

ISOLATION OF HUMAN FUNGI FROM SOIL AND IDENTIFICATION OF TWO ENDEMIC AREAS OF *Cryptococcus neoformans* AND *Coccidioides immitis*.

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SUMMARY

The present study was carried out in two different areas of Province of Cordoba, Argentina, where there was a suspicion of endemic mycosis. The previous data were the presence of a clinical case of pulmonary cryptococcosis in one area (Alta Gracia) and the previous findings of a high incidence of coccidioidin and cryptococcin reactors in the population of the second one (Villa Dolores). In both areas soil samples for fungi were studied and *Cryptococcus neoformans* was found in 2/25 samples from Alta Gracia. In Villa Dolores *Coccidioides immitis* was isolated in 2/40 samples, and *C. neoformans* in 1/40 samples. Delayed hypersensitivity test with cryptococcin was determined in the population from Alta Gracia and it was found to be 5.3%. Positive cutaneous tests with coccidioidin (33.8%) and cryptococcin (31.9%) in Villa Dolores were obtained. With these findings two endemic areas of systemic mycoses in Cordoba, Argentina were delimited.

KEY WORDS: Endemic areas; Human pathogenic fungi; Epidemiology.

INTRODUCTION

Coccidioidomycosis is a mycotic disease caused by a dimorphic fungus, *Coccidioides immitis*. Human infection occurs by inhalation of arthroconidia from the mycelial phase. It has been demonstrated that 60% of the infections are asymptomatic, and they are detected by cutaneous tests of delayed hypersensitivity to coccidioidin. This test is used to quantify the infected people and it helps to determine the endemic area³.

Coccidioidomycosis has a well defined geographic distribution, related to the presence of the fungus of the soil⁷. The fungus has been isolated from soil samples in several areas in the American Continent.

The epidemiology of coccidioidomycosis in Argentina was studied in detail by NEGRONI et al¹³, who made the first investigation in 1966.

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The saprophytic phase of this fungus lives in sandy or clayey soils in certain arid areas with annual rainfall not over 700 — 900 mm; in these regions the wet season is followed by a dry and dusty one with dust storms which disseminate propagules to great distances.

C. neoformans is the etiologic agent of cryptococcosis, a systemic mycosis of universal distribution.

The fungus was isolated from the soil for the first time by EMMONS in 1951⁴. Later on, it was found in pigeons' excrements and soil samples^{2, 5, 8}. The frequency of isolation in the samples oscilated between 41/90 and 72/201. These findings lead to speculate that *C. neoformans* is ubiquitous in nature and may be easily isolated. However, it was found in 1974 by FELTON et al.⁶ in only 4/538 soil samples collected at random in Oklahoma, USA. In samples taken from sites near the houses of patients infected with cryptococcosis, the fungus was isolated in 25/270 samples. The differences in these figures would suggest that distribution in soil is not uniform⁶.

The absence of a specific and sensitive antigen in addition to the particular host parasite relation that induces the excess of *C. neoformans* capsular antigen producing a state of immunotolerance would explain the delay suffered by conductive studies to determinate cryptococcosis infections.

The first epidemiologic survey was made by MUCHMORE et al¹² in Oklahoma in 1968, who determined the infection index of 32%. Later on in São Paulo, Brasil, LACAZ et al.⁹ obtained 4.3% of positivity. In Argentina the first survey made on individuals in the Province of Cordoba revealed a 14% infection index¹¹.

This paper was based on two former experiences; the study of a case of pulmonary cryptococcosis in a man from Alta Gracia; and the high *Coccidioides* infection index found in Traslasierra (Villa Dolores) in a previous survey. The purposes of this work were to isolate *C. immitis* and *C. neoformans* from soil samples from the places in study and to determine the *Cryptococcus* infection index in the people who live in Alta Gracia (Cordoba).

MATERIALS AND METHODS

Phytogeographic characteristics of the studied areas

The Province of Cordoba is situated in the middle of the Argentina. The region of Traslasierra at which Villa Dolores city belongs is located in the West of the Province of Cordoba, limited at the East by hills with a maximum height of 2.884 m. Alta Gracia city is situated in the centre of Cordoba. Both areas are characterized by low hills and plains with predominantly summer rainfall reaching 600 to 800 mm annually. The mean annual temperature is 18°C with maximum mean temperature of 24-25°C and minimum mean temperature of 10-11°C. The autochthonous vegetation is a xerophilous forest with trees like carob tree quebracho; there are many birds in all the region with predominance of wild pigeons and poultry (Fig. 1).

