Microbiology of Chlamydiae —— with Emphasis on Physicochemistry, Antigenicity and Drug Susceptibility of *Chlamydia pneumoniae* ——

Naoyuki MIYASHITA and Akira MATSUMOTO

Department of Microbiology,
Kawasaki Medical School, Kurashiki 701-01, Japan

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Chlamydiae, obligate intracellular parasites, have been assigned to a single genus, *Chlamydia*, which comprises four spicies, *Chlamydia trachomatis*,[1] *C. pneumoniae*,[2] *C. psittaci*[1] and *C. pecorum*.[3] These organisms multiply through a common, unique developmental cycle in which there are two morphologically and functionally distinct forms: one is the infectious elementary body (EB) and the other is the reproductive reticulate body (RB). The EB penetrates a susceptible host cell by phagocytosis and is converted into an RB, which multiplies by binary fission. Then the RB undergoes maturation through an intermediate body, to form mature progeny EB.[4]

C. trachomatis and *C. pneumoniae* are human pathogens, whereas *C. psittaci* and *C. pecorum* are animal pathogens. However *C. psittaci* causes a severe respiratory disease, psittacosis, in humans. Recent studies of chlamydial infections, especially the sexually transmitted diseases (STD) caused by *C. trachomatis*, revealed the epidemiological status of these infections, which should not be ignored.[5] Reflecting on this status, many test kits for the detection of *C. trachomatis* organisms in clinical specimens have been developed. However, their sensitivity and specificity in the genus *Chlamydia* have not been well investigated.

The prevalence of *C. pneumoniae* infections has a similar, worldwide status to that of *C. trachomatis* infections.[6] However, accumulation of knowledge on the basic microbiology of the former organisms, such as their physicochemical and immunochemical features, has been hampered by the difficulty in isolating them from clinical specimens. Nevertheless, our *Chlamydia* research group was recently able to isolate and maintain several *C. pneumoniae* strains. Consequently, we were able to investigate their microbiological characteristics.

As members of the *chlamydia* research group, we have summarized the results obtained from a series of experiments done over the last four years in this mini-review.

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Ⅰ. Chlamydial strains

The chlamydial strains used in this study are shown in Table 1. *C. pneumoniae* TW-183, AR-39 and AR-388 were purchaced from the Washington Research Foundation (Seattle, WA, USA) and have been maintained in HeLa 229 cells, as have the other *C. pneumoniae* and *C. trachomatis* strains. The Cal 10 strain of *C. psittaci* has been maintained in our laboratory for more than 20 years in the L929 cell line. *C. trachomatis* D/UW-3/Cx and L_2/434/Bu, and *C. psittaci* Budgerigar-1 strains were supplied by the National Institute for Health, Japan, and have been maintained in HeLa 229 or L929 cell lines. The Bo/E58 strain of *C. pecorum* was supplied by Dr. K. Hirai, Faculty of Agriculture, Gifu University and was cultured continuously in the MDBK cell line.

TABLE 1. Chlamydial strains used

species	strain	origin*	reference
C. pneumoniae			
round-shape	KKpn-15	Human, bronchitis, Japan	(7)
	KKpn-1	Human, bronchitis, Japan	(8, 9)
	IOL-207	Human, conjunctivitis, Iran	(10, 11)
	Kajaani-6	Human, pneumonia, Finland	(12)
	YK-41	Human, bronchitis, Japan	(13, 14)
pear-shape	TW-183	Human, conjunctiva, Taiwan	(15, 16)
	AR-39	Human, pharingitis, USA	(15, 16)
	AR-388	Human, ARD, USA	(15, 16)
C. trachomatis	D/UW-3/Cx	Human, cervix, USA	(17)
	L_2/434/Bu	Human, LGV, USA	(18)
C. psittaci	Frt-Hu/Cal 10	Ferret-human, cold, USA	(19)
	Bud/Budgerigar-1	Budgrigar, psittacosis, Japan	(20)
C. pecorum	Bo/E58	Cattle, encephalitis, USA	(21)

* Host (human, lower mammal, or bird), clinical condition or anatomic site, and/or geographic origin. Abbreviations: ARD, acute respiratory disease; LGV, lymphogranuloma venereum

Ⅱ. Purification of EB and EB outer membrane

Because of the low level of synchrony in the chlamydial multiplication stage, EBs and RBs are released at the time when the host cells disintegrate during the late stage of infection. Both EB and RB consist of almost identical components, but the quantity of the components is constant in EBs, whereas it varies in RBs. To get clear results in the many experiments carried out in this study, we established a purification method by which *C. trachomatis* and *C. pneumoniae* EBs were obtained in high purity.[22] Our preliminary experiments indicated that fractions obtained from the cells infected with *C. pneumoniae* and *C. trachomatis* strains by the method of Tamura & Higashi[23] or Caldwell *et al*[24] alone contained a number of RBs and their fragments. Therefore, we

