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Chlamydiosis in birds in Great Britain

2. Isolations of *Chlamydia psittaci* from birds sampled between 1976 and 1984

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SUMMARY

A total of 1531 diagnostic submissions from birds were examined by culture for the presence of *Chlamydia psittaci* between June 1976 and December 1984 by growth in NCTC 929 clone L mouse fibroblast cells, pretreated with an inhibitor of cell division, followed by direct immunofluorescence microscopy. Of these, 196 were found positive. The continued importance of psittacine birds as potential sources of infection was shown by the high number of positives (139) obtained from birds of that order. The percentage of submissions found positive was highest in parakeets (30·1) and was fairly high in psittacines as a group (16·6), but the latter figure was exceeded by the group of collared doves (*Streptopelia decaocto*) and wood pigeons (*Columba palumbus*) (25·0). Domestic poultry generally gave low rates, turkeys being the highest.

Both the numbers of submissions and their rates of positives increased between 1980 and 1984.

Comparing the isolation rates from the various organs sampled, the intestines gave the highest rate (20·4 per cent positive), closely followed by the other internal sites. The superficial swabbed sites (eye, nasal cavity, cloaca) gave lower rates.

MATERIALS AND METHODS

This paper summarizes the results of cultural examinations for *Chlamydia psittaci* carried out on specimens from birds submitted for diagnostic purposes to this laboratory from June 1976 to December 1984. Throughout this period the method used was basically the same, growth in cell culture followed by fluorescence microscopy. Most of the material came from the Veterinary Investigation Centres or direct from practising veterinary surgeons. The purposes were either to attempt to confirm suspicions of chlamydiosis in birds or to search for sources of infection for man, usually prompted by a recent report of a case in a human being who had been in contact with the birds in question.

Sample preparation

Originally pieces of organ were homogenized in an antibiotic solution consisting of streptomycin, kanamycin and vancomycin each at 1 mg/ml in Earle's Balanced Salt Solution (Bevan, Cullen & Read, 1978). Latterly gentamicin at 200 μg/ml has

been preferred in the Chlamydial Transport Medium (CTM) detailed below, and having a final pH of 7·2–7·6.

Earles' balanced salt solution (+phenol red)	100 ml
Fetal calf serum	10 ml
Sorbitol (or inisotol)	10 g
Sodium hydrogen carbonate	0·22 g

After standing in this solution samples were plated on to blood agar, 10% serum agar, malt agar and into thioglycollate broth. These were examined after overnight incubation for evidence of bacterial or mycotic contamination and the thioglycollate replated on to 10% serum agar. If contamination was still present the sample was passed through a filter of 800 nm pore size, Sartorius MINISART NML (Sartorius Instruments Ltd, Sutton, Surrey).

Cell culture method

The cell line used was NCTC 929 clone L, mouse fibroblasts (L929). Confluent L929 cells were trypsinized using 500 mg/l trypsin (1:250) in Earle's Balanced Salt Solution without calcium and magnesium ions but with 200 mg/l EDTA. Diluted to 3×10^5 cells/ml, the L929 were dispensed in 2 ml volumes into sterile 7 ml plastic bijou bottles complete with 13 mm diameter glass coverslips as supplied by Sterilin ('TRACH' bottles code 129AX/1–Sterilin Ltd, Feltham, Middlesex).

These were incubated at 37 °C overnight and the following day were treated with emetine (Paul, 1982) as follows: the cell sheet was drained of medium and 0·5 ml of emetine (0·5 μg/ml) was added. After 5 min the emetine was removed and replaced with 1 ml of growth medium, and the cells were then ready for infection.

Inoculation of cultures

Samples shown to be free of contaminating micro-organisms were inoculated at 0·2 ml per tube into a minimum of six tubes. These were centrifuged in sealed containers at 2000 **g** for 45 min and then incubated at 39 °C. They were examined on days 3 and 6 post inoculation using a direct fluorescent antibody stain. Coverslips for examination were drained of tissue culture fluid and 2 ml acetone was added for fixation, allowing 5 min at room temperature. Staining and examination were as previously described (Bevan *et al.* 1978). Material which was negative after 6 days (referred to as passage One at this stage) was harvested as follows. About five sterile glass beads 2–3 mm diameter) were added to each tube, and mixed for a few seconds on a vortex mixer. Media and cells were harvested, bulked, and checked for absence of micro-organisms. This material was reinoculated on to pretreated cells using four tubes. All further passages were processed as above.