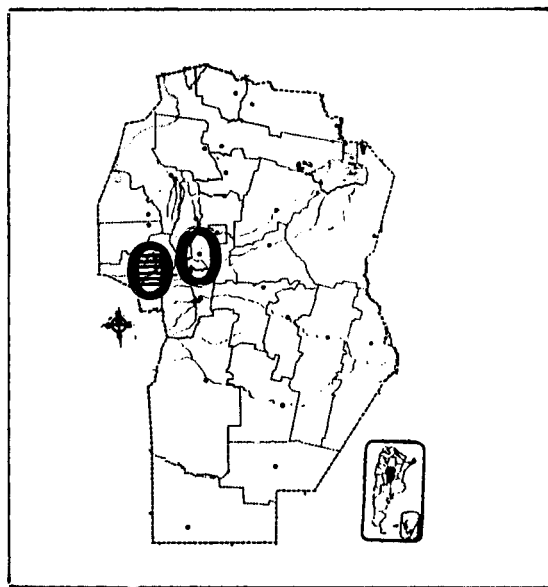


Fig. 1 — Areas under investigation.
▨ Villa Dolores (Traslasierra).
○ Alta Gracia.

Isolation of the fungus from soil

Soil sampling (houses, squares and streets) and sampling from poultry yards and plucking

establishment covered 4 zones of the city of Villa Dolores which represent a homogeneous distribution of its territory. Ten samples collected in each zone were inoculated intraperitoneally into 4 mice.

Isolation of *Cryptococcus neoformans*

Twenty five soil samples mixed with pigeon excrements and vegetal matter collected in different places of the town were studied. Open areas and surrounding buildings were the places chosen for sampling. The collection was made with sterile discarded spatulas. The samples were kept in sterile disposable containers at room temperature until used.

All the samples were processed by duplicated following SHIELDS and AJELLO's method¹⁴ with modifications proposed by CIVILA & CONTI-DIAZ². The material was spread on Petri dish plaques containing the culture media with the following composition: glucose 10 g, creatinine 780 mg, chloramphenicol 50 mg, sunflower seed extract 350 ml, tap water 650 ml, agar 18 g. The preparation of the sunflower seed extract was the following: 70 g of pulverized sunflower seeds are suspended in 350 ml of distillate water. It is autoclaved at 1.5 atm during 10 minutes and it is filtered through gauze. This media pigments colonies of *Cryptococcus neoformans* in a dark chestnutbrown color, making it possible to recognize them macroscopically. The cultures were incubated at 37°C and observed during 10 days.

Test of urease, development in Sabouraud media at 37°C and 21 days old mice intracranial inoculation were carried out in order to corroborate the identity of the pigmented strains developed in the used media.

Isolation of *Coccidioides immitis*

Forty soil samples were taken at Villa Dolores city. Each sample material (10-20 g), collected after discarding the superficial layer, with sterile wooden spatulas were put in sterile containers. Then it was suspended in 50 ml of sterile saline solution and chloramphenicol 300 µg/ml with glass beads. Each sample was shaken vigorously and let it rest for 2 hours, it was taken 0.5 ml

of the middle layer of supernatant and inoculated intraperitoneally in 4 mice. The animals were killed 45 days after inoculation. Lung, liver, spleen and brain homogenates were cultured on Sabouraud-dextrose media and incubated at 37°C during 30 days. The identification of the isolated strains involved the demonstration of the micelial phase *in vitro* and its conversion to parasitic form by intratesticular inoculation of guinea pigs.

Antigen

The cryptococcin used for the delayed type hypersensitivity test was the supernatant of mechanically disrupted cells, prepared from a non-decapsulated *C. neoformans* strain with a proteic concentration of 1.0 mg/ml and with a reaction of latex particle agglutination for polysaccharide antigen until the 1/10.000 dilution¹⁰.

The coccidioidin was elaborated by Professor Ricardo Negroni in the Centro de Micología de la Universidad Nacional de Buenos Aires.

People studied

Tests of delayed, hypersensitivity for coccidioidin and cryptococcin were made in people from Villa Dolores and Alta Gracia. People of both sexes with ages between 16 and 60 without apparent immunologic compromise, not carriers of diabetes or neoplasias and with no corticoid or cytostatic treatment were selected. As far as possible, people with positive reactions to tuberculin were chosen. Double radial immunodiffusion reaction with coccidioidin was made thorax X-Rays were performed on people with positive skin test.