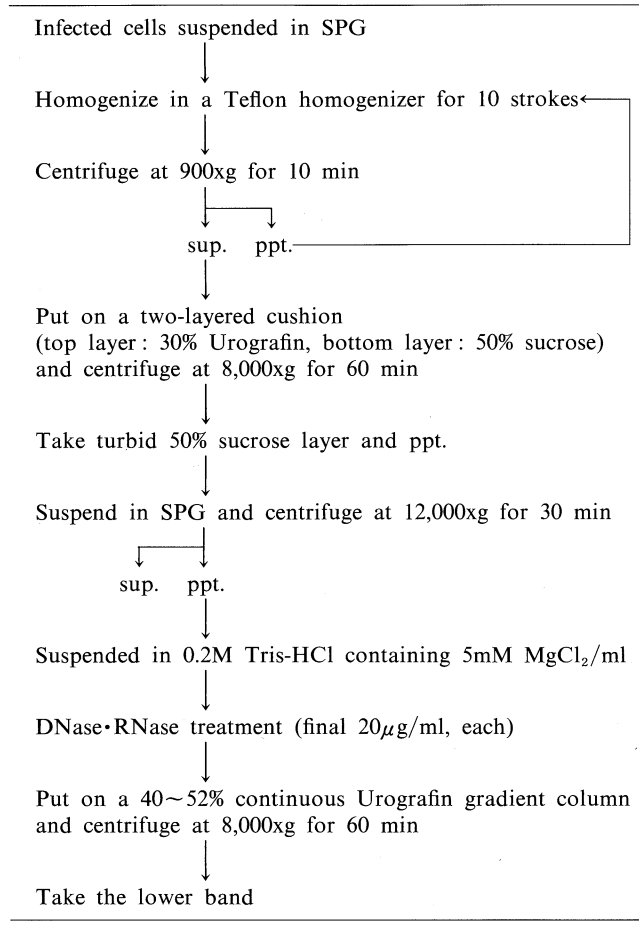

Fig 1. Procedures for purification of EBs of *C. pneumoniae* and *C. trachomatis* strains. Sup. means supernatant and ppt. means precipitate. SPG : Sucrose-phosphate-glutamate

applied a new method for the purification of *C. pneumoniae* and *C. trachomatis* EBs,[22] as shown in Fig 1. The features of this method are two-layer cushioning centrifugation (50% sucrose and 30% Urografin), followed by continuous Urografin gradient centrifugation (40 to 52%). No protenase treatment was done for maintenance of the surface antigen. To enhance the purity of EB preparations, the Urografin gradient centrifugation was repeated three times. Electron microscopy revealed that no debris derived from the host cells or RBs was contained in the EB preparations[22] (Fig 2). On micrographs taken randomly, the rations of intact EBs in individual samples were determined to be almost 100%, 100% and 97~98% in the *C. psittaci*, *C. trachomatis* and *C. pneumoniae* EB suspensions, respectively.

Outer membranes were isolated from the purified EBs by using a 15% sucrose cushion after shaking with 6 g of glass beads (0.1 mm in diameter) and treatment with 0.125% sodium dodecylsulphate (SDS).[14] In a preliminary experiment, it was noted that the EB outer membranes of *C. pneumoniae*

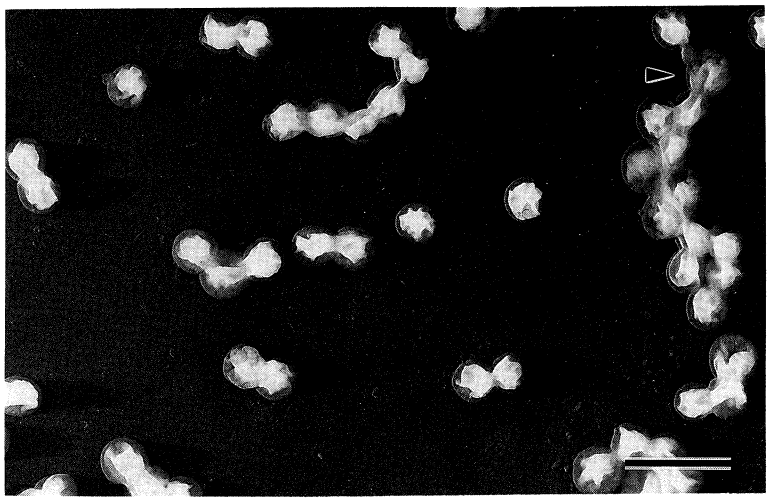

Fig 2. Shadowcast of purified EBs of *C. pneumoniae* KKpn-15 strain. EBs were air-dried on a smooth-surfaced agar plate, transferred to a specimen grid by the pseudoreplica method with collodion, and then shadowcast with Pt-palladium alloy. A few envelopes (arrowhead) are seen in the *C. pneumoniae* EB preparation. The bar indicates 1 μm.

strains broke into small pieces when treated with 0.5% SDS, whereas those of the EBs of strains *C. psittaci* and *C. trachomatis* did not. This suggested a difference in the outer-membrane stability between *C. pneumoniae* and the other two species. Therefore, the SDS concentration was reduced to 0.125% for isolation of the outer membranes of *C. pneumoniae* strains.

Ⅲ. Morphology of EBs and outer membranes

The chlamydial bodies *in situ* inclusions of the *C. pneumoniae* strains at 60 hr postinoculation are shown in Fig 3.[14,25] The EBs of the strains, KKpn-15, KKpn-1, IOL-207, Kajaani-6 and YK-41 (Fig 3a), were round in shape[7,9,11,12,14] with no clear difference in morphology from *C. pneumoniae*, *C. psittaci* and *C. trachomatis* strains. In contrast, the EBs of the TWAR strains had a wide periplasmic space limited by a wavy outer membrane, forming "pear-shaped" profiles[16] (Fig 3b). These results strongly suggest that the "pear-shaped" profiles of EBs in thin sections are not common in *C. pneumoniae* and that the pear-shape is not a morphological criterion for recognizing *C. pneumoniae*.

Further observations on the distribution of host mitochondria in the host cells infected with the *C. pneumoniae* strains revealed no mitochondrial association with the inclusion membrane in any of the *C. pneumoniae* strains examined[7,14,25] (Fig 3c, 3d arrowheads). Therefore, it is likely that the absence of the mitochondria-inclusion association is common during the multiplication of *C. pneumoniae*.

