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# Prevalence of *Chlamydophila psittaci* in Swedish aquatic birds

Maria Blomqvist

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Biology Education Centre, Uppsala University, and Department of Clinical Microbiology, Uppsala University hospital, 751 85 Uppsala
Supervisors: Björn Herrmann and Markus Klint

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# 1. Summary

The intracellular bacterium *Chlamydophila psittaci* is an infectious pathogen primarily targeting avian hosts. It can, however, be transmitted to humans via contaminated bird faeces or dried eye or nasal secretion. Little research has been dedicated to the transmission of this bacterium among wild birds and it is now becoming a topic of interest. With the increasing awareness of the role of wild birds in spreading infectious diseases across the world, the transmission of infectious agents between birds is overall a pressing subject.

The main aim of this project was to investigate the prevalence of *C. psittaci* in Swedish wild birds, mainly mallards but also other aquatic birds. Samples had been obtained from Ottenby bird station on Öland and from Hornborgasjön in Västergötland. The samples were mainly DNA from anal swabs. The prevalence of *C. psittaci* was analysed using qPCR. Following this I wanted to optimize an inexpensive and efficient method for extracting bacterial DNA from bird faeces, the Chelex® 100 method.

Based upon previous studies on birds in other countries the expected occurrence of *C. psittaci* was around 10 percent. A qPCR analysis of 741 samples from a total of 490 birds was made and the prevalence was very low. Seven birds were positive for the bacteria (1.4 %). The Chelex® 100 DNA extraction method was optimized starting with developing a protocol used when extracting DNA for human urine samples. A pathogen not found in birds, *Chlamydia trachomatis*, was added to bird faeces. DNA was extracted with the Chelex® 100 method and optimised according to the results. A total of 92 faecal samples from black-headed gulls were extracted both with MagAttract Virus Mini M48 Kit (192) and Chelex® 100. The samples were then examined for the presence of the pathogen *Escherichia coli*. The Chelex® 100 method was found to be the better extraction method and an acceptable alternative to other extraction methods for faecal samples.

# 2. Introduction

*Chlamydophila psittaci* is a bacterium that primarily infects the respiratory, pharyngeal and cloacal epithelia in birds, but it can also be transferred to humans and cause respiratory infections (Sharples and Baines, 2009). As the bacterium originally was linked to psittacine birds such as parrots and cockatoos, the infections were called parrot-fever, but are now known as ornithosis or avian chlamydiosis. Diseases spread by birds have become an area of great interest over the last years as the threat of pandemics, such as the Severe Acute Respiratory Syndrome (SARS), has become a worldwide issue. In Sweden there are few reported cases of human infection by *C. psittaci* (Table 1) However, the symptoms from the disease are very varied and can easily be mistaken for other respiratory infections so many cases may go unnoticed. However, severe disease may occur and therefore it is mandatory to report *C. psittaci* infections to the Swedish Institute for Infectious Disease Control. Understanding the spread of infectious diseases in wild avian hosts is important regardless of the severity of the disease.

**Table 1. Reported cases of human infection with *Chlamydophila psittaci* in Sweden from 1998-2009.** Adapted from Swedish Institute for Infectious Disease Control. **(Smittskyddsinstitutet, 2009)**

| | Number of cases reported anually | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **County** | **1998** | **1999** | **2000** | **2001** | **2002** | **2003** | **2004** | **2005** | **2006** | **2007** | **2008** | **2009** |
| **Blekinge** | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| **Dalarna** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Gotland** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Gävleborg** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Halland** | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| **Jämtland** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Jönköping** | 2 | 1 | 3 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| **Kalmar** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Kronoberg** | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **Norrbotten** | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Skåne** | 7 | 5 | 4 | 2 | 1 | 1 | 3 | 1 | 1 | 6 | 5 | 3 |
| **Stockholm** | 0 | 2 | 0 | 0 | 1 | 3 | 0 | 0 | 0 | 1 | 0 | 1 |
| **Södermanland** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Uppsala** | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Värmland** | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Västerbotten** | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| **Västernorrland** | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| **Västmanland** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Västra Götaland** | 8 | 11 | 10 | 5 | 5 | 6 | 0 | 0 | 0 | 1 | 4 | 0 |
| **Örebro** | 4 | 4 | 1 | 0 | 1 | 1 | 0 | 2 | 1 | 0 | 1 | 2 |
| **Östergötland** | 2 | 1 | 1 | 1 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **Totalt** | **30** | **29** | **24** | **12** | **13** | **12** | **7** | **5** | **2** | **9** | **11** | **8** |

Because the infection often is linked to domesticated birds there has not been much research on the spread of the bacteria in wild avian hosts. Recently more studies on wild birds have been surfacing from all over the world, but not from Sweden (Schwarzova *et al.*, 2006; Pennycott *et al.*, 2009).

## 2.1 History

In 1880 Dr. J. Ritter identified a disease giving symptoms similar to pneumonia and found it to originate from parrots and finches (Harris and Williams, 1985). In 1893 a disease giving flu-like symptoms was reported to have been transmitted from parrots to humans in Paris. The infectious disease was named psittacosis as it appeared to be linked to psittacine birds such as parrots (Morange, 1895). It was not until the 20$^{th}$ century that *Chlamydia* started to be seriously investigated. The first drawings of cells with *Chlamydia* infection *(Chlamydia trachomatis)* were shown in 1907 by Halberstaedter and von Prowazek. They thought it was a protozoal infection as they saw vacuoles that they mistook for mantled organisms and named them *Chlamydozoaceae* (Halberstaedter and Prowazek, 1907).

In 1929 and 1930 there were pandemic outbreaks of psittacosis in Europe and North America that were traced to the import of infected Amazon parrots from Argentina. In 1930 the causative agent of psittacosis was isolated and classified as a virus of the lymphogranuloma (LGV) type by Bedson *et al*. (1930). Until then psittacosis was only thought to be transmitted from psittacine birds. In 1932 there was a report that the disease had been transmitted from infected chicken to humans (Vanrompay *et al.*, 1995). Soon after that reports of 174 cases of human psittacosis came from the Faeroe Islands where there was a high death rate, especially among pregnant women (Rasmussen-Ejde, 1938; Herrmann *et al.*, 2006). The disease was acquired when fulmars were caught and prepared for cooking. The agent was, however, still believed to be a virus. With the development of electron microscopy in the 1960s it became clear that the psittacosis agent was not a virus. The agent was found to divide by binary fission and to have cell walls and was finally classified as gram-negative intracellular bacterium. In 1966 it was suggested that all organisms of the psittacosis-LGV-trachoma group should be combined into the genus *Chlamydia* belonging to the order *Chlamydiales* (Moulder, 1966). A reclassification of the genus has later been necessary.

