Molecular epidemiology of chlamydiae at the shorebird-human interface in New Zealand

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Abstract

Chlamydiae (plural form of the bacteria, *Chlamydia*) can infect a broad range of avian hosts. The well-recognised *Chlamydia*, *Chlamydia psittaci* can cause avian chlamydiosis; a respiratory, ocular, and enteric disease. *C. psittaci* is also a zoonotic agent causing psittacosis in humans. Worldwide, migratory shorebirds are one of the principal hosts of *C. psittaci.* New Zealand has two Ramsar sites (internationally important wetlands) that serve as significant stopovers for migratory shorebirds using the East Asian-Australasian Flyway. However, there has been no prior surveillance conducted for chlamydiae in migratory shorebirds in New Zealand. The aim of this study was to perform a chlamydiae survey at four estuarine sites in New Zealand, in both shorebirds and the people that handle them.

Molecular methods are currently the most sensitive and rapid tests to detect *C. psittaci* in birds. Most laboratories have designed an in-house molecular assay or applied published protocols, sometimes with slight changes to the original procedure, for their studies with different purposes. Systematic review of 120 articles revealed that the sensitivity and specificity of a molecular test is dependent on the target genes, primer sequences, type of molecular test (quantitative PCR, conventional PCR), DNA extraction methods, and sampling methods. Of the currently available ten genomic targets to detect *C. psittaci* in birds, the *ompA* gene was the most widely used target gene. A testing strategy using a hierarchical approach that includes molecular tests of genus- and species-specific targets is recommended to facilitate detecting the well-recognised *C. psittaci* as well as other avian chlamydial species.

In this study, DNA extracted from choanal and cloacal swabs of 320 live shorebirds of 7 species from New Zealand was screened for chlamydiae by High-Resolution DNA Melt quantitative PCR (qPCR-HRM) analysis. Shedding of chlamydiae was detected in 71 out of 320 (22%; 95%CI 18-27%) shorebirds sampled. Due to low pathogen load, molecular characterisation of avian chlamydial at the species level was possible in only 14 out of 73 positive samples by applying a culture-independent sample preparation method – multiple displacement amplification (MDA). *ompA* (outer

membrane protein A gene) sequencing of the MDA products revealed 100% identity with *C. psittaci* in one pied stilt, and 99.9% identity with the avian *C. abortus* strain in five bar-tailed godwits, four pied stilts, and four South Island pied oystercatchers.

DNA extracted from the nasal swabs of 27 people handling the shorebirds was screened using the same methodologies as above. qPCR-HRM results of the shorebird handlers showed the detection of chlamydiae in 14 out of 27 (52%; 95%CI 33-71%) participants. And the *ompA* sequencing results of human samples revealed 100% identity with *C. psittaci* in one sample, and 99.9% identity with the avian *C. abortus* strain in ten samples. To our knowledge, this is the first report of avian *C. abortus* strains in both shorebirds and people in New Zealand. The outcome from this study suggests potential transmission of chlamydiae among shorebirds populations, and between shorebirds and people. With this, we can raise awareness and inform disease prevention protocol for targeted people, in addition to conservation management strategies for shorebirds and public health plans.

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Introduction, literature review, and research aims

CHAPTER 1 Introduction, literature review and research aims

1.1 Introduction

This section provides an overview of the pathogen (*Chlamydia psittaci*), infection and disease in hosts (birds and people), and diagnostic methods used to identify *C. psittaci* infection. This literature review aims to identify the gaps in our current knowledge of the pathogen and provide context to the research carried out in this thesis.

C. psittaci can cause avian chlamydiosis in birds and psittacosis in humans (Andersen & Franson, 2007). *C. psittaci* is a Gram-negative obligate intracellular bacterium, which undergoes a two-phase lifecycle in host cell's cytoplasm. The elementary body is infectious, metabolically inactive, condense and small; the reticulate body is non-infectious, metabolically active, replicating and big (Sachse, Laroucau, et al., 2015). To date, the nine genotypes of *C. psittaci* described, based on the analysis of the outer membrane protein A (*ompA*) gene are A, B, C, D, E, F, E/B, M56 and WC; genotypes A-F and E/B are linked to avian hosts (Lent et al., 2012). The six provisional genotypes which represent the untypable strains are 1V, 6N, R54, YP84, Mat116 and CPX0308 (Sachse et al., 2008).

In birds, avian chlamydiosis can cause conjunctivitis, coughing, dyspnoea, diarrhoea and death (Knittler et al., 2014) but in some hosts, *C. psittaci* infections result in an asymptomatic carrier state. The disease in birds is most commonly reported in captive pigeons and parrots, however, *C. psittaci* infection is emerging in the poultry industry, especially in the Europe, causing economic losses due to reduced productivity (Gaede et al., 2008). The effect of the pathogen on wildlife populations is less well understood. In general terms, disease can contribute significantly to the decline of some endangered wildlife species or even cause localised extinctions (Smith et al., 2009). Disease can be introduced to wildlife species via illegal importation or uncontrollable animal movement, like migration (Jackson et al., 2000). For example, new hosts may be exposed to *C. psittaci* via migratory birds when infected birds generate contaminated

aerosols during landing and taking flight. In crowded roosting habitats, this aerosolization of infective material can result in potential cross-host transmission, particularly in naïve native wild birds sharing the same habitat (Burnard & Polkinghorne, 2016; Hubalek, 2004).

Avian chlamydiosis can also occur in farmed birds. The epidemiological interactions of *C. psittaci* at a wildlife-livestock interface was illustrated in a *C. psittaci* outbreak in Australia. Farmed ducks were housed in open growing sheds, readily exposed to wild waterfowl (Tiong et al., 2007). The disease transmission possibly occurred when the wild birds as the *C. psittaci* reservoir, were attracted to the feed resources and contaminated the feed in the facility (Burnard & Polkinghorne, 2016; Woodford & Rossiter, 1994). To date, knowledge of *C. psittaci* in birds in New Zealand is rudimentary. At present, there is no epidemiological data on *C. psittaci* in migratory birds, farmed ducks and native wild birds in New Zealand, regarding its occurrence, genotypes, and bacterial shedding level.

In humans, psittacosis starts as flu-like symptoms and may progress to severe pneumonia (Smith et al., 2011). It can be transmitted via occupational, recreational and environmental exposure to *C. psittaci* (Balsamo et al., 2017). Occupational exposure of *C. psittaci* can occur in avian veterinarians, zookeepers, wild bird rehabilitators, pet shop workers, and poultry workers (Balsamo et al., 2017). In a Belgian wild bird refuge centre, three staff were infected with *C. psittaci* as determined by *ompA*-based RT-PCR; two staff had 'flu-like' symptoms for two weeks (Kalmar et al., 2014). In a survey among Belgian chicken famers, 94% (29/31) were *C. psittaci*-positive, based on culture and PCR; with 86% (25/29) having yearly psittacosis-related medical complaints (Lagae et al., 2014). In 2009, four duck farm workers in France had psittacosis and were hospitalised for pneumonia (Vorimore et al., 2015). Human psittacosis is not a notifiable disease in New Zealand, and diagnosis of *C. psittaci* is not a routine test in patients with respiratory tract infection. Hence, there is a lack of epidemiological data and potential underdiagnosed cases of *C. psittaci* infection in humans in New Zealand.

1.2 *Chlamydia psittaci* in New Zealand

In New Zealand, the first case of avian chlamydiosis was reported in 1954 in Dunedin. This disease was observed after a bird fancier imported 66 psittacine birds from Australia, following with 31 deaths. Post mortem findings and pathogen isolation in 13 dead birds confirmed chlamydiosis in 12 of them (Cairney, 1954). In 1959, the first detection of latent carriers of *Chlamydia* sp. via pathogen isolation were described in an apparently healthy wild rosella (*Platycercus* spp.) and nine domestic pigeons (McCausland et al., 1972). Later in 1972, avian chlamydiosis occurred in a suburban aviary in Hamilton, causing the death of 10 out of 150 birds, over one-month period. The affected birds were housed in two neighbouring cages, with a mixture of native and introduced bird species (**Table 1.1**) (McCausland et al., 1972). The diagnosis was made based on clinical signs like anorexia and bilateral serous ocular discharge, pathology like splenomegaly, histopathology like granuloma in liver sinusoids and fibrinous exudate in pulmonary septa, and presence of elementary bodies in egg inoculation (McCausland et al., 1972).

In 1984, *C. psittaci* was isolated in healthy keas (*Nestor notabilis*) sent from New Zealand to the United Kingdom (Johnson et al., 1984). From mid-1984 to 1986, in the 31 bird tissue samples (spleen, liver and lung) submitted to the Central Animal Health Laboratory in Wellington, 11 samples from parrots and pigeons were identified as *C. psittaci*-positive (Bell & Schroeder, 1986). Pathogen detection was done by isolation in embryonated egg, followed by direct fluorescent antibody staining of the inoculated cell cultures (Bell & Schroeder, 1986). Subsequently, regular publication of avian chlamydiosis reports started in 1990s. In 1990, 1991 and 1992, two, five and 19 confirmed cases were reported. Of all the positive samples reported by the Central Animal Health Laboratory, 13% were from pet shops, 42% from aviaries and 45% from pet bird owners (Hemsley, 1995). The samples submitted including faeces and tissues were tested using antigen-detection ELISA (IDEIA™) and direct fluorescent antibody test (Hemsley, 1995). In late 1992, with the diagnosis of chlamydiosis in an adult takahe (*Porphyrio mantelli*), a survey of *C. psittaci* was performed in native endangered birds using immunochromatographic test kit (Clearview®) to identify the susceptible species.

Of the faecal samples, 60% (73/121) were positive, including some collected from the Kakapo (*Strigops habroptilus*) and kiwi (*Apteryx* sp.) (Motha et al., 1995). However, the immunochromatographic test kit (Clearview®) used in this study produced high false positive results, especially when it was tested on cloacal swab (Fudge, 1997). Therefore, cell culture and PCR is needed to confirm the presence of *C. psittaci* in the native bird samples.

Based on the above findings, a prevalence study was designed in 1993, to estimate the *C. psittaci* prevalence in the populations of feral pigeons, captive and wild native birds (Motha et al., 1995). The technique used in this study was cell culture and antigen-detection ELISA (IDEIA™), with a higher specificity than the immunochromatographic test kit (Clearview®). The feral pigeons were sampled from Auckland waterfront, Wellington Zoo and Christchurch coast; captive native birds from Auckland Zoo, Wellington Zoo, Peacock Springs and National Wildlife Centre; wild native birds from Kapiti Island and Little Barrier Island (Motha et al., 1995). In this study, 13% (7/54) of the pigeons were *C. psittaci*-positive, representing all three sampling sites; while of the 62 native birds, only two kaka (*Nestor meridionalis*) from the Kapiti Island were *C. psittaci*-positive (Motha et al., 1995). Following the high prevalence of *C. psittaci* in pigeons, a chlamydiosis outbreak was identified in wild Malay spotted doves (*Streptopelia chinensis*) in Auckland in 2009. This mass mortality event killed several hundred of the wild Malay doves at the Tiraumea reserve in Auckland (Rawdon, 2010).

Later in 2011, as part of a bigger study, disease surveillance for *C. psittaci* was carried out on Little Barrier Island, using the PCR method targeting 16S rRNA. Of 10 samples collected from the wild native passerine birds, one from an adult female hihi (*Notiomyces cincta*) was *C. psittaci*-positive (Gartrell et al., 2013). In a survey among the wild mallards (*Anas platyrhynchos*), by using high-resolution DNA melt quantitative PCR (qPCR-HRM) analysis, 24% (24/100) were *C. psittaci*-positive. This result suggested that wild mallards are a natural reservoir of *C. psittaci in* New Zealand (Soon et al., 2021). Gedye et al. (2018) described the molecular classification of *C. psittaci* genotypes from samples sent to university laboratories, by analysing the sequence variation of *ompA* gene. From this study, genotype A, B and C were identified in samples from both

introduced and native bird species. In native birds, *C. psittaci* was detected in an additional five new host species (**Table 1.1**) (Gedye et al., 2018; Kulkarni, 2018). Recent collaborations with Australian researcher reported the detection of *C. psittaci* genotype B in various host species, including diamond dove (*Geopelia cuneata*), zebra finch (*Taeniopygia castanotis*), superb parrot (*Polytelis swainsonii*), and little blue penguin (*Eudyptula minor*) (Kasimov et al., 2023).

In humans, the first psittacosis in New Zealand was reported among parrot fanciers subsequent to the lifting of the ban on parrot importation from Australia. The Dunedin parrot fancier who brought in the birds showed strong positive results on the complement fixation test for *Chlamydia* (Cairney, 1954). Most reported psittacosis cases involved those who had direct contact with the birds, except for one laboratory worker whose infection was due to accidental laboratory exposure to the pathogen (McCausland et al., 1972). In 1990 to 1992, despite 45% of the *C. psittaci*-positive samples in birds, being from birds kept as pets; no information was available on their zoonotic transmission to humans owners (Hemsley, 1995). In 2010, severe human psittacosis was diagnosed in a veterinarian after occupational exposure to an asymptomatic cockatiel. During the six days of hospitalisation, the veterinarian had meningitis, pneumonia, and hepatitis. An infected veterinary nurse from the same facility had milder symptoms: backache, headache and vomiting (Rawdon, 2010). Human psittacosis is not a notifiable disease in New Zealand; but it is notifiable in the United States of America, Australia and most European countries: Belgium, Denmark, and Germany (Harkinezhad et al., 2009). Worldwide, reported human psittacosis cases range from 0.05 to 8.61 cases per million people. In our neighbouring country, Australia, it occurs at a ratio of 5.54 cases per million people (Harkinezhad et al., 2009). According to the World Organisation of Animal Health (OIE), in Australia, reported psittacosis were 19 cases in 2016, 21 cases in 2017, and 9 cases in 2018 (WOAH, 2018b). It could be under-reported in New Zealand since we share similar features, in terms of socioeconomic, environmental, and ecological factors with Australia.

Table 1.1 A summary of *C. psittaci* detection in birds and people in New Zealand.

Year	City	Species|Genotype of *C. psittaci* detected	Detection method(s)	Disease signs	Reference
1954	Dunedin	Psittacines	Inoculation Histochemical stain	NR	(Cairney, 1954)
1959	Unknown	Rosella (*Platycercus* spp.) Domestic pigeons (*Columba livia domestica*)	NR	N	(McCausland et al., 1972)
1972	Hamilton	Red-crowned parakeet (*Cyanoramphus novaezelandiae*) Red-rumped parrot (*Psephotus haematonotus*) Diamond doves (*Geopelia cuneata*) Squatter pigeon (*Geophaps scripta*) Rock dove (*Columba livia*)	Inoculation Histology	Y	(McCausland et al., 1972)
1984- 1986	Auckland Christchurch Dunedin Palmerston North Whangarei Wellington	Budgerigar (*Melopsittacus undulatus)* Cockatiel (*Nymphicus hollandicus*) Pigeon Turquoisine parakeets (*Neophema pulchella*) Budgerigar Cockatiel Rosella Rosella Turquoisine parakeets	Inoculation Cell culture Immunohistochemistry	NR	(Bell & Schroeder, 1986)
1991 1992	Unknown	Psittacines Java sparrow (*Lonchura oryzivora*) Canary (*Serinus canaria domestica*) Kereru (*Hemiphaga novaeseelandiae*)	IDEIA™ Immunohistochemistry	Y	(Hemsley, 1995)
1992	Mana Island	North Island Takahe (*Porphyrio manfelli*)	NR	NR	(Motha et al., 1995)
1992	Unknown	Kakapo (*Strigops habropfilus*) Kiwi (*Apteryx* sp.)	Clearview®	NR	(Motha et al., 1995)
1995	Auckland Wellington Christchurch Kapiti Island	Feral pigeons Kaka (*Nestor meridionalis*)	Cell culture IDEIA™	NR	(Motha et al., 1995)
2009	Auckland	Malay spotted doves (*Streptopelia chinensis*)	Ulti Med	NR	(Rawdon et al., 2009)
2010	Christchurch	Human (*Homo sapiens*)	NR	Y	(Rawdon, 2010)

Table 1.1 *Cont.*

2011	Little Barrier Island	Hihi (*Notiomyces cincta*)		qPCR-HRM PCR Sequencing	N	(Gartrell et al., 2013)
2018	Unknown	Kaka	A+C	qPCR	Mixed	(Gedye et al., 2018)
		Kereru	A	PCR		
		South Island Takahe (*Porphyrio hochstetteri*)	A	Sequencing		
		Little blue penguin (*Eudyptula minor*)	A			
		Brown teal (*Anas chlorotis*)	A, C			
		Whio (*Hymenolaimus alacorhynchos*)	C			
		Paradise shelduck (*Tadorna variegate*)	C			
		Mallard (*Anas platyrhynchos*)	C			
		Alexandrine parakeet (*Psittacula eupatria*)	A			
		Rosella	A			
		Regent parrot (*Polytelis anthopeplus*)	A			
		Red-tailed black cockatoo (*Calyptorhynchus banksii*)	A			
2018	Auckland	Budgerigar	A	qPCR-HRM	Mixed	(Kulkarni, 2018)
		Cockatiel	A	PCR		
		Masked lovebird (*Agapornis personata*)	A	Sequencing		
		King parrot (*Alisterus scapularis*)	A			
		Crested pigeon (*Ocyphaps lophotes*)	A+C			
		Little blue penguin	B			
2018	Wellington Palmerston North Invercargill	Paradise shelduck Mallard	C A+C C	qPCR-HRM PCR Sequencing	N	(Soon et al., 2021)
2023	Not reported	Diamond dove (*Geopelia cuneata*)	B	WGS	NR	(Kasimov et al., 2023)
		Zebra finch (*Taeniopygia castanotis*)	B	MLST		
		Superb parrot (*Polytelis swainsonii*)	B	Sequencing		
		Little blue penguin	B			

Note. NR, not reported; IDEIA™, antigen detection ELISA (IDEIA™, Dako Diagnostics Ltd, UK); Clearview®, rapid enzyme-immunoassay (EIA) Card Test (Clearview®, Unipath Ltd, UK); Ulti Med, antigen detection ELISA (Ulti Med Chlamydia Antigen Test, Ngaio Diagnostics, Nelson); PCR, conventional polymerase chain reaction; qPCR, quantitative PCR; qPCR-HRM, qPCR-High resolution DNA melting analysis; WGS, whole genome sequencing; MLST, multilocus sequence typing.

1.3 Chlamydia psittaci infections

1.3.1 *Chlamydia psittaci* infections in birds

Worldwide, *C. psittaci* has a broad host range and been identified in more than 460 bird species from 30 orders, including domestic and non-domestic populations (Kaleta & Taday, 2003). The host predilection of seven avian-related genotypes of *C. psittaci* are described as genotypes A (psittacine), B (pigeon), C (duck and turkey), D (turkey), E (pigeons and ducks), F (psittacine), and E/B (duck) (Knittler & Sachse, 2015; Read et al., 2013). It should be noted that the predilections of these genotypes for specific hosts are not exclusive and that there is variation in host-genotype interactions beyond these broad groupings. Natural transmission of *C. psittaci* normally occurs by intake of contaminated materials via aerosol and faecal-oral route, in which the bacteria is excreted in nasal discharges and faeces. As compared to the rare event of vertical transmission of *C. psittaci* via eggs, horizontal transmission via environmental contamination is more common in maintaining the infection (Hulin et al., 2016; Wittenbrink et al., 1993). Disease presentation of *C. psittaci* infections in birds varies, depending on host immune status and strain virulence; presenting as acute, chronic, subclinical or severe manifestations (Andersen & Franson, 2007). Risk factors that will exacerbate pathogen shedding are transportation, handling, overcrowding, nutritional deficiencies and reproductive activities (Harkinezhad et al., 2009).

Primary replication in infected birds begins in mucosal epithelial cells of upper respiratory tract, followed with intense replication in alveolus and macrophages of lower respiratory tract. Within 48 hours, septicaemia occurred, spreading the bacteria to reproductive organs and digestive tracts; resulting in poor egg production and cloacal shedding day 3 post-infection (Page, 1959; Vanrompay et al., 1995). As a systemic illness, the clinical signs of *C. psittaci* infection are non-specific, including respiratory and gastrointestinal signs. The commonly observed clinical signs include weight loss, conjunctivitis, coughing, dyspnoea, serous or mucopurulent nasal and ocular secretions, and diarrhoea with yellowish-green faeces (Andersen & Franson, 2007; Kaleta & Taday, 2003; Page, 1959). Gross pathology will normally include hepatomegaly, splenomegaly,

airsacculitis, bronchopneumonia and pericarditis (Suwa et al., 1990; Vanrompay, Ducatelle, et al., 1993).

With an extensive host range, free-living wild birds are important natural reservoirs of *C. psittaci* (Kaleta & Taday, 2003). In the context of this study, the free-living birds I will study are migratory wildlife (migratory birds). The most common species of peri-domestic wildlife that have been surveyed for *C. psittaci* include feral pigeons, followed by passerines and doves. The most common migratory wildlife bird orders that have been surveyed for *C. psittaci* survey include waterfowl (Anseriformes) and shorebirds (Charadriiformes). Most surveys involved peri-domestic wildlife due to their close proximity to humans (Magnino et al., 2009). In contrast, limited studies of migratory wildlife are available due to their lower opportunities of human contact. Geographically, most *C. psittaci* detection in free-living birds was performed in European countries, followed by Asia and South America. In Europe, large populations of feral pigeons survive very well in hectic cities, and they normally gather in swarms on streets, parks and along rivers (Magnino et al., 2009). The asymptomatic pigeons had a prevalence of *C. psittaci* infection ranging from 2.8% (3/107) to 52.6% (61/116) (**Table 1.2**). Despite the host predilection of *C. psittaci* genotypes for certain bird species, genotypes A, B, C, D, E and E/B were all detected in feral pigeons (Magnino et al., 2009). Hence, molecular investigation allowed a better understanding of the diversity of *C. psittaci* genotypes present in these birds.

Worldwide, migratory birds are one of the principal host of *C. psittaci* (Hubalek, 2004). The common migratory species that are *C. psittaci* reservoirs include waterfowl (Anseriformes) and shorebirds (Charadriiformes) (Reed et al., 2003). The reported prevalence of *C. psittaci* in migratory birds was 10% (6/59) in Slovakia and 1.2% (6/497) in Sweden (Blomqvist, Christerson, Waldenström, Herrmann, et al., 2012; Schwarzova et al., 2006). The variability might be due to intermittent shedding pattern of *C. psittaci* (Takahashi et al., 1988). During migration, the stress in birds may cause immunosuppression and thus increase disease susceptibility. Sometimes the reactivation of latent infections lead to shedding of pathogen, allowing the formation of new disease foci in a novel geographical location (Reed et al., 2003). At the stopover

sites, migratory birds interacted with local populations of free-living birds while feeding and resting. During landing and taking flight, the *C. psittaci*-infected birds generate contaminated aerosols, introducing the pathogen to the local birds sharing the same habitat (Hubalek, 2004). Similar transmission between migratory and local birds was observed for highly pathogenic avian influenza and West Nile fever (Contreras et al., 2016). However, there is limited literature that discusses the details of *C. psittaci* infection at wildlife-livestock interface. Besides sharing habitat, *C. psittaci* can be spread from wild birds to susceptible poultry and pet birds through exposed and contaminated feed equipment (Harkinezhad et al., 2009). Anthropogenic environmental variation allows interactions between domestic, peri-domestic and wild animals, while generally decreasing natural pathogen regulation capacity, and increasing host switching and genetic exchange of pathogens (Wilcox & Colwell, 2005).

Ornamental birds include companion animals and captive birds of exotic origin. Among companion animals, *C. psittaci* most frequently presents in Psittaciformes (cockatiel, budgerigar) and Columbiformes (pigeon, dove), as they are the common pet species. Similarly, studies on *C. psittaci* detection in ornamental birds were readily available in the European countries with an increased interest in Asia, especially China, in the past five years. The reported *C. psittaci* prevalence of racing pigeons, using PCR, ranged from 3% (6/232) to 41% (13/32), predominated with *C. psittaci* genotype B. While in the psittacine birds, the *C. psittaci* prevalence ranged from 2.4% (3/125) to 69% (22/32), predominated with *C. psittaci* genotype A (**Table 1.2**). The samples were obtained from pet shops, private aviaries, pigeon lofts, avian refuge centre, veterinary hospital, bird parks and zoos (Chahota et al., 2006; Kalmar et al., 2014; McElnea & Cross, 1999). When the facilities kept multiple species of birds, an increased diversity of detected *C. psittaci* genotypes was noticeable (Chahota et al., 2006; Kalmar et al., 2014).

Among poultry, *C. psittaci* infection has occurred most frequently in turkeys and ducks. Historically, outbreaks of *C. psittaci* in European and US turkey flocks have caused economic losses but these are now rare. In contrast to earlier cases, the current outbreaks are characterised by a lower mortality rate (Harkinezhad et al., 2009). In turkeys, the mortality rate for untreated illnesses ranges from 5-40%; while in endemic

regions, *C. psittaci* infections present as a mild respiratory disease with low or no mortality (Van Loock, Geens, et al., 2005). In China, the morbidity rate in diseased farms ranged from 20% to 50%, with the presence of clinical signs of economic importance: weight loss, reduced egg production and production of malformed eggs (Yin et al., 2015). Among *C. psittaci* in birds with clinical signs, by using PCR (*ompA*), *C. psittaci* presents in 9/10 (90%) of breeder hens with cystic oviducts; 7/10 (70%) of broilers with pneumonia; 18/20 (90%) of ducks with lower than 50% egg production (Yang et al., 2007). Experimental study is required to confirm if *C. psittaci* is the causative agent of cystic oviducts in the breeder hens although *C. psittaci* was detected in the oviduct membranes in the study by Yang et al. (2007) and similar observation was reported by Page (1959) when the pathogen was spread to avian reproductive organs via septicaemia. More recently, there is an increasing detection of the *C. psittaci* in European and Asian chicken flocks (Lagae et al., 2014; Yin et al., 2015). In 2005, the severe chlamydiosis outbreak in a German poultry farm, affecting more than 1000 young chickens, was initiated by a farmer that illegally bred and traded the infected birds (Gaede et al., 2008). In Belgium, 18/19 (94.7%) of the chicken farms were *C. psittaci*-positive, with genotypes A and D (Lagae et al., 2014). In experimentally infected chickens, *C. psittaci* genotype D is more virulent than genotype B; causing mortality, more severe lesions and clinical signs (Yin et al., 2013).

Historically, mortalities of birds in avian chlamydiosis outbreaks in commercial duck farms has reached 20% in England (Chalmers et al., 1983) and 13% in Australia (Arzey et al., 1990). Later, most of the surveys of *C. psittaci* in farmed ducks were initiated to identify its correlation to human psittacosis outbreak among duck farmers, and to fill in the knowledge gap in some countries, where background data of *C. psittaci* prevalence is not available. *C. psittaci* in ducks has been studied extensively in France, due to the mule duck production for foie gras (Vorimore et al., 2015). To explore the *C. psittaci* circulation from duck hatchery to slaughterhouse in the mule duck production, a study was conducted in seven duck flocks. It confirmed that *C. psittaci* is highly prevalent in French mule duck flocks, with the absence of clinical signs. Besides the long-established vertical transmission of *C. psittaci* in duck farms, the main spreading pathway is environmental contamination; correlating with the dynamic *C. psittaci*

shedding patterns in the ducks (Hulin et al., 2016; Vorimore et al., 2015). Also, in a survey conducted in the duck slaughterhouse; 26/75 (34.7%) of the samples from fattening and broiler ducks were *C. psittaci*-positive (Hulin et al., 2015). At present, there is no data on *C. psittaci* in duck farming industry in New Zealand, regarding its occurrence and genotypes.

1.3.2 *Chlamydia psittaci* infections in humans

Avian strains of *C. psittaci* can infect human, leading to psittacosis, also known as ornithosis, or parrot fever. Human psittacosis of bird origin occurs via mouth-to-beak interaction, handling of infected birds and their tissues, and inhalation of infectious aerosols from dried respiratory tract or ocular secretions, dried faeces or feather dust, from carrier or infected birds (West, 2011). Most of the exposure of *C. psittaci* involved direct contact with companion or cage birds, and the common bird taxa associated with this include psittacine birds and pigeons. Job-related exposure to *C. psittaci* can affect veterinarians, pathologists, taxidermists, zookeepers, avian researchers, wild bird rehabilitators, pet shop workers, pigeon breeders and poultry workers (Balsamo et al., 2017). Among the diagnostic laboratory personnel, out of 3921 reported cases of laboratory-acquired infections around the world, 116 (3%) were psittacosis (Sewell, 1995). Psittacosis can also occur via indirect contact with birds, for example through a transient exposure to their body tissues or excretions (Harkinezhad et al., 2009). Environmental exposure in human cases of psittacosis is normally linked to the excretions of *C. psittaci*-infected wild birds.

With an incubation period of 5 to 14 days, the clinical presentation of psittacosis in humans varies from inapparent and mild, to sometimes fatal systemic disease with severe pneumonia (Balsamo et al., 2017). The common symptoms include sudden onset of headache, malaise, fever, chills, myalgia, non-productive coughing, and dyspnoea. Other complications include keratoconjunctivitis, splenomegaly, hepatitis, arthritis, encephalitis, pericarditis, endocarditis, and myocarditis (Balsamo et al., 2017; Knittler & Sachse, 2015; West, 2011). Adverse outcomes associated with psittacosis during pregnancy have been reported; including fever, sepsis, respiratory distress and

miscarriage or premature delivery (Khatib et al., 1995; Paul et al., 2018). Around 1929 to 1942, prior to the discovery of appropriate antimicrobial agents, 15-20% of human died due to psittacosis (Dunnahoo & Hampton, 1945). More recently, mortality of less than 1% was observed among properly treated patients (Smith et al., 2005). In the first decade of the 2000s, three fatal cases in human psittacosis were reported in Europe: two cases in Hungary and one in Slovakia. The two patients from Hungary were employees in the plucking unit of a poultry processing plant. One died at 9 days and the next at 21 days after the disease onset, while the patient from Slovakia was a pet shop worker, who died 22 days after disease emergence. All of them had severe pneumonia and then respiratory failure (Kovacova et al., 2007; Petrovay & Balla, 2008).

C. psittaci-infected free-living birds can be a source for human psittacosis via direct interactions or by environmental exposure of people to contaminated excretion. In the cities, large urban populations of pigeons, starlings, corvids, and gulls interact with humans on a daily basis. Urban storks and gulls have adapted to forage in garbage dumps, and frequently visit between garbage sites and wetlands like lakes and river estuaries, where the migratory birds gathered. In gardens and parks, these birds interact with susceptible humans like children and elderly people (Contreras et al., 2016). In Europe, among the 101 cases of zoonotic transmission of *C. psittaci* from feral pigeons to humans, half of them were enthusiastic pigeon feeders and the other half were exposed to pigeons' excrements on the buildings and monuments (Haag-Wackernagel & Moch, 2004; Magnino et al., 2009). Similarly in Malaysia, *C. psittaci* was detected in wild spotted doves as the peri-domestic wildlife most commonly seen in open public areas (Phong & AI-Ajeeli, 2006). An increased risk of exposure to *C. psittaci* from waterfowl is also possible via leisure activities such as swimming, fishing and picnics at contaminated water surfaces (Gorham & Lee, 2016). Also, gamebird hunting may expose hunters to zoonotic risks of *C. psittaci* via carcass processing and meat consumption (Horigan et al., 2014). In New Zealand, wild gamebirds (48% of 117, 204 hunters) are the second most popular prey targeted by human hunters (Nugent, 1992).

Even without direct contact to wild birds, grass trimming and lawn mowing may be the cause of psittacosis outbreak (Telfer et al., 2005; Williams et al., 1998). In the

rural areas of Australia, the community outbreak of psittacosis were linked to the mowing practices that disturbed the infectious particles excreted by sick birds on the ground, exposing people to the bacteria through the aerosolisation of droppings (Williams et al., 1998). This outbreak had occurred in a small town surrounded by forest, inhabited with high numbers of native parrots. All the 16 *C. psittaci*-infected humans had fever and 11 of them showed signs of atypical pneumonia. One person with a severe case died in this outbreak, and none of the affected people reported direct contact with birds (Williams et al., 1998). In 2002, a psittacosis outbreak in the Blue Mountains district of Australia resulted in 59 seropositive cases and 2 severe cases requiring intensive care that included intubation and mechanical ventilation. The case-control study performed following the outbreak revealed the risk factors to be direct contact with free-living birds (OR 7.4, 95% CI 2.5–22.0), and lawn mowing without a grass catcher (OR 3.2, 95% CI 1.3–8.0) (Telfer et al., 2005). During the outbreak, the patients reported to observe a higher number of dead wild birds in their yards, and some of them had handled and occasionally mowed over the dead bodies. Wild bird could be a potential source of infection when investigation on the common bird species in the area revealed that *C. psittaci* was detected in the tissue of a king parrot (*Alisterus scapularis*). Also, avian chlamydiosis had been diagnosed among juvenile crimson rosellas the year before the human psittacosis outbreak (Telfer et al., 2005).

Ornamental birds such as pet birds and captive free-living birds can be a source of *C. psittaci* infections in humans. Since 1980s, the imports of exotic birds into North America and Europe have increased due to higher demand. In many cases, these were illegal import, which augmented the risk to introduce zoonoses into general homes (Contreras et al., 2016). Recreational exposure of *C. psittaci* involved direct and transient contact with birds in a home setting or zoos and bird parks. At home, humans have a closer proximity to pet birds and the at-risk groups of *C. psittaci* infection are children with lax hygiene habits and sick people with poor immune system (Contreras et al., 2016). In busy places like zoos, bird parks, bird shows and pet stores, zoonotic transmission may occur when the captured wild birds excreted more pathogens due to stress and immunosuppression (Contreras et al., 2016). In the Netherlands, after a pet shop visit, a middle-aged couple and their daughter developed psittacosis with

pneumonia (De Boeck et al., 2016). Besides recreational exposure, occupational exposure of *C. psittaci* of ornamental birds origin can affect pet shop workers, veterinarians, wild bird rehabilitators, and pigeon breeders (Balsamo et al., 2017). Two similar psittacosis outbreaks occurred during bird fairs in France and the Netherlands. In France, 38% (33/86) of the exhibitors were *C. psittaci*-positive (Belchior et al., 2011; Berk et al., 2008). In a Belgian wild bird refuge centre, three staff were infected with *C. psittaci* as determined by *ompA*-based RT-PCR; two of them had 'flu-like' symptoms for two weeks (Kalmar et al., 2014). An epidemiological study of *C. psittaci* in Belgium recruited humans from 39 parrot-breeding facilities, to collect self-taken pharyngeal swabs, 13% (6/46) of them tested positive for *C. psittaci* in nested PCR (Vanrompay et al., 2007).

In 2005, the zoonotic risk of psittacosis in the poultry industry was demonstrated in an outbreak in Germany, involving 24 persons; of the seven hospitalised patients, three required intensive care and one severely ill patient died. *C. psittaci* genotype A was identified in three severely ill patients and the poultry flocks they were in contact with (Gaede et al., 2008). In a survey among Belgian chicken famers, 94% (29/31) of them were *C. psittaci*-positive, based on culture and PCR, specifically of genotypes A, C and D (Lagae et al., 2014). Zoonotic transmission of *C. psittaci* was also present in workers in poultry hatcheries and slaughterhouses (Dickx, Geens, et al., 2010; Dickx & Vanrompay, 2011). Among the waterfowl, zoonotic transmission of psittacosis from farmed ducks to humans has been reported (Hinton et al., 1993; Laroucau et al., 2009; Newman et al., 1992; Vorimore et al., 2015). Workers at the slaughtering areas of duck abattoirs have a high risk of getting psittacosis due to the exposure and contact with blood or viscera during the process of killing, de-feathering and automated evisceration (Tiong et al., 2007; Williams et al., 2013).

1.4 Diagnosis of *Chlamydia psittaci*

1.4.1 Diagnosis of *C. psittaci* in birds

Diagnosis of avian chlamydiosis and human psittacosis is established by detecting the antigen or antibody, with the presence of related clinical signs or pathological findings (Andersen, 2004). Historically, antigen detection of *C. psittaci* was done via isolation in embryonated egg or tissue culture. Histochemical staining or immunohistochemistry of cytology and histology slides were also a common practice. Currently, advances in the molecular testing such as enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) give rapid results and do not require viable organisms (Andersen, 2004). Serologic testing is commonly available and applied in both humans and birds; but the results are less reliable due to short term antibody response and the need for paired samples for reliable interpretation. For the collection and handling of samples, the techniques differ based on the requirement of individual diagnostic tests (Balsamo et al., 2017).

Antibody detection tests for *C. psittaci* infection in birds can evaluate the seroprevalence of infection and are thus more suitable for epidemiological studies than making diagnosis in individual birds (Sachse, Vretou, et al., 2009). In fact, a single positive serologic test result is just an evidence of previous infection, hence may not indicate an active infection; paired serum samples with a four-fold rise of antibody titre are required to confirm a diagnosis of active infection (Andersen, 2004). Serological assays can detect the antibodies bounded to the surface antigens: elementary bodies (EBs), lipopolysaccharides (LPS), polymorphic outer membrane proteins (POMP) and major outer membrane protein (MOMP) (Sachse, Vretou, et al., 2009). The bounded antibody are observable by using tagged secondary antibodies, fluorescent, and complement fixation. The techniques are known as ELISA, microimmunofluorescence test (MIF), and complement fixation test (Sachse, Vretou, et al., 2009). The results of serological tests can be highly variable, depending on the stages of infection and concentrations of circulating immunoglobulin in birds. False negative results can happen when samples are collected before seroconversion in acute infection, and after antibiotic treatment that reduces the antibody response (Fudge, 1997).

Antigen detection of *C. psittaci* in birds include bacterial culture, immunoassays, and gene-based tests. In live birds, samples came from a combined conjunctival, choanal and cloacal swabs, as *C. psittaci* shed through these anatomical sites. Swabbing is appropriate to recover most of the infected mucosal cells with elementary bodies (Corsaro & Greub, 2006). Other samples such as pharyngeal or nasal swabs, ocular and nasal exudates, and intestinal excrement, are useful for diagnosis too. Although the most reliable sample is pharyngeal swab, sampling from several sites are recommended to improve recovery of bacteria (Andersen, 1996). In dead birds, tissues with lesions are usually sampled, including liver, spleen, air sacs, lung, pericardium and kidney; as these organs harbour the bacteria (Andersen, 2004). In clinically ill birds, *C. psittaci* can be easily isolated from the samples while in asymptomatic birds; multiple samples are required to account for the intermittent shedding behaviour of *C. psittaci* (Takahashi et al., 1988).

The commonly used diagnostic tests in 1990s were serology and immunoassays. Some examples of immunoassays to detect chlamydial antigens included IDEIA™ (Dako Diagnostics, UK), Clearview® (Abbott Unipath, UK), Chlamydiazyme (Abott Laboratories, USA), and SureCell (Eastman Kodak, USA) (Gerlach, 1994). The number of elementary bodies needed for positive detection from these tests ranged from 70 to 4800 particles (0.3125 ng) per gram of samples. Cross reactions with *Staphylococcus aureus* and *Pasteurella multocida* were observed with these tests (Gerlach, 1994). Given the limitations of immunoassays, molecular techniques were later developed for better specificity and sensitivity. In 1990s, in the US, molecular detection of *Chlamydia* was only available in one commercial laboratory – Avian Research Associates, Milford, OH, USA (Fudge, 1997). According to Fudge (1997), there was no "one best test" to detect *C. psittaci* in birds. When choosing a test method, the components to consider were health status of the host, sample types, and the benefits and challenges of the test (Fudge, 1997).

As an obligate intracellular bacterium, viable *C. psittaci* propagates in tissue culture, and immunostaining methods can confirm the chlamydial genus; PCR can identify the chlamydial species. The major limitation of tissue culture is that it requires

live, viable *C. psittaci*. It is a costly, time-consuming, and labour-intensive technique, requiring specialised culture facilities, specialist expertise and adequate protection for microbiologists due to its zoonotic risk concern (Pike, 1976; Sachse, Vretou, et al., 2009; Van Droogenbroeck et al., 2009). In epidemiological investigation, bacterial isolation is essential to recover as little as one viable organism for further molecular testing and strains characterisation (Sachse *et al.*, 2009). Lately, PCR is widely applied to detect *C. psittaci* directly from avian samples (**Table 1.2**).

Table 1.2 Antigen detection of *C. psittaci* in birds in Asia and Australasia.

Country	Species	Laboratory test	Numbers positive/numbers tested (% positive)	Geno type	Reference
Free-living birds					
India, Himachal Pradesh	Pigeons, parrots, crow	EI, IMIFT, dot-ELISA	16/85 (19%)	-	(Chahota et al., 1997)
Japan	Feral pigeons	PCR (*omp*A)	103/463 (22%)	-	(Tanaka et al., 2005)
Japan	Bird sanctuaries	PCR (*omp*A)	5/260 (2%)	A, D	(Chahota et al., 2006)
Korea	43 species	PCR (*omp*A)	4/225 (2%)	E, 1V, 6N	(Jeong et al., 2017)
Malaysia	Spotted doves (*Streptopelia chinensis*)	Clearview®	14/56 (25%)	-	(Phong & Al-Ajeeli, 2006)
Thailand	Feral pigeons	PCR (*omp*A)	44/407 (11%)	B	(Sariya et al., 2015)
Ornamental birds					
Australia	Racing pigeon lofts Pet shops Private aviaries	PCR (*omp*B) Cell culture	66/266 (25%) 30/266 (11%)	-	(McElnea & Cross, 1999)
China, Beijing	Racing pigeon (*Columbia livia*)	IF	55/206 (27%)	B	(Ling et al., 2015)
China	Pet parrots	Semi-nested PCR (*omp*A)	2/21 (10%)	A	(Zhang et al., 2015)
China, Kunming	Psittacines from zoos and pet markets	qPCR (*omp*A)	27/136 (20%)	A	(Feng et al., 2016)
Japan	Pet shops, Bird parks & zoos Vet hospital	PCR (*omp*A)	31/569 (5%) 18/172 (10%) 11/146 (8%)	A, B, D, E, E/B	(Chahota et al., 2006)
Japan	Zoo birds	PCR (*omp*A)	48/668 (7%)	-	(Kabeya et al., 2015)
Thailand	Captive psittacines	PCR (*omp*A)	14/178 (8%)	A	(Suksai et al., 2016)
Farmed poultry					
China	Peafowl (*Pavo cristatus*)	IF	12/20 (20%)	B	(Yang et al., 2011)
China	Duck farms	PCR (*omp*A)	18/20 (90%)	-	(Yang et al., 2007)

Note. -, not reported; EI, egg inoculation; IMIFT, indirect micro-immunofluorescence test; dot-ELISA, dot enzyme-linked immunosorbent assay; IF, immunofluorescence; Clearview®, rapid enzyme-immunoassay (EIA) Card Test (Clearview®, Abbott Unipath, UK); PCR, conventional polymerase chain reaction; qPCR, quantitative PCR; *ompA,* major outer membrane protein A; *ompB,* major outer membrane protein B.

1.4.1.1 PCR

Since 1997, the improved specificity of proposed PCR protocols enhances the ability to identify *C. psittaci* from the genus, species, up to genotype level (Sachse, Vretou, et al., 2009). The DNA amplification of each protocol targets various genes that

include: 16s ribosomal RNA (Messmer et al., 1997), 16s-23s spacer region (Madico et al., 2000), *pmp* genes (Laroucau et al., 2007), and *ompA* gene (Kaltenboeck et al., 1997). The *ompA* gene encodes major outer membrane protein (MOMP), which is an immunodominant protein that displays specific epitopes of genus, species and serovar. In a study comparing PCR of *pmp* genes, the 16s-23s spacer region, and *ompA* gene-based; *pmp* genes-based PCR has a higher sensitivity (Laroucau et al., 2007). Messmer et al. (1997) proposed a two-step protocol; the first step is genus-specific, and the second step is a multiplex to distinguish between *C. psittaci*, *C. pneumoniae* and *C. trachomatis*. A similar two-step nested-PCR targeting *ompA* gene was reported to be robust for routine used (Kaltenboeck et al., 1991). MOMP is defined by four variable domains (VD) separated by five conserved regions (Vanrompay et al., 1998). The conserved regions encoded both the genus- and species-specific epitopes; while the VD2 and VD4 encoded the serovar-specific epitopes (Kaltenboeck et al., 1991; Kaltenboeck et al., 1997; Sachse, Vretou, et al., 2009). Hence, the *ompA* gene is widely used in most PCR-based detection and characterisation of *C. psittaci* (Kaltenboeck et al., 1991).

Genotyping of *C. psittaci* can be done via *ompA* gene sequencing (Bush & Everett, 2001), MOMP serotyping (Morré et al., 1998) and *ompA* restriction fragment length polymorphism (RFLP) analysis (Vanrompay et al., 1997). The *ompA* sequencing uses Sanger sequencing (Bush & Everett, 2001); while MOMP serotyping uses serovar-specific monoclonal antibodies (Morré et al., 1998). The *ompA* RFLP analysis uses Alu1 restriction enzyme for *ompA*-PCR amplicon digestion to detect the DNA sequences of different lengths (Vanrompay et al., 1997). Comparison of the three methods reveals that both RFLP and serotyping failed to detect genotype E/B. However, RFLP is cheaper and faster, and more specific and sensitive, than MOMP serotyping which requires trained interpretation for the immunofluorescence-stained smears (Geens, Desplanques, et al., 2005). Even so, RFLP may not be sensitive enough to detect *C. psittaci* from clinical samples with low number of pathogen (Sachse, Vretou, et al., 2009). Although being more expensive and time-consuming, *ompA* sequencing is the only typing method able to identify all *C. psittaci* genotypes (Geens, Desplanques, et al., 2005). Hence, *ompA* sequencing can be opted for the reference typing method but not for routine diagnosis (Geens, Desplanques, et al., 2005; Sachse, Vretou, et al., 2009). By

incorporating 35 hybridisation probes derived from VD2 and VD4 of the *ompA* gene, the development of DNA microarray technology with high specificity can detect single-nucleotide polymorphism that accurately discriminated the known genotypes, and additionally discovered six new provisional (1V, 6N, Mat116, R54, YP84, CPX0308) *C. psittaci* strains (Sachse et al., 2008).

Bacterial quantification is possible with the development of a *C. psittaci* genotype-specific real-time PCR (qPCR) (Geens, Dewitte, et al., 2005). The target gene used are 23S rRNA (Yang et al., 2006), *incA* gene (Ménard et al., 2006) and *ompA* gene (Geens, Dewitte, et al., 2005; Pantchev et al., 2009). By using a single pair of primers, the 23S rRNA-based qPCR can simultaneously detect and quantify four agents namely *C. psittaci*, *C. pecorum*, *C. pneumoniae* and *C. trachomatis* with the limit of detection of 250fg DNA (10^{-1} IFU) (Yang et al., 2006). The *incA* gene-based qPCR used Taqman probe to distinguish and quantify five strains (GR9, VS1, 225, NJ1 and CP3) of *C. psittaci* (Ménard et al., 2006). Similarly, the Taqman approach applied in *ompA* gene-based qPCR improved its specificity by accurately discriminating the genetically closely related *C. psittaci* and *C. abortus*, and seven genotypes (A, B, C, D, E, F, and E/B) of *C. psittaci* (Pantchev et al., 2009) (Geens, Dewitte, et al., 2005). Taqman identifies genotype E/B; while competitor oligonucleotides discriminate the closely related genotype A, B and E (Geens, Dewitte, et al., 2005). The main limitations of PCR-based assays are the risk of false positive results due to vertical contamination and false negative results due to PCR inhibitors or low sensitivity of the protocol (Corsaro & Greub, 2006). In qPCR, the absence of post-PCR sample handling prevents potential PCR product contamination; resulting in more rapid assays besides providing additional quantification results (Sachse, Laroucau, et al., 2009).

Genotyping and quantification of *C. psittaci* is feasible on a single platform by using qPCR-HRM analysis. High-resolution DNA melting (PCR-HRM) analysis is a fast, simple, and cost-effective post-PCR scanning method. The two major components in a PCR-HRM analysis are high-resolution DNA melting instrument and saturation dye. By applying PCR-HRM in qPCR, this technique can detect, characterise, and quantify *C. psittaci* simultaneously. Prior to PCR amplification, the adding of saturation dye in a

closed tube, can eliminate extra steps for sample processing, as compared to the traditional two-steps protocol; thus, minimising error and contamination (Reed et al., 2007). Mitchell et al. (2009) proposed the application of qPCR-HRM analysis as a simple, cheap, and rapid diagnostic tool of *C. psittaci* in birds and companion mammals. This assay revealed negative cross-reactivity of *C. psittaci* with up to 46 types of bacteria and virus, that include *C. pneumonia, C. trachomatis, C. felis, C. pecorum, E. coli*, and Influenza virus A (Mitchell et al., 2009). In epidemiological investigations, this technique improves the timely reporting of results, identifies the sources of a genotype, and enables analysis of retrospective specimens. During outbreaks, pathogenicity and transmission studies are possible to understand the movement of pathogen across multiple animal facilities (Mitchell et al., 2009). qPCR-HRM analysis was also being applied in a prevalence study of *C. psittaci* in domestic birds in China (Guo et al., 2016).

