Pathogenesis of Chlamydial Infections

（オウム病クラミジアの病原性解析）

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PART II

Investigation of the Emergence of Chlamydial Infection in Animals and Human Disease Conditions

PREFACE

Chlamydiae are the obligate intracellular bacterial pathogens of animals and human beings (185, 188). They replicate within the cytoplasm of host cells, forming characteristic intracellular inclusion bodies. The chlamydiae undergo a unique biphasic developmental cycle characterized by two morphologically distinct forms, the elementary body (EB) and the reticulate body (RB). EBs are small (0.2–0.3 μm in diameter) infectious forms that are either endocytosed or phagocytosed by host cells. The EB within an intracytoplasmic inclusion, transforms into the larger (0.5–1.6 μm in diameter) intracellular non-infectious, metabolically active, RB. The RB multiplies by binary fission and fills the inclusion, which expands in size. RBs re-condense back into EBs via intermediate bodies towards the end of the cycle (24–48 hr, depending on species and strains) and are then released from the host cell by lysis or exocytosis to initiate another cycle of infection (75, 119, 131, 216).

The EB, in contrast to the RB, is structurally rigid, resulting from the formation of extensive disulphide linkages between various cysteine-rich proteins in or associated with the outer membrane. The chlamydial outer membrane consists of lipopolysaccharide (LPS), polymorphic outer membrane proteins (POMPs), peptidoglycan and cysteine-rich proteins like major outer membrane protein (MOMP), OmcA and OmcB. The most important of these is the MOMP, which makes up 60% of the weight of the outer membrane. MOMP is about 40- to 45-Kda in mass and contains serovar- and species-specific epitopes. MOMP acts as porins, channels to allow exchange of molecules including nutrients. The high rigidity results in EBs being resistant to both chemical and physical factors and therefore, adapted for prolonged extracellular survival, an important factor in terms of chlamydial pathogenesis and control or treatment of chlamydial infections (75, 176).

Earlier all chlamydiae were placed in order *Chlamydiales*, family *Chlamydiaceae* and one genus *Chlamydia* that included 4 species: *C. trachomatis*, *C. psittaci, C.*

pneumoniae and *C. pecorum* (52, 64, 132). Following reclassification of the order *Chlamydiales* in 1999, the family *Chlamydiacaeae* is now divided into two genera, *Chlamydia* and *Chlamydophila* (43). The genus *Chlamydia* comprises of *Chlamydia trachomatis*, *Chlamydia suis* and *Chlamydia muridarum* species. The genus *Chlamydophila* contains the species *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, *Chlamydophila abortus*, *Chlamydophila pecorum*, *Chlamydophila felis* and *Chlamydophila caviae.*

Chlamydiae are responsible for a diverse range of diseases in birds and mammals including humans. Among human chlamydial infections, *C. trachomatis* causes blindness (trachoma), sexually transmitted disease and infertility. *C. pneumoniae* is a cause of acute respiratory infection, coronary heart disease and chronic inflammatory lung diseases *viz.* chronic obstructive pulmonary disease and asthma (71, 105, 177).

Animal chlamydial infections are caused by many chlamydial species. In genus *Chlamydia*, *C. suis* is associated with either asymptomatic infections or cause conjunctivitis, enteritis, pneumonia and genital tract infections in domesticated and wild porcine (99, 165, 183). *C. muridarum* causes either no disease or asymptomatic pneumonitis among mice and hamsters (40, 48). In genus *Chlamydophila*, *C. pecorum* is associated with chronic conditions *viz.* pneumonia, polyarthritis, conjunctivitis, enteritis, encephalomyelitis, metritis and mastitis among ruminants, swine and marsupials (40, 52). *C. felis* is responsible for "feline chlamydiosis", which is characterized by rhinitis and conjunctivitis in cats, particularly kittens (192). Studies conducted in Japan have shown *C. felis* to be more widespread in stray cats than in pet cats (26, 57, 222). *C. abortus* is a cause of abortion in sheep, goats and responsible for reproductive failure in bovine, equine and porcine (40, 113, 160). *C. psittaci* causes "avian chlamydiosis" among many avian species (reported 460 avian species) and can also infect mammalian species (8, 94, 210). Avian chlamydiosis is characterized by diarrhea, anorexia, respiratory distress, sinusitis, rhinitis

and conjunctivitis (8, 188). *C. caviae* is a host specific pathogen of guinea pigs and causes either self-limiting or asymptomatic inclusion conjunctivitis among young (4-8 weeks old) guinea pigs (63, 101, 135). *C. pneumoniae* is also reported from marsupials and equines (62, 218).

Animal chlamydial infections also have public health hazard significance. Among all chlamydial species infecting animals, *C. psittaci*, *C. abortus* and *C. felis* are potential zoonotic pathogens (113). *C. abortus* can cause severe, life-threatening disease in pregnant women. Infection in women during pregnancy can result in spontaneous abortion or stillbirths, which are typically preceded by several days of acute influenza-like illness, as well as renal failure, hepatic dysfunction, disseminated intravascular coagulation, and possibly death (25, 87). Cases of *C. felis* infection associated with conjunctivitis and atypical pneumonia in humans have been reported over the years (19, 113), but it is only recently that compelling evidence of transmission from cats to humans has been provided by way of molecular classification techniques (74). In Japan serological evidence of human *C. felis* infection has been reported (222).

The zoonotic infection by *C. psittaci*, is particularly a significant occupational health hazard to the workers in poultry industry, aviaries, zoos and also pet shop owners, farmers and veterinarians. Infection in humans can vary in severity but is usually associated with respiratory disease. But multiple organs may become infected resulting in endocarditis, myocarditis, hepatitis, encephalitis or meningitis and result in deaths (113, 181, 210). The financial losses resulting from this disease, particularly those incurred in the poultry and pet industries, combined with the fact that this is the most common animal chlamydiosis transmissible to man, highlights the economic importance and public health significance of avian chlamydiosis. The incidences of zoonotic infection by *C. caviae*, *C. pecorum*, *C. suis* and *C. muridarum* are unknown (113).

Defining the distribution of natural chlamydial infections in animals and human and its relationship to pathogenesis provides the fundamental basis for management and control

of chlamydial diseases. The final outcome of chlamydial infection in animals and human depends on large numbers of host and organism specific factors (217). Genetic and biological differences among various chlamydial species/strains are well known. Different pathobiotypes of *C. trachomatis* strains have been recognized, which include trachoma, urogenital and lymphogranuloma venereum (LGV) biovars. These biovars varies in tissue tropism and host specific cellular response which indicates variation in disease causation mechanism (128, 137, 214). In *C. pneumoniae* also, human, equine and koala biovars are known. The virulence and genetic distinction of *C. abortus* strains causing abortion in ewes from non abortifacient strains has also been established (38, 159, 162). However, in *C. psittaci* no such relationship has been explored among various genetically diverse strains even though it has been detected from diverse host rage and disease conditions. On the other hand, some studies have found varied virulence of *C. psittaci* strain to avian species (212). Therefore, detailed molecular epidemiological studies of animal chlamydial species by screening variety of host species and disease conditions is important to identify association of particular species/strain with specific disease syndrome.

The diagnosis of chlamydial infections and analysis of chlamydial diversity are done by isolation, serological and biotyping studies (175, 184, 220). But these procedures are time consuming and high technical skills are required for testing samples. Therefore, the recent epidemiological studies are mostly contemplated by detecting species/strain specific gene (DNA) by nucleic acid amplification using polymerase chain reaction (PCR). By this technique even a very small amount of EBs can be detected. The genetic diversity among the prevalent chlamydial species/strains can be studied by sequencing and genetically analyzing the amplified DNA fragment (59, 80, 83, 96, 97, 148).

The differentiation at the species level among various chlamydiae is usually done by genetic analysis of highly conserved genes like 16S ribosomal RNA, 16S-23S

ribosomal RNA intergenic spacer region, *rnpB* and *groEL-1* genes (24, 41, 54, 79, 149, 193, 205). However, for the identification at strain level, *ompA* gene coding MOMP has been targeted in many studies (38, 47, 50, 60, 173, 191).

For identification and differentiation of *C. psittaci* isolates especially of avian origin, *ompA* gene was analyzed by many researchers (50, 98, 174, 191, 207). Depending on serovar specific monoclonal antibody typing, 8 serotypes named A to H have been identified in *C. psittaci* (4, 56, 206, 207). Based on AluI restriction fragment length polymorphism (RFLP) pattern of *ompA* gene, *C. psittaci* strains are also divided into 7 genotypes from A to F and E/B (60, 174, 207), but serotyping and RFLP studies do not reflect the actual genetic diversity (60).

Therefore, the detailed investigations about natural distribution, molecular identification, genetic diversity and virulence of various chlamydial species/strains of animal origin are necessary to understand disease pathogenesis. Hence, to elucidate some of these questions the present study was undertaken with the following objectives:

1) Study of molecular epizootiology of *Chlamydophila psittaci* among captive and feral avian species on the basis of VD2 region of *ompA* gene.
2) Analysis of genetic diversity and molecular phylogeny of *Chlamydophila psittaci* strains prevalent among avian fauna and those associated with human psittacosis.
3) Examination of virulence patterns of *Chlamydophila psittaci* strains predominantly associated with avian chlamydiosis and human psittacosis using BALB/c mice.
4) Investigation of the emergence of chlamydial infection among animals and human disease conditions.

These findings will help to identify predominant/emergent chlamydial genotypes prevalent among domesticated and wild fauna, with/without disease symptoms and also those with high zoonotic potential for better understanding of disease pathogenesis.

PART I

Molecular Epidemiology, Genetic Diversity, Phylogeny and Virulence Analysis of *Chlamydophila psittaci*

CHAPTER-I

Study of molecular epizootiology of *Chlamydophila psittaci* among captive and feral avian species on the basis of VD2 region of *ompA* gene

INTRODUCTION

Chlamydiae are obligate intracellular pathogens and widely prevalent among avian species, animals and human beings (7, 51, 132, 188). According to the recent classification based upon 16S rRNA gene sequences, all the chlamydiae are divided into four families (43). *Chlamydophila* species belonging to the family *Chlamydiaceae* are often involved in the infection of avian species and domestic animals (43, 53).

The avian chlamydiosis is caused by *Chlamydophila psittaci*. The disease is characterized by clinical and/or subclinical infection. The clinical signs vary in severity and disease is usually systemic with up to 30% mortality (8, 188). *C. psittaci* has been reported in more than 400 avian species and occasionally in non-avian hosts (8, 94, 143, 182, 209). *C. psittaci* is usually transmitted horizontally to in-contact animals and human beings by diseased or subclinically infected birds (35, 112, 113, 115, 181). People are frequently exposed to diverse type of domestic and/or feral avian fauna in daily life either incidentally or occupationally. Furthermore, the feral birds congregated in urban public habitations also contaminate the environment with *C. psittaci* laced aerosols and droppings (30, 55, 70, 202). In Japan, 45 cases of human psittacosis in 2002, 44 in 2003 and 36 in 2004 were reported (136) and in USA 935 cases were reported from 1988 to 2003 (181). Many more cases may have occurred but are neither correctly diagnosed nor reported due to confusing symptoms.

Immunodiagnostic and biotyping studies (31, 184, 220) were reported to identify various avian species harboring *C. psittaci* and to detect chlamydial diversity. Depending on serovar specific monoclonal antibody typing, 8 serotypes named A to H have been identified in *C. psittaci* (4, 56, 206, 207). In the last decade, for identification and differentiation of *C. psittaci* isolates especially of avian origin, *ompA* gene coding major outer membrane protein (MOMP) was analyzed by many researchers (50, 98, 174, 191, 207). On the basis of AluI restriction fragment length polymorphism (RFLP) pattern of *ompA* gene, *C. psittaci* strains are also divided into 7 genotypes from A to F and E/B (60, 174, 207), but serotyping and RFLP studies do not reflect the actual genetic diversity (60). The *ompA* gene consists of genetically conserved fragments called conserved/constant domains (CD) flanking 4 genetically variable domains (VD). The VDs of *ompA* gene are reported to have species/strain specific sequences and thus can be analyzed for identification of chlamydial species/strains and also to study the genetic diversity directly from field samples after PCR amplification (12, 86, 96, 148, 225).

The detailed epizootiological study of avian chlamydiosis is essential to know the prevalence of different chlamydial strains/species among various avian species and to ascertain the possibility of health hazard risk to in-contact animals and human beings. Moreover, limited information is available in literature on the current molecular epizootiology and genetic diversity of *C. psittaci* after reclassification of avian and mammalian isolates of the previous *Chlamydia psittaci* group (43). Therefore, the present research was planned to investigate the molecular epizootiology and genetic diversity among *C. psittaci* strains and/or other chlamydial species prevalent in diverse avian fauna. It appears that various genetically diverse chlamydial species and strains may cause avian chlamydiosis but some strains of *C. psittaci* are highly prevalent and are frequently associated with clinical/subclinical infections.

MATERIALS AND METHODS

Samples examined: A total of 1,147 samples from 11 avian orders were collected from 4 avian pet shops, 3 bird sanctuaries/wild animal rehabilitation centers, 3 bird parks/zoos and 14 veterinary hospitals located in 11 prefectures of Japan from January 2003 to December 2004. The collected samples included the cloacal swabs or freshly voided feces and/or whole blood in heparin and/or pieces of visceral organs (1-2 g) including lung, liver, spleen, heart and posterior intestinal loop from dead birds. The avian fauna screened in the study is shown in Table 1. The distribution of tested samples according to clinical history and sources are shown in Table 2. In total, 11 avian orders include 28 genera and 81 species from psittacine birds and 25 genera comprising of 32 species from non-psittacine birds.

DNA extraction: SepaGene DNA extraction Kit (Sanko Junyaku, Tokyo, Japan) was used to extract DNA from samples according to the manufacturer's instructions. In brief, about 200 mg of fecal/tissue material or 50 μl of blood or cloacal swabs suspended in 700 μl of phosphate-buffered saline (PBS), pH 7.4 were processed for DNA extraction. DNA was finally dissolved in 30 μl Tris-EDTA (TE) buffer, pH 7.4 (100 mM Tris-HCl, pH 7.4 and 10 mM EDTA, pH 8.0) and stored at –30°C. DNA extracted from purified elementary bodies of GPIC strain of *Chlamydophila caviae* (ATCC VR-813) was used as a positive control in the test.

Nested PCR: Two sets of consensus oligonucleotide primers based on *ompA* gene were used in a two-steps procedure. An outer pair of primers CMGP-1F (5'-CCTTGTGATCCTTGCGCTACTTG-3'; nucleotide (nt) 138 to 160 in *ompA* gene sequence of 6BC strain of *C. psittaci* with accession number X56980) and CMGP-1R (5'-GTGAGCAGCTCTTTCGTTGAT-3'; nt 1184 to 1164) and an inner pair of primers

Table 1. Details of the avian species screened in the study.

Order	Family	Genus (sample no.)	Avian species and number of samples tested[a]
Psittaciformes	*Cacatuidae*	*Cacatua* (98)	*C. alba* (Umbrella or White-crested Cockatoo)-27; *C. galerita* (Sulphur-crested Cockatoo)-3; *C. triton* (Tritone Cockatoo)-3; *C. tenuirostrius* (Long-billed Corella)-1; *C. sulphurea* (Yellow-crested Cockatoo)-26; *C. sanguinea* (Little Cockatoo)-1; *C. leadbeateri* (Pink Cockatoo)-2; *C. sulphurea citrinocristana* (Citron-crested Cockatoo)-17; *C. moluccensis* (Moluccan or Salmon-crested Cockatoo)-18
		Eolophus (9)	*E. roseicapillus* (Roseate Cockatoo or Galah)-9
		Nymphicus (77)	*N. hollandicus* (Cockatiel)-77
	Psittacidae	*Agapornis* (11)	*A. roreicollis* (Peached-faced Lovebird)-10; *A. lilianae* (Lilian Lovebird)-1
		Amazona (13)	*A. aestiva* (Blue-fronted Amazon)-6; *A. aestiva xanthopteryx* (a type of Blue-fronted Amazon)-1; *A. ochrocephala* (Yellow-crowned Amazon)-3; *A. auropalliata* (Yellow-naped Amazon)-1; *A. xanthops* (Yellow-faced Amazon)-1; *A. farinosa* (Mealy Amazon)-1
		Ara (55)	*A. nobilis* (Red-shouldered Macaw)-2; *A. chloroptera* (Green-winged Macaw)-10; *A. ararauna* (Blue-and-Yellow Macaw)-11; *A. severa* (Chestnut-fronted Macaw)-7; *A. auricollis* (Yellow-collard Macaw)-23; *Ara* sp. (Harlequin Macaw)-1; *A. macao* (Scarlet Macaw)-1
		Aratinga (44)	*A. aurea* (Peach-fronted Conure)-3; *A. wagleri* (Scarlet or Red-fronted Conure)-2; *A. jandaya* (Jandaya Conure)-6; *A. solstitialis* (Sun Conure)-9; *A. erythrogenys* (Red-masked Conure)-5; *A. weddellii* (Dusky-headed Conure)-5; *A. acuticaudata* (Blue-crowned Conure)-7; *A. pertinax* (St. Thomas Conure)-1; *A. guarouba* (Golden Conure)-2; *A. rubritorquis* (Red-throated Conure)-4
		Bolborhynchus (1)	*B. lineola* (Barred Parakeet)-1
		Brotogeris (2)	*B. chrysopterus* (Golden-winged Parakeet)-2
		Cyanoliseus (1)	*C. patagonus* (Patagonia Conure)-1
		Eclectus (10)	*E. roratus* (Eclectus Parrot)-10
		Eos (3)	*E. bornea* (Red Lory)-1; *E. reticulata* (Blue-streaked Lory)-2
		Forpus (1)	*F. coelestis* (Pacific Parrotlet)-1
		Lorius (11)	*L. lory* (Black-capped Lory)-5; *L. garrulus* (Chattering Lory)-1; *L. chlorocerus* (Yellow-bibbed Lory)-5
		Melopsittacus (59)	*M. undulatus* (Budgeriger)-59
		Myiopsitta (1)	*M. monachus* (Quaker or Monk Parrot)-1
		Neophema (2)	*N. bourkii* (Bourke's Parrot)-2
		Pionites (14)	*P. melanocephala* (Black-headed Caique)-6; *P. leucogaster* (White-bellied Caique)-8
		Pionus (12)	*P. senilis* (White-crowned Parrot)-4; *P. fuscus* (Dusky Parrot)-1; *P. menstruus* (Blue-headed Parrot)-4; *P. chalcopterus* (Bronze-winged Parrot)-3
		Platycerus (1)	*P. elegans* (Crimson Rosella)-1
		Poicephalus (121)	*P. cryptoxanthus* (Brown-headed Parrot)-26; *P. meyeri* (Meyer's Parrot/Broun Parrot)-19; *P. rueppellii* (Rüppell's parrot)-7; *P. rufiventris* (Red-bellied Parrot)-4; *P. senegalus* (Senegal Parrot)-42; *P. gulielmi* (Red-crowned/Jardine's Parrot)-23
		Polytelis (3)	*P. anthopeplus* (Regent Parrot)-2; *P. alexandrae* (Alexandra's Parrot)-1
		Pseudeos (7)	*P. fuscata* (Dusky Lory)-7
		Psittacula (12)	*P. krameri manillensis* (Indian Ring-neck Parakeet)-2; *P. eupatria* (Alexandrine Parakeet)-4; P. derbiana (Derbyan Parakeet)-3; *P. cyanocephala* (Plum-headed Parakeet)-3
		Psittacus (163)	*P. erithacus* (African Grey Parrot)-154; *P. erithacus timneh* (Timneh Grey Parrot)-9
		Pteroglossus (2)	*P. aracari* (Black-necked Aracari)-2
		Pyrrhura (21)	*P. molinae* (Green-cheecked Conure)-4; *P. egregia* (Fiery-shouldered Conure)-3; *P. rupicola* (Rock or Black-capped Conure)-2; *P. frontalis* (Red-bellied Conure)-3; *P. rhodocephala* (Rose-headed Conure)-1; *P. perlata lepida* (Pearly Conure)-5; *P. hypoxantha sallvadori* (Yellow-sided Conure)-3
		Trichoglossus (52)	*T. haematodus* (Green-naped Lorikeets or Rainbow Lory)-49; *T. haematodus capistratus* (Edward's Lorikeet)-3

Anseriformes	*Anatidae*	*Anas* (3)	*Anas* sp. (Duck)-3
		Branta (1)	*B. sandvicensis* (Hawaiian Goose)-1
		Cairina (1)	*C. moschata* (Muscovy Duck)-1
Ciconiiformes	*Ardeidae*	*Nycticorax* (1)	*N. nycticorax* (Black-crowned Night Heron)-1
	Ciconiidae	*Ciconia* (256)	*C. ciconia* (White Stork)-256
Columbiformes	*Columbidae*	*Columba* (2)	*Columba livia* (Pigeon)-2
Cuculiformes	*Musophagidae*	*Tauraco* (10)	*T. persa* (Guinea Turaco)-4; *T. livingstonii* (Livingstone's Turaco)-5; *T. hartlaubi* (Hartlaub's Turaco)-1
		Musophaga (7)	*M. violacea* (Violet Turaco)-7
Galliformes	*Phasianidae*	*Lophura* (1)	*L. nycthemera* (Silver Pheasent)-1
		Gallus (5)	*G. domesticus* (Chicken and Chicken Silkie Bantom)-5
		Coturnix (3)	*C. japonica* (Japanese Quail)-3
		Pavo (2)	*P. cristatus* (Common Pea Fowl)-2
Gruiformes	*Gruidae*	*Grus* (14)	*G. japonensis* (Red Crowned Crane)-14
Passeriformes	*Estrildidae*	*Padda* (5)	*P. oryzivora* (Java Sparrow)-5
	Fringillidae	*Coccothraustes* (1)	*C. coccothraustes* (Hawfinch)-1
		Lagopus (1)	*L. mutus* (Rock Ptarmigan)-1
	Hirundinidae	*Hirundo* (1)	*H. rustica* (Barn Swallow)-1
	Ploceidae	*Passer* (1)	*P. montanus* (Eurasian Tree Sparrow)-1
	Pycnonoyidae	*Hypsipetes* (1)	*H. amaurotis* (Brown-eared Bulbul)-1
	Sturnidae	*Gracula* (2)	*G. religiosa* (Southern Grackle or Hill Mynah)-2
		Sturnus (1)	*S. cineraceus* (Grey Starling)-1
Piciformes	*Ramphastidae*	*Ramphastos* (11)	*R. toco* (Toco Toucan)-3; *R. tucanus* (Red-billed Toucan)-3; *R. sulfuratus* (Keel-billed Toucan)-1; *R. vitellinus* (Channel-billed Toucan)-3; *R. swainsonii* (Chestnut-mendibled Toucan)-1
Sphenisciformes	*Sphaniscidae*	*Spheniscus* (5)	*S. demersus* (Cape Penguin)-5
Strigiformes	*Strigidae*	*Bubo* (5)	*B. bengalensis* (Bengal Eagle Owl)-3; *B. virginianus* (Horned Owl)-2
	Tytonidae	*Tyto* (1)	*T. alba* (Barn Owl)-1

[a] Common names of avian species are shown in the parentheses.

Table 2. Type of avian fauna screened and distribution according to clinical history and sources of sampling.

Classification of the screened avian fauna			Source and clinical status wise division of the samples												Total samples
			Veterinary hospitals[a]			Pet shops[b]			Bird parks and zoos[c]			Bird sanctuaries and wild fauna[d]			
Order (sample no.)	Family (sample no.)	Genus	Normal	Sick[e]	Dead	Normal	Sick[e]	Dead	Normal	Sick[e]	Dead	Normal	Sick[e]	Dead	
Psittaciformes (806)	*Cacatuidae* (184)	*Cacatua*	7	0	0	91	0	0	0	0	0	0	0	0	98
		Eolophus	3	0	0	4	0	0	1	0	1[g]	0	0	0	9
		Nymphicus	24	10	0	36	0	0	7	0	0	0	0	0	77
	Psittacidae (622)	*Agapornis*	7	3	0	1	0	0	0	0	0	0	0	0	11
		Amazona	4	1	0	8	0	0	0	0	0	0	0	0	13
		Ara	5	4	0	12	0	0	7	20	7[f]	0	0	0	55
		Aratinga	1	0	0	42	0	0	1	0	0	0	0	0	44
		Bolborhynchus	0	0	0	1	0	0	0	0	0	0	0	0	1
		Brotogeris	0	0	0	2	0	0	0	0	0	0	0	0	2
		Cyanoliseus	0	0	0	1	0	0	0	0	0	0	0	0	1
		Eclectus	0	0	0	9	0	1	0	0	0	0	0	0	10
		Eos	0	0	0	3	0	0	0	0	0	0	0	0	3
		Forpus	0	1	0	0	0	0	0	0	0	0	0	0	1
		Lorius	0	0	0	11	0	0	0	0	0	0	0	0	11
		Melopsittacus	15	25	4	1	0	0	14	0	0	0	0	0	59
		Myiopsitta	0	0	0	1	0	0	0	0	0	0	0	0	1
		Neophema	0	1	0	1	0	0	0	0	0	0	0	0	2
		Pionites	2	1	0	10	0	1	0	0	0	0	0	0	14
		Pionus	3	0	0	9	0	0	0	0	0	0	0	0	12
		Platycerus	0	0	0	1	0	0	0	0	0	0	0	0	1
		Poicephalus	1	0	0	118	2	0	0	0	0	0	0	0	121
		Polytelis	0	0	0	3	0	0	0	0	0	0	0	0	3
		Pseudeos	0	0	0	7	0	0	0	0	0	0	0	0	7
		Psittacula	0	0	0	12	0	0	0	0	0	0	0	0	12
		Psittacus	6	1	0	154	1	0	1	0	0	0	0	0	163
		Pteroglossus	0	0	0	2	0	0	0	0	0	0	0	0	2
		Pyrrhura	2	1	0	18	0	0	0	0	0	0	0	0	21
		Trichoglossus	0	0	0	6	0	0	29[g]	4	13[g]	0	0	0	52
Anseriformes (5)	*Anatidae* (5)	*Anas*	1	0	0	0	0	0	2	0	0	0	0	0	3
		Branta	1	0	0	0	0	0	0	0	0	0	0	0	1
		Cairina	1	0	0	0	0	0	0	0	0	0	0	0	1

Ciconiiformes (257)	*Ardeidae* (1)	*Nycticorax*	0	0	0	0	0	0	0	0	0	0	1	0	1
	Ciconiidae (256)	*Ciconia*	0	0	0	0	0	0	0	0	0	254	0	2	256
Columbiformes (2)	*Columbidae* (2)	*Columba*	0	0	0	0	0	0	2	0	0	0	0	0	2
Cuculiformes (17)	*Musophagidae* (17)	*Tauraco*	0	0	0	0	0	0	10	0	0	0	0	0	10
		Musophaga	0	0	0	0	0	0	7	0	0	0	0	0	7
Galliformes (11)	*Phasianidae* (11)	*Lophura*	0	0	0	0	0	0	1	0	0	0	0	0	1
		Gallus	3	0	0	0	0	0	2	0	0	0	0	0	5
		Coturnix	0	0	0	0	0	0	3	0	0	0	0	0	3
		Pavo	0	0	0	0	0	0	2	0	0	0	0	0	2
Gruiformes (14)	*Gruidae* (14)	*Grus*	0	0	0	0	0	0	14	0	0	0	0	0	14
Passeriformes (13)	*Estrildidae* (5)	*Padda*	4	0	0	0	0	0	1	0	0	0	0	0	5
	Fringillidae (2)	*Coccothraustes*	0	0	0	0	0	0	0	0	0	0	1	0	1
		Lagopus	0	0	0	0	0	0	0	0	0	0	0	1	1
	Hirundinidae (1)	*Hirundo*	0	0	0	0	0	0	1	0	0	0	0	0	1
	Ploceidae (1)	*Passer*	1	0	0	0	0	0	0	0	0	0	0	0	1
	Pycnonoyidae (1)	*Hypsipetes*	1	0	0	0	0	0	0	0	0	0	0	0	1
	Sturnidae (3)	*Gracula*	1	0	1	0	0	0	0	0	0	0	0	0	2
		Sturnus	0	0	0	0	0	0	0	0	0	0	1	0	1
Piciformes (11)	*Ramphastidae* (11)	*Ramphastos*	0	0	0	0	0	0	11	0	0	0	0	0	11
Sphenisciformes (5)	*Sphaniscidae* (5)	*Spheniscus*	0	0	0	0	0	0	5	0	0	0	0	0	5
Strigiformes (6)	*Strigidae* (5)	*Bubo*	0	0	0	0	0	0	5	0	0	0	0	0	5
	Tytonidae (1)	*Tyto*	0	0	0	0	0	0	1	0	0	0	0	0	1
		Total	93	48	5	564	3	2	127	24	21	254	3	3	1147

[a] Include samples from hospitals located in Nara, Tokyo, Gifu, Aichi, Saitama, Okinawa, Kanagawa, Kyoto and Fukuoka prefectures of Japan and represents birds those were individually or pair wise caged and kept as pets in households.

[b] Mostly birds were imported from South Africa, Singapore, Thailand and USA or in house bred by pet shops (located at Tokyo, Aichi, Osaka and Hyogo prefectures) and sold to various clients.

[c] Include birds kept in large enclosures as colonies in artificial habitats and are exposed to human contact continuously. These bird parks or zoos are located in Okinawa, Hokkaido (Kushiro), Hyogo and Shimane prefectures of Japan.

[d] The samples represent birds living in natural or near natural habitats and also includes oriental white storks imported from Russia. Samples were collected from Hokkaido and Gifu prefectures.

[e] The birds showing the chlamydia specific or unrelated clinical signs and are not clinically normal.

[f] Include samples from outbreak-I in a bird park at Shimane prefecture.

[g] Include samples from outbreak-II in another bird park at Okinawa prefecture.

CMGP-2F (5'-GCCTTAAACATCTGGGATCG-3'; nt 384 to 403), CMPG-2R (5'-GCACAACCACATTCCCATAAAG-3'; nt 634 to 613) was used for the first and second steps, respectively. Five μl of the first step PCR product was used for the second step PCR. This test is able to detect 2 to 10 copies of genomic DNA of diverse types of *Chlamydophila* sp. in a 50 μl reaction mixture (data not shown). The primers were synthesized by the Rikaken Co., Nagoya, Japan. In both steps, reaction was performed in 50 μl reaction mixture containing 0.15 μM of each forward and reverse primer, 250 μM of each dATP, dTTP, dGTP, dCTP, 100 μM of Mg^{2+} in buffer and 2.5 units of TaKaRa *Ex-Taq* (Takara Bio Inc., Otsu, Shiga, Japan) and 2.0 to 5.0 μg of template DNA of each sample. The thermo cycling conditions used were initial denaturation at 94°C for 3 min, then 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 60 sec, then final extension for 5 min at 72°C and soaking at 4°C. In the second step PCR; the same thermal cycle conditions were used except shorter extension period of 30 sec. The DNA of *C. caviae* was used as positive control and PBS (pH 7.4) and water were used as negative controls.

Cloning of PCR product and sequencing: The PCR products after second step were purified by gel electrophoresis using low melting agarose gel in Tris-acetate-EDTA (TAE) buffer, pH 7.4 followed by QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The DNA fragment was cloned in pGEM-T vector (Promega, Madison, Wisconsin) and DH5α strain of *E. coli* (Tyobo Co., Osaka, Japan) was used for transformation by heat shock method (171). DNA insert containing clones were selected on Luria-Bertani (LB) medium plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) (23.8 μg/ml), ampicillin (50 μg/ml) and 5-Bromo-4-Chloro-β-D-Galactoside (X-gal) (40 μg/ml) (171). From each sample, 3 to 5 clones with expected size DNA insert were taken for sequencing. We preferred cloning and sequencing over direct sequencing

because DNA of normal intestinal flora (fecal samples) interfered with direct sequencing. The sequencing was done using the dye-terminator method and performed by a commercial resource (Dragon Genomics Co., Yokkaichi, Mia, Japan). Both strands were read. The sequences were assembled and edited using Genetyx-Mac/ATSQ 4.2.3 and Genetyx-Mac, version 13.0.6 (SDC, Tokyo, Japan).

Analysis of sequences and construction of phylogenetic trees: The chlamydia species and strains were identified by NCBI-BLAST (http://www.ncbi.nlm.nih.gov) search of nucleotide and deduced amino acids sequences. For phylogenetic analysis, the *ompA* gene sequences of *C. psittaci* strains and each representative species of genus *Chlamydophila* as well as *Chlamydia trachomatis* were retrieved from the DNA Data Bank of Japan (DDBJ). Multiple alignments of the trimmed sequences were done using ClustalX, version 1.83 (198).

Phylogenetic analysis was done with programs in the Phylogeny Inference Package software (PHYLIP) (version 3.6a3; [http://evolution.genetics.washington.edu /phylip.html]). The distance matrix between species was computed by DNADIST using F84 model (46, 102) and clustering of lineages was done by NEIGHBOR using neighbor-joining method (170). The phylogenetic tree of inferred amino acid sequences was also constructed by neighbor-joining method with the distance matrix calculated using PROTDIST by Jones-Taylor-Thornton model (91). The bootstrap values were calculated to evaluate the branching reliability of trees from a consensus tree constructed by generating 1,000 random data sets using SEQBOOT (45). *C. trachomatis* strain D/IC-Cal-8 (accession no. X62920) was used as out-group.

RESULTS

The epizootiological and genetic data were analyzed to identify the chlamydial genotypes prevalent among different avian species kept or living in different habitats and having variable disease manifestations. Out of 1,147 samples screened, 68 (5.93%) were found positive by the nested PCR among clinically normal, sick and dead birds of all avian species (Table 3). By sequencing and analyzing the genetic composition of VD2 region of *ompA* gene, genetic diversity and phylogenetic relationship among detected strains of chlamydiae was examined.

Genetic variation and relative prevalence of chlamydial species and strains: The genetic analysis of VD2 region of *ompA* genes from 68 samples showed prevalence of 64 *C. psittaci* (94.12%), 3 *C. abortus* (4.41%) and 1 unknown type of *Chlamydophila* sp. (1.47%) among all avian species (Table 3).

Table 3. Prevalence of chlamydiae according to the sampling sources and clinical status of the host avian species.

Source	Clinical status wise prevalence rate			Overall positive	Chlamydial species (sample no.)
	Normal	Sick[a]	Dead		
Vet. hospitals	2/93 (2.15%)[b]	7/48 (14.58%)	2/5 (40%)	11/146 (7.53%)	*C. psittaci* (11)
Pet shops	30/564 (5.32%)	0/3 (0%)	1/2 (50%)	31/569 (5.45%)	*C. psittaci* (31)
Bird parks and zoos	6/127 (4.72%)	0/24 (0%)	12/21 (57.14%)	18/172 (10.46%)[c]	*C. psittaci* (17) *C. abortus* (1)
Bird sanctuaries and wild fauna	8/254 (3.14%)	0/3 (0%)	0/3 (0%)	8/260 (3.08%)	*C. psittaci* (5) *C. abortus* (2) Unknown *Chlamydophila* species (1)
Total	46/1038 (4.43%)	7/78 (8.97%)	15/31 (48.39%)	68/1147 (5.93%)	

[a] Indicate the birds showing the chlamydia specific or unrelated clinical signs.

