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DISTRIBUTION OF CRYPTOCOCCUS NEOFORMANS IN A PIGEON HABITAT

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Abstract. Samples of pigeon droppings were taken from 7 sites in a church tower contaminated with *C. neoformans*, and the distribution patterns of the fungus were studied. From various sites, 0 to 3×10^5 viable *C. neoformans* particles were recovered per one gram of dry excreta. The factors causing the different density of *C. neoformans* population in the habitat were: uric acid share of the total nitrogen, creatinine content, sunlight and probably pH. Chemical composition of the substrate is the primary factor in the distribution of *C. neoformans* in droppings.

Association of *C. neoformans* with pigeon droppings is well established now (Emmons 1955; Ajello 1967; Littman and Walter 1968; et al.). There are many laboratory studies about the resistance of *C. neoformans* to several adverse environmental factors (Staib 1962, 1963; Ishaq et al. 1968; Littman and Borok 1968; Sethi and Randhawa 1968; Walter and Yee 1968; Böhm et al. 1970; Weiland 1970) but only very few remarks were published on distribution patterns of the fungus in a natural habitat.

In a foregoing paper (Hubálek et al. 1971) we reported about the isolation of *C. neoformans* from pigeon excreta in southern Moravia (Czechoslovakia). In the present contribution, an attempt has been made to determine *C. neoformans* distribution in the same habitat.

MATERIAL AND METHODS

Habitat. The pigeon habitat is located in the small village Popice (district Břeclav) with 950 inhabitants, with 53 % working in agriculture. The village is surrounded by fields and is situated at 195 m above sea-level. The main population of feral pigeons resides in the church (about 20 pairs of birds in 1970), smaller breeding places are scattered throughout the village. The church population has persisted for at least 3 years. The church tower is about 30 m high, and in its inside parts a quite good air circulation and an access of restricted diffuse day light are provided through 4 great skylights with Venetian blinds, seven very little windows about 15×15 cm and one opening 70×70 cm at the top of the dome. The latter serves the pigeons as the sole entrance into the tower. The stone tower is build in a baroque style, in the inner part abundant rafters are present, and the dome is covered with a metal-plating (Fig. 1).

Sample collections. Samples of droppings were collected on 26th January 1971 in the church tower, and the sites where collections were made are shown in Fig. 1. All the microlocalities except No. 7 (the outer surface of the dome) were situated inside the church tower. The weather was mild for several days (daily temperature of about +7 °C), without a snow cover. Monthly weather values in the area are recorded in Table 1.

The droppings were collected with flamed tweezers into sterile cork-stoppered tubes and processed on the following day after a storage at +4 °C.

***C. neoformans* counts.** 0.50 g of each sample was weighted into a sterile 100 ml-Erlenmeyer flask with glass beads; 25 ml of saline solution supplemented with Tween 80 (0.05 %), penicillin (500 i.u./ml) and streptomycin (500 μg/ml) was added and shaken for 10 min. Serial tenfold dilutions were prepared and each 0.2 ml were streaked on 5 plates of Sabouraud glucose agar with yeast extract (0.1 %) and chloramphenicol (0.01 %), and on 3 plates of Guizotia agar (Shields and Ajello 1966). Colony counts were read after 4-day incubation at 37 °C; all types of yeast colonies were microscopically screened and the suspicious ones were isolated and further determined. The criteria for the identification of *C. neoformans* included: spherical cells, no pseudomycelium, good growth at 37 °C, urease production, brown pigment on Guizotia agar, and creatinine assimilation. In selected strains, the mouse pathogenicity was verified.

