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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland
EPIDEMIOLOGY AND LABORATORY DIAGNOSIS
OF DOMESTIC PULMONARY MYCOSES

(Aspergillosis, Mucormycosis,
Cryptococcosis and Candidiasis)

Praxis
(Practice)
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Pages 1118-1124

ABSTRACT

Review of the epidemiology and laboratory diagnosis of the four bronchopulmonary mycoses which occur with appreciable frequency in Switzerland. Emphasis is placed on the importance of microscopic examination of fresh material which should be performed in the clinical laboratory. Coordination of the efforts of clinicians, serologists and mycologists is recommended in order to improve the diagnosis of bronchopulmonary candidiasis. The report lists the sources of available skin-test antigens and the laboratories accepting sera or serological tests, including those for the more important extra-European pulmonary mycoses.

Habitat of Pathogens and Epidemiology

None of the pulmonary mycoses can be regarded as contagious. Except under very extreme conditions, transmission from man to man or from animal to man does not occur. With the exception of candidiasis, infection arises from exogenous sources.

Aspergillus fumigatus is one of the most common lab moulds. The very light spores, highly resistant to dehydration, are found everywhere in the air, even in large cities, with seasonal peaks in late fall and winter (Ref. 27). The fungus itself vegetates by preferences on straw or hay and other slowly decomposing vegetative material. Highly resistant to heat, it is not affected but rather favored by the so-called spontaneous
heating of such material (Ref. 18). The air in barns, grain silos, etc., is frequently amply charged with spores. Although bronchopulmonary infection of man is due to inhalation of the spores, the risk of infection is relatively low even under intense exposure. A certain occupational disposition of farmers, silo workers and others for primary pulmonary aspergillosis is unmistakable and should be taken into account in relation to Workman’s Compensation (Ref. 9). However, this is much less frequent than might be expected. If sensitivity exists, the relatively less exposed urban dweller appears to be infected as easily as the rural dweller. In other words, reduced local or general resistance of the host -- e.g., pulmonary tuberculosis, leukemia, corticosteroid treatment -- is the decisive factor in many cases. Such pulmonary aspergillosis should be considered as secondary.

A limited occupational disposition should also be considered for some non-agricultural occupations such as workers in flour mills (Ref. 28) and furriers (Refs. 22, 41). Pulmonary aspergillosis of pigeon breeders and wig makers in Paris (Ref. 32) now appears to be merely of historic interest.

Aspergillus flowers, A. niger, A. nidulans and some other species of the family aspergillus which -- much less frequently than A. fumigatus -- may cause pulmonary mycosis, have similar habitats and basically the same epidemiology.

Pathogens of the mucormycosis are thermophile or thermotolerant club moulds (Mucoraceae) from the families Absidia, Rhizopus and Mucor (important pathogenically: Absidia corymbifera, Rhizopus oryzae, Mucor pusillus). Their distribution is only less wide than that of the Aspergillus and the habitat is the same, i.e., straw and hay (particularly under spontaneous heating) but in addition also humus and leaf mould (Ref. 18). A "popular" substrate is spoiled bread. The spores have frequently been demonstrated in the air and are probably ubiquitous. Pulmonary mucormycosis is acquired by inhalation of such spores.

However, in the classical sense, it is not possible to speak of an epidemiology of the mucormycoses. Sensitivity and/or reduced resistance of the host is so obviously decisive for infection that the factor of exposure can be disregarded. The most frequent predisposition for pulmonary mucormycosis are malign diseases of the hematopoietic system and decompensated diabetes mellitus (Refs. 3, 20, 5).

The exclusive pathogen in cryptococcosis is Cryptococcus neoformans, an anaseeeporogenic yeast, which forms a polysaccharide capsule under many environmental conditions -- also in tissues. The path of entry of the pathogen is aerogenic-pulmonary in most and probably practically all such diseases (Ref. 25).
At some given time, the lungs may be the only organ affected but hematogenic transmission to the meninges and brain generally occurs sooner or later and there are cases of cryptococcosis meningeal encephalitis where the primary focus is no longer demonstrable (Ref. 40).

