensity.

Staining of organisms in formalin-fixed tissue. The sensitivity and specificity of the unadsorbed and adsorbed conjugates were tested with sections of tissue containing *Prototheca* species and with sections containing morphologically similar fungi. As with the cultures, the unadsorbed conjugates demonstrated a lack of specificity while possessing the desired sensitivity. The adsorbed (monofactor) conjugates possessed the desired sensitivity and specificity and stained the homologous organisms at a level of intensity similar to that in culture (Fig. 2).

The labeled preimmunization globulins did not stain the *Prototheca* species or fungi in the sections of tissue.

Preparation of a screening conjugate for Prototheca species in tissue. An FA reagent that would selectively stain all *Prototheca* species in tissue would be of value for rapidly

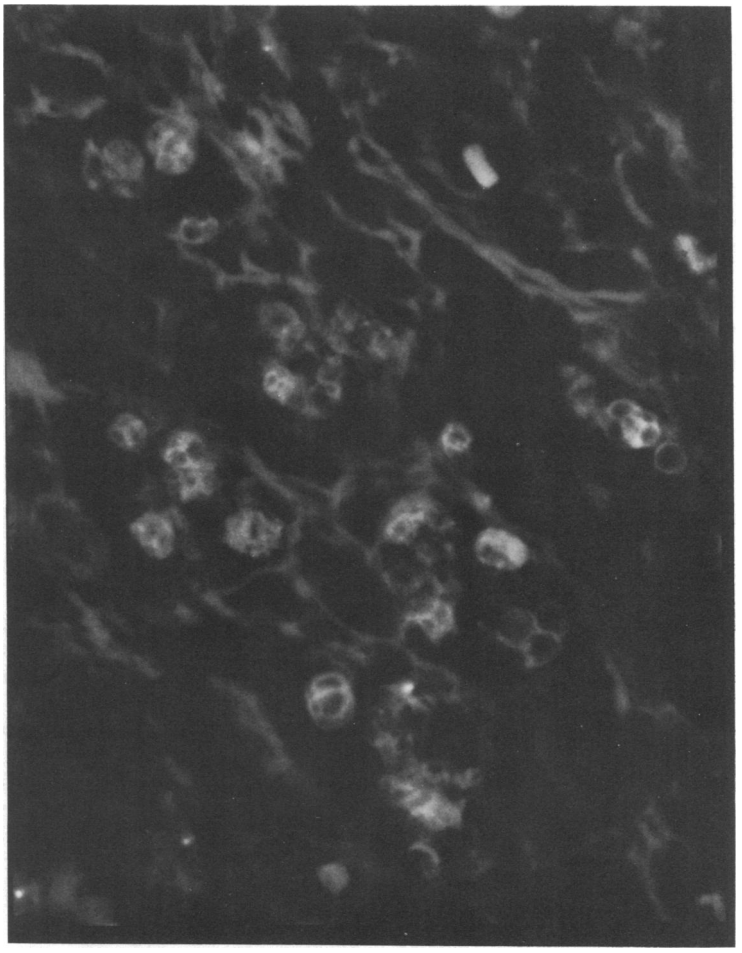

FIG. 2. *P. wickerhamii cells in tissue stained with fluorescein-labeled globulins to P. wickerhamii. Daughter cells are discerned. Magnification at ×500.*

screening specimens for the presence of protothecae. The unadsorbed fluorescein-labeled antiglobulins to *P. stagnora* were selected for use because, in contrast to the other conjugates, they intensely stained all of the *Prototheca* species in cultures and in tissue. This unadsorbed reagent, however, brightly stained tissue-form elements of *C. immitis* and the yeast form of *B. dermatitidis*. Adsorption of this conjugate twice with *C. immitis* mycelial-form cells yielded a reagent that brightly stained all of the *Prototheca* species, except *P. filamenta*, in culture and tissue without staining any of the morphologically similar fungi.

Counterstaining sections of tissue stained by conventional stains. Sections of formalin-fixed tissue containing *Prototheca* species, previously stained by Gridley, Gomori, and hemotoxylin and eosin (H and E) stains, were treated with the unadsorbed and with the adsorbed conjugates. Organisms in sections stained with H and E were stained by the respective conjugates at a level comparable to that in sections not previously stained by conventional methods. Organisms stained with Gomori and Gridley stains could not be counterstained by FA.

Diagnostic application of the adsorbed conjugates. Tissues from seven animal cases of protothecosis diagnosed solely on the basis of histopathology were tested with the screening conjugate and also with the conjugates specific for the *Prototheca* species. Organisms present in tissues from five of the seven cases were brightly stained by the screening conjugate, whereas those in the remaining two failed to stain. Sections of tissue from the latter two cases were stained with Gridley, Gomori, and H and E. After critical examination it was found that although the organisms resembled protothecae, they were not members of this genus.

The staining results with the specific conjugates indicated that in tissues from two of the five protothecosis cases the protothecae were *P. wickerhamii* and in three cases, *P. zopfii*.

Fourteen cultures that were tested for the diagnostic applications of the conjugates had been received with the following designations: five as *Prothotheca* sp., four as *O. moriformis*, one as an achloric *Chlorella*, one as *P. chlorelloides*, one as *P. pastoriensis*, one as *P. trispora*, and one as *P. ubrizsyi*. The five cultures of *Prototheca* sp., the four cultures of *P. moriformis*, and the one achloric *Chlorella* were all specifically stained with the *P. wickerhamii* species-specific conjugate and were morphologically and biochemically compatible with *P. wickerhamii*. *P. chlorelloides*, *P. pastoriensis*, *P. trispora*, and *P. ubrizsyi* were specifically stained with the *P. zopfii* species-specific conjugate and were morphologically and biochemically compatible with *P. zopfii* (Table 6).