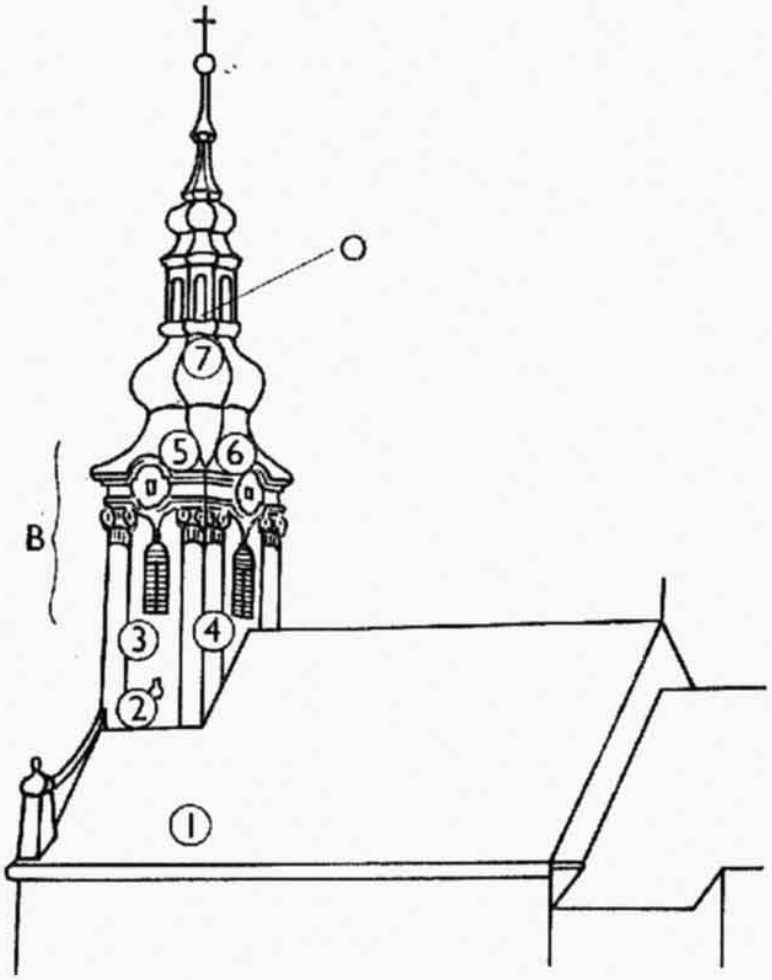

Fig. 1. The pigeon habitat with the sampling sites recorded
The sites No. 1—6 are inside the tower, while site No. 7 is placed on the outer surface of the dome. O = opening (in-and-out opening), B = belfry.

Dropping extracts. 4 % (w/v) suspensions in 0.01n—HCl were prepared of pulverized dry droppings, heated in a 95 °C water bath for 30 min., neutralized and centrifuged. The supernatant was used as the sole nitrogen source for the growth of *C. neoformans:* each 2.5 ml of serial dilutions were added to 2.5 ml of the basal medium (glucose 4.0 %, KH_2PO_4 0.3 %, $MgSO_4 . 7H_2O$ 0.1 %, $CaCl_2 . 2H_2O$ 0.05 %, NaCl 0.05 %, $FeSO_4 . 7H_2O$ 0.002 %, yeast extract Difco 0.004 %) in aluminium-capped tubes, autoclaved at 120 °C for 20 min. and inoculated with 0.06 ml of *C. neoformans* 722 (an isolate from the same habitat) saline suspension with 2×10^7 cells/ml. This inoculum was grown on Sabouraud glucose agar at 26 °C for 72 hours, suspended in saline, starved for 12 hours at 26 °C and washed three times (5 min. at 400 g). Incubation of the test cultures was strictly stationary, for 72 hours at 26 °C in a dark thermostat. The density of cells was then counted in Bürker's haemocytometer, plotted against the individual extract concentrations, and the value c_{10d_0} of extract dry weight (in % [w/v] of the suspension), which resulted in a tenfold, number of *C. neoformans* cells in comparison with the blank (d_0, the inoculum in the medium with no extract of droppings) after the incubation, was read from the graph.

Table 1. Climatic conditions of the area studied

	1970						1971	
	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.
Air temperature (°C):								
Average	18.8	18.3	13.9	8.7	6.6	0.5	—2.8	1.2
Maximum	32	30	28	23	21	10	11	11
Minimum	9	7	—3	—4	—2	—15	—22	—10
Relative air humidity (%)	72	77	74	81	80	86	83	76
Total precipitation (mm)	69	41	15	48	68	15	19	25
Sunshine (hours)	224	205	199	113	44	26	59	108

Water content of droppings was determined by the standard method, and expressed on the wet weight basis. pH was measured in a small volume of distilled water, using pH-meter PHK (ZPA Praha).