Cutaneous test

A conventional technique was applied, 0.1 ml of antigen on the anterior side of the forearm; reactions were read between 24 and 72 h, indurations over 8 mm were considered positive.

Clinical case

Patient O. F. Male, 53 years old, was born in Alta Gracia (Cordoba) where he lives present-

tly; occupation: broker. He started 2 months before the first appointment with pains in the anterior and side region of left hemithorax, without cough, expectoration, dyspnea, fever, anorexia or weight loss. A thorax X-Ray showed a dense homogeneous, round shaped imagen of tumoral appearance with sharp edges and a diameter of approximately 8 cm in mid peripheric location in the left hemithorax (Figs. 2-3).

The possibility of neoplasia was ruled out by bronchofibroscopy: the biopsy revealed elements compatible with mycosis. The diagnosis could not be confirmed due to the limited size of the sample. Fifteen days later a palpable tumor became evident in the left hemithorax which coincided in the localization with the X-Ray image. Due to this fact an exploratory thoracotomy was made and upon exposing the ribs it was possible to observe in the 5th intercostal espace the protrusion of a gelatinous tumoral mass which had destroyed the 4th rib. Several biopsic samples were taken and sent to the mycologic examination. The mycology laboratory reported the presence of a large number of yeast cells with colorless capsule when examined di-

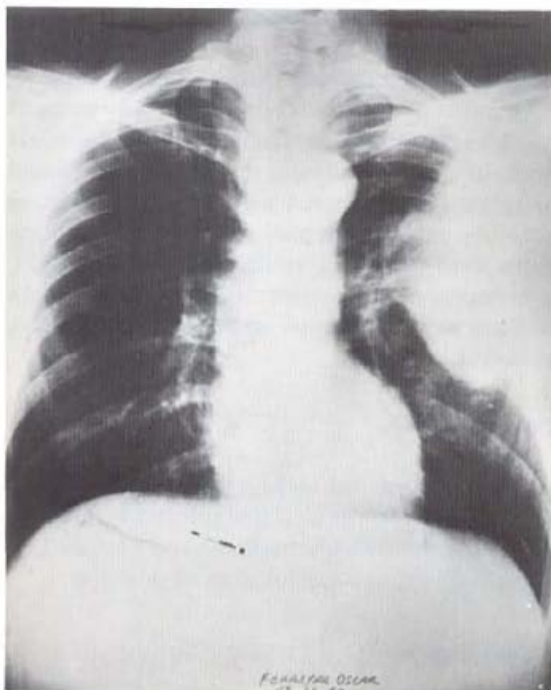


Fig. 2 — Pulmonary cryptococcosis. Chest roentgenogram showing large mass in the left upper lobe.

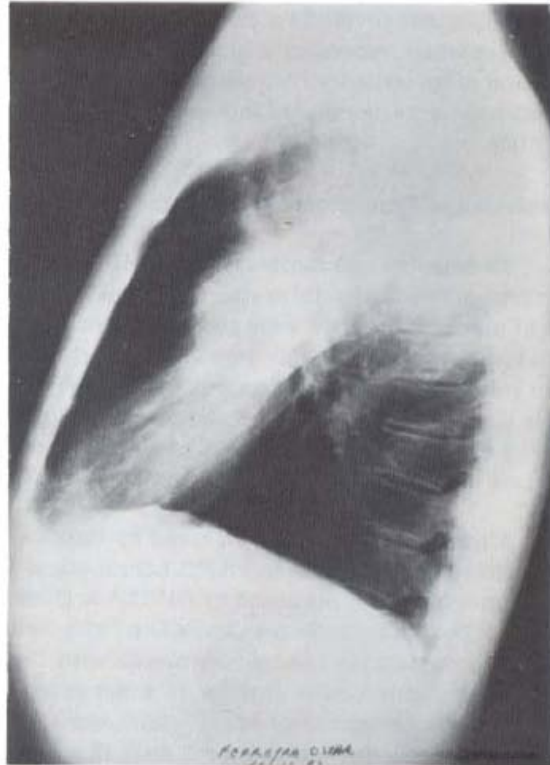


Fig. 3 — Pulmonary cryptococcosis. Lateral view showing the same mass in a posterior location.

rectly with Indian ink. *C. neoformans* was identified in cultures. This diagnosis was later confirmed by the pathologic examination of the lung biopsy. Counterimmunoelectrophoresis (with cryptococcin) was performed to evaluate antibodies. The results were negative.