1.4.1.2 MLST and beyond

Although *omp*A genotyping with a qPCR-HRM protocol is quick and easy for *C. psittaci* epidemiological investigations, it provides limited information on the genetic diversity of *C. psittaci* and subsequently inadequate understanding on the spread of the disease. Currently in New Zealand, qPCR-HRM protocol using EvaGreen® revealed that genotype A and B shared a similar range of dissociative temperature on the melt curve (Kulkarni, 2018). Similarly, Mitchell et al. (2009) using qPCR-HRM, with Lux chemistry, reported to have genotypes D and F sharing the same melt curve pattern. To overcome the limitations of examining a single gene, the more discriminating multi-locus sequence typing (MLST) is required; examining the sequences of multiple loci in a bacterial genome to sufficiently identify genetic variants (Maiden, 2006). The protocol by Pannekoek et al. (2008) is currently mostly applied for MLST of *C. psittaci*; analysing the seven housekeeping genes: *enoA, fumC, gatA, gidA, hemN, hlfX and oppA*. The loci adequately distances from individual on the chromosome, each loci exhibits a comparable level of variation in nucleotide sequence; not located near to genes encoding putative outer membrane and secreted proteins. This protocol has revealed an association between host species and individual *C. psittaci* sequence types (Pannekoek et al., 2010).

The conventional method of using MLST to analyse *C. psittaci* requires DNA extracted from single isolate cultures. However, *C. psittaci* is an obligate intracellular bacteria, in which it is laborious to yield adequate genomic material for sequencing through tissue culture (Taylor-Brown et al., 2018). A culture-independent approach is achievable, using multiple displacement amplification (MDA). It allows for intense increases of genomic DNA without prior knowledge of the sequence; providing enough template DNA to perform MLST and leading to the discovery of novel species (Taylor-Brown et al., 2018). In epidemiology, transmission pathways of infectious diseases hypothesise based on the combination of contact patterns, relevant risk factors and genetic characterisation of pathogens (Kao et al., 2014). Whole-genome sequencing (WGS) or next-generation sequencing (NGS) is a high-throughput sequencing technology that allows extraction of genetic materials from small pathogen samples, while capturing the whole genomes of entire populations of pathogens (Kao et al., 2014). For multi-host pathogens, a combination of epidemiological data and phylogenetic information to detect single nucleotide polymorphism is valuable to differentiate between pathogen spillover and emergence (Kao et al., 2014; Land et al., 2015).

1.4.2 Diagnosis of *C. psittaci* in humans

C. psittaci detection in human can be performed by using methods like those in birds. In most routine diagnostic schemes in New Zealan, *C. psittaci* testing is not offered. When specific testing is carried out, the commonly performed laboratory procedures include serology, PCR, and culture. In humans, the samples required for diagnosis are blood and respiratory specimens such as pharyngeal swab, sputum, and pleural fluid (Balsamo et al., 2017). In psittacosis outbreak investigations, reviewed by Nieuwenhuizen et al. (2018), serology testing was performed in all the cases reviewed. However, to exclude cross-reactivity with other chlamydial species (*C. pneumoniae*, *C. trachomatis*), combinations of multiple tests were recommended to confirm a diagnosis. More recently, the use of molecular tests like PCR has increased with higher reliability to specifically identify *C. psittaci*. However, PCR is only highly sensitive for samples collected during acute infection. Culture is also possible, but not widely available in

diagnostic laboratories, because of the technical difficulty and occupational hazard concerns related to handling human samples that potentially have a biosafety level 3 pathogen (Harkinezhad et al., 2009).

The available serology tests to diagnose psittacosis are immunostaining methods (MIF, IP, IF), complement fixation test (CFT) and ELISA. Historically, CFT and micro-immunofluorescence test (MIF) had been commonly used to test paired sera (21-days apart) of patients with clinical symptoms. Although MIF is more sensitive and specific than CFT, but the MIF test still exhibits cross-reactivity with other chlamydial species such as *C. trachomatis* (Harkinezhad et al., 2009). Therefore, a titre less than 1:128 should be interpreted carefully; true acute and convalescent (2–4 weeks later) sample tests are required for precise interpretation. Antibiotics treatments can diminish or delay the antibody response; a third serum sample should be collected 4–6 weeks after the acute specimens (Balsamo et al., 2017). To ensure results consistency, all serologic testing should be performed within the same laboratory. Although serology tests are more commonly available than molecular tests, results can often be vague, resulting in subjective interpretation. Hence, serology should be used as a supportive test that supplements the results of other more reliable tests (nucleic acid–based tests) (Bourke et al., 1989; Nieuwenhuizen et al., 2018).

In 2004, PCR was first used in a *C. psittaci* outbreak in addition to serology tests to identify *C. psittaci* in humans (Heddema, van Hannen, et al., 2006). Since then, molecular tests consisting nucleic acid amplification, has increased its availability and reliability. The PCR tests were mainly implemented on hospitalised patient samples. The protocols differed in DNA targets, amplification techniques, species and genus specificity (Nieuwenhuizen et al., 2018). The samples collected included sputum, bronchoalveolar lavage, blood, urine, throat, nasal, and pharyngeal swabs. Nasal swabs were the preferred samples due to the ease of collection and recovery rate of bacteria (Nieuwenhuizen et al., 2018; Verminnen et al., 2008). In a psittacosis outbreak in a veterinary teaching hospital, human diagnosis was carried out with a blend of testing strategies, including real-time PCR (targeting *ompA* gene), serology (ELISA and CFT on paired samples) and sequencing of *ompA* gene; revealing 34% (10/29) positive cases

(Heddema, van Hannen, et al., 2006). In another outbreak related to a bird fair, real-time PCR (targeting *incA* gene) and serology (MIF) were used to confirm the diagnosis (Belchior et al., 2011). In epidemiological investigations, it is more efficient to do early confirmation of *C. psittaci* outbreak based on PCR test of epidemiological-linked individuals and genotyping of PCR-positive samples for source tracing (Nieuwenhuizen et al., 2018) (**Table 1.3**).

Table 1.3: Human cases of psittacosis of various bird origins in Asia and Australasia.

Country	Suspected sources	Laboratory test	Human cases (positive/total number)		Reference
Free-living birds					
Australia	Native psittacine	Indirect IF	16 cases, 1 died		(Williams et al., 1998)
Australia	Crimson rosellas (*Platycercus elegans*) King parrots	MIF	59/95 (62%)		(Telfer et al., 2005)
Australia	Crimson Rosella	MIF qPCR (*omp*A)	3 cases <10 copies		(Branley et al., 2008)
Ornamental bird					
China	Racing pigeon (*Columbia livia*)	ELISA IF	19/79 (24%) 16/67 (24%) Genotype B		(Ling et al., 2015)
Hong Kong	Mealy Parrots (*Amazona farinose*)	qPCR MIF	5 cases		(To et al., 2014)
Israel	Pet parrots	Cell culture Indirect IP	4 cases		(Samra et al., 1991)
Japan	Cockatiel	MIF	4 cases		(Kaibu et al., 2006)
Netherlands	Various bird species	PCR (*omp*A)	10 cases Genotype A, B, C		(Heddema, van Hannen, et al., 2006)
Turkey	Pet parrots	MIF	2 cases		(Ciftci et al., 2008)
Vietnam	Passerines, psittacines at home, shops, eating establishments	qPCR	5 cases (1 died, 3 ICU) Genotype A, E/B		(Ngan et al., 2013)
Farmed poultry					
China	Peacock	Direct IF IF	1/4 4/4		(Yang et al., 2011)
Australia	Duck farm	CFT	Recent infection Past infection	3/25 (12%) 16/25 (64%)	(Hinton et al., 1993)
Australia	Rural duck abattoir	IF	Abattoir Maintenance Farm office	37/57 (65%) 7/15 (47%) 8/19 (42%) 1/6 (17%)	(Tiong et al., 2007)

Note. IF, immunofluorescence; MIF, micro-immunofluorescence; indirect IP, indirect immunoperoxidase assay; ELISA, enzyme-linked immunosorbent assay; PCR, conventional polymerase chain reaction; qPCR, quantitative PCR; ompA, major outer membrane protein A; CFT, complement fixation test.

1.5 Aims and structure of the thesis

What is the molecular epidemiology of chlamydiae at the shorebird-human interface in New Zealand? Three research aims were established to answer this question.

The first research objective was to systematically review the globally available molecular tests, to identify the most commonly published molecular detection of *C. psittaci* in birds for different study purposes (Chapter 2). When I was preparing for Chapter 1, I observed the application of a wide range of various molecular tests in different studies. This review was anticipated to assist in making informed decision when choosing a suitable molecular test for a chlamydial study.

The second research objective was to perform targeted surveys of migratory and resident shorebirds, and people handling the shorebirds to determine the prevalence of *C. psittaci* (Chapter 3). The third objective was to identify molecular strain variation in *C. psittaci* using MLST and DNA sequencing to examine the diversity of pathogens and hosts in New Zealand (Chapter 4). The knowledge on the presence of chlamydiae in shorebirds and people handling them may provide essential information to conservation management strategies for shorebirds and public health plans for targeted people.

This thesis consisted of five chapters, including an introduction, three research chapters, and a general discussion. The three research chapters was presented as individual manuscripts intended for publication in peer-reviewed journals.

CHAPTER 2

Molecular detection of *Chlamydia psittaci* in birds: a systematic review

CHAPTER 2 Molecular detection of *Chlamydia psittaci* in birds: a systematic review

2.1 Introduction

Chlamydia psittaci is a Gram-negative, obligate intracellular bacterium with a global distribution that can infect a broad range of domestic and non-domestic avian hosts (Kaleta & Taday, 2003). In birds, chlamydiosis causes respiratory, ocular, and enteric disease. This leads to economic losses in the poultry industry and conservation implications to endangered native wildlife (Burnard & Polkinghorne, 2016; Gaede et al., 2008). At the time of writing, the ten characterised *Chlamydia* species detected in birds are *C. psittaci*, *C. avium*, *C. gallinacea*, *C. buteonis*, *C. abortus, C. muridarum, C. pecorum, C. trachomatis,* and *C. suis* (Zaręba-Marchewka et al., 2020). In this review, we focused on the detection of *C. psittaci* in birds because it is one of the foremost well-documented *Chlamydia* species in birds (Sachse, Laroucau, et al., 2015), as compared to other newly discovered avian chlamydial species.

The detection of *C. psittaci* in birds comprises of two main approaches: antigen detection and antibody detection. Antigen detection of *C. psittaci* directly detects the bacteria from samples using bacterial isolation, cytochemical staining, histochemical and immunohistochemical staining, immunoassays, and nucleic acid amplification tests (molecular tests) (Sachse, Vretou, et al., 2009). Antibody detection of *C. psittaci* uses serology tests to detect anti-chlamydial antibodies from blood samples. The serological test is a less preferred approach because the results can be inconsistent due to short term antibody response and paired samples are required for reliable interpretation (Balsamo et al., 2017). Recent systematic reviews on chlamydiae from Sukon et al. (2021) and Hogerwerf et al. (2020) reported that PCR (polymerase chain reaction) was preferred to detect *C. psittaci* infection in birds, when compared to serology and culture, with PCR performed in 48 out of 74 studies (Sukon et al., 2021), and in 37 out of 45 studies (Hogerwerf et al., 2020). However, these studies were focusing on different aims, with Sukon et al. (2021) on global prevalence of chlamydial infections and

Hogerwerf et al. (2020) source of zoonotic transmission. Hence, in this review, we chose to focus on laboratory methods which detect *C. psittaci* antigens in birds.

To date, two review papers, Fudge (1997) and Sachse, Vretou, et al. (2009) on the available detection methods for *C. psittaci* have been published. Fudge (1997) compared the specificity and sensitivity of various detection methods, evaluated the results from different samples, and listed the available tests in relevant laboratories in the US in 1997. Meanwhile, Sachse, Vretou, et al. (2009) described the technologies to diagnose chlamydial infections in 2009 with more focus on the molecular tests. In 1990s, there were only 4 species (*C. trachomatis*, *C. psittaci*, *C. pneumoniae*, *C. pecorum*) of *Chlamydia* being identified, and the *C. psittaci* strains were determined by serologic analysis, reporting five serovars (A, B, C, D, E) (Vanrompay, Andersen, et al., 1993). At that time, the major outer membrane protein (*ompA*) was newly known as the genus-specific lipopolysaccharide antigen of *Chlamydia*. For sample collection, swabs from the pharynx and conjunctiva were recommended to enhance the detection of *Chlamydia* (Andersen, 1996). In 1990s, culture was still considered the gold standard among all the other available diagnostic tests like serology, immunoassays, and DNA-based tests (Fudge, 1997).

Then, Sachse, Vretou, et al. (2009) reviewed the further developments in laboratory diagnosis of *Chlamydia* infections in the next 10 years, following Fudge (1997). In 1999, based on DNA sequence analysis of 16S and 23S rRNA genes, Everett et al. (1999) proposed the taxonomic reclassification of the 9 species in family *Chlamydiacaea* into two genera, *Chlamydia* (*C.*) and *Chlamydophila* (*Ch.*). However, comparative genome analysis of chlamydial species revealed that the host-divergent strains of family *Chlamydiaceae* are biologically closely related, leading to the reuniting of the members into single genus *Chlamydia* in 2015 (Sachse, Bavoil, et al., 2015). The further developments of molecular-based detection methods of *Chlamydia* supported the emendation of *Chlamydia* classification, and also provided sufficient genomic data for source tracing in epidemiological investigation (Sachse, Vretou, et al., 2009). Some of the available molecular based methods were conventional PCR, real-time PCR (qPCR), and DNA microarray technology (Sachse, Vretou, et al., 2009). The selection of a suitable

test method depended on sample types, bacterial viability, presumptive diagnosis based on clinical signs and pathology, and clinical history (Sachse, Vretou, et al., 2009).

According to the World Organisation for Animal Health (WOAH) Terrestrial Manual 2018 on the detection of *C. psittaci* in birds, the preferred method is no longer bacterial isolation. This is due to its cost, being time-consuming and labour-intensive, the need for viable bacteria and specialised culture facilities, and its zoonotic risk to laboratory personnel (WOAH, 2018a). Therefore, the currently recommended *C. psittaci* detection method is a molecular test. In the manual, the three main molecular methods available for various purposes were conventional PCR, qPCR, and DNA microarray assay. To confirm clinical cases, the recommended and validated method was real-time PCR (qPCR), while the suitable methods that needed further validation were conventional PCR and DNA microarray assay. For *C. psittaci* surveillance, WOAH suggested that the suitable test method was qPCR. In addition, conventional PCR and DNA microarray assay can be used, but its application could be limited by cost or reliability (WOAH, 2018a).

Given that qPCR is the preferred method for diagnosis and surveillance, WOAH (2018a) described a recommended hierarchical approach using qPCR in detail. The first test in this approach was a *Chlamydiaceae*-specific qPCR based on 23S rRNA gene (DeGraves, Gao, Hehnen, et al., 2003; Ehricht et al., 2006; Everett et al., 1999), followed by *C. psittaci*-specific qPCR based on *ompA* gene (Pantchev et al., 2009) and *incA* gene (Ménard et al., 2006). Then, genotyping can be done via *ompA*-based genotype-specific qPCR protocol from Geens, Dewitte, et al. (2005) and Heddema et al. (2015). However, the choice of laboratory test can be affected by the purpose of the study, availability of the equipment and expertise in individual facilities. Hence, we aim to systematically review the globally available molecular tests, to identify the most commonly published molecular detection of *C. psittaci* in birds for different study purposes.

2.2 Methods

2.2.1 Literature search strategy

A search strategy was designed to identify studies that describe laboratory methods of detection of *C. psittaci* in birds with an emphasis on antigen detection. We searched the databases Scopus, PubMed, SciQuest (Kokako and New Zealand Veterinary Journal) and Web of Science (Web of Science Core Collection, Biological Abstracts, CABI: CAB Abstracts, Current Contents Connect, FSTA – the food science resource, KCI-Korean Journal Database, MEDLINE, Russian Science Citation Index and SciELO Citation Index).

The key elements that this review considered were the population (birds), index test (molecular detection), and target condition (*C. psittaci* infection). Key words for the literature search were selected based on the key elements (**Table 2.1**). We searched for electronic literature published between 1st January 2000 and 22nd July 2020. Selecting to begin in 2000 was due to the major adjustment in taxonomy and nomenclature of the family *Chlamydiaceae* in 1999 (Everett et al., 1999). Language was restricted to English. No limitations were applied on the study designs. The search results from all article databases were combined into one EndNote X9 (Clarivate Analytics USA) file. Duplicates were removed by using EndNote and by hand.

Table 2.1 Key words included in the search string for literature search.

Key elements	Search string
Population	bird* or avian
Index test	molecular or detection or laborator* or diagnos* or epidem* or surve* or prevalence
Target condition	"*chlamydia psittaci*" or *chlamydia* or *psittaci* or *chlamydophila* or chlamydiosis or ornithosis or psittacosis
Exclude	*rickettsia* or *giardia* or *felis* or *pneumoniae* or *trachomatis* or *caviae* or *abortus* or *muridarum* or *suis* or *pecorum*

* The asterisk is used as a wildcard symbol to broaden a search to variations of words, for example diagnos* searches for diagnose, diagnosis, diagnoses, etc.

2.2.2 Inclusion and exclusion criteria

Titles and abstracts of the articles were screened to include studies that described the molecular detection of *C. psittaci* in birds. According to the PRISMA 2020 statement, a record is the title or abstract of a report catalogued in a database (Page et al., 2021). In this review, the selection included records that mentioned various molecular methods to detect *C. psittaci*. Whenever the titles or abstracts were inconclusive to decide on full-text assessment eligibility, the full-text articles were revised to decide on relevancy. The studies without complete supportive information were still selected due to their sufficient descriptions on the molecular methods used.

Full-text assessment was completed by one investigator (XqS) and uncertainties about inclusion or exclusion of an article were resolved by discussion with other authors (BG, KG and JB). At full-text assessment, reports were excluded if they described any of the following: insufficient information on laboratory methods, avian chlamydiosis due to *Chlamydia* species other than *C. psittaci*, only serological testing in birds, only human cases without detection in birds, guidelines, review articles, articles presenting unoriginal data and languages other than English. For the purposes of this review, a report is a journal article selected for full-text assessment. And the reports to be included in this review are known as studies.

2.2.3 Data extraction

The type of information extracted from the selected studies was chosen to address the study aim, illustrate the study background, provide spatio-temporal context, and identify the factors affecting test selection and test outcome. To address the study aim, we extracted details on the study purpose and molecular methodology in the study. To illustrate the study background, we extracted details on host population, study location, and time of the study being conducted. For possible factors affecting test selection and test outcome, we extracted details on sample type (Fudge, 1997; Sachse, Vretou, et al., 2009), sample handling and storage (Sachse, Vretou, et al., 2009), swab type, DNA extraction methods, number of birds tested and the associated results.

Data extraction was done by XqS. Uncertainties of extracted information were discussed and achieved consensus between authors (XqS, BG, KG and JB). When extracting the information, the definitions of the original authors in the articles were followed. When the desired information from selected studies were not available, we looked for relevant publications from the authors and extracted related details from these as appropriate. For example, Chahota et al. (2006) was not cited in Tanaka et al. (2005), but we matched the primers name and extracted the primer system of nested PCR protocol used. When the desired information was completely unavailable, it is regarded as "not reported".

2.2.4 Data cleaning and analysis

Data cleaning was performed to ensure the uniformity of usage of a specific vocabulary to describe the same species, sample types, and laboratory techniques. For instance, the bird species *Columba livia,* was mentioned by various nouns in the articles. These include pigeon, feral pigeon, broiler pigeon, meat pigeon, domestic pigeon, urban pigeon, racing pigeon, rock dove, feral rock dove, silver king pigeon and columbiformes. In this case, we resolved the confusion by using only three nouns to differentiate their husbandry settings and assigned these to the subjects accordingly. We defined feral pigeons as free-living pigeons, racing pigeons as captive exotics for sports, and broiler pigeon as farmed poultry for human consumption. Bird species were categorised based on their order in bird taxonomy (e.g., columbiformes, passeriformes, psittaciformes etc.).

Firstly, the characteristics and main information of all the included studies were tabulated to allow transparent and complete reporting of the results (Page et al., 2021). In **Table 2.2**, we included the individual citation of each study, host population, time of study, study location, study purpose, sample types and molecular tests. The countries of the study were classified by regions and income levels based on World Bank (2022), to provide an economic context for analytical purposes. Next, quality assessment was carried out to evaluate the reporting completeness of each study (Page et al., 2021).

Quality assessments included the availability of targeted information in the main study characteristic table (**Table 2.2**), the availability of other supportive information such as swab type, sample handling and storage, DNA extraction method and NCBI accession number.

The data on study purposes and types of molecular test was stratified into four-year groups (of five years duration each) in individual table, to describe the trend over years. Then, to address the study aim, we combined both the data on study purposes and corresponding molecular tests in a single table. The categorisation of study purposes and types of molecular method was adapted from WOAH (2018a). For study purposes, we added nine items on top of the two original study aims in the manual – surveillance and clinical cases, to be more comprehensive when presenting the information. To further investigate the different protocols used in the multiple types of molecular tests, we produced four tables based on the main classification of the types of tests – qPCR, conventional PCR, nested PCR, and the remaining molecular tests. The information included in the table were, the detection target, amplicon size, primer sequence, and times the protocol was cited in the included studies. For studies that had performed DNA sequencing, MLST, WGS, and NGS, we examined the protocols used and NCBI assession number as appropriate.

2.3 Results

2.3.1 Study selection and study characteristics

Our search strategy yielded 982 records from four databases. After removing the duplicates, 698 records were unique for title and abstract screening. Of these, 212 records were potentially relevant, and 120 articles were included in this review after full-text assessment (**Figure 2.1**). The selected articles described the antigen detection technique of *C. psittaci* in birds from 39 countries (**Table 2.2**). In further classification based on income level, over half of the studies (n = 74, 62%) were conducted in high income countries, 39 (33%) in upper middle-income countries, six in lower middle-income countries, and one in a low-income country. The most studied bird species were

Columbiformes (n = 29), followed by Psittaciformes (n = 22) and domestic chickens (n = 12).

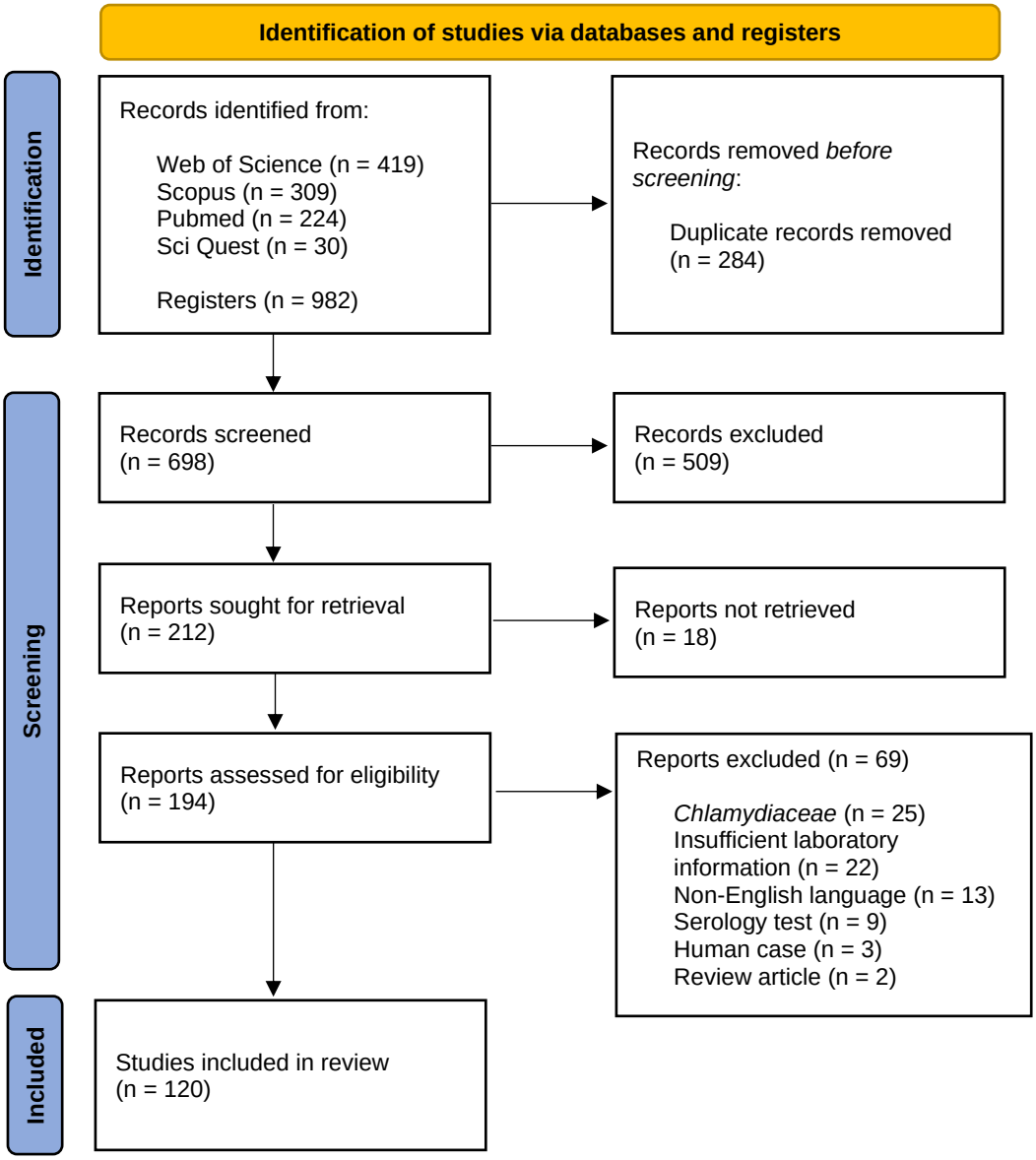

Figure 2.1 PRISMA 2020 flow diagram used to select studies in this systematic review.

Table 2.2 Characteristics of the selected studies on molecular methods to detect *Chlamydia psittaci* in birds, sorted first by date of study published, then by descending alphabetical order.

Reference	Bird orders	Year of study	Country	Purpose	Sample types	Molecular detection method (genomic target)
Wang et al. (2020)	Fulmar	2016	Denmark	Surveillance	Swab	qPCR (23, A), MLST, WGS
Stokes, Martens, Jelocnik, et al. (2020)	Psittaciformes	2016-2018	Australia	Surveillance	Swab	PCR (16, c), Seq (16)
Perez-Sancho et al. (2020)	Columbiformes	2005-2014	Spain	Surveillance	Swab	PCR, qPCR
Mahzoonieh et al. (2020)	Mixed	NR	Iran	Surveillance	Swab	qPCR, nPCR, PCR, Seq
Li et al. (2020)	Pelecaniformes	NR	China	Surveillance	Faeces	qPCR (23, A), PCR (16, IGS, A), Seq (16, IGS, A)
Crispo et al. (2020)	Passeriformes	2018	USA	Clinical case	Tissue	qPCR (23), nPCR, Seq
Amery-Gale et al. (2020)	Mixed	2014-2015	Australia	Surveillance	Swab, Tissue	qPCR-HRM (16), MLST
Wang et al. (2019)	Mixed	2015-2016	China	Surveillance	Tissue	PCR, Seq
Vogler et al. (2019)	Turkey	2016-2017	Switzerland	Surveillance	Swab	qPCR (23, A), MicAr (23), PCR (16), Seq (16)
Vilela et al. (2019)	Psittaciformes	2009-2011	Brazil	Characterise	Tissue	PCR, nPCR, Seq
Sutherland et al. (2019)	Psittaciformes	2015-2016	Australia	Surveillance	Swab	qPCR-HRM (16), NGS, MLST
Plaza et al. (2019)	Raptors	2014-2017	Argentina	Surveillance	Swab	nPCR (16), Seq (16)
Origlia et al. (2019)	Psittaciformes	2013-2015	Argentina	Surveillance	Swab, Tissue	qPCR (23, A), nPCR, Seq
Mina et al. (2019)	Mixed	2015-2016	Iran	Surveillance	Swab, Tissue	nPCR, PCR, Seq
Mattmann et al. (2019)	Columbiformes	2014-2018	Switzerland	Surveillance	Swab, Tissue	qPCR (23, A), MicAr (23), MLST, PCR (16, A), Seq (16, A)
Liu et al. (2019)	Mixed	2014-2017	Taiwan	Surveillance	Swab, Faeces	nPCR (16, A), PCR, Seq
Lin et al. (2019)	Duck	2015	China	Poor egg prod.	Tissue	qPCR (23), PCR, Seq
Hamzah et al. (2019)	Mixed	NR	Iraq	Evaluate test	Swab	PCR
Wang et al. (2018)	Columbiformes	2015-2016	China	Surveillance	Faeces	nPCR, Seq
Szymańska-Czerwińska et al. (2018)	Mixed	NR	Poland	Avian outbreak	Swab	qPCR (23, iA), PCR, Seq
Heijne et al. (2018)	Chicken	2015-2016	Netherlands	Surveillance	Faeces	qPCR (23, A)
Gedye et al. (2018)	Mixed	NR	New Zealand	Characterise	Swab, Tissue	qPCR (23), PCR, Seq
Donati et al. (2018)	Chicken	2016	Italy	Surveillance	Swab	qPCR (23, iA)
Cechova et al. (2018)	Chicken	NR	Slovakia	Surveillance	Swab	PCR (23), Seq (23)
Burt et al. (2018)	Columbiformes	2017	Netherlands	Surveillance	Faeces	qPCR (23, A)
Yao et al. (2017)	Columbiformes	2015	China	Surveillance	Faeces	nPCR, Seq
Vaz et al. (2017)	Psittaciformes	2013-2014	Brazil	Surveillance	Swab	PCR (p)
Szymańska-Czerwińska et al. (2017)	Multiple species	2014-2015	Poland	Surveillance	Swab	qPCR (23), PCR (16, IGS, A), Seq (16, IGS, A)
Jeong et al. (2017)	Mixed	2016	Korea	Surveillance	Swab	qPCR (23), PCR (16, A), Seq (16, A)
Hegazy et al. (2017)	Mixed	NR	Egypt	Surveillance	Tissue, EI	nPCR, Seq
Gartrell et al. (2017)	Penguins	2013	New Zealand	Avian outbreak	Tissue	qPCR-HRM (16), PCR (16), Seq (16)
Ferreira et al. (2017)	Mixed	NR	Brazil	Human outbreak	Swab	PCR (p)
Cadario et al. (2017)	Psittaciformes	2010-2015	Argentina	Human outbreak	Swab	nPCR (16, A), Seq

Table 2.2 *Cont.*

Vasconcelos et al. (2016)	Psittaciformes	2011	Brazil	Surveillance	Swab	PCR (p)
Tatari et al. (2016)	Turkey	2013-2014	Iran	Surveillance	Swab, Tissue	qPCR (23), nPCR
Suksai et al. (2016)	Psittaciformes	2013-2014	Thailand	Surveillance	Swab	nPCR, Seq
Razmyar et al. (2016)	Psittaciformes	NR	Iran	Clinical case	Swab	nPCR, PCR, Seq
Ornelas-Eusebio et al. (2016)	Psittaciformes	2015	Mexico	Clinical case	Swab, Tissue	nPCR, PCR, Seq
Mahzounieh et al. (2016)	Mixed	NR	Iran	Surveillance	Swab	qPCR, nPCR, PCR, Seq
Konicek et al. (2016)	Mixed	2012-2014	Austria, Czech	Surveillance	Swab	qPCR (23, A), PCR (IGS, A), Seq (IGS, A)
Hulin et al. (2016)	Duck	2013	France	Surveillance	Swab, Tissue	qPCR (23, iA), PCR, Seq
Ferreira et al. (2016)	Columbiformes	NR	Brazil	Surveillance	Swab	PCR (p)
Feng et al. (2016)	Psittaciformes	NR	China	Surveillance	Faeces	qPCR, PCR, Seq
Dovč et al. (2016)	Mixed	NR	Slovenia	Surveillance	Swab, Faeces	qPCR (23, A)
Cechova et al. (2016)	Columbiformes	NR	Slovakia	Surveillance	Swab	PCR (23), Seq (23)
Vorimore et al. (2015)	Duck	NR	France	Surveillance	Swab	qPCR (23, A), PCR, Seq
Shivaprasad et al. (2015)	Turkey	NR	USA	Clinical case	Tissue	qPCR (23, iA), PCR, MLVA, MLST
Ling et al. (2015)	Columbiformes	2008-2010	China	Surveillance	Swab	nPCR, Seq
Laroucau et al. (2015)	Chicken, Duck	2013	France	Human outbreak	Swab	qPCR (23, iA), MicAr (23), PCR, Seq
Khodadadi et al. (2015)	Columbiformes	2013-2014	Iran	Surveillance	Tissue	nPCR
Hulin et al. (2015)	Mixed poultry	2013	France	Surveillance	Swab	qPCR (23, iA), PCR (16, A), Seq (16, A)
Ghorbanpoor et al. (2015)	Columbiformes	NR	Iran	Surveillance	Swab	PCR (16-23, p)
Donati et al. (2015)	Columbiformes	2010-2013	Italy	Surveillance	Swab	qPCR (23, iA), MLVA, MLST, nPCR, Seq
Aaziz et al. (2015)	Seabirds	2011-2014	France	Surveillance	Swab	qPCR (23, iA, A), MLST, PCR (16, IGS, A), Seq (16, IGS, A)
To et al. (2014)	Psittaciformes	2012	China	Human outbreak	Swab, Tissue	qPCR (ITS)
Stenzel et al. (2014)	Columbiformes	2010-2013	Poland	Surveillance	Swab, Tissue	qPCR (iA), nPCR, Seq
Kalmar et al. (2014)	Mixed	NR	Belgium	Surveillance	Swab	qPCR
Elezi et al. (2014)	Mixed	NR	Albania	Surveillance	Blood	PCR
Beckmann et al. (2014)	Mixed	2005-2011	UK	Surveillance	Tissue	qPCR (23, A), MicAr (23), PCR
Tomić, Laroucau, et al. (2013)	Columbiformes	NR	Croatia	Surveillance	Faeces	qPCR (23, A), PCR-RFLP, MLVA
Tomić, Gottstein, et al. (2013)	Chicken	2010	Croatia	Clinical case	Swab	qPCR (23, A), Seq
Tel et al. (2013)	Pelecaniformes	NR	Turkey	Surveillance	Faeces	PCR (p)
Szymańska-Czerwińska et al. (2013)	Chicken	NR	Poland	Clinical case	Swab	nPCR, qPCR (23), PCR, Seq
Sheleby-Elías et al. (2013)	Psittaciformes	2009	Costa Rica	Surveillance	Swab	nPCR, PCR, Seq
Perelman et al. (2013)	Mixed poultry	2008-2011	Israel	Clinical case	Swab	PCR (p), qPCR
Madani and Peighambari (2013)	Mixed	NR	Iran	Surveillance	Swab, Tissue, Faeces, CC	nPCR, PCR-RFLP, PCR, Seq
Gartrell et al. (2013)	Passeriformes	2010-2011	New Zealand	Surveillance	Swab	qPCR-HRM (16), PCR (16), Seq (16)
Dickx et al. (2013)	Canada Geese	NR	Belgium	Surveillance	Swab	qPCR
Zocevic et al. (2012)	Chicken	NR	France, Greece	New method	Swab	qPCR (23, iA), MLST, PCR (16, A), Seq (16, A)
Šatrović et al. (2012)	Turkey	2007-2008	Bosnia	Surveillance	Swab	qPCR
Piasecki et al. (2012)	Psittaciformes	2007-2012	Poland	Surveillance	Swab	PCR (p, A), PCR-RFLP
Marhold et al. (2012)	Mixed	2010-2011	Slovenia	Surveillance	Swab	qPCR (23, A), MLVA
Križek et al. (2012)	Mixed	NR	Croatia	Surveillance	Swab, Faeces	PCR

Table 2.2 *Cont.*

Gilbert et al. (2012)	Mixed	2007-2009	Cambodia	Surveillance	Swab	qPCR
Geigenfeind et al. (2012)	Columbiformes	2007-2009	Switzerland	Surveillance	Swab, Faeces	nPCR-EIA, qPCR
Deem et al. (2012)	Raptors	2009	Ecuador	Surveillance	Swab	PCR-RFLP
Colvile et al. (2012)	Passeriformes	2005-2011	UK	Avian outbreak	Tissue	PCR
Blomqvist, Christerson, Waldenström, Lindberg, et al. (2012)	Raptors	2006-2007	Sweden	Surveillance	Swab	qPCR (23), PCR (16), nPCR, Seq (16, A)
Yanga et al. (2011)	Columbiformes	2004	Mexico	Surveillance	Swab, Tissue	PCR
Yang et al. (2011)	Peacock	2009	China	Human outbreak	Swab, Tissue	PCR
Gasparini et al. (2011)	Columbiformes	2009	France	Surveillance	Swab	qPCR (23, A), PCR (16, A), Seq (16, A)
Doosti and Arshi (2011)	Columbiformes	NR	Iran	Surveillance	Faeces	nPCR
Zhou et al. (2010)	Chicken	NR	China	Poor egg	Tissue	PCR, Seq
Vázquez et al. (2010)	Columbiformes	2006-2007	Spain	Surveillance	Cloacal content	PCR, Seq
Tarsitano et al. (2010)	Columbiformes	2006-2007	Italy	Surveillance	Faeces	PCR (p)
Robertson et al. (2010)	Mixed	2007-2008	Australia	Evaluate test	Swab, Tissue	PCR (16), Seq (16)
Geigenfeind and Haag-Wackernagel (2010)	Columbiformes	NR	Switzerland	Surveillance	Faeces	MicAr (IGS)
Dickx, Geens, et al. (2010)	Chicken, Turkey	2007	Belgium	Zoonotic risk	Swab	nPCR-EIA, micoA, PCR
Dickx, Beeckman, et al. (2010)	Columbiformes	2008	Belgium	Surveillance	Swab, Faeces	nPCR-EIA, PCR
Christerson et al. (2010)	Sea birds	NR	USA	Surveillance	Swab, Faeces	qPCR (23), PCR (16, rB), snPCR, Seq (16, rB, A)
Zweifel et al. (2009)	Mixed	2005-2006	Switzerland	Surveillance	Swab, Tissue	qPCR (23), MicAr (23), PCR (16, 23, IGS, A), Seq (16)
Song et al. (2009)	Mixed	NR	China	Characterise	Inoculated egg	PCR, Seq
Sharples and Baines (2009)	Mixed	NR	UK	Surveillance	Swab	PCR-dotblot (B)
Sareyyupoglu and Cantekin (2009)	Passeriformes	NR	Turkey	New method	Swab, Tissue	PCR (p)
Sachse, Laroucau, et al. (2009)	Mixed	NR	Germany	Evaluate test	Swab, Tissue, Faeces, CC	PCR, PCR-RFLP, Seq
Mitchell et al. (2009)	Mixed	2004-2007	USA	New method	Swab, Blood	qPCR-HRM, PCR, Seq
Laroucau et al. (2009)	Duck	2006	France	Human outbreak	Swab	qPCR (23), PCR-RFLP, MLVA, Seq
Droogenbroeck et al. (2009)	Turkey	NR	Belgium	Zoonotic risk	Swab	nPCR-EIA, qPCR
Zhang et al. (2008)	Chicken	NR	China	Poor egg prod.	Swab, Tissue	nPCR, Seq
Verminnen et al. (2008)	Turkey	NR	Belgium	Zoonotic risk	Swab	nPCR-EIA, PCR, Seq
Laroucau et al. (2008)	Mixed	NR	Belgium	Characterise	NR	PCR-RFLP, MLVA
Gaede et al. (2008)	Mixed poultry	NR	Germany	Surveillance	Swab, Faeces	qPCR (23), MicAr (IGS), nPCR, Seq
Branley et al. (2008)	Psittaciformes	NR	Australia	Human outbreak	Swab, Tissue	qPCR (23, A), nPCR (16), PCR (16), Seq (23)
Yang et al. (2007)	Chicken, Duck	NR	China	Surveillance	Swab, Tissue	PCR
Vanrompay et al. (2007)	Psittaciformes	NR	Belgium	Zoonotic risk	Swab, Faeces	nPCR-EIA, qPCR
Schenker and Hoop (2007)	Mixed	1991-2005	Switzerland	Atherosclerosis	Tissue	PCR (23), Seq (23)
Sareyyupoglu et al. (2007)	Mixed	NR	Turkey	Surveillance	Faeces	PCR (p)
Harkinezhad et al. (2007)	Psittaciformes	NR	Belgium	Zoonotic risk	Faeces	nPCR-EIA, qPCR
Assuncao et al. (2007)	Pelecaniformes	NR	South Africa	Surveillance	Swab	PCR
Travis et al. (2006)	Cormorant	2003	Ecuador	Surveillance	Swab	PCR
Herrmann et al. (2006)	Fulmar	1999	Denmark	Surveillance	Swab	qPCR (23), nPCR, Seq
Heddema, van Hannen, et al. (2006)	Psittaciformes	2004-2005	Netherlands	Human outbreak	Faeces	qPCR, nPCR, Seq
Heddema, Ter Sluis, et al. (2006)	Columbiformes	2005	Netherlands	Surveillance	Faeces	qPCR, nPCR, Seq

Table 2.2 *Cont.*

Van Loock, Verminnen, et al. (2005)	Turkey	2001	France	New method	Swab	PCR, Seq
Van Loock, Geens, et al. (2005)	Turkey	2001	Belgium, France	Other pathogens	Swab	nPCR-EIA
Tanaka et al. (2005)	Columbiformes	2003-2004	Japan	Surveillance	Faeces	nPCR, Seq
Greco et al. (2005)	Psittaciformes	2005	Italy	Clinical case	Faeces	PCR (p)
Dovc et al. (2005)	Passeriformes	2001	Slovenia	Experimental	Tissue	nPCR
Bonner et al. (2004)	Canada Geese	2002	Germany	Surveillance	EI	nPCR
Schettler et al. (2003)	Raptors	1994-1997	Germany	Surveillance	Tissue	nPCR

Note. NR, not reported; EI, egg inoculation; CC, cell culture; PCR, conventional polymerase chain reaction; PCR-RFLP, PCR-restriction fragment length polymorphism; qPCR, quantitative PCR; qPCR-HRM, qPCR-High resolution DNA melting analysis; nPCR, nested PCR; snPCR, semi-nested PCR; nPCR-EIA, nested PCR-enzyme immunoassay; MicAr, DNA microarray technology; MLVA, multiple-locus variable number tandem-repeat analysis; MLST, multilocus sequence typing; Seq, Sanger sequencing; NGS, next-generation sequencing; WGS, whole genome sequencing; A, *ompA* (major outer membrane protein A); B, *ompB* (major outer membrane protein B); p*, pmp* (putative outer membrane protein); rB, *rnpB* (RNase P RNA gene); iA, *incA* (inclusion membrane protein); ITS, *ITS* (internal transcribe spacer); c, *Cpsit_0607* (hypothetical A protein); 16, 16s rRNA; 23, 23s rRNA; IGS, IGS-23S rRNA (16S rRNA-23S rRNA intergenic spacer together with 23S rRNA domain I).

*All molecular detection methods with unspecified detection target in the table were by default targeting *ompA* gene.

2.3.2 Study quality assessment

All the selected studies (n = 120) reported study purposes, molecular detection methods and study location (**Table 2.3**). Since only 75 studies reported the year of study conducted, the analysis involving time used the publication year instead. For species of the host population, Song et al. (2009) did not report the details of bird species. For sample type, Laroucau et al. (2008) received the extracted DNA from other facilities, so the details of the sample origin were not reported. Information regarding swab type was only applicable to samples which involved swabbing (n = 103), and not applicable to other sampling methods such as collecting tissue samples. Since not all studies reported on swab type, sample handling and storage, and DNA extraction methods, unintended bias should be taken into consideration when interpreting the findings, especially in relation to the prevalence differences reported between studies.

Table 2.3 Study quality assessment (11 items) of the selected 120 studies in a systematic review on molecular detection of *Chlamydia psittaci* in birds.

Items	**No. of studies**		
	Yes	No	Not applicable
Study purpose was clearly stated.	120	0	0
Molecular detection method was cited or described in detail for reproducibility purposes.	120	0	0
Location of the study was reported.	120	0	0
When the study conducted was reported.	75	45	0
Species of the host population was reported.	119	1	0
Sample type was reported.	119	1	0
Swab type was reported.	36	67	17
Sample handling and storage were reported.	75	45	0
DNA extraction method was reported.	103	17	0
NCBI accession number was reported.	38	27	55

2.3.3 Study purposes

We identified twelve purposes from all the studies (**Table 2.4**). Most studies were conducted to identify the prevalence of *C. psittaci* infection via surveillance (n = 79), to diagnose clinical cases (n = 8), and to investigate the animal source of human psittacosis outbreaks (n = 8). To aid in epidemiological studies of *C. psittaci,* we found articles that investigate avian chlamydiosis outbreaks (n = 3), characterise *C. psittaci*

strains (n = 4), and assess the diseases zoonotic risk (n = 5). Both human outbreak investigation and zoonotic risk assessment consisted of samples from birds and humans, but the latter studies collected both samples at the same time, while the putative source of a human outbreak were traced back retrospectively. There were also articles that described the development of new detection methods (n = 4) and evaluated the accuracy of the diagnostic tests (n = 3). There were articles examining the link of *C. psittaci* with poor egg production (n = 3) (Lin et al., 2019; Zhang et al., 2008; Zhou et al., 2010), atherosclerosis (n = 1) (Schenker & Hoop, 2007), and the presence of other respiratory pathogens (n = 1) (Van Loock, Geens, et al., 2005). There was only one article of experimental infection of *C. psittaci* that describe the clinical signs and pathology in birds (Dovc et al., 2005). Over the years, publication on the molecular detection of *C. psittaci* in birds had increased, with a similar trend observed in publication reporting avian surveillance.

Table 2.4 The study purposes (12 purposes) of a total of 120 selected articles on the molecular detection of *Chlamydia psittaci* in birds by publishing years.

Study purposes	**2000-2005**	**2006-2010**	**2011-2015**	**2016-2020**	**Total**
Avian surveillance	3	14	28	34	79
Avian clinical case	1	-	4	3	8
Human outbreak investigation	-	3	3	2	8
Zoonotic risk assessment	-	5	-	-	5
Characterise *C. psittaci* strains	-	2	-	1	4
Develop new method	1	2	1	-	4
Evaluate diagnostic test	-	2	-	1	3
Avian outbreak investigation	-	-	1	2	3
Poor egg production	-	2	-	1	3
Link with atherosclerosis	-	1	-	-	1
Link with other pathogens	1	-	-	-	1
Experimental infection	1	-	-	-	1
Total	**7**	**31**	**37**	**45**	**120**

2.3.4 Molecular detection methods

2.3.4.1 Types of molecular tests

Based on the three main molecular methods as identified by WOAH (2018a), namely conventional PCR, qPCR, and DNA microarray assay, we classified the fourteen molecular methods identified in this review into three main groups (**Table 2.5**). The first main group is quantitative PCR (qPCR), which includes probe-based qPCR and dye-based qPCR-High resolution DNA melting analysis (qPCR-HRM). The second main group is gel electrophoresis-based molecular tests. The eight molecular tests in this group were conventional PCR, nested PCR (nPCR), semi-nested PCR (snPCR), nested PCR-enzyme immunoassay (nPCR-EIA), PCR-dot blot, PCR-restriction fragment length polymorphism (PCR-RFLP), multilocus sequence typing (MLST), and Sanger sequencing. The third main group is for the tests using other instruments – DNA microarray assay, multiple-locus variable number tandem-repeat analysis (MLVA), next generation sequencing (NGS), and whole genome sequencing (WGS).

Overall, the most commonly used molecular tests were conventional PCR (n = 71), Sanger sequencing (n = 65), qPCR (n = 55), and nPCR (n = 35). All three gel electrophoresis-based molecular tests were used more frequently from 2006 to 2020, while qPCR was increasingly adopted from 2006 to 2020. Interestingly, the five types of molecular tests that were not in use in the published studies, in the 2016-2020, were snPCR, nPCR-EIA, PCR-dotblot, PCR-RFLP, and MLVA. Two newer molecular tests were performed only in 2016-2020 – NGS and WGS.

Table 2.5 The molecular tests (14 tests) identified in 120 selected articles on the detection of *Chlamydia psittaci* in birds by publishing years.

Types of molecular tests	2000-2005	2006-2010	2011-2015	2016-2020	Total
qPCR					
Quantitative PCR (qPCR)	-	11	22	22	55
qPCR-High resolution DNA melting analysis (qPCR-HRM)	-	2	1	3	6
Gel electrophoresis-based					
Conventional PCR	2	18	22	29	71
Nested PCR (nPCR)	4	6	9	16	35
Semi-nested PCR (snPCR)	-	1	-	-	1
Nested PCR-enzyme immunoassay (nPCR-EIA)	1	6	1	-	8
PCR-dot blot	-	1	-	-	1
PCR-restriction fragment length polymorphism (PCR-RFLP)	-	3	4	-	7
Multilocus sequence typing (MLST)	-	-	4	4	8
Sanger sequencing	2	17	15	31	65
Others					
DNA microarray assay	-	4	2	2	8
Multiple-locus variable number tandem-repeat analysis (MLVA)	-	2	4	-	6
Next generation sequencing (NGS)	-	-	-	1	1
Whole genome sequencing (WGS)	-	-	-	1	1

*84 studies performed more than one molecular test.

2.3.4.2 Study purposes and types of molecular tests

To identify the most published method of molecular detection of *C. psittaci* in birds for different study purposes, we combined the data on the study purposes and corresponding types of molecular tests in **Table 2.6**.