Total nitrogen was determined according to Kjeldahl.

Uric acid. 5 ml of 0.05n-NaOH was added to 50 mg of a dry sample, the suspension was heated at 95 °C for 15 min., supplied with water to 5.0 ml, and centrifuged. 0.2 ml of phosphowolframic acid ($Na_2WO_4 \cdot 2H_2O$ 4.0 %, H_3PO_4 5.5 %) and 0.8 ml of 20 % Na_2CO_3 were added to 1.6 ml of the neutralized and diluted supernatant. The extinction was measured after 10 min. at 650 nm, using Spekol (VEB Carl Zeiss Jena). Standard (uric acid puro A. R. Koch-Light) was treated as the samples.

Creatinine. 1 ml of 0.04 m-picric acid and 0.2 ml of 10 % NaOH were added to 3 ml of the supernatant, and measured at 520 nm after 15 min. Standard (creatinine puriss. Fluka) was treated as the samples.

Tower microclimate was analysed on 17th February 1971 (this was a cloudy day with drizzle and temperature of 3 °C). Air humidity was measured by Assmann's aspiration psychrometer, air temperature by a mercury thermometer divided at 0.1 °C. Light intensity was determined using the luxmeter Metra.

RESULTS

The frequency of *C. neoformans* is shown in Table 2. The fungus was not uniformly distributed throughout the habitat: its population was the highest on site No. 5 (2.93×10^5 cells per one gram of dry droppings), lower counts were obtained from sites No. 4, 3, 6, very low from the microlocality No. 2, while on sites No. 1, 7 no viable cells of *C. neoformans* were found. The belfry (No. 3, 4, 5, 6) was the most satisfactory space for the recovery of *C. neoformans*.

In the last column of Table 2, a coincidence is evaluated between the numbers of *C. neoformans* and variable changes of the habitat, using rank correlation coefficient τ (Kendall 1943). From this statistical comparison follows the striking and significant ($P < 0.001$) positive correlation between the population density of *C. neoformans* and the nutritive value of the dropping extract for the fungus. This is conditioned predominantly by the content of nitrogenous compounds, the major part of which forms uric acid in pigeon excreta (according to our results, about 2/3 or 3/4 of total N is uric acid N). A significant ($P < 0.01$) correlation was found between uric acid share in total N and the counts of *C. neoformans*, and also between the concentration of creatinine and the counts in droppings ($P < 0.05$). Moreover, we disclosed a correlation ($P < 0.01$) between the *C. neoformans* counts and the frequency of the new excreta dropped on the site (this ensures a supply of nutrients). The other correlations were statistically insignificant, but certain trends may be followed. On the whole the tower microclimate responded to the outdoor climate, and the temperature and humidity differential of

the air inside the tower is so small, that it should not be the cause of count differences in the individual samples. The illumination, however, plays a role in the distribution patterns of *C. neoformans:* the microlocalities exposed to direct sunlight (No. 7, 2) were free or almost free of this fungus. Water content of the positive samples was 50 to 60 % and pH 6.4—7.2. In our first report (Hubálek et al. 1971), however, *C. neoformans* was isolated from a sample (collect. Aug. 1, 1970) with as little as 9.6 % of water and pH 6.1. It is possible that the reaction of pH 7.2 and higher was an adverse factor in the distribution of *C. neoformans* (see samples No. 1, 2).