## 2.2 The order *Chlamydiales*

*C. psittaci* belongs to the order *Chlamydiales* and to the family *Chlamydiaceae* (Figure 1). The *Chlamydiaceae* is a family of obligate intracellular gram-negative bacteria that give infections in both humans and animals. First the genus *Chlamydia* was only thought to include two species, *Chlamydia trachomatis* and *C. psittaci*. Later the genus was branched into four species with the addition of *Chlamydia pneumoniae* and *Chlamydia pecorum.* In 1999 a taxonomic reclassification of the *Chlamydiales* was made (Everett *et al.*, 1999) and the family *Clamydiaceae* was suggested to consist of two genera, *Chlamydia* and *Chlamydophila.*

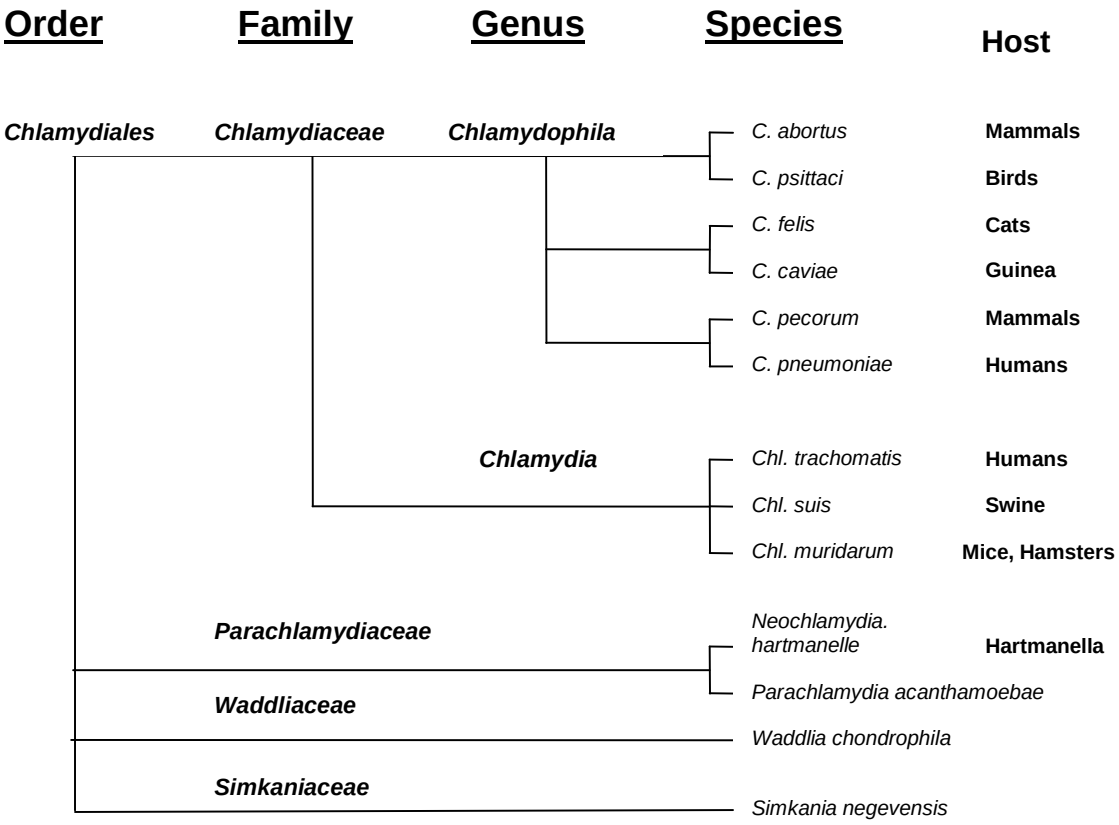


**Figure 1. Taxonomy over the order *Chlamydiales*** (Adapted from Bush and Everett (2001))

*C. psittaci* has six known avian serovars (A-F) that are distinguished by monoclonal antibodies (mAbs). The mAbs recognize specific epitopes on the major outer membrane protein (MOMP) of the bacteria. The serovars correspond to six genotypes that are relatively host-specific. A seventh genotype was identified in 2005, genotype E/B (Vanrompay *et al.*, 1993; Geens *et al.*, 2005; Harkinezhad *et al.*, 2007). Genotype A is endemic among psittacine birds, genotype B is common among pigeons and has also been found in turkeys, genotype C has primarily been isolated from ducks and geese but has been found in different types of birds, for example, swans and turkeys. Genotype D is mainly found in turkeys, genotype E is the most diverse and is frequent in pigeons, ducks, and turkeys and has occasionally been isolated from humans. Genotype F has been identified in psittacine birds and turkeys. Genotype E/B has been found in turkeys, pigeons and ducks (Harkinezhad *et al.*, 2009).This genotype reacts with monoclonal antibodies (mABs) from both serovar E and serovar B but differs from both genotypes by a difference in the major outer membrane protein.

## 2.3 Developmental cycle

*Chlamydiaceae* bacteria, including *C. psittaci*, have an interesting developmental cycle with two distinct cell forms. The bacteria have an extracellular form called elementary body (EB) that is approximately 0.2 to 0.3 µm in diameter and is the infectious agent. It also has a larger (0.6 to 0.8 µm in diameter) intracellular form, the reticulate body (RB), which is the replicating form (Costerton *et al.*, 1976). *C. psittaci* infection occurs when the EB attaches to the host cell and then enters (Figure 2). Inside the cell the bacterium undergoes a biphasic developmental cycle within an inclusion, a non-acidified vacuole. The inclusion allows the bacterium to avoid fusion with lysosomes and other organelles. The tightly packed, spherical EB undergoes nucleoid decondensation and chromatin dispersal as the bacterium becomes transcriptionally active. Within a few hours after entering the cell, the EB expands and differentiates into an RB. Approximately 8 hours after infection the RB starts replicating by binary fission. Replication takes place near the inclusion membrane as the bacterium does not have free access to cytosolic nutrients, and must import them across the inclusion membrane. When the inclusion becomes overcrowded with reticulate bodies the bacteria start to differentiate back to EBs, and approximately 50 hours after infection the inclusion is lysed along with the host cell, or the EBs are released to the surroundings by reverse endocytosis. Sometimes persistent growth is favoured instead of leaving the cell, depending on the current environment. In that case, the bacteria do not complete the cycle and stay as non-replicating persistent bodies instead of differentiating to EBs. This may lead to chronic infection in the host but may not give any symptoms at all (Vanrompay *et al.*, 1995; Harkinezhad *et al.*, 2009).