The data on epidemiology still have many gaps although important findings have been gathered in recent years. The pathogen has repeatedly been demonstrated from soil, root juice, milk of cows with manifest or latent cryptococcal mastitis, and, with particular constancy, from the hardened droppings of such birds as starlings, canaries and predominantly pigeons (Ref. 15, 16, 36, 43). However, the birds themselves are not the carriers of cryptococcosis but their droppings form an excellent culture medium which is subsequently colonized by Cryptococcus neoformans. In pigeon droppings from a sidewalk in Washington, D.C., Ref. 18 counted fifty million propagating cells per gram. The yeasts were also found at a high density in histological sections of the droppings (Ref. 18). In the dry state, such yeasts exhibit thermotolerance and longevity unusually high for vegetative germs (Ref. 44).

By reason of the frequent demonstration in pigeon droppings, the New York daily press recently demanded destruction of the pigeons in the city. Epidemiologically, such a step cannot yet be justified (Ref. 33). In cities with many pigeons, there are on the average no more cases of cryptococcosis than in cities with few pigeons. It was possible only very infrequently to establish a credible correlation between particularly high exposure and the illness (a case with almost confirmed source of infection (pigeon droppings) was communicated shortly before printing of this article (Ref. 31). According to the present state of our knowledge, cryptococcosis involves a sporadic infrequent illness almost always fatal without therapy and, in roughly one-third of the cases, there exist predisposing disorders, generally malign lymphomatoses or reticuloses such as Hodgkin's disease.

By epidemiologically, a relatively frequent occurrence of benign and clinically neutral pulmonary cryptococcoses may be suspected (Ref. 17). Individual host factors probably decide whether this turns into the progressing disease which is today the only one known as cryptococcosis. For possible support of this hypothesis, more attention should be given to the possibility of cryptococcosis in illnesses involving contact with pigeons.

As pathogen of pulmonary candidiasis, I here consider only Candida albicans which is probably responsible for more than ninety percent of the cases, without disregarding that some other species such as C. tropicalis, C. parapsilosis and C. pseudotropicalis may be pathogenic. C. albicans, an anaerobicogenic yeast forming threads (pseudomycelium and mycelium) in a suitable milieu inclusive of tissues, is a saprophyte.
and opportunistic parasite on the mucosae of many warmblooded organisms (Ref. 2). Within our present knowledge, there is no appreciable pathogen reservoir in nature. Depending on the author and method, *C. albicans* can be demonstrated by culture from the buccal cavity or the feces in twenty to sixty percent of healthy individuals (Ref. 5) and not infrequently also from a barely inflamed vaginal mucosae particularly during pregnancy (Ref. 12). It is probable that infection of the newborn child frequently takes place during the act of birth. If subsequently a buccal thrush or even a pulmonary candidiasis develops, we might speak of infection. However, in probably all other cases, candidiasis is caused by strains which have long been resident as saprophytes in the patient.

The number of the yeasts demonstrable by culture from healthy intestinal mucosae is always relatively low. An illness is characterized by the coincidence of the following two conditions: (a) high increase of the number of yeasts; (b) destructive invasion of the mucosae with the formation of (pseudo-) mycelium (Ref. 21). Without doubt, the first condition may occur under treatment with antibiotics which interfere with the ecolagical equilibrium in favor of the yeasts. Sufficient data are lacking which would indicate that the antibiotics can produce the second condition. However, the causative factor of the invasion does not lie in the pathogen -- whose virulence does not change as shown by animal experimentation -- but in the host. The resistance-reducing effect of corticosteroid treatment has probably been sufficiently demonstrated. Due to the interference with cellular defense, leukemia and other malign hemopathies are a further important predisposition. The significance of the absence of humoral resistance factors has not yet been clarified (Refs. 21, 26).

In the practically sterile bronchial tree of the healthy individual, yeasts evidently do not exist. Their entry takes place as a rule candidularly from the pharinx although a manifest pharyngeal thrush does not need to exist. The pathogenesis of bronchial and pulmonary candidiasis has some features in common with that of the intestinal thrush but is more complicated and still insufficiently known. Since a "habitat" flora is absent, ecological arguments are only conditionally applicable and, in addition to the general resistance-reducing factors (e.g., leukemia), the local (bronchial and pulmonary) factors are predominant. Of possible significance is disturbance of the ciliary current. Primary pulmonary candidiasis without predisposition is probably infrequent.

