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Immunochemical Diversity of the Major Outer Membrane Protein of Avian and Mammalian *Chlamydia psittaci*

HIDETO FUKUSHI* AND KATSUYA HIRAI

Department of Veterinary Microbiology, Faculty of Agriculture, Gifu University, Yanagido 1-1, Gifu 501-11, Japan

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Immunochemical properties of the major outer membrane protein (MOMP) of 16 strains of *Chlamydia psittaci* isolated from psittacine birds, budgerigars, a pigeon, turkeys, humans, cats, a muskrat, sheep, and cattle and a strain of *C. trachomatis*, L2/434/Bu, were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by immunoblotting analysis with hyperimmunized rabbit antisera to strains of parrot, turkey, feline, and bovine origin. The MOMPs of the strains showed variation in molecular weights and immunological specificities. Fifteen of the *C. psittaci* strains were classified into two avian and two mammalian types based on immunological specificity of the MOMP, whereas the other strain was not classified in this study. Immunological classification based on specificity of the MOMP by immunoblotting proved to be a valuable method to classify various strains of *C. psittaci*.

Chlamydia psittaci is an obligatory intracellular parasitic microorganism isolated from a variety of birds and animals (27). Pathogenicity of this microorganism varies with host animals and their physical conditions (35). Biochemical and immunological characterization of *C. psittaci* strains is insufficient in spite of their importance in the field of veterinary and medical science.

Caldwell et al. (6) described 16 antigens of *C. psittaci* elementary bodies (EBs) and 19 antigens of *Chlamydia trachomatis* EBs, although they did not investigate *C. psittaci* antigens in detail. Banks et al. (3) indicated that different avian isolates with a common source had similar antigenic reactivity by plaque reduction test. Schachter et al. (31, 32) classified bovine and ovine isolates into two serotypes, which were related to pathogenicity, by plaque reduction test. Spears and Storz (37) and Perez-Martinez and Storz (29) classified mammalian isolates into eight biotypes by inclusion body morphology and nine immunotypes by the microimmunofluorescence (MIF) test, respectively. However, the researchers did not analyze the biochemical and immunochemical basis of the classifications.

C. trachomatis, the other species of the genus *Chlamydia*, is classified into 15 serotypes by the MIF test (16). The type-specific antigen is believed to be the major outer membrane protein (MOMP), although recent analyses with monoclonal antibodies have shown that the MOMP possessed genus-, subgenus-, and subtype-specific determinants to add to type-specific determinants (4, 7). Therefore, it may be possible to classify a variety of *C. psittaci* strains according to the immunological specificities of the MOMPs.

We recently reported a monoclonal antibody typing system for *C. psittaci* (15). The typing system was mainly applied to avian isolates because the monoclonal antibodies used were prepared against a single avian strain. The results suggested an immunological diversity of avian *C. psittaci* and a vast difference between the avian and mammalian strains examined. The immunological relationships, however, among avian and mammalian *C. psittaci* strains are still unclear.

In the present report, we compared the polypeptide and antigen composition of chlamydial outer membrane complexes (COMCs), especially the MOMPs, of 16 strains of *C. psittaci* which were derived from various birds and mammals. The immunochemical analysis of the MOMPs indicated at least four types in the avian and mammalian *C. psittaci* strains examined.

MATERIALS AND METHODS

Chlamydiae. The *C. psittaci* and *C. trachomatis* strains examined are listed in Table 1. These strains were isolated in Canada, Japan, and the United States between 1936 and 1987. The strains obtained as infected yolk sacs were serially passaged to grow in cell culture three to five times. Most strains were directly adapted to L cells in suspension form. The Mu/M56 and Ov/IPA strains were grown in MDCK cells and HeLa 229 cells, respectively, at first. Both of the strains were finally propagated in L cells in suspension form. The Hu/Borg strain was adapted to grow in HeLa 229 cells, and its EBs were obtained in HeLa 229 cells. Two strains, Frt-Hu/Cal10 and *C. trachomatis* L2/434/Bu, were obtained as an infected L cell culture and a HeLa 229 cell culture, respectively, and were grown in each line of cells. The cells were treated with Eagle minimal essential medium (Nissui, Japan) containing 30 μg of DEAE-dextran (Pharmacia, Upsala, Sweden) per ml for 5 min at room temperature before adsorption. Maintenance medium for L cells in suspension form was 0.1% yeast extract, 0.5% lactalbumin hydrolysate, Earle balanced salt solution (38) supplemented with 5% fetal bovine serum, and that for other line cells was minimal essential medium supplemented with nonessential amino acids, 0.1% glucose, and 5% fetal bovine serum. Cycloheximide was supplied at 0.5 μg/ml for most strains.

