

Antigenic Analysis of Feline and Bovine *Chlamydia psittaci* with Monoclonal Antibodies

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ABSTRACT. Monoclonal antibodies were established for antigenic analysis of feline and bovine *Chlamydia psittaci*. The monoclonal antibodies recognized lipopolysaccharide (LPS), 56–64, 84 or 86 kDa antigens. At least 5 antibody-binding sites were detected on LPS with the monoclonal antibodies. The 56–64 kDa antigen was suggested to have both polypeptide and carbohydrate antibody binding sites. Immunoblotting analysis of cat and cattle sera indicated that the 56–64 kDa antigen is an important antigen in host immune response. The monoclonal antibodies are extremely useful tools to analyse the structure and function of chlamydial antigens.—**KEY WORDS:** antigenic analysis, *Chlamydia psittaci*, monoclonal antibody.

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Chlamydia psittaci has been isolated from a variety of birds and animals including over 140 species of birds, cattle, sheep, goat, cats, mice, guinea pigs, muskrats and koalas [17, 22]. Chlamydial infection in domestic animals has mainly been reported in diseases of cattle and sheep, which include pneumonia, enteritis, conjunctivitis, polyarthrititis, encephalitis and abortion [21, 22]. In Japan, during the period from 1950 to 1960, Omori *et al.* [19] reported the isolation of chlamydial organism in pneumonia of goats, and diarrhea and encephalitis of cows. Recent serological and etiological investigations by our laboratory [12] and by Ishida *et al.* [14] suggested the persistence of chlamydial infection in cattle and sheep in Japan. Chlamydial infection in companion animals was at first reported in pneumonia of cats in England [1, 2], and then in the United States, Canada, Australia and other countries, and on dogs and horses [21, 22]. Lately, Wills *et al.* [24] reported conjunctivitis by *C. psittaci* in a colony of cats in a laboratory. Our serological investigation also indicated possible chlamydial infection in cats in Japan [11].

Antigenic structures of feline and bovine strains of *C. psittaci* are, however, not understood well, although knowledge on the structures is essential for resolving pathogenicity and immunogenicity of mammalian *C. psittaci*. Our genetic analysis indicated that mammalian *C. psittaci* differs from avian *C. psittaci* in antigenicity [8] and DNA-DNA homology [9]. Perez-Martinez and Storz classified

mammalian *C. psittaci* with polyclonal antisera [20]. DeLong and Magee distinguished into ovine abortion and ovine arthritis *C. psittaci* strains with 2 monoclonal antibodies [6]. In the present study, we investigated the immunochemical structure of feline and bovine *C. psittaci* and established monoclonal antibodies to characterize antigens of mammalian *C. psittaci*.

MATERIALS AND METHODS

Organisms: *C. psittaci* Fe/Pn-145 and Bo/Yokohama strains were used to prepare monoclonal antibodies. Purified elementary body (EB) and chlamydial outer membrane complexes (COMC) were prepared with differential and sucrose density gradient centrifugations, and differential extraction with sodium N-laurylsarkocinate, respectively [5, 8]. Protein assay was examined by the method of Lowry *et al.* [16].

Other materials: Re-LPS and lipid A of *Salmonella choleraesuis* serovar *minnesota* were kindly provided by Dr. K.-I. Amano (Akita University, Akita, Japan). *Acinetobacter calcoaceticus* (biovar anitratus) was the gift of Dr. Y. Ashihara (Kyorin University, Tokyo, Japan). Boiled extract of the *A. calcoaceticus* cells was obtained from bacterial suspension incubated in boiling water for 4 hrs followed by centrifugation at 10,000 rpm for 15 min.

Establishment of monoclonal antibody producing cell lines: A murine myeloma cell line, P3X63Ag8.653 (purchased from Flow Laboratory, Inc., Tokyo, Japan) was fused with immune spleen cells in 50% polyethylene glycol solution as de-

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scribed previously [10]. Specific antibody producing cells were screened by an enzyme-linked immunosorbent assay (ELISA) using COMC or Triton X-100 treated EB as a coating antigen [7, 10]. The antibody producing cells were cloned in Dulbecco's modified Eagle medium containing 0.35% agar and 20% fetal bovine serum. Cloned cells were injected into the peritoneal cavity of pristane primed BALB/c mice to produce ascites.

