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Diagnosis of *Chlamydia suis* infection in pigs and humans

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences (Cell and Gene Biotechnology)

Nederlandse vertaling titel:

Diagnose van *Chlamydia suis* infecties bij het varken en de mens.

Refer to this thesis:

De Puysseleyr, L. (2015). Diagnosis of *Chlamydia suis* infection in pigs and humans. PhD thesis, Ghent University, Ghent, Belgium

ISBN-nummer:

978-90-5989-807-3

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Abbreviations

ADP	Adenosine Diphosphate
AGP	Antibiotic Growth Promoter
AI-3	Autoinducer 3
ANOVA	Analysis Of Variance
ATCC	American Type Culture Collection
ATP	Adenosine Tri-Phosphate
BGM	Buffalo Green Monkey
bLF	bovine Lactoferrin
BSA	Bovine Serum Albumin
Caco-2	Human Colon Adenocarcinoma Cells
CADD	*Chlamydia* protein associating with death domains
C.	*Chlamydia*
COMC	*Chlamydia* Outer Membrane Complex
Cp.	*Chlamydophila*
CRP	Cystein Rich Protein
CSO	Mean Spot Occupancy per Cell (an estimate for the overall replication)
Ct	Threshold Cycle
CTB	*Chlamydia* Trac Bottles
DAPI	4',6-Diamidino-2-Phenylindole
DFA	Fluorescent Antibody tests
DMEM	Dulbecco’s Modified Essential Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EB	Elementary Body
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EMEM	Eagle’s Minimal Essential Medium
FCS	Fetal Calf Serum

FITC	Fluorescein Isothiocyanate
FtsZ	Filamentation Temperature Sensitive Z
ELISA	Enzyme-linked Immunosorbent Assay
GAG	Glucosaminoglycans
GDP	Guanosine Diphosphate
GlcN	Glucosamine
HD-11	Chicken macrophage cells
hEGF	human Epidermal Growth Factor
hLF	human Lactoferrin
HRP	Horseradish Peroxidase
HSV-2	Herpes Simplex Virus type 2
Hsp	Heat shock protein
HPPTP	High Pure PCR Template Preparation
IB	Intermediate Body
IFN	Interferon
IFU	Inclusion Forming Units
Ig	Immunoglobuline
IMDM	Iscove's Modified Dulbecco's Medium
Inc	Inclusion membrane protein
IPEC-J2	Intestinal Porcine Epithelial cells
ITS	Insuline-Transferrine-Selenium
Kdo	3-deoxy-D-manno-oct-2-ulopyranosonic acids
LED209	N-phenyl-4-[(phenylamino) thioxomethyl]amino-benzenesulphonamide
LF	Lactoferrin
LGV	Lymphogranuloma venereum
LPS	Lipopolysaccharide
MC	Mitochondrial Carrier
McCoy	Mouse fibroblast cells
MEM	Minimal Essential Medium

MIP	Macrophage Infectivity Potentiator
MOMP	Major Outer Membrane Protein
mRNA	messenger RNA
MSA	Mean Spot Area (a measure for inclusion size per cell)
MSN	Mean Spot Number (inclusion number per cell)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MZN	Modified Ziehl-Neelsen
NAAT	Nucleic Acid Amplification Test
Npt1Ct	Nucleoside Phosphate Transporter 1
Npt2Ct	Nucleoside Phosphate Transporter 2
NTTs	Nucleotide Transport Proteins
OD	Optical Density
OEA	Ovine Enzootic Abortion
Omc	Outer membrane complex protein
Omp	Outer membrane protein
OvoTF	Ovotransferrin
P.aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate Buffered Saline
PB	Persistent Body
PCR	Polymerase Chain Reaction
PCV-2	Porcine Circovirus type 2
PEDV	Porcine Epidemic Diarrhea Virus
p.i.	Post Infection
PMWS	Postweaning Multisystemic Wasting Syndrome
POMP or Pmp	Polymorphic membrane protein
Por	Porine
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
QS	Quorum Sensing
QSI	Quorum Sensing Inhibitor

RB	Reticulate Body
RNA	Ribonucleic Acid
rRNA	ribosomal RNA
RR	Response Regulator
SE	Standard Error
SEP	Septum
SK-6	Swine kidney cells
SNP	Single Nucleotide Polymorphism
SPF	Specific Pathogen Free
SPG	Sucrose Phosphate Glutamate Buffer
SS	Sensor kinase
Tarp	Translocated actin-recruiting phosphoprotein
Tc	Tetracycline
$TCID_{50}$	Tissue Culture Infective Dose
Tc^R	Tetracycline Resistant
Tc^S	Tetracycline Sensitive
TCSS	Two-Component Signal Transduction System
tRNA	transfer RNA
TET	Tetracycline
T3S	Type III secretion
T3SS	Type III Secretion System
T3SE	Type III Secretion Effector
Vero	African green monkey kidney cells
VD	Variable domain
WHO	World Health Organization
2-SP	*Chlamydia* transport medium

Study Objectives

Chlamydiaceae infections are common in pig farming worldwide, and frequently cause economic losses (Schautteet and Vanrompay 2011; Schautteet *et al.* 2013). Four chlamydial species have currently been isolated from swine: *Chlamydia (C.) suis, C. abortus, C. pecorum* and *C. psittaci*. The pig is considered as the natural host for *C. suis*, which is also the most prevalent chlamydial species occurring in these animals. The primary pathogenicity of *C. suis* for the conjunctiva, respiratory system, gastrointestinal and urogenital tract has been demonstrated in various experimental infection studies in gnotobiotic and conventionally raised pigs (Rogers and Andersen 1996; Rogers *et al.* 1996; Rogers and Andersen 1999; 2000; Sachse *et al.* 2004; Reinhold *et al.* 2008; Guscetti *et al.* 2009). Moreover, natural chlamydial infections in pigs have been associated with conjunctivitis, arthritis, pericarditis, polyserositis, pneumonia, enteritis, diarrhea and reproductive failure (Willigan and Beamer 1955; Sarma *et al.* 1983; Woollen *et al.* 1990; Zahn *et al.* 1995; Andersen 1998; Eggemann *et al.* 2000b). Nevertheless, the majority of chlamydial infections in pigs is believed to be subclinical (Pospischil and Wood 1987; Szeredi *et al.* 1996; Nietfeld *et al.* 1997; Hoelzle *et al.* 2000; Camenisch *et al.* 2004a; Englund *et al.* 2012). *Chlamydia suis* is highly related to the human pathogen *C. trachomatis* (Everett *et al.* 1999). Therefore, *C. suis* is believed to have zoonotic potential. Interestingly, *C. suis* was recently detected in the eyes of Nepalese villagers suffering from trachoma (Dean *et al.* 2013), and in two healthy employees of a Belgian abattoir (De Puysseleyr *et al.* 2014a). However, the significance and clinical impact of *C. suis* infections in humans is still largely unknown. Therefore, the first aim of this study was to evaluate the presence of *C. suis* in pig farmers, on nine Belgian farms. Pigs and farmers were examined for the presence of *C. suis* DNA and viable *C. suis* bacteria.

Chlamydial infections in livestock and humans are primarily treated with tetracycline (Tc), or one of its derivatives (Chopra and Roberts 2001; Michalova *et al.* 2004). *Chlamydiaceae* are generally highly sensitive to Tc, however, stably tetracycline resistant (Tc^R) *C. suis* strains have been isolated in the U.S. in 1998, and since then also in Italy, Cyprus, Germany, Israel, Switzerland and Belgium, leading to treatment failure (Andersen 1998; Di Francesco *et al.* 2008; Borel *et al.* 2012; Schautteet *et al.* 2012). The resistant phenotype is associated with the presence of a resistance gene *tet(C)* in the chlamydial chromosome (Dugan *et al.* 2004). The extensive in-feed use of Tc in commercial pig herds likely established a favorable environment for acquisition and maintenance of the *tet(C)* gene by *C. suis* (Dugan *et al.* 2004). Emergence of Tc resistance in *C. suis* might also present a hazard to human health.

Suchland *et al*. (2009) demonstrated the *in vitro* transfer of the *tet(C)* gene among and within chlamydial species, including from *C. suis* into clinical isolates of *C. trachomatis,* resulting in a stable Tc^R phenotype. Given the possible zoonotic potential of *C. suis*, the transmission of Tc^R *C. suis* strains from pigs to *C. trachomatis* infected humans treated with Tc, would facilitate the transfer of Tc resistance into *C. trachomatis*. Once a resistant *C. trachomatis* strain is established, cross-serovar transmission through a patient population might occur rapidly (Suchland *et al.* 2009), leading to treatment failure in human medicine as well. This would affect millions of people worldwide suffering from ocular or genital *C. trachomatis* infections (WHO 2012). Therefore, we also examined the presence of the *tet(C)* gene in the identified porcine and human *C. suis* isolates on the Belgian farms. The identification of viable bacteria through isolation of chlamydial pathogens in cell culture remains crucial for the generation of new isolates and their characterization and pathogenesis studies. However, the reports on growth conditions of *C. suis* strains are limited, and isolation of *C. suis* from field samples is often fastidious (Sandoz and Rockey 2010). Hence, as second aim, the growth characteristics of a conjunctival, respiratory and intestinal *C. suis* strain were studied in cell culture. The growth of these strains was examined in six different cell lines, and two chlamydial growth media were compared.

Tetracycline and its derivatives are critical therapeutic agents in the fight against chlamydial infections. However, the introduction and distribution of Tc resistance in *C. suis* creates a therapeutic challenge. New Tc analogs and antibiotics of other classes are frequently examined. Yet, resistance can evolve rapidly in microorganisms, further limiting treatment options. Therefore, alternative therapies are being investigated based on the inhibition of virulence rather than bacterial growth. This approach is considered to impose a lower selective pressure. Promising virulence blocking compounds have already been described. The third aim of this study was the evaluation of the anti-bacterial effect of two proteins towards extra- and intracellular *C. suis* bacteria *in vitro*. However, for reasons of confidentiality in view of valorization, the results of these experiments were not disclosed in this thesis.

Chapter I

Chlamydial infection biology and associated virulence blockers

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Part of this chapter has been published as:

Beeckman, D., De Puysseleyr, L., De Puysseleyr, K. & Vanrompay, D. (2012). Chlamydial biology and its associated virulence blockers. *Critical Reviews in Microbiology*, 40(4), 313-28.

1 INTRODUCTION TO *CHLAMYDIACEAE*

Chlamydiaceae are Gram-negative obligate intracellular bacteria causing disease in mammals and birds. Chlamydial bacteria show a unique biphasic life cycle characterized by two distinct morphological forms, the elementary bodies (EB) and the reticulate bodies (RB). These pathogens are widely spread and mainly replicate in macrophages, epithelial cells of the respiratory, gastrointestinal and urogenital tract, and in the conjunctiva (Pospischil *et al.* 2010). Chlamydial infections in animals can lead to conjunctivitis, encephalomyelitis, respiratory disease, enteritis, arthritis, infertility, or abortion (Longbottom and Coulter 2003). *Chlamydia (C.) trachomatis* and *C. pneumoniae* are the most common chlamydial pathogens in humans, whereas the other species mainly infect other animals and birds. In the so-called developing countries, *C. trachomatis* is the leading cause of infectious blindness and sexually transmitted disease, which can induce pelvic inflammatory disease, infertility and ectopic pregnancy. *Chlamydia pneumoniae* causes respiratory disease, and chronic infections could contribute to atherosclerosis (Belland *et al.* 2004; Campbell and Kuo 2004). Several other chlamydial species may provoke zoonotic diseases, including *C. psittaci* (birds) and *C. abortus* (mainly in sheep and goats). Transmission of *Chlamydiaceae* occurs through direct contact or via aerosols without the need for an alternate vector (Everett 2000).

2 TAXONOMY

The taxonomy of the family of *Chlamydiaceae* (domain *Bacteria*, phylum *Chlamydiae*, class *Chlamydiae*) has been reclassified several times the last decades. Before 1999, the order of *Chlamydiales* only consisted of the family of *Chlamydiaceae.* In 1999, Everett *et al*. (1999) proposed a reclassification based on phylogenetic analyses of the 16S and 23S ribosomal RNA (rRNA) genes and genetic, phenotypic and morphological data. The family of *Chlamydiaceae* was divided into two genera, *Chlamydia* and *Chlamydophila*. The genus *Chlamydia* comprised the three species *C. trachomatis*, *C. muridarum* and *C. suis*. The genus *Chlamydophila (Cp.)* consisted of six species, *Cp. psittaci*, *Cp. pneumoniae*, *Cp. abortus*, *Cp. pecorum*, *Cp. felis* and *Cp. caviae*. Moreover, the order of the *Chlamydiales* was expanded with three additional families (*Parachlamydiaceae, Simkaniaceae, Waddliaceae*). However, comparative genomic analysis revealed that host-divergent strains of *Chlamydiae* are biologically and ecologically closely related and that the taxonomic separation of the *Chlamydiaceae* into two genera was inconsistent with the natural history of the organism. Therefore, the family of *Chlamydiaceae* is currently reunited into a single genus, *Chlamydia*, containing nine species (Table I-1) (Stephens *et al.* 2009). Recently, based on phylogenetic

Table I- 1 Members of the family *Chlamydiaceae* [according to Greub *et al.* (2010a; b) (adapted from Kerr *et al*. (2005))]

Species	Host	Clinical signs
Chlamydia trachomatis	Humans	Chronic conjunctivitis and blindness (trachoma) Sexually transmitted disease (STD) Infection of the urogenital tract, infertility
Chlamydia muridarum	Mice Hamsters	Respiratory tract infection Genital tract infection
Chlamydia suis [b]	Pigs	Diarrhea, pneumonia, conjunctivitis, reproductive disorders
Chlamydia pneumoniae	Humans Koala	Pneumonia, bronchitis, encephalomyelitis, laryngitis, atherosclerosis, reactive arthritis
Chlamydia psittaci [a]	Birds	Respiratory tract infection
Chlamydia gallinaceae [b]	Chicken Guinea fowl Turkey	Respiratory tract infection
Chlamydia avium [b]	Pigeons Psittacine birds	Respiratory tract infection
Chlamydia abortus [a]	Ruminants Pigs	Reproductive disorders, abortion and bad semen quality
Chlamydia pecorum	Ruminants Pigs Koala	Reproductive disorders, infertility, infection of the urine tract (koala) and abortion, enteritis, polyarthritis, encephomyelitis, metritis, conjunctivitis and pneumonia (other animals)
Chlamydia felis [b]	Cats	Conjunctivitis and respiratory tract infection
Chlamydia caviae [b]	Guinea pigs	Ocular and urogenital tract infection

[a] Zoonotic pathogen
[b] Potential Zoonotic pathogen

analysis of rRNA and *ompA* genes and multi-locus sequence analysis, two new species were added (Figure I-1). These species are referred to as *C. avium* sp. nov., consisting of strains originating from pigeons and psittacine birds, and *C. gallinacea* sp. nov., comprising of strains from poultry (Sachse *et al.* 2014b).

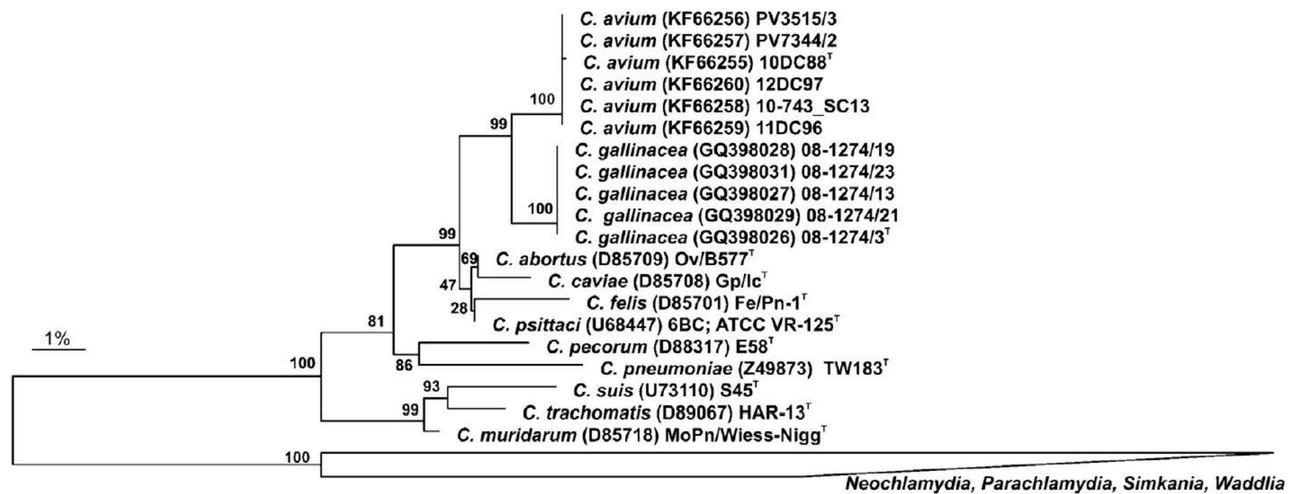

Figure I-1 Phylogenetic reconstruction of the *Chlamydiaceae* classification, including the two new species *Chlamydia* avium and *Chlamydia gallinaceae* based on the alignment of almost complete 16S rRNA genes. The numbers on the nodes indicate the bootstrap support of each branch after 100 replicates. The bar indicates 1% sequence divergence. Adapted from Sachse *et al*. (2014a).

3 MORPHOLOGY

3.1 Developmental forms

Chlamydiaceae possess a unique biphasic developmental cycle during which two morphologically distinct structures can be observed, the infectious elementary body (EB) and the replicating reticulate body (RB). An overview of the most important discriminative characteristics is represented in table I-2. Elementary bodies are usually small (0.2-0.3 µm), spherical, electron dense structures with a dense eccentric core of condensed DNA and chromatin (Costerton *et al.* 1976; Longbottom and Coulter 2003). They are surrounded by a lipid cytoplasmic membrane and a rigid outer membrane (both ~8 nm) with extensive disulfide bridging between cysteine and methionine residues of outer membrane proteins, including the ‘Major Outer Membrane Protein’ (MOMP) (Newhall and Jones 1983). Therefore, EBs are osmotically more stable and less permeable than RBs, allowing them to survive up to several months outside the host cell (Longbottom and Coulter 2003). Elementary bodies are metabolically inert until attachment to the host cell and subsequent internalization.

Upon uptake by the host cell, the elementary bodies differentiate into non-infectious replicating reticulate bodies. During this transition, disulphide bonds between outer membrane proteins are reduced, rendering the outer membrane more permeable to facilitate nutrient uptake by the RBs (Newhall and Jones 1983). Compared to EBs, RBs are also

spherical but have a larger diameter (0.5-1.6 µm). Their cytoplasm is less electron dense and the nucleus is not clearly distinguishable. The RBs become transcriptionally more active, leading to higher amounts of RNA and ribosomes in the cytoplasm, required for protein synthesis (Ward 1988). Reticulate bodies are metabolically active and replicate intracellularly by binary fission. During maturation from RBs back to EBs, morphologically intermediate bodies (IBs) can be formed, which are capable of infecting host cells *in vitro* (Litwin *et al.* 1961; Costerton *et al.* 1976; Vanrompay *et al.* 1996; Rockey and Matsumoto 2000).

Table I-2 Characteristics of chlamydial elementary and reticulate bodies.