The studies identifying *C. psittaci* prevalence via surveillance (n = 79), with publishing year ranged from 2003 to 2020, incorporated all thirteen types of molecular tests. The more frequently used molecular tests in surveillance studies were conventional PCR (n = 44), Sanger sequencing (n = 40), qPCR (n = 38), nPCR (n = 25), DNA microarray assay (n = 6), and MLST (n = 6). For conventional PCR, the most commonly used PCR protocols were from Denamur et al. (1991) (n = 12) targeting *ompA* gene, and Everett et al. (1999) targeting 16S rRNA gene (n = 6), IGS-23S (n = 4), and 23S rRNA gene (n = 2). For qPCR, the most commonly used qPCR protocols were from Ehricht et al. (2006) (n = 24) targeting 23S rRNA gene, Pantchev et al. (2009) targeting *ompA* gene (n = 16), and Ménard et al. (2006) (n = 7) targeting *incA* gene. For nPCR, the most frequently used nPCR protocols were from Heddema, Ter Sluis, et al. (2006) (n = 10) and Sachse and Hotzel (2003) (n = 5), both targeting *ompA* gene. Of all the 41 studies performing

Sanger sequencing, the most common sequencing gene was *ompA* gene while the common protocols were from Denamur et al. (1991) (n = 8) and Heddema, Ter Sluis, et al. (2006) (n = 8).

The studies diagnosing avian clinical cases of *C. psittaci* infection (n = 8), were published from 2005 to 2020. The six types of molecular tests that were conducted in these studies were conventional PCR (n = 6), qPCR (n = 5), Sanger sequencing (n = 5), nPCR (n = 4), MLVA (n = 1), and MLST (n = 1). For conventional PCR, the common PCR protocols used were from Laroucau et al. (2001) (n = 2) targeting *pmp* gene, and Sachse et al. (2008) (n = 2) targeting *ompA* gene. For qPCR, the common qPCR protocols used were from Ehricht et al. (2006) (n = 4), and Pantchev et al. (2009) (n = 2). The sequencing references used were from Sachse et al. (2008) (n = 2), Denamur et al. (1991) (n = 1), Kaltenboeck et al. (1991) (n = 1), Kaltenboeck et al. (1993) (n = 1), and Pantchev et al. (2009) (n = 1).

The studies investigating human outbreaks of *C. psittaci* infection (n = 8), were published from 2006 to 2017. The seven types of molecular tests that were performed in these studies were qPCR (n = 5), Sanger sequencing (n = 5), conventional PCR (n = 4), nPCR (n = 3), PCR-RFLP (n = 1), DNA microarray assay (n = 1), and MLVA (n = 1). The commonly used qPCR protocols were from Ehricht et al. (2006) (n = 2) and Geens, Dewitte, et al. (2005) (n = 2). For conventional PCR, protocol from Denamur et al. (1991) (n = 2) were most frequently used. For Sanger sequencing, there were two sequencing genes used – *ompA* gene (n = 4), and 23S rRNA gene (n = 1). The *ompA* sequencing references were from Sachse and Hotzel (2003) (n = 2), Denamur et al. (1991) (n = 1), Geens, Dewitte, et al. (2005) (n = 1), and Heddema, Ter Sluis, et al. (2006) (n = 1). The 23S rRNA sequencing reference was from DeGraves, Gao, Hehnen, et al. (2003).

The studies assessing zoonotic risk of *C. psittaci* (n = 5), were published from 2007 to 2010. The four types of molecular tests performed were nPCR-EIA (n = 5), qPCR (n = 3), DNA microarray assay (n = 1), conventional PCR (n = 2), and Sanger sequencing (n = 1). All five studies opted for nPCR-EIA from Van Loock, Verminnen, et al. (2005). Additionally, three studies performed qPCR from Geens, Dewitte, et al. (2005), and one

study performed DNA microarray assay from Sachse et al. (2008). Only one study (Verminnen et al., 2008) performed *ompA* sequencing from Denamur et al. (1991). The studies investigating avian outbreaks of *C. psittaci* infection (n = 3), were published from 2012-2018. The four types of molecular tests used were conventional PCR (n = 3) targeting 16S rRNA and *ompA* gene, Sanger Seqeuncing (n = 2) targeting 16S rRNA and *ompA* gene, qPCR (n = 1) targeting 23S rRNA and *incA* gene, and qPCR-HRM (n = 1) targeting 16S rRNA gene.

Table 2.6 The fourteen molecular methods identified in 120 selected articles on the detection of *Chlamydia psittaci* in birds based on their study purposes.

Study purposes / Types of molecular tests	qPCR	qPCR-HRM	PCR	nPCR	nPCR-EIA	PCR-RFLP	MicroA	MLVA	MLST	Others	Seq
Surveillance	38	3	44	25	2	4	6	3	6	4*	40
Avian clinical case	5	-	6	4	-	-	-	1	1	-	5
Human outbreak investigation	5	-	4	3	-	1	1	1	-	-	5
Zoonotic risk assessment	3	-	2	-	5	-	1	-	-	-	1
Characterise *C. psittaci* strains	1	-	3	1	-	1	-	1	-	-	3
Develop new method	1	1	3	-	1	-	-	-	1	-	2
Evaluate diagnostic test	-	1	2	-	-	1	-	-	-	-	2
Avian outbreak investigation	1	1	3	-	-	-	-	-	-	-	2
Poor egg production	1	-	2	1	-	-	-	-	-	-	3
Link with atherosclerosis	-	-	1	-	-	-	-	-	-	-	1
Link with other pathogens	-	-	1	-	-	-	-	-	-	-	1
Experimental infection	-	-	-	1	-	-	-	-	-	-	-
Total	**55**	**6**	**71**	**35**	**8**	**7**	**8**	**7**	**8**	**4**	**65**

*snPCR, PCR-dotblot, NGS, WGS

2.3.4.3 Detection target and specificity level

Of all the PCR tests, the ten detection targets used were *ompA* (major outer membrane protein), *ompB* (major outer membrane protein B)*, pmp* (putative outer membrane protein), *rnpB* (RNase P RNA gene), *incA* (inclusion membrane protein), *ITS*, *Cpsit_0607* (hypothetical A protein), 16S rRNA, 23S rRNA, and IGS-23S rRNA (16S rRNA-23S rRNA intergenic spacer together with 23S rRNA domain I). Some studies use a single detection target (63%; 75/120) in their methodology, while some used two (20%; 24/120), and three (12%; 14/120) detection targets. Only a few studies used four (n = 5) and five (n = 2) detection targets. The most common single detection targets used were *ompA* (n = 54), *pmp* (n = 9), 16S rRNA (n = 6), and 23S rRNA (n = 3). The most common combination using two detection targets were 23S rRNA and *ompA* (n = 16), and for three detection targets were 16S rRNA, 23S rRNA, and *ompA* (n = 7).

When classifying the PCR methods into their specificity level of genus, species, and genotype, we observed that a variety of tests were available to obtain the desired results. To detect *Chlamydia* at the genus level, *Chlamydiaceae*-specific test were done via conventional PCR, qPCR, and DNA microarray assay. Next, to detect *C. psittaci* at the species level, the *C. psittaci*-specific tests were done via conventional PCR, PCR-dotblot, qPCR, qPCR-HRM, nPCR, snPCR, nPCR-EIA and DNA microarray assay. Finally, genotyping was done via PCR-RFLP (Sayada et al., 1995), qPCR (Geens, Dewitte, et al., 2005), qPCR-HRM (Mitchell et al., 2009), nPCR (Heddema, Ter Sluis, et al., 2006), and DNA microarray

assay (Sachse et al., 2008). MLVA typing was done via the MLVA protocol (Laroucau et al., 2008) while sequence typing was done via MLST (Pannekoek et al., 2010).

When investigating if the studies had conducted a hierarchical approach to detect *C. psittaci* as recommended by WOAH (2018a), a total of 42 studies (35%) opted for this approach. Of these 42 studies, six studies (16%; 6/38) were from 2000-2010, and 36 studies (44%; 36/82) were from 2011-2020. In detail, 31 studies were for surveillance (n = 79), four studies were to diagnose avian clinical cases (n = 8), three studies to investigate human psittacosis outbreak (n = 8), and one study to investigate avian *C. psittaci* outbreak (n = 3). For instance, Aaziz et al. (2015) started the molecular analysis by screening genus-specific *Chlamydiacaea* DNA using 23S rRNA-based quantitative PCR (qPCR) (Ehricht et al., 2006). The positive samples from the first test were further analysed to detect species-specific *C. psittaci* DNA using qPCR targeting the *incA* gene (Ménard et al., 2006) and *ompA* gene (Pantchev et al., 2009). Then, positive samples from *incA*-qPCR were subjected to MLST (Pannekoek et al., 2008) to determine the sequence type (ST). Finally, to generate phylogenetic trees, sequencing of the positive samples were done by targeting 16S rRNA (Pudjiatmoko et al., 1997; Thomas et al., 2006), 23S rRNA (Everett et al., 1999), and *ompA* gene (Denamur et al., 1991). For visual purposes, Mattmann et al. (2019) (7 molecular tests) and Vogler et al. (2019) (5 molecular tests) had included figures illustrating the decision tree using hierarchical approach to detect *C. psittaci* in their studies.

In detail, for the first main group of quantitative PCR (qPCR), we identified ten qPCR protocols and two qPCR-High resolution DNA melting analysis (qPCR-HRM) protocols (**Table 2.7**). Of all the ten qPCR protocols, two were genus-specific, seven were species-specific and one was genotype-specific. The commonly performed qPCR protocol for a genus-specific assay was from Ehricht et al. (2006) (n = 33), while the species-specific protocols were from Pantchev et al. (2009) (n = 18) and Ménard et al. (2006) (n = 11). The only genotype-specific qPCR protocol was from Geens, Dewitte, et al. (2005) (n = 11). For qPCR-HRM protocol, the species-specific protocol was Robertson et al. (2009) while the genotype-specific protocol was from Mitchell et al. (2009).

For the second main group of gel electrophoresis-based molecular tests, we separated the protocols of the eight molecular tests identified in this group into two tables. **Table 2.8** included 15 conventional PCR protocols, and one protocol each for PCR-dot blot and PCR-restriction fragment length polymorphism (PCR-RFLP). Of the 15 conventional PCR protocols, one was family-specific, four were genus-specific, and ten were species-specific. Overall, the commonly performed genus-specific conventional PCR protocols were from Everett et al. (1999) (n = 13) targeting 16S rRNA gene (n = 7), IGS-23S (n = 4), and 23S rRNA gene (n = 2). For species-specific conventional PCR, the frequently used protocol were from Denamur et al. (1991) (n = 20) targeting *ompA* gene, and Laroucau et al. (2001) (n = 12) targeting *pmp* gene. The PCR-dotblot protocol (McElnea & Cross, 1999) was species-specific, while the PCR-RFLP protocol (Sayada et al., 1995) was genotype-specific.

In the table of PCR using gel electrophoresis (**Table 2.9**), we identified ten nested PCR (nPCR) protocols, and one protocol each for semi-nested PCR (snPCR), nested PCR-enzyme immunoassay (nPCR-EIA). Of all the ten nPCR protocols, nine can detect both genus and species level of *C. psittaci*, while one was genotype-specific, from Heddema, Ter Sluis, et al. (2006) (n = 12). The commonly performed nPCR protocols were from Sachse and Hotzel (2003) (n = 9) and Kaltenboeck et al. (1991) (n = 7). The protocol in Sachse and Hotzel (2003) was modified from Kaltenboeck et al. (1997), so both protocols shared the same primers. The nPCR-EIA protocol from Van Loock, Verminnen, et al. (2005) (n = 8) was commonly used too. However, the nPCR-EIA method was last reported in a study in 2009 (Geigenfeind et al., 2012).

The protocols we have defined as other are displayed in **Table 2.10**, these include DNA microarray, multiple-locus variable number tandem-repeat analysis (MLVA), and multilocus sequence typing (MLST). In this category, all methods were still reported Of all the three DNA microarray protocols, they were genus-specific (Borel et al., 2008) (n = 5), species-specific (Sachse et al., 2005) (n = 2), and genotype-specific (Sachse et al., 2008) (n = 13). Interestingly, of all the thirteen studies that had cited Sachse et al. (2008), only three studies (Dickx, Beeckman, et al., 2010; Dickx, Geens, et al., 2010;

Zweifel et al., 2009) performed DNA microarray technology, while the rest applied the primers from Sachse et al. (2008) in conventional PCR.

From the 65 studies that performed Sanger sequencing, 40 studies have submitted sequence data to NCBI and have been assigned accession numbers. From the 27 studies without NCBI accession numbers, two studies (Gartrell et al., 2017; Vogler et al., 2019) obtained negative results from testing the samples. Most studies (n = 63) performed one type of molecular test, while two studies (Blomqvist, Christerson, Waldenström, Lindberg, et al., 2012; Christerson et al., 2010) performed two types of molecular tests to obtain the PCR product for Sanger sequencing. The types of molecular tests performed before Sanger sequencing were conventional PCR (n = 43), nPCR (n = 22), and qPCR (n = 4). Most studies (n = 54) sequenced single gene, while some studies (n = 11) sequenced more than one gene and one study (Laroucau et al., 2015) sequenced one gene (*ompA*) using two protocols.

The five genes used for sequencing were *ompA* (n = 54), 16S rRNA (n = 17), 23S rRNA (n = 4), IGS-23S (n = 4), *rnpB* (Herrmann, Pettersson, et al., 2000) (n = 1), and *pmp* (Laroucau et al., 2001) (n = 1). The sequencing of *ompA* gene was done using 16 PCR protocols. Among all, five of the most commonly used primers were CTU and CTL (Denamur et al., 1991) (n = 16), CPsittGenoFor and CPsittGenoRev (Heddema, Ter Sluis, et al., 2006) (n = 9), (Sachse & Hotzel, 2003) (n = 5), CTU and ompA rev (Sachse et al., 2008) (n = 7), 5GPF and 3GPB (Kaltenboeck et al., 1991) (n = 5). For the sequencing of 16S rRNA, the primers commonly used were 16SIGF and 16SIGR (Everett et al., 1999) (n = 8), 16S1 and rp2 (Pudjiatmoko et al., 1997) (n = 7). For the sequencing of 23S rRNA and IGS-23S, the primers commonly used were U23F and 23SIGR (Everett et al., 1999) (n = 2), and 16SF2 and 23SIGR (Everett et al., 1999) (n = 4).

Overall, we observed three author groups led by Kaltenboeck, Sachse, and Heddema, in chronological order, who had contributed multiple protocols of molecular tests to detect *C. psittaci* in this review. The author group led by Kaltenboeck contributed four species-specific nPCR protocols. They were Kaltenboeck et al. (1991) (n = 7), Kaltenboeck et al. (1992) (n = 2), Kaltenboeck et al. (1993) (n = 3), and

Kaltenboeck et al. (1997) (n = 5). The author group led by Sachse contributed three protocols. One was species-specific nPCR protocol (Sachse & Hotzel, 2003) (n = 9), and the other two were DNA microarray assay – species-specific (Sachse et al., 2005) (n = 2), and genotype-specific (Sachse et al., 2008) (n = 13). The author group led by Heddema contributed to three protocols to detect *C. psittaci*. The most cited protocols were from Heddema, Ter Sluis, et al. (2006) (n = 11) using genotype-specific nPCR, followed by Heddema, Beld, et al. (2006) (n = 5), and Heddema et al. (2015) (n = 1), both using species-specific qPCR.

Table 2.7 The quantitative PCR methods and the protocols identified in 120 selected articles on the molecular detection of *Chlamydia psittaci* in birds.

Specificity	Ref First author	Gene	Amplicon size (base pairs)	Primer, Probe & Sequence (5'-3')	Cited times
Quantitative PCR (qPCR)					
Chlamydiaceae	(DeGraves, Gao, Hehnen, et al., 2003)	23S rRNA	168	CHL23SUP: GGGGTTGTAGGGTYGAGRAIAWRRCATC	4
				CHL23SDN: GAGAGTGGTCTCCCCAGATTCARACTA	
				CHL23LCR: CCTGAGTAGRRCTAGACACGTGAAAC	
				CP23FLU: ACGAAARAACAARAGACKCTAWTCGAT	
Chlamydiaceae	(Ehricht et al., 2006)	23S rRNA	111	Ch23S-F: CTGAAACCAGTAGCTTATAAGCGGT	33
				Ch23S-R: ACCTCGCCGTTTAACTTAACTCC	
				Ch23S-p: CTCATCATGCAAAAGGCACGCCG	
C. psittaci	(Feng et al., 2016)	*ompA*	NR	F: TGTGATTCACAAACCAAGAGGCTATA	1
				R: CGAGGCCTACTTGCCATTCA	
				p: TATGTTTAGGCATCTAAAAC	
C. psittaci	(Heddema, Beld, et al., 2006)	*ompA*	82	CPsittF: CGCTCTCTCCTTACAAGCC	5
				CPsittR: AGCACCTTCCCACATAGTG	
				CPsitt-p: AGGGAACCCAGCTGAACCAAGTTT	
				CPsitt-Icp: TCGAGACAGTGCAACGTAAGCCTA	
C. psittaci	(Heddema et al., 2015)	*ompA*	174-183	CPVDF: GTCAAGAGCAACTTTTGATGC	1
				CPVDR: ATTTTGTTGATCTGAATCGAAGC	
C. psittaci	(Ménard et al., 2006)	*incA*	74	F1-incA-Cpsi: GCCATCATGCTTGTTTCGTTT	11
				R1-incA-Cpsi: CGGCGTGCCACTTGAGA	
				Cpsi-incA-NM: TCATTGTCATTATGGTGATTCAGGA	
C. psittaci	(Nordentoft et al., 2011)	23S rRNA	301	Cpsi-F: GATTAATCATCTACCATTATACGT	1
				Chuni4-R: AGACTAGGTTTCACGTGTCTAG	
C. psittaci	(Pantchev et al., 2009)	*ompA*	76	CppsOMP1-F: CACTATGTGGGAAGGTGCTTCA	18
				CppsOMP1-R: CTGCGCGGATGCTAATGG	
				CppsOMP1-P: CGCTACTTGGTGTGAC	
C. psittaci	(To et al., 2014)	ITS	145	F: TTGGTCTGTAAATTATTGATCC	1
				R: CATTTAGTTTACGATCAAGTATG	
				p: ATGCAAGTTAACWTCACCTAAAGACAT	
Genotype	(Geens, Dewitte, et al., 2005)	*ompA*	151	CpPsSSfor: TTATTAAGAGCTATTGGTGGATGCC	11
				CpPsSSrev: AACGTATAATGGTAGATGATTAATCTACCG	
qPCR-High resolution DNA melting analysis (qPCR-HRM)					
C. psittaci	(Robertson et al., 2009)	16S rRNA	460	16SG-F: TGATGAGGCATGCAAGTC	5
				16SG-R: TTACCTGGTACGCTCAAAT	
Genotype	(Mitchell et al., 2009)	*ompA*	109	Ppac-F: TATTGTTTGCCGCTACGGGT	1
				Ppac-R: TCCTGAAGCACCTTCCCACA	
			274	GTpc-F: TGTGCAACTTTAGGAGCTGAGTC	
				GTpc-R: GCTCTTGACCAGTTTACGCCAATA	
			98	GT-F: CATTCGTGAACCACTCAGCGC	
				GT-R: CTCCTACAGGAAGCGCAGCA	

Table 2.8 The conventional PCR methods identified in 120 selected articles on the molecular detection of *Chlamydia psittaci* in birds.

Specificity	Ref First author	Target	Amplicon size (base pairs)	Primer, Probe & Sequence (5'-3')	Cited times
Conventional PCR					
Chlamydiales	(Borel et al., 2006)	16-23S spacer	352	CAAGGTGAGGCTGATGAC TCGCCTKTCAATGCCAAG	1
Chlamydiaceae	(Everett et al., 1999)	16S rRNA	298	16SIGF: GATGAGGCATGCAAGTCGAACG 16SIGR: CCAGTGTTGGCGGTCAATCTCTC	7
		23S rRNA	600	U23F: GATGCCTTGGCATTGATAGGCGATGAAGGA 23SIGR: TGGCTCATCATGCAAAAGGCA	2
		IGS-23S rRNA	1000	16SF2: CCGCCCGTCACATCATGG 23SIGR: TGGCTCATCATGCAAAAGGCA	4
Chlamydiaceae	(Pudjiatmoko et al., 1997)	16S rRNA	260	16S1: CGGATCCTGAGAATTTGATC rp2: GCAAGGATCGCAAGATC	7
Chlamydiaceae	(Soldati et al., 2004)	23S rRNA	92	23SQAPF2: GAACCTGAAACCA(AG)TAGC 23SAPR: CTGGCTCATCATGCAAAAGG	1
Chlamydiaceae	(Thomas et al., 2006)	16S rRNA	1470	16SIGF: GATGAGGCATGCAAGTCGAACG rP2Chlam: CTACCTTGTTACGACTTCAT	4
C. psittaci	(Denamur et al., 1991)	*ompA*	1050	CTU: ATGAAAAAACTCTTGAAATCGG CTL: CAAGATTTTCTAGAYTTCATYTTGTT	20
C. psittaci	(Herrmann, Pettersson, et al., 2000)	*rnpB*	325	BH3: TGGACTTCATAAGAAAAGAT BH2: (AG)TAAGCCGGGTTCTGT	1
C. psittaci	(Hewinson et al., 1997)	*ompA*	1170	Cpf: GCAAGACACTCCTCAAAGCC Cpr: CCTTCCCACATAGTGCCATC	7
C. psittaci	(Jelocnik, Islam, et al., 2017)	*Cps*_0607	263	F3: AGAACCGGATTAGGAGTCTT B3: GCTGCTAAAGCGAGTATTGA	1
C. psittaci	(Laroucau et al., 2001)	*pmp*	300	CpsiA: ATGAAACATCCAGTCTACTGG CpsiB: TTGTGTAGTAATATTATCAAA	12
C. psittaci	(Madico et al., 2000)	16S rRNA 16S-23S spacer	111	CPS100: CCCAAGGTGAGGCTGATGAC CPS101: CAAACCGTCCTAAGACAGTTA	1
C. psittaci	(Mahzounieh et al., 2016)	*ompA*	157	Cp.sG1F: GCCTTAAACATTTGGGATCG Cp.sG1R: CGTTAGGAAGTTGCATTGGA	2
			1024	Cp.sG2F: GACACTCCTCAAAGCCATTA Cp.sG2R: CGCCAATATATGGAACAAGC	
			622	Cp.sG3F: TGCAACTTTAGGAGCTGA Cp.sG3R: GTTCTGATAGCGGGACAA	
C. psittaci	(Olsen et al., 1998)	*ompA*	245	Or1: TTTCGATCGTGTATTAAAAGTT Or2: AGAAAATGTCGAAGCGATCCA	1
C. psittaci	(Zhou et al., 2010)	*ompA*	1170	F: AGGAGATCTATGAAAAAACTCTTGAAATCG R: TGGGTCGACTTAGAATCTGAATTGAGCATTC	1
C. psittaci	(Vanrompay et al., 1998)	*ompA*	1200	DV-1: CGGAATTCATGAAAAAACTCTTGAAATCGG DV-2: CGGAATTCAATGTTCGAAAAGACTRAAGTAR 55G2-F: ATTTGGGATCGCTTCGAC 55G2-R: CCTTTATAGCCTCTTGGTTTGTG 1293A5-F: ACCAATGCAGCTTTCCTC 1293A5-R: CCTTTATAGCCTCTTGGTTTGTG	1
PCR-dotblot					
C. psittaci	(McElnea & Cross, 1999)	*ompB*	580	F: CAAACTCATCAGACGAG R: CTTCTTTAAGAGGTTTTACCC	1
PCR-restriction fragment length polymorphism (PCR-RFLP)					
Genotype	(Sayada et al., 1995)	*ompA*	1050	CTU: ATGAAAAAACTCTTGAAATCGG CTL: CAAGATTTTCTAGACTTCATTTTGTT	8

Table 2.9 The nested PCR methods identified in 120 selected articles on the molecular detection of *Chlamydia psittaci* in birds.

Specificity	Ref First author	Target	Amplicon size (base pairs)	Primer, Probe & Sequence (5'-3')	Cited times
Nested PCR (nPCR)					
C. psittaci	(Buxton et al., 1996)	*ompA*	260	cla420 107–129: CAGGACATCTTGTCTGGCTTTAA cla422 349–366: GCAAGGATCGCAAGGATC	1
			165	cla421 202–225: TTAGAGGTGAGTATGAAAAAACTC cla422: GCAAGGATCGCAAGGATC	
C. psittaci	(Herrmann et al., 2006)	*ompA*	1101	FOMPF1: GAAATCGGCATTATTRTTTGCC FOMPR2: CCAGTGATTGACCATTTGTCA FOMPF2: TACGGGTTCCGCTCTCTC FOMPR1: CATTTGTCAGCGTCGATTAACG CpsF2: YGTAGGTGCACGYGGAG 201FAG: GGAGCIGARTTCCAATACGCTCAITC 16SIG-R-new: GGTCAATCTCTCAATCCG	4
C. psittaci	(Kaltenboeck et al., 1991)	*ompA*	1353	5GPF: ACGCATGCAAGACACTCCTCAAAGCC 3GPB: ACGAATTCCTAGGTTCTGATAGCGGGAC	7
			603	MOMPIN: GCIYT[CI]TGGGAITGYGG [CI]TGYGCIAC 3GPB: ACGAATTCCTAGGTTCTGATAGCGGGAC	
C. psittaci	(Kaltenboeck et al., 1992)	*ompA*	1078	9CTROMP: GCTCTGCCTGTGGGGAATCCTGCTGAACC CHOMP371: TTAGAAICKGAATTGIGCRTTIAYGTGIGCIGC	2
			926	29CTROMP: GGAGATCCTTGCGATCCTTG CHOMP336: CAAGMTTTTCTG GAYTTMAWYTTGTT	
C. psittaci	(Kaltenboeck et al., 1993)	*ompA*	1200	-20CHOMP: TTAGAGGTRAGWATGAARAA 9CHOMP: GCIYTGCCTGTIGGGAAYCCIGCIGARCC 119CHOMP: TGGGATIGITTYGAIRTITTYTGYAC 191CHOMP: GCIYTITGGGARTGYGGITGYGCIAC 277CHOMP: CCITAYATHGGIGTIAAITGG	3
			1200	CHOMP371: TTAGAAICKGAATTGIGCRTTIAYGTGIGCIGC CHOMP271: CCAITTIACICCDATRTAIGG CHOMP185: CCIARIGTIGCRCAICCRCATTCCCA CHOMP111: GTICAGAAIAYITCRAAICGRTCCCA	
C. psittaci	(Kaltenboeck et al., 1997)	*ompA*	582-597	191CHOMP: GCIYTITGGGARTGYGGITGYGCIAC CHOMP371: TTAGAAICKGAATTGIGCRTTIAYGTGIGCIGC	5
			440-455	201CHOMP: GGIGCWGMITTCCAATAYGCICARTC CHOMP336: CAAGMTTTTCTGGAYTTMAWYTTGTT	
			389-404	218PSITT: GTAATTTCIAGCCCAGCACAATTYGTG CHOMP336: CAAGMTTTTCTGGAYTTMAWYTTGTT	
C. psittaci	(Messmer et al., 1997)	16S rRNA	436	sense: ACGGAATAATGACTTCGG anti-sense: TACCTGGTACGCTCAATT	4
			127	sense: ATAATGACTTCGGTTGTTATT anti-sense: TGTTTTAGATGCCTAAACAT	
C. psittaci	(Sachse & Hotzel, 2003)	*ompA*	576-597	191CHOMP: GCIYTITGGGARTGYGGITGYGCIAC CHOMP371: TTAGAAICKGAATTGIGCRYIAGTGIGCIGCTT	9
			389-404	218PSITT: GTAATTTCIAGCCCAGCACAATTYGTG CHOMP336: GTAATTTCIAGCCCAGCACAATTYGTG	
C. psittaci	(Tanaka et al., 2005)	*ompA*		CMGP-1F: CCTTGTGATCCTTGCGCTACTTG CMGP-1R: GTGAGCAGCTCTTTCGTTGAT CMGP-2F: GCCTTAAACATCTGGGATCG CMGP-2R: GCACAACCACATTCCCATAAAG	1
Genotype	(Heddema, Ter Sluis, et al., 2006)	*ompA*	1041	CPsittGenoFor: GCTACGGGTTCCGCTCT CPsittGenoRev: TTTGTTGATYTGAATCGAAGC	12
		ompA	894	CPsittFinner: CGCTCTCTCCTTACAAGCC CPsittRinner: GATCTGAATCGAAGCAATTTG	
Semi-nested PCR (snPCR)					
C. psittaci	(Christerson et al., 2010)	*ompA*	931-937	ComOmpAF3: ACTTGGTGCGATGCTTTATC ComOmpAR3: CGTCCTTCAACAGTGATAGC ComOmpAF4: GATGCTTTATCTTTGCGTGC ComOmpAF2: CCGAAATCTCTCARATCAATGTCGT ComSekvR: TGATYTGAGAGATTTCGGGTTTAGC	1
Nested PCR-enzyme immunoassay (nPCR-EIA)					
C. psittaci	(Van Loock, Verminnen, et al., 2005)	*ompA*	472	ML-BbrpI-F01: GCCACGTGCGTCTGCAACACTCAAATATC ML-BbrpI-R01: GGCACGTGCAGTTGTAAGAAGTCAGAGT Sense outer: CCTGTAGGGAACCCAGCTGAA Anti-sense outer: GGTTGAGCAATGCGGATAGTAT Fluorescein-sense inner: GCAGGATACTACGGAGA Biotin-antisense inner: GGAACTCAGCTCCTAAAG	8

Table 2.10 The DNA microarray technology, multiple-locus variable number tandem-repeat analysis (MLVA), and multilocus sequence typing (MLST) identified in 120 selected articles on the molecular detection of *Chlamydia psittaci* in birds.

Specificity	Ref First author	Target	Amplicon size (base pairs)	Primer, Probe & Sequence (5'-3')	Cited times
DNA microarray technology					
Chlamydiaceae	(Borel et al., 2008)	23S rRNA	171	U23F-19: ATTGAMAGGCGAWGAAGGA 23R-22: GCYTACTAAGATGTTTCAGTTC	5
C. psittaci	(Sachse et al., 2005)	IGS-23S rRNA	1000	16SF2: CCGCCCGTCACATCATGG 23SIGR: TGGCTCATCATGCAAAAGGCA	2
Genotype	(Sachse et al., 2008)	*ompA*	1200	CTU: ATGAAAAAACTCTTGAAATCGG ompA rev: TCCTTAGAATCTGAATTGAGC	13
		VD1, VD2	418	VD1-f: ACTACGGAGATTATGTTTTCGATCGTGT VD2-r: CGTGCACCYACGCTCCAAGA	
		VD3, VD4	570	201CHOMP: GGIGCWGMITTCCAATAYGCICARTC ompA-rev: TCCTTAGAATCTGAATTGAGC	
Multiple-locus variable number tandem-repeat analysis (MLVA)					
MLVA typing	(Laroucau et al., 2008)		203	ChlaPsi_222F: GCACTCTCATATCCTATTGAAGTG ChlaPsi_222R: GATGACAGCGGAACAACATACA	6
			647	ChlaPsi_280F: TCAGATAGACTAGGTAATCCTG ChlaPsi_280R: CTCCCTCTACCACAATTGCCTA	
			134	ChlaPsi_281F: AAGATATGCAGGTGAGCCTG ChlaPsi_281R: ACACAGCACAAGTGACCCCA	
			337	ChlaPsi_480F: AGTTTGCAAAGGAGCCGCTGCA ChlaPsi_480R: CCTTTCTCCACCTCTTTCTTTC	
			194	ChlaPsi_605F: CTATAATTCCGCGTTGATTTG ChlaPsi_605R: AAACTCGTGAAGCTTAATCCCC	
			519	ChlaPsi_810F: TGAAAGGCTACTCGCAATATC ChlaPsi_810R: GGCGATAAATACTATGGATACC	
			151	ChlaPsi_929F: CACGGACCCCTAAATTTTCTGG ChlaPsi_929R: TCCGGGAACTCAGGAGCAATAA	
			101	ChlaPsi_1788F: GGCATTCCTGTAATATTCACACC ChlaPsi_1788R: GAGGATGAAGGTAATGGCTTCG	
Multilocus sequence typing (MLST)					
Sequence typing	(Pannekoek et al., 2010)	enoA	450-500	YPenoA3: CCTATGATGAATCTCATTAATGG YPenoA4: CCCAACCATCAAAATCTTCTTCCG	8
		fumC	500-600	YPfumC1: GGGCTCCTGAGGTTATGCC YPfumC2: CGCAAATAATGAATCACCTTATC	
		gatA	500-600	YPgatA3: GCCTTAGAGTTAAGAAATGCCG YPgatA4: CCCCCTGTATCGGAACCTAACGC	
		gidA	500-670	YPgidA1: GCTTATTAGAGAGCTGTCCTGGC YPgidA2: CGCGTTTTCTAACCCACGG	
		hemN	500-630	YPhemN1: GGATCCATTTCGGAGGAGGC YPhemN2: CCTGAAAGGATTTTCTCATGG	
		hflX	500-610	YPhflX3: GAGATTTTTGCTAATCGAGCG YPhflX4: GTAAAACATCTTCATGTAACGC	
		oppA	500-610	YPoppA3: ATGCGCAAGATATCAATGGG YPoppA4: GGCAAGGTTTGGTGTAACTCGC	

2.3.5 Sampling methods

2.3.5.1 Sample types

From all the 119 studies that reported sample types, a variety of samples were collected, only one study (Laroucau et al., 2008) did not describe the sample type, as the samples were obtained from multiple research facilities to characterise *C. psittaci* strains. The main categories of sample types were swabs (n = 84), tissues (n = 34) and faeces (n = 28). For faecal samples, we used the word "faeces" to group the diverse vocabularies (e.g., dry composite, guano, fresh pooled faeces) extracted from the articles. For live bird sampling, the most common swabs collected were cloacal swabs (n = 55). For the swabs collected from various anatomical sites, we grouped throat, nasopharyngeal and oropharyngeal into the same group as pharyngeal swabs (n = 28). Similarly, we grouped both eyelid and ocular swab as the conjunctival swabs (n = 11). In detail, swabs included single individual swab (e.g., cloacal, pharyngeal) and combined swab from multiple anatomical sites (e.g., choanal-cloacal (n = 4), conjunctival-choanal-cloacal (n = 6)).

Ninety studies collected a single type of sample while 29 studies collected multiple sample types. Of all the 90 studies with single sample type, the samples collected were swabs (n = 56), faeces (n = 17), tissues (n = 14), egg inoculation isolates (n = 2) (Bonner et al., 2004; Song et al., 2009), and blood (n = 1) (Elezi et al., 2014). For studies collecting only swabs (n = 56), thirty-six studies (64%) collected swab from single anatomical site while the rest collected from multiple anatomical sites. For studies collected multiple samples types, the combinations included swabs and tissues (n = 17), swab and faeces (n = 8), swab and blood (n = 1) (Mitchell et al., 2009), and all three main types (swab, tissue and faeces) (n = 3) (Madani & Peighambari, 2013; Mina et al., 2019; Sachse, Laroucau, et al., 2009).

For samples collected from dead birds via post-mortem, the common organ tissues collected were liver (n = 24), spleen (n = 19), and lung (n = 14). Some uncommon samples were yolk sac and embryonic organs from inoculated egg (Bonner et al., 2004; Hegazy et al., 2017; Song et al., 2009), blood (Elezi et al., 2014; Mitchell et al., 2009), cell

culture (Sachse, Laroucau, et al., 2009) and cloacal content (Vázquez et al., 2010). Vázquez et al. (2010) collected cloacal content from adult pigeon by introducing 0.5mL of PBS into the cloaca, then recovering the suspension of PBS and cloacal contents.

2.3.5.2 Swab types

From all the 84 studies that had collected swabs, there were 31 studies that reported the types of swabs. We identified three types of tips and shafts being used. Twenty studies reported the tips of the swab which include cotton-tipped (n = 8), dacron-tipped (n = 6), and rayon-tipped (n = 6). Eight studies reported the shafts of the swab such as aluminium-shafted (n = 8), plastic-shafted (n = 2) and wood-shafted (n = 1) (Robertson et al., 2010). There were six studies that described the swab as "sterile swab" without the details on the tip and shaft material. Only 14 studies reported the swab type with manufacturer and location. The four manufacturers were Copan Diagnostic (USA), Fiers (Belgium), MW&E (UK), and Thermo Fisher Scientific (USA). Occasionally, the swab tips were moistened with sterile PBS or sterile normal saline prior to sample collection (Amery-Gale et al., 2020; Sharples & Baines, 2009). For faecal sampling, instead of swabs, two studies reported that sterile gloves and sterile tubes were used for collection (Sareyyupoglu et al., 2007; Yao et al., 2017).

2.3.5.3 Sample handling and storage

From all 68 studies that had reported sample handling and storage, 41 studies reported both the type of transport medium and storage and/or transport temperature, while 14 studies reported type of transport medium only and 13 studies reported the temperature only. For sample handling, 6 studies mentioned dry swab or transport without medium, while 5 studies mentioned swabs in a cryovial (or cryotube or cryogenic vial). For those with transport medium, the reported details were the volume (n = 18) and type (n = 42) of medium. The volume of transport medium ranged from 0.3 ml to 3 ml, with 1 ml (n = 7) as the most reported. We identified 14 types of transport medium. The most common one was sucrose phosphate glutamate (n = 10), followed

by Chlamydia transport medium (n = 11) and phosphate-buffered saline (n = 10). The three references (Madani et al., 2011; Quinn et al., 2004; Vanrompay et al., 1992) of Chlamydia transport medium that were cited in the studies originated from (Spencer & Johnson, 1983). Chlamydia transport medium is made up of 11 ingredients (sucrose (74.6 g/l), KH_2PO_4 (0.512 g/l), K_2HPO_4 (1.237 g/l), L-glutamic acid (0.721 g/l), phenol red (0.015 g/l), gentamycin (50 mg/ml), nystatin (50 mg/ml), vancomycin (100 mg/ml), streptomycin (100 mg/ml), distilled water (1000 ml), and foetal calf serum (10 per cent v/v)) to maintain the bacterial viability of Chlamydia.

For the temperature of the samples, there were transport (n = 16) and storage (n = 49) temperatures reported. Of all the transport condition, 2 studies mentioned dry ice, 5 studies used ice, while the rest (n = 8) used a cooler at 4°C, refrigeration, cold or chilling conditions. Two studies had their samples (cloacal swabs, faeces, tissues) stored in liquid nitrogen cryotank at -196°C in the field (Christerson et al., 2010; Deem et al., 2012). Only one study transported its dry swab at room temperature, but they collected tissues on ice (Robertson et al., 2010). Most samples were stored at -80°C (n = 25) and -20°C (n = 18) until use. Some swab samples were stored at 4°C (n = 5) until use or stored at 4°C temporarily (12 hours to 5 days) until use (Sheleby-Elías et al., 2013; Stokes, Martens, Jelocnik, et al., 2020; Vogler et al., 2019). We identified five studies as irrelevant to report the transport and storage temperature. Of these five, three studies sourced their samples from egg inoculation (Bonner et al., 2004; Hegazy et al., 2017; Song et al., 2009), and the other two sourced theirs from formalin-fixed, paraffin-embedded (FFPE) tissue (Schenker & Hoop, 2007; Vilela et al., 2019). Among the 45 studies that did not report the sample handling and storage, 27 studies collected swabs, 12 studies collected faeces, 9 studies collected tissues, two studies collected blood (Elezi et al., 2014; Mitchell et al., 2009), and one study (Laroucau et al., 2008) that obtained their samples from multiple research facilities.

2.3.6 DNA extraction

From the 120 studies examined in this review, 103 reported the protocol of DNA extraction. Of all the included studies, the majority (n = 97 studies) performed single

DNA extraction methods while only 6 studies (Amery-Gale et al., 2020; Gedye et al., 2018; Perez-Sancho et al., 2020; Sareyyupoglu & Cantekin, 2009; Wang et al., 2020; Zweifel et al., 2009) performed two DNA extraction protocols for different sample types. For example, Sareyyupoglu and Cantekin (2009) used Qiagen DNA stool kit to extract DNA from faeces, and Qiagen DNA tissue kit for swabs and tissues.

Altogether, we observed 46 procedures of DNA extraction. There were 32 types of test kits and 14 types of DNA extraction not using test kits. The common test kits were QIAamp DNA Mini kit (n = 23 studies), DNeasy Blood & Tissue kit (n = 11 studies), QIAamp DNA Stool Mini Kit (n = 8 studies), and Roche High Pure PCR Template Preparation kit (n = 8 studies). The studies that opted for QIAamp DNA Mini kit sourced their samples from swabs (n = 17 studies), tissues (n = 4 studies), faeces (n = 2 studies), blood (n = 1 study), and inoculated egg (n = 1 study). The common DNA extraction methods not using commercial kits were salting-out methods, phenol-chloroform methods (Hewinson et al., 1997; Rasmussen & Timms, 1991; Schenker & Hoop, 2007), and utilising Chelex (Gartrell et al., 2013; Gedye et al., 2018). For the salting-out methods, the chaotropic salts used were guanidium thiocyanate (Boom et al., 2000; Boom et al., 1990), ammonium acetate (Bruford et al., 1992; Rasmussen & Timms, 1991), and sodium iodide (Ishizawa et al., 1991).

Of the 17 studies that did not report DNA extraction protocol, they had collected swabs (n = 8 studies), faeces (n = 4 studies), tissues (n = 3 studies), cloacal content and inoculated egg. Concurrently, seven studies did not report sample handling and storage, while five studies did not report the swab type.

2.4 Discussion

2.4.1 Types of tests

This review shows that, the most commonly published molecular test to detect *C. psittaci* in birds were conventional PCR (n = 71), quantitative PCR (qPCR) (n = 55), nested PCR (n = 35), and Sanger sequencing (n = 65) across all the study purposes. For

C. psittaci detection in birds, from 2000 to 2010, conventional PCR using PCR systems and agarose gel-electrophoresis to visualise the PCR products based on its fragment length, was preferred over bacterial isolation. Besides being a more sensitive *C. psittaci* detection method, molecular tests, such as conventional PCR, do not require high quality viable samples, and long incubation time for bacterial growth, thus avoiding zoonotic risk to laboratory personnel. For instance, typical turn-around time for conventional PCR is short, around 5 hours from DNA extraction to gel electrophoresis (Ehricht et al., 2006; Sachse et al., 2005), while bacterial isolation in cell culture and embryonated eggs took five to ten days (Vanrompay et al., 1992). At the time of writing, even with recent development of advanced molecular detection methods of *C. psittaci*, conventional PCR was still part of the detection methods alongside the main qPCR screening assays (Li et al., 2020). This is because conventional PCR is commonly paired up with Sanger sequencing to amplify longer amplicon size (up to 1000 bp) than qPCR (up to 200 bp) allowing species differentiation. In this review, of all the studies that performed Sanger sequencing, two thirds (64%; 43/67) performed conventional PCR prior to Sanger sequencing. Therefore, conventional PCR is still commonly practiced despite the increased availability of more sensitive molecular test like qPCR in the market.

As the most recommended *C. psittaci* detection method by WOAH (2018a), qPCR is the preferred method due to its high sensitivity, high throughput, rapidity, and potential for quantification. In this review, qPCR is the third most published molecular test to detect *C. psittaci* in birds, with an increasing usage after year 2010. To provide a temporal context, the first qPCR instrument was described in 1993 and was commercialised in 1996 (VanGuilder et al., 2008). The PCR technique was designed in 1983 and then the first automated PCR cycler was commercialised in 1988 with a rapid increase in availability in most diagnostic facilities in the next 20 years (VanGuilder et al., 2008). The qPCR principle is ten years younger than conventional PCR when it comes to its availability and application in the laboratories. To screen large sample sizes, real-time thermal cyclers with multi-well assay plate are available in 96, 384 or 1536 format (Bittker, 2012). With better sensitivity, the limit of detection (LOD) of the genus-specific qPCR protocol from Ehricht et al. (2006) are 1 plasmid DNA (copy number) and 56

chromosomal DNA (equivalent to 0.05 fg DNA or 1.87 IFU (inclusion forming units)) to detect *Chlamydiaceae,* similarly to that of an DNA microarray assay (Sachse et al., 2005). In contrast, conventional PCR and gel electrophoresis based protocols had a LOD of 1000 plasmid and chromosomal DNA (Ehricht et al., 2006). For species-specific qPCR, the protocol from Pantchev et al. (2009) had a LOD of 2 IFU. Unlike gel electrophoresis read-out systems that are laborious, qPCR produces both qualitative and quantitative results that can be visualised and analysed via the instrument software after a qPCR run of about 2 hours (Pantchev et al., 2009). When comparing to nested PCR (nPCR) of an equivalent sensitivity, qPCR of the closed system with only one reaction required less labour and ensured reduced contamination risk. However, qPCR system required specific instruments and sometimes fluorescent-labelled probe, which increased the cost per reaction (Sachse, Vretou, et al., 2009).

As the second most practiced molecular test, Sanger sequencing with three decades of gradual improvement, is a trusted technique with low error rate which is often used to validate the results from other molecular tests (Shendure & Ji, 2008). Although the accuracy is as high as 99.99%, the technique requires a high copy number of the target sequence (Shendure & Ji, 2008). Therefore, Sanger sequencing cannot analyse genomic DNA directly from the samples like conventional PCR, nPCR and qPCR. So, it is normally paired with any of the DNA amplification methods mentioned earlier to increase the target DNA to a sufficient quantity. Instead of the initial screening, Sanger sequencing was performed towards the end of the methodology on positive samples to provide DNA sequence information for confirmation or discovery of new species. For instance, Szymańska-Czerwińska et al. (2017) screened all of their 894 samples for avian chlamydia using qPCR, then the positive samples were subjected to the sequencing of three targets (16S rRNA, IGS-23S, *ompA* gene). Then, the sequences generated from Sanger sequencing were subjected to BLAST (Basic Local Alignment Search Tool) analysis to identify related sequences in the GenBank database (NCBI) (Altschul et al., 1990). Next, the sequences were aligned with a series of references sequences of Chlamydial species and strains of interest to create a dendrogram for phylogenetic analysis (Szymańska-Czerwińska et al., 2017). In our review, for Sanger sequencing, the most commonly performed genus-specific PCR protocol was from

Everett et al. (1999) targeting 16S rRNA, while the species-specific protocol was from Denamur et al. (1991) targeting *ompA* gene. With the cost of DNA sequencing reducing from about USD 5000 per megabase in 2001 to USD 0.01 in 2021 (Shendure & Ji, 2008; Wetterstrand, 2022), we recommend that PCR products from positive samples being submitted to Sanger sequencing when it is available, and submit the sequence data to NCBI, to improve data availability for comparative studies.

2.4.2 Implications for study purposes

Regardless of the study purposes, WOAH (2018a) had recommended a testing strategy using hierarchical approach to detect *C. psittaci*. In this review, we observed a twofold increase in practicing the hierarchical approach in 2011-2020 than in 2000-2010. From 2011-2020, emerging avian Chlamydial species were discovered in birds, like *C. avium, C. gallinacea* (Sachse et al., 2014), avian *C. abortus* (Szymańska-Czerwińska et al., 2017), *Ca. C. ibidis* (Vorimore et al., 2013) and *C. buteonis* (Laroucau et al., 2019). So, by initial screening of the samples with a genus-specific molecular test, we are more likely to detect the diversity of Chlamydial species in a bird population. However, by only using a targeted screening test for *C. psittaci*-specific, there is a chance the presence of atypical chlamydial species in birds will either not be detected or wrongly identified as *C. psittaci*. For instance, Laroucau et al. (2019) reported that a *C. psittaci ompA*-based qPCR was unable to detect *C. buteonis* isolates. On the other hand, Sachse et al. (2014) described the investigation of non-classified chlamydial species with the test results of *Chlamydiaceae*-positive but *C. psittaci*-negative, leading to the characterisation of the novel Chlamydial species – *C. avium* and *C. gallinacea*. We understand that the hierarchical approach using multiple molecular tests in a study is highly influenced by practical constraints like the budget of the study and availability of the tests especially in resource-limited settings. Also, for clinical disease, an additional genotypic differentiation of *C. psittaci* will not change the choice of antimicrobial treatment for *C. psittaci* infections in birds (Bommana & Polkinghorne, 2019). However, in psittacosis outbreaks in both humans and animals, genotyping of the PCR-positive samples from the epidemiologically linked people and animals may assist in source tracing (Nieuwenhuizen et al., 2018). Therefore, the hierarchical approach with both genus and

species differentiation is highly recommended for all study purposes, while genotypic differentiation can be done when required.

In cross-sectional surveys, mass screening is often used to identify the presence of pathogen in a population of apparently healthy individuals (Thrusfield & Christley, 2018). In this review, survey studies often involved large sample sizes (up to 1000 samples) and individual birds of lower pathogen load (Hulin et al., 2016). A qPCR protocol of genus-, species- and genotype-specific would be valuable to accommodate large sample size, low pathogen load, and produce rapid results. When diagnosing avian clinical cases, molecular tests assisted in early accurate diagnosis to ensure prompt targeted treatment for the avian patients. Due to its zoonotic potential, besides providing a definitive result of *C. psittaci* infection in birds, follow-up monitoring on the bird's bacterial shedding after antimicrobial interventions is also essential for public health significance of bird owners (Razmyar et al., 2016). Also, the cloacal shedding in a sick bird with avian chlamydiosis fluctuated along the course of infection (Thierry et al., 2016). This situation is when a molecular test with better sensitivity and ability to quantify bacteria is crucial to diagnose the disease in bird irrespective to its stage of disease. In an outbreak investigation, rapid molecular tests are useful in discriminating the infected animals and facilitating spread tracing to allow further epidemiological evaluation in a timely manner (Thrusfield & Christley, 2018). In an investigation of five dead parrots in a Polish walk-in aviary, qPCR assisted in identifying the pathogen in the outbreak, and later confirming the effectiveness of control measures and pathogen eradication (Szymańska-Czerwińska et al., 2018). In this case, a testing strategy of hierarchical approach using qPCR of genus-specific protocol from Ehricht et al. (2006), species-specific protocol from Pantchev et al. (2009), and genotype-specific protocol from Geens, Dewitte, et al. (2005) is recommended.