Gel electrophoresis and Western blotting: Components of EB were analyzed by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli [15]. SDS-PAGE for LPS was examined on proteinase K treated EB, and the gel slabs were stained with silver reagents as described by Hitchcock and Brown [13]. Procedures of electrophoretic transfer of components to a nitrocellulose sheet and detection of antigen-antibody complexes were described previously [8]. Molecular weights of each protein antigen were estimated using the molecular weight standard markers and prestained markers (Bethesda Research Laboratories, Inc., Gaithersburg, MD, U.S.A.). Molecular weight of the LPS antigen was estimated with the LPS of *S. choleraesuis* serovar *minnesota*.

ELISA with chemical pretreatments: The principal ELISA procedure was described previously [7, 10]. Antigens were prepared as follows: EB was incubated in phosphate-buffered saline containing 1% (w/v) Triton X-100 with or without 5% 2-mercaptoethanol (2-ME) or 20 mM dithiothreitol (DTT) at 37°C for 30 min.

Complement fixation and indirect immunofluorescence tests: Complement fixation test was done with sodium deoxycholate extracted antigen [11, 12]. Indirect immunofluorescence test was conducted as described previously [10]. Briefly, the infected cell suspension was dotted on Microtest slides and fixed in cold acetone for 20 min at -20°C. Monoclonal antibodies, which were diluted with 1% bovine serum albumin-phosphate buffered saline (BSA-PBS) (two-fold serial dilution from 1:100 to 1:12,800), were mounted on the slides and incubated for 1 hr at room temperature. After washing vigorously in PBS, the optimum dilution of secondary anti-mouse IgG labeled with fluorescence isothiocyanate was mounted and incubated for 1 hr at room temperature in the dark. The reciprocal of the highest antibody solution dilution giving specific fluorescence was regarded as the endpoint titer.

RESULTS

Monoclonal antibodies were prepared to analyse the antigens of feline and bovine *C. psittaci*. In the present study, we used Triton X-100 and 2-ME treated EB, formalin inactivated EB, and COMC as immunogen. A total of 45 hybridoma clones producing monoclonal antibodies to feline-derived Fe/Pn145 strain, and a total of 8 clones to bovine-derived Bo/Yokohama were established. All of the monoclonal antibodies were protein A binding IgG.

At first, structural polypeptides and other components of EB of feline strain was analysed by SDS-PAGE with Coomassie brilliant blue and silver staining (Fig. 1). Polypeptide patterns of feline Fe/Pn-145 and bovine Bo/Yokohama strains were reported previously [8]. Proteinase K digestion diminished almost all polypeptides (Fig. 1 Ac and Bc), although a component of 13 kDa was not digested by proteinase K but was stained with Coomassie brilliant blue. COMC of the feline strain contained 10, 39, 56-64, 84 and 86 kDa polypeptides. The 56-64 kDa polypeptide was detected as a diffused band between 56 to 64 kDa of molecular weight. LPS was detected in a region of approximately 5 to 7 kDa as a black band by silver staining (Fig. 1B). The band was not affected by proteinase K digestion but disappeared by sodium periodate oxidation. Sodium periodate oxidation affected the migration rate of various polypeptides in SDS-PAGE (Fig. 1). Antigens on EB of feline strain were investigated with rabbit antiserum by immunoblotting (Fig. 2A). At least 20 antigens were detected in a range of molecular weight 3 to 200 kDa. Their major antigens were 5-7, 16, 39, 56-64, 84 and 86 kDa. The 5-7 kDa antigen was LPS, because it was detected in proteinase K digested sample but not in periodate treated sample. The same results were obtained on structural polypeptides and other components of EB of Bo/Yokohama strain.