I am disregarding pulmonary mycosia which occurs only rarely in Central Europe (e.g., geotrichosis, alleosporiosis) or which are endemic on other continents and are observed by us only in immigrants and returning emigrants. I shall only point out that a suspected histoplasmosis was demonstrated in a badger near Bern some years ago (Ref. 6) so that the occurrence of autochthonous histoplasmosis in man is possible. Confirmed autochthonous cases in man have become known from upper Italy.
Histoplasma capsulatum inhabits the soil and the path of infection for man and animal is a zoonotic (H. capsulatum was isolated from the soil in the vicinity of Bologna very recently Ref. 12).

**Laboratory Diagnosis**

Culture and identification of the pathogens of pulmonary mycosis presents little difficulty in the mycological laboratory. Due to the relatively slow rate of growth of many fungi, the result takes longer than customary in bacteriological diagnosis. Not only to save time but for much more important reasons to which I shall return, primary cultures for fungi should be started in the clinical laboratory. For most purposes, slant-agar tubes with Sabouraud agar incubated at room temperature and the same tubes with brain-heart blood agar incubated at 37°C (Table 1) are satisfactory. It is recommended to add antibacterial antibiotics (100 units penicillin and 200 μg streptomycin per ml or 50 μg chloramphenicol per ml of nutrient medium). However, we must urgently warn against the addition of actidione or the use of commercially available nutrient media for fungi which contain this antibiotic, toxic to Cryptococcus neoformans, Aspergillus and Mucoraceae. Tubes exhibiting fungoid growth can be sent for further investigation to the mycological laboratory.

**Table 1**

**Simple Culture Media for Isolating Pathogens of Pulmonary Mycosis**

(Slant-agar tubes; distribute sputum specimen to optimum number of tubes; eventually homogenize specimen with 1% pancreatin from Fluka AG, Buchs SG)

<table>
<thead>
<tr>
<th>Sabouraud glucose agar (room temperature)</th>
<th>Brain-heart blood agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Agar</td>
<td>25.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0</td>
</tr>
</tbody>
</table>

Addition of either 100 units penicillin and 200 μg streptomycin/ml (prior to solidification) or of 50 μg chloramphenicol/ml (can be set in autoclave). No actidione! (Do not use Mycobiotic Agar from Difco and Mycosel from BBL). If not only fungi but also Nocardia species among the bacteria are to be detected, penicillin, streptomycin and chloramphenicol must not be employed. Cultivation of Actinomyces israelii (also a bacterium) presupposes anaerobic conditions.

-5-
Although reliable diagnosis of a fungoid disease requires identification through culture of the pathogen, the value of such identification should not be overestimated. Successful culture and accurate determination of a possible pathogenic mycosis in many cases proves nothing. This holds true particularly for candidiasis since its pathogens so frequently exist already in the healthy individual. For aspergillosis and mucormycosis, demonstration by culture of the pathogen in the sputum may be difficult or fail for anatomic and other reasons, even with the existence of the disease. On the other hand, we must take into account the possibility that the ubiquitous spores of these moulds have reached the mucosae without producing infection, or that the specimen material has become contaminated through the aerogenic path after having been procured. In all these cases, it must be demonstrated that the fungi have entered the tissue and have here reacted in vital reactions which is obviously easy with specimens from autopsy, surgery and biopsy. In the histological preparation, the fungi cannot only be easily seen with appropriate staining but can generally also be identified by species of family on the basis of morphology and behavior. The task of the investigator then lies in correlating the finding from the culture with a corresponding morphological finding. In any case of doubt, the latter is the better proof. If no histological examination is possible -- which is the rule in the clinic -- the former can be replaced to a certain extent by the so-called direct-microscopic examination of the smear or macerated preparation. The latter should definitely be started and evaluated in the clinical laboratory but with fresh material (cf. Table 2 - Morphological Diagnosis of Aspergillus, Mucoraceae and Candida in the Tissues; Ref. 20).

Further important indices for analysis between host and parasite can be furnished by serological examination (Ref. 35). Unfortunately, no such well standardized serological methods as for coccidioidomycosis and histoplasmosis are available for domestic pulmonary mycosis. Evidently, clinical and roentgenological findings must also be considered.