Characteristic	Elementary body	Reticulate body
Morphology	Spherical	Spherical
Diameter	0.2-0.3 µm	0.5-1.6 µm
Electron density	High	Low
Cell wall	Rigid, cross-linked	Permeable, fragile
Infectivity for the host	High	None
RNA/DNA ratio	1:1	3:1 (more ribosomes)
Metabolic activity	Relatively inactive	Active, binary fission
Trypsin digestion	Resistant	Sensitive
Projections (T3SSs)	11-20, small patch	Up to 83, larger patch

3.2 Outer membrane composition

Similar to other Gram-negative bacteria, *Chlamydiaceae* are surrounded by two membranes, a cytoplasmic inner membrane and an outer membrane, separated by a periplasmic space. The outer membrane of EBs consists of phospholipids, lipids, lipopolysaccharides and proteins. A substantial part of the chlamydial cell wall is insoluble in the ionic detergent sarcosyl, which usually indicates the presence of peptidoglycans covalently linked to lipoproteins. Indeed, genes for peptidoglycan synthesis are present in the genome. However, only negligible amounts of peptidoglycans are present in the cell wall of *Chlamydiaceae*, and yet they are sensitive to antibiotics targeting peptidoglycan synthesis, such as penicillin. This contradiction is known as the ‘chlamydial anomaly’. The cell wall fraction insoluble in sarcosyl is referred to as the ‘Chlamydia Outer Membrane Complex’ (COMC) or cell envelope, mainly consisting of MOMP, the cysteine rich proteins (CRP) Omp2 and Omp3,

and the polymorphic membrane proteins (pmps) (Figure I-2). Furthermore, lipopolysaccharides, PorB, Omp85, heat shock proteins hsp60 and hsp70, and OprB are present in the outer membrane. The most important components will be further discussed in detail below.

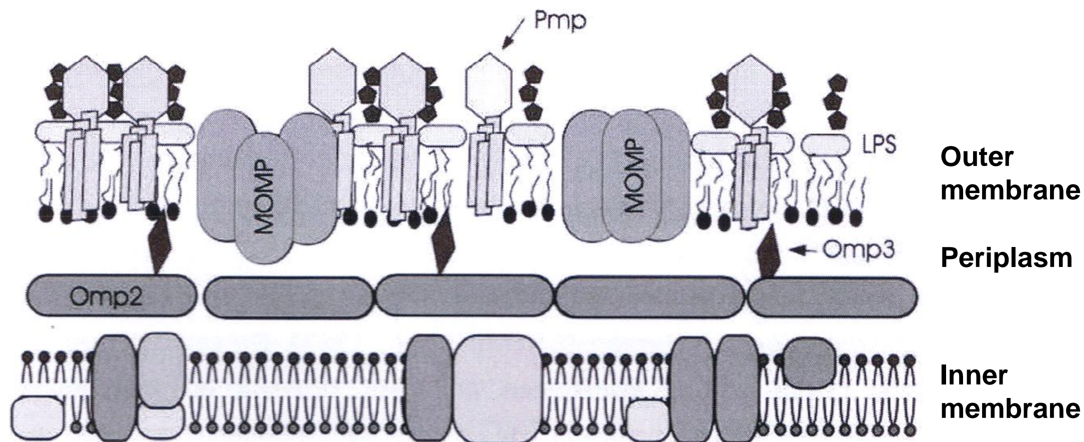

Figure I-2 Schematic representation of the chlamydial EB double membrane. In the outer membrane, MOMP, both CRPs and the pmps are represented. Adapted from Hatch *et al.* (1996).

3.2.1 Major outer membrane protein

The cysteine rich MOMP protein, encoded by the *ompA* gene, has a molecular weight of ~40 kDa and is always present as a trimer. It accounts for about 60% of the outer membrane protein mass of EBs, and nearly 100% of RBs (Caldwell *et al.* 1981). Following reduction of disulphide bonds during the EB to RB transition, MOMP can function as a porin, allowing nutrient uptake by the RB. Moreover, MOMP is presumed to function as an adhesion, involved in nonspecific (electrostatic and hydrophobic) interactions with the host cell (Su *et al.* 1990). The MOMP protein contains four variable domains (VD1-VD4), localized at the outside of the bacterial membrane, flanked by highly conserved hydrophobic regions (Baehr *et al.* 1988a; Yuan *et al.* 1989) (Figure I-3). The variabele domains of MOMP enclose family, genus, species, subspecies and serovar specific epitopes (Caldwell *et al.* 1981; Yuan *et al.* 1989; Everett 2000; Kim and DeMars 2001b). Furthermore, MOMP is an immunodominant protein, and mono- and polyclonal antibodies against MOMP have been shown to neutralize *Chlamydiaceae* infections *in vitro* and *in vivo* (Caldwell and Perry 1982; Zhang *et al.* 1987).

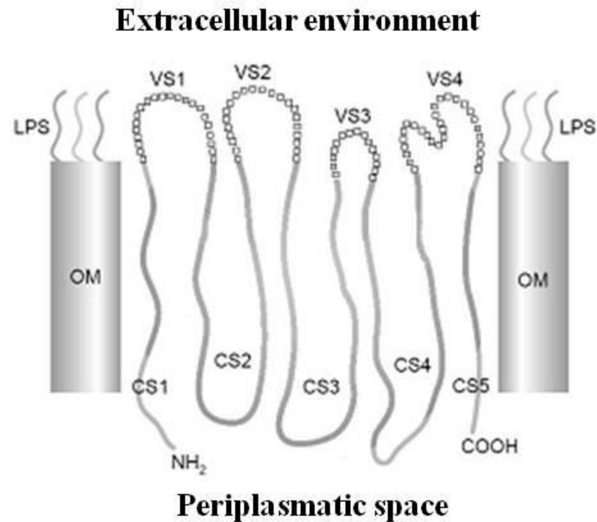

Figure I-3 Model of the positioning of the MOMP protein in the outer membrane of the chlamydial cell wall. The conserved regions are represented as full lines and localized inside the outer membrane. The alternating lines represent the surface-exposed variable domains I to IV [adapted from (Baehr *et al.* 1988b) (Kim and DeMars 2001a)].

3.2.2 Cysteine rich proteins

Two cystein rich proteins (CRP), Outer membrane protein 2 (Omp2, OmcB or EnvB) and 3 (Omp3, OmcA or EnvA) are the second most important proteins present in the COMC. These proteins are highly abundant in EBs, but not in RBs. The omp proteins are expressed late in the growth cycle, when RBs redifferentiate into EBs. Outer membrane protein 2 has a molecular weight of ~60 kDa, is highly conserved, and has been shown to function as an adhesin in *C. trachomatis* LGV1 (Stephens *et al.* 2001; Fadel and Eley 2007; 2008). Since this protein is *Chlamydiaceae* specific and highly immunogenic, it can be used as marker for chlamydial infections (Sanchez-Campillo *et al.* 1999). The omp3 lipoprotein is the smaller CRP with a molecular weight ranging from 9 kDa in *C. trachomatis* to 12 kDa in *C. psittaci* (Everett and Hatch 1995). The omp3 gene sequence is less conserved within the *Chlamydiaceae*, compared to omp2 (Everett and Hatch 1991).

3.2.3 Polymorphic membrane proteins

Longbottom *et al.* (1996) first discovered the pmps at the surface of *C. abortus* S26/3. The number of pmp genes present is variable between species, ranging from 9 pmp genes in *C. trachomatis* to 21 in *C. pneumoniae*, as genome sequencing revealed (Stephens *et al.* 1998; Kalman *et al.* 1999). The pmps represent 3 to 5% of the genome, however, their function has

currently not been fully elucidated. Homology searches, structural comparisons and amino acid sequence analysis strongly suggest that the pmps belong to the family of autotransporters. Based on phylogenetic analysis, pmps fall into six subtypes, implying at least six different roles, probably also including virulence (Henderson and Lam 2001). For some pmps, specific roles in pathogenesis have already been demonstrated, such as adhesion mediated by GGAI motif repeats (Grimwood and Stephens 1999; Kalman *et al.* 1999; Read *et al.* 2000; Wehrl *et al.* 2004; Molleken *et al.* 2010).

3.2.4 Other outer membrane proteins

The PorB (or OmpB) protein has a molecular weight of 37 kDa and is present in the outer membrane. This protein is rich in cysteine residues and highly conserved among chlamydial strains, yet, expressed in low amounts. PorB probably functions as a substrate-specific porin, transferring dicarboxylic acids, and might thus compensate for the incomplete tricarboxylic acid cycle (Iliffe-Lee and McClarty 2000). PorB-specific antibodies have shown to possess neutralizing activity (Sanchez-Campillo *et al.* 1999).

Omp85 is a highly conserved outer membrane protein, widely present in various Gram-negative bacteria. This protein is involved in the insertion and positioning of lipids and proteins into the outer membrane (Genevrois *et al.* 2003), and Omp85 specific antibodies can neutralize chlamydial infections, at least *in vitro* (Stephens and Lammel 2001).

The chlamydial lipopolysaccharide (LPS) has a molecular weight of 10 kDa, is present on both EBs and RBs, and is highly antigenic. It has a lipid A part, with two glucosamines (GlcN) bound to fatty acids. Moreover, it contains a specific tri-saccharide of 3-deoxy-D-manno-oct-2-ulopyranosonic acids (Kdo) of which two residues are linked through a 2→8 linkage, which is unique for *Chlamydiaceae* (Brade *et al.* 1987).

The COMC of EBs and RBs also includes heat shock proteins (Hsps). These proteins are highly conserved within chlamydial species, and are believed to play an important role in chlamydial immunopathology (Zhong and Brunham 1992). The currently identified chlamydial Hsps include Hsp10 (GroES), Hsp60 and Hsp70 (Kornak *et al.* 1991; Brunham and Peeling 1994), which are homologues to GroEL (Hsp60) and DnaK (Hsp70) of *Escherichia (E.) coli* and human mitochondria, with up to 50% protein sequence identity (Brunham and Peeling 1994).

4 DEVELOPMENTAL CYCLE

As obligate intracellular bacteria, *Chlamydiaceae* display a unique biphasic life cycle. An overview of the different stages of chlamydial replication is represented in figure I-4. The acute infection of a host cell initiates with the attachment of EBs to the eukaryotic cell and the subsequent uptake into endocytic vesicles, referred to as inclusions (1 and 2). The EBs tend to attach near microvilli, on the apical surface of the host cell. Since these membrane regions actively transport extracellular material into the host cell, attachment at the base of the microvilli might facilitate entry into the host cell (Escalante-Ochoa *et al.* 1998). Furthermore, attachment might also be associated with clathrin-coated pits, as observed for *C. psittaci* EBs (Vanrompay *et al.* 1996). Several conflicting mechanisms have been described, which possibly occur independently from each other (Byrne and Moulder 1978). Still, the exact mechanism of attachment and entry remains unresolved. Following uptake, the inclusions efficiently avoid lysosomal fusion. For some species, such as *C. trachomatis*, distinct vacuoles can fuse into a larger inclusion (Ridderhof and Barnes 1989; Hackstadt *et al.* 1999), while for other species, including *C. pneumoniae* and *C. psittaci*, fusion of inclusions has not been observed (Rockey *et al.* 1996; Vanrompay *et al.* 1996). The EBs differentiate into RBs, starting from 2 h post infection (p.i.) (3). These RBs migrate to the inclusion periphery, and start to replicate by binary fission, from 8 h p.i. on (4). During this replication phase, host plasma proteins, and lipids and sphingomyelins, acquired from Golgi-derived vesicles, are incorporated into the inclusion membrane, leading to an increase in the inclusion surface (Hackstadt *et al.* 1996; Scidmore *et al.* 1996) (5). Furthermore, also inclusion membrane proteins (Incs) are inserted into the inclusion membrane (Rockey *et al.* 2002). Late in the developmental cycle, RBs detach from the inclusion membrane and re-differentiate into EBs, stored in the lumen of the inclusion. Finally, EBs and remaining non-differentiated RBs are released from the host cell through lysis or reverse endocytosis (6). This release regularly occurs at 24 to 72 h p.i., depending on the host cell and chlamydial species. However, deviations of the replication cycle can be observed, at least *in vitro*. In some cases, chlamydial organisms are present within a host cell, but the life cycle seems interrupted and no visible growth can be observed (7). This phenomenon is known as persistence, and can be induced by several factors, including antibiotics, nutrient deprivation or immune factors, such as interferon gamma (IFN-ɣ) (Mpiga and Ravaoarinoro 2006). During this persistent state, relatively small inclusions and enlarged pleiotrophic RBs or persistent bodies (PBs) are formed (Hogan *et al.* 2004). The PBs accumulate chromosomes, but expression of genes for

cell division does no longer occur (Byrne *et al.* 2001). Persistent bodies revert to normal RBs upon removal of the stress-inducing factor, and the developmental cycle is completed. The role and significance of persistence *in vivo*, and the involvement in chronic infections remains elusive. The major developmental phases will be further discussed below.

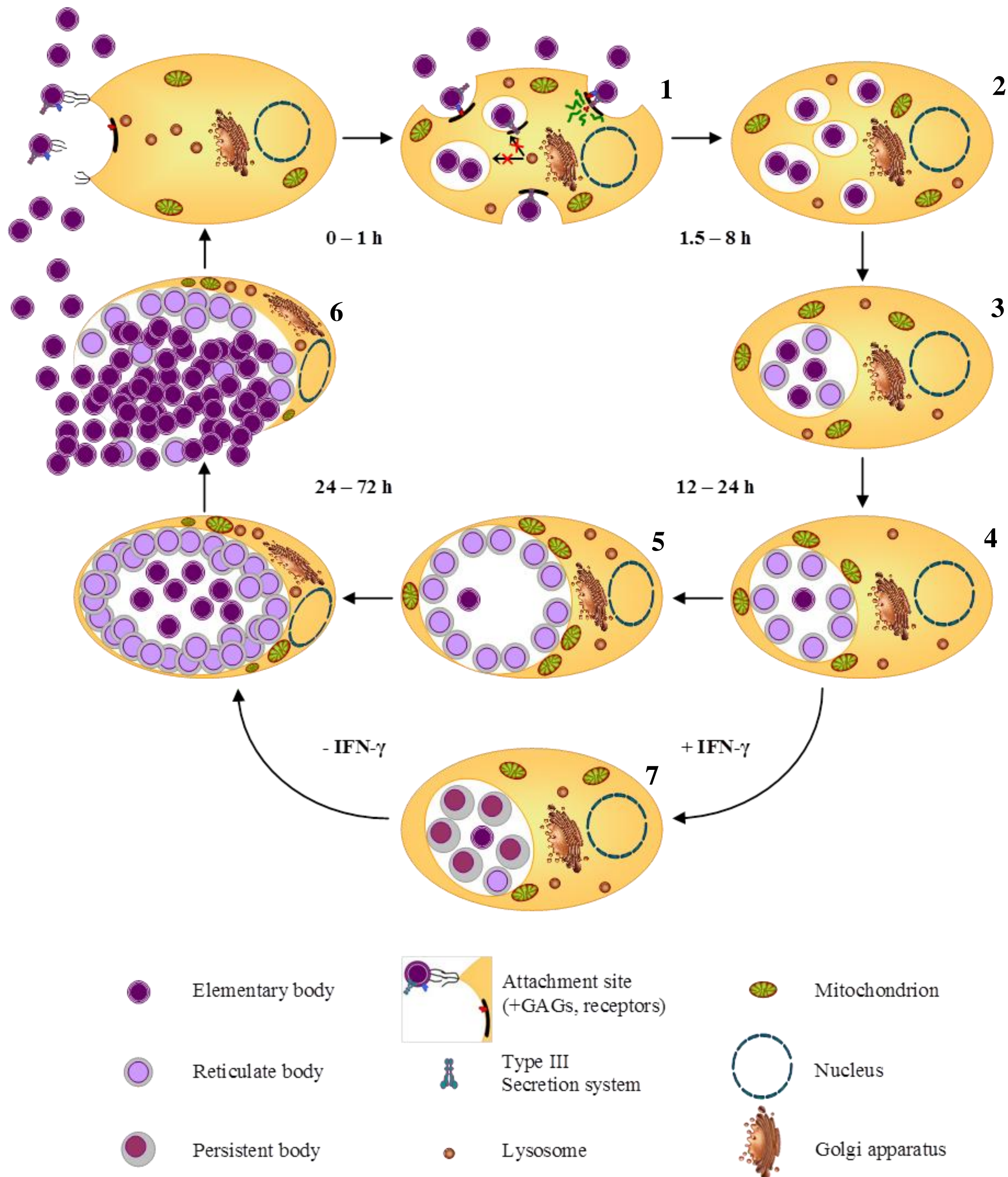

Figure I-4 Schematic overview of the developmental cycle of *Chlamydiaceae*. Bacteria attach preferentially at the base of microvilli and then enter the host cell through parasite specific endocytosis (1). Within the thus formed inclusion, avoiding fusion with host cell lysosomes (2), EBs transform into RBs (3). RBs proliferate at the boundaries of the inclusion by binary fission, until detachment from the inclusion membrane (4-5). RBs revert back to EBs and are stored in the lumen of the inclusion until liberation through lysis or reverse endocytosis (6).

4.1 Attachment: glycosaminoglycans, adhesins and host cell receptors

The attachment of EBs to the host cell probably occurs in at least two steps (Figure I-5). First, EBs attach through a reversible electrostatic interaction with heparin sulphate-like glycosaminoglycans (GAGs) of host origin (Zhang and Stephens 1992; Su *et al.* 1996; Davis and Wyrick 1997). This reversible interaction is followed by a second, irreversible binding of a chlamydial ligand to an unknown host cell receptor, inducing internalization (Carabeo and Hackstadt 2001; Fudyk *et al.* 2002). Since the effect of GAG on the attachment and infectivity varies among *Chlamydiaceae* species, both GAG-dependent and independent mechanisms are likely involved in the attachment process (Zhang and Stephens 1992; Su *et al.* 1996; Rasmussen-Lathrop *et al.* 2000; Fadel 2004). The exact nature of the host cell receptors and chlamydial ligands has not been completely defined. However, several possible bacterial ligands have been identified, including MOMP (Su *et al.* 1990), Hsp70 (Raulston *et al.* 1993), OmcB (Ting *et al.* 1995; Moelleken and Hegemann 2008), the pmp proteins (Wehrl *et al.* 2004; Crane *et al.* 2006; Moelleken and Hegemann 2008), and chlamydial type three secretion system (T3SS) translocon components (CopB, CopD and LcrV) (Watarai *et al.* 1996; Skoudy *et al.* 2000).

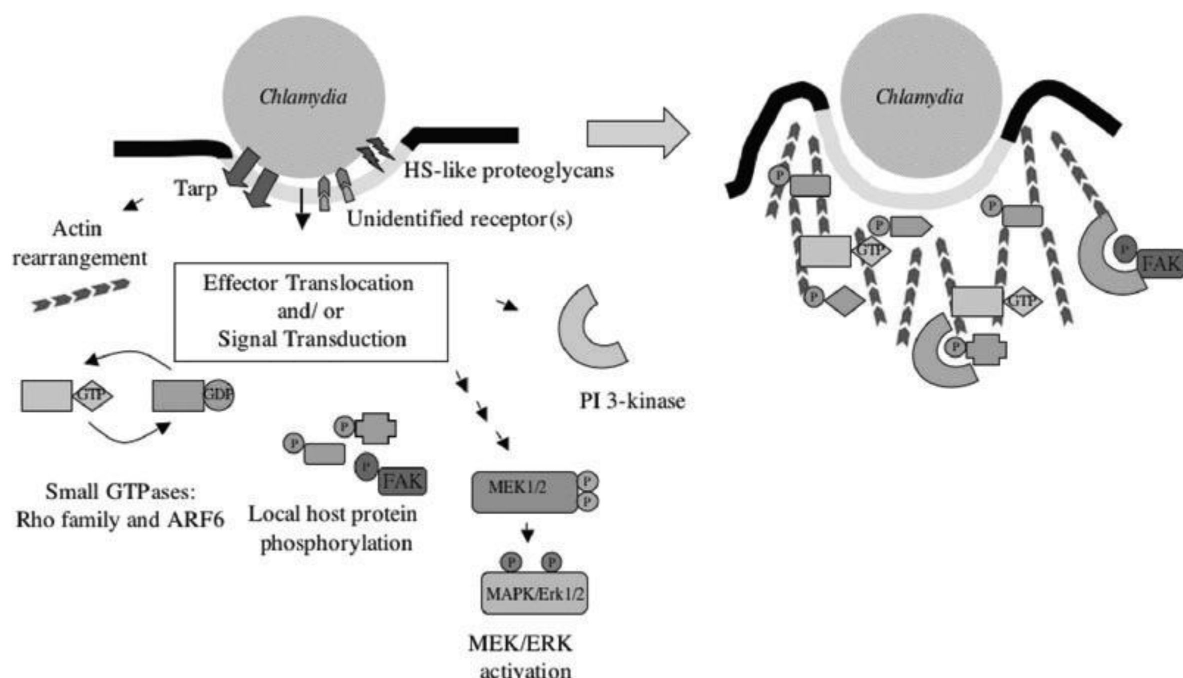

Figure I-5 Attachment and entry of chlamydial EBs. *Chlamydiaceae* interact with the host cell through reversible electrostatic interaction with heparin sulphate-like GAGs on the cell surface, followed by an irreversible interaction with unidentified host cell receptors, possibly associated with cholesterol-rich lipid raft microdomains. Next, host specific signal transduction pathways mediate internalization of the bacteria by actin recruitment and pedestal formation, possibly after injection of T3SS effector proteins (e.g. Tarp). Infection leads to rapid phosphorylation of host cell proteins. Image reproduced from (Dautry-Varsat *et al.* 2005).

