THE ISOLATION OF ASPERGILLUS FUMIGATUS FROM WILD PINK-FOOTED GEESE IN ENGLAND AND SCOTLAND

by J. V. Beer

The survey reported here forms part of the wider investigation into Aspergillosis which Mr Beer has been conducting since 1954, while working for a Ph.D. at the University of Bristol as the holder of the Bristol, Clifton and West of England Zoological Society's Scholarship.

SUMMARY

A SWAB was devised that could be used on a large scale in the field to determine if the wild Pinkfoot carried spores or mycelium of *Aspergillus fumigatus* internally. A selective growth medium based on Czapek-Dox broth allowed the fungus to grow freely with little or no competition from other micro-organisms. Swabbing of the mouth and pharynx of 1188 apparently healthy Pinkfeet over three seasons produced 86 (7·2%) positive cultures of *A. fumigatus*. Controls showed that the technique used was adequate despite the relatively primitive field conditions. No significant difference was found between the three seasons nor between adults and juveniles. However, male Pinkfeet are more likely to harbour the fungus than females. The percentage of positives increased as the season progressed, and there was some evidence to suggest an east–west gradient across the country.

The fungus found was probably derived from the soil and vegetation rather than the internal organs of the birds.

The fungus disease, *Avian Aspergillosis*, is at times fairly common in captive birds, but little is known of its form or occurrence in wild birds. Ainsworth and Rewell (1949) described the disease in captive birds, while among wild birds Ainsworth and Austwick (1955) noted it in a Rook, a Jackdaw and four Woodpigeons; Poulding (1952) in the Herring Gull and the Lesser Black-backed Gull; Quortrup and Shillinger (1941) in 43 (1·4%) wildfowl during the examination of 3000 dead birds for botulism and lead poisoning in the Western Lake areas of the U.S.A., and Venn (1955) in a White-fronted Goose found on the Dumbles at Slimbridge. The disease is caused by the fungus *Aspergillus fumigatus*. Diagnosis is difficult or impossible in the living bird, and a post-mortem examination generally has to be made to show the presence of the disease. The question of the occurrence of the fungus in the healthy wild bird is of primary importance to any study of the disease, and the best that can be done in the field at present is to study the fungus flora of the oro-pharynx.

As early as 1914, Heald and Studhalter found that birds could be carriers of a fungus, and in particular that causing chestnut blight. Sladen (1952 and 1954) examined Penguins, Kelp Geese and Steamer Duck in the wild, but found no evidence of *A. fumigatus*. Tiffany, Gilman and Murphy (1955), while studying Oak Wilt, isolated 16 cultures of the genus *Aspergillus*, comprising some 11 species, including *A. fumigatus*, from Woodpeckers and other birds trapped in the region of the diseased trees. Sladen and Austwick (1955) studied the mycoflora of 317 wild Pinkfeet in Iceland and Britain, but did not isolate any cultures of *A. fumigatus* from these birds. As the technique they used resulted in the isolation of many other fungi, this negative result suggests that *A. fumigatus* is comparatively rare. The work of Tiffany *et al.* (1955) bears this out, as only a few of the 442 isolates were *A. fumigatus*.

If *A. fumigatus* is present in the Pinkfoot, means must be devised to increase the chance of detection. This can be done in four ways:

(1) Sample a larger number of birds.
(2) Improve the swabbing technique.
(3) Provide a selective medium to prevent the possible overgrowth of the fungus being isolated.
(4) Incubate plates at 40° C.

Various improvements were proposed by Sladen and Austwick (1955) along these lines, and the following technique, independently evolved and briefly described in an earlier note (Beer 1955), included several of the suggestions.

METHODS

Source of Material

Pinkfeet were swabbed after trapping in rocket-nets during three seasons in England and Scotland (October and November 1954, October 1955 and October 1956). 1118 birds were examined, in 18 groups varying from 26 to 159 in number. Many controls, samples of soil and of vegetation were taken to obtain some idea of the chances of contamination giving false positives and of the quantity of *A. fumigatus* in the environment of the geese.

The Swab

In previous studies workers have used a cotton-wool swab on a stick stored in a plugged sterile tube. Experiments with ducks showed that it was not easy to swab the oro-pharynx, as the cotton-wool caught on villi at the back of the tongue. Streaking of this type of swab on to media at a later stage does not utilise the maximum amount of inoculum available, and may allow other micro-organisms, particularly bacteria, to overgrow the small amount of *A. fumigatus* present. Ideally, soluble-wool swabs should be used in conjunction with a selective medium, but the former are not easy to use in the field.