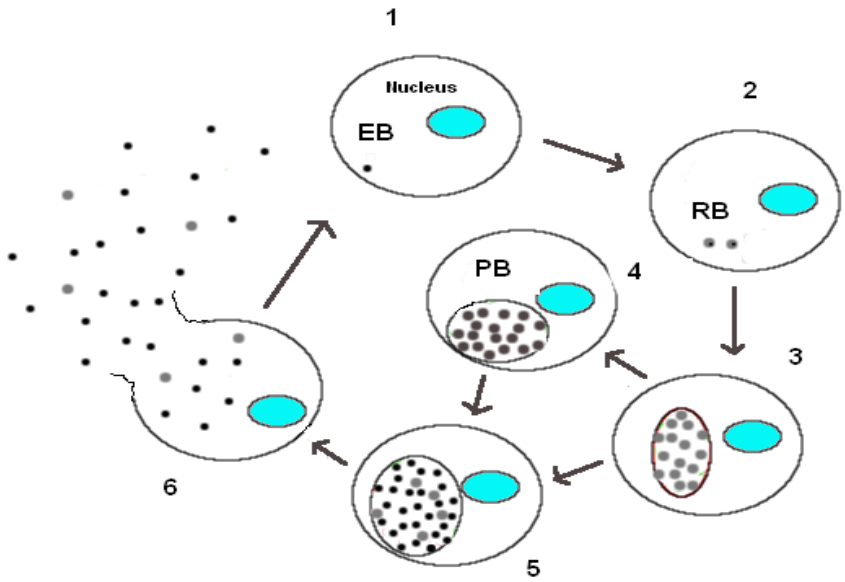


**Figure 2**. **Schematic view of the *Chlamydiales* development cycle.** Infectious elementary bodies (EBs) attach and enter the cell in vesicles (1). Inside the cell the EBs transform into reticulate bodies (RBs) (2) and start to replicate inside an inclusion (3). If the conditions are right the RBs will transform to EBs (5), lyse the cell and induce another cycle of infection (6). If the conditions are not favourable the RBs may transform to non-replicating persistent bodies (PBs) that can redifferentiate back to EBs when the conditions are right (4). Adapted from Vanrompay *et al.*, (1995)

## 2.4 Transmission and clinical disease

### 2.4.1 Avian hosts

*C. psittaci* as mentioned earlier is an intracellular, gram-negative bacterium that is mainly spread among birds in close contact. The bacteria spread through inhalation of contaminated aerosols from urine, contact with secretion from eyes or exhalation, or contact with faeces. Shedding of the bacteria through faeces occurs occasionally and can continue for several months, the bacteria can survive in faeces for up to 30 days (Harkinezhad *et al.*, 2009). Shedding can be activated by environmental stress such as malnutrition, overcrowding, transport or egg laying. The infection can also be transmitted through contaminated water so that poultry that live in moist soil habitats are at risk of being infected from wild aquatic birds. There can also be transmission between birds via an insect vector such as lice, ticks or flies (Harkinezhad *et al*., 2009). Symptoms and disease following infection of *C. psittaci* vary depending on the bacterial strain and avian host. General symptoms in birds are fever, anorexia, fatigue and diarrhoea. Infection can cause various diseases such as pneumonia, pericarditis, hepatitis and peritonitis (Andersen, 1996). Chronically infected birds often show no clinical symptoms until stressed in some way. The bacteria have been found in high frequency in the respiratory tract of infected birds; they can also be found in the lateral nasal glands and in the plumage (Longbottom and Coulter, 2003).

### 2.4.2 Humans

Humans in close contact with birds, for example poultry farmers, pet shop owners and pet owners with psittacine birds, are naturally the greatest risk for infection (Tsiodras *et al.*, 2008). Infection often occurs from inhalation of dried faeces that has become dust and contaminated aerosols. Thus, people who are not necessarily in close contact with birds can be infected when, for example, mowing the lawn. The infection usually will result in respiratory diseases in humans, but the symptoms can be very variable. Many develop headache, fatigue and fever (Beeckman and Vanrompay, 2009). Since the pathogen most often gives a respiratory infection and is rather rare in humans, it is often misdiagnosed and can lead to more severe consequences than necessary if it is not discovered in time.

## 2.5 Epidemiology

Psittacosis is a widespread disease and can be found all over the world (Table 2.), although probably many cases go unnoticed. There have been numerous studies on *C. psittaci* among domesticated birds as they present a more direct risk for spreading to humans who come in contact with infected birds. Also research in that area has been more extensive due to the economic risks involved with the infection of poultry. Investigations into the prevalence and frequency of the bacteria in wild birds have not been a priority, and until recent years there have been quite few studies performed across the globe.

**Table 2. Reported human cases of psittacosis from different countries** (Adapted from Harkinezhad et al., 2008).

| | Number of reported cases annually | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Country or territory** | **1997** | **1998** | **1999** | **2000** | **2001** | **2002** | **2003** | **2004** | **2005** | **2006** | **2007** |
| Austria | | | 1 | 2 | 3 | 0 | 3 | 6 | | | |
| Argentina | | | | 38 | 1 | | | | | | |
| Australia | 35 | 55 | 81 | 99 | 137 | 213 | 200 | 239 | 164 | 171 | 62 |
| Belgium | 0 | 8 | 12 | 13 | 10 | 23 | 39 | 12 | 7 | 2 | 3 |
| Croatia | 4 | 0 | 5 | 8 | 3 | | 5 | 4 | | | |
| Czech Republic | 5 | 6 | | | 0 | 3 | 0 | 0 | | | |
| Denmark | | | | 31 | 8 | 13 | 14 | 8 | 22 | 7 | 11 |
| Finland | 1 | 2 | 0 | 0 | 0 | | | 0 | | | |
| Germany | 124 | 155 | 109 | 86 | 53 | 40 | 42 | 15 | 33 | 26 | 12 |
| Hungary | 0 | 0 | 1 | 5 | 1 | 6 | 85 | 7 | 140 | 29 | 28 |
| Japan | | | 23 | 18 | 35 | 54 | 44 | 40 | 34 | | |
| Poland | 2 | 0 | 2 | 0 | 5 | | 2 | 2 | | | |
| Slovakia | 0 | 1 | 3 | 10 | 0 | 0 | 1 | 0 | | | |
| Spain | | | | | 5 | 4 | 0 | 1 | | | |
| Sweden | 66 | 30 | 29 | 24 | 12 | 13 | 12 | 7 | 5 | 2 | 9 |
| The Netherlands | 28 | | 25 | 36 | 24 | 17 | 27 | 33 | 49 | 59 | 27 |
| Great Britain | 322 | 293 | 207 | 204 | 106 | 68 | 100 | 62 | 59 | | |
| Northern Ireland | 37 | 0 | 0 | | 44 | 16 | 15 | 13 | | | |
| Ukraine | 2 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | | | |
| USA. | 38 | 54 | 15 | 13 | | 19 | 13 | 11 | | | |

Reports of *C. psittaci* investigations of migrating avian hosts have become more frequent over the last couple of years from all over the world. A study to detect antibodies against *C. psittaci* was undertaken in the Philippines in 2007, where 25 % of the 36 wild birds investigated showed antibodies against the bacteria (Maluping *et al.*, 2007). A recent study of 43 wild birds in the United Kingdom showed *C. psittaci* prevalence in 11.6 % of the birds, all of them pigeons (Sharples and Baines, 2009). Migrating birds are excellent vectors for pathogens as they travel over vast areas and allow the pathogen to spread throughout the world. Investigating the spread of infectious diseases amongst migrating birds is an important and a pressing issue with the current threat of pandemic outbreaks around the world. A better understanding of how migrating birds travel and spread infections can help in predicting the spread of future diseases.