4.2 Internalization

Electron microscopy studies revealed two major possible mechanisms for chlamydial entry into the host cell: zipper-like microfilament dependent phagocytosis (Byrne and Moulder 1978) and clathrin-mediated endocytosis (Hodinka *et al.* 1988). Zipper-like microfilament dependent phagocytosis is induced by binding of chlamydial adhesins to host cell receptors. This entry probably occurs via cholesterol-rich lipid raft domains, which are highly connected to the actin cytoskeleton (Stuart *et al.* 2003; Lillemeier *et al.* 2006). Lipid rafts function as signaling platforms (Simons and Toomre 2000) involved in endocytosis (Parton and Richards 2003), intracellular vesicle trafficking (Helms and Zurzolo 2004), and activation of immune response and apoptosis (Gombos *et al.* 2006). Lipid raft-mediated entry leads to a remodeling of the actin skeleton and the derived endosomes do not enter the lysosomal degradation pathway (Helms and Zurzolo 2004), two features resembling the chlamydial developmental cycle. The exact mechanism of chlamydial internalization through lipid rafts remains elusive, but might be as proposed in figure I-5.

For some chlamydial species, including *C. trachomatis* and *C. psittaci*, association to clathrin-coated pits and uptake into clathrin-coated vesicles has been observed, suggesting entry through receptor-mediated endocytosis (Reynolds and Pearce 1990). However, the occurrence of this type of entry appeared to be dependent upon the inoculation route and culture conditions (Prain and Pearce 1989; Wyrick *et al.* 1989; Reynolds and Pearce 1990). The importance of clathrin-mediated endocytosis for chlamydial entry into the host cell is currently not fully clarified (Balana *et al.* 2005; Dautry-Varsat *et al.* 2005).

Only minutes after chlamydial attachment, actin is recruited to the entry site, albeit transiently, forming an actin-rich pedestal underneath the attachment site. Consequently, the EBs are internalized by the host cell into membrane-bound vesicles, as demonstrated for *C. trachomatis* (Carabeo *et al.* 2002), *C. pneumoniae* (Coombes and Mahony 2002), *C. psittaci* (Beeckman *et al.* 2007), and *C. caviae* (Subtil *et al.* 2004). Clifton *et al.* (2004) identified the translocated actin-recruiting phosphoprotein, also called 'Tarp', to be involved in this process. *Chlamydiae* use their T3SS to translocate Tarp into the cytoplasm of the host cell, thus inducing actin recruitment to the invasion site. Tarp orthologs were already identified for all current pathogenic *Chlamydiaceae* species. Distinct functional domains have been identified into the N- and C-terminal regions of *C. trachomatis* Tarp. The N-terminal region contains tyrosine-rich tandem repeats, phosphorylated inside the host cell, and hereby interacting with guanosine nucleotide exchange factors and small GTPases (Lane *et al.* 2008). In this way, a signal transduction cascade is initiated. However, some species, such as

C. abortus and *C. psittaci*, lack the repeat domain in their Tarp sequence. Accordingly, no tyrosine phosphorylation of Tarp is required to initiate actin recruitment in these species. Meanwhile, the C-terminal part contains a proline-rich domain, promoting Tarp oligomerization, and an actin binding domain (Jewett *et al.* 2006). Both domains are required for Tarp-dependent nucleation of new actin filaments, and are conserved among chlamydial species.

Other chlamydial actin recruitment and entry pathways have already been suggested. Swanson *et al.* (2007) identified the 70-kDa host protein ezrin, a member of the ezrin-radixin-moesin protein family, acting as a physical link between host cell receptors and the actin cytoskeleton. Ezrin colocalizes with actin at the chlamydial attachment and entry sites. Although initial ezrin activation through threonine phophorylation is common among *Chlamydiae*, subsequent tyrosine phosphorylation was only demonstrated for infection of host cells with *C. trachomatis* strains. This might imply species-specificity of chlamydial entry pathways, involving chlamydial specific ligands and host cell receptors. Ezrin is known to interact with the cytoplasmic domain of several receptors, including CD44, member of the integrin superfamily and intercellular adhesion molecules. Other possible ligands identified as chlamydial adhesins include T3SS translocon components, such as CopB, CopD and LcrV, as mentioned earlier. Conclusively, *Chlamydiaceae* probably enter their host cell through more than one pathway, which might be species specific, but possibly partially overlap.

4.3 Inhibition of the phagolysosomal fusion

Chlamydiaceae can efficiently impede fusion of the inclusion to lysosomes, thus preventing subsequent eradication. This ability is exclusively restricted to *Chlamydia*-containing inclusion vacuoles (Eissenberg and Wyrick 1981). However, not all inclusions can escape phagolysosomal fusion, depending on the host cell, chlamydial strain and mode of entry (Moulder 1991). The inclusion membrane has a unique composition, since markers of the plasma cell membrane, early or late endosomes, or lysosomes, such as the vacuolar H^+ ATPase, are absent (Heinzen *et al.* 1996; Scidmore *et al.* 1996; Taraska *et al.* 1996; Al-Younes *et al.* 1999). In accordance with the absence of vacuolar H^+ ATPase, no acidification of the inclusion lumen can be observed. Interestingly, EB-containing vesicles only slowly acquire lysosomal characteristics upon blocking of protein synthesis. A dual mechanism for prevention of the phagolysosomal fusion has been proposed, consisting of an initial phase of delayed maturation depending on an intrinsic property of EBs, followed by an active

modification of the inclusion membrane, wherefore chlamydial synthesis of proteins, such as chlamydial Incs, is demanded (Scidmore *et al.* 2003). The required intrinsic property of EBs might be the translocation of previously produced type three secretion (T3S) effector proteins, as was observed in *Salmonella* (Hackstadt *et al.* 1997; Wyrick 2000). As noted earlier, lipid raft-derived endosomes do not enter the lysosomal degradation pathway. Correspondingly, certain bacteria, such as *Mycobacterium spp.*, are protected against phagolysosomal fusion (de Chastellier and Thilo 2006). Therefore, the underlying chlamydial mechanism for prevention of lysosomal degradation might be similar.

4.4 Proliferation

The RBs, migrated to the periphery of the inclusion, initiate division through binary fission, starting 8 h p.i. Generally, the filamentation temperature sensitive (ftsZ) protein plays a key role in bacterial cell division. Hence, it is highly conserved among eubacteria. However, *Chlamydiae* lack an identifiable *ftsZ* ortholog. Nevertheless, other factors have been identified possibly substituting for the lack of FtsZ during the formation of division septa. As noted earlier, RBs synthesize small amounts of peptidoglycan, in which the chlamydial MurA ortholog (UDP-N-acetylglucosamine enolpyruvyl transferase) catalyzes the first step (Chopra *et al.* 1998). The *murA* gene is expressed during EB to RB differentiation and replication, and the chlamydial murA ortholog appeared to be functional in *murA* deficient *E. coli*. Hence, peptidoglycans might be involved in chlamydial replication (McCoy and Maurelli 2006). Moreover, the SEP (septum) antigen was observed to localize as a ring-like structure beside the chlamydial division plane, resembling the distribution of FtsZ at the septum during bacterial cell division. This localization was only observed in actively dividing RBs and thus may be associated with RB replication (Brown and Rockey 2000). The RBs undergo multiple rounds of division through binary fission, until contact to the inclusion membrane through their T3SS is lost (Wilson *et al.* 2006). Then, the RBs detach from the inclusion membrane and redifferentiate asynchronously into EBs, that are stored in the inclusion lumen until release.

4.5 Nucleotide acquisition

Multiple findings in literature indicate that *Chlamydiaceae* behave as ‘energy parasites’. They absorb a large amount of nutrients from the host cytosol throughout their obligate intracellular lifecycle, eliminating the need for their own *de novo* production. Moreover, they do not exhibit *de novo* nucleotide synthesis and show a restricted nucleotide metabolism and lower ability of ATP generation. However, genome sequencing revealed that *Chlamydiae* do possess

genes allowing production of their own ATP, probably by both the glycolytic pathway and their truncated tricarboxylic acid cycle (Iliffe-Lee and McClarty 1999). Therefore, *Chlamydiae* may not be strict auxotrophic.

To withdraw energy molecules from the host, *Chlamydiaceae* possess nucleotide transport proteins (NTTs). This enables them to perform ATP-ADP counter-exchange and to import nucleotides. The genome of *C. trachomatis,* for instance, contains two genes coding for nucleoside phosphate transporters 1 and 2 (Npt1Ct and Npt2Ct), each performing a different type of transport (Tjaden *et al.* 1999). First, Npt1Ct is an ATP-ADP exchanger, able to function in ATP acquisition from the host cytosol. Secondly, Npt2Ct catalyses transport of nucleotides and H^+ into the cell. Likewise, *C. pneumoniae* (Kalman *et al.* 1999) and *C. psittaci* exhibit an ATP-ADP exchange (Hatch *et al.* 1982). The ribonucleoside triphosphate/H+ transporters of other *Chlamydia* spp. may be of distinct specificity.

Gene expression studies revealed that strong upregulation of the ATP/ADP anti-porter gene of *C. trachomatis* occurs early in the developmental life cycle. Indeed, uptake of ATP from the host cell would be most relevant early in the infection process, when the whole complement of enzymes for ATP generation via glycolytic and pentose pathway is not present yet, such as during initial differentiation of EBs to RBs shortly after infection of the host cell. Moreover, the substrate for oxidative phosphorylation is now limited in the host cytosol. Under these circumstances, an alternative way for energy generation is an advantage. Later on, only after inclusion niche establishment, structural proteins and proteins of intermediary metabolism are expressed. Once cell division starts, the energy need rises and available energy generation increases by the glycolytic and pentose pathways (Shaw *et al.* 2000). Although *Chlamydia* can transport ATP across the bacterial membrane, the mechanism through which the highly charged ATP molecules pass the inclusion membrane to reach the bacteria is unknown so far, as pores for passive diffusion are absent (Heinzen and Hackstadt 1997).

4.6 Type III secretion

The chlamydial T3SS participates in multiple steps of the chlamydial infection process. At the very start of infection, T3S translocon components might be involved in the irreversible attachment, as mentioned above. The CopB protein likely functions as an adhesin, binding the hyaluron receptor CD44 in lipid rafts, thus inducing assembly of the T3SS translocon in the eukaryotic membrane. In addition, surface exposed T3SS components, such as the outer membrane secretin SctC or needle protein SctF, might also function as ligands in receptor-mediated uptake of *Chlamydiae* (Beeckman and Vanrompay 2010). Shortly after attachment,

the T3SS translocates the effector protein (T3SE) Tarp into the host cytosol, leading to actin recruitment at the attachment site. A pedestal-like structure is formed beneath the attached EB, followed by internalization. In addition to two already identified underlying signaling cascades, other currently uncharacterized T3SEs are likely implicated in additional signaling pathways involved in EB uptake (Jewett *et al.* 2006; Lane *et al.* 2008). Once an inclusion vacuole is established, the inclusion membrane is actively modified through insertion of inclusion membrane proteins or Incs, such as CT229 or IncA, probably through T3S. Multiple functions have been assigned to the Inc proteins, including avoidance of phagolysosomal fusion, and diverting intracellular host cell trafficking to the nascent inclusion to acquire nutrients (Rzomp *et al.* 2006; Cortes *et al.* 2007; Delevoye *et al.* 2008). Other T3SEs, such as the Chlamydia protein associating with death domains (CADD), could be involved in host cell reprogramming and modulation of apoptosis. The CADD protein likely acts through binding to the death domains of tumor necrosis factor receptor (Stenner-Liewen *et al.* 2002). Later in the infection cycle, during proliferation, RBs are closely attached to the inclusion membrane, in which the T3SS is also involved. Near the end of the infection cycle, RBs detach from the inclusion membrane due to spatial limitations in the host cell, inactivating the T3SS and inducing asynchronous re-differentiation of RBs into EBs (Wilson *et al.* 2006; Peters *et al.* 2007).

4.7 Regulation of virulence gene expression - Quorum sensing

Quorum sensing (QS) is a bacterial system involved in the regulation of gene expression in response to environmental cues, such as cell-population density (Miller and Bassler 2001). Though the concept of QS is common among bacteria, the exact molecular mechanism may differ among species. At least six different QS pathways are identified so far (Table I-3) (Surette *et al.* 1999; Schauder *et al.* 2001; Chen *et al.* 2002; Sperandio *et al.* 2003; Henke and Bassler 2004; Higgins *et al.* 2007; Kendall *et al.* 2007). A common signaling pathway containing the membrane-bound QseC histidine sensor kinase (Clarke *et al.* 2006) or its homologues, is present in more than 25 important pathogens in humans and plants. QseC perceives the bacterial quorum sensing signal autoinducer 3 (AI-3) and/or host derived adrenalin and/or noradrenalin. After binding of the signal, QseC increases its autophosphorylation. The following phosphorylation cascade in the bacterial cell regulates the expression of virulence genes (Sperandio *et al.* 2003; Hughes and Sperandio 2008).

Table I-3 Quorum sensing pathways in Bacteria.

Pathway	Signal molecules	Bacteria	References
AHL (AI-1 pathway)	AHLs	Gram-negative	(Salmond *et al*., 1995, Ravn *et al*., 2001, Zavilgelsky and Manukhov, 2001)
4Qs pathway	PQS and HHL	Gram-negative	(Diggle *et al*., 2006)
AI-3 pathway	AI-3	Gram-negative	(Sperandio *et al*., 2003, Kendall *et al.,* 2007)
AI-2 pathway	Two different forms	Gram-negative and Gram-positive	(Surette *et al.,* 1999, Schauder *et al.,* 2001, Chen et al., 2002)
AIP pathway	Oligopeptides	Gram-positive	(McDowell *et al*., 2001)
CAI-1	hydroyketones	Gram-negative	(Henke and Bassler, 2004, Higgins *et al*., 2007)

The genome of *C. trachomatis* comprises two genes, *ctcB* and *ctcC,* with protein sequence similarity to histidine kinase-response regulator pairs of two-component systems (Figure I-6). The latter are a type of QS pathway, which play a role in stage-specific gene expression, such as in- and outside the host cell, two completely different environments in the *Chlamydiaceae* biphasic life cycle. Generally, the histidine sensor kinase component in the bacterial membrane autophosphorylates upon signal perception and subsequently phosphorylates and activates a response regulator, usually a transcription factor, which binds to the promoter of a target gene and initiates transcription upon activation. The sensor kinase and response regulator pair form a genetic network together with a range of downstream molecular factors. This network controls a specific subset of genes, including virulence genes (Novick 2003; Lyon and Novick 2004).

Two-component systems are a primary mechanism to adapt to environmental conditions. Though little is known about transcriptional regulation in *Chlamydiae*, gene expression and development are most likely controlled through recognition of environmental cues or intracellular conditions. Although many bacteria possess several systems to adapt to diverse environmental changes, this is the only complete two-component system identified in *C. trachomatis*. Moreover, this *ctcB-ctcC* system proved to be functional as it is capable of autophosphorylation and phosphotransfer reactions (Koo and Stephens 2003).

The *CtcB* and *CtcC* genes possess a late expression profile and, accordingly, the corresponding proteins are present in EBs, but not in RBs (Koo and Stephens 2003). Most two-component system components, however, are constitutively expressed to adapt efficiently to a changing environment. The late expression profile thus implies involvement in the control of a subset of late genes participating in RB to EB transition. Moreover, the sensor kinase CtcB possesses a redox sensing domain (Koo and Stephens 2003). This could sense the change in redox state when EBs enter the host cells and disulfide-linkage in the outer membrane proteins are reduced, which results in a higher membrane flexibility and increased nutrient uptake. Similarly, a decrease in energy sources or reducing agents results in oxidation of sulfhydrylgroups, hindering RB development and decelerating metabolic activity (Bavoil *et al.* 1984; Hackstadt *et al.* 1985; Ward 1988). To conclude, CtcB and CtcC are developmentally late-expressed proteins with a redox sensing domain. This domain is most likely involved in late gene activation, including the regulation of RBs to EBs differentiation.

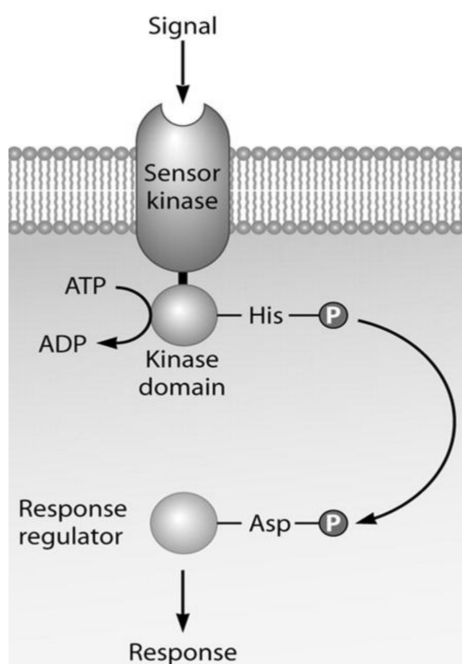

Figure I-6 Organization of the prototypical Two-Component Signal Transduction System (TCSS) in bacteria. The prototypical TCSS is comprised of a single sensor kinase (SK) and a single response regulator (RR). The input domain of the SK recognizes a specific signal(s) from the environment. This recognition results in activation of the kinase domain and autophosphorylation in the output domain of the SK at a conserved histidine residue. The output domain of the phosphorylated SK interacts with the receiver domain of the RR, catalyzing the transfer of phosphate to a conserved aspartate residue within the receiver domain. Phosphorylation of the RR activates its output domain, resulting in conformational changes in the RR that mediate specific biological activities, including DNA binding and transcriptional regulation (adapted from (Mitrophanov and Groisman 2008)).

5 *CHLAMYDIACEAE* INFECTIONS IN PIGS

5.1 *Chlamydiaceae* in pigs

Currently, four chlamydial species have been isolated from pigs: *C. psittaci*, *C. pecorum*, *C. abortus* and *C. suis*. These four species are briefly described below.

Chlamydia psittaci is widely known as an avian pathogen, infecting the conjunctiva, gastro-intestinal and respiratory tract in birds. These infections regularly result in systemic disease, which can be inapparent to severe, and acute or chronic. Transmission of *C. psittaci* among birds mainly occurs through inhalation of contaminated aerosols, but also vertical transmission through the egg is possible (Busch *et al.* 2000; Vanrompay *et al.* 2004). Moreover, *C. psittaci* is transmissible from birds to humans, and even human-to-human transmission has been reported. The clinical signs of human infections can vary widely, from mild flu-like symptoms to severe pneumonia (Beeckman and Vanrompay 2009). Furthermore, *C. psittaci* has been isolated from lung and genital tract tissue of pigs. The reference strain for *C. psittaci* is $6BC^T$ (=ATCC VR 125^T).