Preparation of fluorescein conjugate

The antiserum used was obtained from non-vaccinated sheep which had aborted due to a naturally-acquired infection of *C. psittaci*.

The immunoglobulins (Ig) were precipitated with sodium sulphate (140 g/l) by stirring vigorously at room temperature for 2 h. Centrifugation at 2000 **g** for 20 min at ambient temperature removed the Ig which was then redissolved to the original volume in 0·1 M phophate buffered saline (PBS), pH 7·2. The Ig was reprecipitated with sodium sulphate as described above a further two times. After this it was

dissolved to half the original volume in distilled water and sulphate ions dialysed out against running tap water for 3 h. Molarity was corrected by dialysing overnight at 4 °C against 0·1 M-PBS. Total protein was estimated by spectrophotometer at a wavelength of 280 nm and the solution diluted to give 1 mg/ml protein. This solution was then mixed 2:1 with carbonate/bicarbonate buffer (0·5 M, pH 9·0) and put on ice. Fluorescein isothiocyanate (FITC) on celite (Calbiochem) was added to give 10 mg FITC/60 mg protein and stirred slowly for 30 min on ice. Non-conjugated fluorescein was removed by overnight dialysis against 0·1 M-PBS at 4 °C and the conjugate passed through a column of Sephadex G25 (Medium) to remove fragmented Ig and non-specific proteins. The conjugate was finally purified through a column of DEAE-cellulose equilibrated in 0·01 M-TRIS/HCl buffer, pH 8·7 and eluted with 0·14 M sodium chloride.

The unbound fraction from this column was found to be of good titre and volume. A second fraction could be recovered by raising the molarity of the sodium chloride to 0·28 M in the TRIS/HCl buffer. After checking its specificity and titre, the conjugate was passed through a 450 nm filter and stored at −40 °C in 0·1 ml aliquots, or freeze dried.

RESULTS

Trends in submissions and positive isolations

Table 1 shows the number of samples examined each year, the number of these found positive, and this figure expressed as a percentage. Between 1976 and 1980, the number of samples examined annually remained fairly constant but the percentage positive declined. After 1980, the number examined rose, becoming significantly higher than before, while the percentage positive rose at the same time, returning to approximately the same values as in 1976. The net result has been a big increase in the number of positive isolations in recent years.

Species distribution

Table 2 shows the distribution of the samples received between the species (or groups) of birds, the numbers found positive, and these figures expressed as percentages. In order to shorten the table, the species have been grouped. The full list of species would have comprised 83 identified species, representing 20 families in 11 orders of birds. The psittacines (members of the Order Psittaciformes) have been arranged together at the head of the table, in order to illustrate their dominant position. Of the overall total of 196 positive isolations, 139 were from psittacines.

Pigeons and doves were also sampled in large numbers, and gave similarly high percentages positive.

Smaller numbers of positives were found among turkeys, game birds, finches, canaries, and domestic ducks. The figure does not include the isolations from duck carcases in slaughterhouses and ducks on farms which were obtained from the special investigation reported by Bracewell & Bevan (1982).

Table 1. *Summary of annual submissions of avian material for culture of* C. psittaci

	1976	1977	1978	1979	1980	1981	1982	1983	1984	Total
No. of samples tested	98	84	109	90	116	147	170	307	410	1531
No. of samples positive	14	11	10	1	1	13	27	46	73	196
Percentage of samples positive	14·3	13·1	9·2	1·1	0·9	8·8	15·9	15·0	17·8	12·8