## 2.6 Identifying bacteria with real-time polymerase chain reaction

*C. psittaci* can be identified by different methods such as microarray-based genotyping (Sachse *et al.*, 2009) but the most frequently used and favourable method is real-time polymerase chain reaction (qPCR). The method can estimate the number of bacteria in the sample. The primers and probe used target 168 base pairs of the 23S rRNA ribosomal subunit (DeGraves *et al.*, 2003). One possibility is to use a fluorescence resonance energy transfer probe (FRET) called “adjacent probe”. It consists of two single-stranded hybridization probes where one probe has a donor

fluorophore at the 3' end and the other probe has an acceptor fluorophore at the 5' end. When the probes are not attached in close vicinity of each other no energy transfer can occur and no signal is detected (Marras, 2006). When the two probes are attached a light signal is admitted and the light signal is registered. The cycle at which the fluorescence from a sample crosses the set threshold value is called the cycle threshold, CT. Thus, a lower CT value indicates a stronger infection with a higher amount of bacteria. Another possibility is to use a minor groove binding (MGB) probe that has a hybridization-triggered fluorescent mechanism. The probe has a fluorescent label and a quencher, when the probe binds to a target sequence the probe unfolds, moving the quencher away from the fluorescent label allowing it to admit a signal (Morre *et al.*, 2005).

## 2.7 Aims

The aim of this project was to investigate the occurrence of *C. psittaci* in wild birds in Sweden and, given high occurrence, analyze what type of *C. psittaci* it was.

The study was a follow up of a previous study on fulmars from the Faeroe Islands where prevalence of *C. psittaci* was ten percent (Herrmann *et al.*, 2006). This project was to be done by analyzing anal swab samples and faeces taken from wild birds at Ottenby and Hornborgasjön in Sweden using qPCR. The global aspect of this project was to contribute to the worldwide mapping of infectious diseases spread by animals, typically avian hosts.

In addition to this I wanted to find and optimize an inexpensive and efficient way of extracting bacterial DNA from bird faeces.

# 3. Results

## 3.1 Detecting *Chlamydophila psittaci*

The birds investigated in this study were mainly mallards; these types of birds do not migrate over very large distances. However, the samples were collected from two locations that are regular resting places for migrating birds and there were also other migrating birds, mainly aquatic birds, involved in the study. Anal swabs from wild birds were collected from two different bird stations in Sweden and prepared for DNA detection with MagAttract Virus Mini M48 Kit (192) from Quiagen. The occurrence of *C. psittaci* DNA was estimated by qPCR probes targeting 168 base pairs of the 23S rRNA ribosomal subunit (DeGraves *et al.*, 2003). To get an idea of the sensitivity of the qPCR method, an initial test of two *C. psittaci* strains was performed (Figure 3). The strain 6BC had an initial concentration of $10^6$ DNA copies per µl (1: 6BC) giving the CT value 5.56, and was then diluted in steps of 10 until $10^3$ copies/ µl (4: 6BC) giving a CT value of 22.59 (Table 3). The strain DC5 had the concentrations 500 copies per µl (5: CpsDC5) with the CT value 8.15 and 50 copies per µl (6: CpsDC5) with the CT value 13.02 (Table 3) Due to the large differences in CT values between the two strains, the number of bacterial DNA copies in the positive samples was not estimated. The positive control used in the following experiments *C. psittaci* 6BC sample ($10^5$ copies/µl) gave a CT value of 10.51.

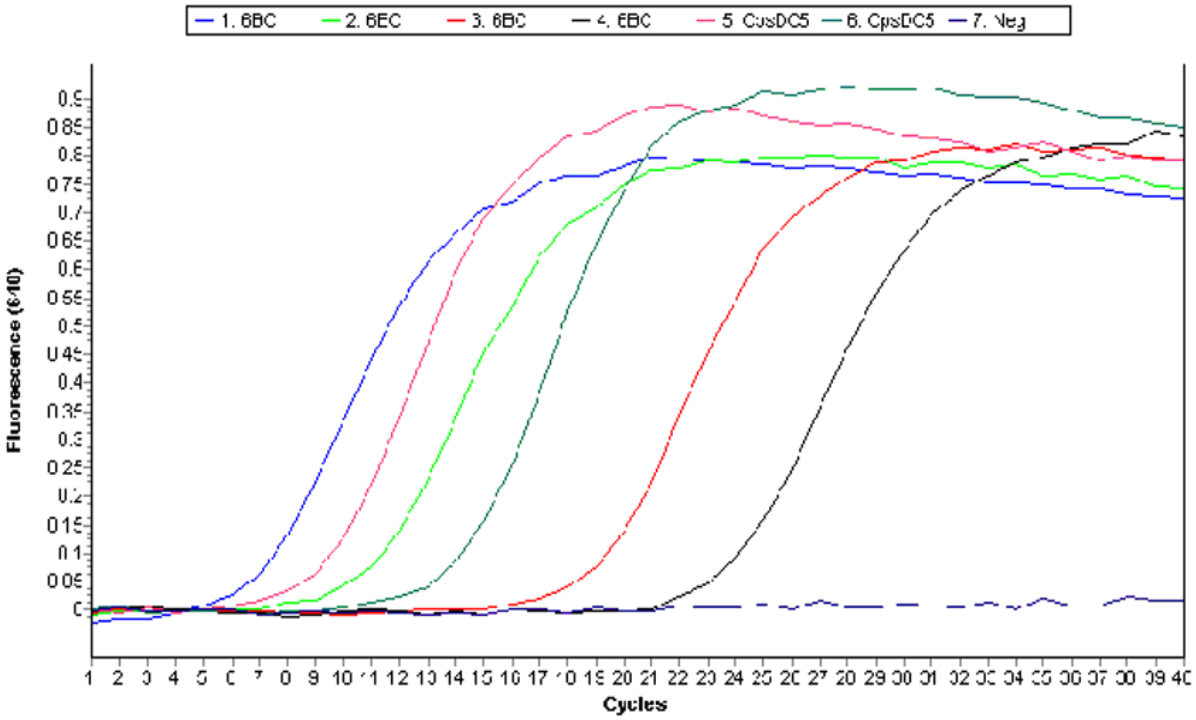


**Figure 3. Initial real-time Polymerase Chain Reaction test of *Chlamydophila psittaci* strains 6BC and DC5.** The strain 6BC had an initial concentration of $10^6$ DNA copies/ µl (1: 6BC) and was then diluted tenfold, 2:6BC represents $10^5$ copies/ µl, 3:6BC represents $10^4$ copies/µl, until $10^3$ copies per µl (4: 6BC). The strain DC5 had the concentrations 500 copies/µl (5: CpsDC5) and 50 copies/µl (6: CpsDC5). Neg represents the negative control. The instrument used was a Roche LightCycler® 2.0.