Preparation of purified EBs and COMCs. EBs were prepared and purified as described by Tamura and Higashi (38) with minor modifications. In brief, a culture fluid was centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatant was saved, and the cell precipitate was homogenized in 0.25 M sucrose–10 mM potassium phosphate–5 mM glutamic acid, pH 7.4 (SPG) and centrifuged at 1,000 × *g* for 10 min. Both supernatants were combined and centrifuged at 10,000 × *g* for 1 h at 4°C. Precipitated crude EBs were suspended in a small volume of SPG. The suspension was sonicated for a few minutes and centrifuged through 30% sucrose–0.01 M Tris hydrochloride, pH 7.4 at 12,000 × *g* for 1 h at 4°C. The

* Corresponding author.

TABLE 1. Strains used

Strain	Host	Place of isolation	Yr of isolation	Known history	Source[a] (reference)
C. psittaci					
Prt/GCP-1	Parrot	Unknown[b]	1980	Systemic infection	A (40)
Prk/Daruma	Parakeet	Unknown[b]	1980	Systemic infection	A (40)
Ckt/Okame	Cockatiel	Unknown[b]	1980	Systemic infection	A (17)
Bd/275	Budgerigar	Aichi, Japan	1987	Normal	A (15)
Bd/286	Budgerigar	Aichi, Japan	1987	Normal	A (15)
Pgn/P1041	Pigeon	Hokkaido, Japan	1981	Normal	B (8)
Tk/CA	Turkey	California			C
Tk/NJ	Turkey	New Jersey			C
Hu/Borg (ATCC VR601)	Human[c]	Louisiana	1941	Pneumonia	D (28)
Hu/Itoh	Human[d]	Tokyo, Japan	1962	Psittacosis	E (18)
Frt-Hu/Cal 10	Ferret (human)[c]	USA	1938	Cold	F (12)
Fe/145	Cat	USA	1969	Conjunctivitis	C (34)
Fe/Pn-1 (ATCC VR120)	Cat	New Jersey	1942	Pneumonia	D (1, 2)
Mu/M56 (ATCC VR630)	Muskrat	Canada	1966	Systemic infection	D (36)
Bo/Yokohama	Cattle	Kanagawa, Japan		Diarrhea	E (19)
Ov/IPA (ATCC VR629)	Sheep	USA	1968	Polyarthritis	D
C. trachomatis L2/434/Bu	Human	USA	1971	Lymphogranuloma venereum	G (33)

[a] Sources of the strains were as follows: A, this laboratory; B, N. Hashimoto; C, J. Schachter; D, American Type Culture Collection; E, Y. Inaba; F, A. Matsumoto; G, National Institute of Health of Japan.
[b] These strains were isolated from imported birds in our laboratory, but the origins of the birds were not known.
[c] These strains were isolated from a human, and their epidermiological relationships to avian strains were obscure (12, 28).
[d] This strain was isolated from a patient with psittacosis whose budgerigar had transmitted the agent.

precipitate was resuspended in SPG and loaded on 30 to 60% (wt/wt) sucrose linear density gradients. After ultracentrifugation at 35,000 rpm for 2 h at 4°C, a white band located at the middle of the gradient was collected with a Pasteur pipette. The fraction was diluted with 0.01 M Tris hydrochloride, pH 7.4, and centrifuged at 12,000 rpm for 1 h at 4°C. The precipitate of purified EBs was finally suspended in a small volume of 0.01 M Tris hydrochloride, pH 7.4, and stored at −80°C until use.

COMCs were prepared as EB material insoluble by sodium *N*-lauryl sarcosinate (5). The protein concentration of each purified preparation was measured by the method of Lowry et al. (23) with bovine serum albumin (BSA) as the standard.