Chlamydia pecorum strains are serologically and pathogenically highly diverse. This species has been isolated from various mammal hosts, including ruminants (cattle, sheep and goats)(Fukushi and Hirai 1992), koalas (Girjes *et al.* 1993) and pigs (Kaltenboeck and Storz 1992). In koalas, *C. pecorum* infections cause urinary tract disease, reproductive disease, and infertility. In other mammals, this species causes conjunctivitis, encephalomyelitis, pneumonia, enteritis, abortion, and polyarthritis. The type strain is *C. pecorum* $E58^T$ (=ATCC VR 628^T).

Chlamydia abortus is the most frequent cause of abortion in sheep and goats in Europe, also known as ovine enzootic abortion (OEA), leading to major economic loss in agriculture (Kerr *et al.* 2005). *Chlamydia abortus* is endemic among ruminants, where it efficiently colonizes the placenta. Moreover, reproductive failure due to *C. abortus* infections has been described in cattle, horses, pigs, deer and mice (Everett *et al.* 1999; Longbottom and Coulter 2003). Zoonotic *C. abortus* infections have been reported, leading to miscarriages and stillbirths in farm women working with sheep (Johnson *et al.* 1985; Wong *et al.* 1985) and goats (Pospischil *et al.* 2002; Meijer *et al.* 2004). The type strain is *C. abortus* $B577^T$ (= ATCC VR 656^T).

Chlamydia suis is considered as endemic in the intestinal flora of pigs, which are considered the only natural hosts for *C. suis* (Schautteet and Vanrompay 2011). Among *Chlamydiae* occurring in pigs, *C. suis* is most frequently detected. Infections can remain asymptomatic, or

be associated with conjunctivitis, enteritis, pneumonia, and reproductive disorders. Its primary pathogenicity was proven in multiple experimental infections in gnotobiotic pigs (Rogers and Andersen 1996; 1999; 2000; Reinhold *et al.* 2008; Reinhold *et al.* 2010; De Clercq *et al.* 2014). Resistance to sulfadiazine and/or Tc has been demonstrated in several strains. Sequence analysis suggest a genetically high diversity among *C. suis* strains, compared to other chlamydial species (Everett *et al.* 1999). The reference strain is *C. suis* S45^T (ATCC VR 1474^T).

5.2 Epidemiology

5.2.1 Serodiagnosis

The seroprevalence of *Chlamydiaceae* has been widely reported in Europe. However, the available serodiagnostic tests are based on detection of family specific antibodies, and thus are not able to assess the occurrence of individual species. Nevertheless, the prevalence of *Chlamydiaceae* infections proved to be high. The earliest serological data on the occurrence of chlamydial infections date back to 1966, when Wilson and Plummer (1966) demonstrated antibodies against *Chlamydiaceae* in 23% of sera of pigs in Great Britain. Vanrompay *et al.* (2004) examined 258 Belgian pig farms, and reported a *Chlamydiaceae* seroprevalence of 97%. According to Eggeman *et al.* (2000a), 33% to 72% of the sows and 10 to 47% of the boars in Germany produced *Chlamydiaceae*-specific antibodies. In Switzerland, seroprevalence rates of 62% for sows, 7% for piglets younger than four weeks and 48% for piglets older than four weeks were reported (Camenisch *et al.* 2004b). Moreover, 83% of the tested Swiss finisher pigs were seropositive for *Chlamydiaceae* (Szeredi *et al.* 1996). Similarly, seroprevalence rates were 64 to 81% in Italian finisher pigs (Di Francesco *et al.* 2006). Of interest, *Chlamydiaceae*-specific antibodies were demonstrated in 64% of Italian, free-living boars, suggesting a *Chlamydia* reservoir in wild boar populations (Di Francesco *et al.* 2011). In Lithuania and Poland, anti-chlamydial antibodies were also detected in commercial pigs, albeit to a lesser extent, with reported seroprevalence rates of 8% and 6% respectively (Rypula *et al.* 2014b). These numerous reports demonstrate the high abundance of chlamydial infections in European commercial pigs. Moreover, *Chlamydiaceae* seroprevalence rates in pigs were examined in different provinces of China, with prevalence rates of 63% in the Hunan province, 59% in Jiangxi and 31% in the Guangdong province (Xu *et al.* 2010; Jiang *et al.* 2013; Zhang *et al.* 2014). Although variation in reported

seroprevalence rates exists, *Chlamydiaceae* infections are believed to be widespread in commercial pigs worldwide (Schautteet and Vanrompay 2011).

5.2.2 Molecular diagnosis

The development of NAATs enabled species specific examination of chlamydial infections. Implementation of species specific tests identified *C. suis* as the main species involved in chlamydial infections in swine. A high prevalence of *C. suis* was demonstrated in the eyes of Swiss and German pigs without ocular symptoms (23-88%), and pigs suffering from conjunctivitis (79-90%) (Becker *et al.* 2007). Similarly, *C. suis* was detected in Swedish finisher pigs with or without conjunctivitis (Englund *et al.* 2012). Moreover, *C. suis* was involved in the majority of chlamydial intestinal infections in German, Swiss and Belgian pigs (Zahn *et al.* 1995; Szeredi *et al.* 1996), and in growing pigs with and without diarrhea in Sweden (Englund *et al.* 2012). Furthermore, *C. suis* was demonstrated in the intestine of Cypriot and Israeli pigs (Schautteet *et al.* 2012). *Chlamydia suis* was also detected in the urogenital tract of pigs suffering from reproductive disorder in Belgium, Cyprus and Israel (Schautteet *et al.* 2012). Moreover, *C. suis* was also demonstrated in the urogenital tract of pigs in Poland (Szymanska-Czerwinska *et al.* 2011). The urogenital *C. suis* infections might be spread through contaminated pig sperm, since *C. suis* was detected in boar semen intended for export, in a German artificial insemination center (Schautteet *et al.* 2012). In addition, mixed infections of *C. suis* and *C. abortus* occur regularly, as has been demonstrated in the lungs and intestine of German pigs (Hoelzle *et al.* 2000). Although *C. abortus* mainly causes reproductive failure and abortions in pigs, it has also been demonstrated in lung tissue (Sachse *et al.* 2005).

The majority of report on porcine chlamydial infections concern examination on commercial pigs. However, few reports exist on wild boars. Hotzel *et al.* (2004) detected *C. suis, C. abortus* and *C. psittaci* in the lung tissue of wild boars in Germany (Thuringia), while Di Francesco *et al.* (2013) demonstrated the presence of *C. suis* in conjunctival swabs of wild boars in Italy. These findings suggest a possible wildlife reservoir of porcine chlamydial infections.

5.3 Pathogenesis

Chlamydia suis is frequently detected in commercial pigs worldwide, often without clinical manifestation of infection (Hoelzle *et al.* 2000; Camenisch *et al.* 2004a; Englund *et al.* 2012). However, these infections have also been associated with a variety of diseases. The majority

of the 25 *C. suis* strains currently described in literature, were isolated from pigs with clinical symptoms, except *C. suis* S45, 130 and 132 (Table I-4). The pathogenicity of *C. suis* has been demonstrated in experimental infections of gnotobiotic pigs.

Table I-4 *Chlamydia suis* strains isolated from pigs (Schautteet and Vanrompay 2011)

	Isolated from			
Strain	**Location**	**Year**	**Tissue**	**Clinical Symptoms of the pigs**
S45	Austria	1969	Intestines (feces)	Asymptomatic infection
R19	Nebraska	1992	Intestines (feces)	Pneumonia, enteritis, conjunctivitis
R22	Nebraska	1992	Conjunctiva	Conjunctivitis
R24	Nebraska	1992	Respiratory tract (nasal mucosa)	Upper respiratory tract disease
R27	Nebraska	1993	Intestines (colon)	Enteritis
R33	Nebraska	1994	Respiratory tract (nasal mucosa)	Pneumonia
H5	Iowa	1994	Conjunctiva	Conjunctivitis
H7	Iowa	1994	Conjunctiva	Conjunctivitis
130	Nebraska	1996	Intestines (jejunum)	Asymptomatic infection
132	Nebraska	1996	Intestines (ileum)	Asymptomatic infection
DC6	Germany	2004	Conjunctiva	Conjunctivitis
MS1	Italy	2004-2007	Conjunctiva	Conjunctivitis
MS2	Italy	2004-2007	Conjunctiva	Conjunctivitis
MS3	Italy	2004-2007	Conjunctiva	Conjunctivitis
MS4	Italy	2004-2007	Conjunctiva	Conjunctivitis and return to oestrus
MS5	Italy	2004-2007	Conjunctiva	Conjunctivitis and return to oestrus
MS6	Italy	2004-2007	Conjunctiva	Conjunctivitis and return to oestrus
MS7	Italy	2004-2007	Conjunctiva	Conjunctivitis and return to oestrus
MS8	Italy	2004-2007	Conjunctiva	Conjunctivitis and return to oestrus
MS9	Italy	2004-2007	Conjunctiva	Conjunctivitis and return to oestrus
MS10	Italy	2004-2007	Conjunctiva	Conjunctivitis and return to oestrus
MS11	Italy	2004-2007	Conjunctiva	Conjunctivitis and return to oestrus
MS12	Italy	2004-2007	Conjunctiva	Conjunctivitis
MS13	Italy	2004-2007	Conjunctiva	Conjunctivitis
MS14	Italy	2004-2007	Conjunctiva	Conjunctivitis

5.3.1 Conjunctival infection

Conjunctival infection of gnotobiotic piglets with *C. suis* H7, resulted in histological lesions of mild to moderate multifocal conjunctivitis, albeit subclinical (Rogers and Andersen 1999). However, *C. suis* has been associated with clinical signs of conjunctivitis in naturally infected pigs (Rogers *et al.* 1993). Moreover, according to Becker *et al.* (2007), the occurrence of conjunctivitis was correlated to the presence of *C. suis* in extensively kept pigs. In intensive pig-farming systems, however, pigs showed to be predisposed to ocular *C. suis* infections, also leading to a high prevalence in clinically healthy pigs (Becker *et al.* 2007; Schautteet *et al.* 2010).

5.3.2 Respiratory infection

Experimental aerosol challenge in pigs with *C. suis* DC6 resulted in **respiratory infections** leading to pulmonary inflammation, characterized by severe acute bronchiolitis, interstitial pneumonia and dystelectasis, and associated with fever, dry cough, serous nasal discharge and dyspnoea in all infected animals (Sachse *et al.* 2004; Reinhold *et al.* 2008). *Chlamydia suis* DNA was detected in tissue samples from tonsils, and sporadically in spleen and pulmonary lymph nodes of infected pigs, but not in liver samples. The high detection rate of *C. suis* in fecal swabs of infected animals might contribute to airborne transport of C*hlamydiae*. These experimental infections indicate the pathogenic potential of *C. suis* for the porcine respiratory system. Moreover, the involvement of *C. suis* and *C. abortus* in respiratory disease has been demonstrated in naturally infected pigs showing clinical signs of respiratory illness (Hoelzle *et al.* 2000).

5.3.3 Intestinal infection

The pathogenicity of chlamydial isolates for the porcine **intestine** has also been confirmed in experimental infection studies. Although the reference strain *C. suis* S45 was originally isolated from feces of an asymptomatic pig, experimental enteric infection provoked significant enteric disease and lesions in gnotobiotic piglets (Guscetti *et al.* 2009). Histopathological changes included moderate-to-severe villus atrophy, associated with flattened enterocytes and focal villus tip erosions, and moderate mucosal inflammatory cell infiltration in the small intestine of inoculated piglets. Chlamydial replication was observed in the small and large intestinal villus enterocytes, lamina propria, tunica submucosa and mesenteric lymphnodes. No substantial dissemination into extraintestinal sites, such as spleen,

kidney and liver, was demonstrated. Similarly, *C. suis* isolates R27 and R19 caused diarrhea and intestinal lesions in experimentally infected piglets (Rogers and Andersen 1996).
Likewise, moderate-to-severe multifocal villus atrophy was observed in the distal jejunum and ileum of the infected piglets. Chlamydial antigens were also occasionally seen in macrophages in the lamina propria and in foci of inflammation in the submucosa. Extraintestinal dissemination of *C. suis* was not evaluated in this study. Furthermore, experimental enteric infection of gnotobiotic piglets with a *C. psittaci* strain T49/90, of avian origin, elicited enteric infection associated with mild lesions, weak systemic dissemination and fecal shedding. Histopathological lesions consisted again of villus atrophy and an increased number of inflammatory cells in the villus epithelium and lamina propria. Chlamydial replication was observed in the villus enterocytes and lamina propria. Systemic dissemination of *Chlamydia* into mesenteric lymph nodes, spleen and lung occurred to a limited extent. Thus, pigs might also be a potential host for avian *Chlamydiae* (Guscetti *et al.* 2000). Natural intestinal chlamydial infections are frequently detected in both diarrheic and clinically healthy pigs (Pospischil and Wood 1987; Szeredi *et al.* 1996; Nietfeld *et al.* 1997; Hoelzle *et al.* 2000; Camenisch *et al.* 2004a; Englund *et al.* 2012). Therefore, intestinal chlamydial infections are common but the majority is believed to remain subclinical.

5.3.4 Urogenital infection

Recently, De Clercq *et al.* (2014) demonstrated the pathogenic potential of *C. suis* for the female porcine **urogenital tract**. Intravaginal infection of piglets with *C. suis* strain S45 resulted in inflammation of the reproductive system, associated with lesions and congestion of the genital tract. Histopathological changes included degeneration of the epithelium, superficial exfoliated epithelial cells and infiltration of inflammatory cells in the lamina propria. Chlamydial replication occurred throughout the urogenital tract, but extra-urogenital dissemination was not observed. In addition, Vazquez-Cisneros *et al.* (1994) infected sows at 42 days of pregnancy with a *C. psittaci* isolate, originating from aborted ewes. This inoculation caused infection of the fetal membranes, but did not induce abortion. No lesions other than areas of inflammation were observed. Chlamydial species have been linked to various reproductive symptoms in pig farming. Indeed, Eggeman *et al.* (2000b) demonstrated a significant correlation between the presence of chlamydial DNA and the incidence of abortion and litters with stillborn and low viable piglets. According to Hoelzle *et al.* (2000), sows with reproductive disorder showed a significantly higher *Chlamydia* PCR positive rate

compared to healthy controls. Yet, in these cases, *C. abortus* was mainly involved, while *C. suis* was less frequently detected. Furthermore, *C. suis*, *C. abortus* and *C. pecorum* have been regularly demonstrated in aborted fetuses (Schiller *et al.* 1997b; Thoma *et al.* 1997). Besides, *C. suis* has been associated with various reproductive disorders, including vaginal discharge, return to oestrus, endometritis, delivery of weak piglets, increased perinatal and neonatal mortality, abortion and mummification (Woollen *et al.* 1990; Schiller *et al.* 1997b; Camenisch *et al.* 2004a; Kauffold *et al.* 2006b; Schautteet *et al.* 2010; Schautteet *et al.* 2013). In boars, *C. suis* infections have been related to inferior semen quality, orchitis, epididymitis and urethritis (Sarma *et al.* 1983; Schautteet and Vanrompay 2011). However, given the limited number of data on experimental genital tract infections with *C. suis* in pigs, the exact role of *C. suis* in reproductive disorders remains largely elusive. Finally, *Chlamydiae* have also been linked to arthritis, pericarditis and polyserositis in piglets (Willigan and Beamer 1955).

5.3.5 Transmission routes

Although the pathogenicity of *Chlamydiae* in pigs has been demonstrated, the insight into the transmission route of porcine chlamydial infections is currently limited. The pig intestine is assumed to be a reservoir of chlamydial infections. Indeed, fecal shedding of *Chlamydiae* might contribute to oral-fecal transmission or airborne transport of germs on fecal particles. Moreover, chlamydial transmission might also occur through exchange of body fluids, like excretion from the eyes and nose (Becker *et al.* 2007).

5.4 Clinical importance of porcine *Chlamydiaceae* infections

The pathogenicity of chlamydial species in pigs has been demonstrated in various experimental infections, as described above, and natural chlamydial infections in pigs have been associated with numerous clinical manifestations and economic losses (Willigan and Beamer 1955; Sarma *et al.* 1983; Woollen *et al.* 1990; Zahn *et al.* 1995; Andersen 1998; Eggemann *et al.* 2000b; Schautteet *et al.* 2013) However, *Chlamydiae* are frequently detected in clinically healthy animals, and the vast majority of chlamydial infections, especially intestinal infections, is believed to be subclinical (Pospischil and Wood 1987; Szeredi *et al.* 1996; Nietfeld *et al.* 1997; Hoelzle *et al.* 2000; Camenisch *et al.* 2004a; Englund *et al.* 2012), Therefore, *Chlamydiaceae* are assumed to be endemic in commercial pigs. The ambiguous association between infection and clinical disease have raised questions whether

Chlamydiaceae are actual pathogens or commensals (Reinhold *et al.* 2011b). The occurrence of clinical disease upon infection was suggested to depend on the virulence and infectious dose of the chlamydial agent, and the age and immunological status of the host (Leonhard *et al.* 1988; Szeredi *et al.* 1996; Englund *et al.* 2012). Additionally, environmental factors may contribute to the emergence of chlamydial disease. Overcrowding in pig herds induces stress, which can lead to immunosuppression, thereby pre-disposing pigs towards chlamydial infection or a more severe manifestation of infection (Becker *et al.* 2007). Moreover, several cases of pathogenic interaction involving *Chlamydiae* have been reported. Schautteet *et al*. (2010) described a concurrent outbreak of chlamydial disease in boars, sows and gilts and postweaning multisystemic wasting syndrome (PMWS) in weaned piglets on an Estonian pig farm, attributed to co-infection with *C. suis*, *C. abortus* and porcine circovirus type 2 (PCV-2). Carrasco *et al*. (2000) also reported the co-infection of enterocytes with PCV-2 and *Chlamydia* species, associated with intestinal lesions in a 12-weeks-old pig. Therefore, these pathogens might trigger each other's pathology. Moreover, a pathogenic interaction between *Chlamydia* and porcine epidemic diarrhea virus (PEDV) was suggested, based on experimental infections *in vitro* (Stuedli *et al.* 2005) and *in vivo* (Grest *et al.* 2000). Furthermore, Pospischil *et al.* (2009) suggested a synergistic effect between *Salmonella typhimurium* and *C. suis* in the infection of enterocytes in the swine intestine. Co-existence of these organisms might alter their destructive or invasive capacity, possibly leading to a more severe clinical manifestation compared to single infections of these organisms (Pospischil and Wood 1987). Similarly, the coccidium *Eimeria scabra* was suggested to enable *Chlamydiae* to invade and develop in enterocytes (Koudela *et al.* 1990). Moreover, Becker *et al.* (2007) demonstrated the synergistic effect of *Amoebae* and *C. suis* infection in intensive pig farming, leading to serious ocular manifestations. Thus, although C*hlamydiae* are frequently detected in clinically healthy animals, clinical signs of infection may appear through synergistic interaction with other pathogenic bacteria, resulting in clinical disease. Moreover, enteric *Chlamydiae* infections, often subclinical, may induce intestinal lesions that enhance susceptibility for other enteropathogenic agents (Guscetti *et al.* 2009). Therefore, subclinical infections may be more harmful than generally assumed.