**Table 3. Cycle threshold values from initial test of *Chlamydophila psittaci* strains 6BC and DC5.**

| ***C. psittaci* strain** | **DNA conc. Copies/µl** | **CT value** |
|---|---|---|
| 6BC | $10^6$ | 5.56 |
| 6BC | $10^5$ | 9.79 |
| 6BC | $10^4$ | 17.88 |
| 6BC | $10^3$ | 22.59 |
| DC5 | 500 | 8.15 |
| DC5 | 50 | 13.02 |

A total of 741 samples were tested. These samples were collected from 490 birds as many samples were from the same bird at different points in time. Samples were also taken from mallards that were used to attract wild birds to the enclosure where they were captured; these are referred to as "lockand" (Table 4). Of the 741 samples seven were found positive for *C. psittaci* by giving a curve in the qPCR.

**Table 4. Samples tested for the presence of *Chlamydophila psittaci* according to species.**

| **Bird Species** | **Samples** | **Birds** | **Samples positive for *Chlamydophila psittaci*** |
|---|---|---|---|
| Mallard *(Anas platyrhynchos)* | 588 | 350 | 4 |
| "Lockand" *(*Mallard*, Anas platyrhynchos)* | 26 | 15 | 1 |
| Eurasian Wigeon (*Anas penelope)* | 7 | 5 | |
| Northern Pintail (*Anas acuta)* | 1 | 1 | |
| Black-headed Gull (*Chroicocephalus ridibundus*) | 3 | 3 | |
| Common Tern (*Sterna hirundo*) | 56 | 56 | 1 |
| Little Tern (*Sternula albifrons*) | 15 | 15 | |
| Black Tern (*Chlidonias niger)* | 1 | 1 | |
| Caspian Gull *(Larus argentatus cachinnans)* | 2 | 2 | |
| Great Black-backed Gull (*Larus marinus)* | 2 | 2 | |
| Herring Gull (*Larus argentatus)* | 4 | 4 | 1 |
| Greylag Goose (*Anser anser*) | 1 | 1 | |
| Barnacle Goose (*Branta leucopsis*) | 2 | 2 | |
| Common Eider (*Somateria mollissima)* | 4 | 4 | |
| Buff-breasted Sandpiper (*Tryngites subruficollis)* | 1 | 1 | |
| Jack Snipe (*Lymnocryptes minimus)* | 1 | 1 | |
| Grey Heron (*Ardea cinerea*) | 1 | 1 | |
| Tawny Owl (*Strix aluco*) | 3 | 3 | |
| Eurasian Teal (*Anas crecca*) | 8 | 8 | |
| Dunlin (*Calidris alpina)* | 8 | 8 | |
| Wood Sandpiper (*Tringa glareola)* | 4 | 4 | |
| Ruddy Turnstone (*Arenaria interpres*) | 3 | 3 | |
| **Total** | **741** | **490** | **7** |

Many samples were from the same bird at different time points. The positive samples were never from the same bird. An example of a qPCR reaction where a sample was found to be positive can be seen in Figure 4.

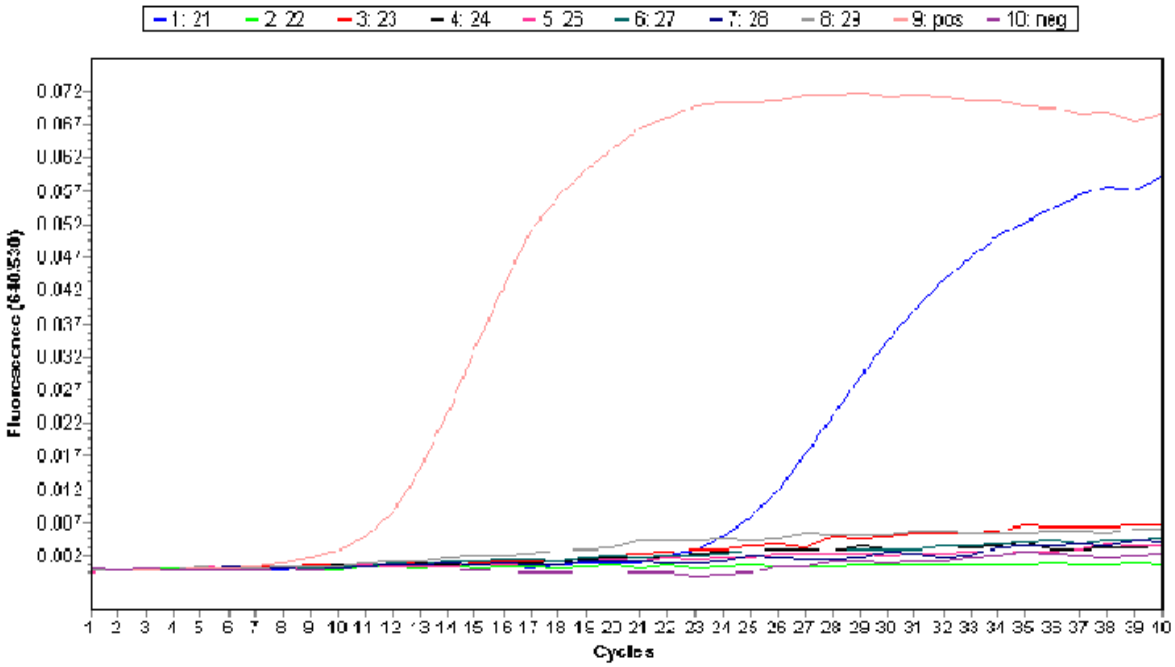


**Figure 4. Real-time Polymerase Chain Reaction identifying *Chlamydophila psittaci* in Swedish wild birds.** The samples were pooled with four samples from mallards in each pool. Sample 9. "pos" represents the positive 6BC DNA $10^5$ copies/µl. Sample 1: 21 showed a cycle of threshold value of 23.33. The instrument used was a Roche LightCycler® 2.0.

The CT values of the positive samples varied in strength (Table 5).

**Table 5. Cycle threshold values in samples from Swedish wild birds found positive for *Chlamydophila psittaci*.**

| Species | CT value |
|---|---|
| Common tern | 22.64 |
| Mallard | 21.22 |
| Mallard | 20.85 |
| Herring gull | 20.00 |
| Mallard | ND |
| Mallard | 24.23 |
| Mallard | 19.16 |

The not determined (ND) CT value for one sample was caused by a complication with the LightCycler® 2.0 instrument.

## 3.2 Optimising Chelex® 100

I was to test the efficiency of the Chelex® 100 DNA extraction method on bird faeces. This method of DNA extraction is cheap and easily performed. Chelex® 100 consists of beads that are mixed with buffer to a slurry solution. The resin consists of styrene-divinylbenzene copolymers containing paired iminodiacetate ions. The resin beads bind polar cell components after lysing the cells and sink to the bottom of the solution whereas non-polar nuclear DNA and RNA are left in the clear solution. Hopefully this method of DNA extraction will prove to be adequate and can be an acceptable alternative to the more expensive methods currently in use to extract bacterial DNA from bird faeces.