[b] The figures are positive samples/total tested samples. The positive percentage is shown in parentheses.

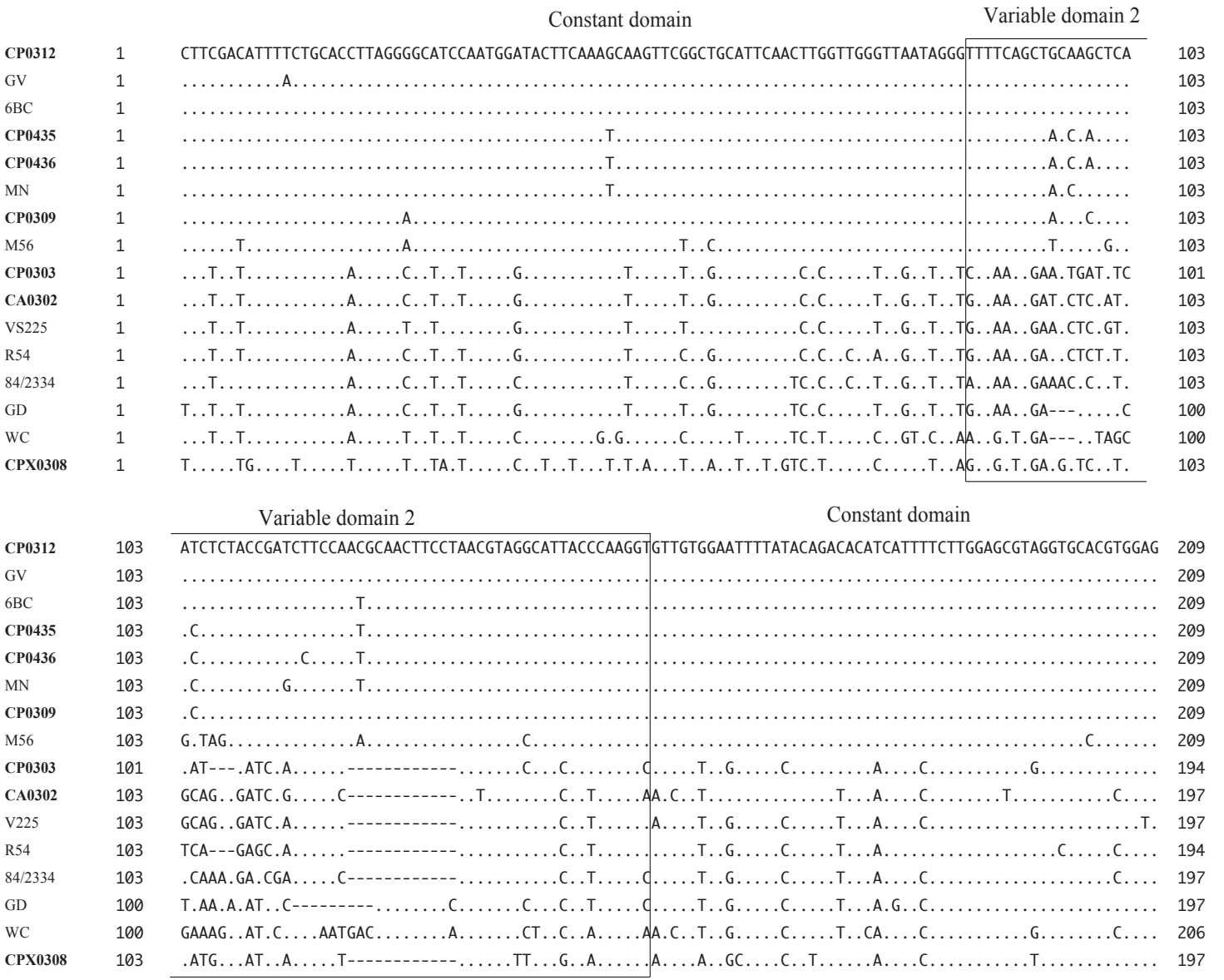
[c] Includes samples of two outbreaks of avian chlamydiosis.

Among sequenced samples, 5 kinds of nucleotide sequences (designated as VD2 sequence types) were detected in VD2 region of *C. psittaci ompA* gene (Fig. 1 and 2; VD2 sequence types 1, 4, 5, 7 and 8 in Table 4). All detected sequence types of *C. psittaci* were grouped into 4 genetic clusters, named from I to IV, based on the genetic distance ≤ 0.014 (Table 4). The genetic distance was calculated by PHYLIP. The majority of detected strains belong only to clusters I (57.35%) and II (19.12%) and these strains were detected mainly among psittacine species and only 2 oriental white storks (Fig. 3). On the basis of phylogenetic and genetic distance analysis, all the known (from data bank) and detected strains of *C. psittaci* were divided into 10 genetic clusters (named I to X) (Table 4 and Fig. 4).

The nucleotide and amino acid sequence based percent identity matrix (Table 5) showed that all the detected and known strains of *C. psittaci* can be broadly divide into two large groups. The first group is represented by the strains of clusters I to III and IX (Table 4). The second group is represented by the strains of clusters IV, V, VI, VII, VIII and X and also CPX0308 strain (Table 4), those are genetically close to *C. abortus* (Fig. 4). The WC and CPX0308 strains appeared to be genetically close to the second group of strains. WC strain showed 88% amino acid homology with GD strain, whereas, CPX0308 showed 79% nucleotide and amino acid homology with *C. caviae*-GPIC and *C. felis*-FP baker strains, respectively.

Phylogenetic analysis: Nucleotide sequence based neighbor-joining (NJ) phylogenetic tree showed distinct genetic clustering of *C. psittaci* strains (Fig. 4). CA0302, CA0306, CA0307 (Table 4) strains form a cluster along with various strains of *C. abortus*. The newly detected strains of clusters III and unknown type of *Chlamydophila* sp., form significantly distinct diverging clusters from other strains.

Fig. 1. Comparative nucleotide sequence alignment of representative variant strains of *C. psittaci*, *C. abortus* and CPX0308 (detected strains in bold letters) in the VD2 region (within box) and flanking constant domain region of *ompA* gene. The representative strains correspond to each group of strains having 100% nucleotide homology in VD2 region (designated as VD2 sequence types) as shown in Table 4. The alignment was done by Genetyx-Mac, version. 13.0.6.


Constant domain
Variable domain 2
Variable domain 2
Constant domain

		Constant domain	Variable domain 2	Constant domain	
CP0312	1	FDIFCTLGASNGYFKASSAAFNLVGLIG	FSAASSISTDLPTQLPNVGITQGV	VEFYTDTSFSWSVGARG	69
GV	1	...Y........................	....	....	69
6BC	1	....	............M...........	....	69
CP0435	1	...............S............	...TN.T.....M...........	....	69
CP0436	1	...............S............	...TN.T.....M...........	....	69
MN	1	...............S............	...T..T..E..M...........	....	69
CP0309	1	....	...TT.T.................	....	69
M56	1	....	...S.AV.....K.....A.....	....	69
CP0303	1	....	LKGTDFN-NQ..----..A.....	.......T.........	64
CA0302	1	....	VKGS.IAADQ..----.......I	.......T.........	65
VS225	1	....	VKGT.VAADQ..----.......I	.......T.........	65
R54	1	....	VKG..LS-EQ..----........	.......T.........	64
84/2334	1	....	IKGNTLTNDR..----........	.......T.........	65
GD	1	....	VKGS.LTND----.....A.....	.......T.........	65
WC	1	...............G..........F.	IAGN.E-.NA.ND.....A....I	.......T.........	68
CPX0308	1	..V......T.....SN....S......	VAGG.LNANE..----..FM...I	..L....T.........	65

Fig. 2. Amino acid homology among representative variant strains of *C. psittaci*, *C. abortus* and CPX0308 (detected strains in bold letters) in the VD2 region of *ompA* gene. The portion within the box represents the genetically variable domain 2 (VD2) flanked by the constant reg--ions of *ompA* gene. Each representative strain corresponds to a group of strains having 100% nucleotide homology in VD2 region as shown in Table 4. The alignment was done by Genetyx-Mac, version 13.0.6.

Table 4. Clusters of detected and known strains of *C. psittaci* based on nucleotide sequence similarity in the VD2 region of the *ompA* gene.

Genetic clusters (≤ 0.014 genetic distance)	VD2 sequence types[a]	Detected strains		Known strains		Chlamydial species
		Name	No.	Name (accession no.)	No.	
I	1	CP0312[b], CP0315, CP0316, CP0317, CP0318, CP0320, CP0321, CP0322, CP0323, CP0324, CP0325, CP0326, CP0327, CP0328, CP0329, CP0330, CP0331, CP0369, CP0370, CP0371, CP0372, CP0373, CP0374, CP0375, CP0376, CP0432, CP0434, CP0448, CP0449, CP0450, CP0451, CP0452, CP0453, CP0454, CP0455, CP0456, CP0457, CP0458, CP0462	39	MN Zhang (AF269281), 90/1051 (AY762608)	2	*C. psittaci*
	2	Not detected[c]		6BC[b] (X56980)	1	*C. psittaci*
	3	Not detected[c]		GV[b] (L25437)	1	*C. psittaci*
II	4	CP0435[b], CP0437, CP0438, CP0439, CP0440, CP0441, CP0443, CP0444, CP0445, CP0446, CP0447	11	CP3 (AF269265), 41A12 (AY762609)	2	*C. psittaci*
	5[d]	CP0436[b], CP0442	2	Not reported[e]	0	*C. psittaci*
	6	Not detected[c]		MN[b] (AF269262), MNOs (AF269264), MNRh (AF269263), A22/M (M36703), WS/RT/E30 (AY762613), 3759/2 (AY762611), N352 (L04980), 98AV2129 (AY327465)	8	*C. psittaci*
III	7	CP0309[b], CP0311, CP0314, CP0319 CP0459, CP0460, CP0461	7	Not reported[e]	0	*C. psittaci*
IV	8	CP0303[b], CP0304, CP0305, CP0310, CP0313	5	NJ1 (AF269266), TT3 (AF269267), 7344/2 (AY762610)	3	*C. psittaci*
V	9	Not detected[c]		GD[b] (AF269261), CT1 (AF269260), Avian type C (L25436)	3	*C. psittaci*
VI	10	Not detected[c]		VS225[b] (AF269259), 7778B15 (AY762612)	2	*C. psittaci*
VII	11	Not detected[c]		84/2334[b] (AJ310735)	1	*C. psittaci*
VIII	12	Not detected[c]		R54[b] (AJ243525)	1	*C. psittaci*
IX	13	Not detected[c]		M56[b] (AF269268)	1	*C. psittaci*
X	14	Not detected[c]		WC[b] (AF269269)	1	*C. psittaci*
	15	CA0302[b], CA0306K, CA0307K	3	B577 (M73036), EBA (AF269256), OCHL196 (AJ004873), BA1 (L39020), pm326 (AJ004875), LW508 (M73040), S26/3 (X51859), pm225 (AJ005617), wt parakeet[f]	9	*C. abortus*
	16	CPX0308[b]	1	Not reported[e]	0	Unknown *Chlamydophila* species

[a] Detected or known strains having 100% nucleotide homology in VD2 region of *ompA* gene are designated as VD2 sequence type.
[b] The representative strains used for analysis in Fig. 1, Fig. 2 and Table 5.
[c] Strains not detected in our study.
[d] Having 1 nucleotide difference in the VD2 as compared to VD2 type 4 sequences but no change in amino acid residue.
[e] Strains not reported in DNA data banks.
[f] The *ompA* gene of the strain isolated from a diseased parakeet was found 100% homologous to B577 strain (98).

Table 5. Percentage identity matrix (PIM) of detected strains (representative strains only) and known strains of *C. psittaci* and *C. abortus* in VD2 region of *ompA* gene.

	VD2 types	Genetic clusters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1: *C. psittaci*-CP0312	1		**100**	100	100	97	97	97	98	94	79	80	79	77	78	77	73	72	70	72	69	68	60
2: *C. psittaci*-GV	3		99	**100**	99	97	96	97	98	93	79	79	79	77	78	77	72	72	70	72	68	68	60
3: *C. psittaci*-6BC	2	I	99	97	**100**	98	97	98	98	94	79	80	79	77	78	77	73	72	70	72	69	68	60
4: *C. psittaci*-CP0435	4		93	91	94	**100**	100	99	98	92	80	80	79	78	79	77	72	72	70	73	69	67	61
5: *C. psittaci*-CP0436	5		93	91	94	100	**100**	99	97	91	80	80	79	78	79	77	72	72	70	73	69	67	61
6: *C. psittaci*-MN	6	II	93	91	94	97	97	**100**	97	92	79	80	79	78	79	77	72	71	69	72	68	66	60
7: *C. psittaci*-CP0309	7	III	96	94	94	96	96	94	**100**	93	88	81	79	78	79	78	73	72	70	73	70	69	61
8: *C. psittaci*-M56	13	IX	93	91	93	90	90	90	91	**100**	88	80	77	77	75	78	74	72	72	73	71	70	60
9: *C. psittaci*-CP0303	8	IV	86	83	86	86	86	84	80	79	**100**	91	91	92	90	88	81	76	78	80	75	71	64
10: *C. psittaci*-VS225	10	VI	85	83	85	85	85	85	86	83	92	**100**	92	91	89	97	81	77	75	78	74	69	65
11: *C. psittaci*-R54	12	VIII	91	89	91	88	88	88	89	86	93	92	**100**	91	91	92	79	74	74	79	72	69	66
12: *C. psittaci*-GD	9	V	85	83	85	83	83	83	85	86	95	91	94	**100**	92	92	81	77	75	76	73	72	64
13: *C. psittaci*-84/2334	11	VII	83	82	83	82	82	82	83	82	92	88	91	92	**100**	88	83	73	73	75	71	70	63
14: *C. abortus*-CA0302	15	-	86	83	86	83	83	83	85	83	90	97	92	92	88	**100**	79	73	73	76	71	70	63
15: *C. psittaci*-WC	14	X	79	76	79	79	79	79	79	79	86	86	86	88	86	86	**100**	76	70	74	73	69	63
16: Unknown type-CPX0308	16	-	71	69	71	72	72	72	71	69	77	78	78	77	74	78	75	**100**	79	73	71	72	62
17: *C. caviae*-GPIC[a]	-	-	73	70	73	73	73	72	75	75	78	79	79	79	74	77	75	74	**100**	77	70	72	65
18: *C. felis*-FP Baker[a]	-	-	77	73	77	77	77	75	78	77	84	84	80	79	77	82	77	79	78	**100**	70	70	64
19: *C. pneumoniae*-CSF[a]	-	-	69	65	69	69	69	69	69	70	70	71	72	76	76	73	73	69	66	66	**100**	70	67
20: *C. pecorum*-1710S[a]	-	-	66	67	66	68	68	68	68	68	74	72	70	69	69	69	68	74	74	76	69	**100**	65
21: *C. trachomatis*-D[a]	-	-	57	54	57	57	57	57	57	57	59	59	62	59	61	59	60	64	62	59	64	64	**100**

[a] The accession no. of sequences are: *C. caviae*-GPIC (AF269282), *C. felis*-FP Baker (AF269257), *C. pneumoniae*-CSF (AF131889), *C. pecorum*-1710S (AF269279) and *C. trachomatis*-D (X62920). For *C. psittaci* strains accession no. are shown in Table 4.

The upper right triangular half of the matrix is nucleotide based and lower left triangular half is amino acid based. The bold figures separating two triangular halves indicate the 100% identity based on nucleotide and amino acid sequences. The strains having ≥90% identity are boxed together in the solid line. The numbers (1 to 21) in the top row correspond to the respective species/strains listed in the first column. The matrix was constructed by ClustalX (version 1.83). The comparative PIM of *C. psittaci* strains with other *Chlamydophila* species and *C. trachomatis* infecting mammals and human (non-avian hosts) is also shown.

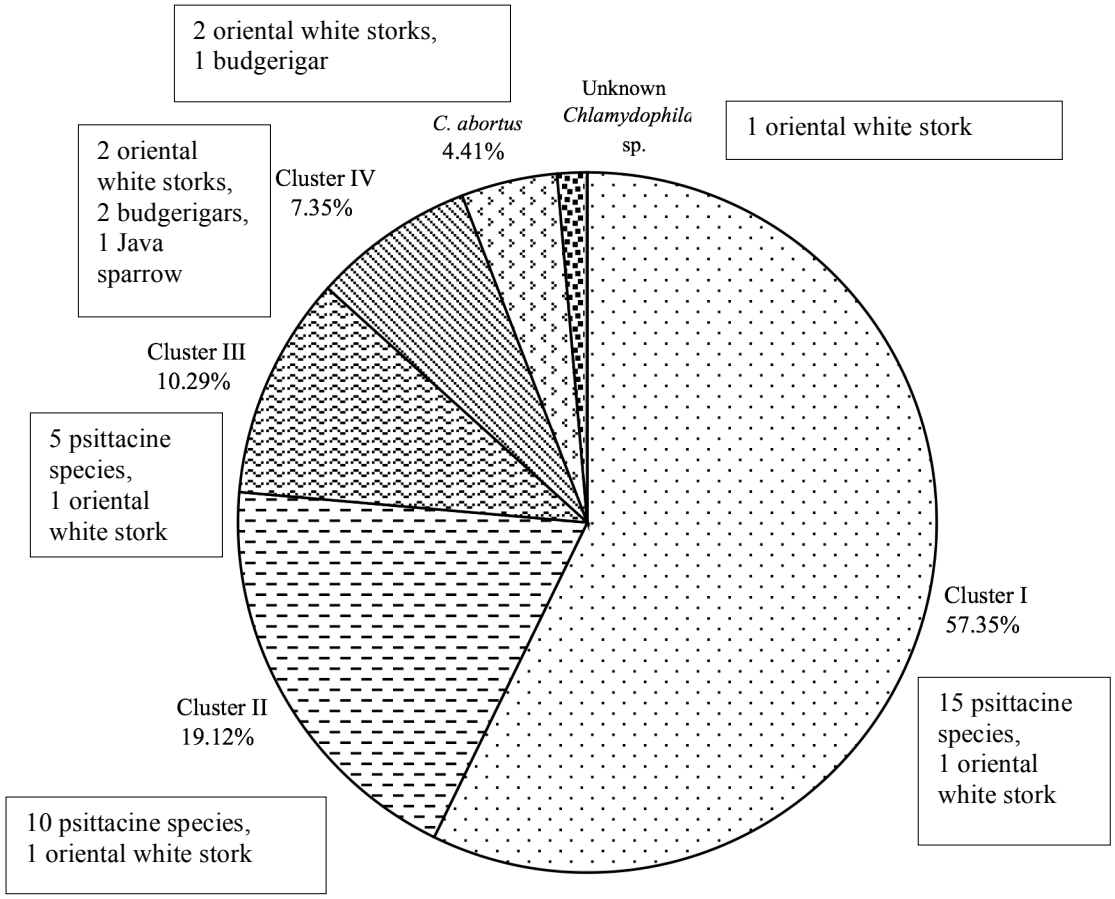


Fig. 3. Diagram showing the comparative prevalence of chlamydial strains detected in this study. The types of avian species (psittacine species are members of *Cacatuidae* and *Psittacidae* families) tested chlamydia positive are shown in boxed text near each cluster of strains. Two largest clusters of *C. psittaci* strains are cluster I (57.35%) and cluster II (19.12%) respectively.

Epizootiological analysis: The cases of avian chlamydiosis were mainly due to *C. psittaci* strains and detected among 58 samples from *Psittaciformes* birds, 5 from oriental white storks and 1 from Java sparrow. The *C. abortus* was detected in 1 budgerigar and 2 oriental white storks samples. One unknown species, CPX0308 was also found in an oriental white stork imported from Russia to a bird sanctuary in Japan (Table 6). The strains of cluster I were mainly detected in chlamydiosis cases including 2 outbreaks (Fig.

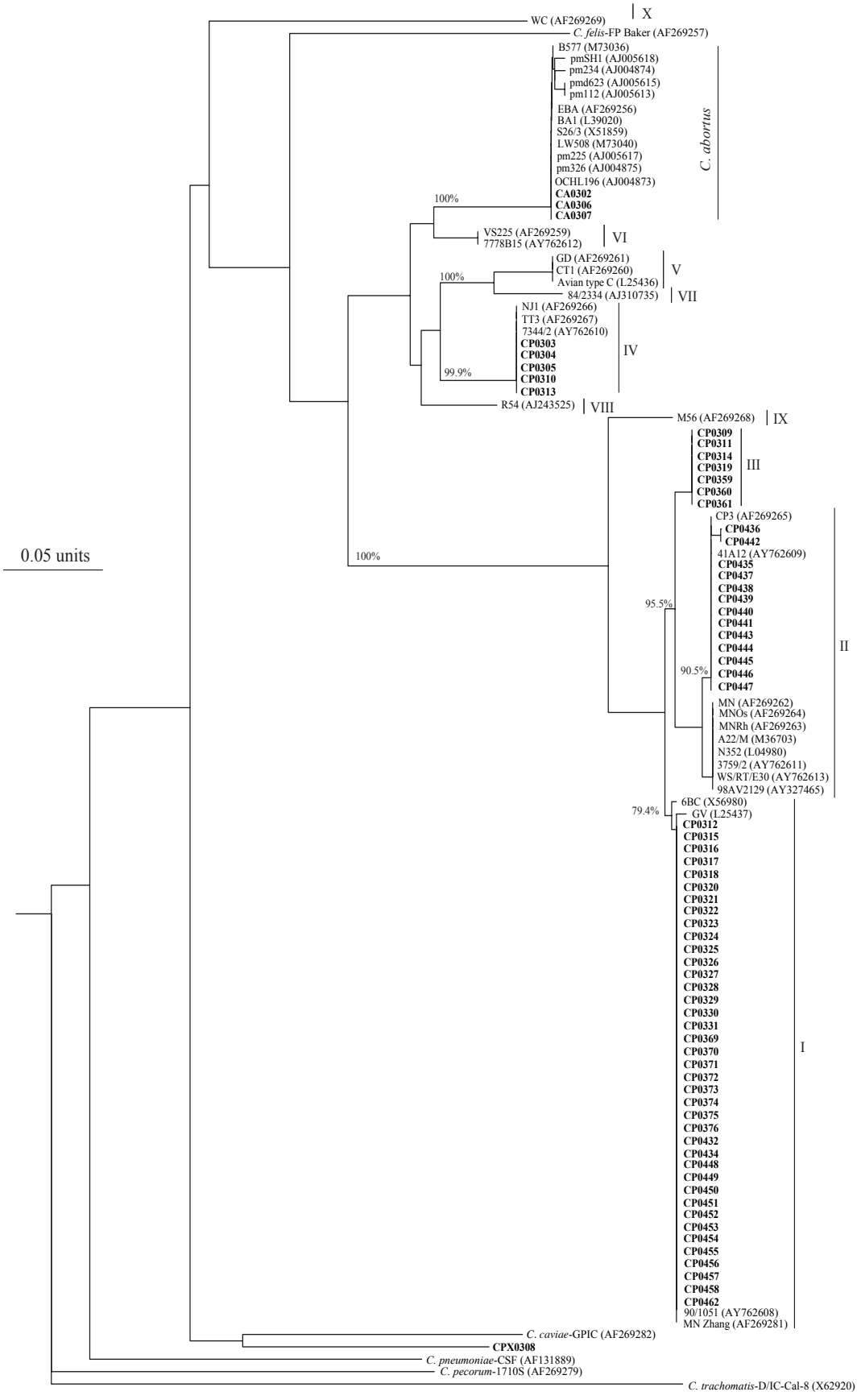
5). However, other genotypes were also detected occasionally including an outbreak with strains of cluster III (mixed infection with cluster I strains). The month wise incidence rate of chlamydiosis due to various genotypes during 2003 and 2004 is shown in Fig. 5. The chlamydiosis cases were detected throughout year but slight rise was observed in chlamydiosis incidences in the beginning of winter and spring seasons including 2 outbreaks.

The prevalence rate according to clinical status, sources of sampling, avian species, sex, age and geographical locations is as follow:

(i) Clinical status wise prevalence. Among 4.43% (46/1038) clinically normal birds all 6 types of *C. psittaci* genotypes (genetic clusters) were detected in the feces including one case (CP0432) in which only blood sample gave positive PCR result. The strains of clusters I and II were detected from 8.97% (7 out of 78) sick birds showing chlamydia specific and non-specific symptoms. 48.39% (15 out of 31) dead birds, those showed typical clinical symptoms of chlamydiosis before death and histopathological lesions, were found to carry chlamydial strains of clusters I, II and III (Tables 3 and 6).

(ii) Avian habitat wise prevalence. According to the habitats of avian hosts (represented by the sources of sampling) that also epitomize managemental conditions and avian-human interactions, the highest 10.46% cases were detected from bird parks/zoos (including 2 outbreaks by strains of clusters I and III), followed by 7.53% from individually/pair wise caged birds, mostly kept as pets in households. However, 5.45% and 3.08% cases were detected respectively from pet shops and bird sanctuaries (Table 3). The cluster II strains were detected mostly from one pet shop. The strains of cluster I and II were detected most often among samples from veterinary hospitals and pet shops. The chlamydial infection in the bird parks and zoos were mostly due to strains of clusters I, III, and IV of *C. psittaci* and *C. abortus* strains, whereas, among samples from bird sanctuaries

Fig. 4. Neighbor-joining (NJ) phylogenetic tree, based on nucleotide sequences of VD2 region of *ompA* gene of different strains of *C. psittaci*, *C. abortu*s and other *Chlamydophila* species. The strains detected in this study are shown in bold letters and vertical lines mark genetic clusters of *C. psittaci* strains from I to X. The genetic distance is indicated in 0.05 unit bar. Bootstrap values are shown at respective nodes. The *C. trachomatis* is used as out-group.


0.05 units
X
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C. felis-FP Baker (AF269257)
B577 (M73036)
pmSH1 (AJ005618)
pm234 (AJ004874)
pmd623 (AJ005615)
pm112 (AJ005613)
EBA (AF269256)
BA1 (L39020)
S26/3 (X51859)
LW508 (M73040)
pm225 (AJ005617)
pm326 (AJ004875)
OCHL196 (AJ004873)
CA0302
CA0306
CA0307
C. abortus
100%
VS225 (AF269259)
7778B15 (AY762612)
VI
GD (AF269261)
CT1 (AF269260)
Avian type C (L25436)
V
100%
84/2334 (AJ310735)
VII
NJ1 (AF269266)
TT3 (AF269267)
7344/2 (AY762610)
CP0303
CP0304
CP0305
CP0310
CP0313
IV
99.9%
R54 (AJ243525)
VIII
M56 (AF269268)
IX
CP0309
CP0311
CP0314
CP0319
CP0359
CP0360
CP0361
III
CP3 (AF269265)
CP0436
CP0442
41A12 (AY762609)
CP0435
CP0437
CP0438
CP0439
CP0440
CP0441
CP0443
CP0444
CP0445
CP0446
CP0447
II
100%
95.5%
90.5%
MN (AF269262)
MNOs (AF269264)
MNRh (AF269263)
A22/M (M36703)
N352 (L04980)
3759/2 (AY762611)
WS/RT/E30 (AY762613)
98AV2129 (AY327465)
6BC (X56980)
GV (L25437)
79.4%
CP0312
CP0315
CP0316
CP0317
CP0318
CP0320
CP0321
CP0322
CP0323
CP0324
CP0325
CP0326
CP0327
CP0328
CP0329
CP0330
CP0331
CP0369
CP0370
CP0371
CP0372
CP0373
CP0374
CP0375
CP0376
CP0432
CP0434
CP0448
CP0449
CP0450
CP0451
CP0452
CP0453
CP0454
CP0455
CP0456
CP0457
CP0458
CP0462
90/1051 (AY762608)
MN Zhang (AF269281)
I
C. caviae-GPIC (AF269282)
CPX0308
C. pneumoniae-CSF (AF131889)
C. pecorum-1710S (AF269279)
C. trachomatis-D/IC-Cal-8 (X62920)

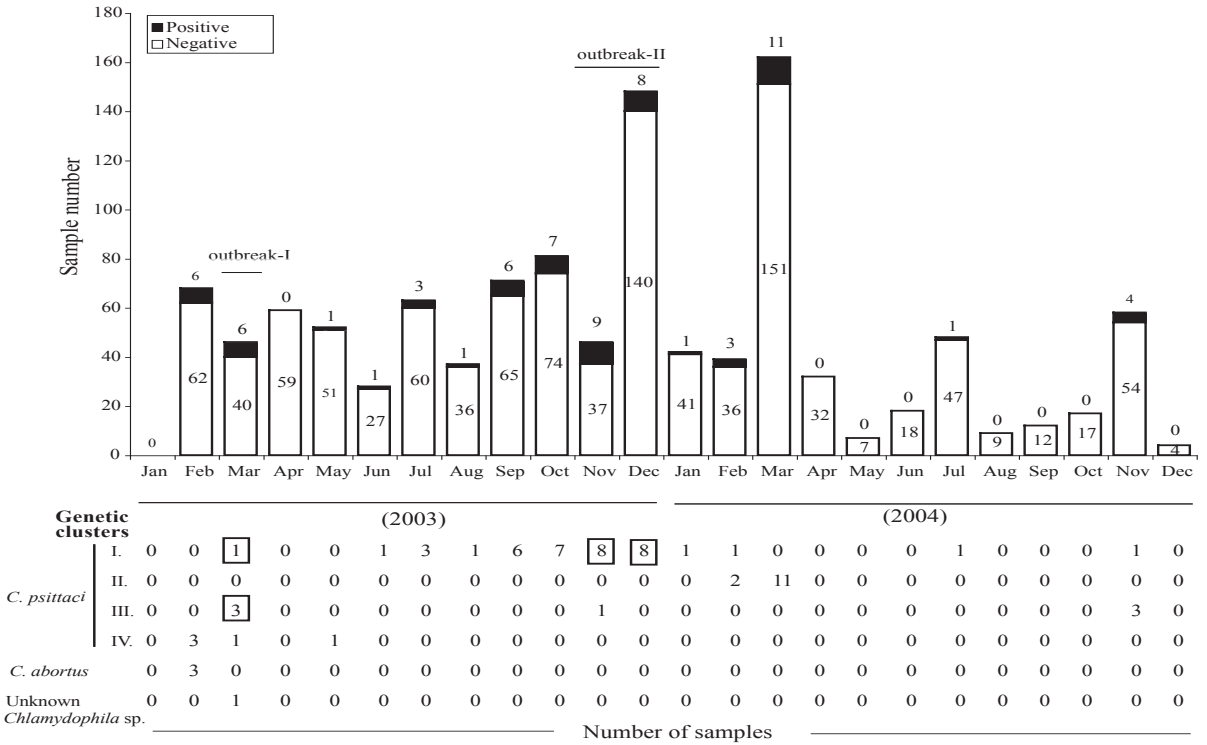


Fig. 5. Month wise incidences of avian chlamydiosis cases detected by PCR during 2003 and 2004 among all avian species. The numerical figures shown inside and above the bars are actu--al numbers of chlamydiae positive and negative cases. The numbers shown in columns below each bar indicatethe distribution of samples and detected strain type in each month. Outbreak-I and outbreak-II took place at two different bird parks and boxes mark the types of *C. psittaci* strains involved.

strains of clusters I, III, IV and unknown type of the *Chlamydophila* sp. were detected (Table 6).

(iii) Avian species wise prevalence. The incidences of chlamydiosis were predominantly detected among *Psittaciformes* birds (detected among 28 psittacine avian species). It includes chlamydia positive rates of 7.61% (14 out of 184) in family *Cacatuidae* and 7.23% (45 out of 622) in family *Psittacidae*. However, some psittacine bird species such as *Trichoglossus haematodus* 12 out of 52 (23.08%), *Nymphicus hollandicus* 11 out of 77 (14.28%), *Aratinga* sp. 5 out of 44 (11.36%), *Ara* sp. 5 out of 55 (9.09%) and *Mesopsittacus undulates* 5 out of 59 (8.47%) showed high incidences (Tables 1, 2 and 6). In the *Cacatuidae* family mainly the cluster I strains were detected; however, in *Psittacidae*, strains of clusters I, II, III, IV and *C. abortus* were detected. Whereas, among non-psittacine species, 3.12% (8 out of 256) samples from oriental white storks (*Ciconia boyciana*) belonging to *Ciconiidae* family of order *Ciconiiformes,* were found having strains of clusters I, II, III, IV, *C. abortus* and unknown type of *Chlamydophila* sp. A single case of chlamydiosis due to a strain of cluster IV was also detected in a Java sparrow (*Padda oryzivora*) belonging to *Estrildidae* family of *Passeriformes* order (Table 6).

(iv) Avian sex and age wise prevalence. The incidences of chlamydiosis were found higher among birds around the age group of 3 months due to different genotypes, irrespective of their sexes (Table 6).

(v) Worldwide locations wise prevalence. All type of genotypes were detected among the avian species imported to Japan from Singapore, Indonesia, USA, South Africa and Russia as well as from different places in Japan, indicating ubiquitous presence of various chlamydia genotypes infecting different avian fauna (Table 6).