2.4.3 Detection target

The specificity of molecular tests to detect *C. psittaci* is closely related to its detection target, be it a gene or intergenic spacer region. As a component of the ribosome, ribosomal RNA (rRNA) has been used to study the phylogenetic relationships

of bacteria. It consists of highly conserved variable domains, has constant functions and presents in all bacteria (Vandamme et al., 1996). Generally, direct sequencing of partial or full-length 16S or 23S rRNA molecules via PCR provides taxonomic information from family level, genus level up to species level (Vandamme et al., 1996). For *Chlamydia*, 16S rRNA genes are 93 to 97% identical among the *Chlamydiaceae* family, leading to limited differentiation in terms of *Chlamydia* species and strains (Everett & Andersen, 1997). Similarly, from our review, we observed the investigation of 16S and 23S rRNA genes in various PCR methods, was mostly used to identify *Chlamydia* at the genus level. Exceptionally, the 16S rRNA gene was applied in two nPCR methods (Hotzel et al., 2005; Messmer et al., 1997) to identify *Chlamydia* at the species level, by using an additional pair of inner primers. For better sensitivity, Everett and Andersen (1997) used the IGS-23S rRNA gene coding the region of the 16S rRNA-23S rRNA intergenic spacer and 23S rRNA domain I, which successfully identified *Chlamydia* at species and strain level (serovar A, B, C, D, E). This segment of the rRNA operon contained highly conserved regions flanked by less conserved regions of species-specific sequence deletions or polymorphisms. The IGS-23S rRNA gene was applied in conventional PCR (Madico et al., 2000), qPCR (Nordentoft et al., 2011) and DNA microarray technology (Sachse et al., 2005).

As the most commonly used detection target, the *ompA* gene encodes major outer membrane protein which harbours both conserved regions and variable domains (Caldwell et al., 1981; Dutilh et al., 1989). The conserved regions encoded antigenic determinants targeting at genus and species level, while the variable domains (VD) encoded the serotype-specific epitopes, mainly VD2 and VD4 (Conlan et al., 1988). In early 1990s, the classification of serotypes were defined by monoclonal antibodies, which were later revealed to be corresponding to the genotyping classification based on *ompA* genes (Vanrompay et al., 1997). The heterogenous structure of *ompA* gene makes it a good candidate to differentiate *Chlamydia* at species and genotype level (Sachse, Vretou, et al., 2009). Besides the common genes, there were also PCR protocols using other genes such as *ompB* gene (Hartley et al., 2001; McElnea & Cross, 1999), and *pmp* gene (Laroucau et al., 2001). When tested on vaginal swabs from ewes, conventional PCR protocol from Laroucau et al. (2001) was 10 times more sensitive (limit of detection

of 0.005ng) than Denamur et al. (1991) that was targeting *ompA* gene. In Laroucau et al. (2007), when tested on the six major avian strains (serovars A-F) of *C. psittaci*, Laroucau et al. (2001) had similar or increased sensitivity on individual strain when compared to Everett and Andersen (1997), that was amplifying 16S–23S rRNA intergenic spacer.

2.4.4 Sampling and DNA extraction

Besides type of molecular tests, sampling methods can also affect the accuracy of a survey results. In this review, more than half of the studies collected samples from multiple anatomical sites. The type of sample collected is closely related to the pathogenesis of chlamydial infections, that includes adhesion, replication, spreading and finally bacterial shedding. For *C. psittaci* infection via aerosol, the primary adhesion and replication site is at the epithelial cells of upper respiratory tract with intense replication occurred in the lung (Vanrompay et al., 1995). Within 48 hours, the bacteria was detected in the circulation system, spreading to the kidney, reproductive organs, and digestive tract via septicaemia (Page, 1959; Vanrompay et al., 1995). Pharyngeal and cloacal shedding of *C. psittaci* in chickens was detected at day 2 and day 3 post-infection (Page, 1959; Yin et al., 2013). In diseased birds, WOAH (2018a) recommended to collect the samples in accordance to the clinical signs, like faeces, ocular and nasal exudates. Generally, in live birds, pharyngeal and nasal swabs are preferred, while cloacal and conjunctival swabs can also be taken (WOAH, 2018a). To detect the obligate intracellular Chlamydiae, swabbing of mucosal specimens can recover sufficient amount of infected host cells (Corsaro & Greub, 2006). Given that chlamydia can affect both the respiratory and digestive system, studies that collected only samples from a single shedding site may introduce sampling bias and thus incurring the possibility of false negative results. Therefore, collecting samples from multiple potential shedding sites allows better accuracy of results when determining *C. psittaci* status of birds.

For transport medium, we cannot make any conclusion from this review as there is no one most used type of transport medium in the studies. This is the same for transport temperature of the sample as only a small number of studies (n = 16) reported this information. Corsaro and Greub (2006) recommended that the chlamydial samples

should be stored at 4 to 8°C for immediate processing or at -70°C when processing happens after more than 24 hours. Although the storage conditions can influence DNA stability, the common practice of storing extracted DNA samples in frozen aqueous solutions (-20 or -80°C) still allowed DNA recoveries of about 100% after 2 years, keeping in mind that DNA degradation was accelerated after repetitive of multiple freeze-thaw processes (Matange et al., 2021). For instance, specimens with low pathogen load before freezing experienced a rapid loss of chlamydial DNA and became negative after cryostorage. Stabilising reagents to preserve the DNA should be considered when there is a delay in sample processing (DeGraves, Gao, & Kaltenboeck, 2003). In most cases, cloacal swab and faeces were more commonly collected to detect *C. psittaci* in birds due to its minimally invasive nature. However, DNA extraction from avian faeces may be more challenging as compared to mammals, due to its high content of uric acid from the mixing of urinary and faecal material in the cloaca (Eriksson et al., 2017). In addition, birds have huge variations in their diets, like granivore, omnivore and carnivore, which required different DNA extraction methods for better performance on microbial relative abundance and cell lysis capacity. For the detection of Chlamydiae, bead-based DNA extraction methods were the best to obtain better DNA yield and DNA integrity (Hou et al., 2021). For specific bird species, further optimisation of the commercial DNA extraction kit by adding specific pre-treatment protocols and slight modifications of the kit, ensured high-quality DNA output for downstream applications (Eriksson et al., 2017). Therefore, negative detection of *C. psittaci* in a bird population should be interpreted carefully, taking into consideration deficiencies in sample collection, sample handling and processing, and DNA extraction methods.

2.4.5 Limitations

This review did have some limitations. With the inclusion of only one language in our search strategy, the geographical distribution of studies included in this review is limited. For instance, most studies are from the European region, and they are predominantly high-income countries. Similar search results were reported by the systematic review done by Hogerwerf et al. (2020) even when they included six languages in their search strategy. In our review, this may reflect in the availability and

application of the newer molecular tests like qPCR to detect *C. psittaci* in birds in high-income countries. On the country level, of the two countries with the highest number of studies included, Belgium had a greater research and publication interest in psittacosis as it is a notifiable disease in the country (Harkinezhad et al., 2009), while China is proposing to include psittacosis into their list of notifiable disease (Liu et al., 2022). Besides, we did not contact the main authors for clarification of some details, thus excluding 25 potentially relevant studies with unidentified chlamydia species and 22 studies with insufficient information on laboratory methods.

Besides, data extraction was challenging when a reference for the molecular protocols described more than one protocols with multiple DNA targets. For instance, the conventional PCR protocols from Everett et al. (1999) targeted the 16S rRNA, IGS-23S and 23S rRNA genes, while the DNA microarray protocols from Sachse et al. (2008) targeted the full length *ompA* gene, partial *ompA* gene of VD1 and VD2, and partial *ompA* gene of VD3 and VD4. Sometimes, the primers of molecular protocols were adapted in a different molecular test than the one applied in the original reference. For example, Vilela et al. (2019) screened parrots for *C. psittaci* by using primers from DNA microarray protocols targeting at the partial *ompA* gene of VD1 and VD2 (Sachse et al., 2008) in conventional PCR. Also, Gedye et al. (2018) used the same set of primers from Sachse et al. (2008) in conventional PCR to amplify and sequence the partial length of *ompA* gene. In thirty-six studies, more than one protocol was used for one type of molecular tests. These details were entered separately in the supplementary tables in Appendices but summarised in the main table (**Table 2**) for presentation.

CHAPTER 3

Determination of chlamydiae carriage in shorebirds and bird handlers in New Zealand

CHAPTER 3 Determination of chlamydiae carriage in shorebirds and bird handlers in New Zealand

3.1 Introduction

Chlamydiae are Gram-negative, obligate intracellular bacteria within a single genus *Chlamydia* (*C.*) from the family *Chlamydiaceae.* They are detected in a wide host range, including human, livestock, companion, and wild animals (Borel et al., 2018). To date, the genus *Chlamydia* comprises 14 species, namely *C. abortus, C. avium, C. buteonis, C. caviae, C. felis, C. gallinacea, C. muridarum, C. pecorum, C. pneumoniae, C. poikilothermis, C. psittaci, C. serpentis, C. suis,* and *C. trachomatis* (Zaręba-Marchewka et al., 2020). Of all, *C. psittaci* is the most-studied avian chlamydial species and most well-documented zoonotic chlamydial species. In birds, clinical signs of avian chlamydiosis include conjunctivitis, respiratory distress, diarrhoea, anorexia, and weight loss (Andersen & Vanrompay, 2008). *C. psittaci* infection is commonly reported in pet birds like parrots and pigeons, and in domestic poultry like turkeys and ducks (Sachse, Laroucau, et al., 2015).

In free-living birds, chlamydial bacteria have been detected in at least 70 species of birds. In addition to the established wild hosts like the Psittaciformes and Columbiformes, the Corvidae and Accipitridae families with high *C. psittaci* prevalence, are emerging as significant hosts (Stokes et al., 2021). For the shorebirds (or waders) from the order Charadriiformes, molecular detection of chlamydiae was positive in at least 57 species from eight different families, including Alcidae, Burhinidae, Chionidae, Charadriidae, Haematopodidae, Laridae, Scolopacidae, and Stercorariidae. Of all, the two most studied families were Laridae (28 bird species) and Scolopacidae (13 bird species) (Aaziz et al., 2015; Blomqvist, Christerson, Waldenström, Herrmann, et al., 2012; Christerson et al., 2010; Herrmann, Rahman, et al., 2000; Isaksson et al., 2015; Jeong et al., 2017; Kalmar et al., 2014; Kasimov et al., 2021; Navarro et al., 2019; Padilla et al., 2006; Schwarzova et al., 2006; Szymańska-Czerwińska et al., 2017; Valdebenito et al., 2020).

New Zealand has two Ramsar sites (internationally important wetlands) that serve as significant stopovers for migratory shorebirds using the East Asian-Australasian Flyway – Firth of Thames and Manawatū estuary. This is where the arctic breeders – bar-tailed godwit (*Limosa lapponica*) and red knot (*Calidris canutus*) from family Scolopacidae spend their time during the non-breeding season (Riegen & Sagar, 2020). Bird migration can potentially contribute to the transmission of infectious disease across national and intercontinental borders when the birds act as the long-range carriers for any pathogen (Reed et al., 2003). Some of the major zoonotic pathogens of migratory birds in North America include West Nile virus, Influenza A virus, *Salmonella spp*., *Campylobacter jejuni* and *C. psittaci*. *C. psittaci* was the second highest priority of emerging infectious pathogens (one of the nine non-foodborne pathogens), listed by the United States National Institute of Allergy and Infectious Diseases (NIAID, 2018; Reed et al., 2003). Disease surveillance for avian influenza in both arctic breeders was performed in New Zealand from 2004 to 2010 and found no evidence of avian influenza carriage (Watts et al., 2016). However, there has been no prior surveillance conducted for *C. psittaci* in migratory shorebirds in New Zealand.

Wild birds with *C. psittaci* infection can be a source of human psittacosis, besides the more common source of infection from pet birds and poultry (Nieuwenhuizen et al., 2018). Zoonotic transmission normally occurs via direct mouth-to-beak interactions, handling of infected birds and their tissues, inhalation of infectious aerosols, or by environmental exposure to contaminated excretion (West, 2011). With an incubation period of 5 to 14 days, the clinical presentation of psittacosis in humans varies from inapparent and mild flu-like symptoms, to sometimes fatal systemic disease with severe pneumonia (Balsamo et al., 2017). For psittacosis occurring via direct handling of sick wild birds, two cases have been reported, one in an Australian veterinary clinic, and another in a Belgian wild bird refuge centre (Branley et al., 2008; Kalmar et al., 2014). For non-direct contact with wild birds, zoonotic transmission of *C. psittaci* has been identified via inhaling the infectious aerosols from pigeons' excrements on buildings and monuments in Europe (Haag-Wackernagel, 2006; Magnino et al., 2009), and lawn mowing over the droppings or the dead wild birds in Australia (Telfer et al., 2005; Williams et al., 1998). From the limited publications on psittacosis linking to free-living

birds, most reports described outbreak investigation, but not *C. psittaci* surveillance of wild birds and human in the same setting. Unlike *C. psittaci* zoonotic risk assessment that was commonly performed in poultry settings (Dickx, Geens, et al., 2010), it is insufficiently studied in any wild birds, including wild aquatic birds, and their handlers.

In New Zealand, positive detection of *C. psittaci* in birds has been mainly in Psittaciformes, Columbiformes, Passeriformes, and Anseriformes (Gartrell et al., 2013; Gedye et al., 2018; Soon et al., 2021). In 2011, using a PCR method targeting 16S rRNA gene, one adult female hihi (*Notiomyces cincta*) (from a sample of 10 birds) was *C. psittaci*-positive (Gartrell et al., 2013). Recently in 2016, 24% (24/100) of the wild mallards (*Anas platyrhynchos*) were tested positive in high-resolution DNA melt quantitative PCR (qPCR-HRM) analysis (Soon et al., 2021). For human psittacosis in New Zealand, three surveys of zoonotic infections among Auckland Zoo staff in 1991, 2002 and 2010, showed low-levels of antibodies indicating past exposure to chlamydiae in 7 (n = 49), 1 (n= 42), and 3 (n = 46) zoo staff, respectively (Forsyth et al., 2012). In 2010, a veterinarian had severe psittacosis with meningitis, pneumonia, and hepatitis, after occupational exposure to an asymptomatic cockatiel (Rawdon, 2010). Human psittacosis is not a notifiable disease in New Zealand; but it is notifiable in the United States of America, Australia, and most European countries: Belgium, Denmark, and Germany (Harkinezhad et al., 2009). Worldwide, reported human psittacosis cases range from 0.05 to 8.61 cases per million people. In Australia, it occurs at a ratio of 5.54 cases per million people (Harkinezhad et al., 2009). According to the World Organisation of Animal Health (WOAH), in Australia, reported psittacosis were 9 cases in 2018, 21 cases in 2017 and 19 cases in 2016 (WOAH, 2018b). In New Zealand, *C. psittaci* is not routinely tested for among patients with acute respiratory illness, hence it could be under-diagnosed and under-reported.

A One Health surveillance system using transdisciplinary approaches and collaborative efforts from multi domains is essential to efficiently manage zoonotic diseases at the human-wildlife interface (Bordier et al., 2020). *C. psittaci* is transmissible through a multi-host system with an ability to cross boundaries between wildlife, livestock, and humans; affected by the conditions of pathogen, host, environment, and

socioeconomic factors. By using a One Health approach to study *C. psittaci* infections as a multi-host system, the epidemiological interactions at human-wildlife interface can be incorporated into disease models and management strategies for early detection and rapid response (Miller & Olea-Popelka, 2013). An example of pathogen flow of *C. psittaci* at wildlife-livestock-human interface was reported as a psittacosis outbreak in Australia in 2003. In this outbreak, *C. psittaci* was cultured from farm ducks housed in the open growing sheds, that had been exposed to wild waterfowl. This cluster of bird cases was linked to a psittacosis outbreak of 10 cases from 61 abattoir workers, with 5 being hospitalised, in a 5 months period (Tiong et al., 2007). To date, most epidemiological studies of *C. psittaci* in humans were initiated following a psittacosis outbreaks, with a lack of active surveillance in both humans and birds.

The aim of this study was to perform a survey at four estuarine sites, to estimate the prevalence of chlamydiae in both shorebirds and the people that handle them in New Zealand. These four estuarine sites are important habitat for shorebirds of various species, including domestic and international migrants (Riegen & Sagar, 2020).

3.2 Materials and methods

3.2.1 Ethics

The study methodology for live birds was in accordance with the Code of Ethical Conduct for the Use of Live Animals for Research, Testing and Teaching and was approved by the Animal Ethics Committee at Massey University, Palmerston North, New Zealand (MUAEC protocol number 19/27). Permission to collect samples from shorebirds species protected under the Wildlife Act 1953 was granted by Department of Conservation, New Zealand (permit 71359-FAU). Iwi consultation was conducted with the members of Te Awahou hapū and Rangitane o Manawatū.

The study protocol involving people was in accordance with the Code of Ethical Conduct for Research, Teaching & Evaluations Involving Human Participants and was

approved by Human Ethics Committee at Massey University, Palmerston North, New Zealand (MUHEC protocol number SOA 20/38).

3.2.2 Study site and target population

3.2.2.1 Study site

In this study, the four study sites were the geographical locations used for annual banding studies of waders in New Zealand (**Figure 3.1**). These estuarine sites support a large population of both migratory and resident waders, while having accessible coastal tidal flats that allow bird capture for banding during summer. Two of the study sites were in North Island, while the other two were in South Island. In the North Island, our study sites were at the Firth of Thames (37°09'56.3"S, 175°18'58.8"E) in the Waikato region and at Manawatū estuary (40°28'02.2"S, 175°13'48.0"E) in the Manawatū-Whanganui region. In the South Island, our study sites were at Pakawau Beach, Golden Bay (40°35'55.3"S, 172°41'15.1"E) and Moturoa Rabbit Island, Tasman Bay (41°15'46.8"S, 173°08'46.1"E) (**Figure 3.2**), both in Nelson region. Sampling was carried out in October to November 2019 and January to April 2021.

Figure 3.1 Locations of the four study sites selected for chlamydiae survey in shorebirds in New Zealand. (Retrieved May 5, 2022, from https://www.google.com/maps)

Figure 3.2 Photograph of a flock of South Island Oystercatchers about 300 to 400 birds (black and white dots in the middle of the photo) in Moturoa Rabbit Island, Tasman Bay, Nelson, New Zealand. (Photo taken on 15th April 2021)

3.2.2.2 Shorebirds' population

The seven species of shorebirds that were included in this study were bar-tailed godwit (*Limosa lapponica*), red knot (*Calidris canutus*), wrybill (*Anarhynchus frontalis*), South Island pied oystercatcher (SIPO; *Haematopus finschi*), variable oystercatcher (*Haematopus unicolor*), pied stilt (*Himantopus leucocephalus*), and black-backed gull (*Larus dominicanus*). Shorebirds in this study were categorised into three main groups, namely the arctic migrants, domestic migrants, and residents.

The arctic migrants included in this study were the bar-tailed godwits and red knots. They travel from the tundra of Alaska and Siberia and arrive at New Zealand in mid-September to early October, via the East Asian-Australasian flyway (Williams et al., 2006). To date, bar-tailed godwit is the most abundant while red knot is the second most abundant arctic breeding shorebird species that visits New Zealand yearly. The domestic migrants like South Island pied oystercatcher (SIPO) and wrybill, travel within New Zealand, from the inland breeding grounds in South Island to the non-breeding coastal areas in North Island (Sagar & Geddes, 1999; Sagar et al., 2000). The resident birds reside and breed in the same area throughout the year. They are the pied stilt, variable oystercatcher, and black-backed gulls (Heather & Robertson, 2015).

The population size of shorebirds is monitored by Birds New Zealand (formerly the Ornithological Society of New Zealand (OSNZ)) by conducting the biannual national shorebird counts at 35 major estuaries in North Island and 39 major estuaries in South Island, from 2005-2019 (Riegen & Sagar, 2020). Based on their shorebird counts, the estimated population of bar-tailed godwit in New Zealand was about 82,000 birds, and red knot was around 29,000 birds (Riegen & Sagar, 2020). Based on the OSNZ shorebird counts done in 2008-2012, the estimated population of SIPO in New Zealand was 79,000 birds, wrybill was 5,300 birds, pied stilt was 30,000 birds, and variable oystercatcher was 6,000 birds (Heather & Robertson, 2015). In detail, the average count of shorebirds sighted in summer 2005-2019 (except wrybill counts collected during winter), in the four estuarine sites included in this study were shown in **Table 3.1** (Riegen & Sagar, 2020).

Table 3.1 Shorebird counts at four estuarine sites in New Zealand during summer* 2005-2019.

Family	Bird species	North Island		South Island	
		Thames	Manawatū	Golden	Tasman
	Arctic Migrants				
Scolopacidae	Bar-tailed godwit (*Limosa lapponica*)	6425	-	2227	4007
	Red knot (*Calidris canutus*)	3257	-	-	721
	Domestic Migrants				
Haematopodidae	South Island pied oystercatcher (SIPO; *Haematopus finschi*)	1441	-	1881	961
Charadriidae	Wrybill (*Anarhynchus frontalis*)	2164	19	-	44
	Residents				
Recurvirostridae	Pied stilt (*Himantopus leucocephalus*)	746	-	-	-
Haematopodidae	Variable oystercatcher (*Haematopus unicolor*)	107	-	157	343

Note. Adapted from Riegen and Sagar (2020); -, data not available.
*All bird counts were a mean of (>2,000) bar-tailed godwits, (>500) red knot, (>500) SIPO, (>300) pied stilt, and (>100) variable oystercatcher counted during summer, while (>10) wrybill counted during winter 2005–2019.

3.2.2.3 Human participants

The participants were invited to consent and participate in this study at the end of a shorebird banding event. The participants recruited in this study included personnel working closely with shorebirds, regardless of the regularity of their bird handling. Some of them were experienced banders, trainee banders, researchers, and volunteers. At the banding event, personnel were often assigned with multiple tasks, but most participants had close contact with the birds in the capture or processing of the birds (**Figure 3.3**) before the people were sampled.

Figure 3.3 Photograph of participants handling and banding the birds in a group of five. (Photo was taken at the Firth of Thames, New Zealand on 15th January 2021.)

3.2.3 Sample collection

3.2.3.1 Bird sampling

Shorebirds were mostly caught using cannon netting, however, on one occasion, at the Firth of Thames, mist netting was used. After being captured, birds were removed from the net for processing and sampling out of the holding boxes, one at a time. After weighing the bird using bird bag and Pesola scale, the bird was passed to the dedicated bander. The bill length and wing length was measured using calliper and wing ruler. Finally, birds without a band were banded using metal bird bands and coloured wrap-around bands. The whole process took around 10 to 15 minutes.

After routine measurement and banding processes, the birds were gently restrained by wrapping in a towel. Samples were collected separately from the choanal slit and cloaca of each bird using individual sterile plain dry swabs, with rayon tip and plastic shaft in labelled tubes (CLASSIQSwabs; Copan Diagnostics, Corona CA, USA). The samples were then held on ice before being transported to and processed at the Hopkirk

Research Institute, Massey University, Palmerston North. There, the samples were stored at 4°C until DNA extraction was performed within 72 hours of receipt.

3.2.3.2 Human sampling

Participants provided a self-taken nasal swab. A small, soft-tipped rayon swab (CLASSIQSwabs; Copan Diagnostics, Corona CA, USA) was inserted into each of the nostrils with a slow and steady motion. Then, sideways pressure was placed on the swab to collect epithelial cells from the nasal mucosa. Next, the swab was withdrawn with a rotating motion for a few times until it is covered in mild secretions. Finally, the swabs were transported and stored the same way as the bird samples until further processed. Participants were sampled once.

3.2.4 DNA extraction

Genomic DNA was extracted from the swabs using a Chelex® 100 extraction protocol, with minor modification (Martin-Platero et al., 2010). The individual swab was placed in a 1.5ml Eppendorf tube with 100 µl MilliQ water and left to resuspend for 5 minutes. An additional 50-µl MilliQ water was added into the tube if the swab absorbed all the water. The swab was removed after vortexing the tube for 5 seconds twice and the tube was centrifuged at 14000xG for 3 minutes. The supernatant was discarded, leaving approximately a 10 µl solution of cells in the tube. After adding 40 µl of 6% Chelex® 100 w/v (Bio-Rad, Hercules, CA, USA) and 1 µL of Proteinase K 20 mg/mL (Roche, Switzerland), the tube was vortexed for another 10 seconds. This mixture was incubated for 1 hour at 56°C, boiled for 8 minutes and vortexed again for 10 seconds. The samples were stored in the freezer at -20°C for later use in qPCR-HRM analysis. Prior to the analysis, the samples were thawed in room temperature and centrifuged at 14000 RCF (Relative Centrifugal Force) for 3 minutes. The supernatant of the samples contained the DNA was used as the template in the analysis, leaving the Chelex® settled at the bottom of the tube.

3.2.5 Quantitative PCR (qPCR) and HRM analysis (qPCR-HRM)

Primers previously designed by Sachse et al. (2008), which amplify the variable domain one and two region (418bp) of the *C. psittaci* outer membrane protein A gene (*ompA*), were used to develop a high-resolution melt (HRM) analysis quantitative PCR protocol. Primers VD1-f (5'-ACT ACG GAG ATT ATG TTT TCG ATC GTG T-3') and VD2-r (5'-CGT GCA CCY ACG CTC CAA GA-3') were obtained from IDT (Iowa, USA), and used to amplify the *ompA* variable domain one and two region under the following conditions: 1x HOT FIREpol® EvaGreen® HRM master mix (Solis Biodyne, Tartu, Estonia), 200nM of each primer and 2 µl of DNA template in a final volume of 20µl. Quantitative PCR (qPCR) was performed in a Rotor-Gene Q qPCR Cycler (QIAGEN, Hilden, Germany) under the following cycling conditions: activation cycle at 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 20 seconds, with data acquired on the green channel. HRM was pre-conditioned at 95°C for 60 seconds and 40°C for 60 seconds while HRM analysis was performed between 70°C and 90°C in 0.3°C/second increments. Water blanks were included as negative controls to validate a lack of contamination throughout the process. Positive controls and a standard curve were produced from previously cloned *C. psittaci* genotype A provided by K. Gedye (SoVS, Massey University, NZ). *C. psittaci* genotype A was isolated from an Alexandrine parakeet in New Zealand. The standard curve was produced from the titration of the cloned *C. psittaci* genotype A at 10-fold serial dilutions from 0.1 ng to 1×10^{-8} ng.

3.2.6 Interpreting qPCR-HRM results

Detection was determined by both the melting temperature (Tm) in melt-curve analysis and the quantification cycle (Cq) in the qPCR-HRM run. Based on the expected range of melting temperature (Tm) for *C. psittaci* from Soon et al. (2021) (genotype A, Tm 86.36 - 87.36°C; genotype C, Tm 84.81 - 85.81°C), melt curves with peaks at Tm 83 - 88°C were considered positives in this study. Samples with melting temperature within the range (Tm 83 - 88°C) and a quantification cycle (Cq) less than 38 in the qPCR-HRM run were deemed positives. All the bird samples were tested twice to screen for the presence of *C. psittaci*. Positive samples from any of the two qPCR-HRM screening were

tested again. All human samples were tested thrice on the same qPCR-HRM assay. All samples with at least one positive (meeting both Tm and Cq requirement) in the qPCR-HRM run were classified as positives.

When analysing the melt curve of the samples in this study, a non-specific product (Tm <80°C), most probably primer dimer, was observed in the reaction. It could be clearly distinguished from the desired PCR product due to its lower melting temperature (Tm value). The presence of primer dimer peak in positive samples could possibly be due to low target DNA. For instance, we provided the examples of positive and negative detection in both bird samples (**Figure 3.4**) and human samples (**Figure 3.5**). In this study, these three melt curves were considered as negative samples: no peak (b411), one peak at Tm <80°C (b398), and multiple peaks not within the range of Tm 83 - 88°C (b409) (Figure 3.4). For positive bird samples, we observed melt curves with one peak in sample b388 (Tm = 83.35°C) and sample b394 (Tm = 85.75°C) and melt curve with multiple peaks in which each was primer dimer (Tm = 77.74°C) and target product (Tm = 85.6°C) in sample b397 (Figure 3.4). Similarly, for human samples, the three negative samples produced melt curves with no peak in sample h22, one peak (Tm = 77.05°C) in sample h17, and multiple peaks (Tm = 77.65°C, 81.04°C) in sample h27. For positive human samples, the examples here were melt curves with one peak (Tm = 87.85°C) in sample h4, and multiple peaks (Tm = 77.2°C, 85.06°C, 87.4°C) in sample h18 (Figure 3.5).

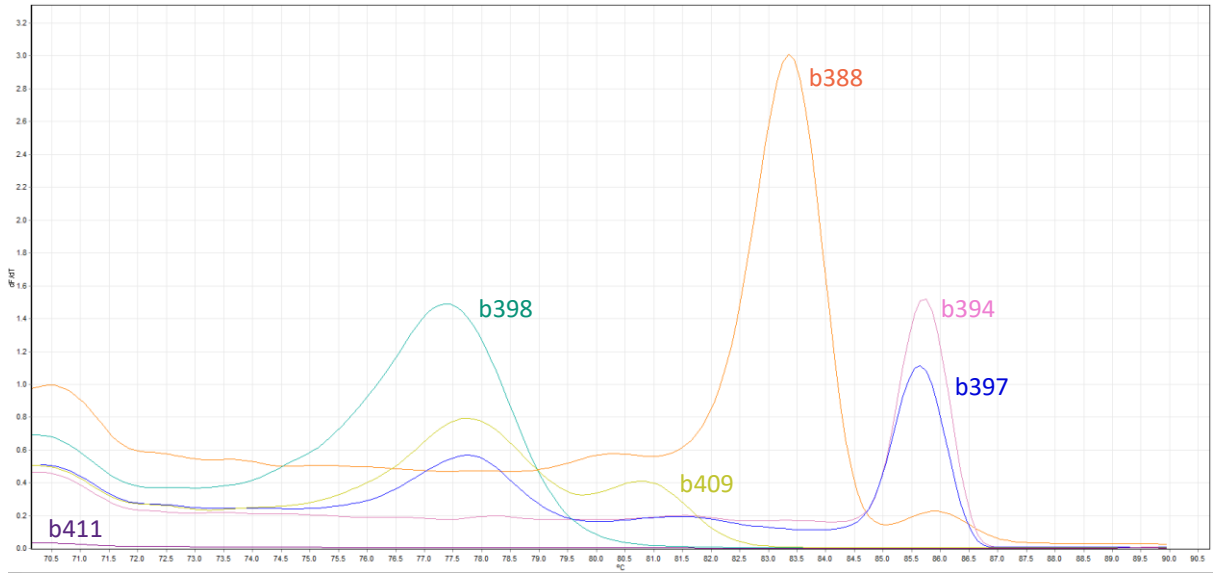

Figure 3.4 Melt curves of fluorescence (-dF/dT) against melting temperature (Tm) from qPCR-HRM of *C. psittaci ompA* gene from bird samples. Negative samples: amplicon from sample b411 produced no peak, sample b398 produced one peak (primer dimer), and sample b409 produced two peaks (primer dimer and Tm not within target range Tm 83 - 88 °C). Positive samples: amplicon from sample b388 produced one peak (Tm = 83.35°C) and sample b394 (Tm = 85.75°C), and sample b397 produced two peaks (primer dimer, Tm = 77.74°C; target product, Tm = 85.6°C). (HRM run 22)

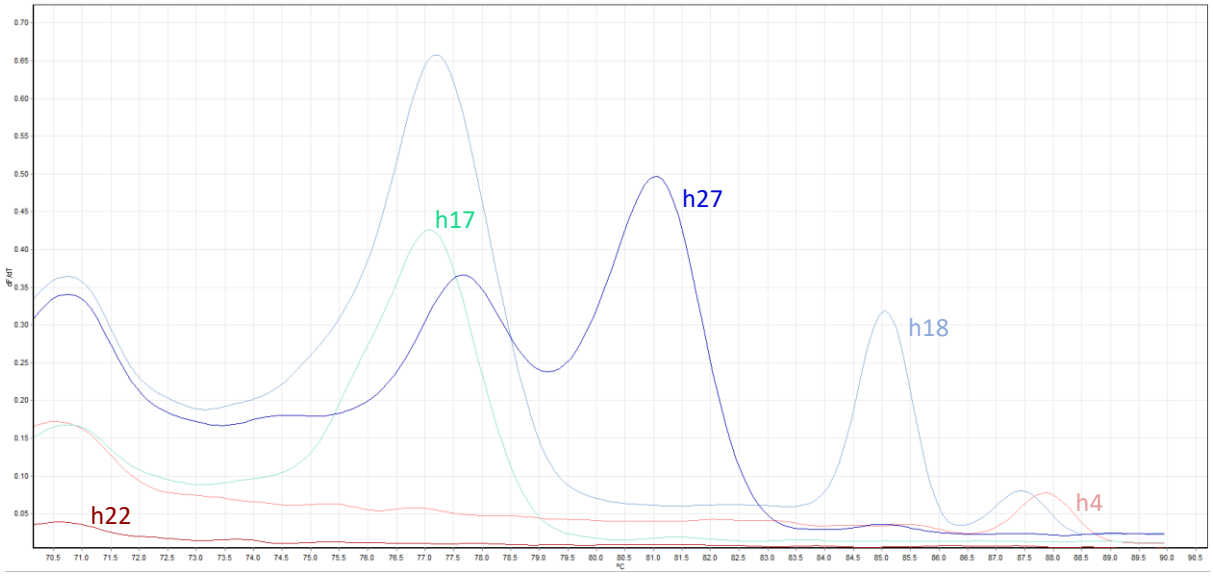

Figure 3.5 Melt curves of fluorescence (-dF/dT) against melting temperature (Tm) from qPCR-HRM of *C. psittaci ompA* gene from human samples. Negative samples: amplicon from sample h22 produced no peak, sample h17 produced one peak (primer dimer), and sample h27 produced two peaks (primer dimer and Tm not within target range Tm 83 - 88 °C). Positive samples: amplicon from sample h4 produced one peak (Tm = 87.85°C), and sample h18 produced multiple peaks (primer dimer, Tm = 77.2°C; target product, Tm 85.06°C, Tm 87.4°C). (HRM run 18)

3.2.7 Statistical analysis

The statistical analyses were conducted in R (R Core Team, 2017). The apparent prevalence for positive detection of chlamydiae and the 95% confidence interval was established for individual bird species. Correlation between dependent variables of interest such as bird species, the apparent prevalence of chlamydiae shedders among bird species was calculated using the chi-squared test with Bonferroni correction. The odds ratios were also calculated to assess the likelihood of detection of chlamydiae in different bird species. The bird species with a sample size less than thirty birds were excluded from statistical analysis.

3.3 Results

A total number of 320 birds and 27 people were sampled from the four estuarine sites in New Zealand.

3.3.1 Shorebirds' sampling

A total of 640 swabs were collected from 320 birds of seven bird species. At sample level, both choanal and cloacal samples were successfully collected from all birds sampled. At bird level, out of the 320 birds, there were 39 bar-tailed godwits (*Limosa lapponica*) from Firth of Thames and six from Manawatū Estuary, 10 red knots (*Calidris canutus*) from Firth of Thames, 126 South Island pied oystercatchers (SIPO; *Haematopus finschi*) from Firth of Thames, 30 from Golden Bay and 21 from Tasman Bay, 21 wrybills (*Anarhynchus frontalis*) from Firth of Thames, 63 pied stilts (*Himantopus leucocephalus*) from Manawatū Estuary, one variable oystercatchers (*Haematopus unicolor*) from Firth of Thames and two from Tasman Bay, and one black-backed gull (*Larus dominicanus*) from Firth of Thames. DNA was extracted from all 640 swabs and subjected to *Chlamydiaceae*-specific qPCR-HRM targeting *ompA* gene.

3.3.2 Chlamydiae detection in shorebirds

Results of *Chlamydiaceae*-specific qPCR-HRM for seven bird species are presented in **Table 3.2** and **Table 3.3**. At sample level, among 640 samples, 74 were chlamydiae-positive, with 23 positives (23/320; 7%; 95% CI 4-10%) from choanal swabs and 51 positives (51/320; 16%; 95% CI 12-20%) from cloacal swabs. When comparing different anatomical locations, the positive detection from cloacal swabs was higher (χ^2 = 11.139; *df* = 1, $p < 0.001$) than from choanal swabs.

Of all the 74 positive swabs, not all samples showed positive result in all three qPCR-HRM runs. Most samples (n = 55) were positive once out of the three qPCR-HRM run, while the rest were positive twice (n = 12) or thrice (n = 7). Of all the chlamydiae-positive choanal swabs, the samples were from bar-tailed godwit (n = 1), red knots (n = 2), SIPOs (n = 7), pied stilts (n = 12), and black-backed gull (n = 1). For chlamydiae-positive cloacal swabs, the samples were from bar-tailed godwits (n = 16), red knots (n = 2), SIPOs (n = 11), pied stilts (n = 18), and wrybills (n = 4). Of all the 74 positives, only three birds had chlamydiae detected from both choanal and cloacal swabs. All the three birds were pied stilts from Manawatū Estuary.

The results translate to a total of 71 chlamydiae-positive (71/320; 22%; 95% CI 18-27%) birds. The highest percentages of chlamydiae-positive swabs were obtained from two bird species: pied stilt (27/63; 43%; 95% CI 31-55%) and bar-tailed godwit (17/45; 38%; 95% CI 24-52%). The positives from pied stilts were from either the choana (n = 12) or cloaca (n = 18). For bar-tailed godwits' most positive samples were from the cloaca (n = 16) and only one sample was positive from the choana (n = 1). As the bird species with the largest sample size in this study, the percentage of positive samples in SIPOs was 10% (18/177; 95% CI 6-15%) with the positive swabs from either the choana (n = 7) or cloaca (n = 11).

The four bird species with sample sizes below thirty were red knot, wrybill, variable oystercatcher, and Southern black-backed gull. From the ten red knots sampled, chlamydiae were detected in four birds (4/10; 40%; 95% CI 10-70%), in which

two were from choana and the other two were from cloaca. The percentage of positive samples in wrybills was 19% (4/21; 95% CI 2-36%) and all positives were from cloacal swabs. In the only Southern black-backed gull sampled, chlamydiae was detected in the choanal swab. Chlamydiae were not detected in the three variable oystercatchers sampled.

3.3.3 Statistical analysis of apparent chlamydiae prevalence in shorebirds

The chi-squared test showed statistically significant differences of chlamydiae prevalence between bird species, for SIPOs and bar-tailed godwits ($p = 0.0002$), and between SIPOs and pied stilts ($p < 0.001$). The odds ratio (OR) revealed that the odds of chlamydiae shedding in bar-tailed godwits was 4.8 times (OR = 4.8; 95% CI 2.1-11.2) higher than in SIPOs. Similarly, the probability of chlamydiae shedding in pied stilts was 6.2 times (OR = 6.2; 95% CI 3.0-13.2) higher than in SIPOs.

Table 3.2 The positive detection of chlamydiae in two anatomical sites of shorebirds in New Zealand using qPCR-HRM of partial *ompA* gene.

Swab site	Positive	Total	Prev	95% CI
Choanal	23	320	0.07	0.04-0.1
Cloacal	51	320	0.16	0.12-0.2
Total	74	640	0.12	0.09-0.14

Table 3.3 The positive detection of chlamydiae in shorebirds in New Zealand at bird level using qPCR-HRM of *ompA* gene.

Bird species	**North Island**		**South Island**		**Total**		
	Thames	Manawatū	Golden	Tasman	Pos/N	Prev	95% CI
Arctic Migrants							
Bar-tailed godwit (*Limosa lapponica*)	13/39	4/6	-	-	17/45	0.38	0.24-0.52
Red knot (*Calidris canutus*)	4/10	-	-	-	4/10	0.40	0.10-0.70
Domestic Migrants							
South Island pied oystercatcher (*Haematopus finschi*)	15/126	-	1/30	2/21	18/177	0.10	0.06-.015
Wrybill (*Anarhynchus frontalis*)	4/21	-	-	-	4/21	0.19	0.02-0.36
Residents							
Pied stilt (*Himantopus leucocephalus*)	-	27/63	-	-	27/63	0.43	0.31-0.55
Variable oystercatcher (*Haematopus unicolor*)	0/1	-	-	0/2	0/3	0	0
Southern black-backed gull (*Larus dominicanus*)	1/1	-	-	-	1/1	1	1
Total (Pos/N)	37/198	31/69	1/30	2/23	**71/320**	0.22	0.18-0.27
Total (Prev)	0.19	0.45	0.03	0.09			
Total (95% CI)	0.13-0.24	0.33-0.57	0-0.1	0-0.2			

Note. Pos/N, positive/total number; Prev, prevalence; 95%CI, 95% confidence interval; -, samples not collected.

3.3.4 Infection prevalence of chlamydiae in shorebird handlers

Nasal swabs were collected from 27 people handling shorebirds. Of all, nineteen of them were collected at the Firth of Thames, one at the Manawatū Estuary, and seven at Golden Bay. In total, 14 out of 27 (52%; 95%CI 33-71%) nasal swabs showed positive detection of chlamydiae (**Table 3.4**). Most samples (n = 8) were positive once out of the three qPCR-HRM run, while the rest were positive twice (n = 3) or thrice (n = 3). There were eleven chlamydiae-positive samples from the participants at the Firth of Thames, two from Golden Bay, and one from Manawatū Estuary. Of all the positives, only four samples showed positive result on all three qPCR-HRM runs. The rest showed positive once (n = 7) or twice (n = 3) on the qPCR-HRM runs.

Table 3.4 The positive detection of chlamydiae in shorebird handlers and shorebirds in New Zealand using qPCR-HRM targeting partial *ompA* gene.

Location	Date	Human sample	SIPO	Godwit	Pied Stilt	Wrybill	Red knot	Gull
North Island								
Firth of Thames	15 Jan 2021	10/12	9/10	2-	-	-	-	-
	2 Mar 2021	1/7	0/2	2/5	-	0/7	1/1	1/1
Manawatū estuary	2 Feb 2021	1/1	-	4/6	27/63	-	-	-
South Island								
Golden Bay	14 Apr 2021	2/7	1/30	-	-	-	-	-
Total		14/27						

Note. n/N, positive/total number; human sample, nasal swab; SIPO, South Island pied oystercatcher (*Haematopus finschi*); godwit, bar-tailed godwit (*Limosa lapponica*); pied stilt (*Himantopus leucocephalus*); wrybill (*Anarhynchus frontalis*); red knot (*Calidris canutus*); gull, black-backed gull (*Larus dominicanus*); -, samples not collected.

3.4 Discussion

The prevalence of chlamydiae shedders among shorebirds (Charadriiformes) in this study was 22% (71/320). When comparing with other studies, at the bird order level, the reported prevalence of chlamydiae detection in Charadriiformes varied in different studies, with a range from 2% to 18%. Specifically, the prevalence was reported as 2% (2/106) in Sweden (Blomqvist, Christerson, Waldenström, Herrmann, et al., 2012), 3% (1/37) in a Subantarctic Island (Bird Island, South Georgian archipelago) (Herrmann, Rahman, et al., 2000), 3% (2/63) in Poland (Szymańska-Czerwińska et al., 2017), 5% (10/219) in Spain (Valdebenito et al., 2020), 10% (55/554) in United States of America (Christerson et al., 2010), 11% (15/142) in France (Aaziz et al., 2015), and 18% (46/257) in Antarctic Peninsula (Base Bernardo O'Higgins, King George Island, and Livingston island) (Isaksson et al., 2015). It is important to note that the reported prevalence values might not be directly comparable due to differences in the sampling methods, sample sizes, detection techniques, geographic locations, and host species of the studies. In this case, all the studies mentioned above collected cloacal swabs and performed qPCR with high sensitivity, except for Herrmann, Rahman, et al. (2000), that collected faeces and performed nested PCR. Also, the annual variations in climate, food availability and population density may affect the detection rates of chlamydiae in shorebirds. For instance, higher detection rates may occur when the bacteria survive better in warmer climates, increased pathogen transmission may occur in flocks with higher bird population densities due to successful breeding season, and scarcity in food resources may encouraging encourage birds to congregate closer to each other hence promoting bacterial spread. Due to low sample sizes and species variations between years, we were unable to determine if an annual effect was present in the birds we sampled. Future research in this area should aim to collect comparable data sets between years.

In our study, the prevalence of shorebirds shedding chlamydiae in New Zealand (22%) was slightly higher than the reported prevalence from other countries (2-18%) (Aaziz et al., 2015; Blomqvist, Christerson, Waldenström, Herrmann, et al., 2012; Christerson et al., 2010; Herrmann, Rahman, et al., 2000; Isaksson et al., 2015; Szymańska-Czerwińska et al., 2017; Valdebenito et al., 2020). This could be due the

sample collection of two separate swabs (choanal and cloacal) from each individual birds in this study, as compared to single swab (cloacal) collection in the other studies. In our study, of all the 71 chlamydiae-positive birds, only three birds had positive detection in both choanal and cloacal swabs, while the rest had positive detection in one swab. As a more commonly collected sample, cloacal swab did yield more positive detections than choanal swab in our study. Generally, chlamydiae can affect both the respiratory and digestive system, resulting in both pharyngeal and cloacal shedding in birds (Page, 1959; Yin et al., 2013). So, when possible, collecting samples from multiple potential shedding sites can reduce sampling bias and the following potential false negative results, thus improving the accuracy of determining pathogen occurrence and chlamydiae status in birds.

At the bird family and species level, this study reported the first detection of chlamydiae in six bird species from five families namely South Island pied oystercatcher (Haematopodidae), wrybill (Charadriidae), pied stilt (Recurvirostridae), southern black-backed gull (Laridae), bar-tailed godwit, and red knot (Scolopacidae). In our study, chlamydiae detection in South Island pied oystercatcher (SIPO) revealed a low prevalence of 10% (18/177). In published articles, positive detection in Haematopodidae was only reported in Australian pied oystercatcher (*Haematopus longirostris*) (n= 1) (Kasimov et al., 2021), and negative detection in Eurasian oystercatcher (*Haematopus ostralegus*) (n = 1) (Kalmar et al., 2014). For Charadriidae, we reported chlamydiae detection of 19% (4/21) in wrybill. In published articles, positive chlamydiae detection in Charadriidae were reported in northern lapwing (*Vanellus vanellus*) (1/1) in Belgium (Kalmar et al., 2014), Kentish plover (*Charadrius alexandrines*) (10/219; 5%) in Spain (Valdebenito et al., 2020), and masked Lapwing (*Vanellus miles*) (1/8) in Australia (Kasimov et al., 2021). Although at the bird family level, our results for Charadriidae was higher than the study in Spain (Valdebenito et al., 2020), with both studies performing qPCR, only cloacal swabs were collected in the study in Spain. Also, host species differences should be taken into consideration (Stokes, Martens, Walder, et al., 2020).

For the resident or nomadic shorebirds, the detection of 43% (27/63) chlamydiae-positive pied stilts in our study showed the first detection of this bacteria in

both the species and family. There were no previous studies of chlamydiae detection in Recurvirostridae, but only positive serology result using complement fixation test in Spain (Astorga et al., 1994). The chlamydiae detection in the only southern black-backed gull (Laridae) in this study was also the first detection in this species. As the most-studied shorebird family (n = 28 species), the chlamydiae prevalence in Laridae ranged from 3% (3/100) in Franklin's gulls (*Leucophaeus pipixcan*) (Isaksson et al., 2015), to 14% (11/81) in European herring gulls (*Larus argentatus*) (Aaziz et al., 2015), 15% (22/146) in Glaucous-winged gulls (*Larus glaucescens*) (Christerson et al., 2010), and 34% (33/96) in Kelp gulls (*Larus dominicanus*) (Isaksson et al., 2015). Since Laridae consists of the largest number (n = 99 bird species) of bird species in Charadriiformes (Billerman et al., 2022), there is more yet to be discovered regarding chlamydiae detection in this family. The types of shorebird species being selected as study subjects for chlamydiae detection varies greatly between studies, probably due to convenience sampling based on availability, and geographic distribution of certain bird species. Therefore, studies including more shorebird species with larger sample size are warranted, to identify the true prevalence of chlamydiae in specific shorebird species.

For the arctic migrants, detection rate of chlamydiae was 38% (17/45) in bar-tailed godwits and 40% (4/10) in red knots, both from the family Scolopacidae. This was the first detection in both bird species. In other bird species from the family Scolopacidae, chlamydiae was detected in woodcock (*Scolopax rusticola*) (2/3) in Korea (Jeong et al., 2017), red-necked stint (*Calidris ruficollis*) (1/7) in United States of America (USA) (Christerson et al., 2010), common snipe (*Gallinago gallinago*) (3/11), and wood sandpiper (*Tringa glareola*) (n=1) in Slovakia (Schwarzova et al., 2006). Negative detection of chlamydiae in the family Scolopacidae was also reported in four studies, in different countries, namely Poland (n = 1) (Krawiec et al., 2015), Sweden (n = 17) (Blomqvist, Christerson, Waldenström, Herrmann, et al., 2012), USA (n = 101) (Christerson et al., 2010), and Slovakia (n = 5) (Schwarzova et al., 2006). In our study, the higher detection rate of chlamydiae in bar-tailed godwits was sampled in both Firth of Thames and Manawatū Estuary, with the presence of red knots and pied stilts that both had similar high detection rates. As an important Ramsar site, Firth of Thames serves as wintering sites for several indigenous-breeding shorebird species like SIPO, pied stilts,

and wrybills (Dowding & Moore, 2006). The positive detection of chlamydiae in all arctic migrants, domestic migrants, and resident shorebirds suggests that a potential pathogen spillover between these shorebird species sharing the same habitat. Then, further pathogen spread within the country is possible when domestic migrants travel between wintering sites (Dowding & Moore, 2006). However, further characterisation of the species and genotypes of chlamydiae, with phylogenetic analysis is needed to provide more supportive information on this theory (see Chapter 4).