Preparation of hyperimmunized rabbit antisera. Antisera to four strains, including Prt/GCP-1, Tk/NJ, Fe/145, and Bo/Yokohama, were prepared as described by Caldwell et al. (6). Briefly, 100 μg of EB protein was treated with 1% Triton X-100 in 1 ml of phosphate-buffered saline (PBS) at 37°C for 1 h and sonicated. The EBs were emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) and given intramuscularly. After 3 weeks, a series of five intravenous injections of treated antigen were given at 3-day intervals for the first four injections and 7 days for the fifth, in progressively increasing doses (10, 10, 15, 20, and 50 μg of protein). Rabbits were bled 1 week after the last injection.

All the antisera were titrated by the enzyme-linked immunosorbent assay described elsewhere (14). Titers of the antisera were 1:12,800 in this assay.

PAGE and immunoblotting. Polypeptide profiles of EBs and COMCs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (22). Polypeptide bands were visualized by Coomasie brilliant blue R-250 staining. Apparent molecular weights were calibrated with molecular weight standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

Antigens were detected by immunoblotting with the hyperimmune antisera. After SDS-PAGE, the SDS-polyacrylamide gel was layered on a nitrocellulose sheet and electrophoretically transferred with Semi-Dry Electroblotter (Sartorius GmbH, Göttingen, Federal Republic of Germany) by the method of Kyhse-Andersen (21). The nitrocellulose sheet was dipped in PBS containing 3% BSA at 4°C overnight. Then the sheet was incubated with the hyperimmune antiserum, which was diluted at 1:20 with PBS containing 0.3% BSA, at 4°C overnight. Then the sheet was incubated with horseradish peroxidase-conjugated *Staphylococcus aureus* protein A (7, 31) in PBS containing 0.3% BSA at 37°C for 2 h. Finally, the sheet was incubated in 0.5 mg of 4-chloro-1-naphthol per ml and 0.009% H_2O_2 in 16.7% (vol/vol) methanol–0.01 M Tris hydrochloride (pH 7.2) (11).

RESULTS

Comparison of polypeptide profiles. We first compared constituents of EBs of 16 *C. psittaci* strains and a *C. trachomatis* strain by SDS-PAGE to ascertain biochemical relationships among *C. psittaci* strains. However, the complex polypeptide composition (over 50 polypeptides) made strain comparison difficult. Therefore, the COMCs were chosen because of the simple composition, which included the MOMP, a 56,000- to 64,000-M_r (56K to 64K) polypeptide, and several other polypeptides (Fig. 1). A distinct difference was noted in the molecular weights of the MOMPs of each strain (Fig. 1, Table 2). The *C. psittaci* were classified into two groups based on their MOMP molecular weights. A group which consisted of most of the avian isolates and the L2 strain possessed the 39.2K to 40K MOMP, whereas the other group, which consisted of three avian isolates and all the mammalian isolates, had 38K to 38.5K MOMP.

Comparison of immunological specificities of the MOMP. We prepared antisera to four strains of *C. psittaci*, including Prt/GCP-1, Tk/NJ, Fe/145, and Bo/Yokohama, which were chosen as representatives of avian and mammalian *C. psittaci*. Immunoblotting analysis with the antisera showed several antigens with molecular weights of under 10,000, 38,000 to 40,000, and 56,000 to 64,000 and some other minor