Table 6. Types of chlamydial species/strains detected in relation to the zoological position, sampling source, clinical status, sex, age and import source of avian host species

Order, family and scientific name (common name) of the host avian species (no. of positive/tested samples)	Species wise positive/tested samples	Details about the PCR positive and sequenced samples							Strains designated	Genetic clusters	Accession no.
		No.	Type	Clinical history	Source	Age:sex of the host[a] (M: male, F: female)	Source of import[b]	Chlamydial species			
Order-*Psittaciformes* (59/806)											
Family-*Cacatuidae* (14/184)											
Cacatua moluccensis (Salmon-crested Cockatoo)	1/18	1	Clotted blood	Normal	Pet shop	6 months	Singapore	*C. psittaci*	CP0432	I	AB239867
C. sulphurea (Yellow-crested Cockatoo)	1/26	1	Cloacal swab	Normal	Pet shop		Indonesia	*C. psittaci*	CP0457	I	AB239878
Eolophus roseicapillus (Roseate Cockatoo or Galah)	1/9	1	Pooled visc. orgn.[c]	Dead (outbreak-I)[d]	Bird park/zoo			*C. psittaci*	CP0312	I	AB239842
Nymphicus hollandicus (Cockatiel)	11/77	2	Feces	Normal	Vet. Hospital	3 months		*C. psittaci*	CP0315	I	AB239843
								C. psittaci	CP0316	I	AB239844
		4	Feces	Sick[e]	Vet. Hospital	6 months		*C. psittaci*	CP0322	I	AB239848
						2 years 10 months		*C. psittaci*	CP0460	III	AB239897
								C. psittaci	CP0448, CP0449	I	AB239869, AB239870
		5	Cloacal swabs	Normal	Pet shop		Domestically bred (Japan)	*C. psittaci*	CP0451, CP0452	I	AB239872, AB239873
									CP0453, CP0454		AB239874, AB239875
									CP0455		AB239876
Family-*Psittacidae* (45/622)											
Amazona aestiva (Blue-fronted Amazon)	1/6	1	Feces	Sick[e]	Vet. hospital	1 year		*C. psittaci*	CP0462	I	AB239880
Ara ararauna (Blue-and-yellow Macaw)	1/11	1	Cloacal swab	Normal	Pet shop			*C. psittaci*	CP0444	II	AB239888
A. auricollis (Yellow-collard Macaw)*	2/23	2	Cloacal swabs	Normal	Pet shop	2 years	Singapore	*C. psittaci*	CP0445, CP0446	II	AB239889, AB239890
A. severa (Chestnut-fronted Macaw)*	2/7	2	Pooled visc.orgn.[c]	Dead (outbreak-I)[d]	Bird park/zoo			*C. psittaci*	CP0311, CP0314	III	AB239893, AB239894
Aratinga aurea (Peach-fronted Conure)	1/3	1	Cloacal swab	Normal	Pet shop	1 year:F	South Africa	*C. psittaci*	CP0328	I	AB239855
A. pertinax (St. Thomas/Brown-throated Conure)*	1/1	1	Cloacal swab	Normal	Pet shop	1 year:M	South Africa	*C. psittaci*	CP0329	I	AB239856
A. acuticaudata (Blue-crowned Conure)	1/7	1	Cloacal swab	Normal	Pet shop		South Africa	*C. psittaci*	CP0330	I	AB239857
A. wagleri (Scarlet or Red-fronted Conure)*	1/2	1	Cloacal swab	Normal	Pet shop	M	South Africa	*C. psittaci*	CP0439	II	AB239884
A. jandaya (Jandaya Conure)*	1/6	1	Cloacal swab	Normal	Pet shop	3 months	South Africa	*C. psittaci*	CP0442	II	AB239908
Lorius lory (Black-capped Lory)*	1/5	1	Cloacal swab	Normal	Pet shop	1 year	Singapore	*C. psittaci*	CP0441	II	AB239886
Melopsittacus undulatus (Budgerigar)	5/59	1	Feces	Normal	Bird park/zoo			*C. abortus*	CA0302	-	AB239904
		2	Feces	Normal	Bird park/zoo			*C. psittaci*	CP0303, CP0304	IV	AB239899, AB239900
		1	Spleen	Dead	Vet. hospital	4 months		*C. psittaci*	CP0447	II	AB239891
		1	Liver	Dead	Vet. hospital			*C. psittaci*	CP0438	II	AB239883
Neophema bourkii (Bourke's Parrot)	1/2	1	Feces	Sick[e]	Vet. hospital	6 months:M		*C. psittaci*	CP0458	I	AB239879
Poicephalus rueppellii (Rüppell's Parrot)*	2/7	2	Cloacal swabs	Normal	Pet shop	M	South Africa	*C. psittaci*	CP0326	I	AB239853
							South Africa	*C. psittaci*	CP0450	I	AB239871
P. senegalus (Senegal Parrot)	1/42	1	Cloacal swab	Normal	Pet shop	3 months	South Africa	*C. psittaci*	CP0319	III	AB239895
P. cryptoxanthus (Brown-headed Parrot)*	2/26	1	Cloacal swab	Normal	Pet shop	3 months:M	South Africa	*C. psittaci*	CP0323	I	AB239850
		1	Cloacal swab	Normal	Pet shop	3 months:M	South Africa	*C. psittaci*	CP0436	II	AB239907
P. meyeri (Meyer's Parrot/Broun Parrot)	1/19	1	Cloacal swab	Normal	Pet shop	3 months:M	South Africa	*C. psittaci*	CP0435	II	AB239881

P. gulielmi (Red-crowned/Jardine's Parrot)	1/23	1	Cloacal swab	Normal	Pet shop		South Africa	*C. psittaci*	CP0443	II	AB239887
Pionites leucogaster (White-bellied Caique)	1/8	1	Pooled visc.orgn.[c]	Dead[f]	Pet shop	3 months:M	USA	*C. psittaci*	CP0461	III	AB239898
P. melanocephala (Black-headed Caique)	1/6	1	Cloacal swab	Normal	Pet shop			*C. psittaci*	CP0456	I	AB239877
Psittacula derbiana (Derbyan Parakeet)	1/3	1	Cloacal swab	Normal	Pet shop		Domestically bred (Japan)	*C. psittaci*	CP0327	I	AB239854
Psittacus erithacus (African Grey Parrot)	3/154	3	Cloacal swabs	Normal	Pet shop	3 months:M	South Africa	*C. psittaci*	CP0317, CP0318	I	AB239845, AB239846
						3 months	South Africa	*C. psittaci*	CP0434	I	AB239868
Pyrrhura hypoxantha sallvadori (Yellow-sided Conure)	1/3	1	Cloacal swab	Normal	Pet shop	5 months		*C. psittaci*	CP0440	II	AB239885
P. perlata lepida (Pearly Conure)*	1/5	1	Feces	Sick[g]	Vet. hospital	1 year 9 months:M		*C. psittaci*	CP0459	III	AB239896
Trichoglossus haematodus (Green-napped Lorikeet)	1+11[h]/49	8	Pooled visc. Orgn.[c]	Dead (outbreak-II)[h]	Bird park/zoo			*C. psittaci*	CP0320, CP0369	I	AB239847, AB239859
									CP0370, CP0371		AB239860, AB239861
									CP0373, CP0374		AB239863, AB239864
									CP0375, CP0376		AB239865, AB239866
		1	Feces	Dead (outbreak-II)[h]	Bird park/zoo			*C. psittaci*	CP0324	I	AB239851
		2	Feces	Normal (outbreak-II)[h]	Bird park/zoo			*C. psittaci*	CP0321, CP0372	I	AB239849, AB239862
		1	Cloacal swab	Normal	Pet shop	M		*C. psittaci*	CP0325	I	AB239852
Order-*Ciconiiformes* (8/257)											
Family-*Ciconiidae* (8/256)											
Ciconia boyciana (Oriental White Stork)*	8/256	2	Feces	Normal	Bird sanctuary		Russia	*C. abortus*	CA0306, CA0307	-	AB239905, AB239906
		1	Feces	Normal	Bird sanctuary		Russia	Unknown *Chlamydophila* sp.	CPX0308	-	AB239931
		3	Feces	Normal	Bird sanctuary		Russia	*C. psittaci*	CP0309	III	AB239892
							Russia	*C. psittaci*	CP0331	I	AB239858
							Russia	*C. psittaci*	CP0437	II	AB239882
		2	Cloacal swabs	Normal	Bird sanctuary		Russia	*C. psittaci*	CP0310, CP0313	IV	AB239902, AB239903
Order-*Passeriformes* (1/13)											
Family-*Estrildidae*(1/5)											
Padda oryzivora (Java Sparrow)	1/5	1	Feces	Normal	Bird park/zoo			*C. psittaci*	CP0305	IV	AB239901

[a] Only the available data about age and sex of avian species are shown.
[b] Only the confirmed records about the source of importation and breeding place of host species are shown.
[c] Pooled visceral organs includes pieces lung, liver, spleen and heart.
[d] Samples are from outbreak-I.
[e] Include those birds showing chronic weight loss, yellowish-green diarrhea, anorexia, cachexia and rise in body temperature.
[f] Died with acute symptoms and also found positive for avian polyoma virus by PCR.
[g] Had crop inflammation due to yeast infection along with clinical symptoms of chlamydiosis.
[h] Samples from outbreak-II.
* The avian species detected chlamydia positive first time when compared to referred latest updated list (94).

DISCUSSION

C. psittaci mainly infects avian species and also has potential zoonotic importance (8, 181, 188). In this chapter, we investigated the strain level prevalence of various chlamydiae among diverse avian fauna and examined various situations those contribute to the maintenance, precipitation and/or horizontal transmission of infection. Further genetic and phylogenetic relationships among prevalent species/strains were also examined.

We analyzed partial *ompA* gene (VD2 region) for identification of chlamydial species and strains. This locus has been reported to be associated with the phylogenetic divergence of *Chlamydophila* spp. and *Chlamydia* spp. and has been used in other phylogenetic studies. Although the phylogenetic analyses of conserved 16S rRNA, *ompA* and *rnpB* genes of chlamydiae group are often used to classify and differentiate *C. psittaci* strains from other species of family *Chlamydiaceae* (60, 79, 149, 191, 193, 205), the analysis of *ompA* gene is advantageous in epizootiological strain typing due to presence of strain/serovar/genotype specific motifs in 4 genetically variable domains (12, 86, 148, 225). Sequence analysis of highly conserved 16S rRNA gene may not detect minor strain variations. Direct sequencing of 16S rRNA gene was also not suited in our study due to the interference by normal intestinal microflora DNA as we tested many samples of fecal/fecal swab or intestinal loops origin. By our approach, small DNA fragment of *ompA* gene can be conveniently amplified from clinical samples by nested PCR and sequenced even in lower EB concentration in samples (2 to 10 EBs). This approach of direct strain typing from clinical samples particularly in large-scale epizootiological/epidemiological studies is less cumbersome as serotyping and isolation is time consuming.

To survey the host range of *C. psittaci*, total 113 avian species from 11 avian orders examined. The chlamydiae were detected among 28 species of psittacine birds and 2 species of non-psittacine birds (oriental white stork and Java sparrow). Out of total 28

psittacine bird species 10 were detected first time to harbor chlamydiae in this study along with 1 oriental white stork species (Table 6). Till date the chlamydiae have been detected among 460 avian species from 30 avian orders that include 153 *Psittaciformes* species (94). Therefore, the chlamydiae have been detected among 163 (47.66%) out of total 342 *Psittaciformes* species prevalent worldwide including results of present study. Some species popularly kept as pets such as *Trichoglossus haematodus* (Green-napped Lorikeet), *Nymphicus hollandicus* (Cockatiel), *Aratinga* sp. (Parakeet or Conure), *Ara* sp. (Macaw) and *Mesopsittacus undulates* (Budgerigar) showed exceptionally high incidences of chlamydiosis. From the results of this study and earlier reports those documented high chlamydiosis incidences among *Psittaciformes* species (18, 69) it appears that *Psittaciformes* are predominant reservoirs of chlamydiae among all avian species. Therefore, *Psittaciformes* avian species may be responsible for maintenance or transmission of infection in the population.

Although there are many reports regarding the pigeons as natural carriers of *C. psittaci* and source of infection to human being (6, 16, 20, 45), in this study only few samples from pigeons were tested, those were found negative. As the main thrust of this study was analyzing samples from captive avian fauna, either imported or indigenously bred and kept under near natural or caged conditions and are often exposed to human beings and animal fauna.

C. psittaci strains are divided into 8 serotypes named A to H (4, 56, 206, 207) and 7 genotypes from A to F and E/B (60, 174, 207), based on monoclonal antibody typing and AluI RFLP pattern of *ompA* gene respectively. In the present study, clustering pattern of *C. psittaci* strains in relation to grouping on the basis of serological and RFLP studies was also analyzed phylogenetically using nucleotide sequences and deduced amino acid sequences as described in materials and methods. Genetic cluster I includes *C. psittaci*

strains with *ompA* gene AluI RFLP pattern and serotype type A; clusters II includes RFLP types B, E and E/B and serotype types B and E; cluster IV includes RFLP and serotype type D; whereas, strains of cluster III and CPX0308 are unknown types.

The genetic and phylogenetic analysis of chlamydial strains detected in the screened avian fauna revealed that VD2 sequence type 1 and 4 form clusters I and II along with genetically related strains classified as serovars/genotypes-A and B by others (4, 174). Clusters I and II were found predominantly prevalent especially among *Psittaciformes* birds. High prevalence of serotypes/genotypes A and B have also being reported in recent studies (60, 191, 207).

Cluster III strains were also detected from 6 psittacine birds and 1 oriental white stork. Cluster III strains have 3 amino acid substitutions in VD2 region as compared to VD2 sequence types 1 and 4 clusters of strains. Cluster III strains appeared to be recently evolved from cluster II strains.

Strains of cluster IV which are genetically the same as serovar/genotype-D strains of *C. psittaci* (4, 60, 174) were detected from 2 oriental white storks, 2 budgerigars and 1 Java sparrow. These strains were usually detected from non-psittacine avian species and are highly pathogenic to domesticated poultry (4, 7, 8, 207). Our results indicate that these strains may have broad host range and may dormantly exist among other free-living avian fauna those can be potential source of infection to domesticated/commercial poultry.

CA0302, CA0306 and CA0307 strains detected from 3 samples, genetically resembled to a cluster of *C. abortus* strains. Similarly a “wt parakeet” strain, isolated from a diseased parakeet by Kaltenboeck *et al.* (96), was found to have *ompA* gene sequence 100% homologous to that of B577 strain of *C. abortus* (98). The genetic data based on RFLP pattern and 16S rRNA and 16S-23S rRNA inter genic spacer region sequencing studies have also indicated that the genetic relatedness among *C. psittaci* and *C. abortus* is

quite high and there are some isolates having the characteristic of both groups (43, 51, 149, 193, 205). Recent studies have also pointed toward this fact (191). The *C. abortus* seems to be latest diverging group still having genetic roots in the avian hosts.

From the epizootiological and genetic data of this study and other reports, it can be inferred that all chlamydial strains responsible for avian chlamydiosis can be broadly divided into two groups: major *C. psittaci* strains and minor *C. psittaci* strains. The major group includes those strains which are highly prevalent among avian species, evolutionally conserved and are genetically adapted to class aves. The examples are strains of clusters I, II, III and IV (Tables 4 and 5) and serovar/genotypes-A, B, C, D, E and E/B (4, 56, 60, 206, 207). Whereas, the minor group include very rarely detected strains among avian and mammalian species and genetically intermediate between other mammalian *Chlamydophila* species and *C. psittaci* such as strains of CA0302, CA0306 and CA0307 strains and unknown type of *Chlamydophila* sp. (CPX0308 strain) (Tables 4 and 5) and serovar-F, G, H and 84/2334, VS225 strains (4, 56, 206, 207) and R54 strain (79), V448, V351 strains (191) and Daruma strain (51).

Finally, it can be concluded that incidences of avian chlamydiosis are more among psittacine birds especially under captive conditions. Though disease incidences are high in some avian species but the *C. psittaci* is maintained and propagated in the population by a large variety of inapparently infected avian fauna. Many strains of *C. psittaci* are genetically well adapted to avian fauna and associated with disease or simply co-exist in dormant stage but only some of the strains are predominantly prevalent among birds.

SUMMARY

For studying the genetic diversity and occurrence of *Chlamydophila psittaci*, a total of 1,147 samples from 11 avian orders including 53 genera and 113 species of feral and captive birds were examined using *ompA* gene based nested PCR. Three types of chlamydiae: *C. psittaci* (94.12%), *C. abortus* (4.41%) and unknown *Chlamydophila* sp. (1.47%) were identified among 68 (5.93%) positive samples (*Psittaciformes*-59, *Ciconiiformes*-8 and *Passeriformes*-1). On the basis of nucleotide sequence variations in the VD2 region of *ompA* gene, all 64 detected *C. psittaci* strains were grouped into 4 genetic clusters. Clusters I, II, III and IV were detected from 57.35%, 19.12%, 10.29% and 7.35% samples respectively. A single strain of unknown *Chlamydophila* sp. was found to be phylogenetically intermediate between *Chlamydophila* species of avian and mammalian origin. Among *Psittaciformes*, 28 out of 81 tested species including 10 species previously unreported were found chlamydiae positive. Chlamydiosis was detected among 8.97% sick and 48.39% dead birds as well 4.43% clinically normal birds. Therefore, it was concluded that, though various genetically diverse chlamydiae may have caused avian chlamydiosis, only a few *C. psittaci* strains were highly prevalent and frequently associated with clinical/subclinical infections.

CHAPTER-II

Analysis of genetic diversity and molecular phylogeny of the *Chlamydophila psittaci* strains prevalent among avian fauna and associated with human psittacosis.

INTRODUCTION

Chlamydophila psittaci is an obligate intracellular pathogen, mainly causing clinical or subclinical infections among avian species and is also potentially pathogenic to other mammals and human beings (8, 181). The disease of avian species is called "avian chlamydiosis" and human infection is known as "psittacosis" (181). The clinical avian chlamydiosis is either acute or subacute and characterized by respiratory distress, diarrhea, ocular and nasal discharge, nervous signs or systemic infection with varying severity and mortality up to 30% (8). The infectious elementary bodies (EBs) of *C. psittaci* can survive for months in feces and secretions in the environment and human get infection by inhaling air borne infectious particles (181).

Avian chlamydiosis has been reported from 471 avian species but mainly from psittacine birds (33, 94). Human infections are typically acquired from exposure to pet psittacine birds, however, infection from poultry, free ranging birds have also been reported (9, 113, 127, 197). The morbidity and mortality among the infected populations is attributed to many factors. In human psittacosis, a wide spectrum of illness is possibly ranging from asymptomatic infection to mild influenza-like illness to a fulminate disease with involvement of several extra pulmonary sites (67, 181).

Attempts have been made in past to serotype or biotype *C. psittaci* strains of avian origin by using polyclonal sera, plaque reduction neutralization, microimmunofluroscence

and examining inclusion morphology (11, 183). Biological difference has also been observed among various *C. psittaci* strains. Two different nucleic acid precursor utilization patterns and variation in susceptibility to sulfonamides and 5-fluorouridine were observed (125). Winsor and Grimes also divided 17 *C. psittaci* strains into low infective and high infective groups on the basis of infectivity and cytopathology to L929 cells (219). These biological differences are also well supported by later genetic studies based on gene specific or whole genome restriction endonuclease analysis and DNA-DNA hybridization (3, 6, 51, 126, 200).

After the reclassification of order *Chlamydiales* on the basis of the 16S rRNA gene, all the avian and 2 mammalian origin strains of the old *Chlamydia psittaci* group are now classified as *Chlamydophila psittaci* (43). Alternatively, *ompA* gene that encodes major outer membrane protein (MOMP), which is immuno dominant, is used for serotyping and genotyping of the *C. psittaci* strains in epidemiological studies. Various *C. psittaci* strains are classified into 8 serovars from A to H using MOMP specific monoclonal antibodies (Mab) (4, 56, 206, 207) and into 7 genotypes on the basis of the AluI restriction fragment length polymorphism (RFLP) of the *ompA* gene (60, 174, 207). But the recent epidemiological studies and some previous reports have shown the existence of genetically diverse strains among avian fauna (33, 51, 79, 191, 205). The phylogenetic positions of these strains have not been established yet. Though the host specificity of different *C. psittaci* serotypes and genotypes has been reported (7), but their prevalence across many avian/mammalian species has been detected (33, 174, 207). Therefore, it is imperative to genetically characterize all the genetically variant *C. psittaci* strain*s* irrespective of host species and to design a suitable and more accommodative classification scheme. Further identification of *C. psittaci* strains with higher potential for transmitting and causing infection of human and other mammalian livestock is also needed.

Therefore, in this chapter, diverse *C. psittaci* strains detected from various avian and mammalian hosts including human beings were genetically analyzed in *ompA* gene locus to know the phylogenetic relationships, pathobiotic implications and for improved strain categorization. *C. psittaci* strains with the histories of causing human psittacosis are either isolated/detected directly from clinically sick patients or from in-contact avian hosts. Phylogenetic positions of highly diverse strains were confirmed by additionally analyzing the16S rRNA gene. It was observed that all the genetically diverse *C. psittaci* strains could be grouped into 4 broad lineages and 8 small genetic clusters, which encompass all the 7 known and 4 new genotypes as well as all 8 known serotypes. The majority of *C. psittaci* strains responsible for the human infection belongs to genetic cluster I.

MATERIALS AND METHODS

Chlamydial species and strains: The *C. psittaci* strains used for genetic analysis are listed in the Table 7 along with brief historical background. The *C. psittaci* strains with the history of human psittacosis includes those isolated in Japan in last 40 years and those available in DNA data banks.

Chlamydial culture and purification of EBs: McCoy cell lines were used for primary isolation and multiplication of new and laboratory strains of *C. psittaci*. The cells were maintained in Eagle's minimum essential medium-1 (MEM-1) (Nissui, Japan) containing 5% fetal bovine serum (FBS), 10 μg/ml gentamicin and 1 μg/ml of cycloheximide. The EBs were harvested 3 to 4 days after inoculation. Chlamydial EBs were partially purified as reported by Tamura and Higashi (194). Briefly, harvested cell suspension was centrifugation at 1,000 × *g* for 10 min at 4°C to remove cell debris. The supernatant was then again centrifuged at 12,000 × *g* for 60 minutes at 4°C. The pellets

Table 7. *C. psittaci* strains used to study *ompA* gene.

Strains	Source (sample type)	Human infection	Geographical location	Reference	Data bank accession no.
N-1	Budgerigar (fecal swab)	Psittacosis[a]	Japan	This study	AB284055
Itoh	Human (blood and sputum)	Psittacosis[b]	Japan	This study[c]	AB284056
Izawa-1	Budgerigar (feces)	Psittacosis[a]	Japan	This study[d]	AB284057
Mat116	Chestnut-fronted Macaw (feces)	Psittacosis[h]	Japan	This study	AB284058
CP0315	Cockatiel (feces)	Psittacosis[a]	Japan	This study	AB284059
Nosé	Budgerigar (feces)	Psittacosis[a]	Japan	This study[d]	AB284060
30A	Human (ear swab	Psittacosis[b]	Japan	This study[c, d]	AB284061
KKCP1	Human (nasopharyngeal swab)	Psittacosis[b]	Japan	This study[d]	AB284062
KKCP2	Human (bronchoalveolar fluid)	Psittacosis[b]	Japan	This study[d]	AB284063
CPX0308	Oriental White Stork (feces)	Unknown	Japan	This study	AB284064
Daruma	Parakeet (viscera)	Unknown	India	This study[g]	AB284065
Borg	Human (lung)	Pneumonitis[b]	USA	This study[e]	AB284066
6BC	Parakeet	Unknown[f]	USA	(42)	M73035
MN Zhang	Human/ferret	Psittacosis[b]	USA	(49)	AF269281
CP3	Pigeon	Unknown	USA	(145)	AF269265
TT3	Turkey	Psittacosis[h]	USA	(144)	AF269267
NJ1	Turkey	Psittacosis[h]	USA	(142)	AF269266
MN/Cal10	Human/ferret	Psittacosis[b]	USA	(49)	AF269262
MNOs	Ostrich	Unknown	USA	(5)	AF269264
MNRh	Rhea	Unknown	USA	(5)	AF269263
WC	Bovine (intestinal tissues)	Psittacosis[h]	USA	(143)	AF269269
VS225	Orange-fronted Parakeet	Unknown	USA	(24)	AF269261
CT1	Turkey	Unknown	USA	(141)	AF269260
41A12	Wild turkey	Unknown	Belgium	(60)	AY762609
7778B15	Wild turkey	Unknown	Belgium	(60)	AY762612
90/1051	Amazon parrot	Unknown	Belgium	(60)	AY762608
92-1293	Turkey	Unknown	Belgium	(208)	Y16562
84-55	Parakeet	Unknown	Belgium	(208)	CPS16561
98AV2129	Swine	Unknown	Belgium	(211)	AY327465
84/2334	Amazon parrot (lung)	Unknown	Germany	(205)	AJ310735
GD	Duck	Unknown	Germany	(88)	AF269261
WS/RT/E30	Duck	Unknown	Germany	(60)	AY762613
A22/M	Sheep	Unknown	UK	(147)	M36703
N352	Duck	Unknown	UK	(157)	L04980
Avian type C	Unknown avian sp.	Unknown	UK	Storey (1994)[j]	L25436
GV	Goat (vaginal sample)	Unknown	UK	Storey (1994)[j]	L25437
7344/2	Pigeon	Unknown	Italy	(60)	AY762610
3759/2	Pigeon	Unknown	Italy	(60)	AY762611
M56	Muskrat	Unknown	Canada	(182)	AF269268
R54	Brown skua (feces)	Unknown	Sub Antarctic Island	(79)	AJ243525
Fulmar#10	Fulmar (cloacal sample)	Unknown	Faroe Islands	(78)	AM050561
05/02	Human	Psittacosis[i]	Netherlands	Heddema *et al*. 2005)[j]	DQ324426
OSV	Human (sputum/throat wash)	Psittacosis[i]	Netherlands	(77)	DQ230095
CLV	Pigeon	Unknown	Netherlands	(77)	DQ230096
Orni	Pigeon (feces)	Unknown	Netherlands	(76)	DQ435299
CLA	Pigeon(feces)	Unknown	Netherlands	(76)	DQ267973

[a] Confirmed by detecting chlamydia specific antibodies in psittacosis patients and isolation from companion birds.
[b] Isolation from human patients having all the typical clinical signs of psittacosis.
[c] Itoh strain was isolated by Jo *et al.* 1962 (90) and 30A strain was isolated by Mukai *et al.* 1985 (134).
[d] Isolated and kindly provided by Dr. Akira Matsumoto, Kawasaki School of Medicine, Japan (120).
[e] Isolated by Olson and Treuting, 1944 (ATCC VR-601) (140).
[f] DNA similar to 6BC (ATCC VR125) was detected in vaginal sample (81).
[g] Isolated by Yamashita and Hirai, 1981 (221).
[h] Doubtful history of psittacosis.
[i] Confirmed by *C. psittaci* DNA detection in clinical samples of human psittacosis patients.
[j] Unpublished work.

were then resuspended in 0.01M Tris-HCl, pH 7.4, in appropriate volumes and each strain was stored at -80°C in aliquots. These aliquots were later used for DNA extractions.

DNA extraction: SepaGene DNA extraction Kit (Sanko Junyaku, Tokyo, Japan) was used to extract DNA from samples according to the manufacturer's instructions. In brief, about 100 µl partially purified EB were suspended in 700 µl of phosphate-buffered saline (PBS), pH 7.4, were processed for DNA extraction. DNA was finally dissolved in 50 µl of Tris-EDTA buffer, pH 7.4 (100 mM Tris-HCl, pH 7.4 and 10 mM EDTA, pH 8.0) and stored at –30°C. The *ompA* and 16S rRNA genes were amplified in overlapping fragments by PCR using pair of forward and reverse primers as shown in Table 8.

PCR amplification: The PCR was done in 50 µl reaction mixture containing 0.15 µM of each forward and reverse primer, 250 µM of each dATP, dTTP, dGTP, dCTP, 100 µM of Mg^{2+} in buffer and 2.5 units of TaKaRa *Ex-Taq* (Takara Bio Inc., Shiga, Japan) and 2.0 to 5.0 µg of template DNA of each strain. The thermo cycling conditions were: denaturation for 3 min at 94°C, then 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at a temperature depending on melting temperature of forward and reverse primer pairs and extension at 72°C for 60 sec, then final extension for 5 min at 72°C and soaking at 4°C.

DNA sequencing: The cloning of PCR amplified products was done as described in CHAPTER-I, *i.e.* 3 to 5 clones with expected size DNA insert were taken for sequencing. The sequences were assembled and edited using Genetyx-Mac/ATSQ 4.2.3 and Genetyx-Mac, version 13.0.6 (SDC, Tokyo, Japan).

Phylogenetic analysis: For phylogenetic analysis, all the available *ompA* gene sequences of *C. psittaci* strains and each representative species of genera *Chlamydophila* and *Chlamydia* were retrieved from DDBJ. The *ompA* and 16S rRNA gene sequences were trimmed to the uniform length to included all the known, genetically diverse *C. psittaci*

Table 8. Oligonucleotide primers used to amplify *ompA* and 16S rRNA genes.

Name	Sequence	Positions[a]	Tm (°C)[b]	Size (bp)
ompA gene				
M/0PF	5` GCAAGTATAAGGAGTTATTGCTTG 3`	-275 to -252	54-56	352
M/0PR	5` CCTACAGGCAAGGCTTGTAAG 3`	56-77	57-60	
M/1PF	5` CGGCATTATTGTTTGCCGCTAC 3`	20-41	59	390
M/1PR	5` CGAAGCGATCCCAAATGTTTAAGGC 3`	409-385	59-61	
M/2PF	5` CGCTTACGGAAGGCATATGCAAGATG 3`	330-355	62	397
M/2PR	5` GTGAATCACAAATTGTGCTGGGCT 3`	726-703	58-60	
M/3PF	5` CGTGGAGCTTTATGGGAATGTGGTTG 3`	607-632	62	330
M/3PR	5` GAGCAATGCGGATAGTATCAGCATC 3`	937-913	60-62	
M/4PF	5` GGCGTAAACTGGTCAAGAGCAAC 3`	886-908	60-62	387
M/4PR	5` TCCCAGGTTCTGATAGCGGGACAA	1272-1246	61-63	
16S rRNA gene				
CPR/P1F	5` CTGAGAATTTGATCTTGGTTCAG 3`	5-27	54-56	400
CPR/P1R	5` CGCTTCGTCAGACTTTCGTCCATT 3`	404-381	60	
CPR/P2F	5` CGTCTAGGCGGATTGAGAGATTG 3`	291-313	60-62	418
CPR/P2R	5` CGCATTTCACCGCTACACGTGGAAT 3`	708-684	60-62	
CPR/P3F	5` GCGTGTAGGCGGAAAGGAAAGTT 3`	585-607	60-62	413
CPR/P3R	5` CCAGGTAAGGTTCTTCGCGTTGCAT 3`	997-973	59-61	
CPR/P4F	5` CCGTGTCGTAGCTAACGCGTTAAGT 3`	860-884	60-62	444
CPR/P4R	5` GGCTAGCTTTGAGGATTTGCTCCAT 3`	1403-1279	59-61	
CPR/P5F	5` CGAGGATGACGTCAAGTCAGCAT 3`	1191-1213	60-62	367
CPR/P5R	5` TGTCGACAA AGGAGGTGATCCAG 3`	1557-1537	55-57	

[a] Based on *ompA* gene open reading frame (ORF) of *C. psittaci*-6BC strain (accession no. X56980) and 16S rRNA gene of *C. psittaci*-Prk/GCP-1 strain (D85713). The negative values indicate the nucleotide position upstream of respective ORF.
[b] Averages of melting temperatures of forward and reverse primer sets were used in the PCR amplifications.

strains. A total of 1,492 bp fragment of 16S rRNA was analyzed.

The analyzed *ompA* gene sequences include about 898 to 940 bp fragment extending from 161 bp downstream from starting codon of *ompA* gene to 1,098 bp position. It includes all four hyper variable domains (VDs). The combined constant domains (CDs) of 631 bp and variable domains of 267 to 309 bp were analyzed separately. Calculation of percent identity matrix and multiple alignments of the trimmed sequences was done using ClustalX, version 1.83 (198). Phylogenetic analysis was done with programs in the PHYLIP (version 3.6a3; [http://evolution.genetics.washington.edu/phylip.

html]). The distance matrix between species was computed by DNADIST using Jukes-Cantor model (93) and clustering of lineages was done by NEIGHBOR using neighbor-joining method (170). The bootstrap values were calculated to evaluate the branching reliability of trees from a consensus tree constructed by generating 1,000 random data sets using SEQBOOT (45).

***in-silico* AluI random fragment length polymorphism (RFLP) map:** The comparative AluI RFLP maps of different *C. psittaci* strains were generated by using Genetyx-Mac, version 13.0.6 (SDC, Tokyo, Japan).

RESULTS

We initially analyzed relative evolution of whole *ompA* gene among all species of family *Chlamydiaceae vis-à-vis* species of new *C. psittaci* group. Then comparative analysis of constant/conserved domains (CDs) and variable domains (VDs) of all genetically variable *C. psittaci* strain was done for genetic clustering and comparison.

Evolution of *ompA* gene among the members of *Chlamydiaceae* family: The neighbor-joining (NJ) tree in Fig. 6 is showing phylogenetic distances among the *Chlamydophila* spp. and *Chlamydia* spp. infecting human and various types of animal and avian species. Clear separation of *Chlamydophila* spp. and *Chlamydia* spp. into two clans irrespective of human and animal chlamydiae was observed. Furthermore on the basis of *ompA* gene, host specific clustering of *Chlamydia* spp. or *Chlamydophila* spp. strains was observed with exception of *C. psittaci* strains of avian origin. The *C. psittaci* strains form distinct small clusters diverging out from other clusters of *Chlamydophila* spp.

***ompA*-gene based genetic clustering of *C. psittaci*:** Various clusters and subclusters of *C. psittaci* strains are resolved in the NJ tree of about 940 bp segment of

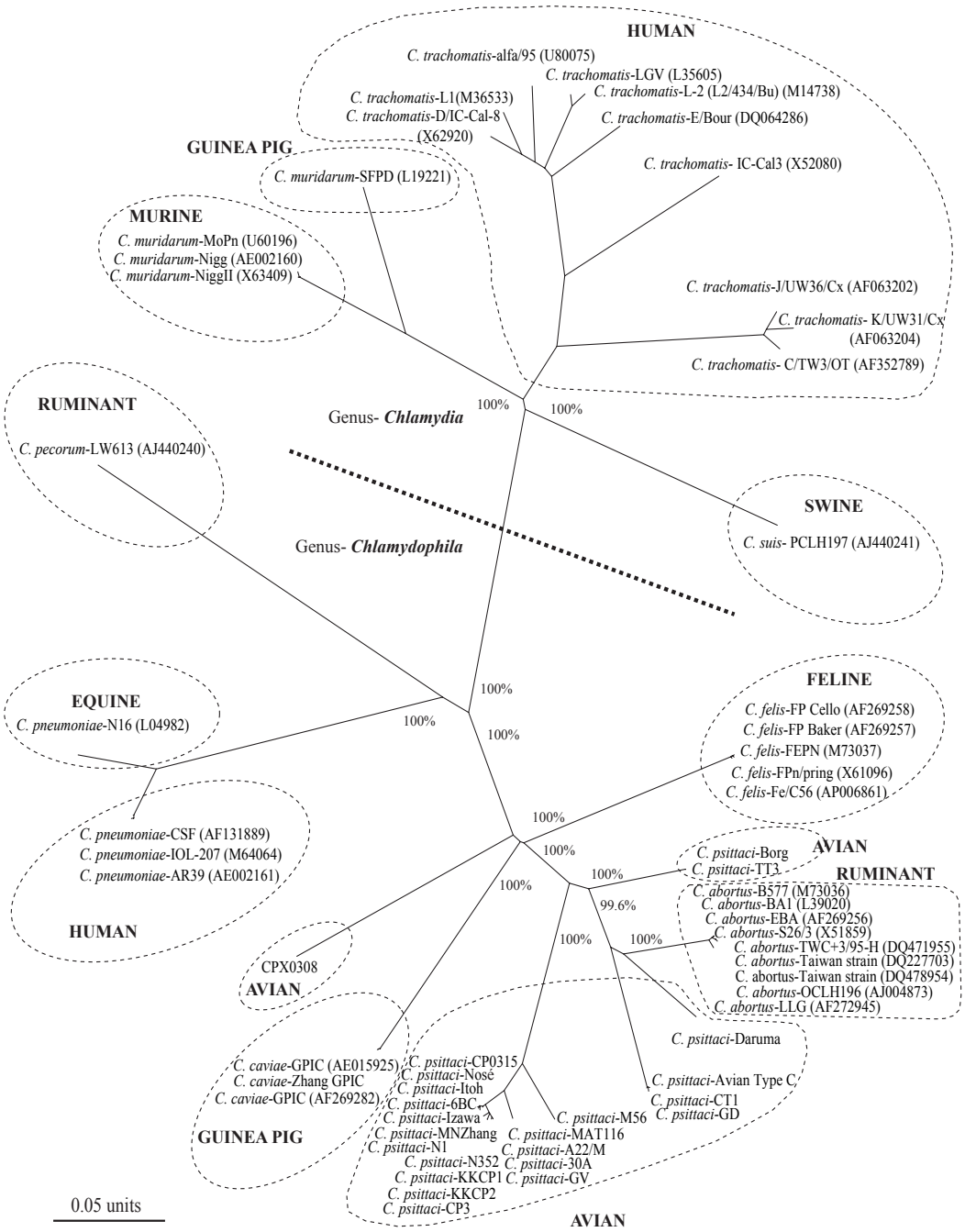


Fig. 6. Neighbor joining (NJ) tree of *ompA* gene ORF showing host species specific clustering of *Chlamydophila* spp. and *Chlamydia* spp. infecting human and various animal species. The tree rev--ealed diverse clusters of *Chlamydophila* spp. of avian origin. The bootstrap values are shown near to respective nodes. The genetic distance is shown in units scale bar. The accession numbers of *C. psittaci* strains are shown in Table 7.