Anti-Fe/Pn145 monoclonal antibodies recognized LPS, 56-64 and 86 kDa antigens (Table 1 and Fig. 2). All of the anti-Fe/Pn145 monoclonal antibodies reacted with Sarkocyl insoluble antigen in a range of 1:400 to 1:12,800 in ELISA. Immunological reactivity of LPS antigen to the monoclonal antibodies was lost by sodium periodate oxidation but not proteinase K digestion of EB. Monoclonal antibodies to 56-64 kDa antigen and a monoclonal antibody to 86

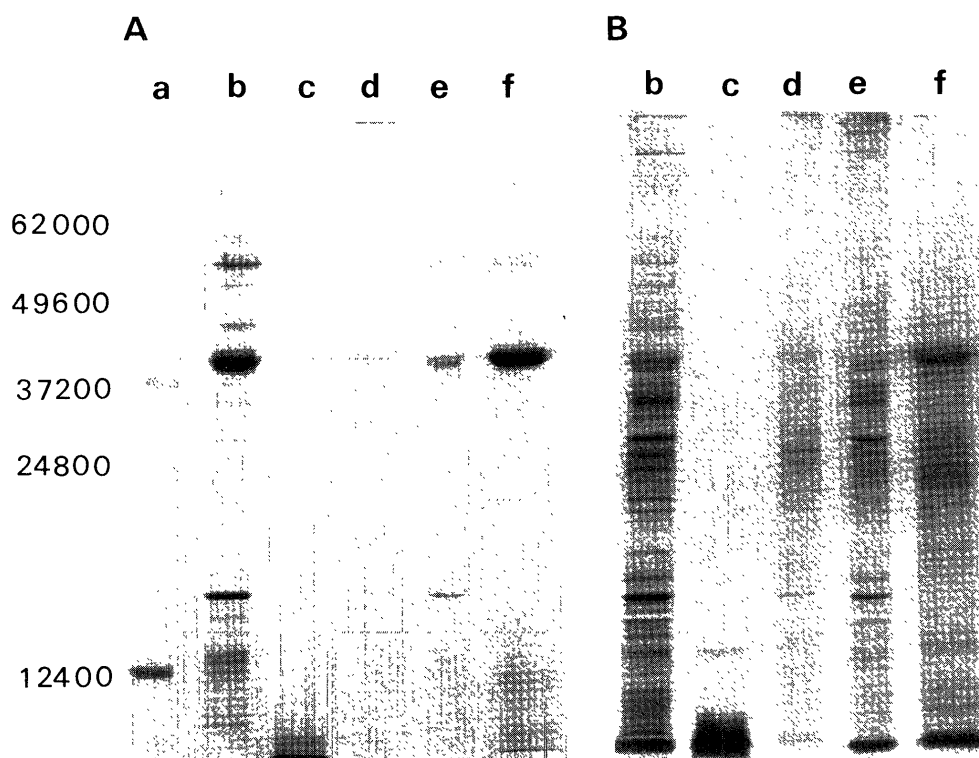


Fig. 1. Polypeptides and LPS composition of feline *C. psittaci* Fe/Pn 145 strain. Polypeptides were detected with Coomassie brilliant blue staining (A). LPS was detected with silver staining (B). Lanes are as follows: molecular weight markers (a), control elementary body (b), proteinase K digested elementary body (c), sodium periodate treated elementary body (d), acetic acid buffer-treated elementary body (e), and an insoluble fraction to Sarkocyl of elementary body (f).