Migratory behaviours like earlier initiation of migration and frequent stopovers throughout the East Asia-Australasian Flyway are a possible route for the birds to introduce disease to new environments (Sullivan et al., 2018). Unlike the bar-tailed godwits that fly non-stop from Alaska to New Zealand (Gill et al., 2009), red knots have a stopover duration of about a month at the major staging site in China (Luannan coast of the Bohai bay) to refuel before arriving in New Zealand (Rogers et al., 2010). At the staging site for migration stops, birds of diverse species normally congregated, and horizontal transmission of pathogens might occur from frequent interactions between species (Hubalek, 2004). For instance, black-headed gulls (*Larus ridibundus*), the domestic migrants that travelled to Bohai Bay in summer, were tested positive on seroprevalence of *C. psittaci* (Chang et al., 2018; Fink D. et al., 2022). In New Zealand, the resident birds inhabiting the Manawatū estuary, such as mallards (*Anas platyrhynchos)*, are a *C. psittaci* reservoir in both North Island and South Island in New Zealand, and could be a source of the pathogen spillover (which may be bidirectional) to both the pied stilts and bar-tailed godwits (Soon et al., 2021). To better understand the risk and direction of spillover or spill-back of chlamydial infections between migratory and resident birds, samples should be taken both on arrival and departure, to identify the pathogen occurrence and then further sequencing and analysis should be carried out to compare the molecular data from the pathogens detected in different species. Like the management of Avian Influenza, by incorporating these data with spatio-temporal data from Global Flyway Network and individual bird data like age, sex, and bodyweight (Battley et al., 2019), a pathogen risk modelling and management project is possible to inform potential transmission risk of chlamydiae.

For the human component of this study, this was the first survey to identify chlamydiae in healthy people after handling wild birds. The positive chlamydiae detection of 52% (14/27) in nasal swabs of healthy people was high. Without wearing a mask when handling the bird, the handler may inhale the aerosolised particles from the birds' respiratory secretions carrying the bacteria. The people we sampled were in close proximity to the birds being banded which may have increased their exposure to respiratory aerosols. This result suggests effective exposure via aerosol transmission from the birds to humans but not necessarily infection, as our results may represent mucosal carriage only. Although it is unusual for general public to have the opportunity to get such close contact with most of the shorebird species mentioned in our study, black-backed gulls appeared to be widely available. As one of the most numerous and widespread shorebirds in New Zealand, the black-backed gulls have a daily commute of 30 km between the roosting sites (riverbeds, lake shores, and coasts), and inland feeding sites (lambing paddocks, rubbish tips, and city parks) (Heather & Robertson, 2015). Black-backed gulls often roost and sometimes nest on roofs in cities, while regularly attracted to food sources from people in urban parks (Miskelly, 2022). Therefore, positive detection of chlamydiae in black-backed gull provided evidence of potential zoonotic risk of chlamydial infection to people sharing the same habitat. Further studies are required to determine if people exposed to the chlamydiae carried by the shorebirds are susceptible to subsequent infection and disease.

Besides the direct handling of wild birds, zoonotic transmission of chlamydiae has been confirmed via occupational exposure of people working in the poultry industry, recreational exposure through the keeping pet birds, feeding feral pigeons, and inhaling infectious aerosols from mowing (Haag-Wackernagel, 2006; Laroucau et al., 2015; Ngan et al., 2013; Telfer et al., 2005). In our study, we cannot confirm that shorebirds handling is the only source of infection as information on other exposure risk was not collected. Future study should include additional exposure information from participants to be more certain of the source of pathogen risk. In a study to investigate the occurrence of *C. psittaci* in psittacine birds and bird handlers, 53% (63/120) of the birds and 6% (4/70) of the people were positive (Tolba et al., 2019). In a study that survey workers from a French poultry slaughterhouse for the presence of chlamydiae from their pharyngeal

swabs occurred in conjunction with the detection of the bacteria in the birds they worked with. With the positive detection of *C. psittaci* in 9/38 duck flocks that were processed in the French poultry slaughterhouse, 18% (2/11) of slaughterhouse workers were *Chlamydiaceae* positive (Hulin et al., 2015). Protective equipment like masks and gloves were provided in the facility but it was sometimes poorly tolerated by the workers (Hulin et al., 2015). In both theirs and our studies, the positive birds were asymptomatic. So, considering its public health significance, it is recommended to wear masks and to take other precautionary measures, such as regular hand hygiene, when handling waders in New Zealand.

As a multi-host pathogen, chlamydiae can easily infect wild birds, humans, and domesticated animals (Burnard & Polkinghorne, 2016). Besides *C. psittaci*, in which wild birds were known as its reservoir, there were also another two *Chlamydia* species with zoonotic potential that were found in wild birds. They were the *C. abortus* detected in a brown skua (*Catharacta antarctica lonnbergi*) from a Subantarctic Island, South Georgian archipelago, and *C. gallinacea* in a wild Australian galah (*Eolophus roseicapillus*) (Herrmann, Rahman, et al., 2000; Stokes et al., 2019). The positive detection of these other chlamydial pathogens was only in wild birds, and to date, there is only evidence of cross-host transmission of these two chlamydial species from domestic ruminants and poultry to humans, but none from the wild birds (Cheong et al., 2019). Although the qPCR protocol used in this study identified all the positive samples as putative *C. psittaci*, further characterisation of the positive samples in this study was required to provide genomic data and describe molecular epidemiology of chlamydiae in shorebirds and people handling shorebirds.

CHAPTER 4

Genomic diversity of chlamydiae in shorebirds and handlers in New Zealand

CHAPTER 4 Molecular characterisation of chlamydiae in shorebirds and handlers in New Zealand

4.1 Introduction

Wildlife diseases can have substantial impacts on conservation and management of biodiversity. The application of genomic tools in wildlife disease epidemiology has advanced our understanding in pathogen detection and characterisation, pathways of disease transmission and spread, host susceptibility and pathogen virulence (Blanchong et al., 2016). By using molecular epidemiology in wildlife disease research, we can conduct wildlife disease surveillance, identify sources of pathogen emergence, investigate host-pathogen dynamics, and manage disease outbreaks (Benton et al., 2015). For example, molecular epidemiology was widely applied in surveillance of avian influenza viruses in wild birds (Hoye et al., 2010), identifying the root of emergence of *Leucocytozoon* blood parasites via phylogenetic investigation of the strains isolated from resident and migratory birds (Yoshimura et al., 2014), and examining the relationship of West Nile virus amplification and host species selection by mosquito vectors (Hamer et al., 2009). Better understanding of the multifaceted interactions between pathogens and wildlife will aid in mitigating the effects of wildlife diseases on global health in humans, livestock and wildlife populations (Blanchong et al., 2016).

As *C. psittaci* is recognised as the sole pathogen causing chlamydiosis in birds, most genomic tools were designed to identify and further characterise *C. psittaci* (Sachse, Vretou, et al., 2009). For instance, genotype classification of the different *C. psittaci* strains can be done by sequencing the major outer membrane protein (*ompA*) gene. The designated genotypes obtained from sequence analysis of *ompA* genes were A-F, E/B, 1V, 6N, Mat116, R54, YP84, CPX0308, I, and J (Geens, Desplanques, et al., 2005; Madani & Peighambari, 2013; Sachse et al., 2008). To date, in New Zealand, genotypes A, B and C have been identified from both introduced and native bird species (Gedye et al., 2018). Although genotyping based on *ompA* gene had been commonly used, *ompA* genotyping has a low resolution, whereas multilocus sequence typing (MLST) provides higher discrimination (Vorimore et al., 2021). For instance, *ompA* gene analysis was

insufficient when molecular characterisation was required for closely related strains. These untypable strains were known as *C. psittaci* Prk/Daruma, *C. psittaci* strain 84/2334, atypical *C. psittaci/C. abortus*, and *C. psittaci/C. abortus* intermediate (Van Loock et al., 2003). To overcome the limitations of examining a single gene, the multi-locus sequence typing (MLST) for *C. psittaci* introduced by Pannekoek et al. (2010), analysing the seven housekeeping genes: *enoA, fumC, gatA, gidA, hemN, hlfX* and *oppA,* has emerged as the new "gold standard" for chlamydial strain characterisation (Anstey et al., 2021). Lately, whole genome sequencing and comparative genome analysis had assisted in identifying genomic features associated with host preference of *C. psittaci* strains (Hölzer et al., 2020; Kasimov et al., 2023; Sachse et al., 2023).

Besides *C. psittaci*, recent studies reported the detection of novel avian chlamydial species such as *C. avium*, *C. gallinacea*, and avian *C. abortus* (Sachse et al., 2014; Szymańska-Czerwińska et al., 2017; Zocevic et al., 2012). *C. avium* was detected in pigeons (Columbiformes), parrots (Psittaciformes), and waterfowl (Anatidae), with *C. avium* causing fatal infection in captive pigeons in the Netherlands, and captive parrots in France (Kik et al., 2020; Popelin-Wedlarski et al., 2020; Szymańska-Czerwińska et al., 2017). Globally, *C. gallinacea* was identified as the dominant chlamydial species in poultry, rather than *C. psittaci* (Guo et al., 2016). Being less pathogenic than *C. psittaci*, *C. gallinacea* infection in chicken was mostly asymptomatic, but may lead to weight loss in broilers (Heijne, Jelocnik, et al., 2021; Heijne, van der Goot, et al., 2021). In shorebirds (Charadriiformes), *C. gallinacea* was detected in two wild woodcocks (*Scolopax rusticola*) in Korea (Jeong et al., 2017). Avian *C. abortus* was detected in multiple wild bird species such as mallards (*Anas chlorotis*), swan (*Cygnus* sp.), Australian pied oystercatcher (*Haematopus longirostris*), short-tailed shearwaters (*Ardenna tenuirostris*), white faced heron (*Egretta novaehollandiae*), and Eurasian magpies (*Pica pica*) (Aaziz et al., 2022; Kasimov et al., 2021; Stalder et al., 2021; Szymańska-Czerwińska et al., 2017). There is some limited evidence of zoonotic potential for *C. gallinacea,* but the zoonotic potential of the other two species is comparatively understudied (Marchino et al., 2022).

As a primary avian chlamydial pathogen, *C. psittaci* had been detected in eight shorebird species, reported in seven published articles. Most of the bird species involved in *C. psittaci* infections were gulls (Laridae), with the studies conducted in Europe (Aaziz et al., 2015; Kalmar et al., 2014; Szymańska-Czerwińska et al., 2017) (Blomqvist, Christerson, Waldenström, Herrmann, et al., 2012; Herrmann, Rahman, et al., 2000; Kasimov et al., 2021; Schwarzova et al., 2006). For studies that reported the genotypes of *C. psittaci* in shorebirds, no host tropism was observed as seven different genotypes were detected. The classical genotypes detected were *C. psittaci* genotype A, D, E/B, and Mat116 (Kalmar et al., 2014; Kasimov et al., 2021; Szymańska-Czerwińska et al., 2017). There were also two previously unidentified strains of *C. psittaci* found in shorebirds, known as strain Prk/Daruma and strain R54, both were closely related to mammalian *C. abortus* (Blomqvist, Christerson, Waldenström, Herrmann, et al., 2012; Herrmann, Rahman, et al., 2000). To date, there is no reported data on the chlamydial species and genotypes in shorebirds in New Zealand.

A sufficient amount of DNA in the samples is required to molecularly characterise *Chlamydiae* (Aaziz et al., 2022). To obtain sufficient DNA material, cultivating *Chlamydiae* in host cells is needed, but this can be costly, time-consuming, labour-intensive, require viable bacteria and specialised culture facilities (WOAH, 2018a). To overcome this, culture-independent genomic approaches have gained more recognition as an alternative in the chlamydial field. Amplification of microbial DNA to effectively increase the DNA yield can be done via both non-targeted and targeted genome capture methods, depending if the target bacterium is known or unknown (Taylor-Brown et al., 2018). The non-targeted genome capture methods like multiple displacement amplification (MDA), allow pathogen discovery, while the targeted methods is ideal for diagnostics and epidemiology, due to its lower cost, higher throughput and better sensitivity. To date, these genome capture methods were mostly applied in the detection of *C. trachomatis* in humans (Taylor-Brown et al., 2018). A published study applied a targeted genome capture method known as sequence capture, followed by Illumina sequencing of core genome, to investigate the genetic diversity of *C. psittaci* strains in isolates derived from one wild parrot and six human (Branley et al., 2016). The phylogenetic analysis revealed that all of the isolates belonged to a same clade, 6BC,

and the isolates were virtually identical, suggesting potential chlamydial transmission from bird to human (Branley et al., 2016).

In investigations of human psittacosis, genotype matching between human and animal samples is normally performed to rapidly identify evidence of zoonotic transmission and potential animal sources of human exposure (Hogerwerf et al., 2020). For instance, the epidemiological investigations of a psittacosis outbreak in a French mixed poultry farm provides evidence to suggest the source of infection was from the duck flocks when *C. psittaci* genotype E/B was detected in both the sputum from farm workers and cloacal swabs from the ducks (Laroucau et al., 2015). Although *C. psittaci* genotypes A, B, C, D, E, F, and E/B had been detected in human, the most common genotypes that match between human and birds were genotypes A, B and E/B (Hogerwerf et al., 2020). Some common bird species as the suspected source for zoonotic transmission of *C. psittaci* were chickens, ducks, turkeys, pigeons, and parrots (Hogerwerf et al., 2020).

Generally, PCR, serology, or a combination of both testing methods were widely applied to diagnose psittacosis, depending on the samples obtained (Nieuwenhuizen et al., 2018). Recently, the application of metagenomic next generation sequencing (mNGS) using Illumina sequencing in human clinical practice had facilitated the rapid detection and identification of atypical pathogens like *C. psittaci*, in patients with pulmonary infection in China (Huang et al., 2023). In New Zealand, *C. psittaci* is not routinely tested for among patients with acute respiratory illness, hence there is no publicly available reports on molecular classification of *C. psittaci* from human samples based on *ompA* genotyping and MLST.

In the previous chapter, we detected chlamydiae in shorebirds and the people that handle them at four estuarine sites in New Zealand using the qPCR-HRM (high resolution melting curve) analysis. The species of chlamydiae found in the birds and humans remained unidentified as qPCR-HRM identifies only to the genus level. In order to further differentiate the infecting chlamydiae and thus, the potential for zoonotic transmission, phylogenetic analyses of both bird and human chlamydiae strains are

required. The aim of this study was, therefore, to investigate the genomic diversity and diversity of *Chlamydiae* in shorebirds and handlers in New Zealand, using the positive samples obtained in the previous chapter.

4.2 Materials and methods

4.2.1 Sample description used for DNA sequencing

For bird samples, of the 74 *Chlamydiaceae*-positive samples (Chapter 3), we selected 43 samples for further investigation using multiple displacement amplification (MDA) and DNA sequencing. The 43 samples were selected as they had been samples with positive result on either all three *Chlamydiaceae* qPCR-HRM runs (n = 7) on two runs (n = 16), or on a single run (n = 20). For human samples, all 14 *Chlamydiaceae*-positive nasal swabs (14/27; 52%) were subjected to MDA for species identification.

MDA using Bst DNA Polymerase (New England Biolabs, Ipswich, USA) was performed on all selected bird (n = 43) and human (n = 13) samples. MDA using ϕ29 DNA Polymerase (Thermo Fisher Scientific, Waltham, USA) was later performed, in addition, on some bird (n = 7) and human (n = 7) samples from the selected samples.

All MDA products were subjected to qPCR-HRM test (same protocol performed in chapter 3), and positive result on qPCR-HRM run determined successful amplification of the samples using MDA. Then, the selected MDA products were subjected to DNA sequencing using Sanger sequencing.

4.2.2 Multiple displacement amplification (MDA)

4.2.2.1 MDA with Bst DNA Polymerase

MDA catalysed by large fragment of *Bst* DNA polymerase was performed using the protocol by Lage et al. (2003), using *Bst* DNA polymerase (New England Biolabs, Ipswich, USA); T4 gene 32 protein (New England Biolabs, Ipswich, USA); and primers

(random 7-mers with two nitroindole residues at the 5’ end and a phosphorothioate linkage at the 3’ end) (Integrated DNA Technologies, IA, USA).

In brief, 1 µL of DNA sample was added to a reaction mixture of 1.5 µL of 10× ThermoPol buffer (New England Biolabs, Ipswich, USA) and random 7-mers primers at a concentration of 100 µM with the whole reaction made to a total volume of 15 µL with nuclease free water. DNA was denatured at 96°C for 2 minutes, cooled at 20°C for 10 minutes, and then put on ice. The reaction mixture was then brought up to 50 µL containing 400 µM deoxynucleotide triphosphate (dNTPs) (New England Biolabs, Ipswich, USA), 1× ThermoPol buffer, and 0.35 units/µL *Bst* DNA polymerase in a 4% w/v solution of dimethyl sulfoxide (DMSO) (Solis BioDyne, Tartu, Estonia) in nuclease free water. T4 gene 32 protein was then added to the reaction mixture in final concentration of 30 ng/µL. The DNA was amplified at 50°C for 6 hours and inactivated at 80°C for 15 minutes using a Mastercycler nexus GX2 (Eppendorf, Hamburg, Germany).

Amplified DNA samples were purified using isopropanol precipitation. Briefly, 100 µL of nuclease-free water, 30 µL sodium acetate, and 300 µL isopropanol were added to the 50 µL amplified DNA samples. The tube was gently inverted at least 7 times and froze at –20°C for 3 hours. The sample was centrifuged at 15,000 rpm at 4°C for 20 minutes. The supernatant was discarded, and the sample was washed with 70% molecular grade absolute ethanol. The sample was then centrifuged at 15,000 rpm at 4°C for 10 minutes to pellet the DNA. The resulting DNA pellet was dried and resuspended in 25 µl of 5 mM Tris-HCl pH 8.5 (Macherey-Nagel, Düren, Germany) overnight at 4°C.

4.2.2.2 MDA with ϕ29 DNA Polymerase

MDA catalysed by ϕ29 DNA polymerase utilised the EquiPhi29 DNA Polymerase (Thermo Fisher Scientific, Waltham, USA), and primers (exo-resistant random primer with two 3'-terminal phosphorothioate (PTO) modifications and 5'- and 3'-hydroxyl ends) (Thermo Fisher Scientific, Waltham, USA).

In brief, 3.5 µL of DNA sample was added to a reaction mixture of 0.5 µL of 1x EquiPhi29 DNA Polymerase reaction buffer (Thermo Fisher Scientific, Waltham, USA), and exo-resistant random primers at a concentration of 100 µM with the whole reaction made to a total volume of 1 µL with nuclease free water. DNA was denatured at 95°C for 3 minutes, and then put on ice. The reaction mixture was then brought up to 20 µL containing 400 µM DTT, 10 µM dNTP mix, 10× EquiPhi29 DNA Polymerase reaction buffer, 10 units/µL EquiPhi29 DNA Polymerase, and nuclease-free water. The DNA was amplified at 45°C for 3 hours and inactivated at 65°C for 10 minutes using Mastercycler nexus GX2 (Eppendorf, Hamburg, Germany).

Amplified DNA samples were purified using Genomic DNA Clean & Concentrator-10 (Zymo Research, Irvine, USA) according to the manufacturer's protocol. First, 40 µL of ChIP DNA Binding Buffer was added to a 0.2ml PCR tube containing 20 µL of the MDA product. The mixture was transferred into Zymo-Spin™ IC-XL Column2 placed in a collection tube. The column was centrifuged for 30 seconds at 13,000xg and the flow-through was discarded. Then, proceed by adding 200 µL of DNA Wash Buffer to the column, in which the column was centrifuged for 1 minute at 13,000xg and the flow-through was discarded. After repeating this wash step using DNA Wash Buffer, the spin column was transferred to a 1.5ml microcentrifuge tube. Then, 10 µL of DNA Elution Buffer was added directly to the column matrix and incubated at room temperature for one minute. The DNA was eluted by centrifuging the column for 30 seconds at 13,000xg.

4.2.3 DNA sequencing

DNA sequencing via Sanger sequencing was performed on amplicons produced from amplification of the *ompA* gene, IGS-23S rRNA, and MLST. The species-specific end-point PCR targeting *ompA* gene of *C. psittaci* utilised the primers VD1-f (5'-ACT ACG GAG ATT ATG TTT TCG ATC GTG T-3') and VD2-r (5'-CGT GCA CCY ACG CTC CAA GA-3') (IDT, Iowa, USA) by Sachse et al. (2008), to amplify the variable domain 1 and 2 region (418bp) of the *C. psittaci* outer membrane protein A gene (*ompA*). The genus-specific end-point PCR targeting IGS-23S rRNA (16S rRNA-23S rRNA intergenic spacer together with 23S rRNA domain I) utilised the primers 16SF2 (5'- CCG CCC GTC ACA TCA TGG -3') and

23SIGR (5'- TGG CTC ATC ATG CAA AAG GCA -3') (IDT, Iowa, USA) by Everett et al. (1999), to amplify the 16S rRNA-23S rRNA intergenic spacer together with 23S rRNA domain I (1000bp). MLST analysis was performed to amplify the seven housekeeping genes (*enoA, fumC, gatA, gidA, hemN, hlfX* and *oppA*) described by Pannekoek et al. (2008) (**Table 4.1**).

Both the PCR and MLST was performed under the following conditions: 1xHOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia), 200nM of each primer and 2 µL of the template, in a total volume of 20µl. The PCR products were separated through a 1.0% (w/v) 0.5x TBE agarose gel containing a 100bp HyperLadder™ (Bioline, London, UK) for size comparison. The PCR amplicon was then cut from the gel and eluted overnight in 25 µl elution buffer (5mM Tris-HCl, pH8.5) and sent for bi-directional Sanger sequencing (Massey Genome Services, Palmerston North, New Zealand).

Table 4.1 The genes and primers used for the multi-locus sequence typing analysis of chlamydiae adapted from Pannekoek et al. (2008).

Gene	Full name	Primer sequences	Annealing temperature
enoA	enolase	F: CCTATGATGAATCTKATCAATGG R: TCTTCTTCRGCWAGMCCATCT	50°C
fumC	fumarate hydratase	F: ATTAAAAAATGTGCTGCT R: CCTTCAGGAACATTYAACCC	50°C
gatA	aspartyl/glutamyl-tRNA amidotransferase subunit A	F: GCTTTAGAATTARSARAWGCT R: GATCCTCCGGTATCYGATCC	50°C
gidA	tRNA (uracil-5-)-methyltransferase	F: GGAGTCWCTACWAAAGAAGG R: TCGTAYTGYACATCRAAAGG	60°C
hemN	coproporphyrinogen III oxidase	F: AGATCTTCTTCWGGRGGWAGAGA R: TTCYTTCAKAACSTAGGTTTT	60°C
hflX	GTP-binding protein	F: GCTTCTARAGTACTTTTAAATG R: TATTTRGAAATYTTTKCSAGYCG	60°C
oppA	oligopeptide-binding protein	F: ATGCGCAAGATATCAGTGGG R: AAAGCTCCRSTWGMTATMGGWAG	60°C

R = A or G; S = G or C; W = A or T; Y = C or T; M = A or C; K = G or T.

4.2.4 Sequence and phylogenetic analysis

The chromatograms for sequences were assessed for quality using Phred quality scores (>30) in Geneious 10.2.6 software (Biomatters, New Zealand). The chosen

sequences were then subjected to BLAST analysis (Altschul et al., 1990) against the GenBank database (NCBI) to compare and identify related sequences. The top BLAST hit, percent (%) sequence identity, and sequence length were recorded. The sequences were aligned to a panel of publicly available sequences (n = 24) as listed in **Table 4.2**. The sequences were *C. felis*, *C. caviae*, *C. psittaci* (A-6BC, A-8455, C, CP3, D-NJ1, E, EB-E30, F, M56, Mat116, MN, VS225, WC, YP84), *C. abortus* (POS, LLG, B577, S26/3), avian *C. abortus* (1V, G1, G2). Prior to constructing dendrograms, sequences were trimmed to approximately 380 bp, removing primer sequences from both forward and reverse strand, to yield only the high-quality target DNA sequence. The dendrogram was constructed using the Neighbour-Joining (NJ) tree-building algorithm, with a Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) of DNA substitution, and the cluster robustness assessed by bootstrapping 1,000 replicates. Mean nucleotide distances among the *C. psittaci* or *C. abortus* genotypes were estimated by using the same model.

Table 4.2 Publicly available sequences (n = 24) of chlamydial reference strains included in the *ompA*-based NJ dendrogram.

Species	Strain	Source	Country	GenBank accession number
C. felis	FP baker	House cat	USA	AF269257
C. caviae	GPIC	Guinea pig	USA	AF269282
C. psittaci	A-6BC	Cell culture	USA	X56980.1
C. psittaci	A-8455	Parakeet	Germany	Y16561.1
C. psittaci	C	Duck	Germany	L25436.1
C. psittaci	CP3	Pigeon	USA	AF269265.1
C. psittaci	D-NJ1	Turkey	USA	AF269266.1
C. psittaci	E	Cell culture	UK	X12647.1
C. psittaci	EB-E30	Duck	Germany	AY762613.1
C. psittaci	F	Orange-fronted parakeet	USA	AF269259.1
C. psittaci	M56	Cell culture	USA	AF269268.1
C. psittaci	Mat116	Chestnut fronted macaw	Japan	AB284058.1
C. psittaci	MN	Cell culture	USA	AF269262
C. psittaci	VS225	Parakeet	USA	CP003793.1
C. psittaci	WC	Cow	USA	AF269269.1
C. psittaci	YP84	King parakeet	Japan	AB284065.1
C. psittaci	1V	Hooded crow	Russia	EF028916.1
C. abortus	POS	Sheep	Greece	HQ637269
C. abortus	LLG	Cell culture	UK	CP018296
C. abortus	B577	Sheep	USA	M73036.1
C. abortus	S26/3	Sheep	UK	CR848038

avian *C. abortus*	1V	Magpie	Poland	KX870484
avian *C. abortus*	G1	Eurasian teal	Poland	KX870485
avian *C. abortus*	G2	Mallard	Poland	KX424655
C. psittaci	15-63/3	Mallard	Poland	MF140918

*NR = Not reported

4.3 Results

4.3.1 Species identification of chlamydiae

Based on the criteria of sequences with high quality chromatograms, a total of 24 *ompA* sequences (GenBank accession: OQ447478-OQ447499, OQ454509-OQ454510) and two 23S rRNA sequences (GenBank accession: OR058535- OR058536) were successfully obtained from 57 samples. All *ompA* sequences were included for downstream analyses. There were 13 *ompA* sequences (**Table 4.3**) and two 23S rRNA sequences (**Table 4.4**) from the bird samples, and 11 *ompA* sequences from human samples (**Table 4.5**). The other 33 sequences failed to fit into the criteria, or sequencing failed, potentially due to mixed infections or low copy numbers.

A Neighbour-Joining dendrogram was constructed using a 380 bp alignment of *ompA* sequences from reference strains of *C. felis*, *C. caviae*, *C. abortus* and *C. psittaci* available from GenBank. When comparing to reference sequences, the 24 sequences obtained from our study were grouped into two clades. In the *ompA* dendrogram (**Figure 4.1**), 2 out of 24 sequences were assigned to one clade representing the previously described *C. psittaci* genotype C (L25436.1), with a bootstrap value of 83.5% and 100% pairwise identity. These sequences were from a pied stilt at the Manawatū Estuary and a human at the Firth of Thames, with only a single nucleotide substitution (**Table 4.6**) when comparing to Germany duck sequence (L25436.1) and Poland mallard sequence (MF140918).

The remaining 22 sequences were assigned to another clade representing the previously described avian *C. abortus* genotype G1. Of the 22 sequences, twelve sequences were from the bird samples and ten were from human samples. The

sequences obtained from bird samples consisted of samples from bar-tailed godwits in Firth of Thames (n = 3), South Island pied oystercatchers in Firth of Thames (n = 3), bar-tailed godwits in Manawatū Estuary (n = 2), pied stilts in Manawatū Estuary (n = 4), and South Island pied oystercatchers in Golden Bay (n = 1). The ten sequences from human samples were collected from Firth of Thames (n = 8) and Golden Bay (n = 2). All the 22 sequences were classified as avian *C. abortus* genotype G1 (KX870485.1) with a maximum 100% bootstrap value. The 22 differed by a maximum of five single nucleotide substitutions (**Table 4.6**) from the Poland Eurasian teal sequence (KX870485.1) and were 99-100% pairwise identity similar to each other.

Table 4.3 Metadata of *ompA* sequences (n = 13) obtained from shorebirds' samples collected in New Zealand.

Sample ID	Species	Anatomical Site	Location	Collection date	Sequence Length (bp)	Sequence Quality (%)	First Hit (Accession Number)	Pairwise Identity (%)	Genbank Accession
mb03	Bar-tailed godwit	Choanal	Thames	30-Oct-2019	393	93.1	av *C. abortus* 15-70d24 (LS450958)	99.7	OQ447479
mb052	Bar-tailed godwit	Cloacal	Thames	01-Nov-2019	398	85.4	av *C. abortus* 15-70d24 (LS450958)	99.7	OQ447480
mb181	South Island pied oystercatcher	Choanal	Thames	15-Jan-2021	390	96.4	av *C. abortus* 15-70d24 (LS450958)	99.7	OQ447481
mb249	South Island pied oystercatcher	Choanal	Thames	15-Jan-2021	350	76.3	av *C. abortus* 15-70d24 (LS450958)	99.6	OQ447482
mb272	Bar-tailed godwit	Cloacal	Manawatū	02-Feb-2021	391	94.1	av *C. abortus* 15-70d24 (LS450958)	99.4	OQ447483
mb276	Bar-tailed godwit	Cloacal	Manawatū	02-Feb-2021	405	93.3	av *C. abortus* 15-70d24 (LS450958)	99.5	OQ447484
mb286	Pied stilt	Cloacal	Manawatū	02-Feb-2021	385	99	av *C. abortus* 15-70d24 (LS450958)	100	OQ447485
mb291	Pied stilt	Choanal	Manawatū	02-Feb-2021	378	98.4	av *C. abortus* 15-70d24 (LS450958)	100	OQ447478
mb337	Pied stilt	Choanal	Manawatū	02-Feb-2021	371	97.3	*Chlamydia* sp. 15-63/3 (MF140918)	100	OQ447486
mb393	Pied stilt	Choanal	Manawatū	02-Feb-2021	375	88.8	av *C. abortus* 15-70d24 (LS450958)	100	OQ447487
mb406	Bar-tailed godwit	Cloacal	Thames	02-Mar-2021	394	92.6	av *C. abortus* 15-70d24 (LS450958)	99.6	OQ447488
mb492	South Island pied oystercatcher	Cloacal	Thames	02-Mar-2021	389	95.1	av *C. abortus* 15-70d24 (LS450958)	99.5	OQ447490
mb557	South Island pied oystercatcher	Choanal	Golden Bay	14-Apr-2021	402	95	av *C. abortus* 15-70d24 (LS450958)	99.1	OQ447489

Abbreviations: Thames, Firth of Thames; Manawatū, Manawatū Estuary.

Table 4.4 Metadata of 23S rRNA sequences (n = 2) obtained from shorebirds' samples collected in New Zealand.

Sample ID	Species	Anatomical Site	Location	Collection date	Sequence Length (bp)	Sequence Quality (%)	First Hit (Accession Number)	Pairwise Identity (%)	Genbank Accession
mb291	Pied stilt	Choanal	Manawatū	02-Feb-2021	923	93.1	av *C. abortus* 15-70d24 (LS450958)	100	OR058535
mb393	Pied stilt	Choanal	Manawatū	02-Feb-2021	951	92.7	av *C. abortus* 15-70d24 (LS450958)	99.9	OR058536

Table 4.5 Metadata of *ompA* sequences (n = 11) obtained from human samples collected in New Zealand.

Sample ID	Location	Collection date	Sequence Length (bp)	Sequence Quality (%)	First Hit (Accession Number)	Pairwise Identity (%)	Genbank Accession Number
mh02	Thames	15-Jan-2021	390	97.9	av *C. abortus* 15-70d24 (LS450958)	99.7	OQ447491
mh03	Thames	15-Jan-2021	298	79.6	av *C. abortus* 15-70d24 (LS450958)	98.7	OQ447492
mh04	Thames	15-Jan-2021	394	96.7	av *C. abortus* 15-70d24 (LS450958)	99.9	OQ447493
mh05	Thames	15-Jan-2021	390	86.2	av *C. abortus* 15-70d24 (LS450958)	100	OQ447494
mh09	Thames	15-Jan-2021	381	96.6	av *C. abortus* 15-70d24 (LS450958)	99.7	OQ447495
mh10	Thames	15-Jan-2021	396	98	*Chlamydia sp.* 15-63/3 (MF140918)	99.7	OQ454509
mh11	Thames	15-Jan-2021	400	95.5	av *C. abortus* 15-70d24 (LS450958)	99.5	OQ447496
mh12	Thames	15-Jan-2021	388	92.5	av *C. abortus* 15-70d24 (LS450958)	99.1	OQ454510
mh18	Thames	03-Mar-2021	382	89.5	av *C. abortus* 15-70d24 (LS450958)	99.6	OQ447497
mh23	Golden	14-Apr-2021	380	98.2	av *C. abortus* 15-70d24 (LS450958)	99.3	OQ447498
mh25	Golden	14-Apr-2021	390	97.9	av *C. abortus* 15-70d24 (LS450958)	99.5	OQ447499

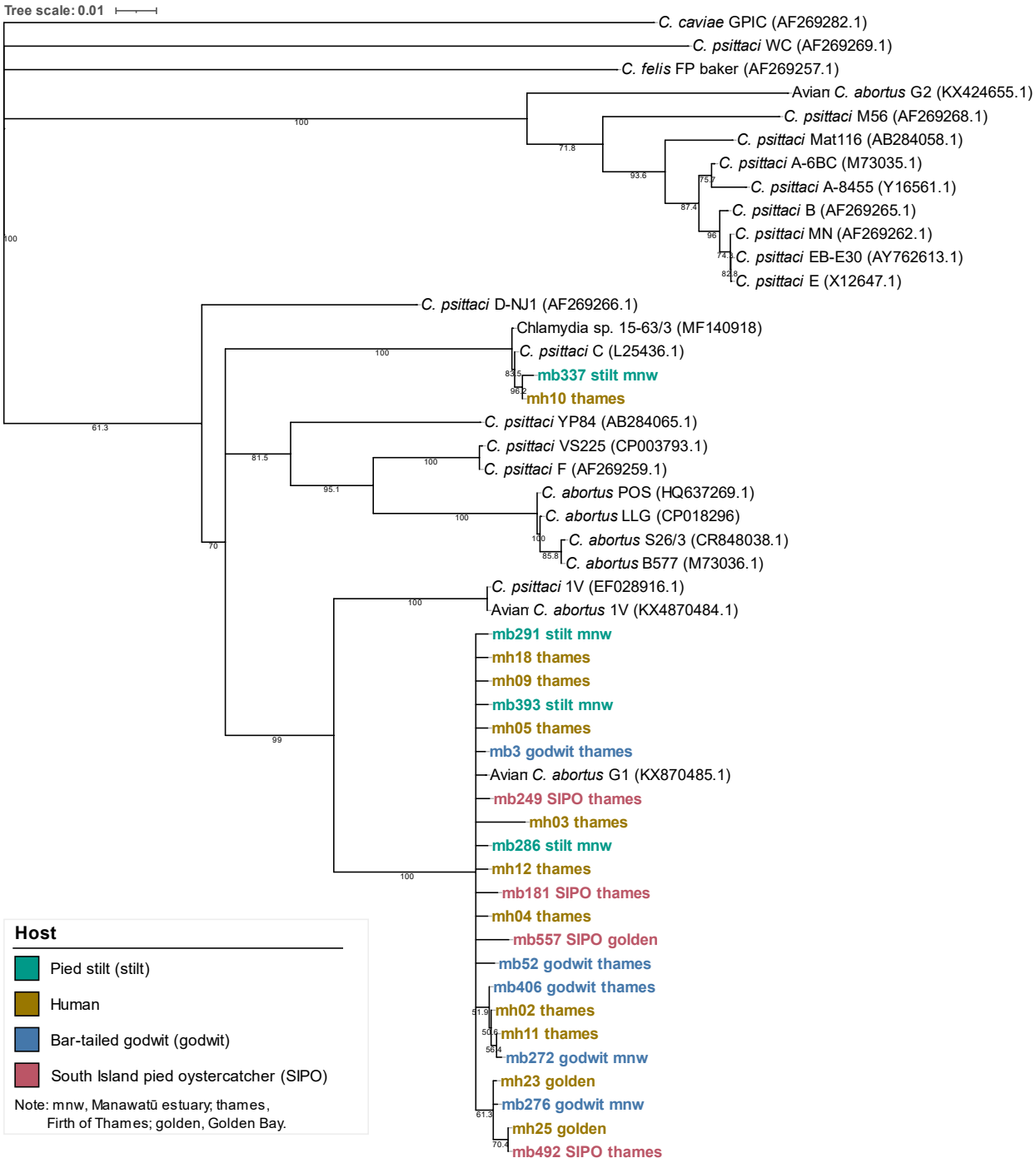

Figure 4.1 *ompA*-based NJ dendrogram of chlamydial reference strains, bird samples, and human samples from this study. This dendrogram was constructed from a MUSCLE alignment of 380bp and rooted on the sequence of *C. Caviae* (GPIC).

Table 4.6 Number of bases or residues which are not identical in a 380bp fragment of the ompA gene among isolates from this study, *C. psittaci* genotype C, and avian *C. abortus* genotypes 1V, G1, and G2.

	mh10	mb337	*C. psittaci* C	*Chlamydia* sp. 15-63/3	av *C. abortus* 1V	*C. psittaci* 1V	mh23	mh03	mh18	mh09	mb557	mb272	mb3	mb406	mb276	mb492	mh25	mb249	mh12	mh02	mb181	mh11	mh04	mb52	mb393	av *C. abortus* G1	mb286	mb291	mh05
mh10_thames		0	1	1	60	60	59	57	58	58	59	59	57	59	57	57	57	55	56	58	57	57	57	57	56	58	56	56	56
mb337_stilt_manaw	0		0	0	59	59	59	57	58	58	58	59	57	59	57	57	57	55	56	58	57	57	57	57	56	56	56	56	56
C. psittaci C	1	0		0	61	61	59	57	58	58	60	59	57	59	58	57	57	55	56	58	57	58	57	57	56	57	56	56	56
Chlamydia sp. 15-63/3	1	0	0		61	61	59	57	58	58	60	59	57	59	58	57	57	55	56	58	57	58	57	57	56	57	56	56	56
av *C. abortus* 1V	60	59	61	61		0	33	29	31	30	32	29	30	31	30	30	30	27	29	30	29	30	29	29	28	29	28	28	28
C. psittaci 1V	60	59	61	61	0		33	29	31	30	32	29	30	31	30	30	30	27	29	30	29	30	29	29	28	29	28	28	28
mh23_golden	59	59	59	59	33	33		10	8	7	7	8	7	8	5	5	5	7	6	7	6	6	6	6	5	5	5	5	5
mh03_thames	57	57	57	57	29	29	10		8	7	7	8	6	8	6	7	7	6	6	7	6	6	6	4	5	5	5	5	5
mh18_thames	58	58	58	58	31	31	8	8		5	5	6	5	6	4	5	5	5	4	5	4	4	4	4	3	3	3	3	3
mh09_thames	58	58	58	58	30	30	7	7	5		4	5	4	5	3	4	4	4	3	4	3	3	3	3	2	2	2	2	2
mb557_SIPO_golden	59	58	60	60	32	32	7	7	5	4		6	4	5	4	4	4	4	3	4	3	4	3	4	2	5	2	2	2
mb272_godwit_manaw	59	59	59	59	29	29	8	8	6	5	6		5	5	4	5	5	5	4	4	4	2	3	4	3	3	3	3	3
mb3_godwit_thames	57	57	57	57	30	30	7	6	5	4	4	5		5	3	4	4	4	3	4	3	3	3	3	2	2	2	2	2
mb406_godwit_thames	59	59	59	59	31	31	8	8	6	5	5	5	5		4	5	5	5	4	3	4	3	2	4	3	3	3	3	3
mb276_godwit_manaw	57	57	58	58	30	30	5	6	4	3	4	4	3	4		1	1	3	2	3	2	2	2	2	1	3	1	1	1
mb492_SIPO_thames	57	57	57	57	30	30	5	7	5	4	4	5	4	5	1		0	4	3	4	3	3	3	3	2	2	2	2	2
mh25_golden	57	57	57	57	30	30	5	7	5	4	4	5	4	5	1	0		4	3	4	3	3	3	3	2	2	2	2	2
mb249_SIPO_thames	55	55	55	55	27	27	7	6	5	4	4	5	4	5	3	4	4		3	4	3	3	3	3	2	2	2	2	2
mh12_thames	56	56	56	56	29	29	6	6	4	3	3	4	3	4	2	3	3	3		3	2	2	2	2	1	1	1	1	1
mh02_thames	58	58	58	58	30	30	7	7	5	4	4	4	4	3	3	4	4	4	3		3	2	1	3	2	2	2	2	2
mb181_SIPO_thames	57	57	57	57	29	29	6	6	4	3	3	4	3	4	2	3	3	3	2	3		2	2	2	1	1	1	1	1
mh11_thames	57	57	58	58	30	30	6	6	4	3	4	2	3	3	2	3	3	3	2	2	2		1	2	1	3	1	1	1
mh04_thames	57	57	57	57	29	29	6	6	4	3	3	3	3	2	2	3	3	3	2	1	2	1		2	1	1	1	1	1
mb52_godwit_thames	57	57	57	57	29	29	6	4	4	3	4	4	3	4	2	3	3	3	2	3	2	2	2		1	1	1	1	1
mb393_stilt_manaw	56	56	56	56	28	28	5	5	3	2	2	3	2	3	1	2	2	2	1	2	1	1	1	1		0	0	0	0
av *C. abortus* G1	58	56	57	57	29	29	5	5	3	2	5	3	2	3	3	2	2	2	1	2	1	3	1	1	0		0	0	0
mb286_stilt_manaw	56	56	56	56	28	28	5	5	3	2	2	3	2	3	1	2	2	2	1	2	1	1	1	1	0	0		0	0
mb291_stilt_manaw	56	56	56	56	28	28	5	5	3	2	2	3	2	3	1	2	2	2	1	2	1	1	1	1	0	0	0		0
mh05_thames	56	56	56	56	28	28	5	5	3	2	2	3	2	3	1	2	2	2	1	2	1	1	1	1	0	0	0	0	

The greyscale heatmap indicates the smallest difference with black (0) and the bigger differences with white.

4.3.2 Genotyping of avian *C. abortus*-positive samples by MLST analysis

Out of the 7 seven housekeeping genes: *enoA, fumC, gatA, gidA, hemN, hlfX* and *oppA,* it was only possible to amplify four gene sequences (*enoA, fumC gatA, oppA*) from two bird samples (mb291, mb393) (**Table 4.7**). The unsuccessful amplification of the three genes (*gidA, hemN, hlfX*) from these two bird samples and other samples in this study did not lead to an appreciable MLST result.

Table 4.7 Metadata of four genes (*enoA, fumC gatA, oppA*) from MLST analysis of pied stilt (*Himantopus leucocephalus*) samples collected in New Zealand.

Sample ID	Gene	Sequence Length (bp)	Sequence Quality (%)	First Hit (Accession Number)	Pairwise Identity (%)
mb291	*enoA*	381	91.5	av *C. abortus* 15-70d24 (LS450958)	99.8
	fumC	465	80.3	av *C. abortus* 15-70d24 (LS450958)	100
	gatA	425	94.9	av *C. abortus* 15-70d24 (LS450958)	100
	oppA	483	93.9	av *C. abortus* 15-70d24 (LS450958)	100
mb393	*enoA*	381	81.4	av *C. abortus* 15-70d24 (LS450958)	99.8
	fumC	621	86.2	av *C. abortus* 15-70d24 (LS450958)	99.8
	gatA	483	87	av *C. abortus* 15-70d24 (LS450958)	98.6
	oppA	557	84.4	av *C. abortus* 15-70d24 (LS450958)	99.8

4.4 Discussion

Our study confirmed the presence of avian *C. abortus* and *C. psittaci* in shorebirds and humans handling the birds in New Zealand, with avian *C. abortus* as the primary species detected. We detected avian *C. abortus* in these three new bird species (bar-tailed godwit, pied stilt, and South Island pied oystercatcher). Further, our detection of avian *C. abortus* in both the North Island and South Island of New Zealand suggests that the organism has likely been present and unrecognised, rather than newly emerged in the country.

With recent whole genome sequencing analysis, the classification of *C. abortus* group was suggested to expand, to incorporate the traditional livestock and emerging avian isolates. The term "avian *C. abortus"*, should be used to describe the emerging avian isolates, previously known as *C. abortus*/*C. psittaci* intermediaries (Longbottom et al., 2021; Szymańska-Czerwińska et al., 2017; Zaręba-Marchewka et al., 2019). For

instance, avian *C. abortus* detection was reported in Polish wildfowl, Italian Eurasian magpie (*Pica pica*), hooded crows (*Corvus cornix*), Australian pied oystercatcher, short-tailed shearwaters, white faced heron, and magpie-lark (*Grallina cyanoleuca*), Galapagos waved albatross (*Phoebastria irrorata*) (Aaziz et al., 2022; Aaziz et al., 2023; Kasimov et al., 2021). These study results showed that avian *C. abortus* is not limited to specific hosts or countries, as it has been identified in bird species from different orders and families, from Europe, Oceania, and South America.

In terms of the genotypic diversity of chlamydial strains present in New Zealand, we identified avian *C. abortus* genotype G1 in pied stilts, bar-tailed godwits, and South Island pied oystercatchers. To date, avian *C. abortus* genotype G1 had been reported mainly in aquatic bird species like swan, Eurasian teal, and Eurasian coot, and Galápagos waved albatross (*Phoebastria irrorata*) (Aaziz et al., 2023; Szymańska-Czerwińska et al., 2017). In our study, we also identified *C. psittaci* genotype C in pied stilt. Generally, *C. psittaci* genotype C are commonly detected in waterfowl like farmed ducks (Vorimore et al., 2015) and wild mallard (Szymańska-Czerwińska et al., 2017). In New Zealand, *C. psittaci* genotype C has been reported in endemic birds like Kākā (*Nestor meridionalis*), brown teal (*Anas chlorotis*), Whio (*Hymenolaimus alacorhynchos*), paradise shelduck (*Tadorna variegata*), and introduced birds like mallard (*Anas platyrhynchos*) (Gedye et al., 2018). In detail, *C. psittaci* genotype C was detected in 22% (11/50) of the wild mallard population in Palmerston North, Manawatū region (Soon et al., 2021). Although the sampling site from Soon et al. (2021) was not the same as our current study, both surveys were done in the Manawatū region. Both host species (pied stilts and mallards) could be the reservoir hosts, or the cases could represent a spillover event from mallards to pied stilts. Both mallards and pied stilts were feeding and sharing the natural habitat at Manawatū Estuary and mallards have long been known to be a natural reservoir of *Chlamydia* species (Evans et al., 1983). Since both avian *C. abortus* genotype G1 and *C. psittaci* genotype C were commonly reported in aquatic birds, future studies investigating the epidemiology of chlamydiae in wild birds in the region should include nearby water bodies to investigate the role of different bird species in disease transmission and spread.

In our study, the shorebirds harbouring avian *C. abortus* genotype G1 and *C. psittaci* genotype C were apparently healthy. Similar observation was reported in Galápagos waved albatross (*Phoebastria irrorata*) in which all birds detected with avian *C. abortus* genotype G1 showed no clinical signs of disease when the sample was taken (Aaziz et al., 2023). Although pathogenicity of the newly discovered avian *C. abortus* is understudied, the Australian pied oystercatcher detected with avian *C. abortus* had clinical signs of disease (Kasimov et al., 2021). Laying hens infected with *C. psittaci* genotype C were observed to have poor egg production due to cystic oviducts (Zhang et al., 2008). However, different disease signs can be observed in host species infected with same chlamydial strains making cross-species extrapolations of pathogenicity difficult. Variability in disease severity and clinical signs between populations can be multifactorial. For instance, when shorebirds experience physiologic stress associated with bird migration, there is a risk of increasing immunosuppression, leading to increased host susceptibility and reduced tolerance of infection (Reed et al., 2003). Also, stressed birds may have increased chlamydial shedding and longer shedding durations, contributing to increased environmental contamination and further transmission (Harkinezhad et al., 2009). Since one of the main study sites in this study – Firth of Thames, is an internationally important destination for Arctic migratory birds using the East-Australasian flyway, it is possible for between-bird transmission of chlamydiae to happen via both national and international migration.