The sensitivity of the Chelex® 100 method was examined by adding a pathogen that is not found in birds (*Chlamydia trachomatis* serotype D, ATCC885) to the faecal samples before DNA extraction with Chelex® 100. The extracted DNA was tested for *Chl. trachomatis* by performing a qPCR targeting the bacterium polymorphic outer membrane protein. The exact concentration of bacteria was not known, it yielded a CT value of approximately 30 when diluted $10^3$-fold (Appendix 1). The bacteria were added in different concentrations to the samples as to determine the sensitivity of the method.

The initial faeces test with the Chelex® 100 method was performed with a protocol that was used for urine samples. The threshold value was set to the value 0.1 which is a standard value used for qPCR. When the samples had been prepared and then run through the qPCR there was a significant difference in CT values between the positive control without faeces (CT value: 25.25) and the faecal sample (CT value 28.98) Therefore a washing step with acetone was added to the protocol according to a protocol used on chicken faeces (Yang *et al.*, 2008). The washing considerably improved the CT values and the washed samples showed almost identical, or higher, CT values to that of the positive control samples. Figure 5 shows the initial test after the washing step had been added where only two dilutions were made.

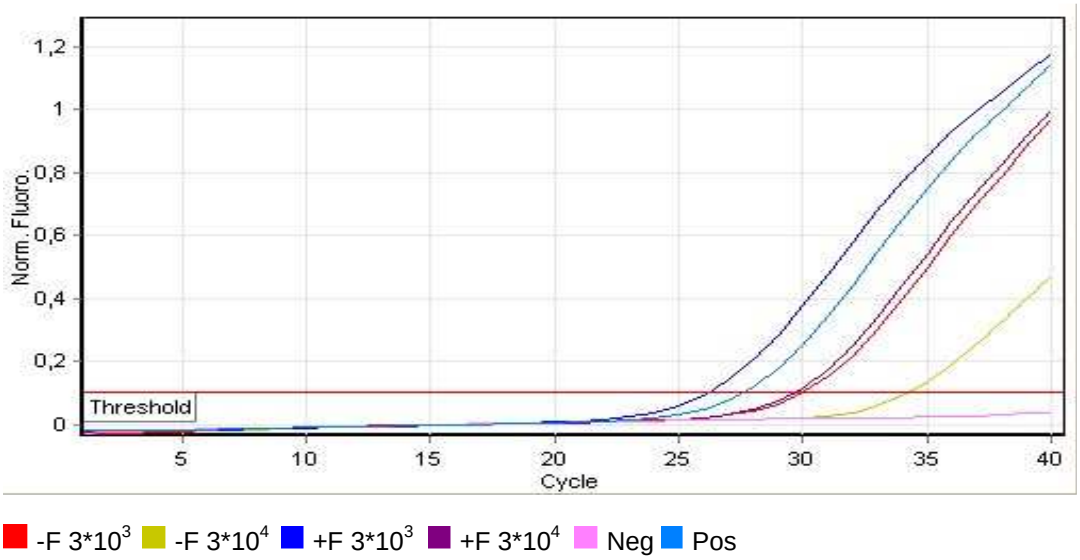


**Figure 5. Real-time Polymerase Chain Reaction results for detecting *Chlamydia trachomatis* in bird faeces.** DNA extraction using Chelex® 100 with acetone washing was done on four samples with added *Chlamydia trachomatis* in the dilutions $10^3$-fold and $10^4$-fold and analysed using qPCR. Two samples were processed without faeces and two processed in bird faeces. The samples processed without bird faeces (–F) had the cycle threshold (CT) values 29.97 (-F 3*$10^3$) and 34.28 (-F 3*$10^4$), whereas the samples processed with bird faeces (+F) had the CT values 26.18 (+F 3*$10^3$) and 29.66 (+F 3*$10^4$). The positive control (Pos) had the CT value 27.59. The instrument used was a Rotor Gene (RG-300) Corbett Research.

Different concentrations of *Chl. trachomatis* bacteria were tested with a starting $10^3$-fold dilution. The exact bacterial concentration was not known. The bacterial suspension was then diluted in steps of ten up to a dilution of $10^7$-fold. The test was repeated several times with similar results. The results showed that the method could be used to prepare the samples for detection of bacterial

DNA, but mainly at higher concentrations since dilutions of $10^6$ and $10^7$ were often not detected at all (Table 6).

**Table 6. Real-time PCR cycle of threshold values when detecting *Chlamydia trachomatis* in faecal samples from birds.**

| Test nr. | Dilutions + F | | | | | Dilutions -F | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $10^3$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| **1** | 26.2 | 29.7 | - | - | - | 30.0 | 34.3 | - | - | - |
| **2** | 25.1 | 30.3 | 35.3 | - | - | 29.0 | 35.0 | 36.0 | - | - |
| **3** | 25.3 | 30.3 | - | - | - | 29.2 | 34.4 | - | - | - |
| **4** | 30.3 | 29.7 | 32.2 | 35.7 | - | 31.4 | 35.4 | - | - | - |
| **Mean** | **26.7** | **30.0** | **33.8** | **35.7** | **-** | **29.9** | **34.8** | **36.0** | **-** | **-** |

All samples shown in table 5 were washed with acetone and DNA extracted with Chelex® 100. The samples called +F represent samples that were processed in bird faeces and -F represent samples that were processed without bird faeces.

### 3.2.1 *C. psittaci* detection from Chelex® 100 prepared samples

One original faecal sample that had tested positive for *C. psittaci* when prepared with MagAttact Virus Mini M48 Kit (192) from Quiagen and 50 original samples that had tested negative were prepared with Chelex® 100. The positive sample was a pooled sample consisting of four original samples so I expected one of the four to be positive. However none tested positive in the qPCR.

The positive sample that had been prepared with the MagAttract Virus Mini M48 Kit (192) was tested again in qPCR and this time tested negative. Due to the confusing results the Chelex® 100 method needed to be tested more thoroughly. Therefore 92 original faecal samples from black-headed gulls were obtained from Kalmar University and prepared with the Chelex® 100 method. The same original samples also had been prepared with the MagAttract Virus Mini M48 Kit (192). The objective was to compare the two methods by analyzing them for a bacterium that is common in bird's digestive system. The gram-negative bacterium *Escherichia coli* was targeted for further analysis by PCR (Tantawiwat *et al.*, 2005) and gel electrophoresis. The Chelex® 100 method identified twelve (13.0 %) samples as positive for *E. coli*, whereas the Quiagen RNA prep kit was able to identify seven (7.6 %) positive samples. This allows a comparison between the two methods where the Chelex® 100 method was found to be the better method.