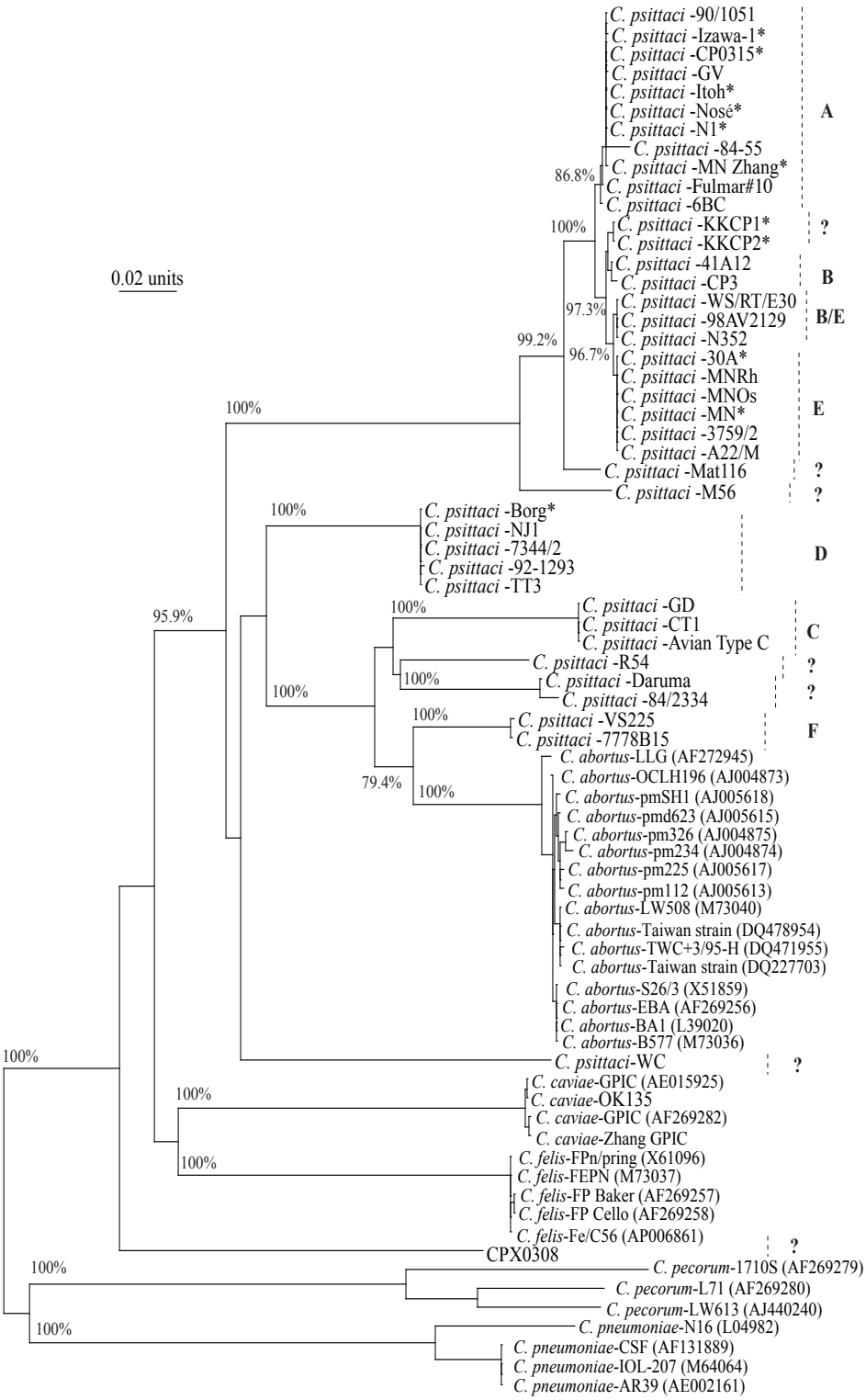


Fig. 7. The partial *ompA* gene (including VDs) based NJ tree showing the relative evolutionary distance among different genetic clusters of *C. psittaci* strains. The known genotypes from A to F and E/B are demarcated by the dotted lines. The unknown/unclassified genotypes are questi--on marked (?). Asterisk mark (*) indicates the *C. psittaci* strains with human psittacosis history. The bootstrap values are shown againt each node. The genetic distance is shown in units scale bar.

ompA gene that includes all 4 variable domains (Fig. 7). The distribution analysis of the genetic distances and percent dissimilarities in *C. psittaci* strains (conserved regions only) showed 4 groups of peaks. The ranges of genetic distance values for these 4 groups are 0.002 to 0.04, 0.061to 0.122, 0.133 to 0.156 and 0.194 to 0.211 (Fig. 8).

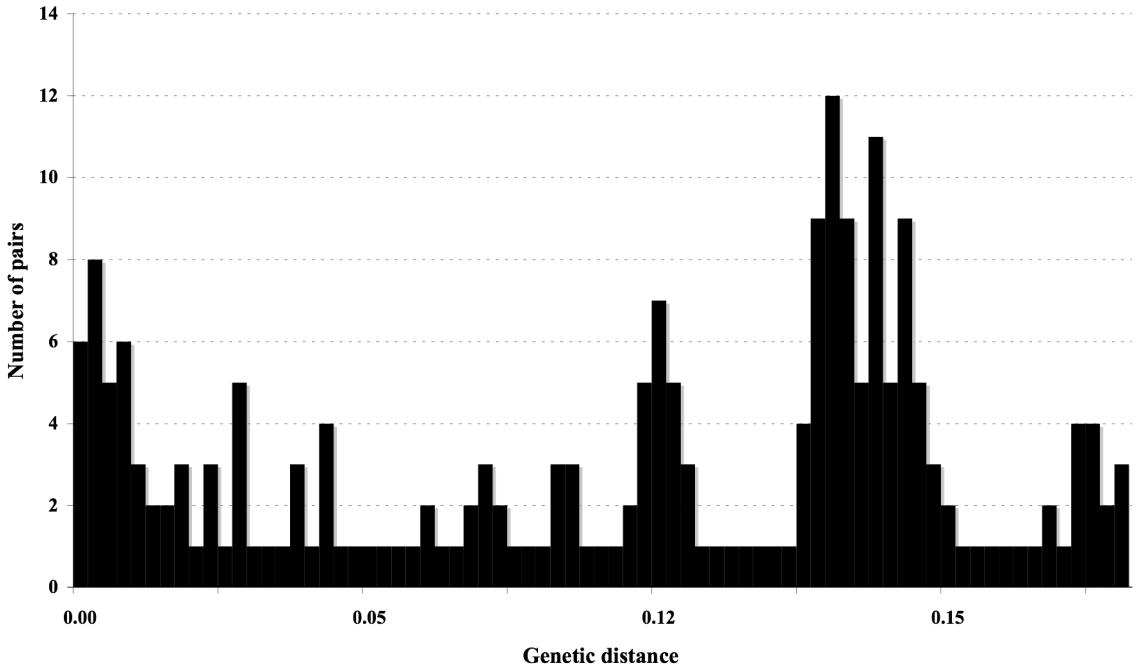


Fig. 8. Distribution of the genetic distances in the conserved regions of *ompA* gene of *C. psittaci* strains. Genetic distances were calculated by Jukes-Cantor method (93).

Then by tabulating the genetic distance and dissimilarity matrices and plotting NJ distance trees did the detailed analysis of clustering patterns of *C. psittaci* strains.

(i) Constant domains. All 21 *C. psittaci* strains with variant CDs were included in analysis along with other representative *Chlamydophila* spp. Previously unclassified Mat116, R54, Daruma, 84/2334 and CPX0308 strains were observed to be phylogenetically diverse. On the basis of genetic distance and dissimilarity matrices and

the NJ tree of CDs, all the *C. psittaci* strains were divided into 4 broad lineages and 8 genetic clusters named from I to VIII (Fig. 9-A, Tables 9 and 10). The genetic distance among strains within a lineage is less than 0.1 (dissimilarity less than 10%). Whereas, within these lineages strains having genetic distance less than 0.05 (dissimilarity less than 5%) are divided into genetic clusters. Lineage-1 has only one large cluster of genetically similar strains mainly detected from psittacine bird, pigeons and ratites. The lineage-2 has 5 genetic clusters of genetically diverse strains mostly detected from poultry, water birds, psittacine and other non-psittacine birds. The lineage-3 is represented by WC strain, a bovine enteritis isolate, and lineage-4 by CPX0308 strain, detected from an oriental white stork. The genetic distance among all *C. psittaci* strains is less than 0.156 except CPX0308 strain that has genetic resemblance to mammalian *Chlamydophila* spp. than avian type. The nearest avian strain is V225 with genetic distance of 0.188. The relative difference in nucleotide and amino acid numbers among 4 lineages and 8 genetic clusters are shown in Table 10. It showed that seemingly large number of nucleotide variations do not reflect the resultant amino acid variation within respective lineages or genetic clusters.

(ii) Variable domains. Total 18 *C. psittaci* strains having variation in VDs regions were analyzed. KKCP1, KKCP2, Mat156, R54, Daruma, 84/2334 and CPX0308 strains were observed to be phylogenetically distinct (Fig. 9-B). All the 8 genetic clusters from I to VIII were also distinguishable but lower degree of percent identity and higher genetic distance was found within each genetic cluster and designated lineages (Fig. 9-B and Table 11). The percent identity between 4 lineages was found to be less than 67%. Each genetic cluster contains the same type of strains as that in CDs based tree.

Phylogenetic positions of CPX0308 and Daruma strain: Two highly genetically diverse strains of avian origin CPX0308 and Daruma were compared with other members of family *Chlamydiaceae*. The NJ tree of 16S rRNA gene is shown in Fig. 10. CPX0308

Fig. 9. NJ trees based on the constant domain regions (A) and the variable domains (B), of *ompA* gene showing genetic clusters of *C. psittaci* strains and other *Chlamydophila* spp. The genetic clusters of *C. psittaci* strains from I to VIII are demarcated by the vertical solid lines and known genotypes from A to F and E/B by dotted lines. The unknown/unclassified genotypes are question marked (?). The *C. psittaci* strains with the history of human psittacosis are marked by asterisk (*). The genetic distance is shown in units scale bar.

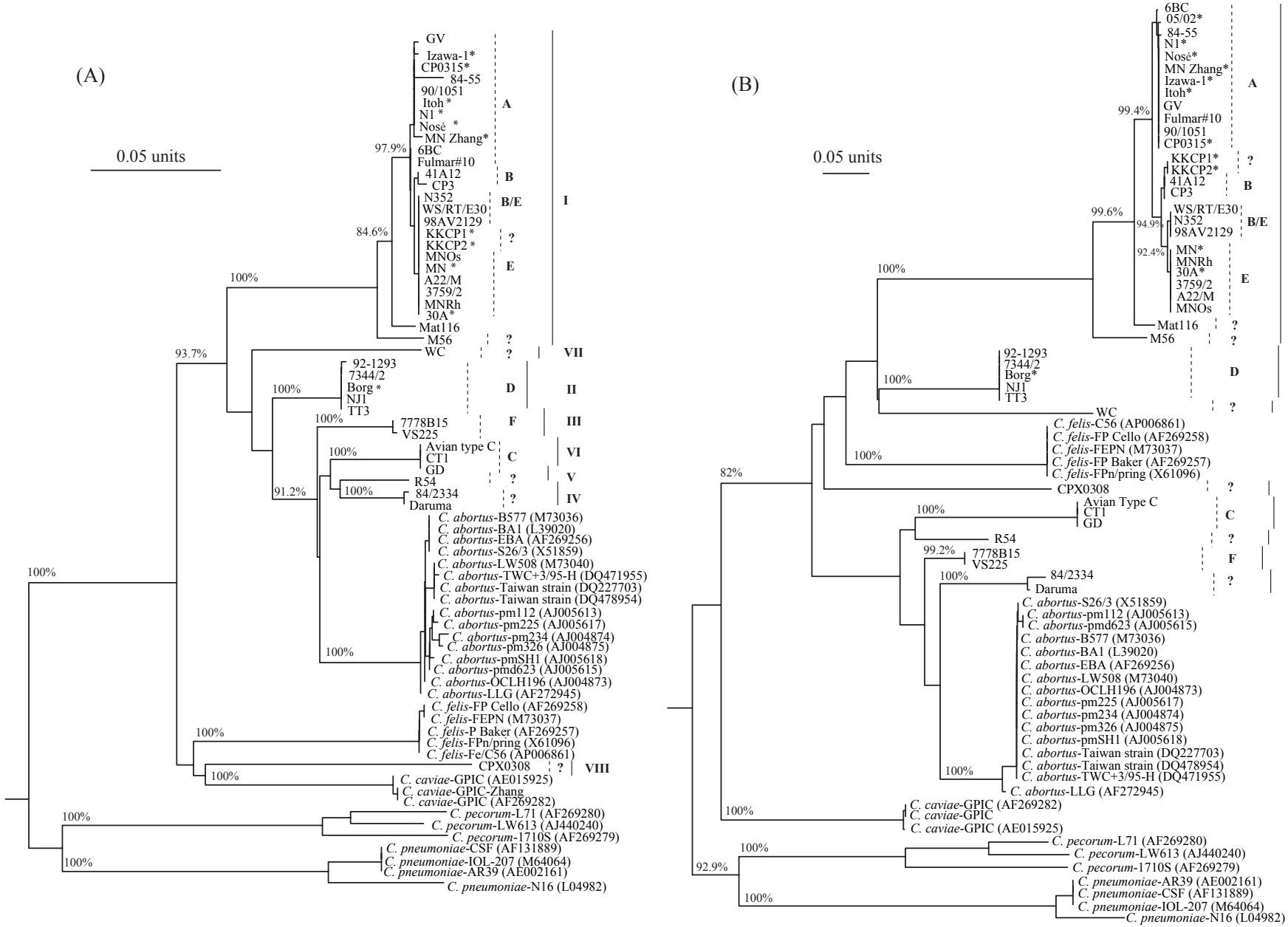

(A)
0.05 units
GV
Izawa-1*
CP0315*
84-55
90/1051
Itoh *
N1 *
Nosé *
MN Zhang*
6BC
Fulmar#10
41A12
CP3
N352
WS/RT/E30
98AV2129
KKCP1 *
KKCP2 *
MNOs
MN *
A22/M
3759/2
MNRh
30A*
Mat116
M56
WC
92-1293
7344/2
Borg *
NJ1
TT3
7778B15
VS225
Avian type C
CT1
GD
R54
84/2334
Daruma
A
B
B/E
?
E
?
?
D
F
C
?
?
I
VII
II
III
VI
V
IV
97.9%
84.6%
100%
93.7%
100%
100%
100%
100%
91.2%
100%
C. abortus-B577 (M73036)
C. abortus-BA1 (L39020)
C. abortus-EBA (AF269256)
C. abortus-S26/3 (X51859)
C. abortus-LW508 (M73040)
C. abortus-TWC+3/95-H (DQ471955)
C. abortus-Taiwan strain (DQ227703)
C. abortus-Taiwan strain (DQ478954)
C. abortus-pm112 (AJ005613)
C. abortus-pm225 (AJ005617)
C. abortus-pm234 (AJ004874)
C. abortus-pm326 (AJ004875)
C. abortus-pmSH1 (AJ005618)
C. abortus-pmd623 (AJ005615)
C. abortus-OCLH196 (AJ004873)
C. abortus-LLG (AF272945)
C. felis-FP Cello (AF269258)
C. felis-FEPN (M73037)
C. felis-P Baker (AF269257)
C. felis-FPn/pring (X61096)
C. felis-Fe/C56 (AP006861)
CPX0308
C. caviae-GPIC (AE015925)
C. caviae-GPIC-Zhang
C. caviae-GPIC (AF269282)
C. pecorum-L71 (AF269280)
C. pecorum-LW613 (AJ440240)
C. pecorum-1710S (AF269279)
C. pneumoniae-CSF (AF131889)
C. pneumoniae-IOL-207 (M64064)
C. pneumoniae-AR39 (AE002161)
C. pneumoniae-N16 (L04982)
100%
100%
100%
100%
100%
100%
100%
?
VIII
(B)
0.05 units
6BC
05/02*
84-55
N1*
Nosé*
MN Zhang*
Izawa-1*
Itoh*
GV
Fulmar#10
90/1051
CP0315*
KKCP1*
KKCP2*
41A12
CP3
WS/RT/E30
N352
98AV2129
MN*
MNRh
30A*
3759/2
A22/M
MNOs
Mat116
M56
92-1293
7344/2
Borg*
NJ1
TT3
WC
C. felis-C56 (AP006861)
C. felis-FP Cello (AF269258)
C. felis-FEPN (M73037)
C. felis-FP Baker (AF269257)
C. felis-FPn/pring (X61096)
CPX0308
Avian Type C
CT1
GD
R54
7778B15
VS225
84/2334
Daruma
C. abortus-S26/3 (X51859)
C. abortus-pm112 (AJ005613)
C. abortus-pmd623 (AJ005615)
C. abortus-B577 (M73036)
C. abortus-BA1 (L39020)
C. abortus-EBA (AF269256)
C. abortus-LW508 (M73040)
C. abortus-OCLH196 (AJ004873)
C. abortus-pm225 (AJ005617)
C. abortus-pm234 (AJ004874)
C. abortus-pm326 (AJ004875)
C. abortus-pmSH1 (AJ005618)
C. abortus-Taiwan strain (DQ227703)
C. abortus-Taiwan strain (DQ478954)
C. abortus-TWC+3/95-H (DQ471955)
C. abortus-LLG (AF272945)
C. caviae-GPIC (AF269282)
C. caviae-GPIC
C. caviae-GPIC (AE015925)
C. pecorum-L71 (AF269280)
C. pecorum-LW613 (AJ440240)
C. pecorum-1710S (AF269279)
C. pneumoniae-AR39 (AE002161)
C. pneumoniae-CSF (AF131889)
C. pneumoniae-IOL-207 (M64064)
C. pneumoniae-N16 (L04982)
A
?
B
B/E
E
?
?
D
?
C
?
F
?
I
II
VII
VIII
VI
V
III
IV
99.4%
99.6%
94.9%
92.4%
100%
100%
100%
82%
99.2%
100%
100%
100%
92.9%
100%

Table 9. Grouping of genetically diverse *C. psittaci* strains based on the genetic distance in constant domains of the *ompA* gene, calculated by Jukes-Cantor method (93). The *C. psittaci* strains having genetic distance less than 0.1 are grouped in a lineage and those less than 0.05 genetic distance are grouped togather into a single cluster. Only the representative genetically variant strains of *C. psittaci* were taken for analysis. The other *Chlamydophila* species are shown with grey background. Different genetic clusters are demarcated by solid lines and lineages by dotted lines.

Chlamydial species	Accession no.	**Lineage-1** 1	2	3	4	5	6	7	8	9	10	11	12	**Lineage-2** 13	14	15	16	17	18	19	20	**Lin.-3** 21	**Lin.-4** 22	23	24	25	26	27
1-*C. psittaci*-GV	L25437	0.000																										
2-*C. psittaci*-Nosé	AB284060	0.002	0.000																									
3-*C. psittaci*-Izawa-1	AB284057	0.003	0.002	0.000																								
4-*C. psittaci*-6BC	M73035	0.003	0.002	0.003	0.000																							
5-*C. psittaci*-MN Zhang	AF269281	0.005	0.003	0.005	0.005	0.000																						
6-*C. psittaci*-41A12	AY762609	0.006	0.005	0.006	0.003	0.008	0.000																					
7-*C. psittaci*-MN	AF269262	0.006	0.005	0.006	0.003	0.008	0.003	0.000																				
8-*C. psittaci*-KKCP1	AB284062	0.006	0.005	0.006	0.003	0.008	0.003	0.000	0.000																			
9-*C. psittaci*-CP3	AF269265	0.010	0.008	0.010	0.006	0.011	0.003	0.006	0.006	0.000																		
10-*C. psittaci*-8455	CPS16561	0.013	0.011	0.013	0.013	0.014	0.016	0.016	0.016	0.019	0.000																	
11-*C. psittaci*-Mat116	AB284058	0.019	0.018	0.019	0.016	0.021	0.019	0.019	0.019	0.023	0.029	0.000																
12-*C. psittaci*-M56	AF269268	0.034	0.032	0.034	0.031	0.036	0.034	0.034	0.034	0.031	0.044	0.031	0.000															
13-*C. psittaci*-Borg	AB284066	0.116	0.114	0.116	0.113	0.114	0.116	0.116	0.116	0.120	0.124	0.129	0.118	0.000														
14-*C. psittaci*-92-1293	Y16562	0.118	0.116	0.118	0.114	0.116	0.118	0.118	0.118	0.122	0.126	0.131	0.120	0.002	0.000													
15-*C. psittaci*-7778B15	AY762612	0.137	0.135	0.137	0.133	0.135	0.137	0.137	0.137	0.141	0.145	0.141	0.150	0.071	0.073	0.000												
16-*C. psittaci*-VS225	AF269261	0.139	0.137	0.139	0.135	0.137	0.139	0.139	0.139	0.139	0.147	0.143	0.148	0.073	0.075	0.002	0.000											
17-*C. psittaci*-Daruma	AB284065	0.139	0.137	0.139	0.135	0.137	0.139	0.139	0.139	0.143	0.147	0.139	0.143	0.082	0.084	0.080	0.082	0.000										
18-*C. psittaci*-84/2334	AJ310735	0.141	0.139	0.141	0.137	0.139	0.141	0.141	0.141	0.145	0.148	0.141	0.145	0.084	0.086	0.082	0.084	0.002	0.000									
19-*C. psittaci*-R54	AJ243525	0.145	0.143	0.145	0.141	0.143	0.145	0.145	0.145	0.148	0.152	0.141	0.145	0.077	0.078	0.063	0.065	0.051	0.053	0.000								
20-*C. psittaci*-GD	AF269261	0.150	0.148	0.150	0.147	0.148	0.150	0.150	0.150	0.154	0.158	0.147	0.145	0.073	0.075	0.061	0.063	0.070	0.071	0.058	0.000							
21-*C. psittaci*-WC	AF269269	0.148	0.147	0.148	0.145	0.145	0.148	0.148	0.148	0.152	0.156	0.154	0.152	0.105	0.107	0.113	0.114	0.114	0.116	0.128	0.120	0.000						
22-*C. psittaci*-CPX0308	AB284064	0.211	0.209	0.211	0.207	0.211	0.205	0.207	0.207	0.205	0.209	0.196	0.205	0.196	0.198	0.190	0.188	0.207	0.207	0.205	0.194	0.192	0.000					
23-*C. abortus*-S26/3	X51859	0.152	0.150	0.152	0.148	0.150	0.152	0.152	0.152	0.156	0.160	0.148	0.148	0.091	0.093	0.071	0.073	0.087	0.089	0.065	0.080	0.128	0.203	0.000				
24-*C. caviae*-GPIC	AE015925	0.176	0.174	0.176	0.172	0.174	0.176	0.176	0.176	0.180	0.184	0.166	0.170	0.164	0.166	0.170	0.172	0.182	0.184	0.174	0.172	0.166	0.174	0.174	0.000			
25-*C. felis*-C56	AP006861	0.188	0.186	0.188	0.184	0.182	0.188	0.188	0.188	0.192	0.200	0.180	0.180	0.160	0.162	0.160	0.162	0.172	0.174	0.154	0.166	0.174	0.186	0.168	0.170	0.000		
26-*C. pneumoniae*-AR39	AE002161	0.284	0.282	0.284	0.280	0.282	0.280	0.280	0.280	0.280	0.296	0.273	0.289	0.280	0.282	0.284	0.282	0.293	0.293	0.296	0.289	0.282	0.312	0.305	0.301	0.277	0.000	
27-*C. pecorum*-L71	AF269280	0.313	0.311	0.313	0.308	0.313	0.308	0.308	0.308	0.313	0.313	0.316	0.316	0.289	0.292	0.304	0.306	0.316	0.318	0.311	0.311	0.308	0.320	0.320	0.304	0.333	0.269	0.000
Designated genetic clusters		**I**												**II**		**III**		**IV**		**V**	**VI**	**VII**	**VIII**	**Other *Chlamydophila* spp.**				

Table 10. The difference in number of nucleotides (upper right triangular half) and amino acids (lower triangular half) among different lineages and genetic clusters of *C. psittaci* strains in constant domains of the *ompA* gene. Only the representative genetically variant strains of *C. psittaci* were taken for analysis. Other *Chlamydophila* species are demarkated by grey background. Different genetic clusters are demarcated by solid lines and lineages by dotted lines.

		Lineage-1												**Lineage-2**								**Lin.-3**	**Lin.-4**					
Chlamydial species	Accession no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1- *C. psittaci*- GV	L25437	0	1	2	2	3	4	4	4	6	8	12	21	68	69	79	80	80	81	83	86	85	116	87	99	105	150	163
2- *C. psittaci*- Nosé	AB284060	1	0	1	1	2	3	3	3	5	7	11	20	67	68	78	79	79	80	82	85	84	115	86	98	104	149	162
3- *C. psittaci*- Izawa-1	AB284057	2	1	0	2	3	4	4	4	6	8	12	21	68	69	79	80	80	81	83	86	85	116	87	99	105	150	163
4- *C. psittaci*- 6BC	M73035	2	1	2	0	3	2	2	2	4	8	10	19	66	67	77	78	78	79	81	84	83	114	85	97	103	148	161
5- *C. psittaci*- MN Zhang	AF269281	1	0	1	1	0	5	5	5	7	9	13	22	67	68	78	79	79	80	82	85	83	116	86	98	102	148	163
6- *C. psittaci*- 41A12	AY762609	4	3	4	2	3	0	2	2	2	10	12	21	68	69	79	80	80	81	83	86	85	113	87	99	105	148	161
7- *C. psittaci*- MN	AF269262	4	3	4	2	3	1	0	0	4	10	12	21	68	69	79	80	80	81	83	86	85	114	87	99	105	148	161
8- *C. psittaci*- KKCP1	AB284062	4	3	4	2	3	1	0	0	4	10	12	21	68	69	79	80	80	81	83	86	85	114	87	99	105	148	161
9- *C. psittaci*- CP3	AF269265	5	4	5	3	4	1	2	2	0	12	14	19	70	71	81	80	82	83	85	88	87	113	89	101	107	147	169
10- *C. psittaci*- 84-55	CPS16561	4	3	4	4	3	6	6	6	7	0	27	18	72	73	83	84	84	85	87	90	89	115	91	103	111	155	163
11- *C. psittaci*- Mat116	AB284058	4	3	4	2	3	4	4	4	5	6	0	19	75	76	81	82	80	81	81	84	88	109	85	94	101	145	164
12- *C. psittaci*- M56	AF269268	4	3	4	2	3	4	4	4	3	6	4	0	69	70	86	85	82	83	83	83	87	113	85	96	101	152	164
13- *C. psittaci*- Borg	AB284066	10	9	10	8	9	10	10	10	11	12	10	10	0	1	43	44	49	50	46	44	62	109	54	93	91	148	153
14- *C. psittaci*- 92-1293	Y16562	11	10	11	9	10	11	11	11	12	13	11	11	1	0	44	45	50	51	47	45	63	110	55	94	92	149	154
15- *C. psittaci*- 7778B15	AY762612	13	12	13	11	12	13	13	13	14	15	11	13	7	8	0	1	48	49	38	37	66	106	43	96	91	150	159
16- *C. psittaci*- VS225	AF269261	13	12	13	11	12	13	13	13	14	15	11	13	7	8	0	0	49	50	39	38	67	105	44	97	92	149	160
17- *C. psittaci*- Daruma	AB284065	9	8	9	7	8	9	9	9	10	11	9	9	3	4	4	4	0	1	31	42	67	114	52	102	97	154	162
18- *C. psittaci*- 84/2334	AJ310735	10	9	10	8	9	10	10	10	11	12	10	10	4	5	5	5	1	0	32	43	68	114	53	103	98	154	163
19- *C. psittaci*- R54	AJ243525	10	9	10	8	9	10	10	10	11	12	9	10	4	5	4	4	1	2	0	35	74	107	74	94	98	145	161
20- *C. psittaci*- GD	AF269261	11	10	11	9	10	11	11	11	12	13	9	11	6	7	6	6	4	5	4	0	70	113	39	98	88	155	160
21- *C. psittaci*- WC	AF269269	12	11	12	10	11	11	11	11	12	14	12	12	7	8	10	10	8	9	9	20	0	108	48	97	94	152	162
22- *C. psittaci*- CPX0308	AB284064	31	30	31	29	30	28	28	28	29	33	29	31	27	28	26	26	28	28	28	29	27	0	112	98	104	161	165
23- *C. abortus*-S26/3	X51859	12	11	12	10	11	12	12	12	13	14	11	12	8	9	5	5	5	6	4	8	9	27	0	98	95	159	166
24- *C. caviae*- GPIC	AE015925	20	19	20	18	19	20	20	20	21	22	16	20	19	20	15	15	18	19	18	20	19	28	18	0	96	158	157
25- *C. felis*-C56	AP006861	21	20	21	19	20	21	21	21	22	23	19	21	16	17	18	18	16	17	17	19	18	27	18	22	0	146	163
26- *C. pneumoniae*-AR39	AE002161	45	44	45	44	44	44	44	44	45	47	42	46	43	44	41	41	41	41	41	42	42	42	42	42	39	0	145
27- *C. pecorum*-L71	AF269280	44	43	44	43	43	43	43	43	44	46	44	45	43	44	44	44	41	42	41	43	43	46	43	47	45	38	0
Designated genetic clusters		**I**												**II**		**III**		**IV**		**V**	**VI**	**VII**	**VIII**	**Other *Chlamydophila* spp.**				

Table 11. Percentage identity matrix (PIM) of nucleotides in the genetically variable regions (VDs) of *ompA* gene of different lineages and genetic clusters of *C. psttaci* strains. The PIM was calculated by ClustalX, version 1.83. The other *Chlamydophila* species are marked by grey background. The lineages are marked by dotted lines, whereas, the genetic clusters are marked by solid lines.

		Lineage-1										**Lineage-2**						**Lin.-3**	**Lin.-4**					
Chlamydial species	Accession No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1-*C. psittaci*-05/02	DQ324426	100																						
2-*C. psittaci*- Nosé	AB284060	100	100																					
3-*C. psittaci*- 84-55	CPS16561	99	100	100																				
4-*C. psittaci*- 6BC	M73035	99	100	99	100																			
5-*C. psittaci*- CP3	AF269265	98	98	98	98	100																		
6-*C. psittaci*- KKCP1	AB284062	97	98	97	98	100	100																	
7-*C. psittaci*- MN	AF269262	97	97	97	98	99	98	100																
8-*C. psittaci*- WS/RT/E30	AY762613	97	97	97	98	99	98	99	100															
9-*C. psittaci*- Mat116	AB284058	95	95	95	95	95	94	94	94	100														
10-*C. psittaci*- M56	AF269268	89	89	88	89	87	87	87	87	89	100													
11-*C. psittaci*- Borg	AB284066	66	66	67	66	66	66	66	66	66	67	100												
12-*C. psittaci*- VS225	AF269261	60	61	61	61	61	61	60	61	60	60	72	100											
13-*C. psittaci*- R54	AJ243525	60	59	60	59	59	59	59	59	59	60	71	84	100										
14-*C. psittaci*- 84/2334	AJ310735	56	56	56	56	56	56	56	56	56	56	67	83	81	100									
15-*C. psittaci*- Daruma	AB284065	56	56	57	56	57	56	57	56	57	57	68	85	81	98	100								
16-*C. psittaci*- GD	AF269261	55	55	55	55	55	55	55	55	54	56	70	76	79	77	78	100							
17-*C. psittaci*- WC	AF269269	62	61	62	61	61	61	61	61	60	61	70	65	66	65	65	63	100						
18-*C. psittaci*- CPX0308	AB284064	59	58	59	58	58	58	58	58	58	60	67	67	62	60	61	63	62	100					
19-*C. abortus*-S26/3	X51859	59	59	60	59	59	59	58	59	58	60	68	89	82	82	85	73	64	65	100				
20-*C. felis*-C56	AP006861	59	59	59	59	59	60	59	59	59	59	68	69	72	63	65	64	62	62	69	100			
21-*C. pneumoniae*-AR39	AE002161	55	55	55	55	56	55	56	56	56	56	60	61	58	56	58	59	54	62	61	59	100		
22-*C. caviae*- GPIC	AE015925	54	54	54	54	54	55	55	55	55	53	62	69	70	65	67	64	61	59	67	67	58	100	
23-*C. pecorum*-L71	AF269280	52	52	52	52	52	52	52	52	52	52	57	57	57	57	57	53	55	60	56	58	53	61	100
Designated genetic clusters		**I**										**II**	**III**	**IV**	**V**		**VI**	**VII**	**VIII**	**Other *Chlamydophila* spp.**				

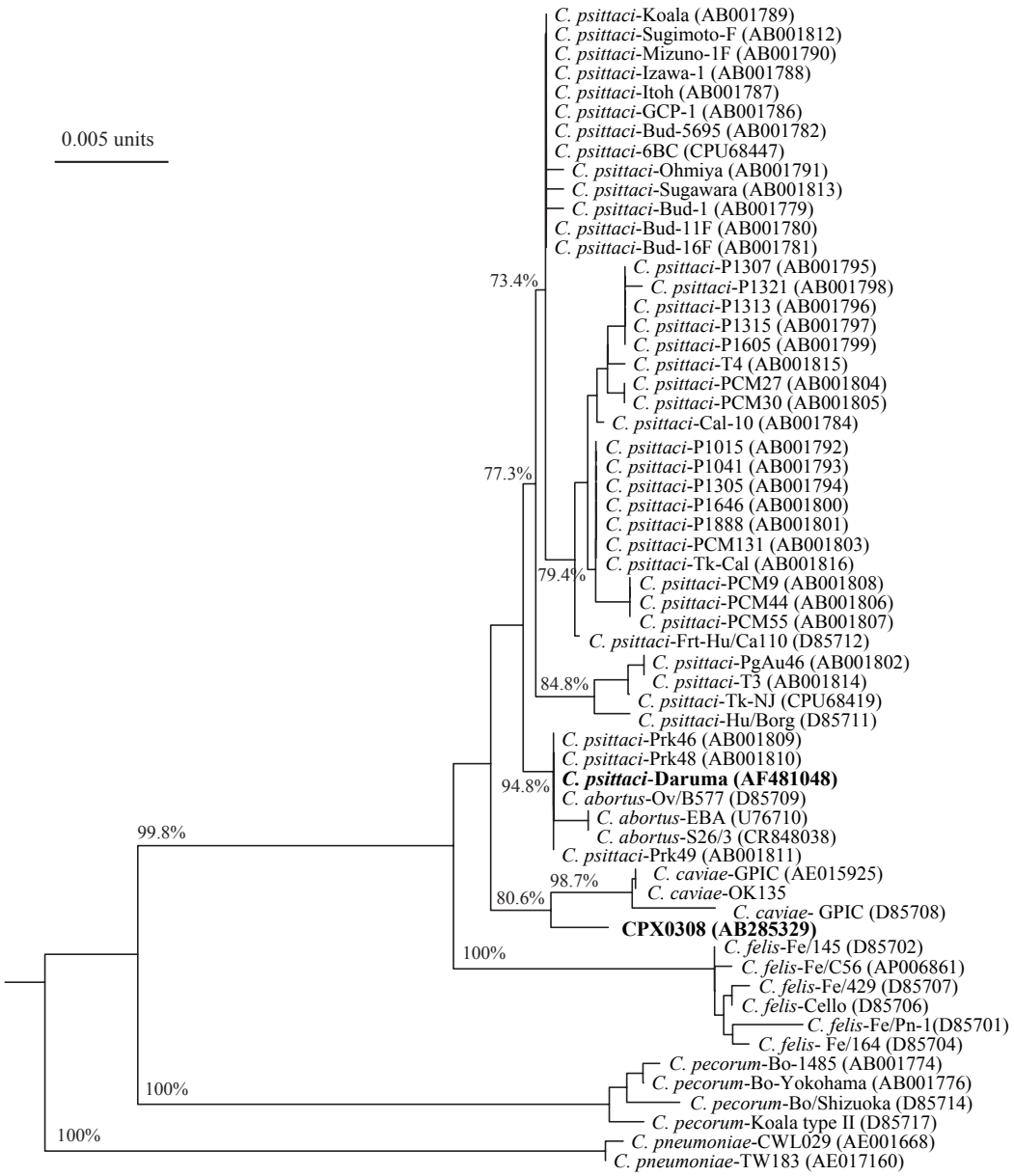


Fig. 10. The 16S rRNA gene based neighbor-joining (NJ) tree showing the evolutionary distance of the CPX0308 and Daruma strains of *C. psittaci* (bold letter) in comparison to the other known species of genus *Chlamydophila*. The accession numbers are shown in parentheses. The genetic distance is shown in units scale bar. The bootstrap values are shown againt nodes.

strain showed genetic distance of 0.006 from the closest relative, Daruma strain, while Daruma strain was genetically closest, that is 0.001 to *C. abortus* S26/3 strain, (Table 12-A). According to the CDs of the *ompA* gene, CPX0308 strain was the closest to *C. caviae* GPIC strain with genetic distance of 0.176, while Daruma strain showed genetic distance of 0.051 from R54 strains of *C. psittaci*. However, on the basis of partial *ompA* gene (including VDs), the closest relatives of CPX0308 and Daruma were V225 and R54 strains of *C. psittaci* with distance of 0.255 and 0.096, respectively (Table 12-B and C). Therefore, Daruma strain was phylogenetically intermediate between the clusters formed by *C. psittaci* and *C. abortus* species, whereas, CPX0308 strain appeared to be intermediate between cluster of *C. psittaci* and *C. abortus* species and that of *C. caviae* and *C. felis* species. Therefore, CPX0308 and Daruma strains are grouped into the lineage-4 (cluster VIII) and lineage-2 (clusters IV), respectively.