To examine the EB envelope and the inclusion membrane, freeze-replicas of the inclusion-bearing cells were prepared at 60 hr postinoculation.[14,26] As shown in Fig 4a, the cleaved faces of the EBs and RBs were exposed as convex or concave faces in the replica membrane. Button structures (B structures)[26] or the craters[27] were clearly seen on a limited area of the concave faces[14] (Fig 4a,

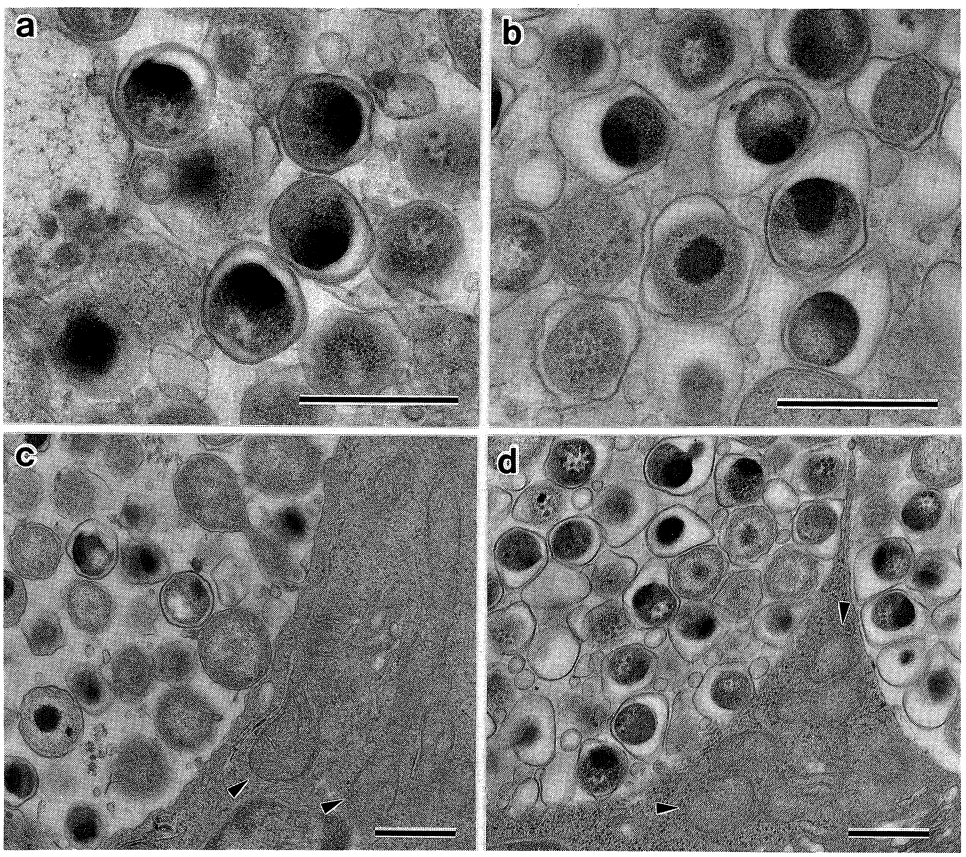

Fig 3. Thin sections of KKpn-15 and AR-39 strains in HeLa 229 cells at 60 hr postinoculation. a, *C. pneumoniae* KKpn-15; b, *C. pneumoniae* AR-39 at higher magnification. KKpn-15 EBs have a narrow periplasmic space and are round in shape, whereas AR-39 EBs are enclosed by a wavy outer membrane and are "pear-shaped" in profile; c, KKpn-15 inclusion; d, AR-39 inclusion. Arrowheads indicate mitochondria located close to inclusions, but not associated with the inclusion membrane. Bars indicate 500 nm.

arrowheads). When the inclusion membrane was replicated, many fine particles grouped together within several areas were frequently observed on the convex face of the inclusion membrane[14] (Fig 4b, arrowheads). The morphological properties of the B structures on the concave faces of *in situ* chlamydial bodies and the particles on the convex faces of inclusion membrane were identical with those mentioned previously.[4] Therefore, the results obtained in the present study strongly suggested the presence of projections on the surface of the *C. pneumoniae* EBs and RBs in inclusions *in situ*.

To confirm directly the presence of projections on the EBs of *C. pneumoniae* strains, purified EBs were dried by the critical point drying method and examined by scanning electron microscopy.[28] Projections were observred on a limited area of the EB surface of *C. pneumoniae* strains[14] (Fig 5). The results, together with those on *C. psittaci* and *C. trachomatis* EBs,[4] strongly suggest that these projections are common to all members of the genus