To overcome some of the disadvantages of previous swabs, one was made of rubber tubing and springy wire. Tubing, $\frac{5}{32}$ inch $\times$ 3 inches, was attached to a hooked length of inert nichrome resistance wire (Fig. 1), and four or five of

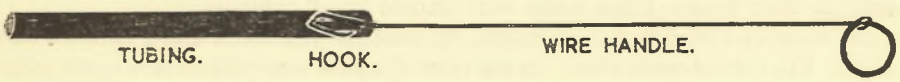

Fig. 1. The Swab

these swabs were placed in a test-tube with a glass cap and a cotton-wool sleeve (Fig. 2). The units were steam sterilised and stored in sterile tins.

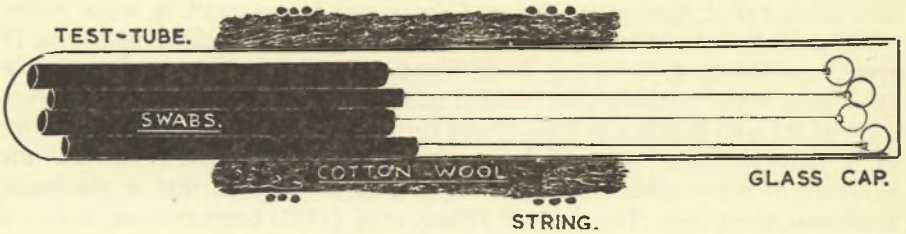

Fig. 2. Storage of Swabs

Medium

The media used by previous workers have not been particularly selective, for *A. fumigatus* and growth of other organisms may well cover up the few colonies present or, even worse, inhibit the growth of the fungus altogether. It is also important that the sample be placed at once into the selective medium to suppress growth of contaminants. A suitable medium consists of a modified Czapek-Dox broth, containing sugar, mineral salts, aureomycin (a wide spectrum antibiotic) and hydrochloric acid to produce a high acidity. Experiments showed that the fungus was capable of rapid growth on this medium. Incubation of this medium at body temperature allows very few other micro-organisms to grow on a culture plate. In the first season a less selective medium was used. Experiments showed that *A. fumigatus* was readily isolated on both media, but the Czapek-Dox grew fewer contaminants and was latterly used in preference to the earlier type. For use in the field, the broth was dispensed in small 2-drachm bijou screw-cap bottles in 4-millilitre amounts and sterilised. The bottles were kept in sterile tins.

Incubation

A. fumigatus grows readily at temperatures between 20° C. and 50° C., with an optimum around 40° C., which coincides with the body temperature of geese. 40° C. was used for incubation. At this temperature most fungi and many bacteria are inhibited, while *A. fumigatus* grows profusely. Prior to incubation, all samples were kept at a low temperature (near 4° C., if possible) to reduce growth of the micro-organisms between collection and culture of the sample.

Collection of Samples

Ringing of the birds was carried out upwind of the rocket-nets, and generally it was possible to arrange the area used for swabbing so that contamination by dust from vegetation, birds, etc., was reduced to a minimum. A clean cloth-covered area of a vehicle comprised the work-bench. Four or five of the bijou bottles of medium were opened and placed on or close to the work area to sample the air during the whole of the operation, which lasted at times for as long as three hours. These tubes were known as air controls.

As soon as a bird had been ringed, its number was noted and a throat swab taken. The cotton-wool sleeve on the tube of swabs was pushed down, the glass cap removed and a swab taken out. Contamination of the remaining swabs

was prevented by immediately replacing the glass cap on the test-tube. The bill of the bird was held open by the fingers, and the swab was rubbed over the back of the mouth and over the entrance of the trachea (photograph, *Eighth Annual Report*, p. 119). Care was taken not to contaminate the swab by any external source, and if there was any suggestion of contamination in any way, the swabs or tubes were discarded. Having obtained the sample from the throat, the swab was washed vigorously in sterile broth contained in the bijou bottles. The rubber tubing buckled up, and it was possible to wash the whole of the tubing in the broth. A proportion of the swabs were waved around in the air, and then washed in the broth to check the probability of contamination of the swab during the time that it was in use.

Soil and vegetation samples were collected in sterile bottles for examination, and all samples were sent by post to Slimbridge and stored at about 4° C. until they could be examined.