Human psittacosis strains: The majority of *C. psittaci* strains isolated from human psittacosis patients or from in-contact avian species was observed to be belonging to lineage-1 and genetic cluster I. These strains are predominantly prevalent among psittacine and pigeons, though also reported from other avian and mammalian species. The recently reported OSV and 05/02 strains (Table 7) from human psittacosis outbreak were also similar to strains of genetic cluster I. OSV strain is 99.78%, 99.02% and 99.78% similar to 6BC, CP3 and MN strains, respectively, whereas, 05/02 strain was 99.80%, 99.09% and 98.88% similar to 6BC, CP3 and MN strains, respectively. Interestingly, KKCP1 and KKCP2 strains showed characteristics of both MN (formerly grouped as genotype/serotype E) and CP3 (formerly grouped as genotype/serotype B) and form intermediate subcluster between them (Fig. 9 B). Second type of *C. psittaci* strains involved in human psittacosis belongs to genetic cluster II that has been known as serotype/genotype D strain. These strains are mostly reported from poultry birds.

Table 12. Genetic distance of CPX0308 and Daruma strains *vis-à-vis* other *Chlamydophila* spp. and *Chlamydia* spp. based on A) 16S rRNA gene, B) constant domains of partial *ompA* gene, C) partial *ompA* gene including variable domains. The distance was calculated by Jukes-Cantor method (93) using Phylip. The two nearest species/strains are shown in grey background.

A)

	C. psittaci CPX0308	*C. psittaci* Daruma	*C. psittaci* 6BC	*C. psittaci* Itoh	*C. psittaci* PCM9	*C. psittaci* Cal10	*C. psittaci* P1307	*C. psittaci* P1015	*C. psittaci* T3	*C. psittaci* Borg	*C. psittaci* Tk-NJ	*C. psittaci* PCM27	*C. psittaci* T4	*C. abortus* S26/3	*C. caviae* GPIC	*C. felis* Fe/C56	*C. pecorum* Bo-1485	*C. pneumoniae* CWL029	*C. trachomatis* D/UW-3/CX	*C. suis* S45	*C. muridarum* Nigg
CPX0308	0.000	0.006	0.007	0.007	0.010	0.009	0.010	0.009	0.010	0.009	0.009	0.010	0.010	0.007	0.009	0.017	0.039	0.046	0.049	0.059	0.045
Daruma	0.006	0.000	0.002	0.002	0.005	0.004	0.005	0.004	0.007	0.006	0.006	0.005	0.005	0.001	0.009	0.016	0.038	0.043	0.051	0.059	0.045
Accession no.	AB285329	AF481048	CPU68447	AB001787	AB001808	AB001784	AB001795	AB001792	AB001814	D85711	CPU68419	AB001804	AB001815	CR848038	AE015925	AP006861	AB001774	AE001668	AE001347	U73110	AE002160

B)

	C. psittaci CPX0308	*C. psittaci* Daruma	*C. psittaci* 6BC	*C. psittaci* Nosé	*C. psittaci* CP3	*C. psittaci* MN	*C. psittaci* Mat116	*C. psittaci* M56	*C. psittaci* R54	*C. psittaci* Borg	*C. psittaci* GD	*C. psittaci* VS225	*C. psittaci* WC	*C. abortus* S26/3	*C. caviae* GPIC	*C. felis* Fe/C56	*C. pecorum* LW613	*C. pneumoniae* AR39	*C. trachomatis* D/UW-3/CX	*C. suis* PCLH197	*C. muridarum* Nigg
CPX0308	0.000	0.207	0.207	0.209	0.205	0.207	0.196	0.205	0.205	0.196	0.194	0.188	0.192	0.203	0.176	0.186	0.296	0.312	0.322	0.339	0.319
Daruma	0.207	0.000	0.135	0.137	0.143	0.139	0.139	0.143	0.051	0.082	0.070	0.082	0.114	0.087	0.184	0.172	0.311	0.293	0.331	0.317	0.351

C)

CPX0308	0.000	0.288	0.293	0.296	0.294	0.297	0.288	0.293	0.281	0.257	0.271	0.255	0.273	0.268	0.260	0.263	0.385	0.377	0.458	0.461	0.401
Daruma	0.288	0.000	0.239	0.239	0.242	0.239	0.234	0.241	0.096	0.160	0.121	0.108	0.198	0.111	0.252	0.252	0.409	0.373	0.448	0.449	0.424
Accession no.	AB284064	AB284065	M73035	AB284060	AF269265	AF269262	AB284058	AF269268	AJ243525	AB284066	AF269261	AF269261	AF269269	X51859	AE015925	AP006861	AJ440240	AE002161	X62920	AJ440241	AE002160

Comparison of genetic cluster and AluI RFLP based genotyping method: The AluI based RFLP maps are shown in Fig. 11. Mat116, KKCP1 and KKCP2 strain have similar RFLP pattern but are genetically diverse with one another (Fig. 7 and Fig. 9). Similarly, some closely related strains having single nucleotide polymorphism (SNP) at a few positions with or without amino acid change could not be resolved by AluI based RFLP genotyping.

DISCUSSION

For differentiation of *C. psittaci* strains, currently serotyping and genotyping methodologies are used which have their own advantages and disadvantages (4, 174, 207). But due to the incessant reports of existence of *C. psittaci* strains with major or minor genetic variation among avian species and human psittacosis cases (33, 60, 77, 191), an alternative and more accommodative strain classification system is desired. Existing methods are also reported to have some ambiguity in strain differentiation (191, 207).

Therefore, in this study, we introduce an alternative genetic cluster based strain classification scheme by using genetic distances and percentage identities. We analyzed the conserved regions of the *ompA* gene, which have genetic evolution trajectory similar to other portion of chlamydial genome (24). The VDs regions with excessive homoplasy were analyzed separately. All the genetically variable strains are grouped into 4 lineages, which are further subdivided into 8 genetic clusters named from I to VIII. This scheme accommodates all *C. psittaci* strains with major or minor genetic differences and groups them according to the actual genetic variation.

The genetic cluster I include 13 types of genetically variant *C. psittaci* strains. It accommodates all closely related subclusters like that formed by KKCP1 and KKCP2 strain due to small variations in VD2 and VD4 regions. Similarly a recently known E/B genotype having only 2-nucleotide variations in VD4 region (60) was clubbed together with other

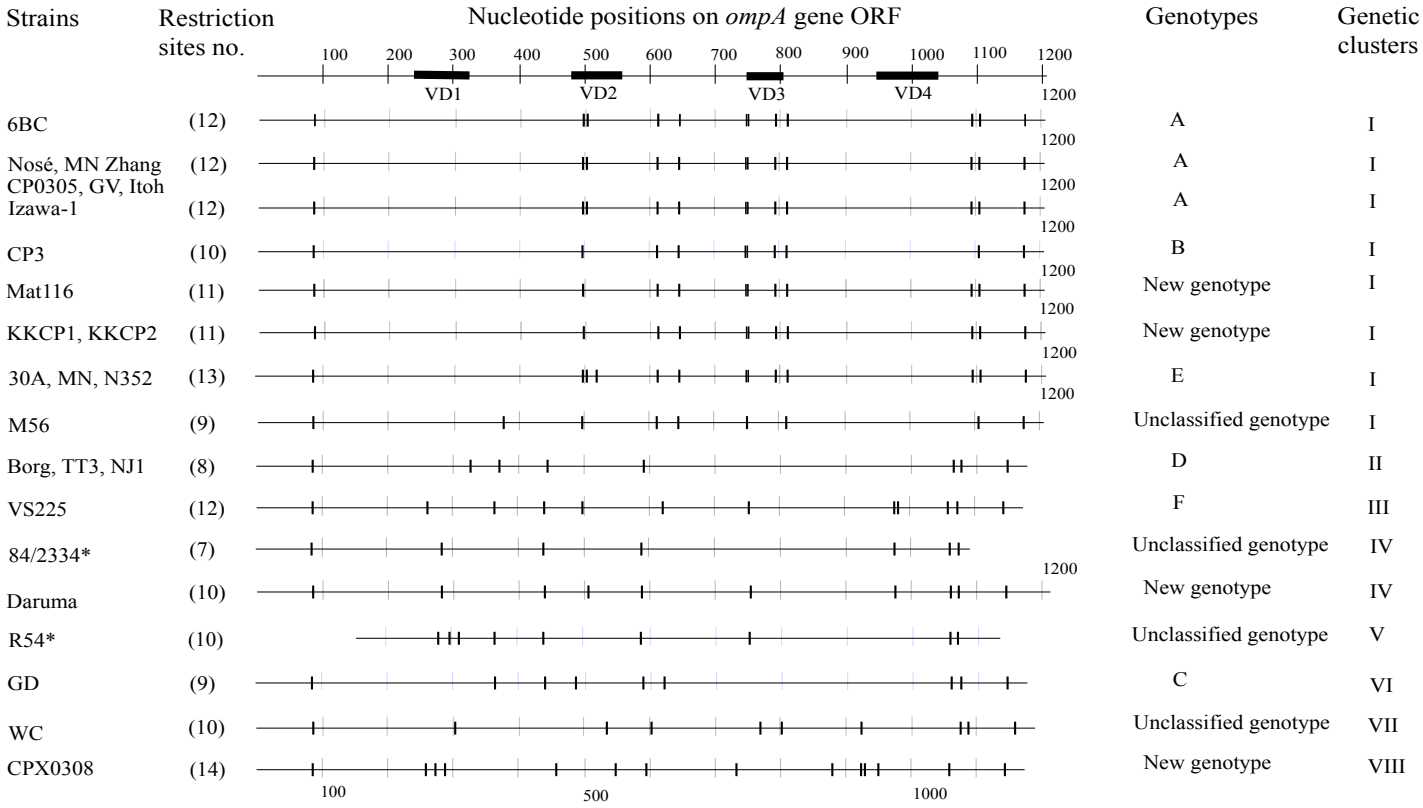


Fig. 11. RFLP restriction map of the *ompA* gene based on AluI enzyme used to categorize various *C. psittaci* strains into A to F genotypes. The unknown/unclassified genotypes and corresponding genetic clusters (I to VIII) are also shown. The asterik (*) indicates that ORF of R54 and 84/2334 strains are incomplete from 3' and 5' ends respectively. The variable domains (VDs) are demarcated by thick lines in the top line diagram of *ompA* gene, which is showing the nucleotide positions in the ORF.

closely related subclusters. The R54 strain detected from brown skua (79) has been placed in genetic cluster V in a group of highly diverse strains (lineage-2). Similarly, we grouped Daruma strain along with 84/2334 strain in genetic cluster IV, which is intermediate in evolution between *C. abortus* and *C. psittaci* groups (205). The 84/2334 strain was previously classified as serotype A (207) based on Mab against MOMP, which include genetically different group of strains. The CPX0308 strain has been grouped as genetic cluster VIII in lineage-4. Hence, proposed scheme of strain classification correctly group different genetically variant strains.

The PCR based chlamydial detection techniques have overcome the problem associated with chlamydial isolations for diagnosing the infection. These procedures are now increasingly used in large-scale epidemiological studies, strain differentiations and for quick clinical diagnosis. The genetic distance based classification can also accommodates uncultured chlamydiae. It also overcomes the constrains associated with serotyping techniques that need isolation of *C. psittaci* strains before typing, which is not possible in some cases due to zoonotic risk. Furthermore, it is known that immunodominat epitopes recognized by monoclonal antibodies used in serotyping studies, reside in the hypervariable regions of *ompA* gene. Therefore, the correspondence between *ompA*-RFLP and MOMP serotyping is not absolute and the results in some strains can be misinterpreted (174, 191). The AluI RFLP pattern based ambiguities among Mat116, KKCP1 and KKCP2 strains also indicate that genotypic classification using only one restriction enzyme is not representative. Therefore, the new strain classification scheme represents much more the actual genetic diversity among *C. psittaci* strains.

The lineages of *C. psittaci* strains also reflect their evolutionary patterns. The lineages-1 strains have adapted to avian species and appear mostly capable of infecting in-contact animals and human beings. The strains of lineage-2 contains 5 genetic clusters of strains that appeared to be recently adapted to avian species as was estimated by their low

prevalence. The *C. abortus* group that has been recognized as distinct pathobiotype (40, 43, 199), has been evolving from hypothetic ancestor belonging to lineage-2. The CPX0308 strain analyzed by *ompA* gene and additional 16S rRNA gene sequences seems to be an intermediate between group of *C. psittaci* and *C. abortus* and other *Chlamydophila* spp. of mammalian origins. This CPX0308 strain may have the common ancestor for this cluster. Further genetic studies of such strains in details are needed to establish their exact phylogenetic positions in relation to other chlamydial species.

The evolutionary basis of epidemiological success and pathobiology of *C. psittaci* strains is difficult to examine due to non-availability of whole genome sequence data. In our study, the analysis of *C. psittaci* strains isolated/detected from human psittacosis patients or in-contact avian hosts revealed that *C. psittaci* strains belongs to genetic clusters I are mostly responsible for human psittacosis. Recently, similar types of strains were reported from an outbreak in Netherlands (77). As these types of strains are mostly prevalent in psittacine birds, there are numbers of reports of sporadic human psittacosis cases or large scale outbreaks from ancient time to very recent time, where the probable source is mentioned as psittacine birds or pigeons, but the genotypes of these strains were not studied (37, 107, 113, 116, 123, 197). These types of strains are also reported from the cases of swine, 98AV2129 strain (211), ewes, A22/M strain (147), Moorish tortoise (209) and dog (68). This reflects the pathogenic potential of this group of strains not only to human but also to other in-contact mammalian and non-mammalian hosts. The Borg strain, which is involved in human psittacosis, belongs to genetic cluster II. Similar genotypes may be involved in human psittacosis cases among the poultry industry workers (8, 117, 127).

Therefore, in this investigation, we grouped the *C. psittaci* strains according to a modified scheme, which is genetically accommodative and appropriately represents actual

genetic diversity among *C. psittaci* strains. It may help in understanding the evolutionary patterns of *C. psittaci* strains and identifying the *C. psittaci* pathobiotypes associated with avian/animals or zoonotic infections.

SUMMARY

Different genetically diverse *C. psittaci* strains detected from various avian and mammalian hosts including human beings were genetically analyzed to evaluate the phylogenetic relationships, pathobiotic implications and to improve the strain categorization. All of the 11 new *C. psittaci* strains together with the previously described 35 strains were genetically analyzed in *ompA* gene locus. Conserved and variable domains of *ompA* gene were analyzed separately and 16S rRNA gene of two highly diverse strains CPX0308 and Daruma was also studied. On the basis of the conserved region of *ompA* gene, 4 lineages of *C. psittaci* strains with genetic distance of less than 0.1 (dissimilarity less than 10%) were identified. The *C. psittaci* strains of 4 lineages, with genetic distance of less than 0.05 (dissimilarity less than 5%) were subdivided into 8 genetic clusters. The *ompA* gene loci of the *C. psittaci* strains of lineage-1 are relatively conserved and the strains were frequently involved in infection of human (psittacosis) and other mammalian species. Lineage-2 strains are genetically diverse and divided into 5 genetic clusters. Lineage-3 and 4, represented by WC and CPX0308 strains, respectively, are genetically more close to *Chlamydophila* spp. causing infection among mammalian hosts. This scheme classifies diverse strains according to their genetic variations and phylogenetic distances, which is more appropriate to accommodate and classify closely and distantly related *C. psittaci* strains. It may be useful to differentiate even uncultured *C. psittaci* strains detected in clinical diagnosis.

CHAPTER-III

Examination of virulence patterns of the *Chlamydophila psittaci* strains predominantly associated with avian chlamydiosis and human psittacosis using BALB/c mice

INTRODUCTION

The *Chlamydophila psittaci* causes "avian chlamydiosis" among diverse avian species, which is characterized by systemic, acute or chronic disease or subclinical/inapparent infection with intermittent shedding of infectious EBs. Different *C. psittaci* strains vary in virulence, which gives rise to variable incubation periods, clinical signs and recovery time. Typical signs of a clinically apparent infection with virulent *C. psittaci* strains include "pneumo-enteritis" with respiratory signs, mucopurulent nasal discharge, diarrhea, polyuria and dullness (fluffed feathers and lethargy). Yellow-greenish diarrhea is a common clinical sign. Occasionally unilateral or bilateral conjunctivitis/keratoconjunctivitis, lameness and central nervous system disturbances may be observed. The precipitation of disease and intensity of clinical signs and mortality also depend upon avian species, chlamydial strains, environmental conditions and concurrent or secondary infections (7, 8, 32, 188, 210).

The human infection by *C. psittaci* is called "psittacosis", which was first named as "psittakos" a Greek word meaning parrot (129). In 1879, Ritter observed this disease among human first time due to exposure to tropical pet birds and named it "pneumotyphous" (72, 158). Later world pandemic occurred in 1929 and 1930 due to shipment of exotic birds from Argentina to Europe and North America and the causative agent was first demonstrated in smears from infected birds and human patients (14, 34, 49,

111). Since then various sporadic cases or large outbreaks have been reported in many countries (155, 197, 224). According to the Office International des Epizooties (IOE) data on zoonotic cases by avian chlamydiosis (http://www.oie.int/hs2/report.asp) from 2000 to 2004 period, 545, 391, 412, 524 and 410 cases were reported by 13, 13, 15, 12 and 13 member countries, respectively. But many more case may have gone undiagnosed owing to confusing symptoms and difficult diagnosis.

Primarily the clinical signs of human psittacosis varies from mild flu like symptoms to severe atypical pneumonia with non productive cough, fever, muscular and joint pain and headache (115, 197, 213, 224) but the secondary complications like myocarditis (109), hepatitis (172), encephalitis/meningitis and neurological disturbances (29, 36, 138, 168), skin erythema (66, 180) or multiple organ failure (201) can increase fatalities. Some suspected human-to-human transmission has also been reported (85). In old literature psittacosis is referred to all human illness caused by all species, which have formerly been grouped under *Chlamydia psittaci* group, but we considered reports those specifically mentioned avian contact or otherwise doubtful histories.

For studying the pathogenesis of chlamydial diseases, animal experimentation models are mostly used, as the genetic manipulation of chlamydiae is not possible at present time. Studies on relative virulence of *C. psittaci* strains of avian origin are very few. In 1958, Page first studied pathogenicity of *C. psittaci* strains from turkey, psittacine and pigeon in mice (141) and in turkeys (142) and reported that some turkey strains are more pathogenic. Subsequently, there have been many reports of experimental animal infection with chlamydiae both in attempt to provide animal models of human disease and to establish etiological relationships between infection and disease. However, the use of animal infections primarily to differentiate between chlamydial strains is limited. The mouse toxicity protection test first provided evidence of strain variations within *C. trachomatis* (2,

15). Rodolakis and colleagues differentiated invasive and non-invasive strains of *C. abortus* by means of mice footpad inoculation experiments and found that invasive strain infect placenta of pregnant mice (159) and sheep (161). These invasive *C. abortus* strains formed a distinct genetic cluster, whereas non-invasive strains formed different clusters on the basis of restriction endonuclease analysis of DNA (162) and RFLP patterns of *ompA* gene (38).

Variation in the infectivity of *C. psittaci* strains to different avian and mammalian hosts was observed in an earlier study (143). Different genetically and serologically variant strains of *C. psittaci* have been identified in the newly classified *Chlamydophila psittaci* group (43). We also divide all genetically diverse strains into 4 lineages and 8 genetic clusters in CHAPTER II on the basis of the composition of *ompA* gene encoding the MOMP. Depending upon MOMP specific panels of Mab and AluI RFLP pattern of *ompA* gene, 8 serotypes and 7 genotypes are known (4, 56, 60, 174, 206, 207). The comparative virulence and pathogenesis of different *C. psittaci* strains (new group) responsible for avian chlamydiosis and human psittacosis has not been investigated in detail yet. The *C. psittaci* strain of serovar D (turkey strains) and serovar A (parakeet strain) were observed to be more invasive in experimentally infected turkeys than was serovar B (pigeon strains) (212).

The murine infection models provide an important set of tools to identify the host and organism factors responsible for disease pathogenesis. Different inbred and knockout mice strains are used to study disease mechanisms of human (16, 21, 133, 223) and animal (22, 124, 159) chlamydial infections. Therefore, in this study we compared the virulence of *C. psittaci* strains predominantly detected form avian species and human psittacosis and belonging to different genetic groups using murine model. Four *C. psittaci* strains were intranasally inoculated in BALB/c mice and appearance of clinical signs, mortalities

(LD_{50}) and clearance of infection was analyzed. Two genotypes found mostly associated with human psittacosis were found to have low LD_{50} for BALB/c mice.

MATERIALS AND METHODS

Chlamydiae: The 4 *C. psittaci* strains tested were, Nosé strain, isolated from a budgerigar (*Melopsittacus undulatus*) that was in-contact with a human psittacosis patient and was kindly provided by Prof. A. Matsumoto; MN/cal10 strain (49), isolated from human/ferret in a human psittacosis outbreak; Mat116 strain isolated from a chestnut fronted macaw (*Ara severa*) in an avian chlamydiosis out break in Japan (This study); Borg strain (ATCC VR-601), isolated from the lung of a human psittacosis patient (140).

Chlamydial culture and purification of EBs: L-suspension cell line was used for multiplying *C. psittaci* strains (50, 194). The cells were maintained in Eagle's minimum essential medium-1 (MEM-1) (Nissui, Japan) containing 5% fetal bovine serum (FBS) and 10 μg/ml of gentamicin. The EBs were harvested 3 to 4 days after inoculation and partially purified as described in CHAPTER II by procedure reported by Tamura and Higashi (194). The aliquots were stored at -80°C and later were used for determining stock EB concentrations and for mice inoculations. The aliquots once thawed were not used again.

Quantification of EBs: The EB concentration in the stock was determined by counting inclusion-forming units (IFU). Two-fold serially diluted, 200 μl suspension of each strain was inoculated in duplicate on confluent McCoy cell monolayers grown (as described in CHAPTER II) on 14 mm diameter glass round cover slips in 24 well plate (Nunc, Roskilde, Denmark). After 24 hr of incubation at 37°C in 5% CO_2, the coverslips were fixed in chilled acetone for 10 min at 4°C and then stained with chlamydia group specific fluorescein isothiocyanate (FITC)-labeled Mab (Denka Seiken Co. Ltd, Tokyo,

Japan). IFU were counted in duplicate slips by using fluorescent microscope (Olympus, model BX50, Tokyo, Japan) using 200 times magnification and concentrations of EBs in the stocks were calculated.

Mice: Specific pathogen free, female, 6 to 8 weeks old BALB/c mice were obtained from Japan SLC Inc. (Shizuoka, Japan). The mice were kept in filtered top cages in isolators under sterilized conditions. The mice were housed under standard environmental conditions and received sterilized food and water *ad libitum* in accordance with the guidelines for animal experiments at Gifu University.

Experimental design and inoculations: Total 96 mice were divided into 4 groups, each consisting of 24 mice for each of 4 *C. psittaci* strains. With in each group, 4 mice were inoculated with single dose and kept together in one cage. Mice were inoculated with 0.5 IFU, 5 IFU, 50 IFU, 5×10^2 IFU, 5×10^3 IFU and 5×10^4 IFU per gm body weight. Mice were inoculated intranasally using 2 μl of inoculum per gram body weight with designated concentration of EBs. A group of 6 mice, inoculated with same volume of MEM, was kept as control. Mice were observed for 21 days post inoculation. The LD_{50} was calculated as the method of Reed and Muench (154). Lung, liver, spleen and heart tissues were collected from mice died before 21 days and those survived after 21 days post inoculation, in PBS, pH 7.4 for DNA extraction and in sucrose-phosphate-glutamate (SPG) medium (218 mM sucrose, 3.8 mM KH_2PO_4, 4.9 mM K_2HPO_4, 4.9 mM L-glutamic acid, pH 7.4) (17) containing kanamycin 100 μg/ml, streptomycin 50 μg/ml, gentamicin 10 μg/ml and vencomycin 100 μg/ml for determining chlamydial burden in lung, liver and spleen. The blood samples were also collected from survived animals for serum extraction and measuring IgG. The experiment was repeated twice with 5×10^2 and 5×10^3 IFU dose rates with the same procedure.

Quantification of chlamydial burden: The 20% suspensions of lung, liver and

spleen tissues in SPG were centrifuged at 1,000 × *g* for 10 minutes at 4°C. After appropriate dilutions, 200 µl of supernatant was inoculated in duplicate on confluent McCoy cell monolayer grown on glass coverslips in 24 well plate and incubated for 24 hr at 37°C in 5% CO_2. The coverslips were fixed in chilled acetone and IFU were counted by the same procedure as mentioned in EB quantification protocol using chlamydia group specific FITC-labeled Mab (Denka Seiken Co. Ltd, Tokyo, Japan).

DNA extraction and nested PCR: The DNA from lung, liver and spleen tissues of mice was extracted by phenol-chloroform-isoamyl procedure (171) and nested PCR was done as reported before (33) for detecting the chlamydial DNA to ascertain the clearance of infection after 21 days post inoculation at different inoculation dose levels of all 4 strains.

Microimmunofluorescence test: The chlamydia specific IgG were detected by microimmunofluorescence test as described before (156, 169) with some modifications. Briefly McCoy cells were grown on Teflon coated 24 well glass plates (Cel-line; Erie Scientific Co., Newfield, New Jersey, USA.) by adding 20 µl of suspension containing 10^5 cells/ml of MEM-1 with 5% FBS on each well and incubating for 24 hr at 37°C in 5% CO_2 under moist conditions. Then after washing slides with MEM-1, 20 µl of suspension containing 10^5 IFU of *C. psittaci* per ml of MEM-1 with 5% FBS and 1 µg/ml of cycloheximide was added on each well of the glass slides and incubated under moist conditions at 37°C in 5% CO_2 for 24 hr. Then after removing medium, the slides were air dried and fixed with acetone for 20 min. Then 20 µl of two fold serially diluted serum, starting from 1:8, was placed on each well and incubated for 60 min at 37°C in moist chamber. After washing thrice with chilled PBS, pH 7.4, the slides were incubated with ICN/Cappel FITC-labeled sheep anti-mouse IgG (whole molecule) (ICN Biomedicals, inc. Aurora, Ohiyo, USA) for 60 min. The slides were washed again thrice with chilled PBS,

pH 7.4 followed by washing with water and finally observed under fluorescent microscope (Olympus, model BX50, Tokyo, Japan) at 200 time magnification after mounting in SlowFade light antifade solution (Molecular Probes, Eugene, Oregon, USA). Preinoculation and serum of negative control mice were used as negative control.

Statistical analysis: The analysis of body weight variation among different groups of mice was done by comparing them with control group at different time points using two tailed Student's *t*-test and Mann-Whitney *U*-test. Results were considered significantly different at the level of *P* value <0.05.

RESULTS

Different indices of virulence like the patterns of appearance/resolution of clinical signs, body weight variations, mortalities and seroconversions at different doses of inoculations with Nosé, MN, Mat116 and Borg strains of *C. psittaci* were monitored. The data of repeated experiments was pooled in final analysis.

Clinical signs: The mice were observed for appearance of clinical signs like general weakness, lethargy, ruffling of fur and respiratory signs. The scoring and pattern of clinical sign are shown in Fig. 12. The appearance of clinical signs started on second to third day post inoculation and the intensity was peaked at 4 to 5 days and persisted up to 9 to 10 days post inoculation among all 4 strains. At the dose rate of 5×10^3 and 5×10^4 IFU/gm body weight, all 4 strains showed the same intensity of clinical signs but at 50 and 5×10^2 IFU/gm body weight dose rate, MN showed milder clinical signs as compared to Nosé, Mat116 and Borg strains. No clinical signs or very mild and transient signs of disease were observed in all strains at dose of 0.5 and 5 IFU/gm body weight. The resolution of clinical signs started after 12 days post inoculation and complete recovery

Fig. 12. The bar diagrams showing the patterns of appearance and resolution of clinical signs in BALB/c mice inoculated intranasally with 0.5 IFU, 5 IFU, 50 IFU, 5×10^2 IFU, 5×10^3 IFU and 5×10^4 IFU per gram body weight with Nosé, MN, Mat116 and Borg strains of *C. psittaci*. The 0-day indicates inoculation day and animals were observed for 21 days. The score 0: no clinical signs or normal, 1: mild weakness, lethargy and ruffling of fur, 2: weakness, lethargy and ruffling of fur, 3: severe weakness, lethargy and ruffling of fur along with labored breathing and hunched back, 4: death. The numbers of mice died out of total inoculated are shown in the parentheses for each dose and strain. The control group (inoculated with MEM-1) did not show any clinical signs. Each bar represents data of a single mouse at particular time point.