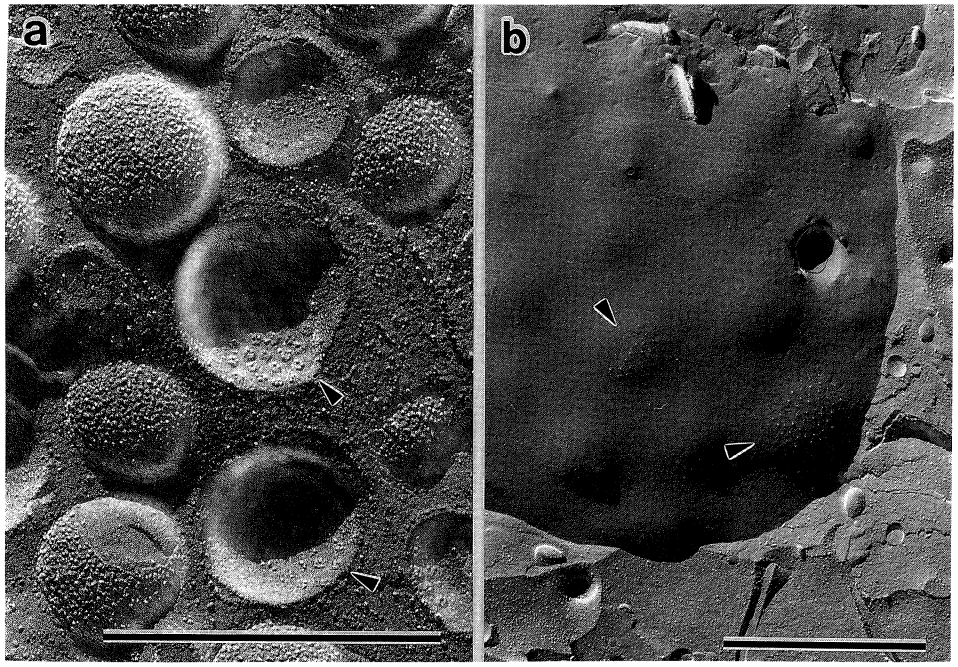

Fig 4. Freeze-replica images of *in situ* chlamydial bodies and inclusion membranes of strain KKpn-15 at 60 hr postinoculation. a, Chlamydial bodies are cleaved into convex or concave faces. Many B structures are seen (arrowheads); b, the inclusion membrane exposed by cleavage. Particles in groups are indicated with arrowheads. The face shows reggendness along RB outlines. Bars indicate 1 μm.

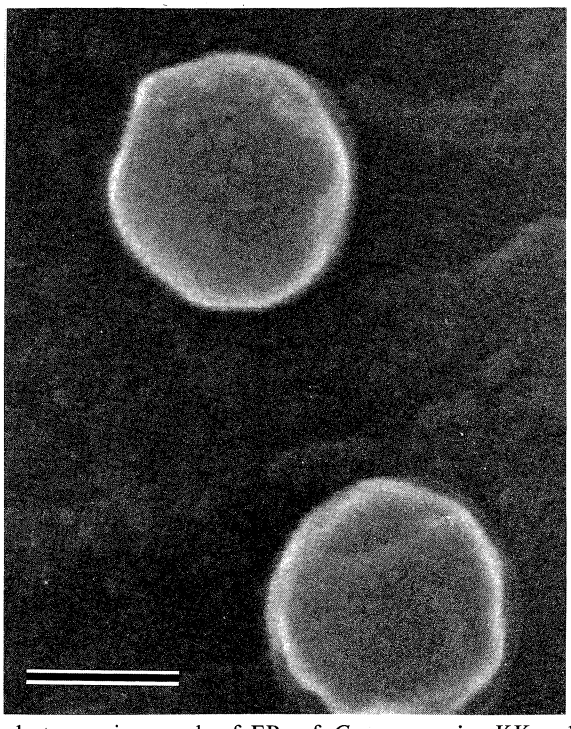

Fig 5. Scanning electron micrograph of EBs of *C. pneumoniae* KKpn-15 strain. The EBs have hexagonally arrayed projections on a limited area of the surface. The bar indicates 200 nm.

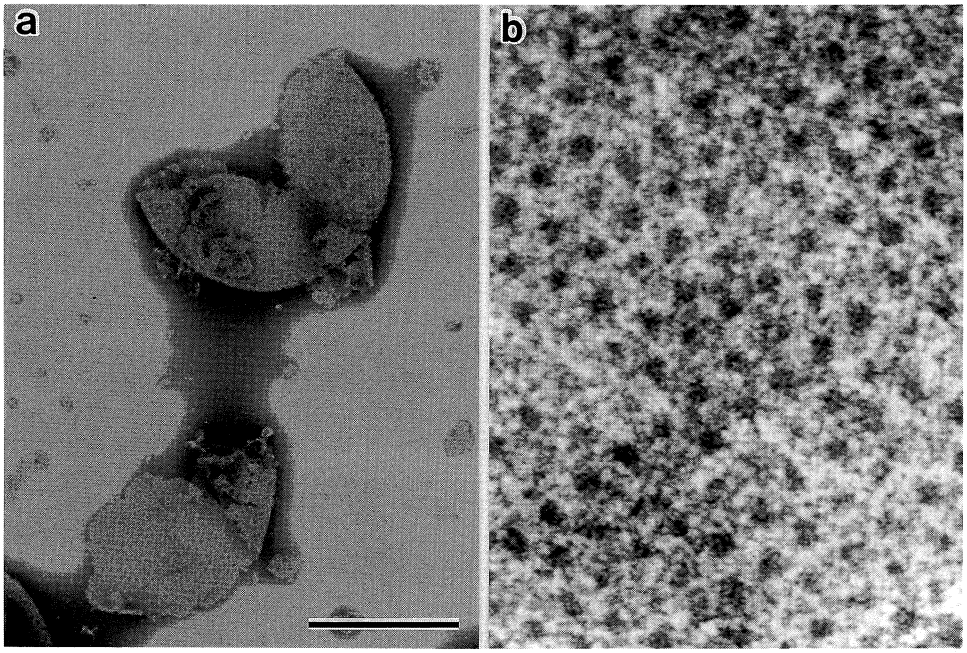

Fig 6. Negatively stained EB outer membrane isolated from purified EBs of *C. pneumoniae* KKpn-15 strain. b, higher magnification obtained from the micrograph shown in a. The hexagonal structure seen throughout the outer membrane. The bar indicates 200 nm.

Chlamydia and this supports the suggestion that they are phenotypic markers for recognizing chlamydiae.[29]

When negatively stained, the hexagonally arrayed structures in the inside layer of the outer membranes of EBs were clearly seen in all specimens.[14,30] (Fig 6). To determine the spacing of the hexagonal structure, a computer Fourier transform was carried out[14] and the results are summarized in Table 2. The average periodicity of all strains was very similar despite the difference in morphology of a round or pear-shaped profile in thin sections.[7,14]

TABLE 2. Comparison of the periodicity between chlamydial species*

Species	strain	Mean (SD) periodicity (Å)
C. pneumoniae		
round-shape	KKpn-15	179 (8)
	KKpn-1	176 (7)
	IOL-207	177 (7)
	Kajaani-6	178 (6)
	YK-41	176 (8)
pear-shape	TW-183	173 (7)
	AR-39	174 (8)
	AR-388	173 (8)
C. trachomatis	L_2/434/Bu	176 (7)
C. psittaci	Frt-Hu/Cal 10	175 (8)

* Periodicity as measured from center to center by Fourier transform

Ⅳ. Antigenic property of proteins in EB

The proteins of chlamydial EBs and EB outer membranes were separated by discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) with 10% acrylamide gels.[7,8] The protein bands were then visualized by silver staining. Figures 7 and 8 show the protein profiles of the purified EBs and EB outer membranes. The profiles of all of the *C. pneumoniae* strains were similar to each other and obviously different in the molecular sizes of proteins and band patterns from those of *C. psittaci* and *C. tarchomatis*.[7,8] The molecular weight of the major outer membrane protein (MOMP) of *C. psittaci* was the highest among all chlamydial strains examined.