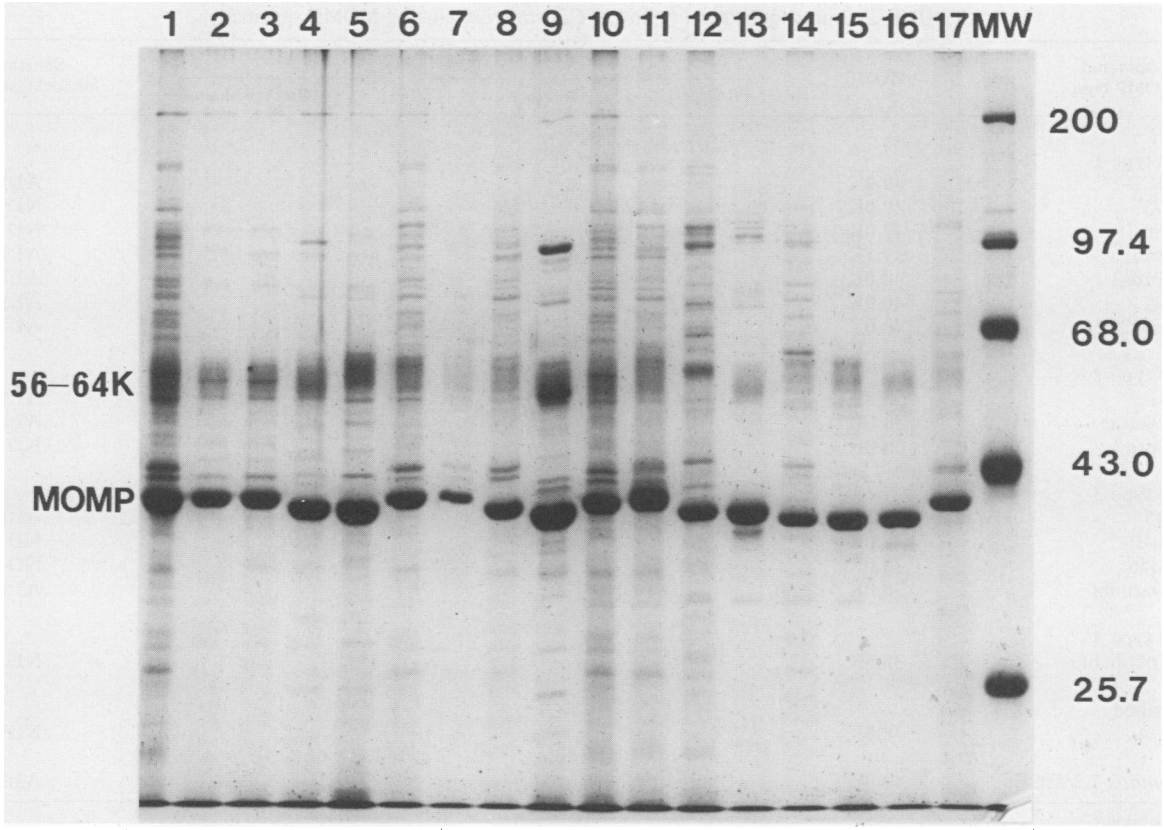

FIG. 1. SDS-PAGE of COMC of chlamydiae. Lanes 1 to 16 are *C. psittaci* strains Prt/GCP-1, Bd/275, Bd/286, Ckt/Okame, Prk/Daruma, Pgn/P1041, Tk/CA, Tk/NJ, Hu/Borg, Hu/Itoh, Frt-Hu/Cal 10, Bo/Yokohama, Ov/IPA, Fe/145, Fe/Pn-1, and Mu/M56, respectively: lane 17 is *C. trachomatis* L2/434/Bu; lane MW, molecular weight standards. The molecular weights of the standards are shown on the right (in thousands): myosine H-chain (200,000), phosphorylase B (97,400), bovine serum albumin (68,000), ovalbumin (43,000), and α-chymotrypsinogen (25,700). Variation in the molecular weights of the MOMPs was observed. The 56K to 64K polypeptides were other prominent ones of the COMC.

antigens in the EBs (Fig. 2). The antigen of molecular weight 38,000 to 40,000 was the MOMP and showed no cross-reactivity among the four strains, although other antigens, including a 56K to 64K antigen, showed the common immunological specificity (Fig. 2). Hence, we immunologically classified the 16 strains of *C. psittaci* according to the specificity of the MOMP.

The 16 strains were classified into four types, MOMP types 1 to 4, and an unclassified strain (Table 2). MOMP types 1 and 2 included avian isolates, while MOMP types 3 and 4 consisted of mammalian isolates and an avian strain. The MOMPs of avian and human chlamydiosis strains were reacted with anti-Prt/GCP-1 (MOMP type 1) or anti-Tk/NJ (MOMP type 2) but not with the other antisera except for strain Prk/Daruma, the MOMP of which reacted with the anti-Fe/145 serum (MOMP type 3). The MOMPs of two feline strains and a muskrat strain reacted with the anti-Fe/145 serum (MOMP type 3), and the MOMP of Bo/Yokohama reacted with the homologous antiserum (MOMP type 4). The MOMP of L2/434/Bu reacted with the anti-Prt/GCP-1, whereas the MOMP of the other ruminant strain did not react with any antiserum examined.