Examination of Samples

The tubes of broth were plated out with a similar but solid medium, and incubated for seven days at 40° C. The presence or absence of *A. fumigatus* was noted (large green colonies with certain microscopic characteristics), and pure cultures were made of a large proportion of these isolates. Most plates were sterile, while a few grew other fungi and bacteria, but in no case was the growth of any contaminant so great as to overgrow *A. fumigatus*.

RESULTS

The picture obtained from the swabbing showed that a small proportion of the birds carried the fungus in the oro-pharynx. Some 86 cultures were obtained from 1188 birds, a percentage of 7·2. The results of three seasons are given in Table I.

TABLE I

Year	Total Swabbed	*A. fumigatus* Present	Percentage of Total with *A. fumigatus*
1954	348	31	8·9
1955	289	15	5·2
1956	551	40	7·3
	1188	86	7·2

Although the percentage varies between 5·2% and 8·9%, an analysis of the figures shows that the proportion of birds carrying the fungus is much the same from year to year.

A note was made of the age of the bird—either adult or juvenile. The age of some adult birds was known accurately, but as so few were involved no age analysis was attempted on a yearly basis. These birds were lumped together with the other adults. Table II gives the breakdown of the results in terms of age.

TABLE II

	Total Swabbed		*A. fumigatus* Present		Percentage of Total with *A. fumigatus*	
Year	Adults	Juveniles	Adults	Juveniles	Adults	Juveniles
1954	237	111	20	11	8·4	9·0
1955	211	78	9	6	4·3	7·7
1956	254	97	36	4	7·9	4·3
	902	286	65	21	7·2	7·3

Analysis shows that there is no significant difference between the two age-groups.

In 1954 most of the birds were sexed; the results are in Table III.

TABLE III

Sex	Total Swabbed	*A. fumigatus* Present	Percentage of Total with *A. fumigatus*
Male ..	142	21	14·3
Female ..	129	8	6·1

On analysis this difference is found to be greater than would be likely to occur by chance, and suggests that males are more likely to harbour the fungus than females.

It had been hoped that a large number of suitable recaptures would be available for repeat swabbing. In practice only nine birds were swabbed twice, and in each case the fungus was not isolated. To obtain any useful results from recaptures, a very much larger sample would have to be taken.

As the percentage of positives is quite small, the results obtained from the controls are quite important. To begin with, some 85% of the plates were completely sterile, which indicates that at least gross contamination by organisms capable of growing under the provided selective conditions has not occurred. The results from various controls are given in Table IV.

TABLE IV

Year	Air Samples	+ ves	Swabs Samples	+ ves	Fingers Samples	+ ves	Feathers Samples	+ ves	Net Samples	+ ves
1954	11	0	10‡	0	12	0	1	0	2	0
1955	18	0*	2	0	—	—	—	—	—	—
1956	31	1†	40	0	—	—	—	—	—	—
Totals	60	1	52	0	12	0	1	0	2	0

* Two tubes produced a mucor-type fungus.
† Tube exposed for 3 hours. One colony of the fungus produced.
‡ Unused swabs examined after the trip.

The lack of positive cultures in all the controls except one air tube indicates that the technique was adequate for the purpose, i.e. the swabs and sample tubes were not exposed to too great a risk of contamination. The single positive tube was exposed for some 3 hours. It seems reasonable to conclude that the chances of a swab, exposed for no more than a minute, becoming contaminated are very small. The only other contaminated tubes were exposed for 1–2 hours and grew mucoraceous fungi.

Samples of soil and vegetation were collected in sterile bottles from the fields where catches were made. On culture, the following picture was obtained:

TABLE V

Year	Total Samples	Samples with *A. fumigatus*
1954	28	13
1955	17	17
1956	47	10

The variation is quite large, and some fields did not produce any cultures of the fungus. In 1955 all samples from all fields were positive.

DISCUSSION

The Technique

The failure of other workers to isolate *A. fumigatus*, except incidentally in the Oak Wilt survey by Tiffany *et al.* (1955), may be due to a variety of reasons, which are implied in the four suggested improvements in the introduction and are now discussed in the light of the results.