The antigen-specific serological response to *C. pneumoniae* was studied in 60 patients with acute respiratory tract infections. All patients were serologically positive (IgG titer of ≧512) to *C. pneumoniae* EBs by the micro-immunofluorescence test.[17] The antigen-specific reactivity of the sera was analyzed by immunoblotting.[7,8,13] An example of the immunoblotting test with

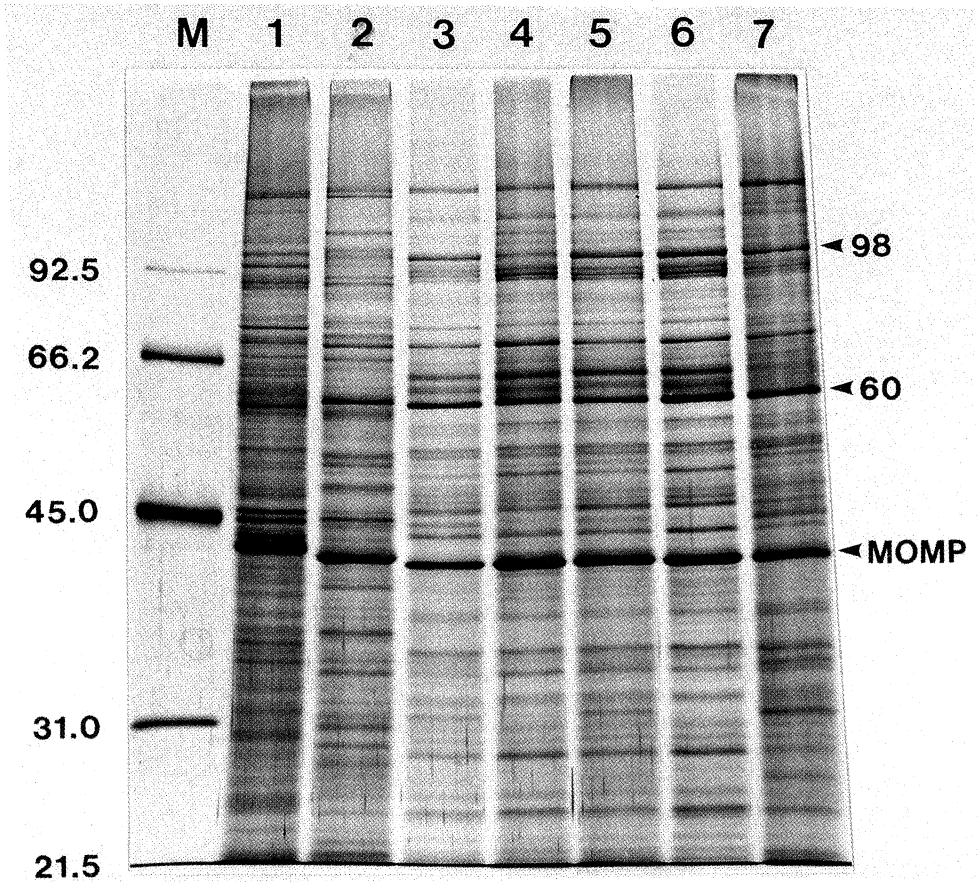

Fig 7. Protein analysis of whole EBs The purified EBs (0.15 μg of proteins) were electrophoresed by SDS-PAGE in 10% acrylamide gel. The proteins were visualized by the silver staining method. Lanes: M, molecular weight markers; 1, *C. psittaci* Frt-Hu/Cal 10; 2, *C. trachomatis* L_2/434/Bu; 3, *C. pneumoniae* KKpn-15; 4, TW-183; 5, AR-39; 6, Kajaani-6; 7, IOL-207. Numbers on both sides indicate the molecular weight (Kilodaltons).