In our previous chapter (3), all the isolates were initially identified as *C. psittaci* using qPCR based on variable domain (VD) 1 and 2 of the *ompA* gene. The more discriminatory gene sequencing using *ompA* gene in this study has revealed the presence of avian *C. abortus* and *C. psittaci* in the shorebirds in New Zealand. This confusion between closely related chlamydial species is consistent with a previous study, when the avian *C. abortus* was identified as *C. psittaci* using an *incA*-based qPCR (Szymańska-Czerwińska et al., 2017). The closely related *C. psittaci* and *C. abortus* were also indistinguishable using qPCR based on VD1 of the *ompA* (Mitchell et al., 2009). Later, the investigation of the VD4 region of the *ompA* gene in qPCR was effective in

differentiating *C. psittaci* and *C. abortus*, while maintaining its high sensitivity, simplicity and cost-effectiveness (Heddema et al., 2015). For species-specific detection, it is recommended to use CPSIT_0607 (hypothetical protein) gene for *C. psittaci* (Opota et al., 2015; Voigt et al., 2012) and *sucB* (dihydrolipoamide acetyltransferase) gene for *C. abortus* (both avian and mammalian strain) (Aaziz et al., 2023), in which both genes were found to be unique to their own species. We opted for *ompA* gene instead of both targeting genes mentioned above because it was the most used targeting genes to detect *C. psittaci* (Chapter 2). Since avian *C. abortus* is only newly discovered, its detection has been done mostly via gene sequencing (*ompA*, partial 16S rRNA gene, intergenic spacer, and partial 23S rRNA gene) which requires a large amount of DNA in the samples (Kasimov et al., 2021; Stalder et al., 2020; Szymańska-Czerwińska et al., 2017). Genomic tools with better sensitivity and specificity are needed to allow rapid detection and to establish the prevalence and diversity of chlamydial species in susceptible bird populations.

The phylogenetic classification of avian *C. abortus* has been unclear, but it has improved significantly with the advancement of molecular techniques. Due to their close genetic relationship, the taxonomy of both *C. psittaci* and *C. abortus* has undergone multiple revisions. By analysing the 16S and 23S rRNA gene sequences phylogenetically, *C. psittaci* and *C. abortus* were grouped as separate species (Everett et al., 1999). Later, to investigate some unresolved cluster of *C. psittaci* strains (84/2334, Prk/Daruma), three different genes (*ompA*, *rnpB*, and *rrn* intergenic spacer) were analysed. This study recommended to use multiple genes when characterising *C. psittaci* and *C. abortus* strains, as there is an evolutionary link between these two species (Van Loock et al., 2003). Then, a species-specific qPCR test, targeting *ompA* gene, to detect *C. psittaci* and *C. abortus* was developed and is still widely applied (Pantchev et al., 2009). Traditionally, *C. abortus* is considered to be a ruminant pathogen causing ovine enzootic abortions and thus significant economic impacts (Borel et al., 2018). Besides its mammalian hosts, *C. abortus* has also been reported in budgerigars (*Melopsittacus undulatus*) and oriental white stork (*Ciconia boyciana*) (Japan), and feral pigeon (*Columba livia*) (Germany) when characterised by the *ompA* gene (Chahota et al., 2006; Sachse et al., 2012). By analysing the 16S and 23S rRNA genes, *C. abortus* was detected

in feral pigeon and white stork (*Ciconia ciconia*) in Austria (Konicek et al., 2016). In the case where the molecular data is available, phylogenetic analysis is required to determine whether the detected *C. abortus* strain is of avian or mammalian origin.

In our study, in the MLST analysis, with only four genes (out of seven genes) being sequenced, the MLST typing was not achievable due to low DNA amount recovered. For MLST classification, published data shown that the avian *C. abortus* genotype G1 detected in Polish waterfowl were identified as a MLST type of ST154, while *C. psittaci* genotype C in duck and sheep were ST28 (Sachse et al., 2023; Szymańska-Czerwińska et al., 2017). To date, the chlamydial sequence type (ST) identified in New Zealand were ST24 in budgerigar, and ST27 in diamond dove (*Geopelia cuneata*), zebra finch (*Taeniopygia castanotis*), superb parrot (*Polytelis swainsonii*), and little blue penguin/Kororā (*Eudyptula minor*) from New Zealand (Kasimov et al., 2023). In our study, with a low infection load of chlamydial pathogens in apparently healthy hosts, the non-targeted genome capture method using multiple displacement amplification was insufficient as this method required a higher amount of chlamydial DNA for full genome coverage (Taylor-Brown et al., 2018). The two published studies with successful culture-independent genome sequencing of *C. psittaci* were using targeted sequence capture method on clinical samples from patients that were seriously ill, with correspondingly higher infection loads (Branley et al., 2016; White et al., 2022). In a study where PC3 laboratory and cell culture facility is available, Chlamydiae samples should be cultured to obtain higher amount of DNA (Branley et al., 2016). Future studies should also include samples from clinically ill patients and birds, and perform the targeted sequence capture method to allow whole genome sequencing (WGS) or MLST as an accessible alternative (White et al., 2022).

Chlamydial pathogens can cause diverse wildlife diseases, with two major biological implications: conservation threat in global biodiversity and reservoirs of infectious pathogens to human and domestic animals (Daszak et al., 2000). Firstly, the direct impact of chlamydial infections on biodiversity can occur when highly susceptible hosts are infected with *C. psittaci*, causing mortality and population declines in bird populations (Stokes et al., 2021). Although there are limited publications available on

the impacts of chlamydial infection in wild birds (Burnard & Polkinghorne, 2016), suspected epizootics have occurred in various gull species in the USA, causing a large-scale mortality of more than four hundred birds, mainly fledglings (Franson & Pearson, 1995). Conservation concerns may also arise from the host tropism in chlamydial strains, with certain genotypes more commonly observed in specific orders of birds, with varying pathogenicity (Sachse, Laroucau, et al., 2015). Concurrent infection and immunosuppression due to additional stressors in chlamydial infection in birds often increase disease severity, leading to mortality events (Stokes et al., 2021). In a retrospective survey of mortality incidents in British wild passerines, about half of the *C. psittaci*-positive birds had concurent infections, including avian pox (Beckmann et al., 2014). In New Zealand, it has been associated with a mortality cluster in wild Malay doves (*Streptopelia chinensis*) (Rawdon et al., 2009). So, I recommend *Chlamydiae* testing should be included when investigating unusual mass mortality events in wild birds in New Zealand.

With limited evidence, *C. psittaci* spillover from wildlife to domestic animals and humans is possible (Burnard & Polkinghorne, 2016). The epidemiological interactions at wildlife-livestock interface was illustrated in a *C. psittaci* outbreak in Australia. The farm ducks were housed in open growing sheds, readily exposed to wild waterfowl (Tiong et al., 2007). The disease transmission possibly occurred when the wild birds as the *C. psittaci* reservoir, were attracted to the feed resources and contaminated the feed in the facility (Woodford & Rossiter, 1994; Burnard & Polkinghorne, 2016). In 2014, the presence of *C. psittaci* in equine foetal membranes leading to foal loss was likely associated with a cluster of five cases of human *C. psittaci* infection at a rural veterinary school in New South Wales, Australia (Chan et al., 2017). A follow up investigation using MLST to characterise the *C. psittaci* strain linked to the equine placentitis revealed that the isolate denoted to *C. psittaci* genotype A (ST24/6BC clade). This molecular description suggested that the native Australian parrots may have been the original reservoir (Jelocnik, Branley, et al., 2017). A bigger study involving 161 cases of equine abortion conducted in 2016 in New South Wales revealed a *C. psittaci* positivity of 21.1% (Jenkins et al., 2018). Besides *C. psittaci* of parrot-origin, in a stud farm in Queensland where pigeons were sighted, a pigeon-type *C. psittaci* (ST27/genotype B) was detected

in the placenta and aborted equine foetus lung tissue in an equine abortion case (Jelocnik et al., 2018). Finally, a survey of *C. psittaci* in a wide range of hosts (parrots, pigeons, horses and humans) using MLST, confirmed that clonal ST24 strains Clonal ST24 strains predominantly cause infections in Australian parrots and horses (82/88; 93.18%) (Anstey et al., 2021). In New Zealand, wild waterfowl (peri-domestic wildlife) commonly appear on farm waterways and pastures, sometimes visiting rural backyard poultry flocks (Zheng et al., 2010). The mallards also share the feeding sites with the migratory birds at the estuaries and wetlands. So, the potential disease transmission pathway of *C. psittaci* in New Zealand could be from the migratory shorebirds to peri-domestic mallards to free-range poultry and other livestock.

In the human samples, we detected *C. psittaci* genotype C and avian *C. abortus* genotype G1 in the nasal swabs which was identical to the strains identified from the bird samples. This is suggestive of the potential exposure risk of both chlamydial strains to humans handling the birds. In a systematic review of human psittacosis, genotypical matches between human and animal samples were reported in 14 articles (n = 80). The most commonly reported genotypes with genotypical match between human and birds were genotypes A, B and E/B, with no report on the genotypical matches of *C. psittaci* genotype C (Hogerwerf et al., 2020). However, potential zoonotic risk was observed when *C. psittaci* genotype C was detected in the air samples from turkey hatchery and pharyngeal swabs from hatchery workers (Dickx & Vanrompay, 2011). In a psittacosis outbreak in a British poultry processing plant, the common symptoms of *C. psittaci* genotype C infection in human were fever, cough, sore throat, and headache (Williams et al., 2013). In our study, the detection of *C. psittaci* genotype C in pied stilt and the human sample were not from the same sampling site as not all positive samples had sufficient DNA amount for further phylogenetic analysis. Besides, additional exposure information such as occupational exposure (farmed poultry) or recreational exposure (pet birds), which could be a source of infection, was not collected from the participants in this study. To compare the bird and human isolates in detail, whole genome sequencing followed by phylogenetic, and SNP (single nucleotide polymorphisms) analysis should be performed to confirm if the isolates were identical (Branley et al., 2016).

In conclusion, the genomic diversity of Chlamydiae in the three estuarine sites in New Zealand, important wetlands for breeding shorebirds, was investigated in this study using DNA sequencing. In this study, we identified avian *C. abortus* genotype G1 and *C. psittaci* genotype C in both humans and shorebird samples. The first detection of avian *C. abortus* in New Zealand indicated that there is more than one species of Chlamydia present in New Zealand shorebirds. This result suggests that shorebirds are a possible reservoir of avian *C. abortus* for humans, and wild aquatic birds sharing the habitats. A collaborative One Health approach like routine targeted surveillance of Chlamydiae at human-wildlife-livestock interface in New Zealand is needed to improve wildlife health, reduce risk of emerging zoonoses, and mitigate livestock disease transmission.

CHAPTER 5

General discussion

CHAPTER 5 General discussion

5.1 Overview of the key findings

The primary objective of this thesis was to investigate the occurrence of chlamydiae in shorebirds (or waders or Charadriiformes) and people handling the shorebirds at four estuarine sites in New Zealand. In our study, the seven species of shorebirds that were sampled were bar-tailed godwit (*Limosa lapponica*), red knot (*Calidris canutus*), wrybill (*Anarhynchus frontalis*), South Island pied oystercatcher (SIPO; *Haematopus finschi*), variable oystercatcher (*Haematopus unicolor*), pied stilt (*Himantopus leucocephalus*), and black-backed gull (*Larus dominicanus*). Of all four estuarine sites (Firth of Thames, Manawatū estuary, Tasman Bay, Golden Bay) that we had collected our samples from, two of the sites (Firth of Thames and Manawatū estuary) are important Ramsar sites. These two sites are key regions for shorebirds during breeding or wintering seasons (Dowding & Moore, 2006). The spillover risk of pathogens at these estuarine sites between the arctic migrants, endemic shorebirds, and humans due to their potential interactions is of considerable interest.

In Chapter 2 I carried out a systematic review of the molecular detection methods of *Chlamydia psittaci* in birds. Since there were so many different methods available to detect *C. psittaci*, this study aimed to identify the most used molecular detection of *C. psittaci* in birds in published articles, for different study purposes. From the 120 articles (January 2000 to July 2020) included in the review, we identified 12 study purposes and 14 types of molecular tests to detect *C. psittaci* in birds. The most common purpose for the studies I reviewed was surveillance while the most commonly applied molecular tests were conventional PCR and quantitative PCR. There are currently ten genomic targets available to detect *C. psittaci* in birds, and the most widely used target gene was the *ompA* gene. The consensus recommendations from the systematic review were: that a testing strategy should use a hierarchical approach that includes molecular tests of genus- and species-specific targets to facilitate the detection of novel emerging avian chlamydial species besides the well-recognised *C. psittaci*; samples should be sourced from both the respiratory and gastrointestinal tract, instead

of one site, whenever possible to ensure better accuracy of detection; and lastly, standardisation and validation of molecular *Chlamydia* tests is needed to enhance the comparability and reliability of assays to detect *C. psittaci* and other chlamydiae species in birds.

Chapter 3 describes the survey I performed to determine the prevalence of chlamydiae in shorebirds at four estuarine sites which are important wetlands as breeding and wintering sites for Arctic migrant and endemic shorebirds in New Zealand. The detection of chlamydiae in people handling shorebirds was also identified at three of these estuarine sites. Overall, chlamydiae was detected at all four estuarine sites in six species of shorebirds. In the people handling the shorebirds, chlamydiae was detected in samples from handlers at two estuarine sites (Firth of Thames, Golden Bay). This study provides new information on the species of shorebirds infected with chlamydiae in New Zealand and estimates the prevalence of chlamydial infection at that time. The positive detection of chlamydiae from both bird and human samples was suggestive of a zoonotic spillover.

In Chapter 4, I used the samples collected in Chapter 3 to describe the genomic diversity of chlamydiae in shorebirds and their handlers at the four estuarine sites in New Zealand. By using *ompA* sequencing, two species of chlamydiae were identified in both bird and human samples. Most samples were identified as the newly-characterised avian Chlamydia species – avian *C. abortus* genotype G1, while one bird and one human sample were identified as *C. psittaci* genotype C. These findings represent the first detection of avian *C. abortus* genotype G1 in birds and humans in New Zealand and suggests the diversity of chlamydiae present in New Zealand may have been underestimated in previous studies based solely on PCR studies. Phylogenetic analysis in this study revealed that the range of chlamydiae species detected in the shorebirds was similar across all four estuarine sites in New Zealand, which is consistent with the movement of shorebirds between all these sites. The increased resolution in characterising the strain of chlamydiae provided clearer information of the potential for pathogen transmission in the shorebirds and the potential spillover risk between

different hosts at the study sites. Further study is required to understand the role of specific host species in disease transmission.

5.2 Reservoirs of infections

The reservoir species of a multi-host pathogen can be defined as the epidemiologically connected populations in which the pathogen can persist and maintain to act as a continuous source of transmission to a target host species of the study (Haydon et al., 2002). Several practical indicators to identify the reservoir include epidemiologic evidence of association, natural infection in nontarget populations, genetic characteristics of isolated pathogen, and intervention studies (Haydon et al., 2002). Depending on the focus of the study, the target host species may include humans for public health significance, domestic livestock for food security and economic impacts, or wild animal species of conservation interest (Roberts & Heesterbeek, 2020). At times, the target population itself may be part of the reservoir (Haydon et al., 2002). In relation to my study, I was interested in both the possible impact of chlamydiae on shorebird conservation and the public health relevance. With this differing focus, my target population could be Arctic migrants, endemic shorebirds, or human health. I will focus my discussion on bird interactions in this section, so that shorebirds are both the potential target of the study and reservoirs of the chlamydiae pathogens.

In my study, I detected chlamydiae in two species (bar-tailed godwit, red knot) of Arctic migrants and four resident species (South Island pied oystercatcher (SIPO), wrybill, pied stilt, southern black-backed gull) of endemic shorebirds, suggesting a common source allowing both national and international movement of chlamydiae. The positive detection of chlamydiae in the apparently healthy shorebirds provided supportive evidence of natural infection in the population. Further phylogenetic analysis confirmed the presence of *C. psittaci* in pied stilt, and avian *C. abortus* in bar-tailed godwit, South Island pied oystercatcher, and pied stilt. By using *ompA* sequencing, the genetic characteristics of isolated avian *C. abortus* in Arctic migrant (bar-tailed godwit), and endemic shorebirds (SIPO and pied stilt), all identified as genotype G1, was consistent with reservoir-target transmission. At this stage, depending on the target

population of interest, one species of Arctic migrant (bar-tailed godwit) and two species of endemic shorebirds (SIPO and pied stilt) could act as reservoir of avian *C. abortus* in New Zealand. To confirm the role of these shorebird species as reservoir hosts, longitudinal studies of identifiable individual birds with multiple sampling events throughout the year are needed to identify the persistence and maintenance of chlamydiae in the shorebirds.

5.3 Pathogen spillover

Pathogen spillover is a driver of disease dynamics when pathogen transmission occurs from a reservoir to a target population due to high pathogen abundance in the reservoir population (Daszak et al., 2000; Power & Mitchell, 2004). The prevalence of pathogen in reservoir hosts is one of the determinants of spillover risk (Plowright et al., 2017). In our study, we were able to estimate the apparent prevalence of chlamydiae in bar-tailed godwit, South Island pied oystercatcher (SIPO), and pied stilt. These three species of shorebirds could act as a potential source of transmission of chlamydiae to the other susceptible bird populations at the four estuarine sites in New Zealand. For instance, at one of the estuarine sites (Firth of Thames), there is a high degree of spatial and temporal overlap between multiple species of endemic shorebirds (SIPO, pied stilt, wrybill) and Arctic migrants (bar-tailed godwit) (Dowding & Moore, 2006). If a future study is developed to look at the potential direction of pathogen transmission between Arctic migrants and local shorebirds, samples could be collected from the Arctic migrants before their departure to New Zealand, on arrival in New Zealand, and just prior to departure from New Zealand to illustrate the seasonal pathogen dynamics of chlamydiae infection in these international migrants. Such information would allow an evaluation of the role of these birds in the international movement of chlamydiae species.

A feasible transmission route must be established to define pathogen spillover and to confirm a functional reservoir system (Hallmaier-Wacker et al., 2017). Generally, bird-to-bird transmission pathways of *C. psittaci* happens when aerosolised infectious

agents from dried faeces and respiratory secretion were inhaled by susceptible host (Sachse, Laroucau, et al., 2015). Out of the four basic requirements to define a feasible transmission route (Hallmaier-Wacker et al., 2017), I was able to fulfil two criteria. Firstly, I observed direct spatial and temporal connectivity between different shorebird species at the sampling estuarine sites. Then, I detected the DNA of chlamydiae (pathogen involvement) in choana and cloaca of multiple species of the shorebirds. However, my study did not provide proof of pathogen viability which is essential to confirm active infection in the host species. For this purpose, future studies could try to isolate chlamydiae from shorebird samples using Chlamydia transport medium to maintain viability and then cell culture for isolation. However, the laboratory culture of chlamydiae is laborious, which is why I didn't attempt this in my research. Lastly in order to confirm spillover transmission, longitudinal studies are needed to confirm the pathogen presence at multiple times between the non-target and target populations, and within the non-target community as evidence of feasible transmission route.

Describing the natural history of infection in a host is important to fully determine the pathogen dynamics in reservoirs and hence distribution of infection. This includes the level and timing of pathogen shedding from infected hosts, and the severity, intensity, and duration of infection (Plowright et al., 2017). In our study, all shorebirds appeared apparently healthy with no evidence of nasal discharges, conjunctivitis, and diarrhoea. The severity and intensity of *C. psittaci* infection in wild birds are not commonly reported, and reports on the impacts of other chlamydial species in wild birds are sparse (Burnard & Polkinghorne, 2016; Stokes et al., 2021). In captive birds, *C. psittaci* infection can result in severe disease, avian chlamydiosis, with associated clinical signs of upper respiratory tract disease, hepatitis, septicaemia, or sudden death. However, there is variation in disease expression between different orders of birds with parrots and pigeons being more severely affected than other species that may show little evidence of disease following infection (Zaręba-Marchewka et al., 2020). Generally, wild birds in Australia infected with chlamydiae commonly present with emaciation (Kasimov et al., 2021). In a case of severely ill crimson rosella (*Platycercus elegans*) in Australia, emaciation and ocular discharges was observed. Bacterial quantification of the swab samples from bird's conjunctiva revealed high

pathogen load and shedding level (Branley et al., 2008). The susceptibility of shorebirds to avian chlamydiosis following chlamydiae infection is not currently known. In a wild bird population with potential endemic infection of chlamydiae, a seroprevalence study could be conducted to determine antibody persistence. This evidence of chronic infection may have implications on environmental and direct transmission, increasing the potential spillover risk of chlamydial infection from host to host (Stokes, Martens, Walder, et al., 2020). However, antibodies to chlamydiae are generally short-lived in birds and show cross reactivity resulting in false positive results, limiting the usefulness of seroprevalence studies (Fudge, 1997).

5.4 Methodological limitations

The common limitations of wildlife disease surveillance include the challenges to design sampling strategies that sufficiently represent the targeted population, the lack of validated diagnostic tests for wildlife, and missing data leading to inaccurate data interpretation (Stallknecht, 2007). For instance, in Chapter 3, I described a cross-sectional study of Chlamydiae in shorebirds in four selected estuarine sites in New Zealand. Convenience sampling was performed in our study as there were no previous data or reported spillover events of chlamydiae infection in shorebirds in New Zealand. Since the shorebirds were captured as part of regular annual banding studies for species of interest, sample collection was temporally, spatially and species restricted. For instance, of all the 74 estuarine sites in New Zealand, we were only able to collect samples from four estuarine sites where banding events happened. Also, due to the nature of capture performed on wild populations (cannon netting), we obtained unequally distributed sample sizes for individual shorebird species and locations in our study. So, further statistical analysis to compare the pathogen prevalence between host species and locations was not performed. In addition, basic biological data such as age and sex was not collected in this study due to the limitations of aging and sexing wild shorebirds out of breeding season. Since sex was an important predictor of chlamydiae prevalence in crimson rosella (Stokes, Martens, Jelocnik, et al., 2020), future studies should collect this information to improve the precision when comparing prevalence

between or within populations. Future studies should ideally consider stratified random sampling design while capturing the bird species of interest for sample collection.

In wildlife research, most diagnostic tests used were developed for domestic animals and not validated for the species of interest due to logistical difficulties in conducting study in wild animals (Breed et al., 2009). In our study, the qPCR assay used was validated in a study using samples from mallards (*Anas platyrhynchos*) (Soon et al., 2021), but not validated for shorebird species. In Chapter 3, all the isolates were initially identified as *C. psittaci* using qPCR based on variable domain (VD) 1 and 2 of *ompA* gene. However, most of the isolates were recognised as avian *C. abortus* after further phylogenetic analysis based on sequencing of 23S rRNA and *ompA* gene. The qPCR assay based on VD1 of the *ompA* gene is not able to differentiate the closely related *C. psittaci* and mammalian *C. abortus* (Mitchell et al., 2009). Instead, qPCR assay based on VD4 region of the *ompA* gene was effective in differentiating *C. psittaci* and mammalian *C. abortus* (Heddema et al., 2015). Since avian *C. abortus* is only newly discovered, its detection has been done mostly via gene sequencing (*ompA*, partial 16S rRNA gene, intergenic spacer, and partial 23S rRNA gene) which requires a high amount of DNA in the samples (Kasimov et al., 2021; Stalder et al., 2020; Szymańska-Czerwińska et al., 2017). For species-specific detection, future studies should use targeted PCR using the CPSIT_0607 (hypothetical protein) gene for *C. psittaci* (Opota et al., 2015; Voigt et al., 2012) and the *sucB* (dihydrolipoamide acetyltransferase) gene for *C. abortus* (both avian and mammalian strain) (Aaziz et al., 2023).

5.5 Conservation implications

Infectious disease can affect the dynamics of extinction directly or indirectly by causing both reduced reproductive fitness and increased mortality (De Castro & Bolker, 2005; McCallum, 2012). In mammals, population decline in an Australian marsupial, koala (*Phascolarctos cinereus*), have been caused by chlamydial infections (*C. pecorum*, *C. pneumoniae*) leading to *Chlamydia*-associated infertility and mortality (Polkinghorne et al., 2013). There is limited information regarding the effects of chlamydial infections

on reproductive success and survival in wild birds despite it being a significant cause of disease and mortality in captive birds, especially parrots and pigeons. One study of Australian crimson rosella revealed that breeding birds were less likely to be infected by chlamydiae than non-breeding birds. The author suggested multiple possibilities for this observation. The hypotheses included the monogamous behaviour of crimson rosella during breeding season which reduces contact with conspecifics, reduced probability of breeding in infected birds, or purely seasonal variation of the prevalence of Chlamydiae in the birds (Stokes, Martens, Jelocnik, et al., 2020). In domestic birds, there was a report on the isolation of *C. psittaci* in laying hens associated with cystic oviducts, resulting in poor egg production (Zhang et al., 2008). However, current outbreaks in poultry industry had low mortality, affecting mostly young birds (Sachse, Laroucau, et al., 2015). In the wild settings, mass mortality events of wild birds associated with Chlamydial infection were reported in gulls and rosy-faced lovebirds (*Agapornis roseicollis*) (Dusek et al., 2018; Franson & Pearson, 1995). In New Zealand, it has been associated with a mortality cluster in wild Malay doves (*Streptopelia chinensis*) (Rawdon et al., 2009). So, I recommend Chlamydiae testing should be included when investigating unusual mass mortality events in wild birds in New Zealand.

Concurrent infection and immunosuppression due to additional stressors in chlamydial infection in birds often increase disease severity, leading to mortality events (Stokes et al., 2021). In captive settings in Mexico, high mortality associated with *C. psittaci* was reported in a group of wild-caught endangered psittacine birds recovered from illegal trade kept in the same place (Ornelas-Eusebio et al., 2016). In another case of wild-caught birds from illegal trade in Brazil, a mortality rate of 97% reported in a group of 58 blue-fronted Amazon parrot (*Amazona aestiva*) nestlings were tested positive for *C. psittaci*, *Shigella* sp., and *Escherichia coli* (De Freitas Raso et al., 2004). In a retrospective survey of mortality incidents in British wild passerines, about half of the *C. psittaci*-positive birds had concurent infections, including avian pox (Beckmann et al., 2014). Besides, co-infection of circovirus and *C. psittaci* was also reported in wild Australian parrots (beak and feather disease virus) (Amery-Gale et al., 2020) and Polish feral pigeons (pigeon circovirus) (Stenzel et al., 2014; Sutherland et al., 2019). Longitudinal data is limited in wild birds to determine if chlamydial infections is a

predisposing factor to co-infections, or if birds immunosuppressed by other infectious agents are more susceptible to chlamydial infection (Stokes et al., 2021). In chickens, *C. psittaci* infection prior to avian influenza virus (H9N2) contributed to higher mortality, and higer level of virus shedding, when compared to *C. psittaci* infection after H9N2. *C. psittaci* could exhibit immune suppression in chickens, aggravating the effect of viral infection (Chu et al., 2016). Therefore, chlamydiae screening should be included when surveying for other pathogens in wild birds in New Zealand to assess the impact of co-infections. Broad range PCR panels for avian respiratory disease have been recommended to detect co-infections in chickens (Croville et al., 2018), and these techniques would be of benefit in wild bird studies as well.

In our study, all the shorebirds were apparently healthy. Variability of disease severity in host populations may differ with the presence of additional stressors. For instance, when shorebirds travel on the East-Australasian flyway and experience physiologic stress associated with bird migration, there is a risk of increasing immunosuppression, leading to increased host susceptibility (Reed et al., 2003). So, Chlamydiae-positive birds harbouring the pathogen may experience exacerbated impacts of chlamydial infection during migration and this may affect survivability and the bird's ability to complete the migration. Also, stressed shorebirds may shed more chlamydial infectious agents in longer duration, contributing to higher risk of environmental contamination and further spillover to local wild bird populations (Harkinezhad et al., 2009). In New Zealand, the presence of 24% *C. psittaci*-positive wild mallards (*Anas platyrhynchos*) suggested that this most abundant species of waterfowl may be the pathogen reservoir in New Zealand (Soon et al., 2021). Besides, Chlamydiae was commonly detected in both captive and wild Psittaciformes (parrots) and Columbiformes (pigeons), and occasionally in some endangered species of native birds like South Island takahe (*Porphyrio hochstetteri*), little blue penguin (*Eudyptula minor*), brown teal (*Anas chlorotis*) and whio (*Hymenolaimus malacorhynchos*) (Gedye et al., 2018). Further study is needed to determine the direction of transmission between the wild mallards, shorebirds and other native birds to ensure effective conservation management, monitoring the spread of Chlamydiae to naïve host population.

5.6 Zoonotic transmission of chlamydial infection

Wildlife reservoirs have always been considered a major source of emerging infectious disease for human health (Jones et al., 2008). A systematic review reporting human psittacosis outbreaks included three articles on outbreak of wild bird-origin in their study (Nieuwenhuizen et al., 2018). All three human psittacosis outbreaks were in Australia, and the people had direct or indirect contact with sick, infected, wild psittacine birds (Branley et al., 2008; Telfer et al., 2005; Williams et al., 1998). Also, human psittacosis was also reported to link with wild pigeons (Dickx, Beeckman, et al., 2010; Haag-Wackernagel & Moch, 2004; Mair-Jenkins et al., 2018), and other bird species including fulmar birds (*Fulmaris glacialis*), and Charadriiformes (Herring gulls (*Larus argentatus*), and Northern lapwings (*Vanellus vanellus*)) (Fossadal et al., 2018; Kalmar et al., 2014). Transmission of chlamydial infection from wild birds to human may be due to contact with infected bird faeces, while cleaning the cage or wild bird feeder (Chereau et al., 2018; Rehn et al., 2013). On the other hand, infected humans could be the source of chlamydial infection, as human-to-human transmission of *C. psittaci* was confirmed via epidemiological investigation and laboratory metagenomic next-generation sequencing (Zhang et al., 2022). In my research, I detected identical chlamydial species from both the shorebirds and people handling the birds, but the direction of the transmission pathways, if this occurred, was not able to be identified. Since both the shorebirds and humans can be the source of infection, further phylogenetic analysis using next-generation sequencing, to compare the chlamydial strains within and between hosts is needed to confirm the transmission pathways.

Besides the direct transmission from wild birds to human, it is also possible for the indirect transmission of chlamydial infection from wild birds to humans working with poultry and other domestic animals. For instances, wild sea birds may be a potential source of chlamydial infection in French mule ducks, resulting in a hospitalised case of psittacosis in a farm worker (Hulin et al., 2015). As the farm was near the sea, transmission may occur via contaminated water when the free-range ducks share moist soil or aquatic habitats with infected wild aquatic birds, or via contaminated feeding

equipment (Harkinezhad et al., 2009). Similarly, phylogenetic evidence was reported in a potential bird to mammal *C. psittaci* spillover from wild parrots to horses, and then to humans in Australia. This highly virulent strain (ST24 / 6BC clade) of *C. psittaci* was associated with equine abortion and clinical pneumonia in humans (Chan et al., 2017; Jelocnik, Branley, et al., 2017; Jenkins et al., 2018). Although wild birds were usually known as the main reservoir of Chlamydiae, it is also possible for the transmission of Chlamydiae from domestic animals to wild birds. For instance, chickens, as the main reservoir of *C. gallinacea,* could have spread the pathogen to nearby wild Australian galah (*Eolophus roseicapillus*), when being kept in a free-range setting (Stokes et al., 2019). Therefore, future surveys of Chlamydiae in wild birds should consider interactions with free-ranged domestic animals and the people nearby to better illustrate the host pathogen dynamics.

Overall, zoonotic disease dynamics are affected by the spatial and temporal interactions in ecosystem and climates, and between human behaviour, ecology of animal hosts and infectious agents (Lambin et al., 2010). Wildlife are usually portrayed as the zoonotic disease reservoir and fear messages can diminish people's support for wildlife conservation. Wildlife-associated disease messages should be balanced to increase public's knowledge and improve people's confidence to reduce exposure (self-efficacy) and mitigate disease risk by the relevant institutions (e.g., health departments, wildlife agencies) (Buttke et al., 2015). As wildlife professionals, a collaborative One Health approach is needed to improve wildlife health, reduce risk of emerging zoonoses, and mitigate livestock disease transmission (Society, 2020). In this case, routine targeted surveillance of *Chlamydiae* in wild birds in New Zealand is required to monitor and rapidly respond to any chlamydiosis outbreak. Similarly, while monitoring and surveying the zoonotic risk of *Chlamydiae* in humans, preventative intervention like implementing behavioural changes and outreach at the frontline is essential to reduce *Chlamydiae* exposure at the wildlife-human interface. Finally, a sustainable and integrated management in mitigating livestock disease transmission at the livestock-wildlife-human interface is fundamental to ensure balance between ecosystem integrity and global health.

5.7 Recommendations for future work

To overcome our laboratory methodological limitations, we should first consider using DNA extraction kits instead of Chelex to obtain clean and concentrated DNA for more sensitive detection of chlamydiae. Next, the non-targeted genome capture method using multiple displacement amplification was insufficient in our study as we have a low infection load of chlamydial pathogens in healthy hosts. So, for future work we can consider culture-independent genome sequencing using a targeted sequence capture method (White et al., 2022). In cases where a PC3 laboratory and cell culture facility is available, chlamydiae samples should be cultured to obtain more DNA. For surveillance study of chlamydiae, we should always use *Chlamydiaceae* 23S rRNA qPCR from Ehricht et al. (2006) for screening. For species-specific detection, future studies should use targeted PCR using the CPSIT_0607 (hypothetical protein) gene for *C. psittaci* (Jelocnik, Islam, et al., 2017) and the *sucB* (dihydrolipoamide acetyltransferase) gene for *C. abortus* (both avian and mammalian strain) (Aaziz et al., 2023). For molecular typing, we can sequence the full-length *ompA* (Sachse et al., 2008) and perform MLST (Pannekoek et al., 2010). With good quality and high amount of DNA, it is recommended to perform whole genome sequencing to provide a comprehensive view of the entire genome.

To identify the risk factors of chlamydiae carriage in humans, future studies could collect and analyse data such as demographic information and lifestyle factors such as smoking habits or concurrent predisposing disease. It would also be useful to collect additional exposure information such as occupational exposure (farmed poultry) or recreational exposure (pet birds). This is to identify other potential sources of risk of chlamydiae exposure in humans and to be more certain that direct contact with shorebirds is the source of chlamydial infection in the individual participant. Since both the shorebirds and humans can be the source of infection, further phylogenetic analysis using whole genome sequencing, to compare the chlamydial strains within and between hosts is needed to confirm the transmission pathways. To compare the bird and human isolates in detail, whole genome sequencing followed by phylogenetic, and SNP (single nucleotide polymorphisms) analysis can confirm if the isolates were identical. Lastly,

further studies should include a follow-up to determine if people exposed to the chlamydiae carried by the shorebirds are susceptible to subsequent infection and disease. This could help us better understand if there is any chlamydial disease progression, identifying potential risk factors and inform public health guidelines.

For future studies of the prevalence of chlamydiae in wild birds, researchers should ideally consider stratified random sampling design when capturing the bird species of interest for sample collection. We can include more shorebird species with larger sample sizes to reduce uncertainty around the estimates of prevalence of chlamydiae in specific shorebird species. To confirm the role of these shorebird species as reservoir hosts, longitudinal studies of identifiable individual birds with multiple sampling events between and within years are needed to identify the persistence and maintenance of chlamydiae in the shorebirds. To confirm spillover transmission, longitudinal studies are needed to confirm the pathogen presence at multiple times between the non-target and target populations, and within the non-target community as evidence of feasible transmission route. If a future study is developed to look at the potential direction of pathogen transmission between Arctic migrants and local shorebirds, samples could be collected from the Arctic migrants before their departure to New Zealand, on arrival in New Zealand, and just prior to departure from New Zealand to illustrate the seasonal pathogen dynamics of chlamydiae infection in these international migrants. By incorporating all these data with spatio-temporal data from Global Flyway Network and individual bird data like age, sex, and bodyweight (Battley et al., 2019), a pathogen risk modelling and management project is possible to inform potential transmission risk of chlamydiae. Since both avian *C. abortus* genotype G1 and *C. psittaci* genotype C were commonly reported in aquatic birds, future studies investigating the epidemiology of chlamydiae in wild birds in the region should include nearby water bodies to investigate the role of different bird species in disease transmission and spread.

To further study the disease transmission of chlamydiae at the wildlife-livestock-human interface, future surveys of chlamydiae in wild birds should consider

epidemiological interactions between free-ranging domestic animals and the people nearby to better illustrate the host pathogen dynamics. This study can use a One Health approach to initiate field investigation on chlamydial infections, for example investigating the wild birds, farmed ducks and humans from Waikato region in New Zealand. This is an ideal geographical location to study the pathogen flow of chlamydiae at wildlife-livestock-human interface (Figure 5.1). The Waikato region encompasses the Pūkorokoro Miranda Shorebird Centre (migratory wild birds), three wildlife rehabilitation centres (captive wild birds), Waikato Fish and Game (mallards; peri-domestic wildlife), and a free-range duck farm (domestic poultry). This future study can potentially determine the direction of transmission between the wild mallards, shorebirds, other native birds and humans to ensure effective conservation management, monitoring the spread of chlamydiae to naïve host population. Finally, a sustainable and integrated management in disease mitigation is fundamental to ensure balance between ecosystem integrity and global health.

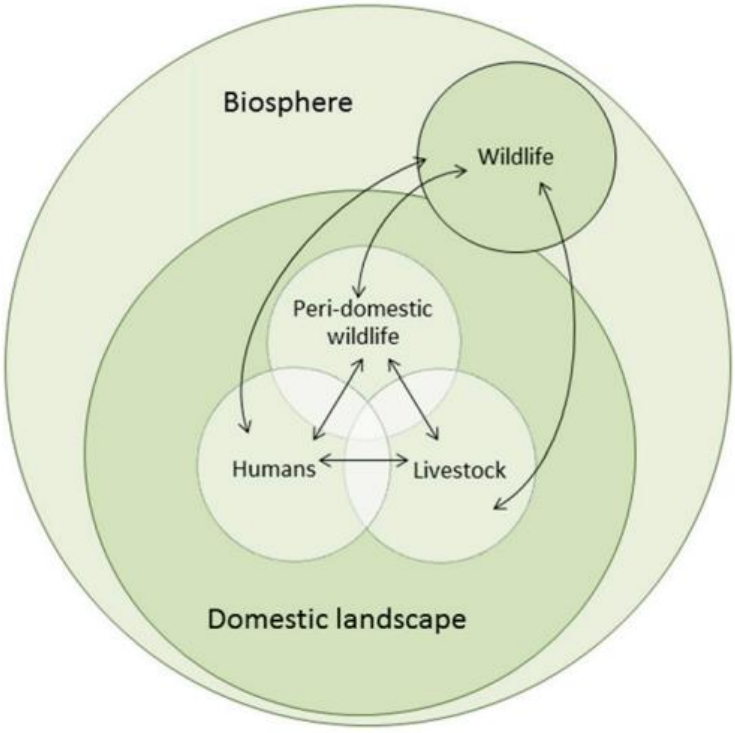

Figure 5.1. Pathogen flow at the wildlife-livestock-human interface adapted from Jones et al. (2013).

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Appendices

Appendices

Appendices for Chapter 2

Appendix 2.1

Characteristics of the selected studies on molecular methods to detect *Chlamydia psittaci* in birds, sorted first by date of study published, then by descending alphabetical order.

Reference, year	**Bird species**	**Year**	**Country**	**Purpose**	**Study design**	**Bird husbandry setting**
Wang et al., 2020	Fulmar (*Fulmarus glacialis*)	2016	Denmark	Surveillance	Cross-sectional	Free-living
Stokes et al., 2020	Crimson Rosella (*Platycercus elegans*)	2016-2018	Australia	Surveillance	Cross-sectional	Free-living
Perez-Sancho et al., 2020	Feral pigeon (*Columba livia*)	2005-2014	Spain	Surveillance	Cross-sectional	Free-living
Mahzoonieh et al., 2020	Feral pigeon (*Columba livia*), House sparrow (Passer domesticus)	NR	Iran	Surveillance	Cross-sectional	Free-living
Li et al., 2020	Crested Ibis (*Nipponia nippon*)	NR	China	Surveillance	Cross-sectional	Captive exotic
Crispo et al., 2020	Gouldian finch (*Erythrura gouldiae*)	2018	United States of America	Clinical case	Case report	Captive exotic
Amery-Gale et al., 2020	Multiple species	2014-2015	Australia	Surveillance	Cross-sectional	Captive exotic
Wang et al., 2019	Psittaciformes, Columbiformes, Multiple species	2015-2016	China	Surveillance	Cross-sectional	Captive exotic
Vogler et al., 2019	Turkey	2016-2017	Switzerland	Surveillance	Cross-sectional	Farmed poultry
Vilela et al., 2019	Blue-fronted Amazon parrot (*Amazona aestiva*)	2009-2011	Brazil	Characterise *C. psittaci* strains	Retrospective	Captive exotic
Sutherland et al., 2019	Cacatuidae	2015-2016	Australia	Surveillance	Cross-sectional	Free-living
Plaza et al., 2019	American black vulture (*Coragyps atratus)*	2014-2017	Argentina	Surveillance	Cross-sectional	Free-living

Origlia, 2019	Psittaciformes	2013-2015	Argentina	Surveillance	Cross-sectional	Captive exotic
Mina et al., 2019	Psittaciformes, columbiformes	2015-2016	Iran	Surveillance	Cross-sectional	Captive exotic
Mattmann et al., 2019	Feral pigeon (*Columba livia*)	2014-2018	Switzerland	Surveillance	Cross-sectional	Free-living
Liu et al., 2019	Domestic waterfowl, Psittaciformes, Columbiformes, Multiple species	2014-2017	Taiwan	Surveillance	Cross-sectional	Farmed poultry, Free-living, Captive exotic
Lin et al., 2019	Laying ducks	2015	China	Poor egg production	Case report	Farmed poultry
Hamzah et al., 2019	Multiple species	NR	Iraq	Evaluate diagnostic test	Cross-sectional	Captive exotic
Wang et al., 2018	Pigeons	2015-2016	China	Surveillance	Cross-sectional	Farmed poultry
Szymańska-Czerwińska et al., 2018	Multiple species	NR	Poland	Avian outbreak investigation	Case report	Captive exotic
Heijne et al., 2018	Chicken	2015-2016	Netherlands	surveillance	Cross-sectional	Farmed poultry
Gedye et al., 2018	Multiple species	NR	New Zealand	Characterise *C. psittaci* strains	Case report	Captive exotic
Donati et al., 2018	Chicken	2016	Italy	Surveillance	Cross-sectional	Farmed poultry
Cechova et al., 2018	Chicken	NR	Slovakia	Surveillance	Cross-sectional	Farmed poultry
Burt et al., 2018	Feral pigeon (*Columba livia*)	2017	Netherlands	Surveillance	Cross-sectional	Free-living
Yao et al., 2017	Broiler pigeon (*Columba livia domestica*)	2015	China	Surveillance	Cross-sectional	Farmed poultry
Vaz et al., 2017	Red-tailed Amazon parrot (*Amazona brasiliensis)*	2013-2014	Brazil	Surveillance	Cross-sectional	Free-living
Szymanska-Czerwinska et al., 2017	Multiple species	2014-2015	Poland	Surveillance	Cross-sectional	Free-living
Jeong et al., 2017	Multiple species	2016	Korea	Surveillance	Cross-sectional	Free-living
Hegazy et al., 2017	Multiple species	NR	Egypt	Surveillance	Cross-sectional,	Free-living,

					Experimental	Captive exotic
Gartrell et al., 2017	Yellow-eyed penguins (*Megadyptes antipodes*)	2013	New Zealand	Avian outbreak investigation	Case report	Free-living
Ferreira et al., 2017	Multiple species	NR	Brazil	Human outbreak investigation	Case report	Captive exotic
Cadario et al., 2017	Psittaciformes	2010-2015	Argentina	Human outbreak investigation	Case report	Captive exotic
Vasconcelos et al., 2016	Blue-and-gold macaws (*Ara ararauna*)	2011	Brazil	Surveillance	Cross-sectional	Captive exotic
Tatari et al., 2016	Turkey	2013-2014	Iran	Surveillance	Cross-sectional	Farmed poultry
Suksai et al., 2016	Psittaciformes	2013-2014	Thailand	Surveillance	Cross-sectional	Captive exotic
Razmyar et al., 2016	African grey parrot (*Psittacus erithacus erithacus*)	NR	Iran	Clinical case	Case report	Captive exotic
Ornelas-Eusebio et al., 2016	Psittaciformes	2015	Mexico	Clinical case	Case report	Captive exotic
Mahzoonieh et al., 2016	Feral pigeon (*Columba livia*), House sparrow (Passer domesticus)	NR	Iran	Surveillance	Cross-sectional	Free-living
Konicek et al., 2016	Pigeons (Columbiformes), Songbirds (Passeriformes), Birds of prey (Accipitriformes, Strigiformes, and Falconiformes), Waterfowl (Anseriformes), Fowl (Galliformes), Storks (Ciconiiformes)	2012-2014	Austria, Czech Republic	Surveillance	Cross-sectional	Free-living
Hulin et al., 2016	Mulard (*Anas platyrhynchos domesticus* × *Cairina moschata*)	2013	France	Surveillance	Cross-sectional	Farmed poultry
Ferreira et al., 2016	Feral pigeon (*Columba livia*)	NR	Brazil	Surveillance	Cross-sectional	Free-living
Feng et al., 2016	Psittaciformes	NR	China	Surveillance	Cross-sectional	Captive exotic
Dovc et al., 2016	Psittaciformes, Columbiformes	NR	Slovenia	Surveillance	Cross-sectional	Captive exotic
Cechova et al., 2016	Feral pigeon (*Columba livia*)	NR	Slovakia	Surveillance	Cross-sectional	Free-living

Vorimore et al., 2015	Mulard (*Anas platyrhynchos domesticus* × *Cairina moschata*)	NR	France	Surveillance	Cross-sectional	Farmed poultry
Shivaprasad et al., 2015	Turkey	NR	United States of America	Clinical case	Case report	Farmed poultry
Ling et al., 2015	Racing pigeon (*Columba livia*)	2008-2010	China	Surveillance	Cross-sectional	Captive exotic
Laroucau et al., 2015	Mule duck, broiler chicken	2013	France	Human outbreak investigation	Case report	Farmed poultry
Khodadadi et al., 2015	Meat pigeons (*Columba livia domestica*)	2013-2014	Iran	Surveillance	Cross-sectional	Farmed poultry
Hulin et al., 2015	Ducks, Mixed poultry (except ducks)	2013	France	Surveillance	Cross-sectional	Farmed poultry
Ghorbanpoor et al., 2015	Pigeon	NR	Iran	Surveillance	Cross-sectional	Captive exotic
Donati et al., 2015	Eurasian Collared Doves (*Streptopelia decaocto*)	2010-2013	Italy	Surveillance	Cross-sectional	Free-living
Aaziz et al., 2015	North Atlantic Seabirds	2011-2014	France	Surveillance	Cross-sectional	Free-living
To et al., 2014	Mealy Parrots (*Amazona farinose*)	2012	China	Human outbreak investigation	Case report	Captive exotic
Stenzel et al., 2014	Domestic pigeon (*Columba livia*), Feral pigeon (Columba livia)	2010-2013	Poland	Surveillance	Cross-sectional	Captive exotic, Free-living
Kalmar et al., 2014	Multiple species	NR	Belgium	Surveillance	Cross-sectional	Captive exotic
Elezi et al., 2014	Multiple species	NR	Albania	Surveillance	Cross-sectional	Captive exotic
Beckmann et al., 2014	Passeriformes, Columbiformes	2005-2011	United Kingdom	Surveillance	Retrospective	Free-living
Tomic et al., 2013	Racing pigeon (*Columba livia*)	NR	Croatia	Surveillance	Cross-sectional	Captive exotic
Tomic et al., 2013	Chicken	2010	Croatia	Clinical case	Case report	Farmed poultry
Tel et al., 2013	Bald ibises (*Geronticus eremita*)	NR	Turkey	Surveillance	Cross-sectional	Captive exotic
Szymańska-Czerwińska et al., 2013	Chicken	NR	Poland	Clinical case	Case report	Farmed poultry

Sheleby-Elías et al., 2013	Psittaciformes	2009	Costa Rica	Surveillance	Cross-sectional	Captive exotic
Perelman et al., 2013	Ostrich (*Struthio camelus*), Chicken	2008-2011	Israel	Clinical case	Case report	Farmed poultry
Madani et al., 2013	Multiple species	NR	Iran	Surveillance	Cross-sectional	Captive exotic
Gartrell et al., 2013	Passeriformes	2010-2011	New Zealand	Surveillance	Cross-sectional	Free-living
Dickx et al., 2013	Canada Geese (*Branta canadensis*)	NR	Belgium	Surveillance	Cross-sectional	Free-living
Zocevic et al., 2012	Chicken	NR	France, Greece	Develop new method	Case report	Farmed poultry
Satrovic et al., 2012	Turkey	2007-2008	Bosnia and Herzegovina	Surveillance	Cross-sectional	Farmed poultry
Piasecki et al., 2012	Psittaciformes	2007-2012	Poland	Surveillance	Cross-sectional	Captive exotic
Marhold et al., 2012	Multiple species	2010-2011	Slovenia	Surveillance	Cross-sectional	Captive exotic
Krizek et al., 2012	Multiple species	NR	Croatia	Surveillance	Cross-sectional	Captive exotic
Gilbert et al., 2012	Multiple species	2007-2009	Cambodia	Surveillance	Cross-sectional	Captive exotic
Geigenfeind et al., 2012	Feral pigeon (*Columba livia*)	2007-2009	Switzerland	Surveillance	Cross-sectional	Free-living
Deem et al., 2012	Galapagos Hawks (*Buteo galapagoensis*)	2009	Ecuador	Surveillance	Cross-sectional	Free-living
Colvile et al., 2012	British songbirds	2005-2011	United Kingdom	Avian outbreak investigation	Case report	Free-living
Blomqvist et al., 2012	White-tailed Sea Eagle (*Haliaeetus albicilla*), Peregrine Falcon (Falco peregrinus)	2006-2007	Sweden	Surveillance	Cross-sectional	Free-living
Yanga et al., 2011	Socorro Ground Dove (*Columbina passerina socorrensis*), Mourning Dove (Zenaida macroura)	2004	Mexico	Surveillance	Cross-sectional	Free-living
Yang et al., 2011	Peafowl (*Pavo cristatus*)	2009	China	Human outbreak investigation	Case report	Farmed poultry