Interestingly, co-infection with other bacteria can induce persistent chlamydial infections, as shown for *C. abortus, C. pecorum* and PEDV, at least *in vitro* (Borel *et al.* 2010). A similar induction of chlamydial persistence *in vitro* was demonstrated for co-infection of *C. trachomatis* with herpes simplex virus type 2 (HSV-2) (Deka *et al.* 2006). During persistence, persistent or 'aberrant' RBs are formed, in which DNA replication and protein

synthesis proceeds, but cell division no longer occurs, as described above. As a result, a small number of very large RBs are present in the inclusions, leading to a chronic infection. These persistent bodies are non-culturable, and insensitive to antibiotic treatment. Other factors known to induce persistence include antibiotics, nutrient deprivation or immune factors, such as interferon gamma (IFN-ɤ) (Mpiga and Ravaoarinoro 2006). Pospischil *et al.* (2009) reported the occurrence of aberrant bodies *in vivo,* in intestinal tissue derived from pigs naturally and experimentally infected with *C. suis*. The high prevalence of subclinical *Chlamydiae* infections in pigs, especially in the intestines, and the occurrence of persistent infections, obscure their potential pathogenic effects (Reinhold *et al.* 2011b). Moreover, although *Chlamydiaceae* have been associated with various chronic diseases in pigs, they are often found together with other pathogens, which make it difficult to attribute the observed pathology to chlamydial infections alone. Furthermore, although the pathogenicity has been demonstrated in pigs, the reported virulence of *C. suis* in pigs appears to be highly variable (Bush and Everett 2001), which may be the consequence of the high degree of genetic diversity in *C. suis* compared to other chlamydial species (Everett *et al.* 1999; Bush and Everett 2001). Currently, the general view is that *Chlamydiae* may act in concert with other potentially pathogenic agents in multifactorial infectious diseases, such as diarrhea in pigs, abortions in sows and genital disorders in boars (Sachse *et al.* 2009).

5.5 Diagnosis of *Chlamydiaceae* in pigs

The diagnosis of chlamydial infections is generally based on (1) the direct detection of the bacterial agent or bacterial components, or (2) screening for *Chlamydiaceae*-specific antibodies produced by the host. The preferred test is dependent upon the type of sample, the viability of the organism in the specimen, potential presumptive diagnosis based on clinical symptoms and pathology, and the clinical history.

Concerning chlamydial diagnosis, **isolation** of the pathogen is historically considered as the ‘gold standard’. Since *Chlamydiaceae* are obligate intracellular organisms, a host system is required to propagate these bacteria. At first, *Chlamydiae* were cultivated in mice and especially developing chicken eggs (Burnet and Rountree 1935; Stamp *et al.* 1950; Tang *et al.* 1957). Therefore, a 10% sample suspension is inoculated into the yolk sac of 6-to-8-day-old embryos, which then die between 4 and 14 days p.i. To verify replication of *Chlamydiae*, smear of the yolk-sac membrane can be prepared and stained using various procedures, including modified Ziehl-Neelsen (MZN) or Giemsa staining, to demonstrate EBs (Stamp *et*

al. 1950). However, egg culture is an expensive and time consuming procedure, lacks reproducibility, and its sensitivity can vary upon chlamydial species and subtype (Sachse *et al.* 2009). Moreover, the regulations concerning experimentation on live embryos have become stricter, and appropriate facilities and expertise are required. Although still used for massive production of antigens, or propagation of fastidious strains, egg culture is largely replaced by cell culture. Various cell lines have been used to propagate *Chlamydiae,* yet the most suitable cell line for chlamydial isolation is species dependent. In case of *C. trachomatis* several cell types can be used, but McCoy, Buffalo Green Monkey Kidney (BGM), and Hela 229 cell lines appear to be most susceptible (Wills *et al.* 1984; Barnes 1989; Thewessen *et al.* 1989; Johnston and Siegel 1992). Similarly, *C. psittaci* grows easily in many cell types, but BGM, African green monkey kidney (Vero), McCoy, HeLa and L cells are commonly used for direct inoculation (Vanrompay *et al.* 1992). For some *C. suis, C. abortus* and *C. pecorum* strains, especially from porcine origin, isolation can be challenging. Little information is available on the culture of these species. Schiller *et al.* (2004) studied the growth characteristics of porcine chlamydial strains, in different cell culture systems. According to this study, human colonic adenocarcinoma cells (Caco) are the most appropriate for isolation of problematic *C. suis* and *C. pecorum* strains. Other cell lines frequently used to propagate laboratory strains of *C. suis* include HeLa, BGM, Vero and McCoy (Rogers *et al.* 1996; Rogers and Andersen 2000; Lenart *et al.* 2001; Sachse *et al.* 2004). Besides the cell line, the infection rate of *Chlamydiae* in cell monolayers is also influenced by the inoculation procedure. Centrifugation (1000 to 3000 x g, 1 h, 30 to 37°C) following inoculation is generally used to enhance the attachment of *Chlamydiaceae* to the cells, increasing the infection rate up to a 1000-fold (Moulder 1991; Schiller *et al.* 2004). Moreover, chemicals blocking host cell replication and metabolism, such as cycloheximide (1-5 µg/ml), may be added to the growth medium during infection to limit the host cell utilization of energy. However, the extent of the effect of cycloheximide treatment on chlamydial replication is species dependent (Schiller *et al.* 2004). Overgrowth of fungi or non-chlamydial bacteria is impeded through addition of antifungal compounds and antibiotics, including gentamycine (10-50 µg/ml) and vancomycin (100 µg/ml), which do not affect chlamydial growth (Sachse *et al.* 2009). After inoculation, the cell cultures are incubated at 37°C for 2-6 days, depending on the species, but an additional passage is frequently applied. Then, inoculated coverslip-monolayers can be fixed and stained, using Giemsa or immunofluorescence staining, for detection of chlamydial inclusions. Careful sampling should avoid contamination with bacteria that can interfere with the isolation of *Chlamydiae*. Although the isolation procedure

has a substantial impact on the infection rate of the cells, successful isolation of chlamydial organisms from biological samples is largely dependent on proper transport and storage of the biological samples, to preserve the viability of the organisms (Sachse *et al.* 2009). Transport of samples at 4°C in a suitable transport medium, such as sucrose-phosphate-glutamate (SPG) supplemented with foetal bovine serum, is recommended (Spencer and Johnson 1983). Although isolation in cell culture has a limited sensitivity compared to nucleic acid amplification tests (NAATs) (Sandoz and Rockey 2010), among other limitations, isolation remains crucial to assess the viability of field strains and to characterize new individual isolates (Sachse *et al.* 2009). However, chlamydial isolation can be challenging in case of persistent infections, when *Chlamydiae* enter a viable but nonculturable state, as described above. This persistent state is induced upon exposure to stress factors, including antibiotics or deprivation of amino acids (Chopra *et al.* 1998; Hogan *et al.* 2004). Persistent RBs are formed, in which DNA replication proceeds but cell division halts, resulting in a prolonged infection refractory to antibiotic treatment (Sandoz and Rockey 2010). Persistence can thus easily be confused with antibiotic resistant infections, or generate false negative results.

Immunohistochemical staining on histological sections is regularly used for diagnostic or epidemiologic examination, and pathogenesis studies (Juvonen *et al.* 1997; Tsakos *et al.* 2001; Hotzel *et al.* 2004; Navarro *et al.* 2004; Borel *et al.* 2006a; Borel *et al.* 2006b). Monoclonal antibodies directed against surface antigens, including LPS or MOMP, are commonly used for detection. These antibodies are either directly linked to the enzyme horseradish peroxidase (HRP), or indirectly detected using a fluorescein-conjugated secondary antibody (Szeredi *et al.* 1996; Buxton *et al.* 2002). In addition to immunodetection in histological sections, various **immunoassays** were developed for detection of chlamydial antigens in clinical specimens. Most of these assays are designed for detection of *C. trachomatis* in human samples. Since these tests target family-specific LPS antigens, other chlamydial species could be detected as well. However, family-specific antigens do not allow species identification. Generally, immunoassays exist in various configurations, including fluorescent antibody tests (DFA), plate-based ELISAs and solid-phase ELISAs (Sachse *et al.* 2009). However, depending on the specimen type, immunoassays can vary in terms of sensitivity and specificity (Eggemann *et al.* 2000a; Bagdonas *et al.* 2005). Hence, molecular or serological methods are generally preferred for chlamydial diagnosis in animals.

More recently, molecular methods were developed for **detection of chlamydial nucleic acids** from tissue or swab samples, which remarkably improved the sensitivity and specificity of

chlamydial diagnosis. Numerous PCR tests currently exist, although part of these tests lack proper validation. Species identification of *Chlamydiaceae* in pigs through PCR and subsequent sequencing is widely used and mainly targets 16S and 23S rRNA signature sequences (Everett *et al.* 1999; Becker *et al.* 2007; Englund *et al.* 2012; Di Francesco *et al.* 2013), as well as the *omp2* and *ompA* genes (Schiller *et al.* 1997a; Hoelzle *et al.* 2000; Kauffold *et al.* 2006b). However, more sensitive species-specific NAATs have recently been developed. Sachse *et al.* (2005) created a 23S rRNA gene based microarray hybridization assay, enabling detection of nine chlamydial species in a single assay. The microarray procedure consists of a biotinylation PCR, amplifying a 1 kbp fragment of the rRNA operon, followed by hybridization of the PCR product to a plastic tube-integrated microchip, containing 11 different probes. Detection is performed using a HRP-streptavidin conjugate, which enables signal amplification. Species identification is performed based on the obtained pattern of coloured spots. Pantchev *et al.* (2010) developed real-time PCR assays for detection of chlamydial species of veterinary importance, including the four species occurring in pigs. For *C. psittaci*, *C. pecorum* and *C. abortus*, the *ompA* gene was targeted, while for *C. suis* the 23S rRNA gene was selected to generate a species specific test. However, this *C. suis* specific real-time PCR was developed for veterinary purposes, and also amplifies *C. trachomatis* DNA. Recently, a 23S rRNA based *C. suis* specific real-time PCR was developed which can differentiate between of *C. suis* and *C. trachomatis,* and is therefore suited for diagnosis of *C. suis* in human samples (De Puysseleyr *et al.* 2014b). Furthermore, real-time PCR assays based on the ompA and 16S rRNA genes have already been used (Schautteet *et al.* 2012) for detection of *C. abortus* (Livingstone *et al.* 2009), *C. psittaci* (Geens *et al.* 2005) and *C. pecorum* (Wan *et al.* 2011) in pig samples.

Serological examination of animal sera, to detect anti-chlamydial antibodies, has been widely used to estimate the prevalence of chlamydial infection in pigs. Since an antibody response may result from a present or prior infection, serology is less suitable in discriminating infected and vaccinated animals. Moreover, a lag period of at least one to two weeks occurs between infection and the appearance of an antibody response. Furthermore, sera of piglets may contain maternal antibodies. Therefore, serology and infection status are not consistently correlated, which might complicate the interpretation of serology results. In addition, the antibody response can vary highly within a pig herd, being also dependent on the age of the animal. Therefore, sampling of multiple animals of different age groups results in a more realistic estimation of the infection status of the herd. Moreover, sampling at multiple

time points enables the detection of titer changes, which are a more accurate indication of infection. Nevertheless, serology has important advantages, since sample collection and transport are relatively straight forward, and the timing of sampling is less critical.
Anti-chlamydial antibodies are commonly captured using inclusions, EBs or chlamydial antigens. Subsequent detection of the bound antibodies is obtained through the evaluation of the consumption of complement (complement fixation test), or through fluorescently labeled secondary antibodies (indirect immunofluorescence and micro immunofluorescence (MIF) tests). Furthermore, other tags for the secondary antibodies are available, including the horseradish peroxidase ezyme (indirect enzyme-linked immunosorbent (ELISA) tests) (Sachse *et al.* 2009).
Several serological assays have been developed to assess the chlamydial seroprevalence in pigs, such as an LPS-based ELISA assay (Wittenbrink 1991), or an ELISA assay based on the recombinant MOMP of *C. psittaci* (Vanrompay *et al.* 2004). In addition, also the LPS-based complement fixation test is widely used to detect anti-chlamydial antibodies in pig sera (Szeredi *et al.* 1996; Rypula *et al.* 2014a). However, these assays are unable to identify the chlamydial species occurring in swine. Moreover, results obtained based on detection of chlamydial LPS or EBs should be interpreted with caution, since cross reactions with antibodies against other pathogens may occur (Caldwell and Hitchcock 1984; Nurminen *et al.* 1984; Brade *et al.* 1987; Yuan *et al.* 1992). A serological assay for the specific and sensitive detection of antibodies produced in response to *C. suis* infection, the major chlamydial species occurring in pigs, is currently unavailable.

5.6 Treatment and Prevention

Chlamydial infections in livestock and humans are primarily treated with tetracycline (Tc) and derivatives (chlortetracycline, oxytetracycline, doxycycline), since they have a low cost, broad spectrum of activity, a low toxicity and an excellent tissue distribution (Chopra and Roberts 2001; Michalova *et al.* 2004). Tetracyclines interfere with the binding of aminoacyl tRNAs on the ribosome, and thus impede bacterial protein synthesis. Besides, also macrolide antibiotics, including azithromycin which also interferes with protein synthesis, are frequently used to treat chlamydial infections in humans, but are more expensive (Martin *et al.* 1992; Rose 1998). However, cases of treatment failure have already been described (Johnson and Spencer 1983; Jones *et al.* 1990; Andersen 1998; Lefevre and Lepargneur 1998; Misyurina *et al.* 2004; Di Francesco *et al.* 2008) and frequently heterotypic resistance, in which only a

small portion of the population displays the resistant phenotype, is observed. In these cases, it is not always clear whether persistence or actual antibiotic resistance is involved. Drug resistance frequently arises through point mutations, altering the expression or the functionality of the antibiotic target, or through insertion of resistance genes into the bacterial genome, as summarized by Sandoz & Rockey *et al*. (2010). Until recently, it was generally believed that the acquisition of antibiotic resistance in *Chlamydia* spp. through lateral gene transfer from other organisms was limited, due to their obligate intracellular life cycle. However, since 1998, tetracycline resistant (Tc^R) *C. suis* strains have been isolated in the U.S., Italy, Cyprus, Germany, Israel, Switzerland and Belgium (Andersen 1998; Di Francesco *et al.* 2008; Borel *et al.* 2012; Schautteet *et al.* 2012). Dugan *et al*. (2004; 2007) demonstrated that stable Tc resistance in *C. suis* is associated with the presence of the *tet(C)* resistance gene. In all examined *C. suis* strains, the *tet(C)* gene is integrated in the *inv*-like gene, of which the function is still elusive. Moreover, *in vitro* studies also demonstrated the transfer of the *tet(C)* gene within and among chlamydial species, including *C. suis, C. trachomatis* and *C. muridarum*, and into clinical isolates from human patients with *C. trachomatis* (Suchland *et al.* 2009). Infections with Tc^R *C. suis* strains can be treated with quinolones, such as enrofloxacin, or macrolides, such erythromycin. However, these antibiotics are more expensive compared to Tc, and Tc^R *C. suis* strains are often resistant to multiple antibiotics frequently used to treat chlamydial infection, such as azythromycin (Lenart *et al.* 2001).

Although the exact mechanism through which *C. suis* acquired the Tc resistance gene *tet(C)* is still unresolved, the addition of antibiotics into animal feeds has promoted the selection of resistant organisms. Although the use of antibiotics as growth promoters is currently no longer allowed in Europe, the supplementation of feeds with antibiotics, especially Tc, was widespread in the poultry, porcine and live stock industry, to promote growth and counter bacterial infections (Sarmah *et al.* 2006; Castanon 2007; Moulin *et al.* 2008; Dewulf *et al.* 2012; Dewulf *et al.* 2013). Considering the high prevalence of Tc resistance in porcine *C. suis* isolates (Schautteet and Vanrompay 2011), it might be also the case in other meat producing industries, resulting in treatment difficulties and potentially severe economic losses. Moreover, perhaps more importantly, there is a potential risk for public health. Contact between Tc^R and Tc sensitive (Tc^S) *Chlamydia* spp. in different settings, including farms, veterinary clinics and slaughterhouses, may lead to transfer of the resistance gene and associated phenotype, which could then be propagated and selected for in patients treated with Tc. This event would interfere with treatment of chlamydial infections, resulting in more severe complications and even a higher mortality rate. In order to combat pathogenic bacteria

which are untreatable using conventional antibiotics, alternative therapies should be developed. Preferably, virulence factors, traits indispensable for pathogenic characteristics, are targeted, rather than merely killing the bacteria. Interestingly, Pollman *et al*. (2005) described a probiotic strain of *Enterococcus faecium* (NCIMB 10415), which reduces the transmission of *Chlamydiaceae* infections from sows to newborn piglets. This strain is licensed by the European Union as an animal feed supplement.

The most optimal approach to protect pigs against *Chlamydiaceae* infections would be vaccine development. However, currently, no vaccines are commercially available. Yet, a few preliminary studies with promising results have been performed. Immunization of breeding sows with an inactivated *C. abortus* strain (OCHL03/99), isolated from vaginal discharge of sows, elicited a primary and secondary IgG serum antibody response (Knitz *et al.* 2003). Moreover, co-vaccination of an *omp1* DNA vaccine and recombinant MOMP lead to a protective immune response against *C. abortus* infections in mice (Zhang *et al.* 2009). Interestingly, De Clercq *et al.* (2014) demonstrated that initial vaginal *C. suis* infection creates partial protection against re-infection. These findings are promising for the development of an effective vaccine.

5.7 Zoonosis

Chlamydiae are highly prevalent microorganisms, infecting a wide range of animal species. *Chlamydia trachomatis* and *C. pneumoniae* are well characterized human chlamydial pathogens. *Chlamydia trachomatis* is the most common cause of bacterial sexually transmitted disease in humans (Bebear and de Barbeyrac 2009), but also induces ocular infections, leading to trachoma and possibly infectious blindness, in millions of people in developing countries (Mabey 2008). *Chlamydia pneumoniae* frequently causes respiratory infections in humans and is possibly associated with atherosclerosis (Grayston 1999). Besides, animals can be a possible zoonotic source of other chlamydial infections in humans. Two of the species occurring in pigs, *C. psittaci* and *C. abortus*, are prominent examples of zoonotic infections. The zoonotic transmission of *C. psittaci* from birds to humans through aerogenic transmission is well described. The clinical outcome of these infections can vary from inapparent to severe pneumonia, with possible fatal outcome without treatment. As mentioned earlier, potentially fatal systemic infections, miscarriages and stillbirths in pregnant women after exposure to infected sheep or goats have been attributed to zoonotic *C. abortus*

infections (Buxton 1986; Kampinga *et al.* 2000; Pospischil *et al.* 2002; Walder *et al.* 2003; Meijer *et al.* 2004; Walder *et al.* 2005).

However, data on cases of zoonotic transmission of porcine chlamydioses and possible modes of transmission to humans are scarce. Interestingly, *C. suis*, the chlamydial species most prominently present in pigs, is phylogentically highly related to *C. trachomatis* (Everett *et al.* 1999). *Chlamydia suis* is frequently detected in the eyes of pigs with conjunctivitis, resembling the ocular infection caused by *C. trachomatis* in humans. Moreover, Dean *et al*. (2013) identified *C. suis* in eye samples of trachoma patients in Nepal. Furthermore, the evaluation of the zoonotic transmission of *C. suis* in a Belgian pig slaughterhouse, identified two human isolates in clinically healthy employees (De Puysseleyr *et al.* 2014a). Therefore, *C. suis* might have zoonotic potential.

6 BLOCKING CHLAMYDIAL VIRULENCE

Virulence blockers can be defined as compounds that specifically target virulence determinants of pathogenic bacteria, thereby preventing the bacteria to colonize the host and allowing the host immune system to clear the infection. As most of these blockers do not directly kill the bacteria -they disarm rather than destroy- it is presumed that the evolutionary pressure for the development of resistant strains is smaller than with classic antibiotics. Popular targets include biofilm formation, bacterial toxins, specialized secretion systems, organism-specific virulence gene expression or cell-to-cell signalling, as Rasko and Sperandio (2010) elegantly reviewed. For the purpose of this review, we will focus on possible mechanisms and compounds that may efficiently block different stages in the chlamydial life cycle.