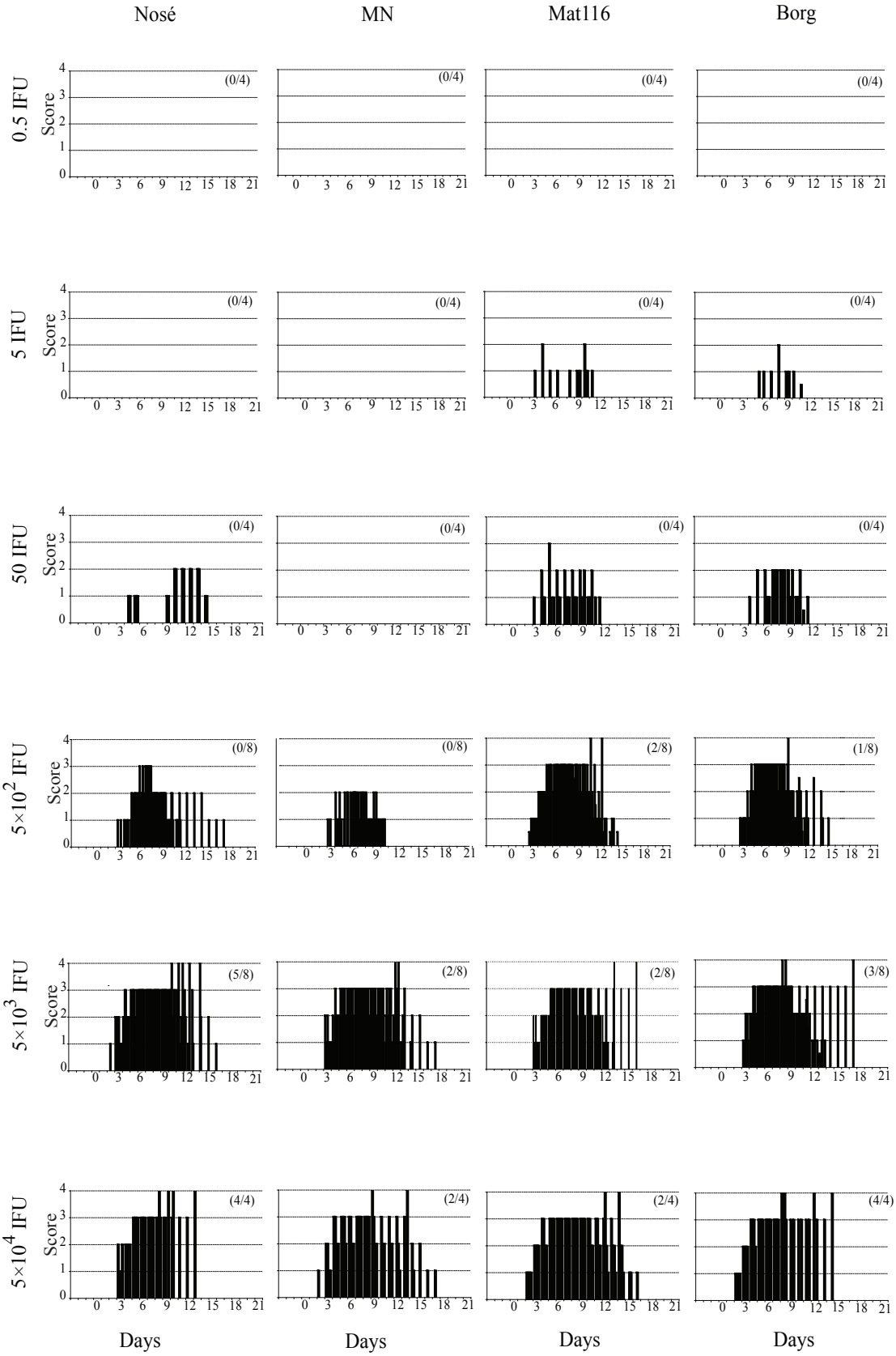
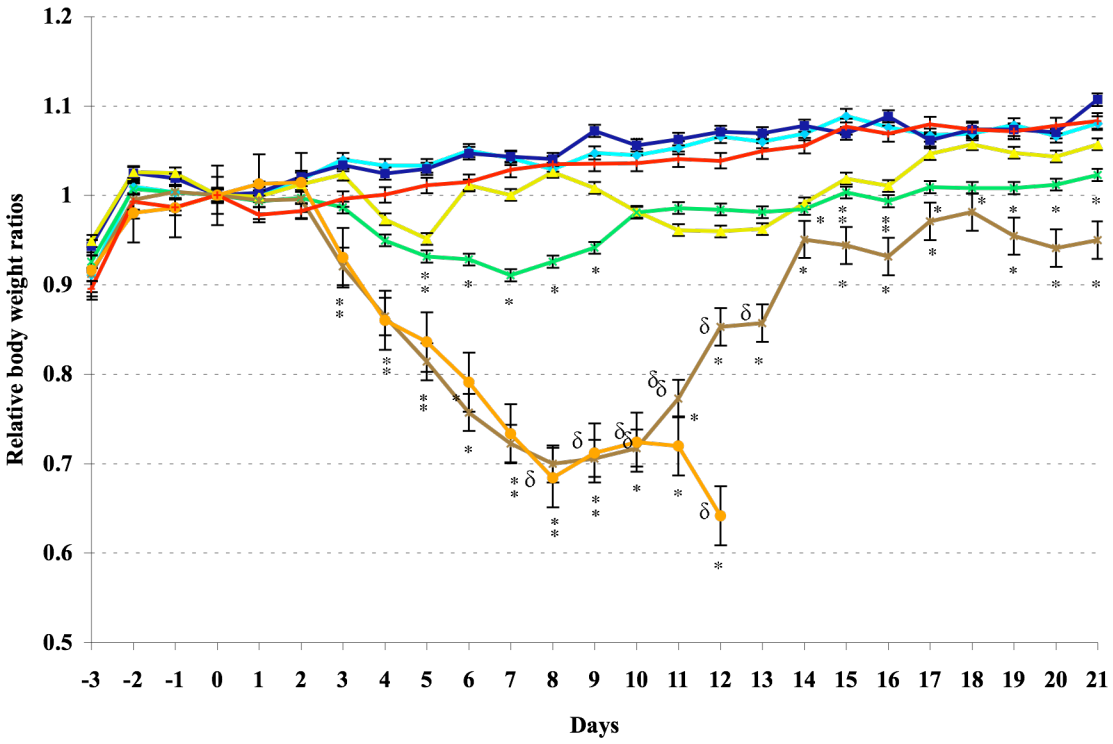
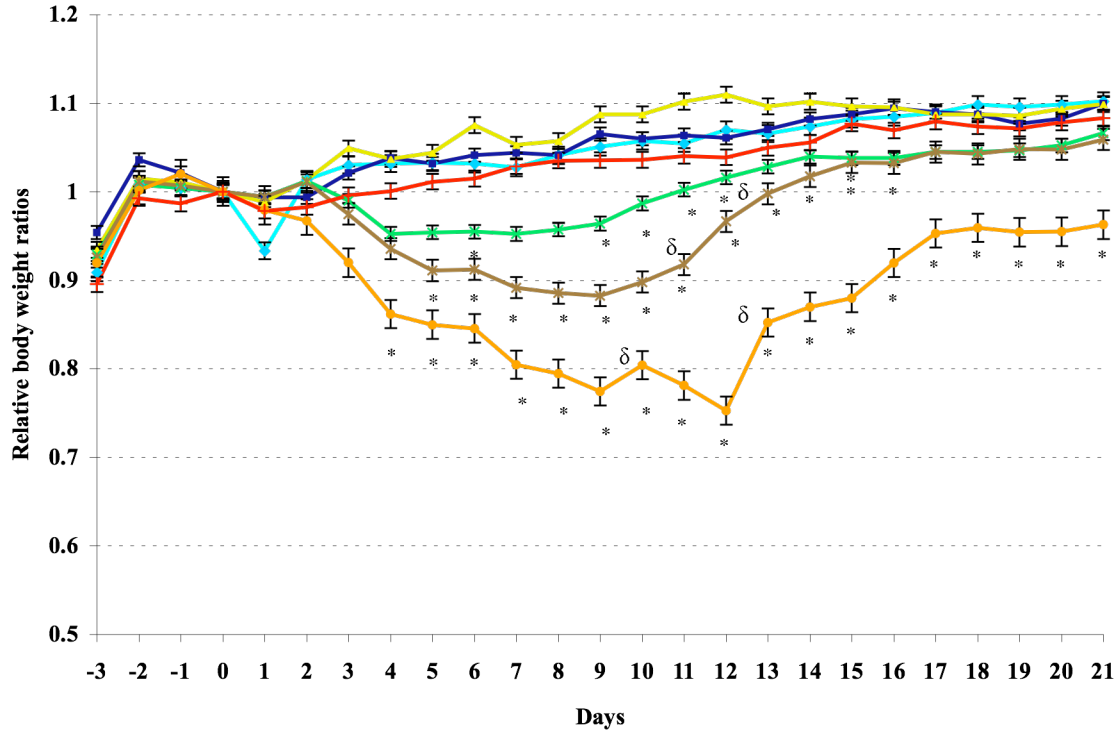

Nosé
MN
Mat116
Borg
0.5 IFU
5 IFU
50 IFU
5×10^2 IFU
5×10^3 IFU
5×10^4 IFU
Score
Days
(0/4)
(0/4)
(0/4)
(0/4)
(0/4)
(0/4)
(0/4)
(0/4)
(0/4)
(0/4)
(0/4)
(0/4)
(0/8)
(0/8)
(2/8)
(1/8)
(5/8)
(2/8)
(2/8)
(3/8)
(4/4)
(2/4)
(2/4)
(4/4)

Fig. 13. Mean relative body weight variations among the BALB/c mice inoculated with Nosé, MN, Mat116 and Borg strains of *C. psittaci*. Mice were inoculated with 0.5 IFU (— line), 5 IFU (— line), 50 IFU (— line), 5×10^2 IFU (— line), 5×10^3 IFU (— line) and 5×10^4 IFU (—line) per gram body weight on 0-day and observed for 21 days post infection. At each time point, represented by 4 to 8 mice, $P < 0.05$ was compared to control group (inoculated with MEM-1 and shown by — line) using Student's *t*-Test and Mann-Whitney *U*-Test. The statistically significant time points are marked with asterisk (*). The error bar shows the standard error. The points of mortality are shown by delta marks (δ) and its numbers represent mice died at that point of time.

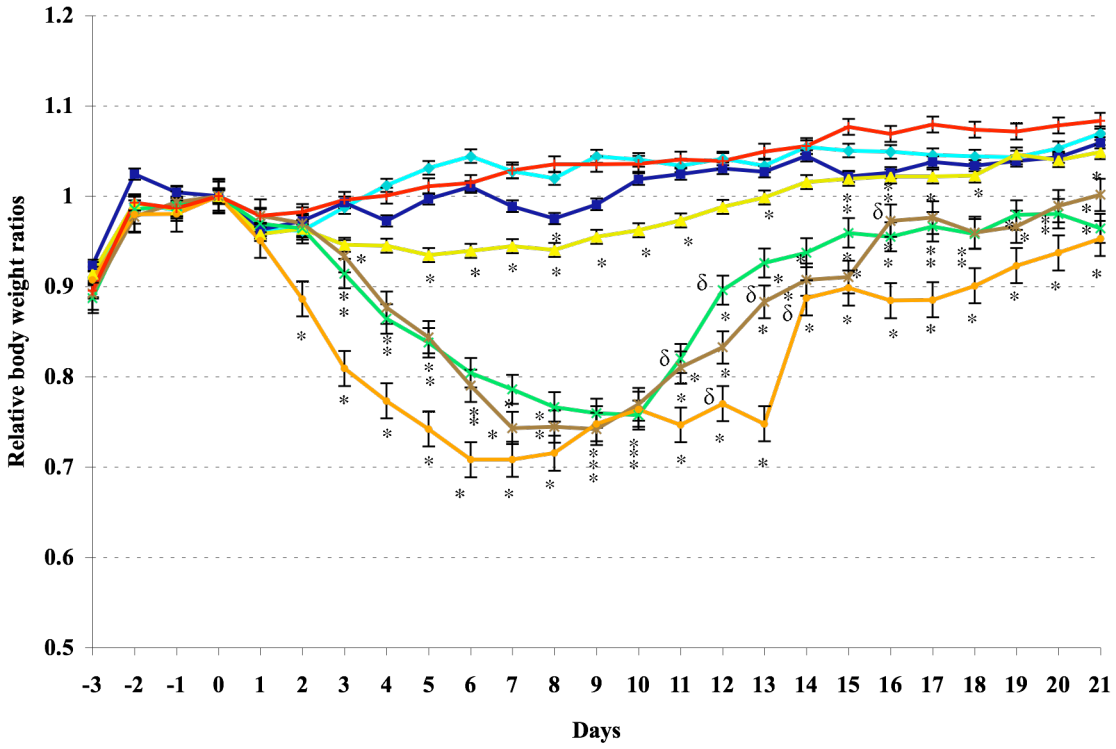
(A) Nosé strain



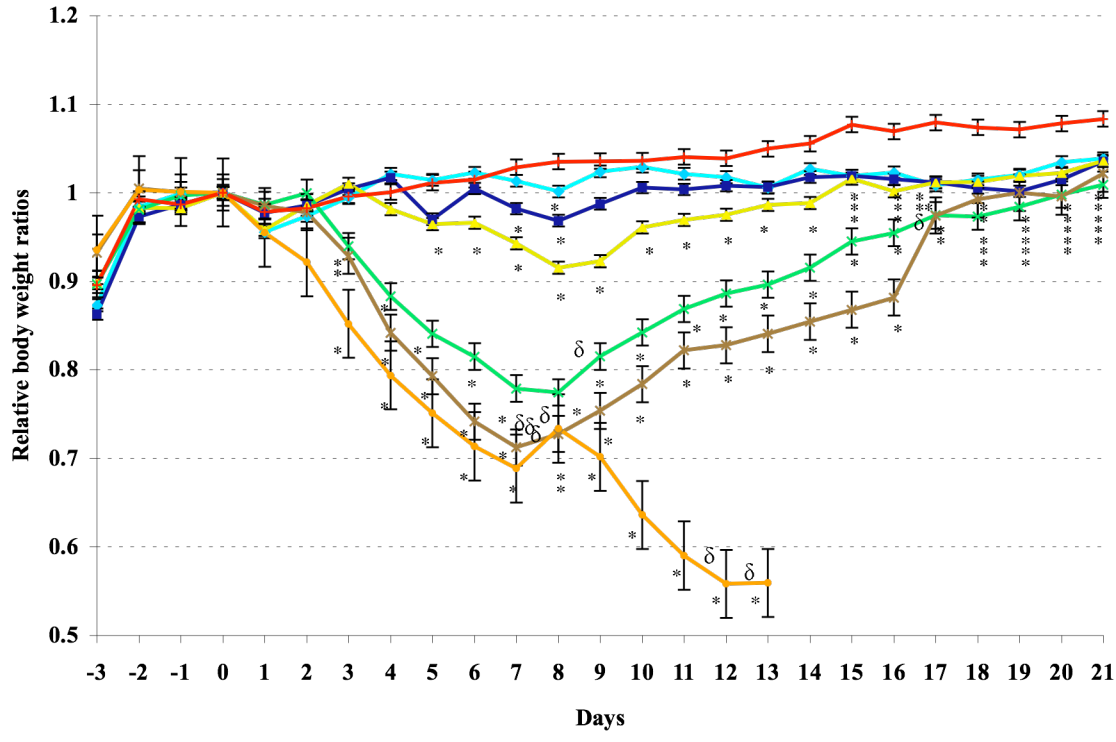
(B) MN/cal110 strain



(C) Mat116 strain



(D) Borg strain



was observed before 21 days, when experiment was terminated (Fig. 12).

Relative body weight variations: The relative body weight variations after the inoculation with each strain is shown in Fig. 13. MN strain showed less variation in the relative body weight at higher doses as compared to Nosé, Mat116 and Borg strains.

Mortality pattern and LD_{50}: All the 4 strains were lethal to BALB/c mice at various dose rates. No particular pattern of mortality was observed among all 4 strains. The LD_{50} of Nosé, MN, Mat116 and Borg were observed to be 3.1×10^3, 19×10^3, 12×10^3 and 6.3×10^3 IFU/gm body weight, respectively.

Chlamydial burden among dead and recovered mice: To study the pneumotropism and lethal lung burden, chlamydial EBs per gram of lung tissue of dead mice were determined among mice inoculated with 5×10^3 IFU/gm body weight. At this dose rate both mortalities and recoveries of mice were observed in all 4 strains. Among all 4 strains Borg strains multiplied in murine lung tissues rapidly and reached to the level of 35.7×10^5 to 71.9×10^5 IFU/gm body weight (Table 13). Among dead mice, infection also spread to other primary organs like liver and spleen but the extent of multiplication was found less than the primary target organ (lung).

Clearance of chlamydial infection: Samples from all dose level from all strains were also screened by nested PCR to test the clearance of chlamydial infection from lung liver and spleen after 21 days of infection. All samples except one infected with Nosé strain, were found PCR negative (Table 13). This indicated that all the *C. psittaci* strains irrespective of genetic group followed the same disease pattern in murine lung infection model.

Chlamydia specific IgG immune response: The geometric mean titers of the chlamydia specific IgG among recovered mice are shown in Table 14. The data showed dose dependent increase in IgG tires among all 4 strains. It also showed that even lowest

Table 13. Chlamydial burden in lung, liver and spleen among the recovered and dead BALB/c mice, inoculated intranasally with 5×10^3 IFU/gm body weight of Nosé, MN, Mat116 and Borg strains of *C. psittaci*.

Strains	Mice[a]	Chlamydia burden[b]						Anti-chlamydial IgG titers
		Lung		Liver		Spleen		
		IFU/gm	PCR[c]	IFU/gm	PCR[c]	IFU/gm	PCR[c]	
Nosé	D5	<1	-	-	-	-	-	512
	D6	<1	-	-	-	-	-	1×10^3
	D18	1.2×10^3	+	<1	-	<1	-	512
	D7∗	8.2×10^5	+	8×10^2	+	9.5×10^2	+	NT
	D8∗	10.5×10^5	+	6×10^2	+	1.4×10^3	+	NT
	D17∗	6.5×10^5	+	<1	+	2×10^2	+	NT
	D19∗	4.4×10^5	+	1.2×10^3	+	1.5×10^2	+	NT
	D20∗	6.4×10^5	+	2.5×10^2	+	1.5×10^2	+	NT
MN	C5	<1	-	<1	-	<1	-	256
	C6	<1	-	<1	-	<1	-	256
	C8	<1	-	<1	-	<1	-	512
	C17	<1	-	<1	-	<1	-	128
	C19	<1	-	<1	-	<1	-	256
	C20	<1	-	<1	-	<1	-	256
	C7∗	8×10^5	+	9.5×10^2	+	1.1×10^3	+	NT
	C18∗	2.9×10^5	+	5×10^2	+	4×10	+	NT
Mat116	A7	<1	-	<1	-	<1	-	256
	A8	<1	-	<1	-	<1	-	512
	A17	<1	-	<1	-	<1	-	256
	A18	<1	-	<1	-	<1	-	1×10^3
	A19	<1	-	<1	-	<1	-	1×10^3
	A20	<1	-	<1	-	<1	-	512
	A5∗	7.8×10^5	+	8.5×10^2	+	<1	+	NT
	A6∗	14.4×10^5	+	<1	+	2×10^2	+	NT
	A14∗	11.4×10^5	+	1×10^4	+	8.2×10^3	+	NT
Borg	B6	<1	-	-	-	-	-	512
	B8	<1	-	-	-	-	-	256
	B17	<1	-	<1	-	<1	-	1×10^3
	B18	<1	-	<1	-	<1	-	512
	B20	<1	-	<1	-	<1	-	512
	B5∗	53.1×10^5	+	1×10^3	+	3.5×10^3	+	NT
	B7∗	71.9×10^5	+	1.9×10^3	+	4.7×10^3	+	NT
	B19∗	35.7×10^5	+	7.2×10^3	+	2×10^3	+	NT

[a] Dead mice are marked by asterisk.
[b] IFU count is the averages of duplicate samples and shown as numbers per gram of tissue and less than one (<1) indicates no IFU in 200µl of 20% tissue suspension.
[c] PCR positive samples are shown as plus sign (+) and PCR negative samples as negative signs (-). Samples of all recovered mice, sacrificed after 21 days post infection, were found PCR negative for all dose groups.
NT indicates not tested.

Table 14. Geometric mean of IgG titers detected among recovered BALB/c mice after intranasal inoculation with *C. psittaci* strains. The numbers of tested sera (*n*) are given in parentheses.

C. psittaci strains	Dose per gram body weight[a]					
	0.5 IFU	5 IFU	50 IFU	5×10^2 IFU	5×10^3 IFU	5×10^4 IFU
Nosé	65.5 (*n*=4)	138.8 (*n*=4)	372.2 (*n*=4)	421.0 (*n*=8)	608.9 (*n*=3)	All mice died
MN	11.3 (*n*=4)	22.6 (*n*=4)	107.6 (*n*=4)	152.2 (*n*=8)	256.0 (*n*=6)	512.0 (*n*=2)
Mat116	22.6 (*n*=4)	26.9 (*n*=4)	53.8 (*n*=4)	143.7 (*n*=6)	512.0 (*n*=6)	724.1 (*n*=2)
Borg	9.5 (*n*=4)	26.9 (*n*=4)	53.8 (*n*=4)	312.1 (*n*=7)	512.0 (*n*=5)	All mice died

[a] The dose rate is inclusion forming units (IFU) per gram body weight of mouse.

dose of 0.5 IFU/gm body weight can leads to seroconversion among inoculated mice.

DISCUSION

The financial losses resulting from the *C. psittaci* infections, particularly those in the poultry and pet industries, combined with the fact that this is the most common animal chlamydiosis transmissible to human beings, highlights the economic importance and public health significance of the avian chlamydiosis (39, 115, 146, 181). Recent molecular epidemiological studies have shown the genetic variations among *C. psittaci* strains (33, 51, 79, 191, 205). It was also observed that though many strains are prevalent among avian fauna, only few types of strains are predominantly responsible for avian chlamydiosis outbreaks and human psittacosis cases (33) (CHAPTER II). Therefore, to know the relative virulence of the 4 representative strains of *C. psittaci*, we used BALB/c mouse model as it would not affect the avian species specific pathogenicity of some strains and results may be more representative of the virulence to non-avian hosts *i.e.* zoonotic infection of human beings. Furthermore, in this study, intra nasal route of inoculation was used with varying

doses to simulate the natural route of infection (mostly infection is by inhalations of infectious aerosol) (142) and extent of exposure which represent the amount of infectious material inhaled that is related to the final outcome human psittacosis infection (140, 197, 224).

It was observed that the Nosé strain that represent a group of strains, mostly prevalent among psittacine birds, is most lethal to the BALB/c mice. The various reports in the literature concerning human psittacosis and horizontal transmission of infection to in-contact animals, support our findings that similar or closely related types of strains are highly virulent and transmissible to non-avian hosts (37, 68, 107, 116, 197, 209, 211, 224). The second most lethal strain to mice was Borg, which represents *C. psittaci* strains mostly prevalent among poultry birds. This type of strains may be associated with human psittacosis cases in the poultry-industry workers (8, 117, 127, 146). Two other strains, Mat116 and MN/cal10 were also found to be lethal to BALB/c mice only at higher dose rate, suggesting that such strains can be potentially pathogenic to avian and non avian hosts when the exposure to the source of infection is prolonged.

The monitoring of appearance and resolution of clinical signs during the course of disease, at varying doses of inoculation, also indicated that Nosé and Borg strains produce severe clinical disease in mice even at lower dose rate as compared to MN. Therefore, the *C. psittaci* strains of these genetic groups may induce clinical disease among in-contact human or other mammalian hosts even after brief exposure to the source of infection. However, similarity in the pattern of appearance/resolution of clinical signs in all tested 4 strains indicated that the disease pathogenesis and protective immune response mounted by hosts to all types of *C. psittaci* strains in murine lung infection model may be the same. This was further confirmed by testing IgG immune response and chlamydial burden in lung, liver and spleen among recovered and died mice. It showed that IgG titers are dose

dependent and chlamydial infection is almost cured within 21 days. The IgG also appear at 10 to 14 days of infection in mice, experimentally infected with human biovars of *C. trachomatis* and *C. pneumoniae* (73, 104, 223). The variations in levels of morbidity and mortality indicate that, though the pattern of resolution of infection is the same in all *C. psittaci* strains, the level of protection to primary infection is variable. It may be due to the difference in virulence factors associated with infecting strain.

Human psittacosis is not a major public health problem in term of numbers of cases reported but it remains an important occupational hazard to the workers of poultry industries, pet shops or aviaries staff, veterinarians and avian pets owners (39, 115, 146). Many cases of human psittacosis occur without any history of avian contacts. Therefore, it is necessary to test for *C. psittaci* in suspected cases of psittacosis and atypical pneumonia (39, 224). Our study will provide a platform for further studies to identify the virulent factors associated with highly pathogenic strains for better protection of avian and human health.

SUMMARY

For evaluating the degree of virulence of *C. psittaci* strains predominantly responsible for avian chlamydiosis and human psittacosis, BALB/c mice were used as experimental infection model. Four representative *C. psittaci* strains: Nosé, MN, Mat116 and Borg were intranasally inoculated at dose rate of 0.5 IFU, 5 IFU, 50 IFU, 5×10^2 IFU, 5×10^3 IFU and 5×10^4 IFU per gram body weight and different parameters of virulence were monitored. The LD_{50} of Nosé, MN, Mat116 and Borg strains were 3.1×10^3, 19×10^3, 12×10^3 and 6.3×10^3 IFU/gm body weight, respectively. The intensity of clinical signs against the infectious doses of 5×10^3 and 5×10^4 IFU/gm body weight was the same among

the 4 strains, however, at 50 and 5×10^2 IFU/gm body weight doses, the MN strain showed milder clinical signs. The animals inoculated with the MN strain at higher doses also showed less variations in the relative body weights as compared to those inoculated with Nosé, Mat116 and Borg strains. Among the 4 strains, Borg strain rapidly multiplied in murine lung tissues and infection reached up to 35.7 to 71.9×10^5 IFU/gm body weight level at the point of death. Lateral spread of infection to other primary organs including liver and spleen was also observed. The complete recovery and clearance of chlamydial infection was verified at 21 days post infection by detecting chlamydial DNA and/or chlamydial IFU in lung, liver and spleen tissues. Chlamydia specific IgG immune response was found to be dose dependent. This study indicated that the *C. psittaci* strains frequently associated with avian and human psittacosis cases were highly virulent to mice and that the immune responses lead to variable degrees of protection. Our murine lung infection model can be used for further virulence analysis studies at molecular level.

PART II

Investigation of the Emergence of Chlamydial Infection Among Animals and Human Disease Conditions

CHAPTER-IV

Involvement of multiple *Chlamydia suis* genotypes in porcine conjunctivitis in commercial farms of Japan

INTRODUCTION

Porcine chlamydial infections are either clinical or subclinical in nature. In the past, *Chlamydia trachomatis* like strains were detected from various types of porcine specimens in USA (99, 165, 183). These genetically distinct and host specific strains have been classified as *Chlamydia suis* under family *Chlamydiaceae* in recent classification on the basis of 16S rRNA gene (43). *C. suis* has been mainly detected either from conjunctivitis, pneumonitis, pericarditis, vaginitis, mastitis and enteritis cases or from lymph nodes, semen, feces and lungs of normal pigs (82, 84, 99, 166, 196). Other chlamydiae such as *Chlamydophila abortus*, *Chlamydophila pecorum*, *Chlamydophila psittaci* and *Chlamydia*-like organisms are also reported in pigs with respiratory and reproductive tract disorders, polyarthritis and semen from healthy boars (82, 84, 178, 179, 196). Mixed infection of *C. suis* with *C. pecorum* in aborted porcine fetal livers, and with *C. abortus* in intestinal, respiratory and reproductive tract samples have also been reported (82, 179).

Epidemiological studies on porcine chlamydiosis have indicated high prevalence rate among sick and normal pigs in Germany (82, 84), USA (95, 165) and Switzerland (178, 179). Recent studies have also indicated prevalence of chlamydial infection among both male and female porcine used for breeding purpose in Belgium, Germany and Switzerland (100, 196, 211). In Japan there is only one report of abortion due to *Chlamydia psittaci* in a pig farm (139) and another report of prevalence of complement

fixing antibodies among 0.7% pig samples (58). There is no study that has identified involvement of specific chlamydial species or strain in the porcine clinical infections.

For detection and identification of chlamydial species, 16S-23S ribosomal RNA intergenic spacer region was reported as a suitable target (41, 44). The *ompA* gene that encodes MOMP is also the most preferred target for chlamydial epidemiological studies and analysis of genetic diversity of the chlamydiae detected directly from field samples (33, 82, 96). Chlamydial *ompA* gene consists of genetically conserved domains (CD) flanking 4 genetically variable domains (VD). The VDs of *ompA* gene are reported to have species/strain specific sequences, which encode immunologically specific epitopes on MOMP (12, 98). Genetic analysis of *ompA* gene of *C. suis* strains of lungs and intestinal origin has shown 20% nucleotide variation (82).

In the present study, the etiology of frequent conjunctivitis incidences and some reproductive disorder cases occurring among 5 commercial farms in 3 prefectures of Japan was investigated. Initial screening was done by chlamydia group specific direct fluorescent antibody test (FAT) on impression smears. Then followed by nested PCR tests targeting 16S-23S rRNA intergenic spacer region and *ompA* gene for detection, identification and analysis of genetic diversity in chlamydial spp./strains. Complement fixation test (CFT) was done to screen serum samples. Diverse *C. suis* genotypes were identified among conjunctivitis cases by nucleotide sequences analysis of 16S-23S rRNA intergenic spacer and *ompA* gene fragments.

MATERIALS AND METHODS

Samples analyzed: A total of 61 samples were taken from 49 animals of 5 commercial pig farms in 3 prefectures of Japan from 2003 to 2005. The samples were 35

ophthalmic swabs taken from pigs with mucopurulent conjunctivitis and keratoconjunctivitis (Fig. 14), 9 vaginal swabs from aborted animals, 2 tonsilar swabs and 3 seminal fluid samples from breeding boars. Twelve serum samples were also collected (Table 15).

Table 15. List of the farms and samples analyzed.

Farms (prefectures)	Disease condition	Type of samples	Sample no.
Farm-I (Chiba)	Conjunctivitis	Eye swabs	10
	Normal	Tonsillar swabs	2
Farm-II (Miyagi)	Conjunctivitis	Eye swabs	10
	Abortion	Vaginal swabs	9
	Normal	Seminal fluid	3
	Abortion and normal	Serum samples	12
Farm-III (Kanagawa)	Conjunctivitis	Eye swabs	5
Farm-IV (Kanagawa)	Conjunctivitis	Eye swabs	5
Farm-V (Kanagawa)	Conjunctivitis	Eye swabs	5
		Total	61

Chlamydial and other bacterial species: For standardizing the PCR test, DNA was extracted from S45 strain of *C. suis* (ATCC VR 1474) and other *Chlamydophila* spp. *viz*. *C. psittaci*- 6BC and MN strains, *C. felis*- FePn Baker strain, *C. abortus*- B577 strain and *C. caviae*- GPIC strain. DNA of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas* sp., and *Proteus* sp. was also extracted and used for testing specificity of the PCR. The chlamydial and bacterial strains were obtained from the Laboratory of Veterinary Microbiology, Gifu University, Japan.

Complement fixation test (CFT): Total 12 serum samples collected from a pig farm (farm-II, Table 15) were tested by CFT using *Chlamydia psittaci* CFT kit (Denka Seiken Co. Ltd, Tokyo, Japan) according to the instructions of the manufacturer.

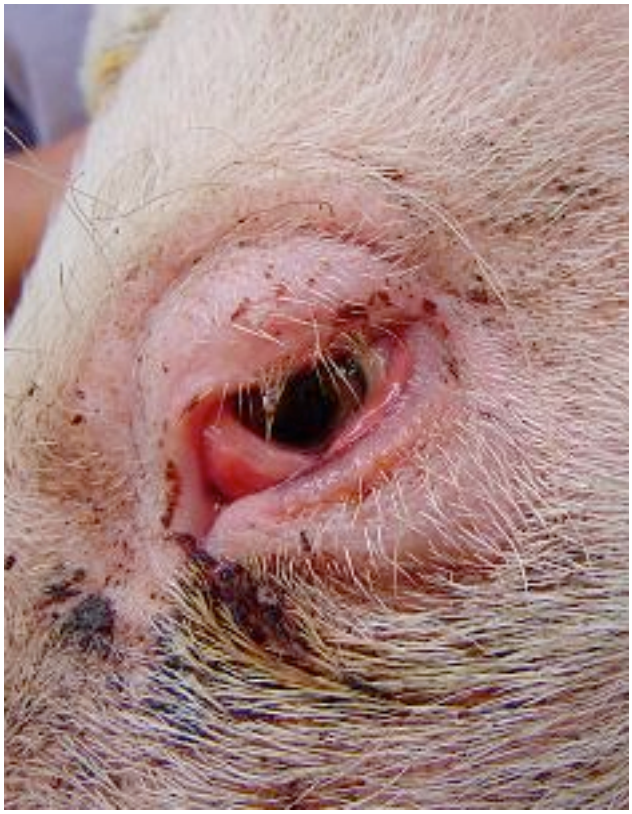


Fig. 14. A domesticated pig (Farm-I, Sample ID PG18) with keratoconjunctivitis and mucopurulent ocular discharge tested *C. suis.* positive by nested PCR.

Direct fluorescent antibody test (FAT): Total 9 ophthalmic swabs and 9 vaginal swabs were stained by chlamydia group specific FITC-coupled Mab (Denka Seiken Co. Ltd, Tokyo, Japan) according to the manufacture's protocol and chlamydial elementary bodies (EBs)/inclusions were observed by fluorescent microscope (Olympus, model BX50, Tokyo, Japan) using 200 times magnification.

DNA extraction: DNA was extracted using SepaGene DNA extraction kit (Sanko Junyaku, Tokyo, Japan) following manufacture's instructions. Briefly ophthalmic, vaginal and tonsillar swabs or 200 µl of seminal fluid were resuspended in 700 µl of phosphate-buffered saline (PBS) pH 7.4 and centrifuged at 16,000 × *g* for 30 min at 4°C and sediments were processed further for DNA extraction. DNA pallets were finally

resuspended in 30 µl of Tris-EDTA (TE) pH 7.4 (100 mM Tris-HCl, pH 7.4 and 10 mM EDTA, pH 8.0) and stored at -30°C until use.

Nested PCR tests: The PCR test targeting 16S-23S rRNA intergenic spacer region was done as reported before (41, 44) with some modifications. 16SF2 and 23R primers were used as outer pair and 16SF3 and IGR as inner pair to amplify 16S-23S rRNA intergenic spacer segment. The *Chlamydophila* genus specific test was also performed as reported before (33). *Chlamydia* genus specific nested PCR was standardized in this study. The oligonucleotide primer sequences used in the study are shown in Table 16. PCR primers were synthesized by Rikaken Co., Nagoya, Japan. The conditions for maximum DNA amplification were adjusted using variable annealing temperatures, primer concentrations and thermocycling conditions. All the PCR tests were performed in 50 µl reaction mixture containing 0.20 µM of each forward and reverse primers, 250 µM of each dATP, dTTP, dGTP, dCTP, 100 µM of Mg^{2+} in buffer and 2.5 units of TaKaRa *Ex-Taq* (Takara Bio Inc., Shiga, Japan) and 2.0 to 5.0 µg of template DNA of each sample. DNA of *C. suis* S45 strain was used as a positive control. Five microliters of the first step PCR product was used for the second step PCR. The thermocycling conditions used in both steps of *Chlamydia* genus specific PCR were: 3 min at 94°C then 35 cycles of 30 sec at 94°C, 20 sec annealing at 58°C and extension for 90 sec at 72°C (60 sec in second step PCR), followed by 5 min at 72°C and soaking at 4°C. For the amplification of 16S-23S rRNA intergenic spacer region, same thermocyclic conditions were used except annealing for 30 sec at 55°C (50°C in second step PCR) and extension of 60 sec at 72°C (30 sec in second step PCR) during 35 cycles. PBS and water were used as negative controls. Sensitivity and specificity were verified under the determined conditions with DNA templates of different bacteria and *Chlamydophila* spp.

Table 16. Oligonucleotide primers used in the study.

Primer name	Nucleotide sequence	Positions[a]	Melting temperature (°C)	Reference
16S-23S rRNA intergenic spacer region[b]:				
16SF2	5'-CCGCCCGTCACATCATGG-3'	1398-1415 (16S rRNA)	60-62	(41)
23R	5'-TACTAAGATGTTTCAGTTC-3'	212-193 (23S rRNA)	47-49	
16SF3	5'-TCGTAACAAGGTAGCCC-3'	1496-1512 (16Sr RNA)	52-54	
IGR	5'-AGCTCTTA(T/G/A)(C/T)AACTTGGTCTGTA-3'	23-1 (23S rRNA)	53-55	(44)
ompA gene:				
Genus *Chlamydia* specific				This study
CS-1PF	5'-GGAATCCTGCTGAACCAAGCCTTATGAT-3'	77-104	61-63	
CS-1PR	5'-GCGAGTCTCAACAGTAACTGCGTATT-3'	1107-1082	60-62	
CS-2PF	5'-GGCGTTGAATATTTGGGATCGTTTTG-3'	366-391	58-60	
CS-2PR	5'-CTTGCTCGAGACCATTTAACTCCAATG-3'	872-846	59-61	
Genus *Chlamydophila* specific				(33)
CMGP-1F	5'-CCTTGTGATCCTTGCGCTACTTG-3'	138-159	60-62	
CMGP-1R	5'-GTGAGCAGCTCTTTCGTTGAT-3'	1184-1164	57-60	
CMGP-2F	5'-GCCTTAAACATCTGGGATCG-3'	384-403	56	
CMPG-2R	5'-GCACAACCACATTCCCATAAAG-3'	634-613	57	

[a] The positions of the primers are based on the *ompA* gene sequence of *Chlamydia suis*, PCLH197 strain (accession no. AJ440241) and *Chlamydophila psittaci*, 6BC strain (accession no. X56980).
[b] The primer positions are based on 16S rRNA and 23S rRNA genes of R22 strain of *C. suis* (accession no. U68420).