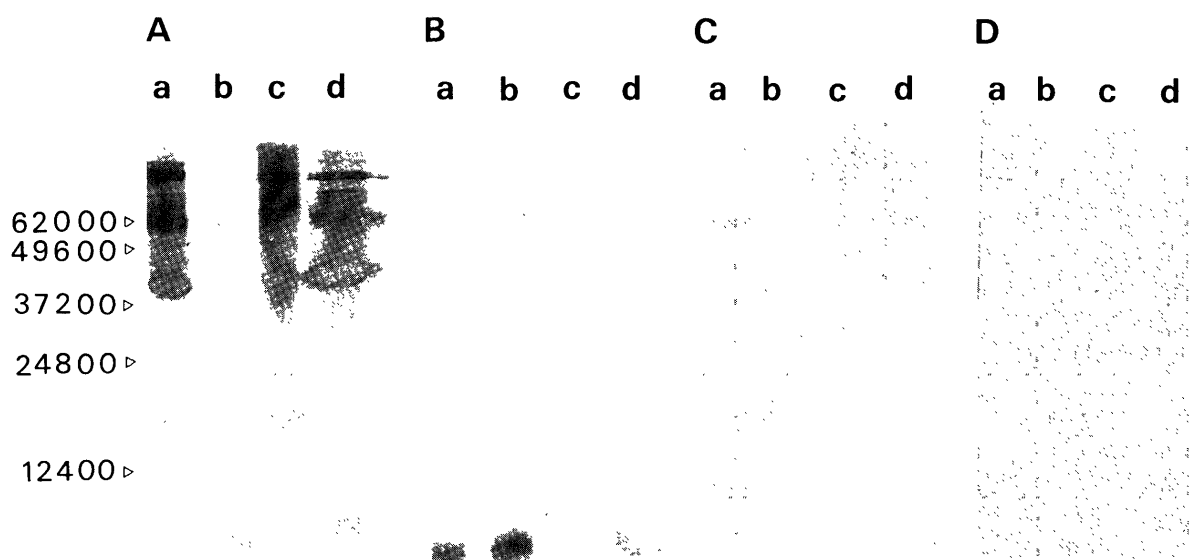


Fig. 2. Antigens of *C. psittaci* Fe/Pn145 strain detected by immunoblotting with hyperimmune serum (A), monoclonal antibodies of B-3 (B), B-5-1 (C), and C-6-2 (D). Lanes are as follows: control elementary body (a), proteinase K digested elementary body (b), sodium periodate treated elementary body (c), and an insoluble fraction to Sarkocyl of elementary body (d). The left most figures are positions and molecular weights of markers.

Table 1. Susceptibility to chemical treatments of feline and bovine *C. psittaci* antigens recognized by monoclonal antibodies

Monoclonal antibodies	Recognized Antigens	Untreated EB	Periodate Oxidized EB	proteinase K digested EB
Anti-Fe/Pn 145 monoclonal antibodies				
B-3 ^{*1}	LPS	12800 ^{*5}	<100	12800
B-5-1 ^{*2}	56-64 kDa	12800	400	3200
C-6-2	86 kDa	400	100	100
Anti-Bo/Yokohama monoclonal antibodies				
G-10-2	LPS	12800	<100	12800
A-10-1 ^{*3}	56-64 kDa	12800	12800	<100
G-3-1 ^{*4}	84 kDa	400	<100	<100

*1 And other 16 monoclonal antibodies

*3 And other 3 monoclonal antibodies

*2 And other 2 monoclonal antibodies

*4 And another monoclonal antibody

*5 Monoclonal antibody titers

Table 2. Reactivity patterns of the monoclonal antibodies to mammalian and avian *C. psittaci* and *C. trachomatis*

Monoclonal antibodies	Antigens							
	Fe/Pn145	Bo/Yokohama	Prt/GCP-1	Frt-Hu/Cal10	Hu/Itoh	Tk/NJ	Pigeon	Ct/L2
Anti-Fe/Pn145 anti-LPS								
A-11-2	1600 ^{*2}	1600	1600	800	800	1600	1600	1600
G-5-4	12800	12800	12800	12800	12800	12800	12800	12800
H-8-1	12800	12800	12800	12800	12800	12800	12800	12800
anti-56-64 kDa								
H-5	1600	<100	<100	<100	200	200	—	400
anti-86 kDa								
C-6-2	800	<100	200	200	200	200	200	200
Anti-Bo/Yokohama anti-LPS								
G-10-2	12800	12800	12800	12800	12800	12800	12800	12800
anti-56-64 kDa								
A-10-1 ^{*1}	+++ ^{*3}	+++	+	+	+	+	+	+
anti-84 kDa								
G-3-1 ^{*1}	—	+	—	—	—	+	+	—

*1 These monoclonal antibodies were analysed by immunoblotting because the monoclonal antibodies do not react in immunofluorescent antibody assay.

*2 Monoclonal antibody titer.

*3 Relative reactivities to the homologous reactions: similar signal (+++), weak signal (+), and no signal (—).

kDa antigen showed less reactivity with either proteinase K digested antigen or with sodium periodate oxidized antigen.

Anti-Bo/Yokohama monoclonal antibodies recognized LPS, 56–64 and 84 kDa antigens (Table 1). All of the anti-Bo/Yokohama monoclonal antibodies reacted with Sarkocyl insoluble antigen. Immunological reactivities of LPS and 56–64 kDa antigens of Bo/Yokohama were similar to those of Fe/Pn145. Reactivity of 84 kDa antigen to monoclonal antibodies was lost by both proteinase K digestion and sodium periodate oxidation.