6.1 Inhibition of adhesion

The very first interaction between bacteria and their host cell is the process of adhesion to the cell membrane. Therefore, in order to effectively prevent bacterial colonization of the host, one could already prevent attachment of the pathogen to the host cell membrane. For most bacteria, adhesins such as fimbriae (Type1 and 4 pili) or adhesive autotransporters have been described. Assembly of these pili by the chaperone/usher pathway can be effectively blocked by treatment with so-called pilicides (Aberg and Almqvist 2007). However, the adhesion mechanism in chlamydial species remains rather elusive, and pili do not seem to be involved. Research should therefore focus on already characterized chlamydial adhesins such as MOMP and the pmp-proteins. Such adhesins could effectively be blocked by specific antibodies,

thereby neutralizing chlamydial infectivity and reducing colonization by blocking chlamydial attachment to epithelial cells. In this respect, it has been shown that monoclonal antibodies against MOMP could neutralize chlamydial infection *in vitro* (Peeling *et al.* 1984; Peterson *et al.* 1991) and could provide a modest level of protection against infection when administered passively to mice (Cotter *et al.* 1995). Similarly, antibodies specific to PmpD of *C. trachomatis* and *C. pneumoniae* and Pmp2 and 10 of *C. pneumoniae* were shown to be neutralizing, at least *in vitro* (Wehrl *et al.* 2004; Finco *et al.* 2005; Crane *et al.* 2006). As described above, heparin sulphate-like glycosaminoglycans are also involved in the chlamydial attachment process. Monoclonal antibodies specifically directed against heparan sulphate specifically bind glycosaminoglycans localized to the surface of *C. trachomatis* and *C. pneumoniae* and effectively neutralize their infectivity (Rasmussen-Lathrop *et al.* 2000). However, evidence exists that chlamydial bacteria most likely use different mechanisms of attachment to the host cell (see above), rendering the development of a general anti-adhesion therapy that would completely block chlamydial attachment unlikely.

The immune system of the host provides protection against potential pathogens. The innate immune response serves as first line defense against infection, and is constitutively present and rapidly mobilized upon infection. Therefore, innate immunity is essential to prevent and control the invasion of pathogens (Wira *et al.* 2005). The primary defense mechanism at mucosal surfaces is the mucosal barrier, containing bacteriocidal and bacteriostatic molecules, such as lysozyme and defensins (Quayle 2002; Ganz 2003). Furthermore, commensal bacterial species have a protective function. However, when *Chlamydia* passes through the mucosal barrier, the innate immune effectors provide the next line of defense against invading bacteria. The innate immune system can recognize microbial structures, which are foreign to the host, referred to as pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway 2000). The PAMPs are recognized by the pattern recognition receptors (PRRs), expressed on innate immune cells, including macrophages and dendritic cells. A major group of PRRs are the Toll-like receptors (TLRs). The TLR2 and TLR4 receptors have been shown to be involved in anti-chlamydial host defense. The chlamydial components recognized by TLR4 comprise LPS and Hsp60. However, intact chlamydial organisms can stimulate innate immune cells independently of TLR4. The TLR2 receptor colocalizes with the intracellular chlamydial inclusion, and is likely involved in signaling from this intracellular location. Several components have been suggested as ligands for TLR2, including bacterial lipoproteins, lipopeptides, and bacterial porins (Joyee and Yang 2008). Also TLR9 has been suggested to modulate immune responses in chlamydial infection. Moreover, other PRRs,

such as CD14 or NOD1 (nucleotide-binding oligomerization domain-containing protein 1) are probably involved in the recognition of PAMPs (Welter-Stahl *et al.* 2006; Bas *et al.* 2008; Buchholz and Stephens 2008). The binding of PAMPs on TLRs results in the activation of signaling cascades, leading to expression of effector molecules, such as cytokines and chemokines, which play a crucial role in the activation of the adaptive immune responses. Therefore, TLRs function as a link between the innate and adaptive immune response. The molecular mechanisms mediated by TLRs might be exploited to develop therapeutics or vaccine adjuvants (Joyee and Yang 2008). Indeed, several studies have demonstrated that stimulating TLR9 activity can induce beneficial responses for host protection from chlamydial infection (Bandholtz *et al.* 2002; Pal *et al.* 2002). Although strictly not considered as virulence blocking strategy, TLR activation may be an important mechanism by which vaccines lead to protective immunity. Further understanding of TLR signaling events and the underlying mechanisms to protective immunity would aid in targeted manipulation of immune responses to control chlamydial diseases.

6.2 Inhibition of internalization

As described above, *Chlamydiae* induce actin recruitment to the site of infection, followed by a localized and temporary nucleation, to facilitate uptake into host cell membrane-bound vesicles. The most straightforward way to block internalization would therefore be to interfere with this polymerization by treatment with molecules such as cytochalasin, latrunculin, phalloidin, taxol or colchicines (Peterson and Mitchison 2002). However, as actin is also implicated in other cellular functions such as cell shape or cell migration, the side-effects of a similar treatment would be considerable. One would therefore have to focus on the process of endocytosis itself to prevent *Chlamydiae* from invading the host cells. Research could be directed towards toxins used by pathogenic bacteria such as *Yersinia* spp. to prevent phagocytosis. Especially proteins such as *Yersinia* YopH and YopE and *Pseudomonas (P.) aeruginosa E*xoS and ExoT, interacting with small GTPases, which are also implicated in chlamydial internalization, could be of interest. These proteins, which are T3S substrates, convert Rho family members in an accelerated manner to their GDP-bound, inactive states and thus inhibit endocytotic processes (Ernst 2000).

6.3 Blocking bacterial proliferation

Although bacterial proliferation is no virulence determinant *sensu strictu*, processes currently not targeted by classical antibiotics could open possibilities for the generation of novel antibacterials. Likewise, interest increases in the FtsZ protein as therapeutic target in the antimicrobial research field. This protein is essential for bacterial cell division and thus targeting FtsZ would lead to disruption of cell division and therefore bacterial infection (Awasthi *et al.* 2011). However, as mentioned earlier, *Chlamydiaceae* do not possess an ftsZ-ortholog. Nevertheless, some interesting alternative mechanism to inhibit bacterial proliferation exist, such as the limitation of Fe^{3+} availability, which is crucial in the bacterial metabolism and biofilm formation (Raulston 1997; Cianciotto 2007).

The *Chlamydiaceae* proliferate predominantly in epithelial cells and macrophages (Vanrompay *et al.* 1995). The latter play an important role in the clearance of aged and apoptotic cells and are therefore continuously exposed to high intracellular iron loads. Though the mode of Fe^{3+} scavenging from the environment by *Chlamydia* and other intracellular bacteria such as *Legionella* or *Mycobacterium*, is largely undefined, the cytosolic iron pool is most likely the source. Accordingly, depletion of cytosolic iron could limit the growth of intracellular bacteria. This concept is demonstrated by the incubation of *C. psittaci*– or *L. pneumophila*–infected mouse macrophages with iron chelators deferriprone or desferasirox which results in a reduced level of bacterial infections (Paradkar *et al.* 2008). Both compounds, deferriprone and desferasirox, have previously been approved for human use. They are membrane permeable as they can remove iron from iron loaded macrophages (Paradkar *et al.* 2008). This new generation of chelators has great therapeutic potential for treatment of persistent bacterial infections.

An alternative strategy to limit intracellular Fe^{3+}-levels is the use of the ‘Trojan horse’ transition metal gallium (Ga^{3+}), an ion chemically similar to iron. Unlike Fe^{3+}, it does not undergo redox reactions and thus cannot execute the cellular functions of Fe^{3+} within the bacterial cell (Chitambar and Narasimhan 1991). Through competition with Fe^{3+}, gallium decreases thus bacterial iron uptake. Consequently, the iron need of the bacteria is not fulfilled and bacterial growth is inhibited. Furthermore, gallium proved to be effective both *in vitro* and *in vivo* in treatment of *P. aeroginosa* infections in rabbit and mouse models (Kaneko *et al.* 2007; Banin *et al.* 2008) and is already approved by the Food and Drug Administration for use in large doses to treat hypercalcemia of malignancy (Warrell and Bockman 1989). All together, this hints gallium as a promising treatment strategy in bacterial infections.

6.4 Inhibition of nucleotide transport

As mentioned above, *Chlamydiaceae* scavenge energy molecules from the host using NTTs. These proteins not exclusively constitute bacterial membranes, but are similarly essential to plant chloroplasts where they participate in the import process of cytosolic ATP under certain conditions (Winkler and Neuhaus 1999; Linka *et al.* 2003). Interestingly, the bacterial and plant transporters do not exhibit structural similarity with mitochondrial and peroxisomal adenylate transporters, belonging to the mitochondrial carrier (MC) family. (Klingenberg 1989; Saier 2000; Ren *et al.* 2004). Hence, NTTs are absent in mammalian and human cells and thus represent an attractive target for the development of highly specific anti-chlamydial drugs. However, how the highly charged ATP molecules pass the inclusion membrane to reach the bacteria is unknown so far, as pores for passive diffusion are absent in the inclusion membrane (Heinzen and Hackstadt 1997). As earlier stated, the genome of *C. trachomatis* and *C. pneumoniae* contains genes that might encode enzymes involved in ATP generation (Stephens *et al.* 1998; Kalman *et al.* 1999). However, *Chlamydiae* also possess ATP/ADP anti-porter genes, probably acting to import ATP from the host cell early in the infection cycle, when the enzymes for ATP generation are not present yet (Shaw *et al.* 2000). Since NTTs are not present in human cells, and energy parasitism in the initial phase of the infection process is crucial for the survival of *Chlamydia*, blocking this transport process could be a specific and efficient strategy in controlling chlamydial infections. To our knowledge, there are currently no inhibitors of chlamydial nucleotide transport identified.

6.5 Inhibition of the Type III secretion system

As described above, T3S is involved in different stages of the chlamydial life cycle and mediates translocation of virulence related effector proteins to the host cell cytoplasm (Beeckman and Vanrompay 2010). Consequently, chlamydial disease might be effectively treated by either blocking T3S or inhibiting the interaction with the eukaryotic host. In recent years, several studies describing small molecules specifically inhibiting T3S have been published (Kauppi *et al.* 2003; Keyser *et al.* 2008). These inhibitors have been identified through mass screening of chemical libraries using whole-cell reporter gene assays or ELISA to assess inhibition of T3S and included salicylideneacylhydrazides, salicylanilides, sulfonylaminobenzanilides, salicylideneanilides, phenoxyacetamides, thiazolidones and N-hydroxybenzimidazoles (Keyser *et al.* 2008; Aiello *et al.* 2010; Escaich 2010). In the *Chlamydia* research community, research has predominantly focused on the effects of acylated hydrazones of salicylaldehydes whereby host cell cytokine expression as well as

chlamydial growth and T3S gene expression, but not entry, were shown to be affected at non- or low-cytotoxic concentrations (Muschiol *et al.* 2006; Wolf *et al.* 2006; Bailey *et al.* 2007; Slepenkin *et al.* 2007; Muschiol *et al.* 2009; Prantner and Nagarajan 2009; Chiliveru *et al.* 2010). Wang *et al.* (2011) identified putative target proteins of the salicylideneacylhydrazides, which are involved in the regulation of T3SS gene expression. In addition, the phenoxyacetamide MBX 1641 is capable of inhibiting T3S translocation in *C. trachomatis* infected Hep-2 cells (Aiello *et al.* 2010). Although the exact mode of action has not yet been uncovered, it is very likely that the conserved (structural) elements of the T3SS or its' assembly are targeted, especially given the broad spectrum of bacteria inhibited.

Another strategy is to screen for natural products inhibiting bacterial T3S. Such components have been described, including glycolipids (Linington *et al.* 2002; Linington *et al.* 2006) and transferrins (Gomez *et al.* 2003; Ochoa *et al.* 2003; Ochoa and Clearly 2004; Yekta *et al.* 2010), of which lactoferrin (LF) and ovotransferrin (ovoTF) have proven their potential to inhibit chlamydial infections *in vitro* as well (Beeckman *et al.* 2007). Moreover, ovoTF was shown to efficiently prevent *C. psittaci* infection in experimentally infected SPF turkeys (Van Droogenbroeck *et al.* 2008) and on a commercial turkey farm (Van Droogenbroeck *et al.* 2011). Alternatively, the chlamydial T3S can also be inhibited in a pure mechanical manner. Several studies have been published describing *in vitro* and *in vivo* blockage of T3S using antibodies directed against the translocon adaptor protein LcrV and its analogues in other bacteria (Frank *et al.* 2002; Goure *et al.* 2005; Philipovskiy *et al.* 2005; Gebus *et al.* 2008; Eisele and Anderson 2009; Markham *et al.* 2010; Van Blarcom *et al.* 2010). Whether the LcrV protein is essential in the chlamydial internalization process as well could be studied while infecting epithelial cells and/or macrophages in the presence of anti-LcrV antibodies. If indeed anti-LcrV antibodies could significantly inhibit *C. psittaci* internalization and subsequent replication *in vitro*, one could test whether active immunization with LcrV or passive immunization with anti-LcrV antibodies could provide protection against *C. psittaci* infections *in vivo* as well (Mueller *et al.* 2008). Recently, a chlamydial T3S effector protein (Tarp) was identified as a novel immunodominant antigen in human antisera and immunization with Tarp can induce protective immunity against chlamydial infection and pathology in mice (Wang *et al.* 2009). Information on other T3S effectors in *Chlamydiaceae* is scarce, put potential targets for antibody-mediated inhibition could include the *Chlamydia* protein associated with Death Domains CADD, the serine-threonine kinase Pkn5 or the macrophage infectivity potentiator MIP (Beeckman and Vanrompay 2010).

6.6 Regulation of virulence gene expression -Quorum sensing inhibitors

Blocking QS is increasingly considered as a viable approach for developing therapeutics in the treatment of bacterial infections. The ideal QS inhibitor (QSI) is a chemically stable, low-molecular mass molecule without toxic side-effects on the bacterium or host, and resistant to metabolisation and disposal by the host. It should be specific for the particular regulon and have a significant and similar reduction in expression on all the QS regulon comprised genes, however this is not always the case. The strength of an inhibitor depends on the percentage of QS-controlled genes it targets (Arevalo-Ferro *et al.* 2003; Hentzer *et al.* 2003; Rasmussen *et al.* 2005b). QSIs fall roughly into three categories according to the level of interruption of the signalization: repressors of signal generation, disruptors of the signals or signal molecules and inhibitors of the signal perception. Alternatively, inhibitors are categorized into four different classes: nonpeptide small molecules, peptides, enzymes and antibodies (Pan and Ren 2009).

To our knowledge, no chlamydial QSI compounds are currently known. As there is evidence that *Chlamydiaceae* can sense the redox-state of their environment, blockage or destruction of receptor proteins could be an interesting strategy for therapeutic purposes in this context. One method for receptor blockage is the use of an analogue of the signal molecule. More knowledge about the signal perception is needed to explore this possibility. Generally, a synthetic library of signal molecule derivatives is used to screen for inhibitors. Yet, random compound libraries with natural and synthetic compounds may also be employed (Smith *et al.* 2003b; a; Suga and Smith 2003). In both cases, a screening system and further validation is necessary to be able to identify potential inhibitors. A valuable source for QSI compounds are other bacteria, fungi and plants. These organisms have co-existed for millions of years and some of them probably produce QSI compounds, such as *Penicillium* species for example (Rasmussen *et al.* 2005b). Examples of plants producing QSIs are garlic, carrot, soybean, tomato, among many others (Rasmussen *et al.* 2005a).

Beside the species specific inhibitors discussed above, also broad spectrum inhibitors are already described in literature. Most QS signals only appear in a small number of species. However, certain signaling pathways are common in a range of species, while they are not found in the eukaryotic hosts. A high throughput screen of a library of 150.000 small organic compounds identified the lead structure LED209 (N-phenyl-4-[[(phenylamino) thioxomethyl]amino]-benzenesulphonamide). This non-toxic compound has no effect on pathogen growth but blocks binding of signaling molecules to QseC, thus preventing the autophosphorylation of QseC and consequent activation of virulence genes. LED209 was

tested for its inhibitory effect, and showed a virulence decrease in models of infection for several pathogens both *in vitro* and *in vivo* (Rasko *et al.* 2008). Furthermore, molecular concentrations showed a 10-fold reduction compared to previously characterized virulence inhibitory compounds. LED209 can be considered as the proof of concept that blocking inter-kingdom chemical signaling is a viable strategy to develop novel drugs to control bacterial infection. Unlike the LED209 compound mentioned above, most inhibitors show efficacy *in vitro*, but have not been tested *in vivo* in animal models yet. This partially explains why no QSI is at clinical stage of drug development, and thus no information on their efficacy or toxicity in humans is available. Therefore, more research in the field of QS and *in vivo* testing is required in order to explore the potential, advantages and limitations of QSIs as therapeutics in the control of bacterial infections.

6.7 Conclusion

Antibiotic resistance has been reported in *Chlamydia*. Virulence blockers could fulfill a role in future prevention and/or treatment of *Chlamydia* infections, as they do not directly inhibit the growth of pathogens, but rather target virulence associated processes. Therefore, they are considered to exert a lower selective pressure to develop resistance compared to classic antibiotics. So far, only ovoTF, the avian homologue of mammalian LF, has been tested in an animal (turkeys) model and in veterinary clinical trials. Ovotransferrin efficiently prevented *C. psittaci* respiratory disease in commercially raised broiler turkeys demonstrating its potential for veterinary use.

To our knowledge, anti-virulence strategies for human chlamydial infections have not been implemented in animal models or human clinical trials. Nevertheless, promising virulence blockers such as deferriprone and desferasirox are already approved for human use.

Further *in vivo* testing of innovative candidate virulence blockers as well as *in vivo* testing in animal models and clinical trials is required, not only to assess the potential of *Chlamydia* virulence blockers, but also to study possible limitations and safety.

Chapter II

Transmission of *Chlamydia suis* to pig farmers

This chapter will be published as:
De Puysseleyr, L., De Puysseleyr, K., Braeckman, L., Morré, S.A., Cox, E., and Vanrompay, D. Transmission of *Chlamydia suis* to pig farmers. In preparation.

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Abstract

Chlamydia suis infections are endemic in domestic pigs in Europe, and can lead to conjunctivitis, pneumonia, enteritis and reproductive failure. Moreover, the knowledge of the zoonotic potential of *Chlamydia suis* is limited. Furthermore, the last decades, tetracycline resistant *Chlamydia suis* strains have been isolated, which might interfere with treatment of chlamydial infections in pigs and humans. In this study, the presence of *Chlamydia suis* was examined on nine Belgian pig farms, using a *Chlamydia suis* specific real-time PCR and *Chlamydia* culture in both pigs and farmers. Moreover, farmers were examined using a *Chlamydia trachomatis* PCR. Additionally, the *Chlamydia* isolates were tested for the presence of the *tet(C)* resistance gene. *Chlamydia suis* DNA was demonstrated in pigs on all farms, and eight of nine farmers were positive in at least one anatomical site. None of the farmers tested positive for *Chlamydia trachomatis. Chlamydia suis* isolates were obtained from pigs of eight farms. Nine porcine tetracycline resistant *Chlamydia suis* strains were retrieved, originating from three farms. Moreover, *Chlamydia suis* isolates were identified in three human samples, including one pharyngeal and two rectal samples. These findings suggest further research on the zoonotic transfer of *Chlamydia suis* from pigs to humans.