DISCUSSION

Until recently, identification of the *Prototheca* species was primarily based upon morphologic criteria. However, such criteria are not a sound basis for separation of species because growth conditions affect cell morphology, and isolates within a species show morphologic variation. As a result, a number of invalid species have been described in the literature. Arnold and Ahearn (2) developed a more objective method for the identification of the *Prototheca* species using carbohydrate and alcohol assimilation tests. These tests permitted a reduction in the number of species to five: *P. filamenta*, *P. moriformis*, *P. stagnora*, *P. wickerhamii*, and *P. zopfii*. *P. ubrizsyi* (22), another species, was not included in their study.

The present investigation has shown that the *Prototheca* species share antigens and also possess distinct antigens. The possession of distinct antigens made it possible to prepare species-specific conjugates by selective adsorption. These reagents can be used to differentiate the *Prototheca* species.

The results of the reciprocal adsorption show that *P. moriformis* and *P. zopfii* are very closely related, if not antigenically identical. Our failure to differentiate the two species with FA reagents and the fact that only a single isolate of the little known *P. moriformis* was available made us question the validity of considering *P. moriformis* a distinct species.

It should be pointed out that the two organisms differ in some respects. The single isolate of *P. moriformis* did not grow at 37 C, had smaller, more elliptical cells, and inconsistently assimilated trehalose. On the other hand, the *P. zopfii* isolates grew at 37 C, appeared to have larger, more spherical cells, assimilated propanol, and did not assimilate trehalose. Whether these differences justify placing the organisms into separate species is debatable. We do not believe they do, and suggest that *P. moriformis* and *P. zopfii* be considered conspecific. Based upon the fact that *P. zopfii* is encountered more frequently in the literature and is, therefore, better known, we feel that *P. zopfii* should be retained as the valid species designation, although both were described in the same paper (12).

It is of interest to note that the cultures designated *P. chloroelloides*, *P. pastoriensis*, *P. trispora*, and *P. ubrizsyi* were stained by the

TABLE 6. *Staining properties of the broad-screening and monofactor Prototheca species conjugates in diagnostic staining of Prototheca species in cultures and formalin-fixed tissues*

Organism (original identification)	Broad-screening conjugate	Staining reactions with			
		P. filamenta conjugate	*P. stagnora* conjugate	*P. wickerhamii* conjugate	*P. zopfii* conjugate
Chlorella sp. (achloric) B-1419[a]	+	−	−	+	−
P. chlorelloides B-1420	+	−	−	−	+
p. moriformis B-1414	+	−	−	+	−
P. moriformis B-1444	+	−	−	+	−
P. moriformis B-1445	+	−	−	+	−
P. moriformis B-1446	+	−	−	+	−
P. pastoriensis B-1421	+	−	−	−	+
Prototheca sp. B-1415	+	−	−	+	−
Prototheca sp. B-1416	+	−	−	+	−
Prototheca sp. B-1417	+	−	−	+	−
Prototheca sp. B-1418	+	−	−	+	−
Prototheca sp.	+	−	−	+	−
P. trispora B-1413	+	−	−	−	+
P. ubrizsyi B-1412	+	−	−	−	+
Prototheca sp. T-1049[b]	+	−	−	−	+
Prototheca sp. T-1187	+	−	−	−	+
Prototheca sp. T-1316	+	−	−	−	+
Prototheca sp. T-1341	+	−	−	+	−
Prototheca sp. T-1349	+[c]	−	−	+	−
Prototheca sp. T-1353	−[d]	−	−	−	−
Prototheca sp. T-1354	−	−	−	−	−

[a] B numbers, cultures.
[b] T numbers, tissues.
[c] Positive staining.
[d] Absence of staining.

conjugate specific for *P. zopfii* (Table 6) and were also found to be biochemically and morphologically compatible with *P. zopfii*. Based upon these observations, we believe these species to be conspecific with *P. zopfii*, and suggest that they be considered synonyms with *P. zopfii*.

On the basis of this study and the findings of Arnold and Ahearn (2), the following species are considered valid: *P. filamenta* (Arnold and Ahearn, 1972), *P. stagnora* (Cooke, 1968a), *P. wickerhamii* (Tubaki and Soneda, 1959), and *P. zopfii* (Kruger, 1894). Synonymous species for *P. zopfii* are: *P. chlorelloides*, *P. ciferrii* (Negroni and Blaisten, 1941), *P. moriformis* (Kruger, 1894), *P. pastoriensis* (Ashford et al, 1930), *P. pastoriensis* var. *trispora* (Ashford et al, 1930), *P. segbwema* (Davies et al., 1964), and *P. ubrizsyi* (Zsolt and Novak, 1968).

ACKNOWLEDGMENTS

We thank D. G. Ahearn of Georgia State University for his advice throughout this investigation and Nell Sharman for her assistance in the preparation of this manuscript.

A special thanks is extended to Brenda Tuggle for her patience and assistance in the preparation of the dissertation.

Training was provided by the laboratory Practice Training Program which is supported by a training grant (1 DO4 AH 01126) from the Bureau of Health Manpower Education, National Institutes of Health, and by a Public Health Service research program project grant (CC 00606) from the National Communicable Disease Center. The laboratory research was performed in the Laboratory Division, Center for Disease Control, Atlanta, Ga., under the supervision of the junior author.

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