Animal experimentation is of practically no value for diagnosis of the mycoses discussed here, either for isolation of the pathogens nor for the determination of their pathogenic significance. Pathogenicity for certain laboratory animals is a species characteristic of these fungi which is found both in strains cultivated from non-living substrates and in strains having caused fatal mycosis. Animal experimentation consequently does not permit any decision whether strains isolated from pathological material were, in the respective cases, pathogenic, harmless incidental germs or "contaminants." Only a few more remarks are necessary on laboratory diagnosis of the individual diseases.
Table 2

Direct-Microscopic Demonstration of Pulmonary Mycosis

Very simple method:

KOH (NaOH) 10% or 20% (heat material in lye between slide carrier and cover slide, preferably leave to stand in moist chamber).

Gram

Loeffler's methylene blue

Relatively simple:

FILA from Hotchkiss and McManus (Schiff's reagent obtainable from Merck, Darmstadt)

Best but relatively complicated:

Gomori's methenamine silver.

Cover all preparations and examine with medium to high drying system.

Criteria for diagnosis of bronchopulmonary aspergillosis have been quite clearly defined by French (Refs. 11, 30, 38) and English authors (Refs. 8, 22, 29). As shown in Table 3 prepared by us on the basis of the exemplary work of Ref. 8, two laboratory findings are cumulatively positive in bronchopulmonary "aspergillom": A. fumigatus in the sputum culture and the precipitin test with serum. Diagnosis of allergic bronchial aspergillosis is supported by the coincidence of positive sputum culture, positive skin reaction to scarification with an extract of A. fumigatus, volatile lung infiltrates, and eosinophilia of blood and sputum. It should also be noted that a positive sputum culture alone does not permit assumption of aspergillosis and, inversely, that the sputum culture may be negative when the disease exists, e.g., if an "aspergillom" has lost the open connection with a bronchus or if it has died off. In regard to the two additional forms of bronchopulmonary aspergillosis not included in Ref. 8, non-allergic bronchial aspergillosis and pulmonary aspergillosis (classification from Refs. 23, 39, 47), there are as yet no proved serological methods but a positive precipitin test may be expected in active and recent cases (Ref. 8) (Precipitin Test and Manufacture of the Skin-Test Antigen, cf. Table 4).

The direct-microscopic demonstration of Aspergillus elements in the sputum is even less frequently successful than by culture. Inversely, a positive direct preparation may be regarded as pathognomonic (Fig. 1).
Table 3


<table>
<thead>
<tr>
<th>(b) Schlussdiagnose (mit Patientenanzahl)</th>
<th>(c) positiver Sputumkultur*</th>
<th>(d) positiver Precipitin Test*</th>
<th>(e) Hauttest auf Aspergillen (hauttyp)**</th>
<th>(f) Lungentumoren</th>
<th>(g) Blutfilm erythro- &gt; 10 faden</th>
<th>(h) Sputum- Eosinophilie &gt; 10 faden Lkr</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Aspergillose (27)</td>
<td>83</td>
<td>97</td>
<td>24</td>
<td>4</td>
<td>42</td>
<td>17</td>
</tr>
<tr>
<td>(k) Sicher allergische Aspergillose (87)</td>
<td>98</td>
<td>76</td>
<td>29</td>
<td>4</td>
<td>20</td>
<td>88</td>
</tr>
<tr>
<td>(l) Frische allergische Aspergillose (27)</td>
<td>78</td>
<td>52</td>
<td>77</td>
<td>48</td>
<td>75</td>
<td>70</td>
</tr>
<tr>
<td>(m) Keine Aspergillose, aber Sputumkultur neg. Aspergillose (121)</td>
<td>100</td>
<td>9</td>
<td>90</td>
<td>14</td>
<td>52</td>
<td>65</td>
</tr>
<tr>
<td>(n) Keine Aspergillose, Sputumkultur neg. aber Precipitin Test neg. (15)</td>
<td>0</td>
<td>100</td>
<td>20</td>
<td>0</td>
<td>57</td>
<td>60</td>
</tr>
</tbody>
</table>

(o) * A. fumigatus  ** A. fumigatus-Antigene (p)
(q) Bei 210 (77%) der untersuchten Patienten bestand Asthma bronchiale