Immunological cross reactivities of the monoclonal antibodies to avian and mammalian *C. psittaci* and *C. trachomatis* were preliminarily investigated by indirect immunofluorescence test and immunoblotting (Table 2). Monoclonal antibodies to Fe/Pn 145

LPS and Bo/Yokohama LPS showed genus-specific reactivity. The monoclonal antibody H5, one of the monoclonal antibodies to Fe/Pn145 56–64 kDa antigen, showed cross reactivity with Hu/Itoh, Tk/NJ and Ct/L2 strains. Immunological reactivities of anti-Bo/Yokohama monoclonal antibodies to 56–64 and 84 kDa antigens were investigated by immunoblotting, because they showed no reactivity in indirect immunofluorescence test. The monoclonal antibody to Bo/Yokohama 56–64 kDa antigen reacted with all of the strains examined. The monoclonal antibody to 84 kDa antigen reacted with Bo/Yokohama, Tk/NJ and Pgn/1041. The 56–64 kDa antigen of Bo/Yokohama was immunologically homologous to that of Fe/Pn 145 because of the cross reactivity of the anti-Bo/Yokohama monoclonal antibodies to the 56–64 kDa antigen. On the other

Table 3. Reactivities of the monoclonal antibodies against Re chemotype LPS, lipid A and an extracted antigen of *A. calcoaceticus* in immunoblotting

Monoclonal antibodies ^{a)}	Chlamydial LPS	Re LPS	lipid A	<i>A. calcoaceticus</i>
B-3 ^{b)}	+	—	—	—
G-5-1	+	+	—	—
G-5-4, H-3-1	+	+	+	—
A-4-2, H-3-2	+	—	+	—
F-3-1, F-3	+	+	—	+
G-10-2	+	—	—	+

a) Total 24 monoclonal antibodies against LPS were tested. Twenty three are the monoclonal antibodies to Fe/Pn145 LPS and one (G-10-2) is the monoclonal antibody against Bo/Yokohama LPS.

b) B3 and other 15 monoclonal antibodies.

Table 4. Distribution of recognized antigens of *C. psittaci* by cat and cattle sera

Animals	Samples	Positive	Antigens				
			LPS	31 kDa	39 or 40 kDa	56–64 kDa	84 or 86 kDa
Cat	31	16 (51.6%)	1/16 (6.2%)	1/16 (6.2%)	7/16 (43.7%)	11/16 (68.7%)	9/16 (56.2%)
Cattle	9	6 (66.6%)	0/6 (0%)	0/6 (0%)	1/6 (16.7%)	4/6 (66.7%)	5/6 (83.3%)

hand, monoclonal antibody to Fe/Pn 145 56–64-kDa antigen did not react with 56–64 kDa antigen of Bo/Yokohama. Therefore, at least 2 antibody binding sites were suggested on the 56–64 kDa antigen.

It is well known that chlamydial LPS antigen shows immunological cross reactivity to *A. calcoaceticus* and a part of rough type bacteria of Enterobacteria. We investigated the immunological cross reactivity between the chlamydial LPS and *A. calcoaceticus*, Re-LPS and lipid A using 24 monoclonal antibodies (Table 3). Sixteen monoclonal antibodies represented by B-3 reacted with chlamydial LPS only. One monoclonal antibody, G-5-1, reacted with chlamydial LPS and ReLPS. Two monoclonal antibodies, G-5-4 and H-3-1, reacted with chlamydial LPS, ReLPS and lipid A. Two monoclonal antibodies, A-4-2 and H-3-2, reacted with chlamydial LPS and lipidA. Two monoclonal antibodies, F-3-1 and F-3, reacted with chlamydial LPS, ReLPS and *A. calcoaceticus* antigen. One monoclonal antibody, G-10-2, reacted with chlamydial LPS and *A. calcoaceticus* antigen in immunoblotting.