Cloning of PCR product and sequencing: The second step PCR products were purified by gel electrophoresis using low melting agarose gel in Tris-acetate-EDTA (TAE) buffer, pH 7.4 followed by QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The DNA fragments were cloned in pGEM-T vector (Promega, Madison, Wisconsin) and DH5α strain of *E. coli* (Tyobo Co., Osaka, Japan) was transformed by heat shock method (171). From each PCR product, 5 clones with expected size DNA insert were taken for sequencing. The sequencing was done using the dye-terminator method and performed by a commercial resource (Dragon Genomics Co., Mie, Japan). Both strands were read. The sequences were assembled and edited using Genetyx-Mac/ATSQ 4.2.3 and Genetyx-Mac, version 13.0.6 (SDC, Tokyo, Japan).

Analysis of sequences and construction of phylogenetic trees: The chlamydial species and strains were identified by NCBI-BLAST (http://www.ncbi.nlm.nih.gov) search of nucleotide sequences of 16S-23S rRNA spacer region and *ompA* gene. For phylogenetic analysis, the 16S-23S rRNA spacer region and *ompA* gene sequences of *C. suis* strains and each representative species of genus *Chlamydia* were retrieved from the DDBJ. Multiple alignments of the trimmed sequences were done using ClustalX, version 1.83 (198). Phylogenetic analysis was done with programs in the PHYLIP (version. 3.6a3; [http://evolution.genetics.washington.edu/phylip.html]). The distance matrix between species was computed by DNADIST and clustering of lineages was done by NEIGHBOR using neighbor-joining method. The bootstrap values were calculated to evaluate the branching reliability of trees from a consensus tree constructed by generating 1,000 random data sets using SEQBOOT.

RESULTS

Epidemiological studies: In the preliminary screening, impression smears from conjunctival swabs (*n*=9) and vaginal swabs (*n*=9) from animals with severe clinical signs were examined with FAT. Chlamydial EBs and/or inclusions were detected from 33.3% (3 out of 9) conjunctivitis samples only. Then all the samples (*n*=49) were examined by using nested PCR tests. By 16S-23S rRNA intergenic spacer and *Chlamydia* genus specific PCR, 22 out of 49 (44.9%) samples were found positive. All PCR positive samples were accrued from conjunctivitis cases. In the conjunctivitis cases, 22 out of 35 (62.9%) ophthalmic swab samples were positive including all those which were FAT positive. All samples were found negative by *Chlamydophila* genus specific nested PCR. No chlamydial infection was detected among samples from tonsillar swabs (Table 17).

Table 17. The 16S-23S rRNA intergenic spacer and *ompA* gene based nested PCR results for detection of *Chlamydia* species and *Chlamydophila* species.

Farms	Type of samples	Total samples tested	PCR positive sample			Chlamydial species identified
			Intergenic spacer primers	*Chlamydia* specific primers	*Chlamydophila* specific primers	
Farm-I	Eye swabs	10	6 (60%)	6 (60%)	0	*C. suis*
	Tonsillar swabs	2	0	0	0	
Farm-II	Eye swabs	10	3 (30%)	3 (30%)	0	*C. suis*
	Vaginal swabs	9	0	0	0	
	Seminal fluid	3	0	0	0	
Farm-III	Eye swabs	5	4 (80%)	4 (80%)	0	*C. suis*
Farm-IV	Eye swabs	5	5 (100%)	5 (100%)	0	*C. suis*
Farm-V	Eye swabs	5	4 (80%)	4 (80%)	0	*C. suis*
	Total	49	22 (44.9%)	22 (44.9%)		

The vaginal swab samples from 9 aborted sows and seminal fluid from 3 breeding boars were found negative by all PCR tests. The serum samples analysis of these 12 animals (aborted sows=9 and normal breeding boars=3) by CFT showed 5 aborted sows with antichlamydial immmunoglobulins titers of more than 1:8 (1:8 for 2 animals and 1:16 for 3 animals) indicating possible non-chlamydial etiology of current reproductive problems but may have previously exposed to the chlamydial infection.

Genetic variation analysis: The analysis of 238 to 241 bp nucleotide sequence of 16S-23S rRNA intergenic spacer region of all PCR positive samples by NCBI-BLAST (http://www.ncbi.nlm.nih.gov) search and phylogenetic clustering in NJ tree confirmed the involvement of *C. suis* (Fig. 15 and Table 17). Total 9 variants of 16S-23S rRNA intergenic spacer region nucleotide sequences with less than 3% differences were found (Fig. 16) and were designated as sequence types (Table 18-a). To further confirm the genetic diversity among *C. suis* strain involved in clinical cases of conjunctivitis, nucleotide sequence analysis of *ompA* gene fragment of 454 to 463 bp that includes VD2 and VD3 regions was done. Two PCR positive samples from each farm, including those with multiple 16S-23S rRNA intergenic spacer region nucleotide sequences, were randomly selected and sequenced. Twenty types of nucleotide sequences (sequence types) were detected (Table 18-b). All the *C. suis* strains showed up to 22% nucleotide variations in the sequenced region.

The predicted amino acid sequence alignment of all the detected and already known *C. suis* strains in the sequenced fragment of *ompA* gene (151 to 154 amino acids) revealed the high genetic variations in the form of amino acid substitutions, insertions and deletions leading to gaps formation (varies from 0 to 3 in numbers) in VD2 along with some conserved motifs of amino acid residues in VD2 and VD3 region (Fig. 17).

On the basis of the sequence variation in 16S-23S rRNA intergenic spacer region,

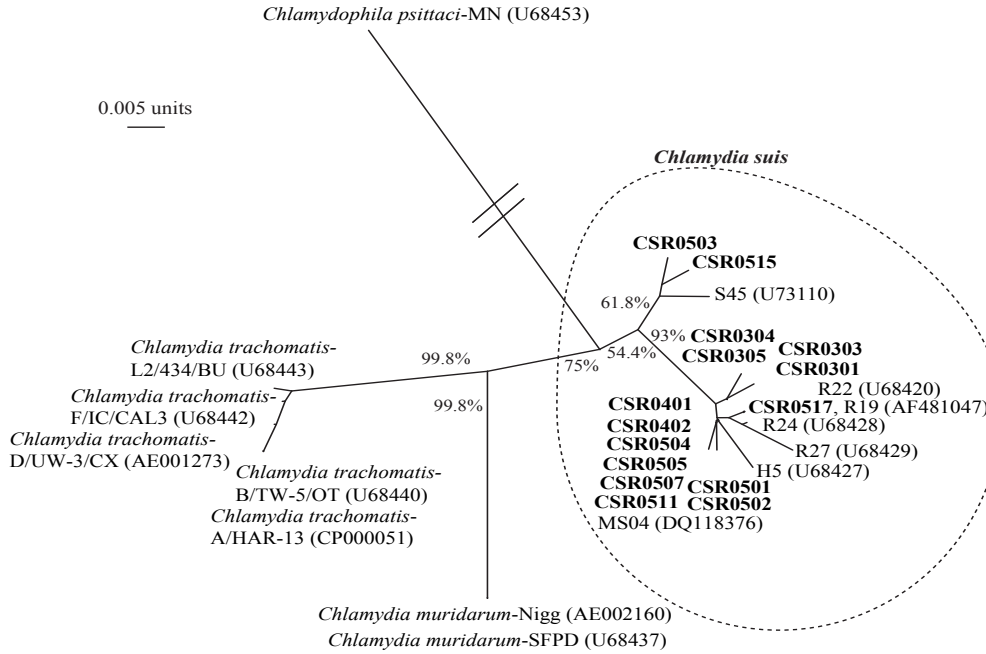


Fig. 15. The NJ tree of 16S-23S rRNA intergenic spacer region of detected (in bold letters) and known strains of *C. suis* showing separate grouping in relation to the *C. trachomatis* and *C. muridarum* species. The branch of *C. psittaci* is reduced three times. The accession numbers of the detected *C. suis* strains are: CSR0301-AB283016, CSR0303-AB283017, CSR0304-AB283018, CSR0305-AB283019, CSR0401 -AB283006, CSR0402-AB284054, CSR0501-AB283007, CSR0502-AB283008, CSR0503-AB283009, CSR0504-AB283010, CSR0505-AB283011, CSR0507-AB283012, CSR0511-AB283013, CSR0515-AB 283014, CSR0518-AB283015 and those of known species and strains are shown against each strain in parentheses in figure. The bootstrap values are shown against each node. The relative genetic distance is shown in 0.005 unit bar.

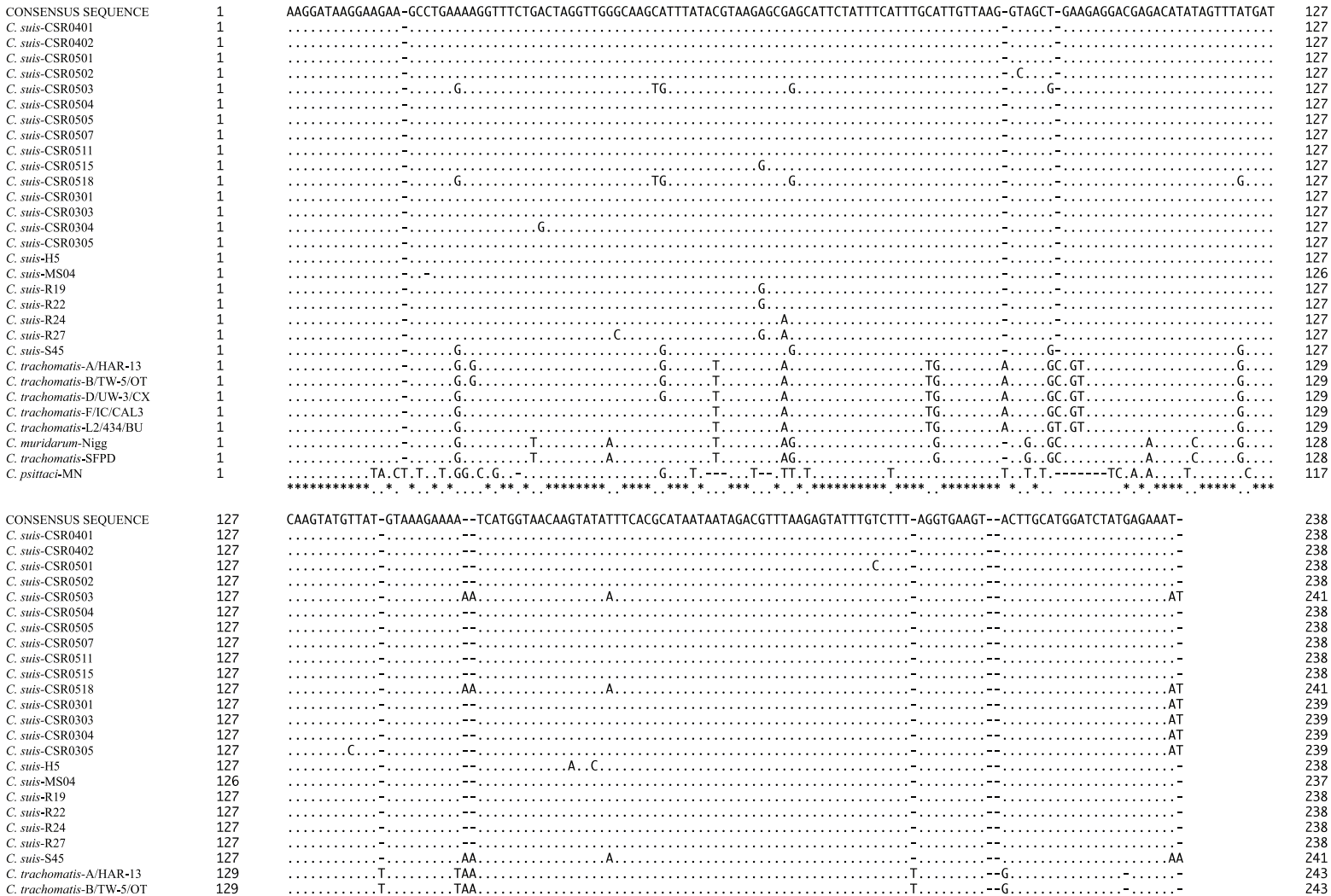


Fig. 16. Multiple alignment of the 16S-23S rRNA spacer region of detected and known *C. suis* strains *vis-a-vis* other *Chlamydia* spp. and the *Chlamydophila psittaci.*

Table 18. Distribution of *C. suis* sequence types in different farms and animals based on (a) 16S-23S rRNA intergenic spacer region and (b) *ompA* gene. The *ompA* gene based genotypic clusters depending on gaps in the VD2 region are also shown.

(a) 6S-23S rRNA intergenic spacer region

Farms (prefectures)	Sample ID[a]	Sequence[b] types	Strains	Accession no.
Farm-I (Chiba)	PG18	1	CSR0301	AB283016
	PG21	1	CSR0303	AB283017
		2	CSR0304	AB283018
		3	CSR0305	AB283019
Farm-II (Miyagi)	PG1	4	CSR0401	AB283006
	PG2	4	CSR0402	AB284054
Farm-III (Kanagawa)	PG5	4	CSR0501	AB283007
		5	CSR0502	AB283008
		6	CSR0503	AB283009
		7	CSR0504	AB283010
	PG6	4	CSR0505	AB283011
Farm-IV (Kanagawa)	PG7	4	CSR0507	AB283012
	PG8	4	CSR0511	AB283013
Farm-V (Kanagawa)	PG9	8	CSR0515	AB283014
	PG10	9	CSR0518	AB283015

(b) *ompA* gene

Farms (prefectures)	Sample ID[a]	Sequence[b] types	Genetic cluster[c]	Strains	Accession no.
Farm-I (Chiba)	PG18	1	A	CS0301	AB270719
		2	A	CS0302	AB270720
	PG21	1	A	CS0303	AB270721
		2	A	CS0304	AB270722
Farm-II (Miyagi)	PG1	3	B	CS0401	AB270723
	PG2	3	B	CS0402	AB270724
Farm-III (Kanagawa)	PG5	4	A	CS0501	AB270725
		5	A	CS0502	AB270726
		6	D	CS0503	AB270727
	PG6	7	A	CS0504	AB270728
		8	A	CS0505	AB270729
		9	D	CS0506	AB270730
Farm-IV (Kanagawa)	PG7	10	A	CS0507	AB270731
		3	B	CS0508	AB270732
		11	A	CS0509	AB270733
		1	A	CS0510	AB270734
	PG8	12	D	CS0511	AB270735
		13	D	CS0512	AB270736
		14	D	CS0513	AB270737
		15	D	CS0514	AB270738
Farm-V (Kanagawa)	PG9	16	B	CS0515	AB270739
		17	B	CS0516	AB270740
		18	A	CS0517	AB270741
	PG10	19	D	CS0518	AB270742
		20	D	CS0519	AB270743

[a] Represents the independent sample taken from the individual animal.
[b] The sequences with same numerical numbers are having 100% homologous nucleotide sequences. The nucleotide sequence types with similar deduced amino acid sequences are bracketed together.
[c] As grouped in Figs. 17 and 18.

only 2 animals (PG21 and PG5) were found harboring multiple genotypes of *C. suis* (Table 18-a), but *ompA* gene based screening detected much more cases of multiple genotypes infection in individual animal or farm. The distribution of different *ompA* gene based *C. suis* genotypes detected among 5 farms and 10 animals are shown in Table 18-b, Fig. 17 and Fig. 18. One animal of farm-I and farm-III each was found to have 3 and 4 types of *C. suis*. In 4 farms, each animal was found to harbor 2 to 4 types of genetically varied *C. suis* strains. Whereas, only one genotype was detected among animals of farm-II. The genetically closely related sequence types 1 and 2 were detected from 2 animals of farm-I and one animal (PG7) of farm-IV. However, animals of farm-III and farm-V and PG7 animal of farm-IV were found harboring highly diverse genotypes. These results indicate the high genetic variation among the *C. suis* stains infecting either a single animal or prevalent in the same farm. The overall numbers of genotypes detected based on sequence analysis of 16S-23S rRNA intergenic spacer and *ompA* gene are shown in Table 19.

Table 19. Number of sequence types detected in different farms and animals based on the 16S-23S rRNA intergenic spacer region and *ompA* gene.

Farms (prefectures)	Sample ID[a]	Sequence types[b]	
		Intergenic spacer	*ompA* gene
Farm-I (Chiba)	PG18	1	2
	PG21	3	2
Farm-II (Miyagi)	PG1	1	1
	PG2	1	1
Farm-III (Kanagawa)	PG5	4	3
	PG6	1	3
Farm-IV (Kanagawa)	PG7	1	4
	PG8	1	4
Farm-V (Kanagawa)	PG9	1	3
	PG10	1	2

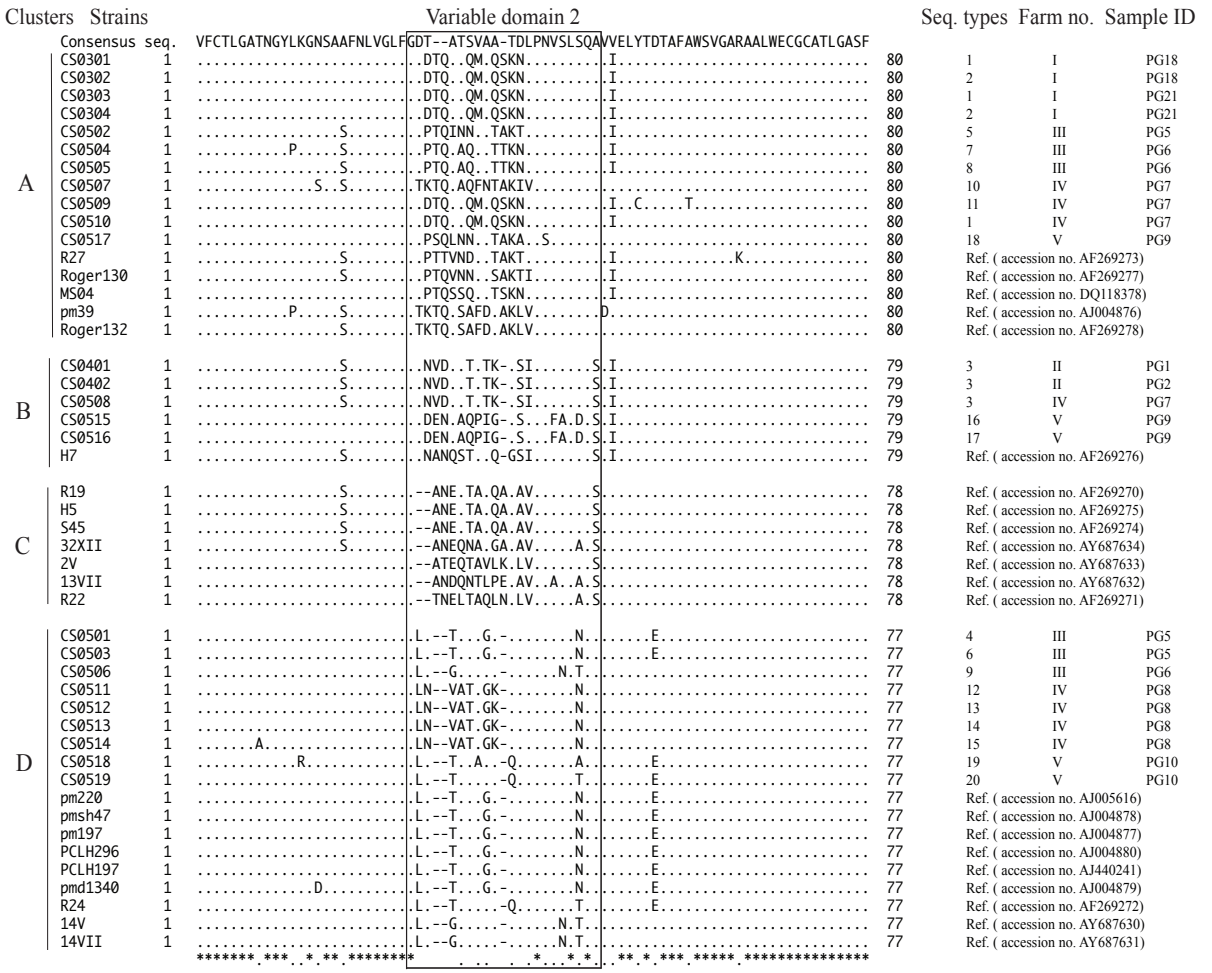
[a] Represents the independent sample taken from the individual animal.
[b] The sequence types with the same numerical numbers are having 100% homologous nucleotide sequences.

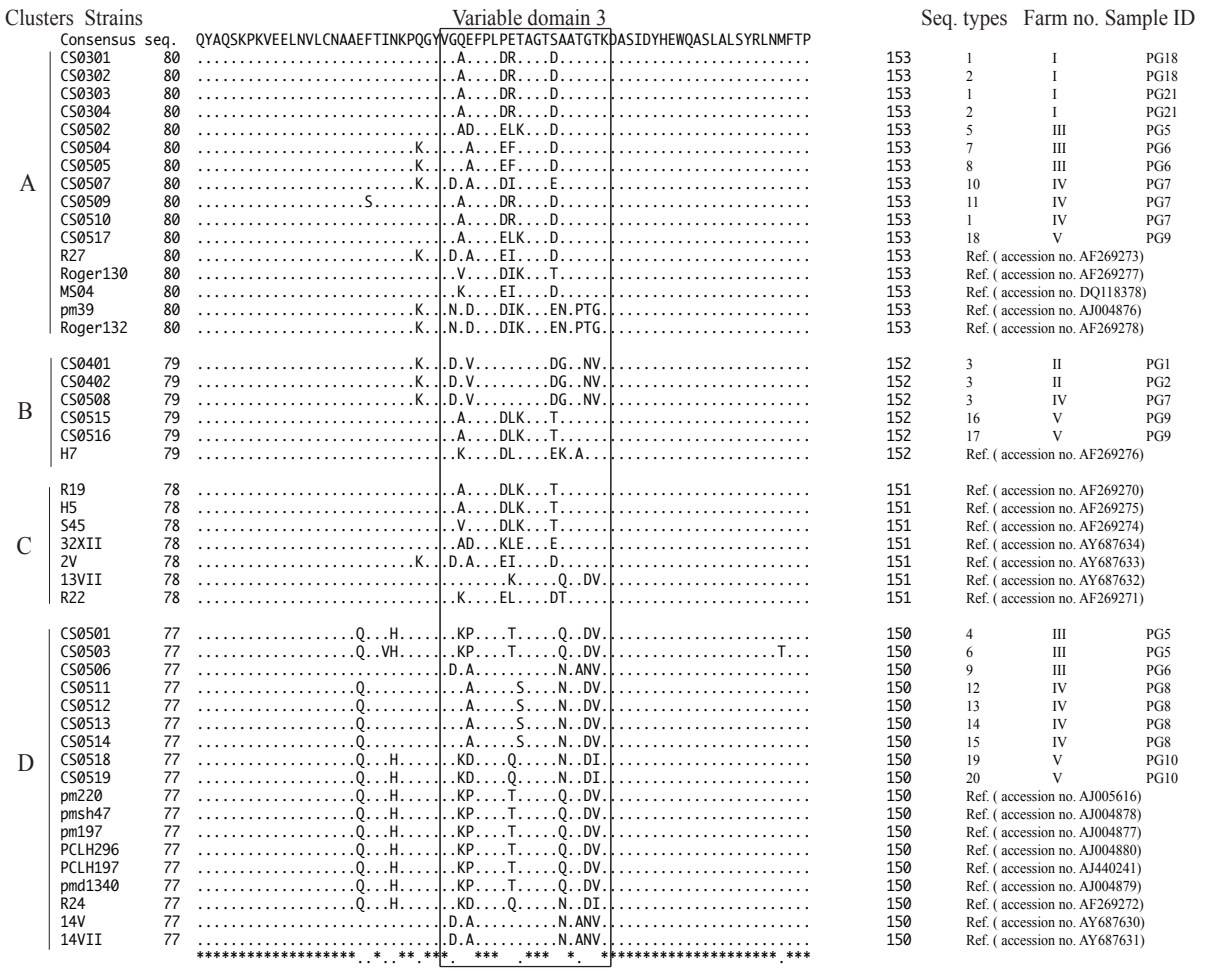
Phylogenic analysis: All the detected and known strains of *C. suis* are clustered together in a group *vis-à-vis* other *Chlamydia* spp. and representative *Chlamydophila psittaci* strain in 16S-23S rRNA intergenic spacer based NJ tree (Fig. 15). For further determination of epizootiologically predominant genotypes of *C. suis*, all the 25 detected *ompA* gene sequences (20 variant types) were compared with all the known strains of *C. suis* derived from lungs, enteric, nasal and ophthalmic samples either from healthy or diseased animals. All the strains were broadly grouped into 4 clusters; named A, B, C and D depending upon the numbers of gaps in the VD2 (Fig. 17 and 18). Genetic cluster A strains have no gap whereas strains of clusters B, C and D have one, two and three gaps, respectively in the VD2. Epizootiologically predominant clusters are D, A, C and B in descending order. In this study, *C. suis* strains only belonging to genetic clusters A, B and D could be detected. Distribution of these genetic clusters among 5 farms and screened animals is shown in Table 18-b. The distributions of *C. suis* strains into 4 genetic clusters do not related to host disease condition or tissue tropism.

DISCUSSION

C. suis is a recently identified pathogen of both domesticated and wild porcine (43, 84, 95, 165). *C. suis* has been demonstrated experimentally to be capable of causing acute pneumonia (166, 167), conjunctivitis (163) and enteric lesions (164) in gnotobiotic pigs. Limited epizootiological studies have indicated that *C. suis* infections in pigs are wide spread but under diagnosed. For detection of porcine chlamydiosis particularly due to *C. suis*, a sensitive *ompA* gene based PCR test was developed and used along with previously reported broad range 16S-23S rRNA based PCR. In the present investigation, *C. suis* was detected among 62.9% conjunctivitis samples by PCR, suggesting higher prevalence of *C.*

Fig. 17. Genetic clusters of *C. suis* strains detected in this study and reported in data bank (referred by accession no.) based on the alignment of amino acid sequences in VD2 and VD3 regions of *ompA* gene and variation in length of sequenced portion of *ompA* gene owing to gaps in VD2 region. The corresponding nucleotide sequence types (from 1 to 20), sample identification and farm numbers are also shown for each detected strain. The portion within the boxes represents the genetically variable domains flanked by the constant regions of *ompA* gene. Dots indicate the identical amino acids and hyphens represent the gaps. Asterisks in the bottom line mark the conserved amino acid residues. The alignment was done by ClustalX ver. 1.83


Clusters Strains
Variable domain 2
Seq. types Farm no. Sample ID
Consensus seq. VFCTLGATNGYLKGNSAAFNLVGLFGDT--ATSVAA-TDLPNVSLSQAVVELYTDTAFAWSVGARAALWECGCATLGASF
A
B
C
D


Clusters Strains
Variable domain 3
Seq. types Farm no. Sample ID
Consensus seq. QYAQSKPKVEELNVLCNAAEFTINKPQGYVGQEFPLPETAGTSAATGTKDASIDYHEWQASLALSYRLNMFTP
A
B
C
D

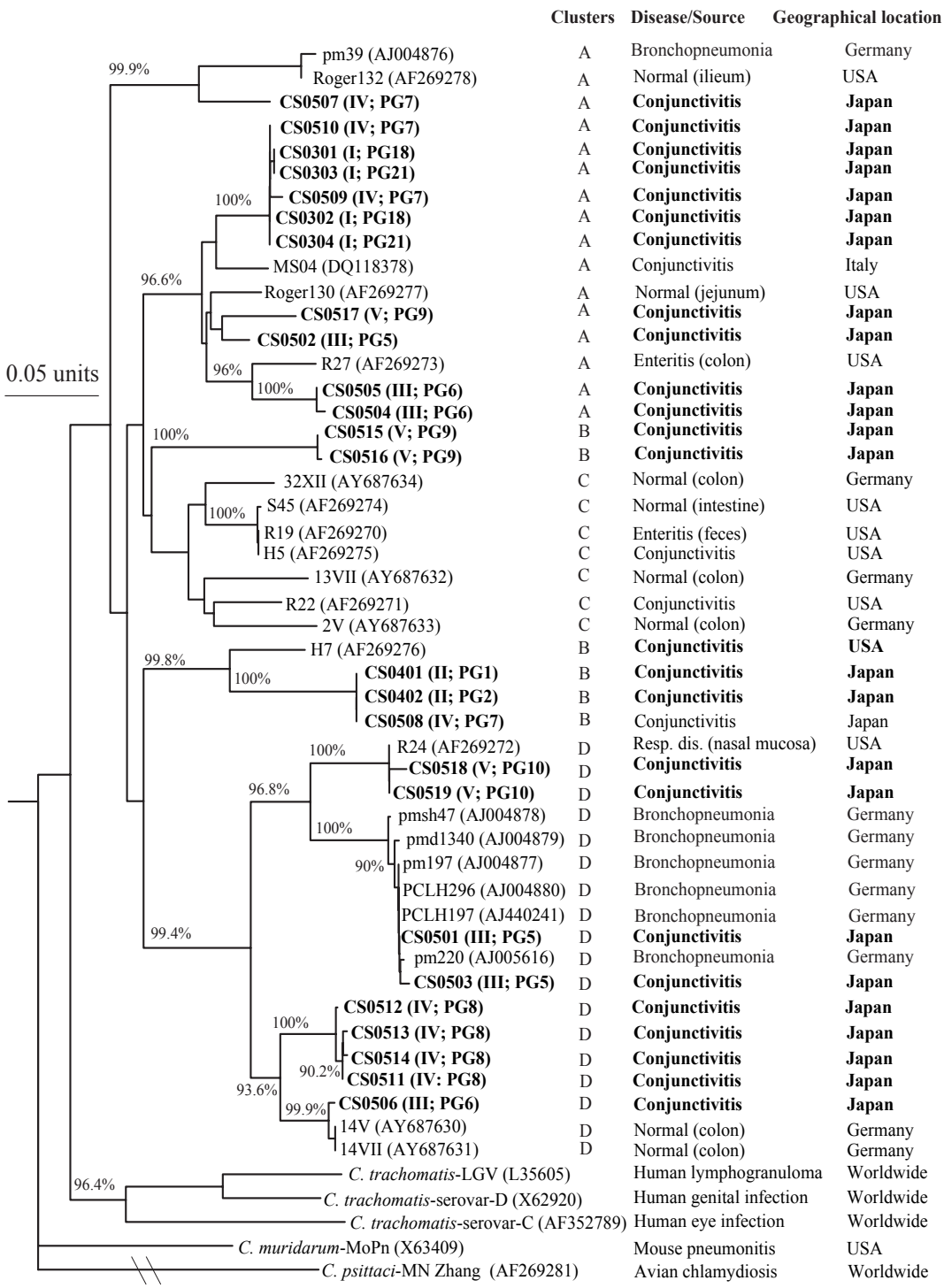


Fig. 18. Neighbor-joining (NJ) phylogenetic tree, based on nucleotide sequences of VD2 and VD3 regions of the *ompA* gene of different strains of *C. suis* and other *Chlamydia* spp. The strains detected in this study are shown in bold letters along with farm and sample identity in parentheses. The corresponding genetic clusters from A to D, isolation source/disease condition and geographical locations are shown against each strain. The genetic distance is indicated in 0.05 unit bar. Bootstrap values are shown at respective nodes. The *C. psittaci* is used as out-group and branch length is reduced to half.

suis among commercial piggeries. PCR based diagnosis is convenient and fast as isolation of *C. suis* is considered as difficult due to antibiotic treatments or feed supplementations (82, 178).

The 16S-23S rRNA intergenic spacer region and *ompA* are subjected to constant systematic selective pressure and functional conservations and are ideal targets for phylogenetic studies (20, 41). The genetic analysis of 16S-23S rRNA intergenic spacer region and partial *ompA* gene revealed high genetic variations among *C. suis* strains. The genetic variations in 16S-23S rRNA intergenic spacer region were less as compared to the *ompA* gene. All the 9 variant 16S-23S rRNA intergenic spacer sequence types showed less than 3% differences, whereas 20 *ompA* gene sequence variants detected from conjunctivitis cases showed 22% genetic variation. The difference in the genetic variations between *ompA* and 16S-23S rRNA intergenic spacer region may be due to the difference in selective pressure at both loci associated with biphasic developmental cycle of chlamydiae (20). In this study all the genetically variable strains of *C. suis* were detected from the conjunctivitis cases but strains detected from gut and lung specimens are also reported to have high degree of genetic variations (82).

Findings of this study are contradictory as compared to those of other chlamydiae causing eye infections in animals, like feline conjunctivitis by *C. felis* (26) and inclusion body conjunctivitis in guinea pigs by *C. caviae* (135). All the known strains of *C. felis* and *C. caviae* have relatively conserved *ompA* loci (173, 227). However our findings correlate with the situation in the human *C. trachomatis* strains, which have divergent *ompA* loci but relatively conserved other portion of genome (20, 98, 189). Immunological selection might promote such a process as change of MOMP peptide sequence, which might help the chlamydiae to escape immune response.

According to the *ompA* gene composition, all genetically diverse strains were divided into 4 groups. But the *ompA* gene based clusters of variant *C. suis* strains did not show specific tissue tropism and disease association. These results are in accordance with other studies suggesting no phylogenetic relationship among serovars that corresponds to various pathobiotypes of *C. trachomatis* (20, 189). The *C. suis* strains of ophthalmic and non-ophthalmic origin could not be differentiate neither in *ompA* gene nor 16S-23S rRNA intergenic spacer region based phylogenetic analysis. Although serotypic variation has not yet been studied in *C. suis*, genetic and biological variations have been reported among *C. suis* strains (82, 95, 99, 183). The pathobiological significance of this high genetic diversity is still not known.

Interestingly multiple genotypes of *C. suis* were involved in either individual animal or a farm. The detection of multiple genotypes in ophthalmic swabs may also indicate the repeated infection. Previous observations have also suggest that the severe clinical symptoms and lesions seen in human trachoma are the results of repeated *C. trachomatis* infection and are immunologically mediated (65, 130, 195). *C. suis* causes mucopurulent keratitis/conjunctivitis under field conditions as observed in the present and previous reports (165). However, the controlled experimental infection of gnotobiotic pigs with H7 strain of *C. suis* (an eye isolate) was observed to be less severe than those of field cases (163). It may be due to the use of single strain or no repetition of inoculations. Besides the repetitive infection, other factors may be playing supportive role in disease precipitation v*iz*. poorly ventilated and dusty housing, houseflies (*Musca domestica*) and other microbes like *Mycoplasma hyorhinis*, *Klebsiella pneumoniae* and *Pasteurella* sp. (165).

In this study, mixed infection of *C. suis* along with other chlamydiae could not be detect. It may be due to lack of sample diversity from non-ophthalmic origin. However, it

also suggests that ophthalmic infection is exclusively caused by *C. suis*. Although *C. suis* has been isolated from porcine ocular samples in America and Italy only, there is no report regarding role of chlamydiae in porcine conjunctivitis and keratoconjunctivitis in Japan or any other Asian country. Further experimental studies are required to access the pathogenicity of various *C. suis* strains and to understand biological significance of high genetic diversity.