1 INTRODUCTION

Chlamydiaceae are obligate intracellullar Gram-negative bacteria causing infections in a broad range of animals, including humans. *Chlamydiaceae* infections are assumed to be widespread and considered as endemic in domestic pigs in Europe (Eggemann *et al.* 2000b; Camenisch *et al.* 2004a; Di Francesco *et al.* 2006), including Belgium (Vanrompay *et al.* 2004). Pigs are the only currently identified natural hosts for *Chlamydia (C.) suis.* Additionally, *C. abortus, C. psittaci* and *C. pecorum* have been isolated from pigs (Schautteet and Vanrompay 2011). *Chlamydia psittaci* and *C. pecorum* are of lower significance, although *C. pecorum* DNA was demonstrated in boar sperm samples, fetuses and pig intestinal tissue (Thoma *et al.* 1997; Kauffold *et al.* 2006b). Meanwhile, *C. abortus* and especially *C. suis* seem to be the main species involved in chlamydial infections in pigs, and mixed infections occur regularly, e.g. in the lung and intestine (Szeredi *et al.* 1996; Hoelzle *et al.* 2000). *Chlamydia abortus* causes abortion in pigs (Schautteet and Vanrompay 2011), but has also been detected in lung tissue (Sachse *et al.* 2005). *Chlamydia suis* was associated with conjunctivitis in intensively kept German, Estonian and Swiss pigs (Becker *et al.* 2007; Schautteet *et al.* 2010). Furthermore, *C. suis* was involved in reproductive failure, including return to oestrus in sows and inferior semen quality in boars, on farrow-to-finish herds in Belgium, Cyprus, Estonia, Germany, Israel and Switzerland (Wittenbrink 1991; Eggemann *et al.* 2000b; Hoelzle *et al.* 2000; Camenisch *et al.* 2004a; Schautteet *et al.* 2010). In addition, intestinal *C. suis* infections are believed to be common in Belgian, German and Swiss pigs, albeit the majority without clinical signs (Nietfeld *et al.* 1993; Rogers and Andersen 1996; Szeredi *et al.* 1996).

Chlamydia suis is phylogenetically highly related to the human pathogen *C. trachomatis*, which causes a sexually transmitted disease in humans, but also keratoconjunctivitis and infectious blindness. This close relation suggests that *C. suis* might cause zoonotic infection. Recently, the presence of *C. suis* was demonstrated in eye infections in Nepalese trachoma patients (Dean *et al.* 2013) and Belgian abattoir employees (De Puysseleyr *et al.* 2014b). Hence, also pig farmers, who are daily in close contact to pigs, might be at risk for *C. suis* infection. Therefore, the presence of *C. suis* DNA and viable *C. suis* organisms was examined in pigs and farmers on nine Belgian farms.

Currently, clinical *C. suis* infections in domestic pigs are complicated by treatment failure. Chlamydial infections are routinely treated with relatively inexpensive and effective tetracyclines (Tc) (Chopra and Roberts 2001). However, since 1998, Tc resistance has been discovered in *C. suis* strains in the US and Europe, possibly due to the feeding of Tc not only

to swine, but also to poultry and cattle, as antibiotic growth promoter (AGP) (Chopra and Roberts 2001). The emergence of Tc resistant (Tc^R) *C. suis* strains required the use of more expensive antibiotics such as enrofloxacin (fluoroquinolone), further increasing the economic impact of *C. suis* infections in pigs. Of interest, recent findings suggest that concurrent infections of Tc^R *C. suis* and *C. trachomatis* in an individual treated with Tc, might lead to the emergence of clinical Tc^R *C. trachomatis* strains (Suchland *et al.* 2009). This would impede treatment of human *C. trachomatis* infections. The emergence of Tc^R *C. suis* strains in pigs and its detection in humans might thus present a risk to public health.

The knowledge on the zoonotic potential of *C. suis* is limited, as described above. Therefore, the prevalence of *C. suis* in pigs and farmers, was investigated on nine Belgian pig farms. Furthermore, the occurrence of *tet(C)* transfer *in vivo* remains poorly understood. Thus, all identified porcine and human *C. suis* isolates were examined for the presence of the *tet(C)* gene. Additionally, the farmers were investigated for *C. trachomatis* infection.

2 MATERIALS AND METHODS

2.1 Samples

In the summer of 2011, pigs and farmers (informed consent) in nine voluntarily participating Belgian pig farms located in East- (n = 3) and West-Flanders (n = 6) were examined (Table II-1). On each farm, 10 finishers (finisher pigs, mean age of five months) and five sows (mean age of three years) were sampled, taking a rectal, vaginal, conjunctival and nasal rayon-tipped aluminum-shafted swab (Copan, Fiers, Kuurne, Belgium). All farmers (eight males and one female) were examined by taking a conjunctival, nasal, pharyngeal and stool swab. Sampling in pigs and humans was performed in duplicate, taking one swab for PCR analysis and an additional one for culture. Swabs for PCR analysis were stored in DNA/RNA stabilization buffer (Roche, Mannheim, Germany), whereas the swabs for culture were stored in *Chlamydia* transport medium (2-SP). Farmers also provided one first void morning urine sample. All samples were transported on ice (4°C) and stored at -80°C until tested.

The farmers were questioned about the general health status, use of medication, and clinical signs/history in their pig herd. Furthermore, the farmers filled out a medical questionnaire, designed to assess information on their professional (work environment) and nonprofessional activities, general health status, smoking habits, use of medication, allergies and clinical signs/history.

Pigs were sampled with the farmers' consent for diagnostic purpose. The study was approved by the medical ethical committee of Ghent University (approval EC UZG 2011/459).

Table II-1 Information on investigated Belgian pig farms

Farm	Location	Farm Type	Sperm source for insemination	Antibiotic Treatment*
1	West-Flanders	Farrow-to-finish	Semen center	Tc
2	West-Flanders	Farrow-to-finish	Semen center	Dc
3	West-Flanders	Farrow-to-feeder	Semen center	Tc
4	West-Flanders	Farrow-to-finish	Semen center	Tc
5	West-Flanders	Farrow-to-finish	Semen center	Tucoprim
6	East-Flanders	Farrow-to-finish	Semen center	Tc
7	East-Flanders	Farrow-to-finish	Semen center	Tc
8	East-Flanders	Farrow-to-finish	Semen center	/
9	West-Flanders	Farrow-to-finish	Semen center	Dc

* Within one month before sampling; Tc= tetracycline; Dc= doxycycline

2.2 DNA extraction

DNA extraction on *Chlamydiae* present in DNA/RNA stabilization buffer and *Chlamydia* positive cell culture harvest was performed as described by Wilson *et al*. (1996). Briefly, specimens were centrifuged (13 000 x g), resuspended in 198 µl STD buffer (0.01 M Tris-HCl [pH 8.3], 0.05 M KCl, 0.0025 M MgCl2.6H20, 0.5% Tween20), and 2 µl proteinase K (20 mg/ml stock solution) was added. The specimens were incubated at 56°C for one hour and subsequently heated at 100°C for 10 min. The DNA samples were further purified by extracting them twice with 200 µl phenol:chlorophorm (1:1). Precipitation was performed by adding 20 µl sodium acetate (3M) and 400 µl of 100% ethanol (1 h, 80°C). After centrifugation (20 min, 4 °C, 16 060 x g), pellets were washed for 5 min with 500 µL of 70% ethanol (4 °C, 16 060 x g) and were finally suspended in 30 µl sterile milli-Q water.

DNA extraction on urine samples was performed with the High Pure PCR Template Preparation (HPPTP) Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturers' protocol.

2.3 PCR analysis on pig samples

Pig samples were examined for the presence of *C. suis* using a recently developed, 23S rRNA based *C. suis*-specific real-time PCR (De Puysseleyr *et al.* 2014b). Samples with a Ct-value below 35 were considered positive. Additionally, the samples were tested for the presence of *C. abortus* and *C. pecorum,* using real-time PCR based on the *ompA* and 16S rRNA gene (Livingstone *et al.* 2009; Wan *et al.* 2011), respectively, and for the presence of *C. psittaci* using an *ompA* based nested PCR (Van Loock *et al.* 2005).

2.4 PCR analysis on human samples

Human samples were tested for *C. suis,* using the recently developed, 23S rRNA based *C. suis*-specific real-time PCR (De Puysseleyr *et al.* 2014b) and for *C. trachomatis*, using the CE-IVD certified PRESTO PCR Kit (Goffin Molecular Diagnostics, Houten, The Netherlands) according to the manufacturer's instructions.

2.5 Cell culture and isolation of *Chlamydia*

All animal and human samples were examined for viable *Chlamydia* by inoculation in Vero cells and *Chlamydia* was identified using the Imagen™ immunofluorescence staining (Imagen, Oxoid, United Kingdom), as previously described (Vanrompay *et al.* 1992). The presence of *Chlamydiae* was scored (Lagae *et al.* 2014). Briefly, positive cells were enumerated in five randomly selected microscopic fields (600 x, Nikon Eclipse TE2000-E, Japan) and results were scored from 0 to 5. Score 0 indicated that no *Chlamydiae* were present; score 1 was given if a mean of 1 to 5 non-replicating elementary bodies (EB's) plus maximum one inclusion (EBs and RBs) was observed; scores 2 to 5 were given when observing a mean of 2 to 5, 6 to 10, 11 to 15, > 15 inclusion positive cells, respectively.

2.6 PCR on *Chlamydia* isolates

Chlamydia positive cell culture harvest of pig or human inocula was first examined by the formerly mentioned *C. suis* specific PCR. Subsequently, the presence of the Tc resistance gene *tet*(C) was examined in PCR positives by a *tet*(C) PCR, as described by Dugan *et al.* (2004).

2.7 Statistical Analysis

Using a *C. suis* specific RT-PCR, the number of positive samples (per farm) was determined on both animal (finishers/sows) and sample type (eye, nose, vagina and rectum) level resulting in a contingency table. The number of positive finishers and sows, testing positive in

at least 1 sample type, was compared to each other. Furthermore, we compared the number of positives in both groups for each sample type separately. Finally, for both animal categories separately, the positive counts were compared between the sample types. Comparisons were performed using the chi-squared test. P-values below 0.05 were considered significant ($p<0.05$). The frequency of detection of *C. suis* isolates in pigs was too low to allow statistical comparison.

3 RESULTS

3.1 PCR on pig samples

Swabs were all negative for *C. psittaci*, *C. pecorum* and *C. abortus*. On the other hand, *C. suis* DNA was detected on all farms (Table II-2). The number of positive pigs (finishers plus sows) per farm ranged from 5 of 15 (33%) to 15 of 15 (100%), respectively. The Ct-values varied between 23.42 and 34.82. Each of the four sample types was *C. suis* positive for at least one animal per farm. Overall, 219 of 540 (41%) samples were positive in the *C. suis* real-time PCR.

3.1.1 Animal level

The number of *C. suis* positive finishers per farm ranged from 2 of 10 (20%) to 10 of 10 (100%). The number of positive sows per farm varied from 0 of 5 (0%) to 5 of 5 (100%). *Chlamydia suis* DNA was detected in 69 of 90 (77%) finishers and 24 of 45 (53%) sows. The frequency of positive finishers was significantly higher compared to sows ($p=0.006$). The results per sample type, for finisher pigs and sows separately, are represented below.

3.1.2 Sample type level

For the finisher pigs, 51 of 90 (57%) nasal, 43 of 90 (48%) rectal, 41 of 90 (46%) conjunctival and 39 of 90 (43%) vaginal swabs tested positive. For the sows, 15 of 45 (33%) rectal, 11 of 45 (24%) vaginal, 10 of 45 (22%) nasal and 9 of 45 (20%) conjunctival swabs were positive. For both the finishers and sows, there was no significant difference between the number of positives per sample type ($p<0.05$). However, when comparing finisher pigs and sows per sample type, the number of *C. suis* positive conjunctival ($p=0.004$), nasal ($p<0.001$), and vaginal ($p=0.032$) samples was significantly higher in finisher pigs.

Table II-2 - Results of *Chlamydia suis* real-time PCR analysis on pig swab samples of nine Belgian farms The number of positive samples is represented. The mean infection rate (%) was determined.

Farm	Eye			Nose			Vagina			Rectum			Tot. N° of FP (n=10)	Tot. N° of Sows (n=5)	Tot. N° of Animals (n=15)
	FP (n=10)	**Sows** (n=5)	**Animals** (n=15)	**FP** (n=10)	**Sows** (n=5)	**Animals** (n=15)	**FP** (n=10)	**Sows** (n=5)	**Animals** (n=15)	**FP** (n=10)	**Sows** (n=5)	**Animals** (n=15)			
1	5	1	6	5	2	7	3	0	3	5	1	6	9	3	12
2	0	2	2	1	1	2	1	1	2	0	1	1	2	3	5
3	4	0	4	3	2	5	2	0	2	2	1	3	5	3	8
4	6	1	7	6	0	6	7	0	7	7	0	7	9	1	10
5	5	0	5	10	0	10	5	5	10	5	5	10	10	5	15
6	4	0	4	6	0	6	3	0	3	4	0	4	7	0	7
7	8	0	8	8	0	8	8	0	8	8	1	9	10	1	11
8	8	4	12	10	4	14	7	3	10	5	5	10	10	5	15
9	1	1	2	2	1	3	3	2	5	7	1	8	7	3	10
Total	41	9	50	51	10	61	39	11	50	43	15	58	69	24	93
Mean (%)	45.56	20.00	37.04	56.67	22.00	45.19	43.33	24.44	37.04	47.78	33.33	42.96	76.67	53.33	68.89

FP: Finisher pigs.

3.2 *Chlamydia* culture on pig samples

Isolation of *Chlamydia* was performed on all pig samples. Viable *Chlamydiae* were present on all nine pig farms. Overall, isolation scores varying between 1 and 2 were obtained, corresponding to 1 to 5 non replicating elementary bodies with maximum one inclusion, and 2 to 5 inclusion positive cells, respectively. In total, 62 samples were positive in culture, showing at least one inclusion positive cell, indicating replicating bacteria.

3.3 PCR on porcine *Chlamydia* isolates

The *C. suis* real-time PCR was performed on all *Chlamydia* isolates. In total, 50 *C. suis* isolates were identified across 8 of 9 (89%) farms (Table II-3). The number of positive animals ranged from 0 of 15 (0%) to 10 of 15 (67%). Further, the results for finisher pigs and sows are reported separately.

3.3.1 Animal level

For the finishers, *C. suis* isolates were identified in seven farms, with the number of positive animals ranging from 0 of 10 (0%) to 7 of 10 (70%). For the sows, *C. suis* isolates were identified in five farms, with the number of positive sows ranging from 0 of 5 (0%) to 4 of 5 (80%). In total, *C. suis* isolates were detected in 30 of 90 (33%) finishers and 12 of 45 (27%) sows. The prevalence of *C. suis* isolates in pigs was too low to allow statistical testing. The results per sample type for finisher pigs and sows are represented below.

3.3.2 Sample type level

For the finishers, 13 of 90 (14%) rectal, 11 of 90 (12%) conjunctival, 8 of 90 (9%) vaginal and 5 of 90 (6%) nasal *C. suis* isolates were identified, whereas for the sows, 5 of 45 (11%) rectal, 4 of 45 (9%) conjunctival, and 4 of 45 (9%) vaginal, but no nasal *C. suis* isolates were identified.

The Tc resistance PCR was performed on all isolates. The presence of the *tet(C)* gene was demonstrated in nine different finishers across three farms, and all nine animals were positive for only one sample type. One vaginal, two nasal, three conjunctival and three rectal samples tested positive.

Table II-3 –Results of *Chlamydia suis* real-time PCR analysis on porcine chlamydial isolates. Pigs of nine Belgian farms were examined for the presence of viable bacteria using *Chlamydia* culture. All isolates were further identified with the *Chlamydia suis* specific real-time PCR. The number of positive isolates is represented. The mean infection rate (%) was determined.

Farm	Eye			Nose			Vagina			Rectum			Tot. N° of FP (n=10)	Tot. N° of Sows (n=5)	Tot. N° of Animals (n=15)
	FP (n=10)	**Sows** (n=5)	**Animals** (n=15)	**FP** (n=10)	**Sows** (n=5)	**Animals** (n=15)	**FP** (n=10)	**Sows** (n=5)	**Animals** (n=15)	**FP** (n=10)	**Sows** (n=5)	**Animals** (n=15)			
1	5	0	5	2	0	2	2	0	2	2	0	2	7	0	7
2	2	1	3	2	0	2	1	2	3	3	1	4	7	3	10
3	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1
4	0	1	1	0	0	0	1	1	2	2	1	3	3	3	6
5	2	1	3	1	0	1	2	0	2	2	3	5	6	4	10
6	2	0	2	0	0	0	0	1	1	1	0	1	2	1	3
7	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	1	0	1	3	0	3	4	0	4
Total	11	4	15	5	0	5	8	4	12	13	5	18	30	12	42
Mean (%)	12.22	8.89	11.11	5.56	0.00	3.70	8.89	8.89	8.89	14.44	11.11	13.33	33.33	26.67	31.11

FP: finisher pigs.

3.4 PCR on human samples

The set of human (n=9) samples for *C. suis* detection consisted of nine conjunctival, eight nasal, nine pharyngeal, and seven stool swabs (sample total n=33). Moreover, four farmers delivered a urine sample, for *C. trachomatis* detection. Eight of 9 farmers (89%) tested positive in the *C. suis* real-time PCR for at least one sample type (Table II-4). Moreover, 5 of 8 (62.5%) farmers tested positive for all sample types. Seven of 9 (78%) conjunctival, 7 of 8 (88%) nasal, 7 of 9 (78%) pharyngeal and 5 of 7 (71%) stool samples were positive for *C. suis*. None of the urine samples tested positive for *C. trachomatis*.

Table II-4 - Results of *Chlamydia suis* real-time PCR analysis on human swab samples The number of positive samples is shown.

Farm	Eye	Nose	Throat	Feces	Total
1	0	0	0	0	0
2	1	NA	1	NA	2
3	1	1	1	1	4
4	1	1	1	1	4
5	1	1	1	1	4
6	0	1	1	NA	2
7	1	1	1	1	4
8	1	1	1	1	4
9	1	1	0	0	2
Total	7	7	7	5	26

NA: not available

3.5 Chlamydia culture on human samples

Twenty of 33 (60%) of the human samples were positive for *Chlamydia* culture. The isolation scores varied between 1 and 2. Four samples showed at least one inclusion positive cell, indicating replicating bacteria.

3.6 PCR on human *Chlamydia* isolates

Three of the human *Chlamydia* isolates were identified as *C. suis*, including one pharyngeal sample and two rectal samples, originating from three different farmers**.** The *tet(C)* gene was not detected in any of the human isolates.

3.7 Medical questionnaire

Based on the questionnaire filled in by the farmers, all pigs appeared healthy. However, 8 of 9 (89%) farmers communicated the antibiotic treatment of their pig herd within one month before sampling.

Five of nine farmers had no health complaints, while four of nine farmers mentioned at least one of the following symptoms: disease of muscles and joints, headache, runny nose, cough or heart disease. The manifestation of these symptoms did not clearly correlate with the presence of *C. suis* organisms.

4 DISCUSSION

The present study investigated the prevalence of *C. suis* DNA and viable *C. suis* bacteria in samples from pigs and farmers on nine Belgian pig farms. An overall prevalence of 69% positive animals in *C. suis* real-time PCR was demonstrated, and 50 porcine *C. suis* isolates were identified, derived from 31% of the animals, across eight farms. Clinical isolates are known to have slower growth rates compared to laboratory strains, and often are present in low numbers. This makes their isolation and subsequent identification challenging, especially for *C. suis* (Sandoz and Rockey 2010; Schautteet and Vanrompay 2011), which could explain the low isolation scores obtained. Nucleic acid amplification tests generally are highly sensitive compared to culture, yet they often cannot equal culture methods concerning specificity, approaching 100%. Therefore, culture methods are often used for confirmatory testing (Johnson *et al.* 2002; Schachter *et al.* 2005). Nevertheless, the current study confirms a high prevalence of *C. suis* in all pig herds, while *C. abortus, C. pecorum* and *C. psittaci* were not involved. However, no clinical signs of infection in the pig herd were reported by any of the farmers, suggesting subclinical infection. These findings are consistent with earlier studies on the prevalence of *Chlamydiaceae* spp. in European domestic pigs (Hoelzle *et al.* 2000; Kauffold *et al.* 2006b; Englund *et al.* 2012). Moreover, these findings confirm the assumption of endemic presence of *Chlamydiaceae* species in the Belgian commercial pigs (Vanrompay *et al.* 2004; Schautteet and Vanrompay 2011), and the widespread occurrence of subclinical intestinal *C. suis* infections (Nietfeld *et al.* 1997).