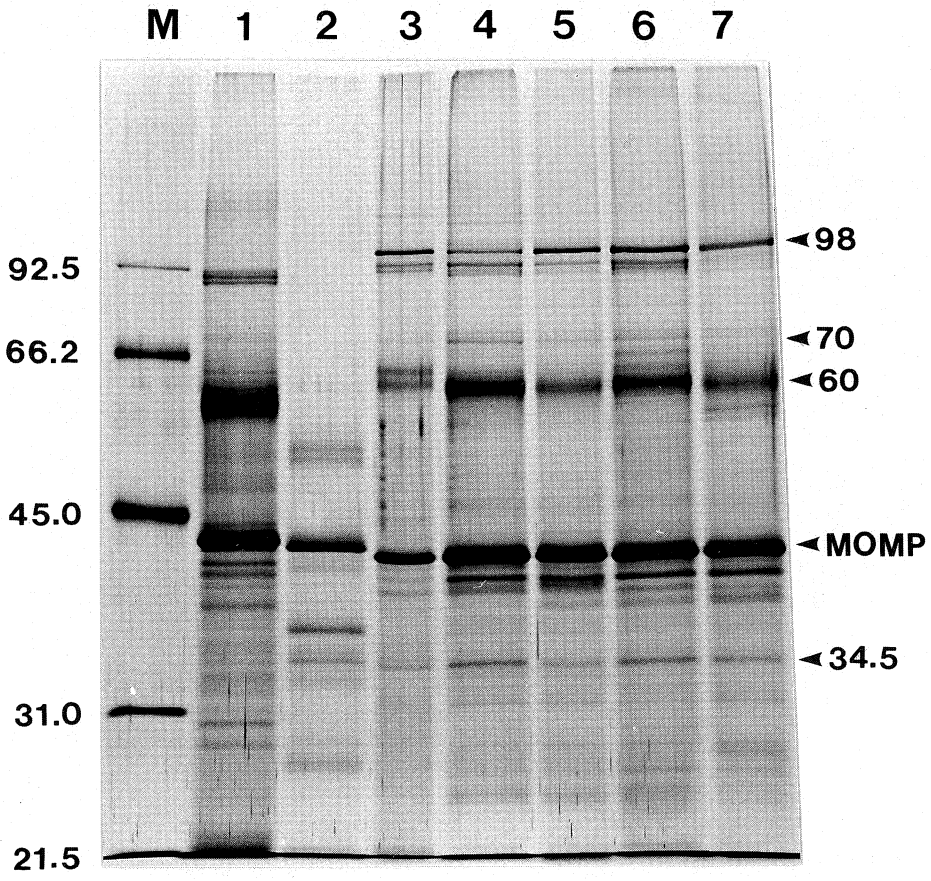

Fig 8. Protein analysis of EB outer membranes (0.2 μg of proteins). The outer membranes were electrophoresed by SDS-PAGE in 10% acrylamide gel. The proteins were visualized by the silver staining method. The lane numbers are identical with those in Fig 7.

sera collected from 11 patients is shown in Fig 9. The sera reacted with proteins in molecular massess of 30, 40 (MOMP), 43, 46, 53, 60, 68, 73 and 98 KDa of *C. pneumoniae* strains. The 43, 46, 53, 60 and 68 KDa proteins were strongly reactive, and the positive ratios to these proteins among the sera of 60 patients were 60, 50, 70, 70, and 70%, respectively. Other proteins, such as 30, 73 and 98 KDa, were also recognized, but their reaction was weak. It was noted that reaction of human sera was different from that of mouse sera. The results led us to the following conclusions; ① The antigenicities of *C. pneumoniae* were strictly conserved. ② The serological responses of humans against *C. pneumoniae* were similar on a qualitative basis, although some quantitative differences were seen. ③ The serological responses of humans who were infected with *C. pneumoniae* differed markedly from those of humans who were infected with *C. trachomatis* or *C. psittaci*. ④ *C. pneumoniae* proteins, such as the 43, 46, 53, 60 and 68 KDa proteins were antigen immunodominant in humans. ⑤ Antibodies against the 40, 60, 68 and 73 KDa proteins of *C. pneumoniae* strains possessed serological cross-reactivities with

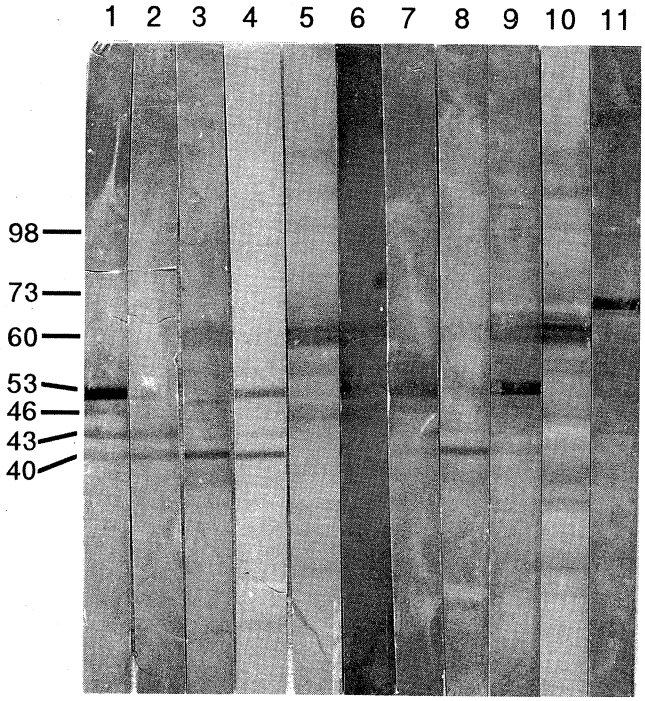

Fig 9. Immunoblot of the EBs of *C. pneumoniae* KKpn-15 with sera from 11 patients with *C. pneumoniae* infections. Each lane contained 1μg of chlamydial protein. The numbers on the left side indicate the molecular weight (kilodaltons).

C. trachomatis and *C. psittaci*. ⑥ The serological responses of humans and mice differed markedly.[7,8,13]

We established some monoclonal antibodies against 53, 60, 73 KDa proteins.[7,8] These monoclonal antibodies reacted specifically with all strains of the *C. pneumoniae* tested, as examined by the ELISA and immunoblotting, indicating that the antigenicities of the *C. pneumoniae* strains were well conserved. One of the monoclonal antibodies, SCP-53, was highly specific against the 53 KDa protein[8] of *C. pneumoniae* KKpn-1 strain. This monoclonal antibody also recognized the 53 KDa proteins of all *C. pneumoniae* strains tested. By immunoelectron microscopy and immunoblotting, it was confirmed that the 53 KDa protein was located on the surface of the outer membranes.