RESULTS

Isolation of fungus from soil

Of all inoculated animals with soil samples of Villa Dolores one died spontaneously after 7 days. The cultures from its organs were negative. Fifteen days after the inoculation, 2 animals inoculated with a sample obtained from the same poultry yard died spontaneously. It was possible to isolate *C. neoformans* from its organs. The remaining animals were sacrificed 45 days after the inoculation. The presence of *C. immitis* was determined in 2 animals inoculated with different soil samples from the same neighbourhood,

both presented microscopic lesions in the liver. The typical spherules were observed in direct examinations. The cultures were positive for both animals.

C. neoformans was isolated in 2 of 25 samples studied from Alta Gracia. Macroscopically, these strains presented a creamy appearance and dark brown pigmentation on both sides of the colony, the color was intensified at room temperature after 4 days of incubation at 37°C. The urease test was positive.

Inoculated mice had to be killed between 20 and 30 days, with no macroscopic lesions in the brain. Due to this fact, this organ was spread on Sabouraud media and was inoculated again in mice. *C. neoformans* developed in all cultures. In some cases it was necessary to perform up to 3 passages through mice in order to obtain the encapsulated cell.

Cutaneous test

Delayed hypersensitivity tests were performed on 65 inhabitants of Villa Dolores, 22 of them were positive to coccidioidin (33.8%) and 22 of 69 tested with cryptococcin were also positive (31.9%). In Alta Gracia city 1/19 positive cutaneous test with cryptococcin was obtained (5.3%), (Table 1).

TABLE 1
C. neoformans and *C. immitis* infection index and isolation in the areas under investigation.

Area	Positive skin test (%)		Isolation of fungus from soil	
	cryptococcin	coccidioidin	<i>C. neoformans</i>	<i>C. immitis</i>
Villa Dolores	31.9	33.8	1/40	2/40
Alta Gracia	5.3	—	2/25	0/40

DISCUSSION

In this paper we were able to identify two endemic areas of systemic mycosis in Cordoba, Argentina. (Fig. 1).

In Alta Gracia three requirements, for the identification of endemic areas have been fulfilled. The first one was the clinical case, characterized by a pulmonary infection in a patient without immunologic deficit. The second condition was the cryptococcin infection index determined in a low percentage of population (5.3%). The third requirement was extensively met, the samples of soil fertilized with pigeons excreta obtained in the city allowed the isolation of *C. neoformans* in two of twenty five samples.

In the previous population survey of Villa Dolores intradermal reactions with coccidioidin and cryptococcin have revealed the existence of a great number of individuals with positive reaction 33.8% and 31.9% respectively.

C. immitis was isolated from soil samples in a 2/40 ratio. Besides it was possible to isolate *C. neoformans* in 1/40 samples. For the first time it was isolated *C. neoformans* from Argentina and *C. immitis* from soil in the province of Cordoba (Villa Dolores).

It is important to remark that the isolation of *C. neoformans* was made from soil fertilized with poultry excrement and not from pigeon excrement as advised by the literature^{2, 5, 8}. Mycotic infections caused by *C. immitis* or *C. neoformans* have not been known in Villa Dolores because there are no specialized laboratories of Mycology in this town.

RESUMEN

Aislamiento de hongos patogenos de suelo. Identificación de areas endemicas de *Cryptococcus neoformans* y *Coccidioides immitis*.

Se presenta un estudio de endemia de micosis causadas por hongos patogenos primarios en dos zonas de la Provincia de Cordoba, Argentina, donde previamente se diagnostico un caso clinico de criptococosis pulmonar en una de las areas (Alta Gracia) y se encontraron altos indices de infección para *C. immitis* y *C. neoformans* en la población de la segunda zona (Villa Dolores).

En ambas regiones se encontraron hongos patogenos en suelos; en Villa Dolores se pudo aislar *C. immitis* en 2/40 muestras de tierra y en 1/40 muestras *C. neoformans*. Mientras que

en la Ciudad de Alta Gracia se pudo aislar *C. neoformans* en 2/25 muestras de tierra. El índice de infección de la población con coccidioidina, en Villa Dolores, fue de 33.8% y con criptococcina de 31.9%. También se determinó el índice de infección de la población con criptococcina en Alta Gracia, el cual fue del 5.3%.

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