Antigen profiles recognized by cat and cattle sera, which possessed CF and/or ELISA antibody, were investigated in immunoblotting to confirm immunogenicity of the LPS, 56–64 and 84 or 86 kDa antigens in host animals (Table 4). Sixteen out of 31

cat sera reacted with LPS, 31, 39, 56–64, and 86 kDa antigens. Six out of 9 cattle sera reacted with 40, 56–64 and 84 kDa antigens. These results indicate strong immunogenicity of 56–64 and 84 or 86 kDa antigens in host immune response.

DISCUSSION

We have established monoclonal antibodies to cat-derived Fe/Pn145 strain and cattle derived Bo/Yokohama strain for antigenic analysis. The monoclonal antibodies recognized LPS, 56–64 and 84 or 86 kDa antigens. Immunoblotting analysis with the monoclonal antibodies indicated the chlamydial LPS contains a structure similar to that of lipid A, which is the chemical skeleton of endotoxin reactivity. The immunoblotting analysis on cat and cattle sera indicated that these antigens induce the immunological response in infected host animals.

Most of the monoclonal antibodies established in the present study were against LPS antigen. Using these monoclonal antibodies, were detected at least five antibody-binding sites on LPS antigen, all of which showed different immunological specificities: (1) chlamydia-specific site, (2) chlamydia and *A. calcoaceticus* cross reactive site, (3) chlamydia, *A. calcoaceticus* and Re chemotype LPS cross reactive site, (4) chlamydia, Re chemotype LPS and lipid A

cross reactive site, and (5) chlamydia and lipid A cross reactive site. Our results indicated that antibody-binding sites on chlamydial LPS possess a structure chemically similar to enterobacterial Re-LPS and lipid A. Using purified LPS from *C. trachomatis* L2 strain and polyclonal antiserum, Brade *et al.* [2, 3] showed at least 2 antibody-binding sites on the LPS including chlamydia specific and chlamydia and Re chemotype LPS cross reactive sites. Caldwell and Hitchcock [4] showed at least 3 antibody-binding sites on the LPS of *C. trachomatis* L2 strain using a monoclonal antibody and antisera against Re chemotype LPS and lipid A. One of the 3 sites, which was recognized by the monoclonal antibody, was chlamydia specific, and the others were cross reactive to LPS from enterobacteria. The discrepancy in the number of antibody-binding sites in these works and our data would be in part due to the direction of the monoclonal antibodies binding to the antigen molecule as well as the number of sites themselves. Young *et al.* [25] described monoclonal antibodies showing varied binding direction to the same antigenic determinant. We did not investigate the effects of our monoclonal antibodies on biological properties of chlamydial LPS and chlamydia itself in the present study. Determining the chemical structure of the chlamydial LPS with the aid of monoclonal antibodies presented here will help resolving the structure and function of chlamydial LPS and the role of pathogenicity.

Antibody to the 56–64 kDa antigen was detected in cat and cattle sera examined at higher percentage than that of MOMP antigen. The 56–64 kDa antigen would be an important antigen in immune response of host animal. Our results with the monoclonal antibodies showed genus- and subgenus-specificities of the 56–64 kDa antigen, although our previous study indicated the 56–64 kDa antigen as a genus-specific antigen [8]. It is interesting that Newhall *et al.* [18] detected antibody to 60–62 kDa antigen in patient sera infected by *C. trachomatis*. They suggested the 60–62 kDa antigen was of value in serological diagnosis. Higher immunogenicity in host immune response and immunological specificity indicated that the 56–64 kDa antigen would be a useful target antigen for serological diagnosis of *C. psittaci* infection. Functions of the 56–64 kDa antigen are still unclear, although one of the monoclonal antibodies to 56–64 kDa antigen diminished a number of inclusion bodies in L cells (unpublished data). Further analysis is required to

resolve its biological function, the role in immune response and the diagnostic significance in seroepidemiology of the 56–64 kDa antigen.

The 84 or 86 kDa antigen would be a glycoprotein of the outer membrane because of its sensitivities to proteinase K digestion and periodate oxidation. Detailed immunochemical properties could not be identified due to the small number of monoclonal antibodies obtained. A role of the 84 or 86 kDa antigen during the infection and the growth cycle of *C. psittaci* should be analysed.

The monoclonal antibodies established in the present study will be an extremely useful tool to investigate the function and the immunochemical structure of *C. psittaci* EB antigens derived from cats and cattle, and also to classify mammalian *C. psittaci* strains.

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