Table 2. A survey of some variables in the pigeon habitat

Sites No.:	1	2	3	4	5	6	7	Rank correlation coefficient r
No. of viable *C. neoformans* particles per 1 g of dry wt. ($\times 10^3$)	0	≦0.1	28.5	69.5	293	21.0	0	
Frequency of pigeon occurrence	—	—	+	++	++	+−	+	
Droppings:								
Layer (cm)	2	6	4	8	4	2	1	
Frequency of falling (sequence)	7	5	4	2	1	3	6	+0.86
Water content (%)	51.8	48.0	40.2	43.2	41.0	59.6	28.3	−0.09
pH	7.9	7.2	7.0	6.4	6.7	6.9	6.5	−0.38
C. neoformans growth in extract (c_{100_0}, %)	0.47	0.39	0.26	0.24	0.21	0.27	0.68	−0.95
Total nitrogen (%dry wt.)	2.15	5.02	3.90	3.17	5.71	8.60	5.59	+0.09
Uric acid (% dry wt.)	1.04	9.19	7.28	7.36	13.14	14.36	8.84	+0.29
% of uric acid N from the total N	16.1	61.1	62.3	77.5	76.7	55.7	52.8	+0.76
Creatinine (% dry wt.)	0.04	0.12	0.16	0.18	0.20	0.17	0.16	+0.67
% of creatinine N from the total N	0.60	0.90	1.47	2.10	1.27	0.75	1.30	+0.48
Microclimate:								
Air temperature (°C)	3.2	3.2	3.6	3.3	3.2	3.2	3.0	+0.38
Relative air humidity (%)	87.7	86.2	84.8	86.2	87.7	90.8	92.2	−0.29
Light intensity (Lx)	<0.1	240	0.3	1.0	0.2	0.1	5400	−0.09

Remark: Correlation coefficients in frame are significant ($P < 0.05$).

Optimal conditions could be specified for the occurrence of *C. neoformans* in the habitat: A site illuminated with markedly restricted, diffuse day light, supplied regularly with fresh excreta; pH of droppings 6.4—7.0, their water content about 40 %, the share of uric acid N in total N about 75 %, creatinine content 0.2 %.

DISCUSSION

Bird excreta are highly concentrated in cations (Ca, K), phosphates, carbohydrates and nitrogen. About 70 % of the nitrogen in fresh avian excreta will be in the form of uric acid; this compound is present at approximately 56 % of the total organic matter (Hutchinson 1950). Wagner and Franzen (1959) analysed nitrogen of chicken droppings and found 56.2 % uric acid N, 21.1 % ammoniacal N, 0.4 % creatinine and/or creatine N, 0.2 % urea N.

Staib (1962), Walter and Yee (1968), and Littman and Borok (1968) observed that pigeon excreta provided nutrients enabling abundant growth of *C. neoformans*. Low-molecular nitrogen substances play a key role in the growth of the fungus in avian droppings (Staib 1962). On the contrary to the majority of other yeasts including nonpathogenic cryptococci, creatinine assimilation is a specific feature of *C. neoformans* (Staib 1962; Kreger-van Rij and Staib, 1963; van Uden et al., 1964). The species hydrolyses or assimilates also other N constituents of avian excreta: uric acid, allantoin, ammonium, urea, xanthine, guanine (Seeliger 1956; Staib 1962; van Uden et al. 1964; Lockwood and Garrison 1968).

Bird droppings constitute one of the most prominent source of *C. neoformans* in nature. The concentration of the fungus in pigeon excreta may be considerable — 3×10^5 viable particles per one gram of dry material were ascertained in this study. As the total amount of droppings on sites No. 3 to 6 was approximately 100 kg, the total number of viable *C. neoformans* particles could be in fact as high as 10^{10} in the whole habitat. This is certainly an enormous number, with a possible epidemiological impact. Powell et al. (1972) counted 7.5×10^5 *C. neoformans* particles per gram of crushed pigeon excreta collected from the belfry of a church. Emmons (1960) estimated that each gram of dry pigeon droppings contained 5×10^7 viable *C. neoformans* cells.

The question arises about the origin of this fungus in pigeon excreta. Feral pigeons were found to be mechanical carriers of *C. neoformans* (Littman and Borok 1968; Weiland et al. 1968) and the fungus survived well the passage through the gastrointestinal tract of birds including pigeons (Staib 1962; Littman and Borok 1968; Sethi and Randhawa 1968; Monga et al., 1971). It is almost certain now that *C. neoformans* may accidentally be inoculated into a previously uncontaminated layer of dropped excreta by a bird ingesting the organism with contaminated food, via defecation. Because pigeon droppings are a favourable substrate for *C. neoformans*, a rapid multiplication of the fungus follows. The selective action of dropping medium could be conditioned either by the stimulatory effect of several basic compounds (N-substances, salts, phosphates) of the excreta on *C. neoformans*, or by an inhibitory effect of these compounds on other organisms. In our opinion, both these mechanisms of action occur simultaneously, enabling thus a high competitive rate of *C. neoformans* on this substrate.