Ⅴ. Diagnosis —evaluation of sensitivity and specificity of test kits—

Isolation of *Chlamydia* is considered the "golden standard" for diagnosis of chlamydial infections. However, cultures for *Chlamydia* isolation are not widely available because the isolation is time-consuming and technically complex. Recently, many non-cultural tests such as direct fluorescent antibody (DFA) techniques including *C. trachomatis* specific monoclonal antibody (MAb), MicroTrak (Syva Co.), genus specific MAb, Cultureset (Ortho Diagnostic Co.), *C. pneumoniae* specific MAb, IMAGEN (DAKO Diagnostic Co.), enzyme immunoassays, Chlamydiazyme (Abott Laboratories), IDEIA

Chlamydia (DAKO Diagnostics Co.),[22] direct nucleic acid hybridization, Gen-Probe PACE 2 (Gen-Probe Inc.),[22] nucleic acid amplification techniques, the polymerase chain reaction (PCR) (AMPLICOR, Roche Diagnostic Systems)[31,32] and the Ligase chain reaction (Abott Laboratories)[33] have been developed. The advantages of Chlamydiazyme, IDEIA Chlamydia, PACE 2, PCR and LCR test kits, which have been designated for detection of *C. trachomatis*, include the fact that the viability of the organisms is not required for detection of organisms, and the tests can be performed rapidly without any technical complexity.

Based on the above mentioned characteristics of chlamydial multiplication, we suspected that the sensitivities of all test kits should be capable of indicating the number of EBs as a test parameter, but not the infectivity (IFU) of a crude preparation containing infectious EBs and non-infectious RBs, because of the constant quantity of the target components in the EBs.

As described above, we established a method for highly purifying the EBs of all chlamydial species. Additionally, we established a method for counting the number of EBs under a scanning electron microscope after sedimentation of EBs on a coverslip by centrifugation.[22] With this method, centrifugal conditions, such as duration and revolution, were carefully determined. Briefly, the purified EB-suspension, diluted appropriately, was centrifuged at 3,300xg (8,000 rpm) in a swing rotor for 1, 3, 5, 7, 10, 15, 30 and 60 min. As shown in Fig 10a, when the sample was centrifuged at 3,300xg for 7 min and longer, the number of EBs reached a plateau. To determine the revolutions, the EBs were spun down at 50xg (1,000 rpm), 210xg (2,000 rpm), 850xg (4,000 rpm), 1,900xg (6,000 rpm), 3,300xg (8,000 rpm), 5,300xg (10,000 rpm) and 7,500xg (12,000 rpm) for 7 min. As shown in Fig 10b, the number of EBs reached a maximum level at 8,000 rpm or 3,300xg. From these results, centrifugation at 3,300xg for 7 min was determined for the counting of the number of EBs by scanning electron microscopy.

The series of purified EBs were prepard by 2- or 10-fold serial dilution on the basis of the number of EBs and then assayed with the antigen detection test

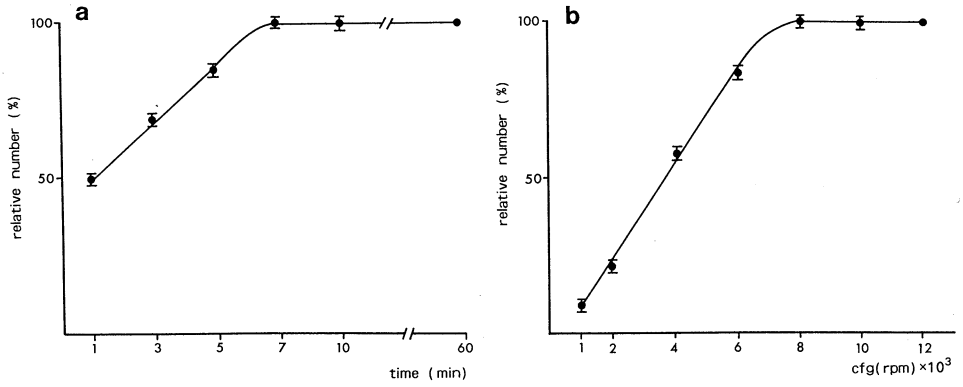

Fig 10. Relative number of EBs spun down by centrifugation. a, Relationship between duration of centrifugation (min) and number of EBs. b, Relationship between revolutions (rpm) and number of EBs. Based on the results, a centrifugal condition at 8,000 rpm for 7 min was determined.

kits described above to compare the number of EBs at the detection limit (cutoff level) of each test kit. The results are shown in Table 3.[22,32,34] The IDEIA test kit is designed for the detection of *C. trachomatis* lipopolysaccharide (LPS), which is a major genus-specific antigen. Therefore, the LPS of *C. psittaci* and *C. pneumoniae* also react in the IDEIA kit. However, the reaction intensity of *C. trachomatis* was higher than that of *C. psittaci* and *C. pneumoniae*. This suggests that the antigenic structure of *C. trachomatis* LPS is somewhat different from that of *C. psittaci* and *C. pneumoniae* LPS. As seen in Table 3, the MicroTrak, PACE2, PCR and LCR were specific for *C. trachomatis*. It was noted that the PCR and LCR were detectable only by the 2 to 4 and 4 EBs/assay, respectively, indicating their extremely higher sensitivities than those of the other kits tested, except MicroTrak which is designated for specific staining of *C. trachomatis* organisms. However, it is very likely that much manual skill in fluorescence microscopy was required to achieve such a high sensitivity with the MicroTrak staining kit.

TABLE 3. Comparison of the sensitivity of test kits to purified EBs (EBs/assay)[a]

Test kits	*C. trachomatis* L_2/434/Bu	*C. pneumoniae* TW-183	*C. psittaci* Cal 10	*C. pecorum* Bo/E58
DFA-MicroTrak	2	—	—	—
Chlamydiazyme	1.6×10^4	N. D.	N. D.	N. D.
IDEIA Chlamydia	1.0×10^3	7.0×10^3	2.7×10^4	N. D.
Gen-probe PACE2	7.5×10^3	—	—	—
PCR-AMPLICOR	2～4	—	—	—
LCR	4[b]	—	—	—

[a]Symbols: —, no reaction. N. D., not done
[b]This date will be examined again and published elsewhere in the near future.