Table 2. *Species distribution of samples cultured for* C. psittaci

Psittacines	No. of samples tested	No. of samples positive	Percentages of samples positive
Macaw	14	0	0·0
Cockatoo	17	2	11·8
African grey parrot	106	22	20·8
Amazon parrot	87	19	21·8
Other parrot	258	45	17·4
Parakeet	83	25	30·1
Lorikeet	2	0	0·0
Cockatiel	92	9	9·8
Conure	9	2	22·2
Love-bird	17	2	11·8
Budgerigar	152	13	8·6
Total	837	139	16·6
Non-psittacines			
Finch	40	3	7·5
Canary	59	3	5·1
Mynah	9	0	0·0
Exotic pigeon	10	1	10·0
British game birds	17	4	23·5
Collared dove and wood pigeon	52	13	25·0
Racing or feral pigeon	192	23	12·0
Chicken	24	0	0·0
Turkey	107	8	7·5
Duck	109	2	1·8
Goose	14	0	0·0
Other birds	61	0	0·0
Total	694	57	8·2
Grand total	1531	196	12·8

Isolation rates from different organs

Table 3 shows the organs examined, those identified being listed in order of their percentage positive. Overall, 1883 sampled yielded 244 positive isolations, equivalent to 12·96% positive

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Table 3. *Isolation rates of* C. psittaci *from organs*

Organ	No. of cultures examined	Number positive	Percentage positive
Intestines	103	21	20·4
Spleen	371	64	17·3
Trachea (or swab)	157	26	16·5
Heart	50	8	16·0
Lung	211	33	15·6
Liver	325	50	15·4
Air sac	35	5	14·3
Oviduct	14	2	14·3
Nasal swab	19	2	10·5
Kidney	30	2	6·7
Eye	40	2	5·0
Cloacal swab	157	7	4·5
Faeces	160	6	3·8
Brain	12	0	0·0
Unidentified	199	16	8·0
Total	1883	244	12·96

DISCUSSION

In 1980 there was a significant increase in the number of reports of *C. psittaci* infection in man (PHLS Communicable Disease Surveillance Centre, 1981). This coincided with the occurrence, in 1979 and 1980, of a number of cases clearly associated with duck-processing plants, involving both regular plant workers and a group of veterinary surgeons visiting a plant for training (Andrews, Major & Palmer, 1981; Ministry of Agriculture, 1981; Palmer, Andrews & Major, 1981; Bracewell & Bevan, 1982). The increased awareness of chlamydiosis as a zoonosis which followed was probably the main cause of the rising number of submissions which have been received by this laboratory since 1980. The percentage positive rose at the same time. This could perhaps be explained by increasing expertise in recognising the disease in the field, and in selecting and despatching suitable samples. The use of gentamicin in the transport medium from 1980 (Bracewell & Bevan, 1982) probably helped considerably to improve isolation rates.

Psittacines have traditionally been regarded as the Order of birds presenting the greatest danger to man. In investigations of suspect psittacosis in man it is to be expected that psittacines will receive the greatest attention. In this study they did provide more samples than all other orders combined (837 out of a total of 1531). It appears that this attention was not misplaced, because the percentage positive (16·6) was higher than from the non-psittacines (8·2). The great majority of the positives obtained from submissions accompanied by a history of disease in man were from psittacines. In contrast, nearly all the positives from birds of other orders were accompanied by histories of disease in the birds themselves, and not in man. However, it is known that humans may become infected from birds of other orders, so it is worth pointing out the especially high proportion of positives found in pigeons and doves, and mentioning the occurrence of positives in turkeys, game birds, finches, canaries, and ducks.

The comparative success rates for isolation from the variety of organs sampled (Table 3) should be treated with caution because the work was not designed to make an objective assessment of the relative value of different sampling sites. For example the high rate given by the oviduct (14·3%) was obtained by only two positives out of 14 samples tested, and these samples themselves would have been biased towards success because they were from turkeys in breeding flocks that had suffered from loss of egg production.

However, where the number of samples of particular organs was much higher (over 100) it is considered that the success rates are probably sufficiently meaningful for them to be compared in a very broad way. Thus, it is probably correct to conclude that the samples obtained from living birds (swabs from the eye or cloaca, and faeces) were less successful than the samples obtained from dead birds; i.e. the samples of internal organs, including intestines, spleen, trachea, lung and liver.

The results illustrate the wide variety of sites in the bird's body from which chlamydia may be isolated, and also the variability of the occurrence in these sites.

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