# 4. Discussion

## 4.1 *Chlamydophila psittaci* in wild birds from Sweden

*C. psittaci* has been found to occur frequently in domestic birds across the world. Recently reports have been appearing of *C. psittaci* found in wild birds, usually not with as high occurrence as in domestic birds but still with noticeable frequency (Schwarzova *et al.*, 2006; Magnino *et al.*, 2009; Pennycott *et al.*, 2009). In this project I wanted to study the occurrence of the bacterium in wild birds in Sweden, mainly mallards. However after testing a large number of samples it was clear that this bacterium is not a frequent pathogen in Swedish mallards. Only seven birds tested positive for *C. psittaci* of a total of 490 birds. The CP values of the positive samples appeared to be quite high and ranging between 19.16 and 22.64. The two reference strains used (6BC and DC5) differed strongly in CT values. The 6BC strain started with $10^6$ copies/µl and had a CT value at 5.56 whereas the DC5 strain started at a concentration of 500 copies/µl giving the CT value 8.15. The 6BC strain had a lower CT at $10^5$ copies/µl than the DC5 strain had at 50 copies/µl. The difference between the two strains is probably due to differences in the initial DNA preparation, but these were the two reference strains available to me. Since I do not know what strain is present in the positive samples from my experiments I was unable to use either reference strain to estimate the actual amount of bacterial DNA.

One mallard tested negative for *C. psittaci* in one sample and then tested positive in a sample taken three days later, with a CT value of 19.16. Since *C. psittaci* is an intracellular bacterium that often persists in a latent form, this would suggest that the bacterium is hard to detect when not shedding. The bacteria shed intermittently and the bird may not even have shown any symptoms at the time of shedding.

Of 365 mallards five tested positive for *C. psittaci*. The other two positive samples were from seafaring birds. As there were much fewer samples tested from these birds it is hard to compare between the species. It is likely that the frequency of infection is higher among aquatic birds, the bacteria can be transmitted via contaminated water and the gulls travel over larger areas than the mallards and there is a difference in diet between the birds. However, Ottenby and Hornborgasjön are common places for migrating birds to pass through and even local birds would be infected if the disease was frequent in the birds travelling farther. This is further illustrated by the fact that a mallard used to attract other birds to the compound was infected. As mentioned, the frequency of the bacteria in different wild birds from other countries has been reported to be around ten percent (Herrmann *et al.*, 2006; Sharples and Baines, 2009). The results from this project suggest that *Chlamydophila psittaci* have lower frequencies in wild avian hosts in Sweden than many other countries but more extensive studies would be preferable.

## 4.2 DNA extraction with Chelex® 100

The Chelex® 100 method is inexpensive and quite simple to perform but had not been evaluated in extracting DNA from faecal samples. During the investigation performed with *Chl. trachomatis* the results seemed very promising, and especially interesting as the samples that contained faeces gave a lower CT value than those without. Yet when an original sample thought to be infected with *C. psittaci* was purified with the Chelex® 100 method the sample tested negative for *C. psittaci* in the qPCR. These results were contradictory, as qPCR is a sensitive method and should be able to detect even low concentrations of bacterial DNA. When the positive sample that had been prepared with the MagAttract Virus Mini M48 Kit (192) from Quiagen was once again screened in the qPCR it now tested negative for *C. psittaci*. Whether the first test was a false positive result from the qPCR or if something went wrong with the Chelex® 100 preparation is unclear. The thawing of samples can be harmful to the bacterial DNA and I did not want to perform too many trials on original samples before the Chelex® 100 method was more thoroughly examined.

Instead a PCR was performed to detect a bacterium quite commonly found in bird's digestive system, *Escherichia coli*. Here the results gave a reasonable comparison between the two methods. The Chelex® 100 method identified twelve (13.0 %) samples as positive for *E. coli*, whereas the MagAttract Virus Mini M48 Kit (192) was able to identify seven (7.6 %) positive samples. These results lead us to believe that the Chelex® 100 method is an acceptable option to more expensive and complicated methods for DNA extraction.

However, the MagAttract Virus Mini M48 Kit (192) has also been questioned as a valid method of bacterial DNA extraction. When my experiments were performed there was little doubt as to the accuracy of the method according to information given by Quiagen Sample and Assay Technology. Illustrated by the results from the comparison between the Chelex® 100 method and the MagAttract Virus Mini M48 Kit (192), the later may be put to question as to efficiency and accuracy. It is, of course, difficult to find a method that is totally efficient for extracting chlamydial DNA as the amount of bacteria in faecal samples is usually quite low.

## 4.3 Future aspects

To be able to acquire a global perspective concerning the spread of diseases amongst wild avian hosts as much knowledge as possible is needed. Mapping the spread of *C. psittaci* all around the world is also important for economic purposes, such as minimizing the risk of the disease in poultry farming. The results from the tests on wild birds from Sweden are interesting in a global perspective and can help with mapping the spread of infections. Hopefully this project and future investigations will contribute to an increased interest in the spread of *C. psittaci*, both in Sweden and around the globe. The Chelex® 100 method has been shown to be as satisfactory as other DNA extraction methods on faecal samples, giving an inexpensive alternative extraction method. In the near future the project will be expanded to examine wild birds from other areas around the globe. Currently there are samples recently collected from penguins in southern Chile and from seagulls in Alaska that are waiting to be investigated.

# 5. Materials and Methods

## 5.1 Faecal material

Samples in the form of anal swabs were collected from wild birds, mainly mallards, at Ottenby bird station on Öland, Sweden and from Hornborgarsjön in Västergötland, Sweden. They were prepared for DNA -and RNA detection before the project started with MagAttract Virus Mini M48 Kit (192) (Quiagen Sample and Assay Technology) according to protocol given by the manufacturer. The *C. psittaci* strains 6BC and DC5 were used to evaluate the qPCR detection from the samples. These strains were donated by Dr. Konrad Sachse at Friedrich-Loeffler-Institut, Germany.

## 5.2 Bacterial detection

### 5.2.1 Detecting *C. psittaci*

Two different types of real-time instruments were used when detecting *C. psittaci* and *Chl. trachomatis.* The real-time-PCR cycling to detect *C. psittaci* was performed on a Roche LightCycler® 2.0 using FRET probes (LightCycler® hybridization probes). The qPCR reaction contained 5 µl of purified *C. psittaci* DNA in a total volume of 20 µl. This included 1 µM of each primer and 1 µM of each probe (Table 7), FastStartHybProbe from Roche containing FastStart Taq DNA Polymerase, reaction buffer dNTP mix (with dUTP instead of dTTP), and 10 mM $MgCl_2$, and additional 4.5 mM $MgCl_2$. The cycling program is shown in Table 8.