Key:
a - Percentage of patients with
b - Final diagnosis (number of patients)
c - Positive sputum culture
d - Positive precipitin test
e - Positive skin test (immediate type)**
f - Volatile Lung infiltrate
g - Blood eosinophilia
h - Sputum eosinophilia
i - Aspergillose (27)
j - Confirmed allergic aspergillosis (87)

1 - Doubtful allergic aspergillosis (27)
m - No aspergillosis but positive sputum culture (121)
n - No aspergillosis, sputum culture negative, precipitin test positive (25)
o - *A. fumigatus
p - ** A. fumigatus antigens
q - 210 (77%) of the patients suffered bronchial asthma
Table 4

Serological and Allergological Tests for Bronchopulmonary Aspergillosis

Agar-gel precipitin test: ship patients' sera to Dr. E. Drouhet, Service de Mycologie, Institut Pasteur, Paris

Or to Dr. J. Pepys, Institute of Chest Diseases, W1, Clinical Immunology Research Group, London

Scarification skin test with A. fumigatus extract: antigen available from Duncan Flockhart and Company, Edinburgh

* Another station has recently been opened in the "Basler Heilanstätte" in Davos Dorf (Dr. R. de Haller).

Figure 1. Bronchial Secretion of Female Patient with Bronchopulmonary Aspergillosis. Threads of Aspergillus fumigatus with typical septation and forked ramification (KOH preparation, phase contrast 500 x)

Mucormycoses of the lungs have so far been diagnosed very rarely in the living individual but only in or after autopsy. Chemotherapeutic animal experimentation and observation in patients with rhinocerebral mucormycosis (Ref. 7) hold out the hope that a therapy with amphotericin B, in the particularly rapid and malignant course of pulmonary forms of the
mycosis, will not be futile if the diagnosis were made early. It may be assumed that the characteristically broad, either not at all, or very little, septate fungus threads could be demonstrated in the sputum, perhaps best by potash-lye preparations (such a preparation from a rhinocerebral illness is shown in the handbook (Ref. 19); Fig. 2 shows the kidney of a mouse with experimental mucormycosis). It should generally also be possible to cultivate a species of Absidia, Rhizopus or Mucor. In some autopsies of man and animal, however, the cultures did not take despite massive direct-microscopic findings. Similar and so far unexplained failures are known from literature (Ref. 13). At the present time, no serological test methods exist for mucormycosis.

Figure 2. Kidney of Mouse with Experimental Mucormycosis.
Broad, irregular, rarely septate threads of Absidia Corymbifera (KOH preparation, phase contrast 250 x)

Demonstration through culture of Cryptococcus neoformans from sputum should in principle present no difficulty. In practice, it always demonstrates the existence of a cryptococcosis. Apparently, the distribution of the pathogen in pigeon droppings, etc., is not so wide that we need to consider a "wrong positive culture" as with aspergillosis and mucormycosis. Direct-microscopic examination permits earlier diagnosis although not with complete reliability (Ref. 37), provided obviously that the sputum contains a sufficient number of germs. The mucous capsules easily shown in India-ink preparations are almost conclusive for Cryptococcus neoformans. Repeatedly proved has been the India-ink method for diagnosis of cryptococcus from cerebrospinal fluid (Ref. 4). Fig. 3 shows a preparation from the peritoneal exudate of an experimentally infected mouse. When examining sputum, it is recommended to add the India ink only after maceration of the material with 10% lye. There are occluded forms of pulmonary cryptococcosis where demonstration of
the pathogen is successful only by (e.g., bronchoscopic) biopsy (Ref. 37). Actually effective serological test methods are not yet available in spite of intensive efforts (Ref. 36). Overlooking cryptococcosis in the clinic is today of particularly grave consequence since it is possible in principle to save the life of the patient by treatment with amphotericin-B.

Figure 3. Peritoneal Exsudate of Mouse with Experimental Cryptococcosis. Encapsulated yeasts of Cryptococcus neoformans (India-ink preparations, ca. 650 x).