Gasparini et al., 2011	Feral pigeon (*Columba livia*)	2009	France	Surveillance	Cross-sectional	Free-living
Doosti et al., 2011	Feral pigeon (*Columba livia*)	NR	Iran	Surveillance	Cross-sectional	Free-living
Zhou et al., 2010	Chicken	NR	China	Poor egg production	Case report	Farmed poultry
Vazquez et al., 2010	Feral pigeon (*Columba livia*)	2006-2007	Spain	Surveillance	Cross-sectional	Free-living
Tarsitano et al., 2010	Feral pigeon (*Columba livia*)	2006-2007	Italy	Surveillance	Cross-sectional	Free-living
Robertson et al., 2010	psittacine birds, herons, magpies, Chicken	2007-2008	Australia	Evaluate diagnostic test	Case report	Captive exotic
Geigenfeind et al., 2010	Feral pigeon (*Columba livia*)	NR	Switzerland	Surveillance	Cross-sectional	Free-living
Dickx Geens et al., 2010	Chicken, Turkey	2007	Belgium	Zoonotic risk assessment	Case report	Farmed poultry
Dickx et al., 2010	Homing pigeon (*Columba livia*), Feral pigeon (Columba livia)	2008	Belgium	Surveillance	Cross-sectional	Captive exotic
Christerson et al., 2010	Sea birds (Laridae, Alcidae)	NR	United States of America	Surveillance	Cross-sectional	Free-living
Zweifel et al., 2009	Feral pigeon (*Columba livia*), Passeriformes, Waterfowl (Anseriformes, Charadriiformes, Pelecaniformes, Gruiformes, Ciconiiformes), Cormorant (Phalacrocorax sp.)	2005-2006	Switzerland	Surveillance	Cross-sectional	Free-living
Song et al., 2009	one duck, 3 wild birds	NR	China	Characterise *C. psittaci* strains	Case report	Free-living
Sharples et al., 2009	Multiple sepcies (columbiformes, passeriformes, strigiformes, anseriformes, lariformes)	NR	United Kingdom	Surveillance	Cross-sectional	Free-living
Sareyyupoglu et al., 2009	Canary	NR	Turkey	Develop new method	Case report	Captive exotic
Sachse et al., 2009	Multiple sepcies	NR	Germany	Develop new method	Case report	Captive exotic
Mitchell et al., 2009	Psittaciformes, columbiformes (not stated how many individually)	2004-2007	United States of America	Develop new method	Case report	Captive exotic

Laroucau et al., 2009	ducks	2006	France	Human outbreak investigation	Case report	Farmed poultry
Droogenbroeck et al., 2009	Turkey	NR	Belgium	Zoonotic risk assessment	Case report	Farmed poultry
Zhang et al., 2008	Chicken	NR	China	Poor egg production	Case control	Farmed poultry
Verminnen et al., 2008	Turkey	NR	Belgium	Zoonotic risk assessment	Cohort	Farmed poultry
Laroucau et al., 2008	Multiple sepcies	NR	Belgium	Characterise *C. psittaci* strains	Case report	Mixed
Gaede et al., 2008	Mixed poultry (chickens, ducks, geese)	NR	Germany	Surveillance	Cross-sectional	Farmed poultry
Branley et al., 2008	Crimson Rosella *(Platycercus elegans*)	NR	Australia	Human outbreak investigation	Case report	Captive exotic
Yang et al., 2007	Mixed poultry (chickens, ducks)	NR	China	Surveillance	Cross-sectional	Farmed poultry
Varompay et al., 2007	Psittaciformes (Cockatoo, parrot, patakeet, lories)	NR	Belgium	Surveillance	Cross-sectional	Captive exotic
Schenker et al., 2007	Multiple species (Psittaciformes, passeriformes, anseriformes, ciconiiformes, others)	1991-2005	Switzerland	Link with atherosclerosis	Retrospective	Captive exotic
Sareyyupoglu et al., 2007	Multiple species (Pheasant (Phasianus colchicus), Canary (Serinus canaria), Canary (Serinus pusillus), parrot (Psittacus erithacus), Budgerigars, Finch (Taeniopygia guttata), parakeet (Nymphicus hollandicus), pigeon, dove (strptopelia risoria)	NR	Turkey	Surveillance	Cross-sectional	Captive exotic
Harkinezhad et al., 2007	Psittaciformes (African grey parrots (Psittacus erithacus), blue and gold macaws (Ara ararauna), greenwinged macaws (Ara chloroptera))	NR	Belgium	clinical case	Case report	Captive exotic
Assuncao et al., 2007	Great White pelicans (*Pelecanus onocrotalus*)	NR	South Africa	Surveillance	Cross-sectional	Free-living
Travis et al., 2006	Flightless cormorant (*Phalacrocorax harrisi*)	2003	Ecuador	Surveillance	Cross-sectional	Free-living
Herrmann et al., 2006	Fulmar (*Fulmarus glacialis*)	1999	Denmark	Surveillance	Cross-sectional	Free-living

Heddema, Hannenm, Duim et al., 2006	Amazon parrots	2004-2005	Netherlands	Human outbreak investigation	Case report	Captive exotic
Heddema, Ter, Buys et al., 2006	Feral pigeon (*Columba livia*)	2005	Netherlands	Surveillance	Cross-sectional	Free-living
Van Loock, Verminnen, Messmer et al., 2005	Turkey	2001	France	Develop new method	Case report	Farmed poultry
Van Loock, Geens, Smit et al., 2005	Turkey	2001	Belgium, France	Link with other pathogens	Cohort - longitudinal	Farmed poultry
Tanaka et al., 2005	Feral pigeon (*Columba livia*)	2003-2004	Japan	Surveillance	Cross-sectional	Free-living
Greco et al., 2005	Red-rumped parrots (*Psephotus hematonotus*)	2005	Italy	clinical case	Case report	Captive exotic
Dovc et al., 2005	Canary and finch	2001	Slovenia	Experimental infection	Experimental	Captive exotic
Bonner et al., 2004	Canada geese (*Branta canadensis)*	2002	Germany	Surveillance	Cross-sectional	Free-living
Schettler et al., 2003	Raptors (Accipitriformes, Strigiformes, and Falconiformes)	1994-1997	Germany	Surveillance	Cross-sectional	Free-living

Appendix 2.2

Molecular tests of the 120 selected studies for systematic review of molecular methods to detect *Chlamydia psittaci* in birds

Reference, year	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7	Test 8	Test 9
Wang et al., 2020	*Chlamydiaceae*-specific PCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Heddema et al. [2006]	MLST - Pannekoek et al. [2010]						
Stokes et al., 2020	*Chlamydiaceae*-specific PCR (16SIG) - Everett et al. [1999]	*C. psittaci*-specific PCR (Cps_0607 gene, F3/B3 primers) - Jelocnik et al. [2017]	Sanger sequencing						
Perez-Sancho et al., 2020	*C. psittaci*-specific PCR (*ompA*) - Hewinson et al. [1997]	*C. psittaci*-specific qPCR (*ompA*) - Geens et al. [2005]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]						
Mahzoonieh et al., 2020	*C. psittaci*-specific PCR (*ompA*) - Heddema et al. [2006]	*C. psittaci*-specific PCR (*ompA*) -Mahzoonieh et al. [2020]							
Li et al., 2020	*Chlamydiaceae*-specific PCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	*Chlamydiaceae*-specific PCR (16SIG) - Everett et al. [1999]	*Chlamydiaceae*-specific PCR (16S rRNA) - Pudjiatmoko et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]				
Crispo et al., 2020	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific PCR (*ompA*)- Pantchev et al. [2009]	*C. psittaci*-specific PCR (*ompA*)- Kaltenboeck et al. [1993] - sequencing						
Amery-Gale et al., 2020	*Chlamydiaceae*-specific qPCR-HRM (16S rRNA) - Robertson et al. [2009, 2010]	MLST - Pannekoek et al. [2008]	Dideoxy sequencing						
Wang et al., 2019	*C. psittaci*-specific PCR (*ompA*) - Hewinson et al. [1997]	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]							
Vogler et al., 2019	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	*Chlamydiaceae*-specific DNA Microarray (23S rRNA) - Borel et al. [2008]	*Chlamydiaceae*-specific PCR (16S rRNA) - Pospischil et al. [2012]	Sanger sequencing				
Vilela et al., 2019	*C. psittaci*-specific PCR (*ompA*) - Andersen et al. [2000]	*C. psittaci*-specific PCR (*ompA*) - Kaltenboeck et al. [1991]	Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, California, USA)						

Sutherland et al., 2019	*Chlamydiaceae*-specific qPCR-HRM (16S rRNA) - Robertson et al. [2009]	NGS	MLST - Pannekoek et al. [2008]						
Plaza et al., 2019	*Chlamydiaceae*-specific modified nPCR-HRM (16S rRNA) - Messmer et al. [1997]	*C. psittaci*-specific PCR-HRM (16S rRNA) - Messmer et al. [1997]							
Origlia, 2019	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (ompA) - Pantchev et al. [2009]	Seq (ompA - Sachse 03)						
Mina et al., 2019	*Chlamydiaceae*-specific nPCR (*ompA*) - Sachse & Hotzel et al. [2003]	*C. psittaci*-specific nPCR (*ompA*) - Sachse & Hotzel et al. [2003]	*C. psittaci*-specific PCR (*ompA*) - Sayada et al. [1995] -sequencing						
Mattmann et al., 2019	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*Chlamydiaceae*-specific DNA Microarray (23S rRNA) - Borel et al. [2008]	*Chlamydiaceae*-specific PCR (16S rRNA) - Pospischil et al. [2012]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	MLST - Pannekoek et al. [2010]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2008]	Sanger sequencing		
Liu et al., 2019	*C. psittaci*-specific nPCR (16S rRNA) - Messmer et al. [1997]	*C. psittaci*-specific PCR (*ompA*) - Heddema et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]	Sequencing					
Lin et al., 2019	Chlamydiaceae-specific qPCR (23S rRNA)-Ehricht et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Vanrompay et al. [1997]							
Hamzah et al., 2019	*C. psittaci*-specific PCR (*ornpA*) - Hamzah et al. [2019]								
Wang et al., 2018	*C. psittaci*-specific nPCR (*ompA*) - Zhang et al. [2014]	Sequencing							
Szymańska-Czerwińska et al., 2018	*Chlamydiaceae*-specific PCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Madani et al. [2013]	Sequencing					
Heijne et al., 2018	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]							
Gedye et al., 2018	*C. psittaci*-specific qPCR (23S rRNA) - Nordentoft et al. [2011]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2009]	bi-directional Sanger sequencing						
Donati et al., 2018	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]							
Cechova et al., 2018	*Chlamydiaceae*-specific PCR (23S rRNA) - Cechova et al. [2018]	Sequencing							

Burt et al., 2018	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	*C. psittaci*-specific PCR (*ompA*) - Heddema et al. [2015]						
Yao et al., 2017	*Chlamydiaceae*-specific snPCR (MOMP) - Buxton et al. [1996]	*C. psittaci*-specific nPCR (*ompA*) - Zhang et al. [2014]	Sequencing						
Vaz et al., 2017	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]								
Szymanska-Czerwinska et al., 2017	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2008]	*Chlamydiaceae*-specific PCR (16S rRNA) - Pudjiatmoko et al. [1997]	*Chlamydiaceae*-specific PCR (16S rRNA) - Thomas et al. [2006]	*Chlamydiaceae*-specific PCR (IGS-23S rRNA) - Everett, Bush et al. [1999]	Seq (16S - Pudjia; IGS-23S - Everett; ompA - Denamur, Sachse 08)	
Jeong et al., 2017	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]	*Chlamydiaceae*-specific PCR (16S rRNA) - Pudjiatmoko et al. [1997]	Sequencing					
Hegazy et al., 2017	*C. psittaci*-specific PCR (*ompA*) - Heddema et al. [2006]	Sequencing							
Gartrell et al., 2017	cited Gartrell 2013								
Ferreira et al., 2017	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]								
Cadario et al., 2017	*Chlamydiaceae*-specific nPCR (16S rRNA) - Messmer et al. [1997]	*C. psittaci*-specific nPCR (16S rRNA) - Messmer et al. [1997]	*C. psittaci*-specific nPCR (*ompA*) - Sachse & Hotzel [2003]	bi-directional nucleotide sequencing					
Vasconcelos et al., 2016	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]								
Tatari et al., 2016	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*Chlamydiaceae*-specific nPCR (*ompA*) - Sachse & Hotzel [2003]	*C. psittaci*-specific nPCR (*ompA*) - Sachse & Hotzel [2003]						
Suksai et al., 2016	*C. psittaci*-specific nPCR (*ompA*) - Heddema Ter et al. [2006]	Nucleotide sequencing							
Razmyar et al., 2016	*Chlamydiaceae*-specific nPCR (*ompA*) - Sachse & Hotzel [2003]	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]	bi-directional automated sequencing						
Ornelas-Eusebio et al., 2016	*Chlamydiaceae*-specific nPCR (*ompA*) - Sachse & Hotzel [2003]	*C. psittaci*-specific nPCR (*ompA*) - Sachse & Hotzel [2003]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2009]	Sequencing					

Mahzooni eh et al., 2016	*C. psittaci*-specific nPCR (*ompA*) - Heddema Ter et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Mahzoonieh et al. [2016]	Sequencing						
Konicek et al., 2016	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2008]	*Chlamydiaceae* -specific PCR (16S/23S rRNA) - Sachse et al. [2005]	Sanger sequencing				
Hulin et al., 2016	*Chlamydiaceae*-specific PCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Kaltenboeck et al. [1993]	Sequencing					
Ferreira et al., 2016	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]								
Feng et al., 2016	*C. psittaci*-specific qPCR (*ompA*) - Feng et al. [2016]	*C. psittaci*-specific qPCR (*ompA*) - Feng et al. [2016]	Sequencing						
Dovc et al., 2016	*Chlamydiaceae*-specific PCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]							
Cechova et al., 2016	*Chlamydiaceae*-specific PCR (23S rRNA) - Cechova et al. [2016] - cited Raso but primers doesn't match	Sequencing							
Vorimore et al., 2015	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	*C. psittaci*-specific PCR (*ompA*) - Kaltenboeck et al. [1993]	Sequencing					
Shivapras ad et al., 2015	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Kaltenboeck et al. [1993]	MLVA - Laroucau et al. [2009]	MLVA - Laroucau et al. [2008]	MLST - Pannekoek et al. [2010]			
Ling et al., 2015	Zhang et al. 2008	Sequencing							
Laroucau et al., 2015	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	*Chlamydiaceae*-specific DNA Microarray (23S rRNA) - Borel et al. [2008]	*C. psittaci*-specific PCR (*ompA*) - Sayada et al. [1995]	*Chlamydiaceae* -specific nPCR (*ompA*) - Sachse & Hotzel [2003]	Sequencing			
Khodadad i et al., 2015	*C. psittaci*-specific nPCR (*ompA*) - Heddema Ter et al. [2006]								
Hulin et al., 2015	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Laroucau,Vorimore et al. [2009]	*Chlamydiaceae* -specific PCR (16S rRNA) -	*Chlamydiaceae* -specific PCR (16S rRNA) -	Sequencing			

				Pudjiatmoko et al. [1997]	Thomas et al. [2006]				
Ghorbanpoor et al., 2015	Borel 2006 - *cannot find relevant info in the original article*	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]							
Donati et al., 2015	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	MLVA - Laroucau et al. [2008]	MLST - Pannekoek et al. [2010]	*C. psittaci*-specific nPCR (*ompA*) - Heddema van Hannen et al. [2006]	Sequencing			
Aaziz et al., 2015	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2010]	MLST - Pannekoek et al. [2008; 2010]	*Chlamydiaceae*-specific PCR (16S rRNA) - Pudjiatmoko et al. [1997]	*Chlamydiaceae*-specific PCR (16S rRNA) - Thomas et al. [2006]	*Chlamydiaceae*-specific PCR (16S rRNA) - Everett et al. [1999]	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]	sequencing
To et al., 2014	*C. psittaci*-specific qPCR (*ITS*) - To et al. [2014]								
Stenzel et al., 2014	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	*C. psittaci*-specific nPCR (*ompA*) - Heddema Ter et al. [2006]	Nucleotide sequencing						
Kalmar et al., 2014	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]								
Elezi et al., 2014	*Chlamydiaceae*-specific DNA Microarray (23S rRNA) - Borel et al. [2008]	*C. psittaci*-specific PCR (*ompA*) - Hewinson et al. [1997]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2009]						
Beckmann et al., 2014	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*Chlamydiaceae*-specific qPCR (23S rRNA) - Zweifel et al. [2009]	*Chlamydiaceae*-specific DNA Microarray (23S rRNA) - Borel et al. [2008]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2008]				
Tomic et al., 2013	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	RFLP - Sayada et al. [1995]	MLVA - Laroucau et al. [2008]					
Tomic et al., 2013	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	Nucleotide sequencing						
Tel et al., 2013	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]								

Szymańska-Czerwińska et al., 2013	*C. psittaci*-specific nPCR (*ompA*) - Kaltenboeck et al. [1997]	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2008]	Sequencing					
Sheleby-Elías et al., 2013	*C. psittaci*-specific nPCR (*ompA*) - Kaltenboeck et al. [1997]	*C. psittaci*-specific nPCR (*ompA*) - Heddema Ter et al. [2006]	Sequencing						
Perelman et al., 2013	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]							
Madani et al., 2013	*C. psittaci*-specific nPCR (*ompA*) - Sachse & Hotzel [2003]	PCR-RFLP genotyping - Sayada et al. [1995]	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]	bi-directional nucleotide sequencing					
Gartrell et al., 2013	*Chlamydiaceae*-specific qPCR-HRM (16S rRNA) - Robertson et al. [2009]	*Chlamydiaceae*-specific PCR (16S rRNA) - Everett et al. [1999]	Sequencing						
Dickx et al., 2013	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]								
Zocevic et al., 2012	*Chlamydiaceae*-specific PCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*incA*) - Menard et al. [2006]	MLST - Pannekoek et al. [2010]	*Chlamydiaceae*-specific PCR (16S rRNA) - Pudjiatmoko et al. [1997]	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]		Sequencing		
Satrovic et al., 2012	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]								
Piasecki et al., 2012	*C. psittaci*-specific PCR (*pomp*) - Longbottom et al. [1998] (cited longbottom but using laroucau 2001 primers)	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]	*C. psittaci*-specific PCR (*ompA*) - Vanrompay et al. [1997]	RFLP - Sayada et al. [1995]					
Marhold et al., 2012	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	MLVA - Laroucau et al. [2008]						
Krizek et al., 2012	*C. psittaci*-specific PCR (*ompA*) - Hewinson et al. [1997]								
Gilbert et al., 2012	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]								

Geigenfeind et al., 2012	*C. psittaci*-specific nPCR (*ompA*) - Van Loock et al. [2005]	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]							
Deem et al., 2012	*C. psittaci*-specific PCR (*ompA*) - Sayada et al. [1995]								
Colvile et al., 2012	*C. psittaci*-specific PCR (*ompA*) - Hewinson et al. [1997]								
Blomqvist et al., 2012	*Chlamydiaceae*-specific PCR (23S rRNA) - DeGraves Gao Kalt et al. [2003]	*Chlamydiaceae*-specific snPCR (16S rRNA) - Christerson et al. [2010]	*C. psittaci*-specific nPCR (*ompA*) - Herrmann et al. [2006]	*C. psittaci*-specific snPCR (*ompA*) - Kaltenboeck et al. [1997]					
Yanga et al., 2011	*C. psittaci*-specific PCR (*ompA*) - Sykes et al. [1997]								
Yang et al., 2011	*C. psittaci*-specific PCR (*ompA*) - Sayada et al. [1995]	yuan 1989							
Gasparini et al., 2011	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific qPCR (*ompA*) - Pantchev et al. [2009]	*C. psittaci*-specific PCR (*ompA*) - Kaltenboeck et al. [1993]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2008] - not cited in article but primers match	*Chlamydiaceae*-specific PCR (16S rRNA) - Pudjiatmoko et al. [1997]	*Chlamydiaceae*-specific PCR (16S rRNA) - Thomas et al. [2006]	Nucleotide sequencing		
Doosti et al., 2011	*C. psittaci*-specific nPCR (*ompA*) - Heddema Ter et al. [2006]								
Zhou et al., 2010	*C. psittaci*-specific PCR (*ompA*) - Hewinson et al. [1997] cited Hewinson but primers matches Zweifel 2009	Sequencing							
Vazquez et al., 2010	*C. psittaci*-specific PCR (*ompA*) - Hewinson et al. [1997]	Sequencing							
Tarsitano et al., 2010	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]								
Robertson et al., 2010	Chlamydiaceae-specific PCR-HRM (16S rRNA)-Robertson et al. [2009]	Nucleotide sequencing							
Geigenfeind et al., 2010	*Chlamydiaceae*-specific PCR (16S/23S rRNA) - Sachse et al. [2005]	*C. psittaci*-specific ArrayTube DNA microarray assay (*ompA*) - Sachse et al. [2005]							

Dickx Geens et al., 2010	*C. psittaci*-specific nPCR (*ompA*) - Van Loock et al. [2005]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2008]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2009]						
Dickx et al., 2010	*C. psittaci*-specific nPCR (*ompA*) - Van Loock et al. [2005]	*C. psittaci*-specific PCR (*ompA*) - Sachse et al. [2008]							
Christerson et al., 2010	*Chlamydiaceae*-specific qPCR (23S rRNA) - DeGraves Gao Hehnen et al. [2003]	*Chlamydiaceae*-specific PCR (16S rRNA) - Everett, Bush et al. [1999]	*C. psittaci*-specific PCR (*rnpB*) - Herrmann et al. [2000]	*C. psittaci*-specific snPCR (*ompA*) - Christerson et al. [2010]	Sequencing				
Zweifel et al., 2009	*Chlamydiaceae*-specific PCR (23S rRNA) - Ehricht et al. [2006]	*Chlamydiaceae*-specific DNA AT Microarray (23S rRNA) - Borel et al. [2008]	*C. psittaci*-specific AT microassay PCR (*ompA*) - Sachse Laroucau Vorimore et al. [2008]	*Chlamydiaceae*-specific PCR (16S rRNA) - Everett et al. [1999]	*C. psittaci*-specific PCR (*ompA*) - Hotzel et al. [2005] - 23S rRNA, intergenic spacer (as well)	Sequencing 16S rRNA			
Song et al., 2009	*C. psittaci*-specific PCR (*ompA*) - Denamur et al. [1991]	Sequencing							
Sharples et al., 2009	*C. psittaci*-specific nPCR (*ompB*) - McElnea et al. [1999]								
Sareyyupoglu et al., 2009	*C. psittaci*-specific multiplex PCR (*pmp*) - Laroucau et al. [2001]								
Sachse et al., 2009	*C. psittaci*-specific duplex PCR DNA microarray (*ompA*) - Sachse et al. [2009]	PCR-RFLP genotyping - Sayada et al. [1995]	Sequencing						
Mitchell et al., 2009	qpCR-HRM from own paper based on primers from Kaltenboeck 1993	Sequencing							
Laroucau et al., 2009	*Chlamydiaceae*-specific qPCR (23S rRNA) - Ehricht et al. [2006]	PCR-RFLP genotyping - Sayada et al. [1995]	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]	Sequencing	MLVA - Laroucau et al. [2008]				
Droogenbroeck et al., 2009	*C. psittaci*-specific nPCR (*ompA*) - Van Loock et al. [2005]	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]							
Zhang et al., 2008	*C. psittaci*-specific nPCR (*ompA*) - Kaltenboeck et al. [1992]	Nucleotide sequencing							

Verminnen et al., 2008	*C. psittaci*-specific nPCR (*ompA*) - Van Loock et al. [2005]	*C. psittaci*-specific PCR (*ompA*) - Vanrompay et al. [1997]	Sequencing						
Laroucau et al., 2008	PCR-RFLP genotyping - Sayada et al. [1995]	MLVA - Laroucau et al. [2008]							
Gaede et al., 2008	*Chlamydiaceae*-specific PCR (23S rRNA) - Ehricht et al. [2006]	*C. psittaci*-specific ArrayTube DNA microarray assay (*ompA*) - Sachse et al. [2005]	*C. psittaci*-specific nPCR (*ompA*) - Sachse & Hotzel [2003]	Sequencing					
Branley et al., 2008	*Chlamydiaceae*-specific PCR (23S rRNA) - DeGraves Gao Kalt et al. [2003]	*C. psittaci*-specific PCR (*ompA*) - Madico et al. [2000]	*C. psittaci*-specific PCR-HRM (16S rRNA) - Messmer et al. [1997]	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]	Sequencing				
Yang et al., 2007	*C. psittaci*-specific PCR (*ompA*) - Sayada et al. [1995]								
Varompay et al., 2007	*C. psittaci*-specific nPCR-EIA (*ompA*) - Van Loock et al. [2005]	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]							
Schenker et al., 2007	*Chlamydiaceae*-specific PCR (23S rRNA) - Soldati et al. [2004]	Sequencing							
Sareyyupoglu et al., 2007	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]								
Harkinezhad et al., 2007	*C. psittaci*-specific nPCR-EIA (*ompA*) - Van Loock et al. [2005]	*C. psittaci* genotype-specific qPCR (*ompA*) - Geens et al. [2005]							
Assuncao et al., 2007	*C. psittaci*-specific PCR (*ompA*) - Hewinson et al. [1997]								
Travis et al., 2006	*C. psittaci*-specific PCR (*ompA*) - Sayada et al. [1995]								
Herrmann et al., 2006	*Chlamydiaceae*-specific PCR (23S rRNA) - DeGraves Gao Kalt et al. [2003]	*C. psittaci*-specific nPCR (*ompA*) - Herrmann et al. [2006]	Sequencing						
Heddema, Hannenm, Duim et al., 2006	*C. psittaci*-specific qPCR (*ompA*) - Heddema Beld et al. [2006]	*C. psittaci*-specific nPCR (*ompA*) - Heddema Ter et al. [2006]	Sequencing						

Heddema, Ter, Buys et al., 2006	*C. psittaci*-specific qPCR (*ompA*) - Heddema Beld et al. [2006]	*C. psittaci*-specific nPCR (*ompA*) - Heddema Ter et al. [2006]	Sequencing						
Van Loock, Verminnen, Messmer et al., 2005	*C. psittaci*-specific nPCR-EIA (*ompA*) - Van Loock et al. [2005]								
Van Loock, Geens, Smit et al., 2005	*C. psittaci*-specific PCR (*omp1*) - Vanrompay et al. [1998]	Sequencing							
Tanaka et al., 2005	*Chlamydiaceae*-specific nPCR (MOMP) - Tanaka et al. [2005]	Sequencing							
Greco et al., 2005	*C. psittaci*-specific PCR (*pmp*) - Laroucau et al. [2001]								
Dovc et al., 2005	*C. psittaci*-specific PCR (*ompA*) - Kaltenboeck et al. [1991]								
Bonner et al., 2004	*C. psittaci*-specific PCR (*ompA*) - Kaltenboeck et al. [1991]								
Schettler et al., 2003	*C. psittaci*-specific nPCR (*omp1*) - Kaltenboeck et al. [1997]								

Appendix 2.3

Summary of sample types in the selected 120 studies on the laboratory methods used for antigen detection of *Chlamydia psittaci* in birds, mainly categorised into swabs, tissues, faeces and others (inoculated egg, blood, cell culture, cloacal content), with some studies collected more than one sample type.

Sample types		**No. of studies**
Swab		
	Cloacal	55
	Pharyngeal	28
	Conjunctival	11
	Tracheal	7
	Choanal	7
	Conjunctival-choanal-cloacal	6
	Choanal-cloacal	4
Tissue		
	Liver	24
	Spleen	19
	Lung	14
	Heart	4
	Kidney	3
	Oviduct	2
	Air sac	2
	Air sac exudates	2
	Others: brain, intestine, main-stem vessels, muscle, nasal gland, oesophagus, ovary	7*
Others		
Faeces		28
Inoculated egg		2
Blood		2
Cell culture		1
Cloacal content		1

* (one study for each sample type)

Appendix 2.4

Sample types and swab types of the selected 120 studies in the systematic review of the laboratory methods used for antigen detection of *Chlamydia psittaci* in birds.

Reference, year	Sample types	Swab types
Wang et al., 2020	Swab - Cloacal	NR
Stokes et al., 2020	Swab - Cloacal	NR
Perez-Sancho et al., 2020	Swab - Cloacal, tracheal (pm)	NR
Mahzoonieh et al., 2020	Swab - Cloacal	NR
Li et al., 2020	Faeces	NR
Crispo et al., 2020	Tissue - Spleen, kidney	NA
Amery-Gale et al., 2020	Swab - Choanal, cloacal (live); Swab - trachea, intestinal swab, liver (pm)	Rayon-tipped
Wang et al., 2019	Tissue - Liver	NA
Vogler et al., 2019	Swab - Cloacal	FLOQ Swabs (Copan Italia, Brescia, Italy)
Vilela et al., 2019	Tissue - Liver, spleen, lung	NA
Sutherland et al., 2019	Swab - Choanal-cloacal	NR
Plaza et al., 2019	Swab - Cloacal	Dacron swabs placed
Origlia, 2019	Swab - CCC; Tissue-liver, lung, air sac, spleen	NR
Mina et al., 2019	Swab - Choanal-cloacal; Faeces; Tissue - Liver, spleen, lung	NR
Mattmann et al., 2019	Swab - Cloacal, choanal-cloacal; Tissue - Liver	NR
Liu et al., 2019	Swab - Cloacal; Faeces	NR
Lin et al., 2019	Tissue - Liver, spleen, heart, ovary	NA
Hamzah et al., 2019	Swab - Cloacal	NR
Wang et al., 2018	Faeces	NR
Szymańska-Czerwińska et al., 2018	Swab - Cloacal	NR
Heijne et al., 2018	Faeces	NR
Gedye et al., 2018	Swab - Cloacal, choanal, conjunctival; Tissue	NR
Donati et al., 2018	Swab - Cloacal	NR

Cechova et al., 2018	Swab - Cloacal, pharyngeal	Cotton swab
Burt et al., 2018	Faeces	NR
Yao et al., 2017	Faeces	NA
Vaz et al., 2017	Swab - Cloacal, oropharyngeal	NR
Szymanska-Czerwinska et al., 2017	Swab - Cloacal	NR
Jeong et al., 2017	Swab - Tracheal (pm)	NR
Hegazy et al., 2017	Tissue - Liver, lung, heart; Inoculated egg - yolk sacs	NA
Gartrell et al., 2017	Tissue - Liver	NA
Ferreira et al., 2017	Swab - Cloacal, ocular	NR
Cadario et al., 2017	Swab - Cloacal, ocular	Dacron swab
Vasconcelos et al., 2016	Swab - Choanal, cloacal	NR
Tatari et al., 2016	Swab - C-C-C; Tissue - Liver, spleen, lung, air sac exudates	Cotton swab
Suksai et al., 2016	Swab - Choanal, cloacal	Cotton swab
Razmyar et al., 2016	Swab - Choanal-cloacal	Sterile swab
Ornelas-Eusebio et al., 2016	Swab - Choanal, cloacal; Tissue - Liver	NR
Mahzoonieh et al., 2016	Swab - Cloacal	NR
Konicek et al., 2016	Swab - C-C-C	NR
Hulin et al., 2016	Swab - Cloacal; Tissue - Liver, spleen, lung, intestine	NR
Ferreira et al., 2016	Swab - Cloacal	NR
Feng et al., 2016	Faeces	NR
Dovc et al., 2016	Swab - Cloacal, oropharyngeal; Faeces	NR
Cechova et al., 2016	Swab - Cloacal, pharyngeal	Cotton swab
Vorimore et al., 2015	Swab - Cloacal	NR
Shivaprasad et al., 2015	Tissue - Nasal gland	NA
Ling et al., 2015	Swab - Throat	NR
Laroucau et al., 2015	Swab - Cloacal	NR
Khodadadi et al., 2015	Tissue - Liver, muscle	NA
Hulin et al., 2015	Swab - Cloacal	NR
Ghorbanpoor et al., 2015	Pharyngeal swab from chuana fossa	NR

Donati et al., 2015	Swab - Cloacal	NR
Aaziz et al., 2015	Swab - Cloacal	NR
To et al., 2014	Swab - Cloacal, pharyngeal, tracheal, conjunctival; Tissue - Liver, spleen, lung, kidney, oesophagus	NR
Stenzel et al., 2014	Swab - Cloacal, pharyngeal; Tissue - Liver	UTM-RT Mini swab system (Copan Diagnostic,Murrieta, CA)
Kalmar et al., 2014	Swab - Pharyngeal	NR
Elezi et al., 2014	Blood	NA
Beckmann et al., 2014	Tissue - Liver, spleen	NA
Tomic et al., 2013	Faeces	NR
Tomic et al., 2013	Swab - Conjunctival, pharyngeal	NR
Tel et al., 2013	Faeces	Sterile swab
Szymańska-Czerwińska et al., 2013	Swab - Throat, tracheal (pm)	NR
Sheleby-Elías et al., 2013	Swab - Cloacal, oropharyngeal	NR
Perelman et al., 2013	Swab - Sinus, trachea, eyelid (live)	NR
Madani et al., 2013	Swab - C-C-C; Faeces; Tissue- air sac exudates, lungs, spleens, livers	NR
Gartrell et al., 2013	Swab - Cloacal	Minitip culture swabs (Copan Diagnostics, Corona CA, USA)
Dickx et al., 2013	Swab - Pharyngeal	NR
Zocevic et al., 2012	Swab - Cloacal	NR
Satrovic et al., 2012	Swab - Cloacal, oropharyngeal	NR
Piasecki et al., 2012	Swab - Tracheal (live)	NR
Marhold et al., 2012	Swab - Cloacal, oropharangeal	Dacron swab
Krizek et al., 2012	Swab - Cloacal, C-C-C; Faeces	NR
Gilbert et al., 2012	Swab - Cloacal	Aluminum handled microswab
Geigenfeind et al., 2012	Swab - Cloacal, pharyngeal; Faeces	Rayon-tipped, aluminium-shafted swabs (Copan)
Deem et al., 2012	Swab - Cloacal	FisherbrandH Sterile Swabs (Fisher Scientific, Pennsylvania, USA)
Colvile et al., 2012	Tissue - Liver, spleen	NA
Blomqvist et al., 2012	Swab - Cloacal	Cotton swab

Yanga et al., 2011	Swab - Cloacal; Tissue - Lung, spleen	NR
Yang et al., 2011	Swab - Pharyngeal; Tissue - Lung	NR
Gasparini et al., 2011	Swab - Cloacal	NR
Doosti et al., 2011	Faeces	NR
Zhou et al., 2010	Tissue - Liver, spleen	NA
Vazquez et al., 2010	Swab - Cloacal	NA
Tarsitano et al., 2010	Faeces	NR
Robertson et al., 2010	Swab - Cloacal, choanal, conjunctival, pharyngeal; Tissue - Liver, spleen	Plastic-, wood-, aluminim-shafted
Geigenfeind et al., 2010	Faeces	NR
Dickx Geens et al., 2010	Swab - Pharyngeal	Rayon-tipped aluminum-shafted swabs (Copan; Fiers, Kuurne, Belgium)
Dickx et al., 2010	Swab - Pharyngeal; Faecal swab from cage floor	Copan rayon-tipped swabs (Fiers)
Christerson et al., 2010	Swab - Cloacal; Faeces	NR
Zweifel et al., 2009	Swab - Cloacal; Tissue - Liver, spleen, lung, kidney, heart	NR
Song et al., 2009	EI isolates	NA
Sharples et al., 2009	Swab - Cloacal	Sterile swab
Sareyyupoglu et al., 2009	Swab - Cloacal, ocular; Tissue - Lliver, spleen, lung	NR
Sachse et al., 2009	clinical samples - swab (cloacal, conjunctival, vaginal), tissue (brain), faeces; BGM cell-grown strains	NR
Mitchell et al., 2009	Swab - Cloacal, choanal, conjunctival; Blood	NR
Laroucau et al., 2009	Swab - Cloacal, tracheal (live)	NR
Droogenbroeck et al., 2009	Swab - Pharyngeal	Rayon-tipped swab (Fiers, Kuurne, Belgium)
Zhang et al., 2008	Swab - Pharyngeal; Tissue - oviduct	NR
Verminnen et al., 2008	Swab - Pharyngeal	Dacron-tipped aluminum shafted swabs (Fiers, Belgium)
Laroucau et al., 2008	NR	NR
Gaede et al., 2008	Swab - Cloacal, nasopharyngeal; Faeces	NR
Branley et al., 2008	Swab - Conjunctival; Tissue - Liver, spleen	NR
Yang et al., 2007	Swab - Throat; Tissue - oviduct	NR
Varompay et al., 2007	Swab - Pharyngeal; Faeces	Dacron-tipped swabs

Schenker et al., 2007	FFPE tissue - heart, main-stem vessel	NA
Sareyyupoglu et al., 2007	Faeces	NA
Harkinezhad et al., 2007	Faeces	Dacron-tipped aluminum shafted swabs (Fiers, Belgium)
Assuncao et al., 2007	Swab - Cloacal, tracheal (live)	Sterile swab
Travis et al., 2006	Swab - C-C-C	Sterile swab (Copan diagnostics, Corona, California, USA)
Herrmann et al., 2006	Swab - Cloacal	NR
Heddema, Hannenm, Duim et al., 2006	Faeces	NR
Heddema, Ter, Buys et al., 2006	Faeces	Sterile cotton swabs (MW&E, United Kingdom)
Van Loock, Verminnen, Messmer et al., 2005	Swab - Pharyngeal	Cotton-tipped aluminium shafted swabs (Fiers, Kuurne, Belgium)
Van Loock, Geens, Smit et al., 2005	Swab - Pharyngeal	Cotton-tipped aluminium shafted swabs (Fiers, Kuurne, Belgium)
Tanaka et al., 2005	Faeces	NR
Greco et al., 2005	Faeces	NR
Dovc et al., 2005	Tissue - Liver, spleen, lung, air sac	NA
Bonner et al., 2004	Eggs: egg-yolk membranes, embryo-liver, spleen, kidney, intestines	NA
Schettler et al., 2003	Tissue - Lung, spleen	NA

Appendix 2.5

Summary of transport medium (14 types) of samples collected in the selected 120 studies on laboratory methods used for antigen detection of *Chlamydia psittaci* in birds, with some studies using more than one type of transport medium.

Transport medium	No. of studies
Chlamydia transport medium	11
Sucrose phosphate glutamate (SPG)	10
Phosphate-buffered saline (PBS)	10
DNA stabilisation buffer	5
Universal transport medium (UTM)	4
Sterile saline	2
Others*: Absolute ethanol, buffered peptone water, Cary-Blair transport medium, cell-growth medium, Hank's solution, Luria broth, viral transport medium, RNA later.	8

* (one study for each transport media type)

Appendix 2.6

Transport medium and sample handling temperature of the selected 120 studies in the systematic review of the laboratory methods used for antigen detection of *Chlamydia psittaci* in birds.

Reference, year	Transport medium	Handling temperature
Wang et al., 2020	swab in SPG solution	transport on dry ice
Stokes et al., 2020	dry cloacal swab	transport at 4°C, store at -80°C
Perez-Sancho et al., 2020	swab in PBS	store at -40°C until analysis
Mahzoonieh et al., 2020	NR	NR
Li et al., 2020	NR	transporte on ice, store at -80°C until use
Crispo et al., 2020	NR	NR
Amery-Gale et al., 2020	dry swabs in sterile PBS	store at -20°C
Wang et al., 2019	NR	transport in cooler, store at -20°C
Vogler et al., 2019	dry flocked swabs	store on site at 4˚C, frozen at -20˚ C in lab
Vilela et al., 2019	FFPE	NA
Sutherland et al., 2019	NR	NR
Plaza et al., 2019	placed in a buffer	store at -20°C
Origlia, 2019	NR	transport swab at 4°C, store tissue at -20°C
Mina et al., 2019	1.5 mL SPG dilution	NR
Mattmann et al., 2019	NR	store at -20˚C, store at -80˚C (Zurich samples)
Liu et al., 2019	1 mL PBS	store at -80°C
Lin et al., 2019	NR	NR
Hamzah et al., 2019	NR	NR
Wang et al., 2018	NR	NR
Szymańska-Czerwińska et al., 2018	NR	store at -20°C
Heijne et al., 2018	NR	NR
Gedye et al., 2018	NR	NR
Donati et al., 2018	NR	NR
Cechova et al., 2018	swab in 0.3 mL sterile saline	store at -80°C
Burt et al., 2018	NR	store at -20°C
Yao et al., 2017	NR	store at -4°C
Vaz et al., 2017	1 mL PBS pH 7.4	NR
Szymanska-Czerwinska et al., 2017	dry swab	store at -20°C
Jeong et al., 2017	swabs in BD Universal Viral Transport (UVT) tubes (BD Biosciences, Baltimore, MD, U.S.A.)	store at -80°C
Hegazy et al., 2017	NR	NR
Gartrell et al., 2017	NR	NR
Ferreira et al., 2017	swab in sterile PBS	NR
Cadario et al., 2017	swab in a 2 mL SPG or UTM (Copan Italia, Brescia, Italy)	store at 4°C

Vasconcelos et al., 2016	swabs in 3 mL absolute ethanol	store at 4°C
Tatari et al., 2016	swabs in plastic bags or SPG	transport in cold, store at -20°C
Suksai et al., 2016	swabs in sterile PBS	transport in cold, store at -20°C
Razmyar et al., 2016	1 mL SPG	store at 4°C
Ornelas-Eusebio et al., 2016	swab in sterile PBS	store at -20°C (swab), -70°C(liver)
Mahzoonieh et al., 2016	NR	NR
Konicek et al., 2016	swab in UTM (universal transport medium; Copan)	NR
Hulin et al., 2016	NR	NR
Ferreira et al., 2016	swabs in PBS	store at -80°C
Feng et al., 2016	NR	NR
Dovc et al., 2016	NR	transport in cooler (4°C), store at -20°C
Cechova et al., 2016	0.3 mL sterile saline	store at -80°C
Vorimore et al., 2015	NR	NR
Shivaprasad et al., 2015	NR	NR
Ling et al., 2015	NR	NR
Laroucau et al., 2015	double swabbing - one in SPG, one dry swab	transport on ice, store at -80°C
Khodadadi et al., 2015	NR	NR
Hulin et al., 2015	dry swab	store at -80°C
Ghorbanpoor et al., 2015	chlamydia transport medium (Quinn et al., 2004) pp:624.)	NR
Donati et al., 2015	placed in sucrose phosphate medium	transport at 4°C
Aaziz et al., 2015	dry swab	store at -80°C
To et al., 2014	in viral transport medium by Chan et al., 2013	NR
Stenzel et al., 2014	NR	NR
Kalmar et al., 2014	NR	transport on ice, store at -80°C
Elezi et al., 2014	NR	NR
Beckmann et al., 2014	NR	tissues were frozen at -20°C
Tomic et al., 2013	NR	NR
Tomic et al., 2013	NR	NR
Tel et al., 2013	Cary-Blair transport medium	NR
Szymańska-Czerwińska et al., 2013	NR	NR
Sheleby-Elías et al., 2013	NR	transport at 4°C, store at -20°C
Perelman et al., 2013	NR	NR
Madani et al., 2013	NR	NR
Gartrell et al., 2013	transport without medium	store at 4°C
Dickx et al., 2013	NR	NR
Zocevic et al., 2012	NR	NR
Satrovic et al., 2012	NR	NR
Piasecki et al., 2012	NR	NR
Marhold et al., 2012	swab in 1 mL 2-sucrose-phosphate	store at -20°C
Krizek et al., 2012	NR	NR

Gilbert et al., 2012	store in cryovial	store at -20°C
Geigenfeind et al., 2012	swabs in 1 mL RNA/DNA Stabilization Reagent for Blood and Bone Marrow (Roche Diagnostics)	transport on ice, store at -80°C
Deem et al., 2012	in cryotubes	frozen in liquid nitrogen (-196°C) in the field and mechanical freezers (80°C)
Colvile et al., 2012	NR	NR
Blomqvist et al., 2012	Luria broth with PBS containing 4.4% glycerol	NR
Yanga et al., 2011	NR	NR
Yang et al., 2011	NR	NR
Gasparini et al., 2011	double swabbing - dry swab; swabs in SPG conservation buffer (Spencer and Johnson, 1983)	store at -80°C
Doosti et al., 2011	NR	store at -20°C
Zhou et al., 2010	NR	NR
Vazquez et al., 2010	introducing 0.5 mL of sterile DNAse- and RNAse-free PBS into cloaca, and then retrieving the PBS together with the cloacal contents. The recovered suspension was diluted with PBS to yield a final volume of 2 mL.	transport under refrigeration
Tarsitano et al., 2010	NR	NR
Robertson et al., 2010	dry swab	transport at room temperature (swab), on ice pack (tissue)
Geigenfeind et al., 2010	NR	NR
Dickx Geens et al., 2010	swab in 2 ml RNA/DNA stabilization reagent (Roche, Brussels, Belgium); swab in 2 mL chlamydia transport medium	transport on ice and store at ⩾80°C
Dickx et al., 2010	2 mL transport medium (Vanrompay et al., 1992); 2 ml DNA stabilization buffer (Roche)	store at -80°C
Christerson et al., 2010	Hank's solution	vials in cryotank liquid nitrogen, transport at -80°C
Zweifel et al., 2009	swab in buffered peptone water	store at -80°C
Song et al., 2009	NR	NR
Sharples et al., 2009	swab in sterile normal saline, transport without medium	NR
Sareyyupoglu et al., 2009	NR	NR
Sachse et al., 2009	NR	NR
Mitchell et al., 2009	NR	NR
Laroucau et al., 2009	store in conservation buffer SPG; dry swab	store at -80°C
Droogenbroeck et al., 2009	chlamydophila transport medium	store at -80°C
Zhang et al., 2008	NR	NR
Verminnen et al., 2008	dry swab; 1 mL of chlamydia transport medium (vanrompay 1992)	store at ⩾80°C
Laroucau et al., 2008	NR	NR

Gaede et al., 2008	NR	NR
Branley et al., 2008	NR	NR
Yang et al., 2007	Chlamydial transport medium (Andersen 1998)	store at -20°C
Varompay et al., 2007	Cp. psittaci transport medium (Vanrompay et al., 1992), DNA stabilisation buffer (Roche, Brussels, Belgium)	store at -80°C
Schenker et al., 2007	NR	NR
Sareyyupoglu et al., 2007	NR	NR
Harkinezhad et al., 2007	2 mL Cp. psittaci transport medium (Vanrompay et al., 1992) or DNAstabilization buffer (Roche)	transport on ice, store at -80°C
Assuncao et al., 2007	cell growth medium RPMI-1640 (Roswell Park Memorial Institute, Sigma, St. Louis, MO, USA) with 10% horse serum.	NR
Travis et al., 2006	stored in cryogenic vials	NR
Herrmann et al., 2006	swabs in PBS	on dry ice
Heddema, Hannenm, Duim et al., 2006	NR	NR
Heddema, Ter, Buys et al., 2006	NR	NR
Van Loock, Verminnen, Messmer et al., 2005	0.2 M sucrose (VWR International, Haasrode, Belgium); 0.015 M Na2HPO4 (VWR International), 0.01 M NaH2PO4 (VWR International) and 20% inactivated foetal calf serum (Integro, Leuvenheim, The Netherlands)	NR
Van Loock, Geens, Smit et al., 2005	2 mL incomplete C.psittaci transport medium, consisting of 0.2 M sucrose (VWR International, Haasrode, Belgium), 0.015 M Na2HPO4 (VWR International), 0.01 M NaH2PO4 (VWR International) and 20% inactivated foetal calf) serum (Integro, Leuvenheim, The Netherlands)	store at -80°C
Tanaka et al., 2005	Dry samples in polyethylene bags; fresh samples in 1 mL of phosphate-buffered saline	NR
Greco et al., 2005	NR	NR
Dovc et al., 2005	NR	NR
Bonner et al., 2004	NR	NR
Schettler et al., 2003	NR	store at -80°C

Appendix 2.7

Summary of DNA extraction methods performed in the selected 120 studies on the laboratory methods used for antigen detection of *Chlamydia psittaci* in birds.

DNA extraction methods		No. of studies
Test kit		
Qiagen	QIAamp DNA Mini Kit	23
	DNeasy Blood & Tissue Kit	11
	QIAamp DNA Stool Mini Kit	8
	QIAamp Tissue Kit	2
	QIAamp Viral RNA Mini Kit	2
	QIAEX II System	2
	QIAamp cador Pathogen Kit	1
	QIAamp MinElute Virus Spin Kit	1
	Qiagen Spin column	1
	BioSprint DNA Blood Kit	1
	EZ1 virus Mini Kit v2.0	1
	MagAttract DNA Mini M48 kit	1
Roche	High Pure PCR Template Preparation Kit	8
	High Pure FFPET DNA Isolation Kit	1
	MagNA Pure LC System	1
Macherey-Nagel	NucleoSpin Tissue kit	3
	NucleoSpin Nucleic Acid/Protein Kit	2
	NucleoMag Tissue Kit	1
Promega	Maxwell 16 Buccal Swab LEV DNA Purification kit	1
	Maxwell RSC Viral Total Nucleic Acid Purification Kit	1
	ReliaPrep gDNA Tissue Miniprep System	1
Biomerieux	NucliSENS easyMAG	3
AmpliSens	DNA-sorbAM nucleic acid extraction Kit	2
SinaClon	DNP DNA extraction Kit	2
Omega Bio-Tek	E.Z.N.A. Stool DNA Kit	2
Sigma-Aldrich	GenElute Mammalian Genomic DNA Miniprep Kit	2
BIONEER	AccuPrep Genomic DNA Extraction Kit	1
A&A Biotechnology	Genomic DNA Prep Plus kit	1
Invitrogen	PureLink Genomic DNA Mini Kit	1
MO BIO	PowerSoil DNA Isolation Kit	1
Zymo	ZR Genomic DNA-Tissue Mini Prep Kit	1
	ZR Quick genomic DNA MiniPrep Kit	1
Perkin Elmer	Magnetic bead method	1
	Van Loock 2005 (SaMag STD DNA Extraction Kit)	5
	DNA extraction kit (CinnaGen, Iran)	1
	PCR Preparation kit (Denazist Asia, Iran)	1
Non test kit		
	Boom 1990 (Salting out- guanidium thiocyanate, silica)	2
	Boom 2000 (Salting out- guanidium thiocyanate, silica)	2
	Gartrell 2013 (Chelex)	2
	Bruford 1998 (Salting-out Ammonium acetate)	1
	Hewinson 1991 (article unretrievable)	1
	Hewinson 1997 (PCI)	1
	InstaGene Matrix (Chelex)	1
	Ishizawa 1991 (Salting-out Sodium iodide, N-Lauroyl sarcosine)	1
	Rasmussen 1991 (PCI, salting-out ammonium acetate)	1
	Schenker 2007 (PCI)	1

Appendix 2.8

DNA extraction methods performed in the selected 120 studies on the laboratory methods used for antigen detection of *Chlamydia psittaci* in birds.