However, many factors limit the population density of *C. neoformans* in droppings. Alkalinization of excreta (in consequence of microbial activity) is an adverse factor for the fungus. Staib (1962) observed no growth of *C. neoformans* in a medium with dropping extract, if pH was 8.0 and more. Walter and Yee (1968) found that a chicken-dropping infusion inhibited the growth of *C. neoformans* at pH 7.5 and more (increased pH of chicken manure is considered to be one of the factors responsible for the absence or rare occurrence of *C. neoformans*). Mosberg and De-Chaudens (1951) and Howard (1961) observed no appreciable growth of *C. neoformans* if the reaction of Sabouraud medium was alkaline (pH 7.3 or more). All these results together with the finding in our study indicate that pH 7.2—7.5 and more is unsatisfactory for the multiplication of *C. neoformans* in droppings; an optimal pH value will be about 6.2—6.9.

Another factor is the temperature. *C. neoformans* is resistant to low temperatures: it grows at +4 °C, and Cramer and Mix (1957) observed a 5-year survival of fungus cultures at —18 °C. Resistance to higher temperatures is also well-developed (Staib 1963; Littman and Borok 1968). However, Ishaq et al. (1968) found that *C. neoformans* survived much longer in the soil at 4—24 °C than at 37 °C; the growth and survival of the fungus was better under winter than under summer conditions in Oklahoma — coupled with cooler winter temperatures, high relative humidity enhanced the proliferation of *C. neoformans*.

Resistance of *C. neoformans* to desiccation was studied by a number of authors. Staib (1963) demonstrated that the fungus survived 420 days storage in dry canary excreta, and Littman and Borok (1968) observed a protective effect of pigeon excreta extract on *C. neoformans* survival when compared with saline: in the former if desiccated the fungus survived 390 to 680 days.

The sensitivity of *C. neoformans* to direct sunlight was indicated by several authors. Emmons (1960) recovered *C. neoformans* more often from specimens of organic debris collected from sheltered sites, and Denton and Di Salvo (1968) confirmed this distribution pattern of the fungus. Also Littman and Schneierson (1959) noted that pigeon faeces collected from outdoor sites were less heavily infected (25 % positive) than specimens taken from indoor sites (57 % positive). Ishaq et al. (1968) inoculated *C. neoformans* into tubes with soil and found a marked decrease in the number of viable cells after 6 months in the tubes exposed to sunlight, while in the tubes covered with a black bucket the decrease was relatively slight. Inhibitory action of direct sunlight was established in an experiment by Böhm et al. (1970): after a 6-day desiccation of *C. neoformans* suspension in sterile sand soil under direct sunlight, a 10—100 fold decrease in the number of viable fungus particles was found in comparison with the desiccation in a dark thermostat. In our study, the samples collected on sites illuminated regularly yielded very low counts or the fungus was absent. Nevertheless, exact data about this influence are still needed because no clear-cut division has usually been made between three components of the sunlight action, viz. radiation (U. V., visible light, etc.); heating; and desiccation.

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РАСПРОСТРАНЕНИЕ ГРИБКА *CRYPTOCOCCUS NEOFORMANS* В МЕСТЕ ОБИТАНИЯ ГОЛУБЕЙ

З. Губалек

Резюме. Из 7 мест в колокольне пораженной грибком *C. neoformans* взяты пробы голубиного помета с целью изучения характера распространения этого грибка. На 1 грамм сухого помета из разных мест находили от 0 до 3×10^5 жизнеспособных частиц *C. neoformans*. Факторами, обусловливающими разную плотность популяции *C. neoformans* в данной среде являлись: доля мочевой кислоты в общем азоте, содержание креатинина, солнечный свет и вероятно pH. Главным фактором распространения *C. neoformans* в помете являлся химический состав субстрата.

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