### 5.2.2 Detecting *Chl. trachomatis*

The qPCR to detect *Chl. trachomatis* was performed in a Rotor Gene (RG-3000) Corbett Research. Initial denaturation at 95°C for 15 min was followed by 50 cycles of denaturating at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72° C for 30 s. The PCR reaction included 5 µl purified DNA of a total volume at 25 µl. The reaction mix contained buffer 10x (final concentration 1x) from Roche, 0.2 mM dNTP, 3.5 mM magnesium chloride, 0.75 U HotStar *Taq* DNA polymerase (Qiagen, Hilden, Germany), 0.6 mM of each primer and 0.2 mM probe (Table 7)

### 5.2.3 Detecting *E. coli* in faecal samples

The regular PCR for detecting *Escherichia* coli was adapted from Tantawiwat *et al*., 2005. Initial denaturation at 95° C for 15 minutes was followed by 30 cycles of 95° C for 30 seconds, annealing temperature 55° C for 30 seconds and elongation at 72° C for 30 seconds and then 72° C for 5 minutes. The PCR reaction included 5 µl purified DNA in a total volume of 25 µl including also: buffer 10x (final concentration 1x) from Roche, 0.2 mM dNTP, 0.5 mM magnesium chloride, 1 U HotStar *Taq* DNA polymerase (Qiagen, Hilden, Germany), and 0.6 mM of each primer (Table 7)

**Table 7. Primers and probes used to detect bacterial DNA.**

| Target species | Primer/Probe | Sequence (5'→3') | Target gene |
|---|---|---|---|
| *C. psittaci* | Forward primer CHL23SUP | GGGGTTGTAGGGTYGAGRAIAWRRGATC | 23S rRNA 168 bp |
| *C. psittaci* | Reverse primer CHL23SDN | GAGAGTGGTCTCCCCAGATTCARACTA | 23S rRNA 168 bp |
| *C. psittaci* | Probe CHL23LCR | LCRed640-CCTGAGTAGRRCTAGACACGTGAAAC | 23S rRNA 168 bp |
| *C. psittaci* | Probe CHL23FLU | ACGAAARAACAARAGACKCTAWTCGAT-6-FAM | 23S rRNA 168 bp |
| *Chl. trachomatis* | Forward primer pmpHF | GGATAACTCTGTGGGTATTCTCCT | Polymorphic outer membrane protein |
| *Chl. trachomatis* | Reverse primer pmpHR | AGACCCTTTCCGAGCATCACT | Polymorphic outer membrane protein |
| *Chl. trachomatis* | Probe SerA-K | FAM-GCTTGAAGCAGCAGGAGCTGGTG–BHQ | Polymorphic outer membrane protein |
| *E. coli* | Forward primer UAL | TGGTAATTACCGACGAAAACGGC | *uid*A gene |
| *E. coli* | Reverse primer UAR | ACGCGTGGTTACAGTCTTGCG | *uid*A gene |

The real-time PCR used to detect *C. psittaci* used two primers and two labelled probes. When detecting *Chl. trachomatis* with qPCR only one labelled probe was used. The *E. coli* PCR detection did not use a qPCR so no probe was necessary.

**Table 8. Cycling steps used for detection of *C. psittaci* in a LightCycler® instrument.**

| Step | Target temp. ºC | Incub. time | Cycles |
|---|---|---|---|
| Denaturation | 95 | 10 min | 1 |
| Amplification 1 | 95 | 0s | |
| | 64 | 12s | 6 |
| | 72 | 8s | |
| Amplification 2 | 95 | 0s | |
| | 62 | 12s | 9 |
| | 72 | 8s | |
| Amplification 3 | 95 | 0s | |
| | 60 | 12s | 3 |
| | 72 | 8s | |
| Amplification 4 | 95 | 0s | |
| | 54 | 8s | 40 |
| | 72 | 8s | |
| Melting | 95 | 0s | |
| | 50 | 20s | 1 |
| | 80 | 0s | |
| Cooling | 35 | 30s | 1 |

## 5.3 Chelex® 100

### 5.3.1 Extracting DNA from faecal samples using Chelex® 100

967µl samples with added 33 µl *Chl. trachomatis*, with or without faeces, was centrifuged at 100 g for 15 min at 4º C to remove faecal pellets. The obtained supernatant was centrifuged at 13000 g for 10 min at 4º C. The pellet was then washed by suspending it in 1, 5 ml acetone. The washing was repeated 3 times. After the last wash the suspension was centrifuged at 20 000 g for 15 min at 4º C and the supernatant was discarded. 300 µl of 20 % w/v Chelex-slurry (Chelex powder from Bio-Rad Laboratories diluted in TE-buffer containing 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) was added and the sample was vortexed heavily for 1 min and then incubated at 95º C for 10 min to lyse the bacterial cells. The preparation was centrifuged at 20 000 g for 5 min and the supernatant which contains the extracted DNA was collected and stored at -20º C.

### 5.3.2 Gel electrophoresis

Agarose gels (1 % w/v) contain 10 µg/ml ethidium bromide in TBE buffer pH 8.3 (1.2 mM EDTA Titriplex, 44.5 mM boric acid and 44.6 mM Tris) were electrophoresed at 4 V/cm for 40 minutes.

# 6. Acknowledgements

I would like to thank Björn Herrmann for making it possible to do this exciting project. I would also like to thank Markus Klint and Linus Christerson for their never ending patience and help. Also, Conny Tolf, Jonas Waldenström and Lovisa Svensson at the University of Kalmar. I would last, but not least, like to thank the whole team at Mikrobiologen for creating a friendly environment to work in.

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The picture on the front page shows *Chlamydia trachomatis* elementary bodies after lysing of a cell, next to a picture of a young mallard. The picture of *Chl. trachomatis* is taken by Björn Herrmann and the picture of the mallard is taken by me.

# 8. Appendix

## Lymfogranuloma venereum-PCR extraktionskontroll

### Författare: Markus Klint

### Bilaga till: LGV detektion med Realtids PCR AL7867

**Beredning**
För att bereda en kontroll krävs en uppodlad stam av *Chlamydia trachomatis* av serotyp A-K. Protokoll för hur *Chlamydia* odlas finns beskrivet i ” Referensmetodik för laboratoriediagnostik vid kliniskt mikrobiologiska laboratorier” (se referens).
Uppodlad stam Chlamydia trachomatis serotyp D (ATCC885) finns nedfryst i –70° i ”SMI-frysen” (inv nr 074383). Spädningsserier av denna kontroll har visat att en 3000 gångers spädning ursprungslösningen ger att CT (cycle of threshold) kring 30 efter extraktion med M48 vilket är önskvärt.

**Stamlösning**
Stamlösning är spädd 100 gånger från ursprungslösningen och uppdelad i rör med 20 ul i varje. Rören är märkta ”CtrD 100x ATCC885 0810 20 ul” och förvaras i -70°C frysen vid spiraltrappan på våning 2.

**Brukslösning**
Brukslösningen bereds genom tillsats av 580 ul TE-buffert till stamlösningsröret. Sedan portioneras lösningen upp i rör med 20 ul i varje som märks ”CtrD 3000x ATCC885 XXYY 20 ul” där XX står för året och YY för månad. Brukslösningen förvaras i -70°C frysen vid spiraltrappan på våning 2.

**Referenser**
Referensmetodik för laboratoriediagnostik vid kliniskt mikrobiologiska laboratorier. I 6. Sexuellt överförbara infektioner (STI). Smittskyddsinstitutet 2:a upplagan 2008.

**Dokumenthistorik**

| Version | Orsak / ändring | Senast ändrad av |
|---|---|---|
| 1 | Kontroll till ny metod | Marcus Klint |