Among all the pulmonary mycoses, candidiasis is by far the most difficult to diagnose. That C. albicans occurs in the healthy buccal cavity and may reach the sputum, has already been pointed out. However, the situation is even further complicated since C. albicans is found not infrequently in the pathological bronchial tree, where it is impossible to assume even a secondary candidiasis (Ref. 37). Although it is to be preferred, bronchoscopic procurement of secretion does not furnish any absolute solution of the problem. Since a positive culture may arise from one single yeast, this single fact obviously means very little. Between bacterial count and pathogenic significance, however, there exists a certain relation so that very dense growth of colonies on solid nutrient medium merits increased attention, provided that the smear obtained constitutes completely fresh material. In a recent communication from Portugal, a method of culture is described for determining, in the pancreatin-digested sputum, the bacterial count of C. albicans per gram of material (Ref. 1). It is to be hoped that this method will make it possible to develop the relevant threshold values. Quantitative indices are also furnished by the direct-microscopic preparation (Fig. 4 - Gram Preparation; unstained and/or potassium-hydroxide preparations are also effective; Ref. 40). As compared to the culture, the preparation has the advantage of lesser sensitivity and only such a preparation makes
possible differentiation between yeast forms and threads (pseudomycelium and mycelium). Threads are relatively significant since they are formed primarily upon penetration of the tissues (Fig. 5).


Figure 5. Pulmonary Candidiasis with Leukemia (from autopsy at Pathological-Anatomical Institute of Basel University). Candida albicans enters alveolar wall with formation of threads (PAS staining, 200 x).
During storing or shipping of sputum, a high increase of the yeast and even the formation of threads may take place (Ref. 19) and it is therefore most important to prepare the smears without delay in the clinical laboratory. A personal communication informs me of the following findings. In a smear of completely fresh sputum, six individual yeasts of C. albicans were counted per fifty fields of vision. In preparations made from the same stored sputum on the second day, six hundred six individual yeasts and thirty-seven small or large groups of yeasts and threads were noted per fifty fields of vision.

In present opinion, serological tests for diagnosis of pulmonary candidiasis are almost useless. It is unfortunately true that we still not have available any standardized methods but the possibilities of serology so far have not been exhausted by any means. Here it is not my opinion primarily that new serological methods are required but that the existing methods should be applied sufficiently consequently and widely in order to define their value as indicator. It has already become apparent that agglutination titers beyond 1 : 160 have significance (Ref. 37) and the precipitation test appears to be of particular value according to some recent work (Ref. 40, Table 5). With a sufficiently strong long-range interest in a large medical center and with close cooperation by clinicians, microbiologists and serologists, it will be possible to improve the diagnosis of pulmonary candidiasis to the same extent as for pulmonary aspergillosis, due to the efforts of French and English teams.

Table 5

Serological Tests for Pulmonary Candidiasis

*Agglutination test*
*Complement-binding reaction*
*Precipitin test (tubes)*

Ship patients' sera to
Dr. H. Seeliger, Institute for Hygiene and Microbiology
of Wurzburg University.

Extra-European pulmonary mycoses should be considered by us for immigrants, tourists, foreign students and others and the occurrence of histoplasmosis in upper Italy has been confirmed. Consequently, Table 6 has been added from which sources for skin-test antigens and some laboratories for the serology of these diseases can be found.
### Table 6

**Allergologic and Serologic Tests for Extra-European Pulmonary Mycoses; Sources of Skin-Test Antigens and Serological Laboratories**

<table>
<thead>
<tr>
<th>Mycosis</th>
<th>Laboratory</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coccidioidomycosis</td>
<td>Cutter Laboratory, Berkeley, California</td>
<td>e.g., Communicable Disease Center, U.S. Department of Health, Education and Welfare, Atlanta 22, Georgia</td>
</tr>
<tr>
<td>Histoplasmosis</td>
<td>Parke Davis</td>
<td></td>
</tr>
<tr>
<td>North American blastomycosis</td>
<td>Parke Davis</td>
<td></td>
</tr>
<tr>
<td>South American blastomycosis</td>
<td>Parke Davis [sic]</td>
<td>Dr. C. Fava Netto, Department of Microbiology and Immunology FMUSP, Sao Paulo (also for precipitin test)</td>
</tr>
</tbody>
</table>

(Centrifuge patients' serum, add merthiolate 1:10,000, ship in fused ampulla by air mail, enclose clinical and epidemiological data.)
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