SUMMARY

Commercial piggeries with frequent ocular and reproductive problems were etiologically investigated. Samples from 49 animals were collected from 5-pig farms located in three prefectures of Japan from 2003 to 2005. Samples were screened preliminarily with FAT and then followed by nested PCR tests. *Chlamydia suis* were detected among 44.9% (22 out of 49) samples in all 5 farms by 16S-23S rRNA intergenic spacer region based PCR and *Chlamydia* genus-specific PCR targeting *ompA* gene. Whereas, all the samples were found negative by *ompA* gene based *Chlamydophila* genus specific PCR. All the PCR positive samples were accrued from conjunctivitis cases only. Total 9 *C. suis* strains with less than 3% nucleotide variation were detected by nucleotide sequence analysis of 16S-23S rRNA intergenic spacer region, whereas, 20 *C. suis* strains with up to 22% nucleotide variation were detected by analyzing *ompA* gene in VD2 and VD3 regions. Multiple genotypes (up to 4 types) were found infecting either individual animals or farms. This study revealed high prevalence of *C. suis* in porcine chlamydiosis and association of genetically diverse, multiple genotypes in ophthalmic infection.

CHAPTER-V

Molecular characterization of the *Chlamydophila caviae* strain OK135, isolated from human genital tract infection, by analysis of some structural and functional genes

INTRODUCTION

The chlamydiae cause a variety of diseases among human beings and animals (185, 188). Genital chlamydial infections are highly prevalent among adults and adolescents in developing and developed countries (1, 89, 92, 204). Mostly *Chlamydia trachomatis* (serovar D to K and L1-L3) and rarely *Chlamydophila abortus* are implicated in human genital tract infections, manifested as salpingitis, cervicitis, pelvic inflammatory disease (PID) and abortions.

Chlamydophila caviae, previously known as a guinea pig inclusion conjunctivitis (GPIC) strain of *Chlamydia psittaci*, has been classified as a separate species under family *Chlamydiaceae* based upon the 16S rRNA gene homology (43). *C. caviae* is a host specific pathogen of guinea pigs and causes either self-limiting or asymptomatic inclusion conjunctivitis among young (4-8 weeks old) guinea pigs (63, 101, 135). *C. caviae* and guinea pig are important small animal experimental models to simulate the antigenic and immune response of human chlamydial infections (118, 150-152). While *C. caviae* has no previous history of infecting human beings, very recently, DNA of *C. caviae* was detected in human, cat, rabbit eyes (114), and also in waste water samples (110), indicating the possible wide ecobiological niche of *C. caviae*. Whole genome sequence of *C. caviae* revealed many species-specific genes (153), although all the known 6 strains of *C. caviae*

isolated from guinea pigs in different continents in different years were found to have identical *ompA* gene sequences (227). In this chapter, characterization of a rare strain of *C. caviae*, (OK135) was done, which was isolated from a woman with cervicitis by Mastumoto *et al.* (unpublished work). Based on phylogenetic analysis of 16S rRNA, *ompA* and *groEL-1* genes, the OK135 strain was found to be genetically closely related to other strains of *C. caviae*. This study highlights the ability of genetically diverse and non-conventional chlamydiae to cause human genital tract infections and their probable escape from detection due to use of diagnostic tests specific for conventional human chlamydial species.

MATERIALS AND METHODS

Case history: OK135 strain was isolated from a 20 years old female suffering from severe cervicitis (Matsumoto *et al.*, manuscript in preparation). The strain was isolated and maintained subsequently in McCoy cell line. Preliminary examination of strain was done by Giemsa and iodine staining and also by using chlamydial group specific Mab in direct immunofluorescence test (Chlamydia FA, Denka Seiken Co. Ltd, Tokyo, Japan). The results of iodine staining and *C. trachomatis* specific direct immunofluorescence staining with Mab (MicroTrak; SYVA Co., Palo Alto, California, USA) showed that OK135 strain is a non-conventional type of chlamydial species (122).

DNA extraction: SepaGene DNA extraction Kit (Sanko Junyaku, Tokyo, Japan) was used to extract DNA according to the instructions of manufacturer. After removing cell debris by centrifugation at 1,000 × *g*, partially purified elementary bodies were used for DNA extraction. DNA was finally dissolved in 50 μl Tris-EDTA (TE) buffer pH 7.4 (100 mM Tris-HCl, pH 7.4 and 10 mM EDTA, pH 8.0) and stored at –30°C until used.

PCR amplification of 16S rRNA, *ompA and groEL-1* genes: Overlapping DNA fragments of 16S rRNA (1445 bp), *ompA* gene (1148 bp) and *groEL-1* gene (1560 bp) were amplified by single step PCR using pairs of primers as shown in Table 20. The 50 μl PCR reaction mixture contained 0.20 μM of each forward and reverse primer, 250 μM of each dATP, dTTP, dGTP, dCTP, 100 μM of Mg^{2+} in buffer and 2.5 units of TaKaRa *Ex-Taq* (TaKaRa Bio Inc., Otsu, Japan) and 2.0 to 3.0 μg of template DNA. Thermocycling conditions were adjusted according to primer sets and in all, 30 amplification cycles were performed.

Cloning and sequencing of DNA fragments: The PCR amplified DNA fragments of each gene were purified by QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). Then each DNA fragment was cloned in the pT7 blue vector (Novagen, Madison, Wisconsin) by transforming *E. coli* DH10β strain by electroporation using Gene Pulser cuvettes, 0.5 cm electrode gap (Bio Rad, Hercules, California). Clones containing DNA insert were selected as in CHAPTER I (171). Total 3 to 5 clones with an expected size DNA fragment insert were selected by using Premix Colony Direct PCR insert check kit (Tyobo Co., Osaka, Japan) and taken for sequencing. Each DNA fragment was sequenced by using thermo sequenase cycle sequencing Kit (USB Corporation, USA) using forward (T7) and reverse (M13) universal fluorescent labeled primers using SEQ4×4 personal sequencing system (Amersham Pharmacia Biotech).

Analysis of sequences and construction of phylogenetic trees: The obtained sequences were assembled and edited using Genetyx-Mac/ATSQ 4.2.3 and Genetyx-Mac, version 13.0.6 (SDC, Tokyo, Japan). For phylogenetic analysis, representative gene sequences of 16S rRNA, *ompA* and *groEL-1* genes of chlamydial species and other bacteria associated with reproductive tract infections of animals and human were retrieved from the DDBJ. Using ClustalX, version 1.83 (198), done the multiple alignments of the

Table 20. Primers used to amplify16S rRNA, *ompA* and *groEL-1* genes.

Name	Sequence	Positions[a]	Tm(°C)[b]	Amplicon
16S rRNA gene				
CPR/P1F	5' CTGAGAATTTGATCTTGGTTCAG 3'	5-27	54-56	400 bp
CPR/P1R	5' CGCTTCGTCAGACTTTCGTCCATT 3'	404- 381	60	
CPR/P2F	5' CGTCTAGGCGGATTGAGAGATTG 3'	291-313	60-62	418 bp
CPR/P2R	5' CGCATTTCACCGCTACACGTGGAAT 3'	708-684	60-62	
CPR/P3F	5' GCGTGTAGGCGGAAAGGAAAGTT 3'	585-607	60-62	413 bp
CPR/P3R	5' CCAGGTAAGGTTCTTCGCGTTGCAT 3'	997-973	59-61	
CPR/P4F	5' CCGTGTCGTAGCTAACGCGTTAAGT 3'	860-884	60-62	444 bp
CPR/P4R	5' GGCTAGCTTTGAGGATTTGCTCCAT 3'	1403-1279	59-61	
CPR/P5F	5' CGAGGATGACGTCAAGTCAGCAT 3'	1191-1213	60-62	367 bp
CPR/P5R	5' TGTCGACAA AGGAGGTGATCCAG 3'	1557 –1537	55-57	
ompA gene				
M/0PF	5' GCAAGTATAAGGAGTTATTGCTTG 3'	-275 to -252	54-56	352 bp
M/0PR	5' CCTACAGGCAAGGCTTGTAAG 3'	56-77	57-60	
M/1PF	5' CGGCATTATTGTTTGCCGCTAC 3'	20-41	59	390 bp
M/1PR	5' CGAAGCGATCCCAAATGTTTAAGGC 3'	409-385	59-61	
M/2PF	5' CGCTTACGGAAGGCATATGCAAGATG 3'	330-355	62	397 bp
M/2PR	5' GTGAATCACAAATTGTGCTGGGCT 3'	726-703	58-60	
M/3PF	5' CGTGGAGCTTTATGGGAATGTGGTTG 3'	607-632	62	330 bp
M/3PR	5' GAGCAATGCGGATAGTATCAGCATC 3'	937-913	60-62	
M/4PF	5' GGCGTAAACTGGTCAAGAGCAAC 3'	886-908	60-62	387 bp
M/4PR	5' TCCCAGGTTCTGATAGCGGGACAA	1272-1246	61-63	
groEL-1 gene				
CVG/1PF	5' GGCAGTTCTC AAGTAAGAGA AATC 3'	-66 to -43	56-58	392 bp
CVG/1PR	5' GCAGTTACGTTTCTCAATCCTTC 3'	326-304	55-58	
CVG/2PF	5' GCAGATAAAGCTGGTGATGGAAC 3'	244-266	58-60	380 bp
CVG/2PR	5' GGATTTGTAGAGAAGTAGCTGGAT 3'	623-600	56-58	
CVG/3PF	5' CGAAACTGTCCTCGACGTTGT 3'	549-569	57-60	405 bp
CVG/3PR	5' CCTAACATAGCTAGAGTTGTGTTC 3'	953-930	56-58	
CVG/4PF	5' GGTCAACTCATCAGCGAAGAG 3'	892-912	57-60	436 bp
CVG/4PR	5' GTGCTCCGATTTGCTCATCTTC 3'	1306-1327	59	
CVG/5PF	5' CGCTGCATCCCTACTTTAGAAG 3'	1261-1282	59	443 bp
CVG/5PF	5' CCCTTTCTTCTCAAGATGGATG 3'	1703-1682	57-59	

[a] Based on 16S rRNA gene of *Chlamydophila psittaci*-Prk/GCP-1 strain (D85713), *ompA* gene of *C. psittaci*-6BC strain (accession no. X56980) and *groEL-1* gene of *Chlamydophila caviae*-GPIC strain (accession no. AE015925). The negative values indicate the nucleotide positions upstream of respective open reading frame.
[b] Averages of melting temperatures of forward and reverse primer sets were used in the direct PCR amplifications.

sequences. Phylogenetic analysis was done with programs available in the PHYLIP (version 3.6a3; [http://evolution.genetics.washington.edu/phylip.html]). The distance matrix between species was computed by DNADIST using Jukes-Cantor model (93) and

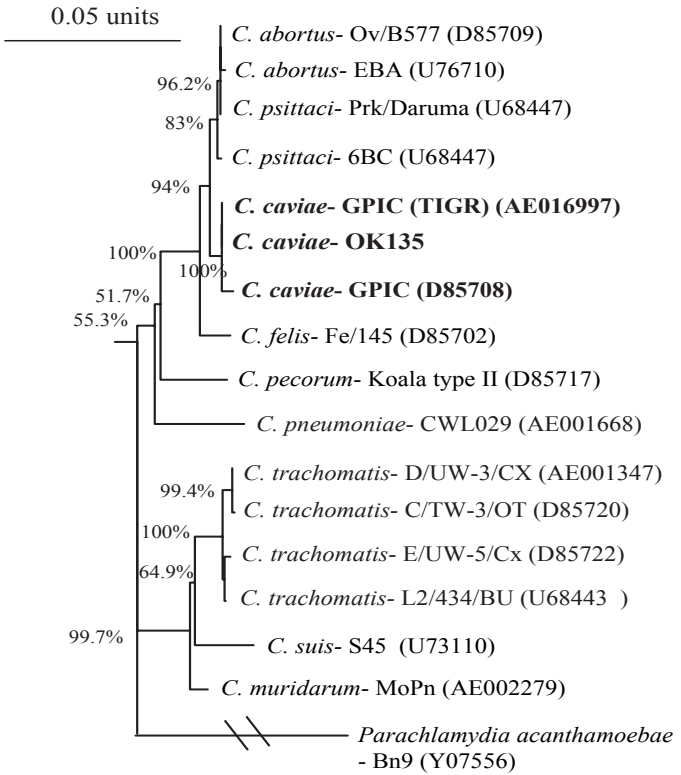
clustering was done using NJ method (170). The bootstrap values were calculated from a consensus tree constructed by generating 1,000 random data sets using SEQBOOT (45). In case of 16S rRNA gene, *Parachlamydia acanthamoeba* was taken as outgroup.

Extraction of chlamydial outer membrane complexes (COMC): Extraction of chlamydial outer membrane complexes (COMC) was done as reported earlier by using 2% sodium N-lauroyl sarcosine (Sarkosyl; Sigma) (27). The protein concentrations of the extracts were determined by Bio-Rad protein assays Kit (Bio-Rad, CA). The proteins profile of extracted COMC was visualized on sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) (108).

RESULTS

The nucleotide sequences and resultant amino acid sequences of 16S rRNA, *ompA* and *groEL-1* genes were compared with sequences in the data bank by NCBI-BLAST search (http://www.ncbi.nlm.nih.gov). The comparative phylogenetic analysis of 3 sequenced genes with previously reported chlamydial species is as follows:

16S rRNA gene: The 16S rRNA gene of OK135 strain was 100% homologous to the *C. caviae* GPIC strain (accession no. AE015925), which was used by the Institute of Genomic Reseach (TIGR) for whole genome sequencing. OK135 strain was also closely related to another *C. caviae* GPIC strain (accession no. D85708 and ATCC No. VR-813, isolated by E.S. Murray in 1964 from the conjunctiva of guinea pigs with conjunctivitis in Massachusetts, USA), showing 99.7% (1509/1514) nucleotide homology (Fig. 19). The NJ analysis of 16S rRNA gene also confirmed that the sequence of OK135 strain is identical to that of *C. caviae* GPIC strain (accession no. AE015925). All the 3 strains of *C. caviae* form a genetically distinct branch diversing from other *Chamydophila* spp. and *Chlamydia*



Species	Disease conditions	Location
Sheep	Abortion	USA
Bovine	Abortion	USA
Parrot	Systemic infection	Japan/India
Parakeet	Systemic infection	USA
Guinea pig	Conjunctivitis	USA
Human	**Cervicitis**	**Japan**
Guinea pig	Conjunctivitis	USA
Cat	Conjuctivitis	USA
Koala	Urogenital infection	Australia
Human	Pneumonitis	Taiwan
Human	Cervicitis	USA
Human	Trachoma	Taiwan
Human	Cervicitis	USA
Human	Lymphogranuloma	USA
Pig	Asymptomatic	Austria
Mouse	Pneumonitis	USA
Acanthamoeba castellanii		Germany

Fig. 19. The NJ phylogenetic tree of the nucleotide sequence of 16S rRNA gene of the OK135 strain as compared to other chlamydial strains of *Chlamydiaceae* family. The *P. acanthamoebae* was taken as out-group and its branch is shortened to half. The genetic dista--nce is shown as unit bar.

spp. This is supported by significantly high bootstrap value (Fig. 19).

***ompA* gene:** The genetic composition of *ompA* gene, which has 4 genetically hyper variable domains (VDs), was found to be identical in OK 135 and GPIC (TIGR) strains. However, when compared to another strain-GPIC (sequence accession no. AF269282 and isolate ATCC No. VR-813), 2 nucleotide differences were found. One at position 843 of ORF (T base changed to C) with no change in amino acid residue and another at position 996 in VD4 (A base changed to T) with conversion of lysine residue to asparagines. Interestingly, in another *ompA* gene sequence of *C. caviae* available in literature (226), only single nucleotide polymorphism (SNP) at position 843 was observed. We also sequenced promoter region of *ompA* gene up to 178 nucleotides upstream and found this region to be conserved among all *C. caviae* strains. The NJ tree of *ompA* gene (without out group) of all the nine species in *Chlamydiaceae* and all *C. caviae* strains, also provided 100% reliability support for the formation of distinct clade of *C. caviae* strains within the family *Chlamydiaceae* (Fig. 20-A). The COMC composition of OK135 strain in SDS-PAGE showed protein bands of MOMP and other proteins with identical sizes to the GPIC (TIGR) strain.

***groEL-1* gene:** The GroEL-1 chaperonin of OK135 strain was 100% homologous to other strains of *C. caviae* but the interspatial differences were observed. In the unrooted NJ tree without out-group, the *groEL-1* gene of other microbes commonly associated with sexually transmitted diseases and members of order *Chlamydials* was found to be phylogenetically distant (Fig. 20-B). The analysis of functional domains of this GroEL-1 chaperonin protein coded by this *groEL-1* gene showed that all the functionally important sites are conserved in family *Chlamydiacae* including this new OK135 strain (Table 21).

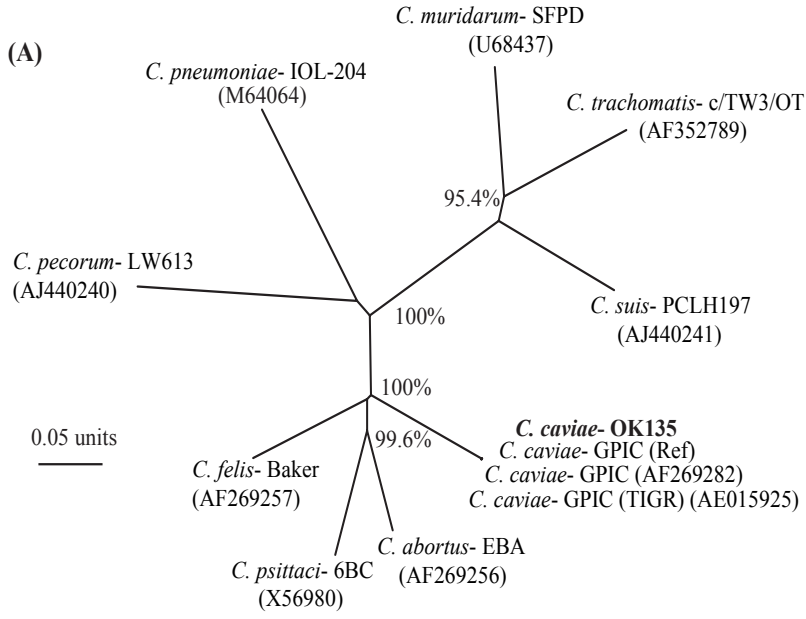


Fig. 20-A. The NJ phylogenetic tree of the nucleotide sequence of the *ompA* gene of OK135 strain (bold letters) and all 9 species of family *Chlamydiaceae* including all reported strains of *C. caviae*.

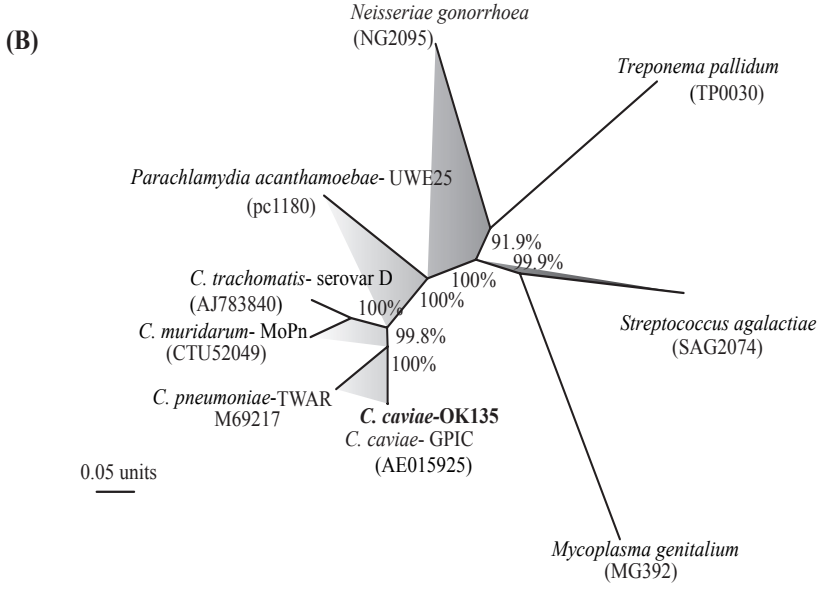


Fig. 20-B. The NJ tree showing the phylogenetic distance in *groEL-1* gene of OK135 strain (bold letters) *vis-à-vis* other *C. caviae* strains, chlamydial species and bacteria frequently in--volved in sexually transmitted infections.

Table 21. Multiple alignment of functional regions of GroEL-1 of OK135 strain *vis-à-vis* other chlamydial species and bacteria associated with reproductive diseases. The residues important for polypeptide binding are boxed in solid line, for GroES contact in dashed line box and for ATPase activity in doted line box.

	Amino acid residue positions[a]																				
Organisms	87	88	89	90	91	150	151	152	199	201	203	204	234	237	259	263	264	265	361	383	405	406
Chlamydia trachomatis	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Chlamydia muridarum	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Chlamydophila pneumoniae	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Chlamydophila caviae-OK135	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Chlamydophila caviae	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Chlamydophila abortus	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Chlamydophila abortus	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Chlamydophila felis	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Chlamydophila pecorum	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Parachlamydia acanthamoeba	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	**Ile**	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
E. coli	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Neisseriae gonorrhoea	Asp	Gly	Thr	Thr	Thr	Ile	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala
Streptococcus agalactiae	Asp	Gly	Thr	Thr	Thr	**Val**[b]	Ser	**Ser**	Tyr	Ser	Tyr	**Met**	Leu	Leu	Leu	Val	**Leu**	Asp	Asp	Ala	Ala	Ala
Mycoplasma genitalium	Asp	Gly	Thr	Thr	Thr	Ile	Ser	**Ser**	Tyr	Ser	Tyr	**Met**	Leu	Leu	**Val**	**Ala**	Val	Asp	Asp	**Gly**	Ala	Ala
Treponema pallidum	Asp	Gly	Thr	Thr	Thr	**Val**	Ser	Ala	Tyr	Ser	Tyr	Phe	Leu	Leu	Leu	Val	Val	Asp	Asp	Ala	Ala	Ala

[a] The numbering of the amino acid residue positions is based on the *E. coli* GroEL-1.
[b] The substituted amino acid residue as compared to *E. coli* are shown in bold.

DISCUSSION

Chlamydiae have been reported in a variety of disease syndromes in human beings and animals (185, 188). Among the *Chlamydiales*, *C. trachomatis* and *C. pneumoniae* are established to be human pathogens. However, other *Chlamydophila* species can occasionally infect human beings (113). In this chapter, a *C. caviae* strain, OK135, isolated from a clinical human cervicitis case was identified and genetically characterized. *C. caviae* usually cause self-limiting or asymptomatic ophthalmic infection in its natural host, guinea pigs (63, 101, 135). Infection in human is unknown except a very recent report of *C. caviae* DNA detection in human eye samples (114). The chlamydial cervicitis is usually caused by *C. trachomatis*, serovars D to K, however, serovar E is reported to be common (190).

Initially, 16S rRNA gene was analyzed for the phylogenetic identification of OK135 strain as this gene formed the basis of recent classification of all the chlamydiae (43). The 16S rRNA gene is reported to have species-specific conserved motifs in the order *Chlamydials*. (43, 54, 149). OK135 strain was found to be genetically identical to GPIC strain. Furthermore, the structural analysis of MOMP, which constitute majority of chlamydial envelope and responsible for serological variations, was done (10, 50, 186, 187). Also genetic makeup of *groEL-1* gene which encodes GroEL-1 (HSP 60) was analyzed as these types of protein are recognized by the host Toll-like receptors (TLRs) as part of innate defense system and implicated in diseases pathogenesis (23, 103).

The *ompA* gene locus of OK135 strain was identical to GPIC strain, and 1 to 2 SNP were detected as compared to two other *C. caviae* sequences available in the data bank. However, in other report, no allelic polymorphism was observed among 6 *C. caviae* strains in partially sequenced (70%) *ompA* locus (227). All the 6 strains were identical to *ompA*

gene sequence of GPIC strain reported by Zhang *et al.* (226), which had a SNP at 843 position between VD3 and VD4 of *ompA* gene as compared to OK135 strain. Therefore, all the *C. caviae* strains have relatively conserved *ompA* locus as compared to other *Chlamydia* spp. and *Chlamydophila* spp., which show high polyporphism in *ompA* gene (98, 189). Lack of high genetic variation among *C. caviae* strains as compared to other chlamydiae with polymorphic *ompA* gene may be due to differences in host-pathogen interaction and selective pressure exerted by host immune response. Furthermore, it has also been observed that anti-MOMP serological response in *C. caviae* is not so protective in experimental ophthalmic and genital experimental infections (13, 203). This is similar to the non-prominent anti-MOMP immune response shown by *C. pnemoniae* that also lack polymorphism in *ompA* gene locus (28). The *C. pneumoniae* infection are widely prevalent among human population (106). Although the *C caviae* GPIC has been studied extensively as surrogate experimental strain to understand pathogenesis and immune responses in human genital infections (150, 215), the *C. caviae* infection among human are uncommon or rather unknown. In the present case, while the circumstantial evidences of contacting *C. caviae* by the said patient from primary host (guinea pig) are lacking, the possible reason for switching of host species may be due to lack of protective immunity in advance or cross protection among human to *C. caviae* infection. Another possible source of infection may be still unknown host or carrier of *C. caviae* as the recent reports are also indicating possible wider ecobiological niche (110, 114).

It was also observed that functional sites in GroEL-1 protein (HPS 60) coded by *groEL-1* gene are conserved among all the members of *Chlamydiaceae* family, it can be speculated that chlamydiae may exhibit a similar disease pathogenesis or ability to infect multiple host species. Therefore, diagnosis of aberrant chlamydial species in various clinical manifestations is important. These types of infection may exist in human

population but remained undiagnosed due to use of species specific diagnostic methods in chlamydial reproductive tract infections *viz*. PCR or LCR Kit are mostly targeted at 7.5-kb plasmid or *C. trachomatis* specific monoclonal antibody based fluorescent tests and in such cases the clinical diagnosis of *C. caviae* can be bypassed (121). It is also observed that severity of clinical manifestation and inflammation of urogenital chlamydia infections is strongly influenced by the number of chlamydiae present in the genital tract rather than the infecting serovar of *C. trachomatis* (61). Hence, undiagnosed infection with such rare chlamydial species can proliferate in genital tract can lead to pelvic inflammatory disease (PID) and its subsequent complications like salpingitis and tubal factor infertility, similar to those occurred by other genital chlamydial species. This study also suggests need for development of new diagnostic tools for chlamydial reproductive infections, encompassing broader *Chlamydiaceae* group.

SUMMARY

Genital tract infections by different serovars of *C. trachomatis* are reported to be alarmingly higher in recent studies. Occasionally *C. abortus* also causes human reproductive disorders. The *C. caviae* is a highly host specific species and causes conjunctivitis and other eye infections in guinea pigs. In our study, we characterize a *C. caviae* strain (OK135) isolated from a woman with acute cervicitis, by sequencing 16S rRNA, *ompA* and *groEL-1* genes. The isolate was consequently confirmed as *C. caviae* and phylogenetically closely related to other strains of *C. caviae*. The *ompA* gene had 100% homology to GPIC strain of *C. caviae* used for genome project by TIGR, but has 1 to 2 nucleotide substitution in the coding region as compared to other already reported *C. caviae* strains. The COMC extracted by sarkosyl detergent showed 38.5 kDa size of

MOMP along with other proteins, similar to those of GPIC strain. The *groEL-1* and 16S rRNA genes also showed 100% homologous to those of GPIC strains. Therefore, the OK135 strain of *C. caviae* appears to be closely related to GPIC strain. This report highlights the ability of non-conventional chlamydiae to cause human infections without many genetic and subsequent structural and physiological adjustments. It suggests the need for broader and not species-specific diagnostic approach for human chlamydial infections.

CONCLUSION

Chlamydiae are the obligate intracellular bacterial pathogens of animals and human beings. They are responsible for a diverse range of diseases in birds and mammals including humans. *C. trachomatis* and *C. pneumoniae* are established to be human pathogens. Animal chlamydial infections are caused by *C. psittaci*, *C. abortus*, *C. suis*, *C. muridarum*, *C. pecorum*, C. *felis* and *C. caviae*. Various studies have shown natural prevalence of genetically diverse strains of chlamydiae within each species. *C. psittaci* is mainly prevalent among avian species and causes either inapparent or clinical infection of varying severity. *C. psittaci* is also considered to be potentially pathogenic to in-contact mammalian hosts including human beings.

This thesis describes the molecular epidemiology, genetic diversity and virulence of *C. psittaci* strains, which were naturally distributed among diverse types of avian fauna, in order to compare the risk to the health of avian and mammalian fauna as well as human beings. This study was also intended to detect the chlamydiae in some diseased animals and humans, as different chlamydial species were detected recently from previously known disease syndromes.

In the molecular epidemiological study to investigate the diversity and natural distribution of *C. psittaci*, total 1,147 samples from 11 avian orders including 53 genera and 113 species of feral and captive birds were examined using *ompA* gene based nested PCR. Three types of chlamydiae including *C. psittaci* (94.12%), *C. abortus* (4.41%) and unknown *Chlamydophila* sp. (1.47%) were identified among 68 birds, which includes 59 from *Psittaciformes*, 8 from *Ciconiiformes* and 1 from *Passeriformes* orders. Based on nucleotide sequence variations in the VD2 region of *ompA* gene, all 64 detected *C. psittaci* strains were grouped into 4 genetic clusters. Clusters I, II, III and IV were detected from

57.35%, 19.12%, 10.29% and 7.35% samples, respectively. A single strain of unknown *Chlamydophila* sp. was found to be phylogenetically intermediate between *Chlamydophila* species of avian and mammalian origins. Therefore, it was concluded that, though various genetically diverse chlamydiae may have caused avian chlamydiosis, only a few *C. psittaci* strains were highly prevalent and frequently associated with clinical/subclinical infections.

All the 46 known and genetically diverse strains of *C. psittaci*, detected in the present and previous studies from various avian and mammalian hosts including human beings were genetically analyzed to evaluate the phylogenetic relationships, pathobiotic implications and to improve the strain categorization. The *ompA* gene loci of 11 new *C. psittaci* strains along with previously known 35 strains were analyzed. 16S rRNA gene of two highly diverse strains CPX0308 and Daruma was also studied. On the basis of the conserved region of *ompA* gene, 4 lineages of *C. psittaci* strains with genetic distance of less than 0.1 (dissimilarity less than 10%) were noted. The *C. psittaci* strains of 4 lineages, with genetic distance of less than 0.05 (dissimilarity less than 5%) were subdivided into 8 genetic clusters. The *ompA* gene loci of *C. psittaci* strains of lineage-1 were relatively conserved and these strains were frequently involved in infection of human (psittacosis) and other mammalian species. Lineage-2 strains are genetically diverse and divided into 5 genetic clusters. Lineage-3 and 4 represented by WC and CPX0308 strains, respectively, are genetically more close to *Chlamydophila* spp. causing infection among mammalian hosts. The proposed scheme classifies diverse *C. psittaci* strains according to the genetic variations and phylogenetic relationships. It may also be useful to classify even uncultured *C. psittaci* strains detected in clinical diagnosis.

Further to evaluate the degree of virulence of *C. psittaci* strains predominantly responsible for avian chlamydiosis and human psittacosis, BALB/c mice were used as experimental infection model. Four representative *C. psittaci* strains: Nośe, MN, Mat116

and Borg were intranasally inoculated at dose rate of 0.5 IFU, 5 IFU, 50 IFU, 5×10^2 IFU, 5×10^3 IFU and 5×10^4 IFU per gram body weight. The LD_{50} of Nosé, MN, Mat116 and Borg strains were 3.1×10^3, 19×10^3, 12×10^3 and 6.3×10^3 IFU/gm body weight, respectively. The intensity of clinical signs against the infectious doses of 5×10^3 and 5×10^4 IFU/gm body weight was the same among the 4 strains, however, at 50 and 5×10^2 IFU/gm body weight doses, the MN strain showed milder clinical signs. The animals incubated with the MN strain at higher doses showed less variation in the relative body weights as compared to those inoculated with the Nosé, Mat116 and Borg strains. Among the 4 strains, complete recovery and clearance of chlamydial infection was verified at 21 days post infection, as no chlamydial DNA and/or chlamydial IFU was detected in lung, liver and spleen tissues. Chlamydia specific IgG immune response was found to be dose dependent. This study indicated that the *C. psittaci* strains frequently associated with avian and human psittacosis cases were highly virulent to mice and that the immune responses lead to variable degrees of protection. Our murine lung infection based model can be used for further molecular virulence analysis studies.

The chlamydial etiological investigation in ophthalmic and reproductive diseases of animals and human showed some interesting results. The detected chlamydial species and strains were genetically characterized by multilocus sequence analysis.

Five commercial piggeries, in three prefectures of Japan, with frequent ocular and reproductive problems were etiologically investigated. Samples from 49 animals were collected and screened preliminarily with FAT and then followed by nested PCR tests. *C. suis* were detected in 44.9% (22 out of 49) samples in all 5 farms by 16S-23S rRNA intergenic spacer region based PCR and *Chlamydia* genus-specific PCR targeting *ompA* gene. Whereas, all the samples were found to be negative by *ompA* gene based *Chlamydophila* genus specific PCR. All the PCR positive samples were accrued from

conjunctivitis cases only. Total 9 *C. suis* strains with less than 3% nucleotide variation were detected by nucleotide sequence analysis of 16S-23S rRNA intergenic spacer region, whereas, 20 *C. suis* strains with up to 22% of nucleotide variation were detected by analyzing VD2 and VD3 regions of the *ompA* gene. Multiple genotypes (up to 4 types) were found infecting either individual animals or farms. This study revealed high prevalence of *C. suis* in porcine chlamydiosis and association of genetically diverse, multiple genotypes in ophthalmic infection.

Human genital tract infections by different serovars of *C. trachomatis* are reported to be alarmingly higher in recent epidemiological studies. Occasionally *C. abortus* also causes human reproductive disorders, whereas, *C. caviae* is a highly host specific species and causes conjunctivitis and other eye infections in guinea pigs. In our study, we characterize a *C. caviae* strain (OK135) isolated from a woman with acute cervicitis, by sequencing 16S rRNA, *ompA* and *groEL-1* genes. The isolate was confirmed as *C. caviae* and phylogenetically closely related to other previously reported strains of *C. caviae*. The *ompA*, *groEL-1* and 16S rRNA genes showed 100% homology to GPIC strain of *C. caviae*. This report highlights the ability of non-conventional chlamydiae to cause human infections without many genetic and subsequent structural and physiological adjustments. It suggests the need for broader and not species-specific diagnostic approach in human chlamydial infections.

The results of this study showed that, though *C. psittaci* is widely distributed among avian fauna, only a few strains/genotypes are epidemiologically successful and are highly virulent to in-contact animals or human beings. In general chlamydiae may be involved in many animal or human disease conditions but either have not yet been investigated in detail or have escaped from clinical diagnosis due to use of very specific diagnostic approaches.

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