DISCUSSION

We established an immunological typing system for *C. psittaci* based on the immunological specificity of the MOMP. The MOMP types found in the present study related to their host of origin; MOMP types 1 and 2 were avian strains, and MOMP types 3 and 4 were mainly mammalian strains. This immunological typing scheme provides an essential tool to investigate the epidemiological and immunological properties of *C. psittaci*.

The results obtained in the present study are in good agreement with those of previous studies on the immunological relationships among a variety of strains. The immunological differences which were clearly shown between the avian and mammalian strains examined in the present study have been indicated by other researchers with the immunofluorescence cross-absorption test (30), plaque reduction test (3), MIF test (9), and cross-immunity test (26). Furthermore, the presence of immunotypes in avian strains has been suggested by the toxin neutralization test (24) and plaque reduction test (3). Serotypes 1 and 2 have been recognized in bovine and ovine strains by the plaque reduction test (31, 32) and MIF test (10). But we could not know the relationship between the MOMP types presented in this study and serotypes 1 and 2, because the bovine and ovine strains used in this study have not been compared with representative strains of the two serotypes shown by the plaque reduction test. Immunological typing of *C. psittaci* of mammalian origin was also reported by Perez-Martinez and Storz (29). The only strain commonly used in their work and ours was Fe/Pn-1. Hence, direct comparison of the results was not possible. The correspondence between the MOMP types found in the present study and the immunotypes found by

TABLE 2. Immunochemical typing of *C. psittaci* strains by MOMP specificity

Strain and MOMP type	MOMP	Reactivity with antiserum				Serovar with monoclonal antibody
		Prt/GCP-1	Tk/NJ	Fe/145	Bo/Yokohama	
C. psittaci						
MOMP type 1						
Prt/GCP-1	40.0K	+	−	−	−	A1:Pa
Bd/275	40.0K	+	−	−	−	ND[a]
Bd/286	40.0K	+	−	−	−	ND
Hu/Itoh	39.2K	+	−	−	−	A1:Pa
Pgn/P1041	40.0K	+	−	−	−	A1:Pa
Tk/CA	40.0K	+	−	−	−	A1:Pb
Frt-Hu/Cal10	40.0K	+	−	−	−	A1:Pb
MOMP type 2						
Tk/NJ	38.5K	−	+	−	−	A1:Pc
Ckt/Okame	38.0K	−	+	−	−	A1:Pa
Hu/Borg	38.0K	−	+	−	−	ND
MOMP type 3						
Fe/145	38.0K	−	−	+	−	A1:Pc
Fe/Pn-1	38.0K	−	−	+	−	ND
Mu/M56	38.0K	−	−	+	−	ND
Prk/Daruma	38.0K	−	−	+	−	A1:Pa
MOMP type 4						
Bo/Yokohama	38.5K	−	−	−	+	ND
Unclassified						
Ov/IPA	38.5K	−	−	−	−	ND
C. trachomatis L2/434/Bu	40.0K	±	−	−	−	A3:Pc

[a] ND, Not done.

Perez-Martinez and Storz may be revealed by the immunoblotting and MIF tests with the same set of strains.

The three strains related to human psittacosis all belonged to the avian MOMP types; Hu/Itoh and Frt-Hu/Cal 10 were included in MOMP type 1 and Hu/Borg was in MOMP type 2. These results seem reasonable. Hu/Itoh was isolated from a psittacosis patient who had had contact with an infected budgerigar from which *C. psittaci* was isolated (18). Al-