Reference, year	**DNA extraction**
Wang et al., 2020	PowerSoil DNA Isolation Kit, QIAamp DNA Mini Kit
Stokes et al., 2020	Ammonium acetate extraction method (Brufor 1998)
Perez-Sancho et al., 2020	QIAamp Viral RNA Mini Kit, QIAamp MinElute Virus Spin Kit
Mahzoonieh et al., 2020	DNP DNA extraction Kit
Li et al., 2020	QIAamp DNA Stool Mini Kit
Crispo et al., 2020	NR
Amery-Gale et al., 2020	QIAamp Viral RNA Mini Kit, Robertson 2009
Wang et al., 2019	QIAamp DNA Mini Kit
Vogler et al., 2019	Maxwell 16 Buccal Swab LEV DNA Purification kit
Vilela et al., 2019	Boom 1990
Sutherland et al., 2019	ReliaPrep gDNA Tissue Miniprep System
Plaza et al., 2019	ZR Genomic DNA-Tissue Mini Prep Kit
Origlia, 2019	ZR Quick gDNA MiniPrep Kit, Invitrogen PureLink Genomic DNA Mini Kit
Mina et al., 2019	High Pure PCR Template Preparation Kit
Mattmann et al., 2019	NucleoSpin Tissue kit
Liu et al., 2019	QIAamp DNA Mini Kit
Lin et al., 2019	NR
Hamzah et al., 2019	QIAamp DNA Stool Mini Kit
Wang et al., 2018	E.Z.N.A. Stool DNA Kit
Szymańska-Czerwińska et al., 2018	QIAamp DNA Mini Kit
Heijne et al., 2018	NucliSENS easyMAG
Gedye et al., 2018	InstaGene Matrix, High Pure FFPET DNA Isolation Kit
Donati et al., 2018	QIAamp DNA Mini Kit
Cechova et al., 2018	DNA-sorbAM nucleic acid extraction Kit
Burt et al., 2018	NucliSENS easyMAG
Yao et al., 2017	E.Z.N.A. Stool DNA Kit
Vaz et al., 2017	NucleoSpin Tissue kit
Szymanska-Czerwinska et al., 2017	QIAamp DNA Mini Kit
Jeong et al., 2017	Maxwell RSC Viral Total Nucleic Acid Purification Kit
Hegazy et al., 2017	QIAamp DNA Mini Kit
Gartrell et al., 2017	Gartrell 2013
Ferreira et al., 2017	NucleoSpin Nucleic Acid/Protein Kit
Cadario et al., 2017	DNeasy Blood & Tissue Kit
Vasconcelos et al., 2016	Ishizawa 1991
Tatari et al., 2016	High Pure PCR Template Preparation Kit
Suksai et al., 2016	NucleoSpin Tissue kit
Razmyar et al., 2016	PCR Preparation kit (Denazist Asia, Iran)
Ornelas-Eusebio et al., 2016	DNeasy Blood & Tissue Kit

Mahzoonieh et al., 2016	DNP DNA extraction Kit
Konicek et al., 2016	QIAamp cador Pathogen Kit
Hulin et al., 2016	QIAamp DNA Mini Kit
Ferreira et al., 2016	NucleoSpin Nucleic Acid/Protein Kit
Feng et al., 2016	QIAamp DNA Stool Mini Kit
Dovc et al., 2016	QIAamp DNA Mini Kit
Cechova et al., 2016	DNA-sorbAM nucleic acid extraction Kit
Vorimore et al., 2015	QIAamp DNA Mini Kit
Shivaprasad et al., 2015	QIAamp DNA Mini Kit
Ling et al., 2015	DNeasy Blood & Tissue Kit
Laroucau et al., 2015	QIAamp DNA Mini Kit
Khodadadi et al., 2015	DNA extraction kit (CinnaGen, Iran)
Hulin et al., 2015	QIAamp DNA Mini Kit
Ghorbanpoor et al., 2015	boiling (hewinson 1991 - article unretrievable)
Donati et al., 2015	QIAamp DNA Stool Mini Kit
Aaziz et al., 2015	QIAamp DNA Mini Kit
To et al., 2014	EZ1 virus Mini Kit v2.0
Stenzel et al., 2014	Magnetic method using the Janus automated workstation, NucleoMag Tissue Kit
Kalmar et al., 2014	NR
Elezi et al., 2014	DNeasy Blood & Tissue Kit
Beckmann et al., 2014	BioSprint DNA Blood Kit (Qiagen)
Tomic et al., 2013	GenElute Mammalian Genomic DNA Miniprep Kit
Tomic et al., 2013	GenElute Mammalian Genomic DNA Miniprep Kit
Tel et al., 2013	QIAamp DNA Stool Mini Kit
Szymańska-Czerwińska et al., 2013	QIAamp DNA Mini Kit
Sheleby-Elías et al., 2013	DNeasy Blood & Tissue Kit
Perelman et al., 2013	NR
Madani et al., 2013	High Pure PCR Template Preparation Kit
Gartrell et al., 2013	Gartrell 2013
Dickx et al., 2013	NR
Zocevic et al., 2012	QIAamp DNA Mini Kit
Satrovic et al., 2012	QIAamp DNA Mini Kit
Piasecki et al., 2012	Genomic DNA Prep Plus kit
Marhold et al., 2012	QIAamp DNA Mini Kit
Krizek et al., 2012	DNeasy Blood & Tissue Kit
Gilbert et al., 2012	NR
Geigenfeind et al., 2012	NR
Deem et al., 2012	NR
Colvile et al., 2012	NR
Blomqvist et al., 2012	MagAttract DNA Mini M48 kit
Yanga et al., 2011	DNeasy Blood & Tissue Kit
Yang et al., 2011	DNeasy Blood & Tissue Kit
Gasparini et al., 2011	QIAamp DNA Mini Kit
Doosti et al., 2011	QIAamp DNA Mini Kit

Zhou et al., 2010	Hewinson 1997
Vazquez et al., 2010	NR
Tarsitano et al., 2010	QIAamp Tissue Kit
Robertson et al., 2010	Robertson 2009
Geigenfeind et al., 2010	NR
Dickx Geens et al., 2010	Van Loock 2005
Dickx et al., 2010	Van Loock 2005
Christerson et al., 2010	NucliSENS easyMAG
Zweifel et al., 2009	MagNA Pure LC System, DNeasy Blood & Tissue Kit
Song et al., 2009	High Pure PCR Template Preparation Kit
Sharples et al., 2009	NR
Sareyyupoglu et al., 2009	QIAamp Tissue Kit, QIAamp DNA Stool Mini Kit
Sachse et al., 2009	High Pure PCR Template Preparation Kit
Mitchell et al., 2009	QIAamp DNA Mini Kit
Laroucau et al., 2009	QIAamp DNA Mini Kit
Droogenbroeck et al., 2009	Van Loock 2005
Zhang et al., 2008	DNeasy Blood & Tissue Kit
Verminnen et al., 2008	Van Loock 2005
Laroucau et al., 2008	QIAamp DNA Mini Kit
Gaede et al., 2008	High Pure PCR Template Preparation Kit
Branley et al., 2008	QIAamp DNA Mini Kit
Yang et al., 2007	DNeasy Blood & Tissue Kit
Varompay et al., 2007	NR
Schenker et al., 2007	Schenker 2007
Sareyyupoglu et al., 2007	QIAamp DNA Stool Mini Kit
Harkinezhad et al., 2007	NR
Assuncao et al., 2007	Boom 1990
Travis et al., 2006	NR
Herrmann et al., 2006	High Pure PCR Template Preparation Kit
Heddema, Hannenm, Duim et al., 2006	Boom 2000
Heddema, Ter, Buys et al., 2006	Boom 2000
Van Loock, Verminnen, Messmer et al., 2005	Van Loock 2005
Van Loock, Geens, Smit et al., 2005	Qiagen spin columns
Tanaka et al., 2005	QIAamp DNA Stool Mini Kit
Greco et al., 2005	NR
Dovc et al., 2005	Rasmussen 1991
Bonner et al., 2004	NR
Schettler et al., 2003	High Pure PCR Template Preparation Kit

Appendices for Chapter 3 & 4

Appendix 3.1

High-resolution melt (HRM) analysis quantitative PCR protocol for the detection of *Chlamydia psittaci*.

Nucleic acid extraction	Kit
DNA	Chelex® 100 extraction protocol

Real-time PCR

Primers	Name	Sequence (5'-3')	Size	Target
Forward	VD1-f	5'-ACTACGGAGATTATGTTTTCGATCGTGT-3'	418	Outer membrane protein A
Reverse	VD2-r	5'-CGTGCACCYACGCTCCAAGA-3'		

Mastermix	HOT FIREpol® EvaGreen® HRM master mix (Solis Biodyne, Tartu, Estonia)

Reagent mix	Volume (µl) 20µl total	Final concentration
Mastermix (5x)	4	1x
500 nM VD1-f primer	0.4	200 nM
500 nM VD2-r primer	0.4	200 nM
DNA	2	20 ng/µl
Sterile distilled water	13.2	

PCR controls	Description	Volume added (µl)
Positive	7 10x standard dilutions, concentrations 0.1 to 10^{-8} ng/µl	2
Negative	Sterile distilled water	2

PCR system: MIC qPCR Cycler (Bio Molecular Systems, Queensland, Australia)

Cycling parameters	Temp (°C)	Time	No. cycle
Denature	95	15 min	1
Denature	95	10 sec	40
Anneal	60	15 sec	
Extension	72	20 sec	
Melt curve	95	60 sec	Pre-melt conditioning
	40	60 sec	
	65-95		0.3 °C/sec increment

Appendix 3.2

Bird samples collection data and molecular test results.

ID	Date	Location	Species	Sample site	HRM1	HRM2	HRM3	qPCR	Bacteria ID	Genbank accession number
B001	30/10/19	Thames	bar-tailed godwit	choanal						
B002	30/10/19	Thames	bar-tailed godwit	cloacal						
B003	30/10/19	Thames	bar-tailed godwit	choanal	85.15			y	av *C. abortus* G1	OQ447479
B004	30/10/19	Thames	bar-tailed godwit	cloacal						
B005	30/10/19	Thames	bar-tailed godwit	choanal						
B006	30/10/19	Thames	bar-tailed godwit	cloacal						
B007	30/10/19	Thames	bar-tailed godwit	choanal						
B008	30/10/19	Thames	bar-tailed godwit	cloacal						
B009	30/10/19	Thames	bar-tailed godwit	choanal						
B010	30/10/19	Thames	bar-tailed godwit	cloacal						
B011	30/10/19	Thames	bar-tailed godwit	choanal						
B012	30/10/19	Thames	bar-tailed godwit	cloacal						
B013	31/10/19	Thames	bar-tailed godwit	choanal						
B014	31/10/19	Thames	bar-tailed godwit	cloacal		84.49	83.86	y		
B015	31/10/19	Thames	bar-tailed godwit	choanal						
B016	31/10/19	Thames	bar-tailed godwit	cloacal						
B017	31/10/19	Thames	bar-tailed godwit	choanal						
B018	31/10/19	Thames	bar-tailed godwit	cloacal						
B019	31/10/19	Thames	bar-tailed godwit	choanal						
B020	31/10/19	Thames	bar-tailed godwit	cloacal						
B021	31/10/19	Thames	bar-tailed godwit	choanal						

B022	31/10/19	Thames	bar-tailed godwit	cloacal						
B023	31/10/19	Thames	bar-tailed godwit	choanal						
B024	31/10/19	Thames	bar-tailed godwit	cloacal						
B025	31/10/19	Thames	bar-tailed godwit	choanal						
B026	31/10/19	Thames	bar-tailed godwit	cloacal						
B027	31/10/19	Thames	bar-tailed godwit	choanal						
B028	31/10/19	Thames	bar-tailed godwit	cloacal						
B029	31/10/19	Thames	red knot	choanal			87.25	y		
B030	31/10/19	Thames	red knot	cloacal						
B031	31/10/19	Thames	red knot	choanal						
B032	31/10/19	Thames	red knot	cloacal						
B033	31/10/19	Thames	red knot	choanal						
B034	31/10/19	Thames	red knot	cloacal						
B035	31/10/19	Thames	red knot	choanal						
B036	31/10/19	Thames	red knot	cloacal		86	86.41	y		
B037	31/10/19	Thames	red knot	choanal						
B038	31/10/19	Thames	red knot	cloacal						
B039	01/11/19	Thames	bar-tailed godwit	choanal						
B040	01/11/19	Thames	bar-tailed godwit	cloacal		83.74		y		
B041	01/11/19	Thames	bar-tailed godwit	choanal						
B042	01/11/19	Thames	bar-tailed godwit	cloacal						
B043	01/11/19	Thames	bar-tailed godwit	choanal						
B044	01/11/19	Thames	bar-tailed godwit	cloacal						
B045	01/11/19	Thames	bar-tailed godwit	choanal						
B046	01/11/19	Thames	bar-tailed godwit	cloacal						
B047	01/11/19	Thames	bar-tailed godwit	choanal						
B048	01/11/19	Thames	bar-tailed godwit	cloacal						
B049	01/11/19	Thames	bar-tailed godwit	choanal						
B050	01/11/19	Thames	bar-tailed godwit	cloacal						

B051	01/11/19	Thames	bar-tailed godwit	choanal						
B052	01/11/19	Thames	bar-tailed godwit	cloacal		84.04		y	av *C. abortus* G1	OQ447480
B053	01/11/19	Thames	bar-tailed godwit	choanal						
B054	01/11/19	Thames	bar-tailed godwit	cloacal						
B055	01/11/19	Thames	red knot	choanal						
B056	01/11/19	Thames	red knot	cloacal						
B057	01/11/19	Thames	red knot	choanal						
B058	01/11/19	Thames	red knot	cloacal						
B059	15/1/2021	Thames	south island pied oystercatcher	choanal						
B060	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B061	15/1/2021	Thames	south island pied oystercatcher	choanal						
B062	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B063	15/1/2021	Thames	variable oystercatcher	choanal						
B064	15/1/2021	Thames	variable oystercatcher	cloacal						
B065	15/1/2021	Thames	south island pied oystercatcher	choanal						
B066	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B067	15/1/2021	Thames	south island pied oystercatcher	choanal						
B068	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B069	15/1/2021	Thames	south island pied oystercatcher	choanal						
B070	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B071	15/1/2021	Thames	south island pied oystercatcher	choanal						
B072	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B073	15/1/2021	Thames	south island pied oystercatcher	choanal						
B074	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B075	15/1/2021	Thames	south island pied oystercatcher	choanal						
B076	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B077	15/1/2021	Thames	south island pied oystercatcher	choanal						
B078	15/1/2021	Thames	south island pied oystercatcher	cloacal						

B079	15/1/2021	Thames	south island pied oystercatcher	choanal						
B080	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B081	15/1/2021	Thames	south island pied oystercatcher	choanal						
B082	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B083	15/1/2021	Thames	south island pied oystercatcher	choanal						
B084	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B085	15/1/2021	Thames	south island pied oystercatcher	choanal						
B086	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B087	15/1/2021	Thames	south island pied oystercatcher	choanal						
B088	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B089	15/1/2021	Thames	south island pied oystercatcher	choanal						
B090	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B091	15/1/2021	Thames	south island pied oystercatcher	choanal						
B092	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B093	15/1/2021	Thames	south island pied oystercatcher	choanal						
B094	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B095	15/1/2021	Thames	south island pied oystercatcher	choanal						
B096	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B097	15/1/2021	Thames	south island pied oystercatcher	choanal						
B098	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B099	15/1/2021	Thames	south island pied oystercatcher	choanal						
B100	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B101	15/1/2021	Thames	south island pied oystercatcher	choanal						
B102	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B103	15/1/2021	Thames	south island pied oystercatcher	choanal						
B104	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B105	15/1/2021	Thames	south island pied oystercatcher	choanal						
B106	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B107	15/1/2021	Thames	south island pied oystercatcher	choanal						

B108	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B109	15/1/2021	Thames	south island pied oystercatcher	choanal						
B110	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B111	15/1/2021	Thames	south island pied oystercatcher	choanal						
B112	15/1/2021	Thames	south island pied oystercatcher	cloacal			84.76	y		
B113	15/1/2021	Thames	south island pied oystercatcher	choanal						
B114	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B115	15/1/2021	Thames	south island pied oystercatcher	choanal						
B116	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B117	15/1/2021	Thames	south island pied oystercatcher	choanal						
B118	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B119	15/1/2021	Thames	south island pied oystercatcher	choanal						
B120	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B121	15/1/2021	Thames	south island pied oystercatcher	choanal						
B122	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B123	15/1/2021	Thames	south island pied oystercatcher	choanal						
B124	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B125	15/1/2021	Thames	south island pied oystercatcher	choanal						
B126	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B127	15/1/2021	Thames	south island pied oystercatcher	choanal						
B128	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B129	15/1/2021	Thames	south island pied oystercatcher	choanal						
B130	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B131	15/1/2021	Thames	south island pied oystercatcher	choanal						
B132	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B133	15/1/2021	Thames	south island pied oystercatcher	choanal						
B134	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B135	15/1/2021	Thames	south island pied oystercatcher	choanal						
B136	15/1/2021	Thames	south island pied oystercatcher	cloacal						

B137	15/1/2021	Thames	south island pied oystercatcher	choanal						
B138	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B139	15/1/2021	Thames	south island pied oystercatcher	choanal	83.1			y		
B140	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B141	15/1/2021	Thames	south island pied oystercatcher	choanal						
B142	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B143	15/1/2021	Thames	south island pied oystercatcher	choanal						
B144	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B145	15/1/2021	Thames	south island pied oystercatcher	choanal						
B146	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B147	15/1/2021	Thames	south island pied oystercatcher	choanal						
B148	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B149	15/1/2021	Thames	south island pied oystercatcher	choanal						
B150	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B151	15/1/2021	Thames	south island pied oystercatcher	choanal						
B152	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B153	15/1/2021	Thames	south island pied oystercatcher	choanal						
B154	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B155	15/1/2021	Thames	south island pied oystercatcher	choanal						
B156	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B157	15/1/2021	Thames	south island pied oystercatcher	choanal						
B158	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B159	15/1/2021	Thames	south island pied oystercatcher	choanal	84.2			y		
B160	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B161	15/1/2021	Thames	south island pied oystercatcher	choanal						
B162	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B163	15/1/2021	Thames	south island pied oystercatcher	choanal						
B164	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B165	15/1/2021	Thames	south island pied oystercatcher	choanal						

B166	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B167	15/1/2021	Thames	south island pied oystercatcher	choanal						
B168	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B169	15/1/2021	Thames	south island pied oystercatcher	choanal						
B170	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B171	15/1/2021	Thames	south island pied oystercatcher	choanal						
B172	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B173	15/1/2021	Thames	south island pied oystercatcher	choanal						
B174	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B175	15/1/2021	Thames	south island pied oystercatcher	choanal						
B176	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B177	15/1/2021	Thames	south island pied oystercatcher	choanal						
B178	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B179	15/1/2021	Thames	south island pied oystercatcher	choanal						
B180	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B181	15/1/2021	Thames	south island pied oystercatcher	choanal		83.8		y	av *C. abortus* G1	OQ447481
B182	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B183	15/1/2021	Thames	south island pied oystercatcher	choanal						
B184	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B185	15/1/2021	Thames	south island pied oystercatcher	choanal						
B186	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B187	15/1/2021	Thames	south island pied oystercatcher	choanal						
B188	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B189	15/1/2021	Thames	south island pied oystercatcher	choanal						
B190	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B191	15/1/2021	Thames	south island pied oystercatcher	choanal			83.41	y		
B192	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B193	15/1/2021	Thames	south island pied oystercatcher	choanal						

B194	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B195	15/1/2021	Thames	south island pied oystercatcher	choanal						
B196	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B197	15/1/2021	Thames	south island pied oystercatcher	choanal						
B198	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B199	15/1/2021	Thames	south island pied oystercatcher	choanal						
B200	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B201	15/1/2021	Thames	south island pied oystercatcher	choanal						
B202	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B203	15/1/2021	Thames	south island pied oystercatcher	choanal						
B204	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B205	15/1/2021	Thames	south island pied oystercatcher	choanal						
B206	15/1/2021	Thames	south island pied oystercatcher	cloacal		84		y		
B207	15/1/2021	Thames	south island pied oystercatcher	choanal						
B208	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B209	15/1/2021	Thames	south island pied oystercatcher	choanal						
B210	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B211	15/1/2021	Thames	south island pied oystercatcher	choanal						
B212	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B213	15/1/2021	Thames	south island pied oystercatcher	choanal						
B214	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B215	15/1/2021	Thames	south island pied oystercatcher	choanal						
B216	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B217	15/1/2021	Thames	south island pied oystercatcher	choanal						
B218	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B219	15/1/2021	Thames	south island pied oystercatcher	choanal						
B220	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B221	15/1/2021	Thames	south island pied oystercatcher	choanal						
B222	15/1/2021	Thames	south island pied oystercatcher	cloacal						

B223	15/1/2021	Thames	south island pied oystercatcher	choanal						
B224	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B225	15/1/2021	Thames	south island pied oystercatcher	choanal						
B226	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B227	15/1/2021	Thames	south island pied oystercatcher	choanal						
B228	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B229	15/1/2021	Thames	south island pied oystercatcher	choanal						
B230	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B231	15/1/2021	Thames	south island pied oystercatcher	choanal						
B232	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B233	15/1/2021	Thames	south island pied oystercatcher	choanal			84.19	y		
B234	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B235	15/1/2021	Thames	south island pied oystercatcher	choanal						
B236	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B237	15/1/2021	Thames	south island pied oystercatcher	choanal						
B238	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B239	15/1/2021	Thames	south island pied oystercatcher	choanal						
B240	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B241	15/1/2021	Thames	south island pied oystercatcher	choanal						
B242	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B243	15/1/2021	Thames	south island pied oystercatcher	choanal						
B244	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B245	15/1/2021	Thames	south island pied oystercatcher	choanal						
B246	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B247	15/1/2021	Thames	south island pied oystercatcher	choanal						
B248	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B249	15/1/2021	Thames	south island pied oystercatcher	choanal	84			y	av *C. abortus* G1	OQ447482
B250	15/1/2021	Thames	south island pied oystercatcher	cloacal						

B251	15/1/2021	Thames	south island pied oystercatcher	choanal						
B252	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B253	15/1/2021	Thames	south island pied oystercatcher	choanal						
B254	15/1/2021	Thames	south island pied oystercatcher	cloacal		84.55		y		
B255	15/1/2021	Thames	south island pied oystercatcher	choanal						
B256	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B257	15/1/2021	Thames	south island pied oystercatcher	choanal						
B258	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B259	15/1/2021	Thames	south island pied oystercatcher	choanal						
B260	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B261	15/1/2021	Thames	south island pied oystercatcher	choanal						
B262	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B263	15/1/2021	Thames	south island pied oystercatcher	choanal						
B264	15/1/2021	Thames	south island pied oystercatcher	cloacal						
B265	02/02/21	Manawatū	bar-tailed godwit	choanal						
B266	02/02/21	Manawatū	bar-tailed godwit	cloacal	84.7	84.1	83.86	y		
B267	02/02/21	Manawatū	bar-tailed godwit	choanal						
B268	02/02/21	Manawatū	bar-tailed godwit	cloacal						
B269	02/02/21	Manawatū	bar-tailed godwit	choanal						
B270	02/02/21	Manawatū	bar-tailed godwit	cloacal						
B271	02/02/21	Manawatū	bar-tailed godwit	choanal						
B272	02/02/21	Manawatū	bar-tailed godwit	cloacal	84.5	83.74	84.01	y	av *C. abortus* G1	OQ447483
B273	02/02/21	Manawatū	bar-tailed godwit	choanal						
B274	02/02/21	Manawatū	bar-tailed godwit	cloacal	84.8	84.16	84.1	y		
B275	02/02/21	Manawatū	bar-tailed godwit	choanal						
B276	02/02/21	Manawatū	bar-tailed godwit	cloacal		84.1		y	av *C. abortus* G1	OQ447484
B277	02/02/21	Manawatū	pied stilt	choanal						

B278	02/02/21	Manawatū	pied stilt	cloacal						
B279	02/02/21	Manawatū	pied stilt	choanal						
B280	02/02/21	Manawatū	pied stilt	cloacal		83.65		y		
B281	02/02/21	Manawatū	pied stilt	choanal						
B282	02/02/21	Manawatū	pied stilt	cloacal						
B283	02/02/21	Manawatū	pied stilt	choanal						
B284	02/02/21	Manawatū	pied stilt	cloacal						
B285	02/02/21	Manawatū	pied stilt	choanal	84			y		
B286	02/02/21	Manawatū	pied stilt	cloacal	84.9	84.46	84.31	y	av *C. abortus* G1	OQ447485
B287	02/02/21	Manawatū	pied stilt	choanal						
B288	02/02/21	Manawatū	pied stilt	cloacal		85.6		y		
B289	02/02/21	Manawatū	pied stilt	choanal						
B290	02/02/21	Manawatū	pied stilt	cloacal		86.26		y		
B291	02/02/21	Manawatū	pied stilt	choanal	86.14	85.36	85.36	y	av *C. abortus* G1	OQ447478
B292	02/02/21	Manawatū	pied stilt	cloacal						
B293	02/02/21	Manawatū	pied stilt	choanal						
B294	02/02/21	Manawatū	pied stilt	cloacal						
B295	02/02/21	Manawatū	pied stilt	choanal						
B296	02/02/21	Manawatū	pied stilt	cloacal						
B297	02/02/21	Manawatū	pied stilt	choanal						
B298	02/02/21	Manawatū	pied stilt	cloacal						
B299	02/02/21	Manawatū	pied stilt	choanal		84.19	86.5	y		
B300	02/02/21	Manawatū	pied stilt	cloacal						
B301	02/02/21	Manawatū	pied stilt	choanal						
B302	02/02/21	Manawatū	pied stilt	cloacal						
B303	02/02/21	Manawatū	pied stilt	choanal						
B304	02/02/21	Manawatū	pied stilt	cloacal						

B305	02/02/21	Manawatū	pied stilt	choanal						
B306	02/02/21	Manawatū	pied stilt	cloacal						
B307	02/02/21	Manawatū	pied stilt	choanal						
B308	02/02/21	Manawatū	pied stilt	cloacal	84.4			y		
B309	02/02/21	Manawatū	pied stilt	choanal	85			y		
B310	02/02/21	Manawatū	pied stilt	cloacal						
B311	02/02/21	Manawatū	pied stilt	choanal						
B312	02/02/21	Manawatū	pied stilt	cloacal						
B313	02/02/21	Manawatū	pied stilt	choanal						
B314	02/02/21	Manawatū	pied stilt	cloacal						
B315	02/02/21	Manawatū	pied stilt	choanal		85.66		y		
B316	02/02/21	Manawatū	pied stilt	cloacal			84.4	y		
B317	02/02/21	Manawatū	pied stilt	choanal						
B318	02/02/21	Manawatū	pied stilt	cloacal						
B319	02/02/21	Manawatū	pied stilt	choanal			83.41	y		
B320	02/02/21	Manawatū	pied stilt	cloacal						
B321	02/02/21	Manawatū	pied stilt	choanal						
B322	02/02/21	Manawatū	pied stilt	cloacal						
B323	02/02/21	Manawatū	pied stilt	choanal						
B324	02/02/21	Manawatū	pied stilt	cloacal	83.4			y		
B325	02/02/21	Manawatū	pied stilt	choanal						
B326	02/02/21	Manawatū	pied stilt	cloacal						
B327	02/02/21	Manawatū	pied stilt	choanal						
B328	02/02/21	Manawatū	pied stilt	cloacal			85.3	y		
B329	02/02/21	Manawatū	pied stilt	choanal						
B330	02/02/21	Manawatū	pied stilt	cloacal						
B331	02/02/21	Manawatū	pied stilt	choanal						
B332	02/02/21	Manawatū	pied stilt	cloacal						
B333	02/02/21	Manawatū	pied stilt	choanal			85.81	y		

B334	02/02/21	Manawatū	pied stilt	cloacal						
B335	02/02/21	Manawatū	pied stilt	choanal						
B336	02/02/21	Manawatū	pied stilt	cloacal						
B337	02/02/21	Manawatū	pied stilt	choanal			85.6	y	*C. psittaci* C	OQ447486
B338	02/02/21	Manawatū	pied stilt	cloacal						
B339	02/02/21	Manawatū	pied stilt	choanal						
B340	02/02/21	Manawatū	pied stilt	cloacal						
B341	02/02/21	Manawatū	pied stilt	choanal			84.25	y		
B342	02/02/21	Manawatū	pied stilt	cloacal						
B343	02/02/21	Manawatū	pied stilt	choanal						
B344	02/02/21	Manawatū	pied stilt	cloacal		86.7	85.84	y		
B345	02/02/21	Manawatū	pied stilt	choanal						
B346	02/02/21	Manawatū	pied stilt	cloacal						
B347	02/02/21	Manawatū	pied stilt	choanal						
B348	02/02/21	Manawatū	pied stilt	cloacal	83.4	86.1	85.3	y		
B349	02/02/21	Manawatū	pied stilt	choanal						
B350	02/02/21	Manawatū	pied stilt	cloacal		86.7		y		
B351	02/02/21	Manawatū	pied stilt	choanal						
B352	02/02/21	Manawatū	pied stilt	cloacal						
B353	02/02/21	Manawatū	pied stilt	choanal						
B354	02/02/21	Manawatū	pied stilt	cloacal						
B355	02/02/21	Manawatū	pied stilt	choanal						
B356	02/02/21	Manawatū	pied stilt	cloacal						
B357	02/02/21	Manawatū	pied stilt	choanal						
B358	02/02/21	Manawatū	pied stilt	cloacal						
B359	02/02/21	Manawatū	pied stilt	choanal						
B360	02/02/21	Manawatū	pied stilt	cloacal						
B361	02/02/21	Manawatū	pied stilt	choanal						
B362	02/02/21	Manawatū	pied stilt	cloacal						

B363	02/02/21	Manawatū	pied stilt	choanal						
B364	02/02/21	Manawatū	pied stilt	cloacal			85.81	y		
B365	02/02/21	Manawatū	pied stilt	choanal						
B366	02/02/21	Manawatū	pied stilt	cloacal						
B367	02/02/21	Manawatū	pied stilt	choanal						
B368	02/02/21	Manawatū	pied stilt	cloacal						
B369	02/02/21	Manawatū	pied stilt	choanal	84.9			y		
B370	02/02/21	Manawatū	pied stilt	cloacal						
B371	02/02/21	Manawatū	pied stilt	choanal						
B372	02/02/21	Manawatū	pied stilt	cloacal			85.45	y		
B373	02/02/21	Manawatū	pied stilt	choanal						
B374	02/02/21	Manawatū	pied stilt	cloacal						
B375	02/02/21	Manawatū	pied stilt	choanal						
B376	02/02/21	Manawatū	pied stilt	cloacal						
B377	02/02/21	Manawatū	pied stilt	choanal						
B378	02/02/21	Manawatū	pied stilt	cloacal						
B379	02/02/21	Manawatū	pied stilt	choanal						
B380	02/02/21	Manawatū	pied stilt	cloacal						
B381	02/02/21	Manawatū	pied stilt	choanal						
B382	02/02/21	Manawatū	pied stilt	cloacal			85.6	y		
B383	02/02/21	Manawatū	pied stilt	choanal						
B384	02/02/21	Manawatū	pied stilt	cloacal						
B385	02/02/21	Manawatū	pied stilt	choanal						
B386	02/02/21	Manawatū	pied stilt	cloacal						
B387	02/02/21	Manawatū	pied stilt	choanal						
B388	02/02/21	Manawatū	pied stilt	cloacal	83.4			y		
B389	02/02/21	Manawatū	pied stilt	choanal						
B390	02/02/21	Manawatū	pied stilt	cloacal	85.5			y		
B391	02/02/21	Manawatū	pied stilt	choanal						

B392	02/02/21	Manawatū	pied stilt	cloacal	86	86.4		y		
B393	02/02/21	Manawatū	pied stilt	choanal	85.5		86.71	y	av *C. abortus* G1	OQ447487
B394	02/02/21	Manawatū	pied stilt	cloacal	85.8			y		
B395	02/02/21	Manawatū	pied stilt	choanal						
B396	02/02/21	Manawatū	pied stilt	cloacal						
B397	02/02/21	Manawatū	pied stilt	choanal	85.6			y		
B398	02/02/21	Manawatū	pied stilt	cloacal						
B399	02/02/21	Manawatū	pied stilt	choanal						
B400	02/02/21	Manawatū	pied stilt	cloacal						
B401	02/02/21	Manawatū	pied stilt	choanal						
B402	02/02/21	Manawatū	pied stilt	cloacal						
B403	03/02/21	Thames	bar-tailed godwit	choanal						
B404	03/02/21	Thames	bar-tailed godwit	cloacal						
B405	03/02/21	Thames	bar-tailed godwit	choanal						
B406	03/02/21	Thames	bar-tailed godwit	cloacal	84.9			y	av *C. abortus* G1	OQ447488
B407	03/02/21	Thames	bar-tailed godwit	choanal						
B408	03/02/21	Thames	bar-tailed godwit	cloacal		83.35	87.85	y		
B409	03/02/21	Thames	bar-tailed godwit	choanal						
B410	03/02/21	Thames	bar-tailed godwit	cloacal						
B411	03/02/21	Thames	bar-tailed godwit	choanal						
B412	03/02/21	Thames	bar-tailed godwit	cloacal						
B413	03/02/21	Thames	bar-tailed godwit	choanal						
B414	03/02/21	Thames	bar-tailed godwit	cloacal			87.85	y		
B415	03/02/21	Thames	bar-tailed godwit	choanal						
B416	03/02/21	Thames	bar-tailed godwit	cloacal						
B417	03/02/21	Thames	bar-tailed godwit	choanal						
B418	03/02/21	Thames	bar-tailed godwit	cloacal	87.31		87.91	y		

B419	03/02/21	Thames	bar-tailed godwit	choanal						
B420	03/02/21	Thames	bar-tailed godwit	cloacal	84.8			y		
B421	03/02/21	Thames	bar-tailed godwit	choanal						
B422	03/02/21	Thames	bar-tailed godwit	cloacal		84.85		y		
B423	03/02/21	Thames	bar-tailed godwit	choanal						
B424	03/02/21	Thames	bar-tailed godwit	cloacal			86.71	y		
B425	03/02/21	Thames	bar-tailed godwit	choanal						
B426	03/02/21	Thames	bar-tailed godwit	cloacal						
B427	03/02/21	Thames	red knot	choanal						
B428	03/02/21	Thames	red knot	cloacal		84.1	86.05	y		
B429	03/02/21	Thames	red knot	choanal						
B430	03/02/21	Thames	red knot	cloacal						
B431	03/02/21	Thames	Wrybill	choanal						
B432	03/02/21	Thames	Wrybill	cloacal						
B433	03/02/21	Thames	Wrybill	choanal						
B434	03/02/21	Thames	Wrybill	cloacal						
B435	03/02/21	Thames	Wrybill	choanal						
B436	03/02/21	Thames	Wrybill	cloacal						
B437	03/02/21	Thames	Wrybill	choanal						
B438	03/02/21	Thames	Wrybill	cloacal						
B439	03/02/21	Thames	Wrybill	choanal						
B440	03/02/21	Thames	Wrybill	cloacal	83.8		87.55	y		
B441	03/02/21	Thames	Wrybill	choanal						
B442	03/02/21	Thames	Wrybill	cloacal						
B443	03/02/21	Thames	Wrybill	choanal						
B444	03/02/21	Thames	Wrybill	cloacal	83.1			y		
B445	03/02/21	Thames	Wrybill	choanal						
B446	03/02/21	Thames	Wrybill	cloacal						
B447	03/02/21	Thames	Wrybill	choanal						

B448	03/02/21	Thames	Wrybill	cloacal						
B449	03/02/21	Thames	Wrybill	choanal						
B450	03/02/21	Thames	Wrybill	cloacal	83.3			y		
B451	03/02/21	Thames	Wrybill	choanal						
B452	03/02/21	Thames	Wrybill	cloacal	83.1	84.4		y		
B453	03/02/21	Thames	Wrybill	choanal						
B454	03/02/21	Thames	Wrybill	cloacal						
B455	03/02/21	Thames	Wrybill	choanal						
B456	03/02/21	Thames	Wrybill	cloacal						
B457	03/02/21	Thames	Wrybill	choanal						
B458	03/02/21	Thames	Wrybill	cloacal						
B459	03/02/21	Thames	south island pied oystercatcher	choanal						
B460	03/02/21	Thames	south island pied oystercatcher	cloacal						
B461	03/02/21	Thames	south island pied oystercatcher	choanal						
B462	03/02/21	Thames	south island pied oystercatcher	cloacal	86.29			y		
B463	03/02/21	Thames	south island pied oystercatcher	choanal						
B464	03/02/21	Thames	south island pied oystercatcher	cloacal						
B465	03/02/21	Thames	south island pied oystercatcher	choanal						
B466	03/02/21	Thames	south island pied oystercatcher	cloacal	83.5			y		
B467	03/02/21	Thames	south island pied oystercatcher	choanal						
B468	03/02/21	Thames	south island pied oystercatcher	cloacal						
B469	03/02/21	Thames	south island pied oystercatcher	choanal						
B470	03/02/21	Thames	south island pied oystercatcher	cloacal						
B471	03/02/21	Thames	south island pied oystercatcher	choanal						
B472	03/02/21	Thames	south island pied oystercatcher	cloacal	84			y		
B473	03/02/21	Thames	south island pied oystercatcher	choanal						
B474	03/02/21	Thames	south island pied oystercatcher	cloacal						
B475	03/02/21	Thames	south island pied oystercatcher	choanal						
B476	03/02/21	Thames	south island pied oystercatcher	cloacal						

B477	03/02/21	Thames	south island pied oystercatcher	choanal						
B478	03/02/21	Thames	south island pied oystercatcher	cloacal						
B479	03/02/21	Thames	south island pied oystercatcher	choanal						
B480	03/02/21	Thames	south island pied oystercatcher	cloacal						
B481	03/02/21	Thames	south island pied oystercatcher	choanal						
B482	03/02/21	Thames	south island pied oystercatcher	cloacal						
B483	03/02/21	Thames	south island pied oystercatcher	choanal						
B484	03/02/21	Thames	south island pied oystercatcher	cloacal						
B485	03/02/21	Thames	south island pied oystercatcher	choanal						
B486	03/02/21	Thames	south island pied oystercatcher	cloacal			83.65	y		
B487	03/02/21	Thames	south island pied oystercatcher	choanal						
B488	03/02/21	Thames	south island pied oystercatcher	cloacal						
B489	03/02/21	Thames	south island pied oystercatcher	choanal						
B490	03/02/21	Thames	south island pied oystercatcher	cloacal						
B491	03/02/21	Thames	south island pied oystercatcher	choanal						
B492	03/02/21	Thames	south island pied oystercatcher	cloacal	87.25			y	av *C. abortus* G1	OQ447489
B493	03/02/21	Thames	south island pied oystercatcher	choanal						
B494	03/02/21	Thames	south island pied oystercatcher	cloacal						
B495	03/02/21	Thames	south island pied oystercatcher	choanal						
B496	03/02/21	Thames	south island pied oystercatcher	cloacal						
B497	03/02/21	Thames	south island pied oystercatcher	choanal						
B498	03/02/21	Thames	south island pied oystercatcher	cloacal						
B499	03/02/21	Thames	south island pied oystercatcher	choanal						
B500	03/02/21	Thames	south island pied oystercatcher	cloacal						
B501	03/02/21	Thames	south island pied oystercatcher	choanal						
B502	03/02/21	Thames	south island pied oystercatcher	cloacal		87.7		y		
B503	03/02/21	Thames	south island pied oystercatcher	choanal						
B504	03/02/21	Thames	south island pied oystercatcher	cloacal						

B505	03/02/21	Thames	south island pied oystercatcher	choanal						
B506	03/02/21	Thames	south island pied oystercatcher	cloacal						
B507	03/03/21	Thames	bar-tailed godwit	choanal						
B508	03/03/21	Thames	bar-tailed godwit	cloacal		84.34		y		
B509	03/03/21	Thames	bar-tailed godwit	choanal						
B510	03/03/21	Thames	bar-tailed godwit	cloacal						
B511	03/03/21	Thames	bar-tailed godwit	choanal						
B512	03/03/21	Thames	bar-tailed godwit	cloacal						
B513	03/03/21	Thames	bar-tailed godwit	choanal						
B514	03/03/21	Thames	bar-tailed godwit	cloacal						
B515	03/03/21	Thames	bar-tailed godwit	choanal						
B516	03/03/21	Thames	bar-tailed godwit	cloacal	85.6		84.19	y		
B517	03/03/21	Thames	red knot	choanal			84.25	y		
B518	03/03/21	Thames	red knot	cloacal						
B519	03/03/21	Thames	Wrybill	choanal						
B520	03/03/21	Thames	Wrybill	choanal						
B521	03/03/21	Thames	Wrybill	cloacal						
B522	03/03/21	Thames	Wrybill	choanal						
B523	03/03/21	Thames	Wrybill	cloacal						
B524	03/03/21	Thames	Wrybill	choanal						
B525	03/03/21	Thames	Wrybill	cloacal						
B526	03/03/21	Thames	Wrybill	choanal						
B527	03/03/21	Thames	Wrybill	cloacal						
B528	03/03/21	Thames	Wrybill	choanal						
B529	03/03/21	Thames	Wrybill	cloacal						
B530	03/03/21	Thames	Wrybill	choanal						
B531	03/03/21	Thames	Wrybill	cloacal						
B532	03/03/21	Thames	Wrybill	choanal						
B533	03/03/21	Thames	south island pied oystercatcher	cloacal						

B534	03/03/21	Thames	south island pied oystercatcher	choanal						
B535	03/03/21	Thames	south island pied oystercatcher	choanal						
B536	03/03/21	Thames	south island pied oystercatcher	cloacal						
B537	03/03/21	Thames	Southern black-backed gull	choanal		85.36		y		
B538	03/03/21	Thames	Southern black-backed gull	cloacal						
B539	14/4/2021	Golden	south island pied oystercatcher	choanal						
B540	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B541	14/4/2021	Golden	south island pied oystercatcher	choanal						
B542	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B543	14/4/2021	Golden	south island pied oystercatcher	choanal						
B544	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B545	14/4/2021	Golden	south island pied oystercatcher	choanal						
B546	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B547	14/4/2021	Golden	south island pied oystercatcher	choanal						
B548	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B549	14/4/2021	Golden	south island pied oystercatcher	choanal						
B550	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B551	14/4/2021	Golden	south island pied oystercatcher	choanal						
B552	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B553	14/4/2021	Golden	south island pied oystercatcher	choanal						
B554	14/4/2021	Golden	south island pied oystercatcher	choanal						
B555	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B556	14/4/2021	Golden	south island pied oystercatcher	choanal						
B557	14/4/2021	Golden	south island pied oystercatcher	choanal	85.3	85.45	83.86	y	av *C. abortus* G1	OQ447490
B558	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B559	14/4/2021	Golden	south island pied oystercatcher	choanal						
B560	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B561	14/4/2021	Golden	south island pied oystercatcher	choanal						

B562	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B563	14/4/2021	Golden	south island pied oystercatcher	choanal						
B564	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B565	14/4/2021	Golden	south island pied oystercatcher	choanal						
B566	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B567	14/4/2021	Golden	south island pied oystercatcher	choanal						
B568	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B569	14/4/2021	Golden	south island pied oystercatcher	choanal						
B570	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B571	14/4/2021	Golden	south island pied oystercatcher	choanal						
B572	14/4/2021	Golden	south island pied oystercatcher	choanal						
B573	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B574	14/4/2021	Golden	south island pied oystercatcher	choanal						
B575	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B576	14/4/2021	Golden	south island pied oystercatcher	choanal						
B577	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B578	14/4/2021	Golden	south island pied oystercatcher	choanal						
B579	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B580	14/4/2021	Golden	south island pied oystercatcher	choanal						
B581	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B582	14/4/2021	Golden	south island pied oystercatcher	choanal						
B583	14/4/2021	Golden	south island pied oystercatcher	choanal						
B584	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B585	14/4/2021	Golden	south island pied oystercatcher	choanal						
B586	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B587	14/4/2021	Golden	south island pied oystercatcher	choanal						
B588	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B589	14/4/2021	Golden	south island pied oystercatcher	choanal						
B590	14/4/2021	Golden	south island pied oystercatcher	cloacal						

B591	14/4/2021	Golden	south island pied oystercatcher	choanal						
B592	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B593	14/4/2021	Golden	south island pied oystercatcher	choanal						
B594	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B595	14/4/2021	Golden	south island pied oystercatcher	choanal						
B596	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B597	14/4/2021	Golden	south island pied oystercatcher	choanal						
B598	14/4/2021	Golden	south island pied oystercatcher	cloacal						
B599	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B600	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B601	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B602	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B603	15/4/2021	Tasman	Variable oystercatcher	choanal						
B604	15/4/2021	Tasman	Variable oystercatcher	cloacal						
B605	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B606	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B607	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B608	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B609	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B610	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B611	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B612	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B613	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B614	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B615	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B616	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B617	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B618	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B619	15/4/2021	Tasman	south island pied oystercatcher	choanal						

B620	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B621	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B622	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B623	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B624	15/4/2021	Tasman	south island pied oystercatcher	cloacal	86.89			y		
B625	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B626	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B627	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B628	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B629	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B630	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B631	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B632	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B633	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B634	15/4/2021	Tasman	south island pied oystercatcher	cloacal						
B635	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B636	15/4/2021	Tasman	south island pied oystercatcher	cloacal			84.19	y		
B637	15/4/2021	Tasman	Variable oystercatcher	choanal						
B638	15/4/2021	Tasman	Variable oystercatcher	cloacal						
B639	15/4/2021	Tasman	south island pied oystercatcher	choanal						
B640	15/4/2021	Tasman	south island pied oystercatcher	cloacal						

Appendix 3.3

Human samples collection data and molecular test results.

ID	Date	Location	HRM1	HRM2	HRM3	qPCR	Sequencing	Bacteria ID	Genbank accession number
h01	15-Jan-21	Thames	87.61	84.85	86.14	y	n		
h02	15-Jan-21	Thames	85.39	0	86.44	y	y	av *C. abortus* G1	OQ447491
h03	15-Jan-21	Thames	0	86.71	0	y	y	av *C. abortus* G1	OQ447492
h04	15-Jan-21	Thames	0	87.85	0	y	y	av *C. abortus* G1	OQ447493
h05	15-Jan-21	Thames	0	84.49	0	y	y	av *C. abortus* G1	OQ447494
h06	15-Jan-21	Thames	86.71	83.2	87.7	y	n		
h07	15-Jan-21	Thames	0	0	0	n	n		
h08	15-Jan-21	Thames	0	0	0	n	n		
h09	15-Jan-21	Thames	84.1	0	0	y	y	av *C. abortus* G1	OQ447495
h10	15-Jan-21	Thames	86.2	0	87.49	y	y	*C. psittaci* C	OQ454509
h11	15-Jan-21	Thames	86.05	0	0	y	y	av *C. abortus* G1	OQ447496
h12	15-Jan-21	Thames	0	85.39	86.5	y	y	av *C. abortus* G1	OQ454510
h13	3-Mar-21	Thames	0	0	0	n	n		
h14	3-Mar-21	Thames	0	0	0	n	n		
h15	3-Mar-21	Thames	0	0	0	n	n		
h16	3-Mar-21	Thames	0	0	0	n	n		
h17	3-Mar-21	Thames	0	0	0	n	n		
h18	3-Mar-21	Thames	85.06	0	0	y	y	av *C. abortus* G1	OQ447497
h19	3-Mar-21	Thames	0	0	0	n	n		
h20	2-Feb-21	Manawatū	0	0	83.89	y	n		
h21	14-Apr-21	Golden bay	0	0	0	n	n		
h22	14-Apr-21	Golden bay	0	0	0	n	n		
h23	14-Apr-21	Golden bay	85.9	0	0	y	y	av *C. abortus* G1	OQ447498
h24	14-Apr-21	Golden bay	0	0	0	n	n		

h25	14-Apr-21	Golden bay	84.7	83.14	84.91	y	y	av *C. abortus* G1	OQ447499
h26	14-Apr-21	Golden bay	0	0	0	n	n		
h27	14-Apr-21	Golden bay	0	0	0	n	n		