The number of birds harbouring the fungus as evaluated by the technique described in this paper is small (7%). Sladen and Austwick (1955) swabbed 40 Pinkfeet caught on stubble-fields and the expected number of positives would be 2 or 3 on the above basis. However, with a sample of this size it is quite likely that no positives would be obtained. Thus the sample size should be increased considerably. A similar argument can be applied to the 254 Iceland birds. Here the sample size should be even larger, as the concentration of spores in central Iceland is probably smaller due to a lower ambient temperature than in Britain, resulting in a lower infection rate of the birds. Thus in any one season it is advisable to swab several hundred birds in Britain and over a thousand in Iceland. Samples of this size with a control swab rate of 1 in 10 or 1 in 5 allow useful statistical analyses to be made. The present study approximated to these figures. With the present level of 7% positives, a total sample of less than 100 is not worth considering except in an initial study.

The swab that was used was easy to prepare and to handle. Rubber tubing slides in and out of the throat very easily and picks up a fair quantity of mucus. This is readily washed off by broth in bijou bottles. There was a tendency for the rubber tubes to stick together or become weak after several sterilisations, and they had then to be discarded.

The use of selective growth conditions increased the chance of detection of the fungus. The medium based on Czapek-Dox agar rather than Sabouraud's provided these conditions, 85% of the plates being sterile. Incubation at 40° C. is near the optimum for *A. fumigatus* and well above the optima of many other

fungi and bacteria, and allows the fungus to grow readily. The selective conditions could be further improved by the use of chloromycetin in place of aureomycin and by the addition of 4–7% sodium chloride.

Discussion of Results

This study has shown that a small proportion (7%) of the wild Pinkfeet in Britain do at times carry *A. fumigatus* in the throat, but it was not possible to show that these birds consistently carried the fungus, as the sample of suitable recaptures was far too small. Sladen and Austwick (1955) have pointed out that the flora of the throat is a reflection of the flora of the stubble-field, and as presumably all the birds in a catch are exposed to the same degree of infection, we would expect to find no difference in the incidence of the fungus between adults and juveniles and between the sexes in any one group of birds. This was the case for the former but not for the latter, and no reasonable explanation can be given for this sex difference. On this evidence and since many of the soil and vegetation samples contained *A. fumigatus*, it is fair to assume that fields are the source of the fungus. This is supported by the fact that cases of Aspergillosis in the birds are rare, and that the nature of the disease is such that transfer of spores from one bird to another is unlikely. Similarly, air samples taken by many workers indicate that the whole genus *Aspergillus* is uncommon in the atmosphere, again pointing to the soil and vegetation as the main source of the fungus.

Although some Pinkfeet can consistently be found carrying *A. fumigatus* internally during October and November, differences and trends can be detected in the samples. The sex difference has already been pointed out though not explained. As the season progresses the incidence of positives increases. The following percentages are calculated from the three years' results, leaving out one small sample of only 26 with 1 positive.

Period ...	... 7–10 Oct.	13–17 Oct.	18–24 Oct.	23–26 Nov.
Per cent positives	3·4	8·8	9·2	10·5

This trend on analysis proves to be significant, but cannot be explained in terms of ambient temperature as the temperature is decreasing during the season, which means that there will be less growth of *A. fumigatus* and, consequently, fewer positives. An increasing humidity coupled with a moderate temperature during the season may allow the fungus to grow more readily on straw and the like, thereby increasing the number of positives. It may also be that a bird takes time to come across and pick up the fungus and, because of the probable transient nature of the infection, the maximum number of positives may take a little while to develop.

If the results are grouped on a regional basis, there is some, but not very convincing, evidence of an east–west gradient but not a north–south gradient. Kinross and the Wash on the east side produced averages of 3·6% and 2·9% positives respectively, and the Solway on the west coast, 12·6%. A low figure for Kinross could be explained by the early sample date. The Humber and Perthshire samples are intermediate, with 9·1% and 6·1% respectively. The trend is significant, but a Southport catch would be most useful to provide a sample from a second west-coast area. This trend could be explained by the warm, damp weather on the west coast and the dry, cool weather on the east coast.

Further Studies

This survey could be extended to confirm or refute the results to date, but to make a fourth sampling worth while a complete season's catch (1500) would have to be utilised. It would be particularly interesting to confirm the seasonal and regional variations. The field technique could be further improved in many small details to reduce further the risk of contamination.

For the present it is proposed to discontinue the swabbing survey, to assume that the results will be much the same in another year, and to study the concentrations of spores in the environment in which the geese live. In particular, regional and seasonal variations will be studied.

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