Ⅵ. Diversity of LPS of *C. pneumoniae*

Observations on the EB morphology of several *C. pneumoniae* strains revealed that the EBs of some strains were round rather than pear-shaped,[7,14] suggesting possible diversity not only in morphology, but also in antigenic properties. Therefore, we also examined the purified EBs of several *C. pneumoniae* strains with the IDEIA test kit. The number of EBs at the cutoff level are summarized in Table 4.[9,35] The results demonstrated that the EBs of *C. pneumoniae* were detected with the IDEIA kit at different reaction intensities ranging from 6.0×10^3 to 4.0×10^4 per assay and that there was no correlation between the number of EBs detectable and morphology. What was not clarified, however, was whether the different reactivities of *C. pneumoniae* strains resulted from differences in the quality or quantity of LPS on the EB surface, although the chemical structure of the antigenic epitope in LPS has been proposed.[36]

TABLE 4. Diversity of *C. pneumoniae* strains in the reaction intensity to the IDEIA test kit[a]

Strain	Shape of EB	EB number per assay
IOL-207	round	6.0×10^3
TW-183	pear-shape	7.0×10^3
AR-388	pear-shape	8.4×10^3
Kajaani-6	round	1.2×10^4
AR-39	pear-shape	2.4×10^4
KKpn-1	round	2.8×10^4
YK-41	round	4.0×10^4

[a]The numbers of EBs are shown in increasing order.

Ⅶ. In vitro susceptibility to chemotherapeuic agents

The *in vitro* susceptibilities to minocycline, doxycycline, erythromycin, three new macrolides (azithromycin, roxithromycin and clarithromycin) and four quinolones (ciprofloxacin, ofloxacin, tosufloxacin and sparfloxacin) were examined by minimum inhibitory concentration (MIC) determined in accordance with the standard method of the Japan Society of Chemotherapy.[37] The MICs of the agents examined are summarized in Table 5. All chlamydial strains showed almost identical susceptibility to the antimicrobial agents tested.[7,38] Minocycline and clarithromycin were the most effective drugs tested, although the other macrorides, doxycycline and the quinolones was also effective on the chlamydial growth *in vitro*. These results suggest that despite the morphology, *C. pneumoniae* are equally sensitive to chemotherapeuic drugs, although some drug-resistant of *C. trachomatis* have been reported.[39,40]

TABLE 5. Activities of antibiotics against chlamydiae

	MIC (μg/ml)											
	C. pneumoniae								*C. psittaci*		*C. trachomatis*	
	KKpn -15	KKpn -1	IOL -207	Kajaani -6	YK -41	TW -183	AR -39	AR -388	Bud. -1	Cal 10	D	L_2
Erythromycin	0.125	0.125	0.125	0.125	0.25	0.25	0.25	0.25	0.125	0.25	0.125	0.125
Roxithromycin	0.063	0.063	0.063	0.063	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
Azithromycin	0.125	0.25	0.125	0.125	0.25	0.125	0.125	0.125	0.125	0.125	0.125	0.25
Clarithromycin	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
Ciprofloxacin	1.0	2.0	1.0	1.0	2.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0
Ofloxacin	0.5	0.5	1.0	0.5	1.0	1.0	0.5	1.0	0.5	0.5	0.5	1.0
Tosufloxacin	0.063	0.125	0.125	0.063	0.125	0.125	0.125	0.125	0.125	0.125	0.063	0.125
Sparfloxacin	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.125	0.063	0.125	0.063	0.125
Doxycycline	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.031
Minocycline	0.016	0.016	0.016	0.016	0.031	0.016	0.016	0.031	0.031	0.016	0.016	0.031

CONCLUSION

The purification method for *C. psittaci* EBs was established by Tamura & Higashi in 1963.[23] Using their method, the final EB fractions of *C. trachomatis* and *C. pneumoniae* contained, however, a lot of RB and RB debris. RBs vary in size, are noninfectious and are fragile to mechanical shock. On the other hand, EBs are uniform in size and mechanically rigid. Although EBs are infectious, the infectivity is readily inactivated under laboratory conditions. These facts strongly suggest the appropriateness of examination of the protein composition, antigenicity and of sensitivity evaluation of the test kits using purified EBs. The newly established method for purification of *C. trachomatis* and *C. pneumoniae* EBs was also applicable to *C. psittaci* EBs. The EB counting method was applicable to EBs of all chlamydial species. The reproducibilities of both methods were confirmed by repeated experiments. Therefore, we may emphasize that the results shown here, especially protein analysis by electrophoresis, immunoblotting, and evaluation of various test kits, are reliable.

C. pneumoniae was classified in 1989 as a new species on the basis of the EB morphology, serology and DNA analysis of the TWAR strains.[2] Since then, several *C. pneumoniae* strains, including our isolates, have been established from patients with respiratory diseases. It is noteworthy that in spite of the striking difference in EB morphology, only one serotype has been recognized.[2] Although humans have been thought to be the natural reserver, similar *chlamydiae* have been isolated from a horse[41] and Koala bear.[42] These facts, together with the different reactivities of EBs of various *C. pneumoniae* isolates in the IDEIA kit, led us to speculate the presence of different serotypes of *C. pneumoniae*.

Recent investigations have suggested a direct relation between *C. pneumoniae* infection and coronary artery disease.[43–46] We also investigated the relation between *C. pnemoniae* IgG antibody titers and angiographically diagnosed coronary artery disease. Based on standardization for age and gender, the geometric mean antibody titer in the cases was higher than that in the controls (data not shown). These results may support an association between infection with *C. pneumoniae* and coronary artery disease. However, final verification of a pathogenetic role of the chlamydial infection in the development of coronary heart disease has not been made.

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