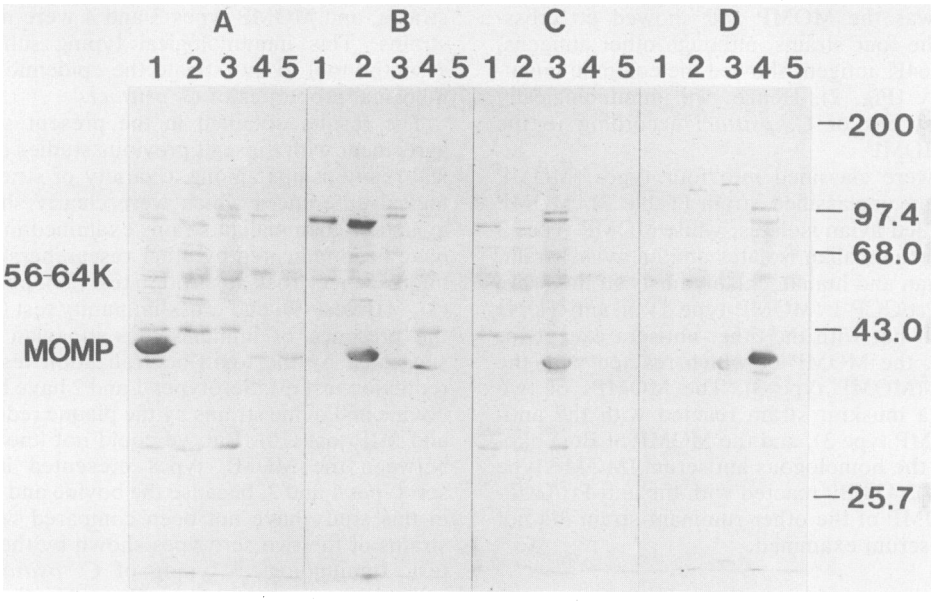

FIG. 2. Immunoblots of four *C. psittaci* strains, Prt/GCP-1 (lanes 1), Tk/NJ (lanes 2), Fe/145 (lanes 3), and Bo/Yokohama (lanes 4), and *C. trachomatis* L2/434/Bu (lanes 5) with antisera to Prt/GCP-1 (A), Tk/NJ (B), Fe/145 (C), and Bo/Yokohama (D). The MOMPs of the four strains showed little cross-reactivity. The 56K to 64K bands were common to all the *Chlamydia* spp., although variation in the molecular weight of this antigen was observed. The positions and molecular weights (in thousands) of the molecular weight standards are shown on the right. The standards are the same as in Fig. 1.

though no relationship between the Frt-Hu/Cal10 and Hu/Borg strains and birds or animals had been found in epidemiological studies (12, 28), later investigations indicated close relationships between Hu/Cal 10 and pigeons and among Hu/Borg and Tk/NJ and egrets (25). Recently, the *C. psittaci* strains which are completely human specific were isolated and designated the TWAR strains (20). The uniqueness of the TWAR strain was recognized by the specific reactivity of a monoclonal antibody to the strains and not investigated with conventional polyclonal antibody. It would be interesting to determine the MOMP type of the TWAR strains by the immunochemical typing system described here.

The close relationships between the feline and muskrat strains of MOMP type 3 were unexpected. Spalatin et al. (36) described the immunological uniqueness of the muskrat strain from the results of the type-specific complement fixation test by Fraser and Berman (13), although they found a weak cross-reaction between Mu/M56 and Fe/Pn-1. Immunoblotting analysis used in the present study was a qualitative, all-or-none assay. Therefore, quantitative analysis would reveal the difference between the two. This could be applied to other strains belonging to a single MOMP type.

The MOMP types established in this study do not always correspond to the serovars defined by the monoclonal antibody typing system in our previous study (15). This discrepancy may be caused by use of different antigens and antibodies in the two systems; the previous monoclonal antibody typing system was based on the combined immunological specificity of two antigens, including the lipopolysaccharide and the MOMP antigens, and only two monoclonal antibodies to the MOMP were used. The MOMP types established in the present study will be further divided into subtypes by application of more monoclonal antibodies to the MOMP of the representative strains of each MOMP type, as indicated by Wang et al. (39) in *C. trachomatis*.

In this study, we ignored the minor polypeptides of the COMC in spite of their different molecular weights among the strains examined, as shown in Fig. 1. It is obvious that these differences in the minor polypeptide profile would have the potential for further classification of the strains. The classification will provide useful epidemiological markers.

The immunological typing system newly established in this study is a simple and feasible method. An immunoblotting method has definite advantages in that pure samples and monospecific antiserum are not always required and that objective judgment is possible. Also, no special apparatus such as a fluorescence microscope is needed. Our recent DNA fingerprinting analysis shows good agreement with both MOMP and genetic types (H. Fukushi and K. Hirai, manuscript in preparation). Therefore, the MOMP types will serve as good markers for strain identification by expanding our immunological typing system to include the TWAR strains and more mammalian strains. We hope to establish a systematic immunological classification of *C. psittaci*.

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