In this study, the infection rate of finisher pigs showed to be significantly higher compared to sows. Furthermore, the presence of *C. suis* DNA was compared between finishers and sows for each sample type separately. The frequency of *C. suis* DNA in conjunctival, nasal, and

vaginal samples was significantly higher for finishers, compared to sows. These observations could be attributed to the housing differences of both groups. At sampling, the sows were housed separately in boxes with their piglets. The finisher pigs, however, were group housed with direct access to feces and body fluids of group members, implying a higher risk for oral-fecal transmission. According to Becker *et al.* (2007), the knowledge on the transmission routes of these micro-organisms is limited, but it presumably occurs mainly through exchange of body fluids, like excretion from the eyes and nose possibly leading to aerosol formation. Therefore, housing conditions might contribute to the transmission of chlamydial infections in pigs.

As mentioned earlier, *C. suis* is highly related to the human pathogen *C. trachomatis*, and thus the zoontic potential of *C. suis* is likely. Indeed, few reports exist on the detection of *C. suis* in human samples. Dean *et al.* (2013) already demonstrated the involvement of *C. suis* in eye infections of villagers of Nepal leading to follicular or intense trachomatous inflammation, and De Puysseleyr *et al.* (2014b) demonstrated the presence of viable *C. suis* bacteria in the eyes of two Belgian pig abattoir employees, albeit asymptomatic. In this study, viable *C. suis* bacteria were detected in one pharyngeal and two rectal samples, originating from three different farmers. Further clinical and *Chlamydia suis* specific serological examination of the farmers might confirm a *C. suis* specific serologic response and give more insight into the clinical significance of *C. suis* infections in humans. Furthermore, sequence analysis of the identified porcine and human *C. suis* isolates can clarify if the isolates demonstrated in the farmers were the result of a zoonotic transfer.

As noted earlier, Tc is still the drug of choice in the treatment of chlamydial infections. However, since 1998, Tc^R strains have been isolated in the United States, Italy, Cyprus, Germany, Israel, Switzerland and Belgium (Andersen 1998; Di Francesco *et al.* 2008; Borel *et al.* 2012; Schautteet *et al.* 2012). Many of these farms suffered from severe reproductive failure, leading to economic loss, such as a drop in conception rate from 90% to 65% at 50 days following artificial insemination (Schautteet *et al.* 2012). Moreover, the Tc^R *C. suis* strains are often also resistant to more than one antibiotic used in anti-chlamydial treatment, such as azithromycine (Lenart *et al.* 2001). In the present study, the *tet(C)* gene was detected in nine viable *C. suis* isolates from three distinct farms, which suggests that the presence of the *tet(C)* gene in *C. suis* is common in the Belgian domestic pig population. As shown by the questionnaire, the three corresponding farmers communicated antibiotic treatment of the pig herd shortly before sampling. This treatment probably selected for Tc^R *C. suis* strains, since Borel *et al.* (2012) already reported rapid selection for Tc^R *C. suis* strains following antibiotic

treatment. Since the resistance was demonstrated in three of the eight farms where pigs were treated, selection of Tc resistance upon treatment might be common.
The emergence of Tc^R strains not only complicates the treatment of chlamydial infections in domestic pigs, these strains could also imply major public health concerns, as described earlier. Tetracycline is used to treat millions of patients with sexually transmitted or ocular *C. trachomatis* infections, in particular in the developing countries, according to the World Health Organization (WHO) estimates for 2008 (WHO 2012). Of interest, Suchland *et al.* (2009) already demonstrated the *in vitro* transfer of the *tet(C)* resistance gene from naturally resistant *C. suis* R19 strain into a clinical *C. trachomatis* isolate, leading to a stable Tc^R phenotype. Moreover, Lenart *et al.* (2001) reported that *C. suis* R19 and *C. trachomatis* L2 are present together within the same inclusion after sequential infection. Thus, *C. suis* and *C. trachomatis* can grow within close contact in patients infected with both species. This contact might enable the transfer of the Tc^R to *C. trachomatis*, leading to Tc^R *C. trachomatis* strains, which could be selected in patients treated with Tc. However, in this study, no *tet(C)* gene could be detected among the identified human *C. suis* isolates. Currently, there are no clinical *C. trachomatis* isolates identified yet, expressing a stable Tc^R phenotype. A study on a larger, statistically representative human population is recommended. Moreover, research should be promoted on preventive or alternative therapeutic measures, such as probiotics or vaccines, to tackle *C. suis* infections in domestic pigs.
In conclusion, this study demonstrated the presence of viable *C. suis* bacteria in pharyngeal and rectal samples of pig farmers. Moreover, the *tet(C)* gene was demonstrated in porcine *C. suis* isolates on three pig farms. Possible transfer of this gene into *C. trachomatis* might impose a risk for public health. Therefore, these findings demand further epidemiologic and clinical research on (Tc^R) *C. suis* infections in pigs and humans.

ACKNOWLEDGEMENTS

A. Dumont and L. Devlieger are acknowledged for technical assistance. This study was funded by the Federal Public Service of Health, Safety of the Food Chain and Environment (convention RF-10/6234), Ghent University (IOF/STARTT/002) and MSD Animal Health (Boxmeer, The Netherlands).

Chapter III

Study of the growth characteristics of *Chlamydia suis* in cell culture

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This chapter will be published as:

De Puysseleyr, L., De Puysseleyr, K., De Vos, W., and Vanrompay, D. Study of the growth characteristics of *Chlamydia suis* in cell culture. In preparation.

Abstract

Chlamydia suis is a porcine pathogen widespread in pig farming worldwide. Although the majority of *Chlamydia suis* infections is believed to remain subclinical, *Chlamydia suis* has also been associated with conjunctivitis, pneumonia, reproductive disorder and inferior semen quality in pigs. Several *Chlamydia suis* strains have been isolated from the intestine, conjunctiva and respiratory tract of pigs in Europe and the United States. Isolation in culture remains crucial for the generation of new isolates and their characterization and pathogenesis studies. However, the reports on the growth conditions of *Chlamydia suis* strains are limited, and isolation of *Chlamydia suis* from field samples is often fastidious. Therefore, the growth characteristics of a conjunctival, respiratory and intestinal *Chlamydia suis* strain were examined in six different cell lines, and two chlamydial growth media were compared. The results of this study suggest that the preferred cell line for propagation of *Chlamydia suis* differs among strains, and may be divergent from the cell lines currently applied. Furthermore, the use of IMDM as chlamydia culture medium may increase the replication of *Chlamydia suis*, yet, this effect is strain and cell type dependent. According to these results, an adaptation of the currently used isolation methods to the origin of the concerning *Chlamydia suis* isolate would be appropriate.

1 INTRODUCTION

Chlamydia suis (*C. suis*) is an obligate intracellular Gram-negative bacterium, belonging to the order of the *Chlamydiaceae.* Since the pig is the only natural host currently identified, *C. suis* is generally known as a porcine pathogen. However, recently, *C. suis* isolates were detected in the eye of Nepalese villagers and Belgian pig slaughterhouse employees (Dean *et al.* 2013; De Puysseleyr *et al.* 2014b). Nevertheless, the knowledge on the zoonotic potential of *C. suis* is limited. The reference strain, *C. suis* S45, was isolated from feces of an asymptomatic pig in Austria in the late 1960s (Koelbl 1969). Intestinal *C. suis* infections are assumed to be widespread but the majority probably is subclinical (Nietfeld *et al.* 1997). However, the enteric pathogenicity of the reference strain was demonstrated in gnotobiotic piglets (Guscetti *et al.* 2009). Moreover, *C. suis* infections in pigs have also been associated with conjunctivitis, pneumonia and reproductive disorder and inferior semen quality (Nietfeld *et al.* 1993; Rogers *et al.* 1993; Rogers and Andersen 1996; Rogers *et al.* 1996; Rogers and Andersen 1999; Eggemann *et al.* 2000b; Rogers and Andersen 2000; Schautteet *et al.* 2010), and several *C. suis* strains have been isolated from the intestines, conjunctiva and respiratory tract of pigs in Europe and the U.S. (Schautteet and Vanrompay 2011). *Chlamydiaceae* were originally cultivated in fertile hen's eggs. Although this technique is still used for isolating fastidious samples, it is largely replaced by cell culture, which is more sensitive compared to egg culture. Different cell lines have been used to culture *Chlamydiae*, but the success of isolation is influenced by the cell line, chlamydial species and sample type. Nevertheless, successful propagation of *Chlamydiae* is dependent on the viability of the bacteria, for which adequate transport and storage of biological samples is crucial. More recently, numerous nucleic acid amplification tests (NAATs) for the detection of chlamydial DNA have been developed. Since these tests are not dependent upon the viability of the bacteria and no biohazard containment facilities are required to perform analysis, they are far more sensitive and convenient, and less labour-intensive and expensive compared to culturing of *Chlamydiae*. Although NAATs are more sensitive, they often cannot equal culture concerning specificity, approaching 100%. Furthermore, isolation in culture remains crucial for the generation of new isolates and their characterization and pathogenesis studies. Several cell lines have been used for chlamydial isolation, with varying success depending on the chlamydial species being cultured. The McCoy (mouse fibroblasts), BGM (monkey kidney cells), Vero (monkey kidney cells) and HeLa (human cervix cancer cells) cell lines are most commonly used (Rogers *et al.* 1996; Rogers and Andersen 2000; Lenart *et al.* 2001; Sachse *et al.* 2004). However, some species, such as *C. suis*, are more difficult to grow, especially from

tissue and rectal samples (Wittenbrink *et al.* 1991; Rogers *et al.* 1993; Sandoz and Rockey 2010). Moreover, the recovery rate can differ significantly among *C. suis* strains, which could reflect the high genetic diversity observed within this species (Everett *et al.* 1999). The knowledge on the culture of porcine *Chlamydiaceae* is limited. Schiller *et al*. (2004) studied the growth characteristics of porcine chlamydial strains, including *C. suis* S45, in different cell culture systems. According to these results, *C. suis* inclusions were markedly increased in number and size in Caco (Human colon adenocarcinoma) cells, compared to Vero cells. Moreover, the use of Iscove's modified Dulbecco's medium (IMDM) instead of Eagle's minimal essential medium (EMEM) significantly increased the number of *C. suis* inclusions in Vero cells.

Currently, the isolation of *C. suis* in culture is laborious and complicated. Moreover, the culture recovery rate of *C. suis* isolates can vary widely. However, isolation of viable bacteria is highly valuable for the characterization of individual strains from a diagnostic viewpoint. In order to improve culture conditions for *C. suis* isolates, the growth characteristics of a conjunctival, respiratory and intestinal *C. suis* strain were examined in six different cell lines. The BGM, McCoy and Vero cell lines were included, since they are sensitive artificial cells routinely used for chlamydial diagnosis and propagation. Moreover, the Caco-2 cells were investigated based on the results obtained by Schiller *et al*. (2004), as described above. There are currently no data available on the replication of characterized *C. suis* strains in cell lines from porcine origin. Moreover, the pig intestine is considered as the natural habitat for *C. suis* (Shewen 1980; Englund *et al.* 2012). Therefore, the swine kidney SK-6 cell line, frequently used for porcine virus propagation (Kasza *et al.* 1972), and the porcine intestinal IPEC-J2 cell line (Schierack *et al.* 2006) were also examined in this study. Moreover, two chlamydial growth media were compared.

2 MATERIALS AND METHODS

2.1 Chlamydial strains

Three *C. suis* strains were used in this study. The origin of the strains is listed in table III-1. The strains were propagated in McCoy cells, using standard techniques (Vanrompay *et al.* 1992). The 50% Tissue Culture Infective Dose ($TCID_{50}$) of the bacterial stock was determined by the method of Spearman & Kaerber (Mayr 1974). The TCID50/ml correlates with the number of inclusion forming units (IFU/ml) (Beeckman *et al.* 2009), and is regularly used for

titration of intracellular organisms in cell culture, such as viruses. Strains were diluted in sucrose-phosphate-glutamate (SPG) storage medium (218 mM sucrose, 38 mM KH_2PO_4, 7 mM K_2HPO_4, 5 mM L-glutamic acid) and stored at -80°C.

Table III-1 Origin of the *Chlamydia suis* strains used to study their growth characteristics in cell culture

	Isolated from			
Strain	**Location**	**Year**	**Tissue**	**Clinical Symptoms of the pigs**
H7	Iowa	1994	Conjunctiva	Conjunctivitis
R24	Nebraska	1992	Respiratory tract (nasal mucosa)	Upper respiratory tract disease
S45	Austria	1969	Intestines (feces)	Asymptomatic infection

2.2 Cell cultures

Chlamydiaceae were cultured on six cell lines: McCoy (Mouse fibroblast cells, CRL-1696 American Type Culture Collection), Vero (African Green Monkey kidney cells, CRL-1586 American Type Culture Collection), BGM (Buffalo Green Monkey kidney cells, America Culture Type Collection), IPEC-J2 (Intestinal porcine epithelial cells) and SK-6 (Swine kidney cells, both obtained from professor Eric Cox, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University), and Caco-2 (Human colon adenocarcinoma cells, HTB-37 American Culture Type Collection). The McCoy, Vero and BGM cells were culture in Eagle's minimal essential medium (EMEM, Life Technologies) supplemented with 10 % calf serum (Life Technologies), 2 mM L-glutamine (Life Technologies), 1% MEM vitamins (Life Technologies), and 0.1 mg/ml streptomycin and vancomycin. For the Caco-2 cells, this medium was additionally supplemented with 1% non-essential amino acids (Life Technologies). The SK-6 cells were cultured in EMEM supplemented with 2 mM L-glutamine and 0.1 mg/ml streptomycin and vancomycin. The IPEC-J2 cells were cultured in Dulbecco's modified Eagle medium/Nutrient Mixture F-12 (DMEM/F-12, Life Technologies) supplemented with 5% calf serum (Life Technologies), 4 mM L-glutamine, 1% Insuline-Transferrin-Selenium (ITS, Life Technologies), 0.1 mg/ml streptomycin and vancomycin, and 5 ng/ml human epidermal growth factor (hEGF, Life Technologies).

2.3 Infection Forming Unit Curve of *Chlamydia suis* S45

Depending on the chlamydial species and cell type, EBs and some non-differentiated RBs are released from the host cell at 24 to 72 h post infection (p.i.) through lysis. To assess the appropriate time point for quantification of replication, shortly before cell lysis, the IFU curve

of *C. suis* S45 was determined. Therefore, McCoy cells were seeded in Chlamydia Trac bottles (CTB) at a concentration of 200 000 cells/ml, and infected with 100 µl *C. suis* S45 inoculum (10^5 IFU/ml), following standard procedures (Vanrompay *et al.* 1992) . The CTBs were incubated at 37°C, and *Chlamydiae* were harvested at 12, 24, 36, 48, 54, 60 and 72 h p.i. The IFU/ml of the resulting culture harvest was determined using the method of Spearman & Kaerber (Mayr 1974).

2.4 *Chlamydia* culture and immunofluorescence staining

Cells were seeded in 24-well plates (Greiner) and incubated for 24 h at 37°C and 5% CO_2. Subsequently, culture medium was removed and cells were infected with 10^4 IFU diluted in SPG. All inoculations were performed in duplicate in two independent experiments. The monolayers were centrifuged for 1 h at 1300 x g and 37°C. The bacteria were subsequently removed and maintenance medium was added, which differed from the growth medium by containing 5% fetal calf serum, 2.2 µg/ml cycloheximide and 5.5 mg/ml glucose. Additionally, a second maintenance medium was used for comparison, based on Iscove's modified essential medium (IMDM) (further referred to as MEM and IMDM culture medium, respectively). For McCoy, Vero, BGM, Caco-2 and SK-6 cells, IMDM was supplemented with 5% fetal calf serum, 0.1 mg/ml streptomycin and vancomycin, 2 mg/ml glucose and 2.2 µg/ml cycloheximide. Additionally, for the IPEC-J2 cells, 1% ITS and 5 ng/ml hEGF was added. Cells were incubated at 37°C. At 40 h p.i., maintenance medium was removed and monolayers were washed twice with PBS (Sigma) to remove unattached bacteria. The cells were fixed with methanol for 10 minutes at -20°C. After washing once more, the cells were incubated overnight at 4°C with 40 mg/mL BSA (Sigma) in PBS. *Chlamydia suis* was detected using the Imagen™ *Chlamydia* immunofluorescence staining (Oxoid, United Kingdom), as described by Vanrompay *et al.* (1992). The kit contains a monoclonal antibody directly conjugated to fluorescein isothiocyanate (FITC) and Evans Blue pancellular counterstain. The cell nuclei were stained using 1 ng/ml DAPI (4',6-diamidino-2-phenylindole) (Life Technologies, Belgium) and slides were mounted with Vectashield Mounting Medium (Vector Labs, United States).

2.5 High content microscopy and image analysis

A fully automated inverted Nikon Ti widefield fluorescence microscope (Nikon Instruments, Paris, France) was used, equipped with motorized XYZ stage, filter cube turret and shutters. Samples were magnified with a 40x Plan Fluor oil objective (numerical aperture of 1.3) and

images were acquired with an Andor Ixon EM- CCD camera, yielding a pixel size of 0.276 µm/pixel. To obtain a representative sample of each condition with minimal edge effects, three separate but sufficiently central regions were chosen per slide. Per region a 5-dimensional hyperstack was recorded, consisting of 16 fields (acquired in a 4x4 mosaic), 5-7 z-slices (separated by 1µm) and 3 channels (corresponding with the DAPI, Evans Blue and FITC channels).

To analyze the multidimensional files, dedicated macro scripts were written in FIJI image analysis freeware (http://fiji.sc, (Schindelin *et al.* 2012)), which are essentially based on a pipeline described before (De Vos *et al.* 2010). Briefly, the analysis consists of a stepwise segmentation of the features of interest, followed by a quantification of the regions of interest. Before commencing segmentation, hyperstacks are flattened by means of a maximum projection along the Z-axis. Then, presumed *Chlamydia* containing foci are segmented. To this end, the FITC channel images are convolved with a Laplacian filter to enhance the signals and automatically thresholded using the isodata algorithm. Only spots larger than 3 pixels are taken into account. Subsequently nuclei are segmented with an algorithm that consists of a smoothing step (Gaussian blur), an autothreshold (isodata) and a watershed procedure. Finally, cells are delineated by direct segmentation of the Evans Blue channel. Cell segmentation occurs by seeking a local minimum in the intensity histogram that separates background from true signal. Optionally, cells can be separated by conditional region growing from the nuclear regions of interest. Once all regions of interest are retrieved, the following metrics were derived: mean spot area (MSA, a measure for inclusion size per cell), mean spot number (MSN, inclusion number per cell) and mean spot occupancy per cell (CSO, ratio of inclusion and cellular surface, an estimate for the overall replication).

Since less then three biological replicates were generated in this study, no statististical analysis was performed on the data. Instead, the observed trends in the data were described.

3 RESULTS

3.1 Infection forming unit curve *Chlamydia suis* S45

The IFU curve of *C. suis* S45 was established to determine the appropriate time point for quantification of *C. suis* replication (Figure III-1). The IFU/ml started to increase from 24 h p.i. on, and reached a maximum, when cell lysis occurs, at 48-60 h p.i., and decreased again after 72 h p.i. Therefore, replication of the *C. suis* strains was examined at 40 h p.i.

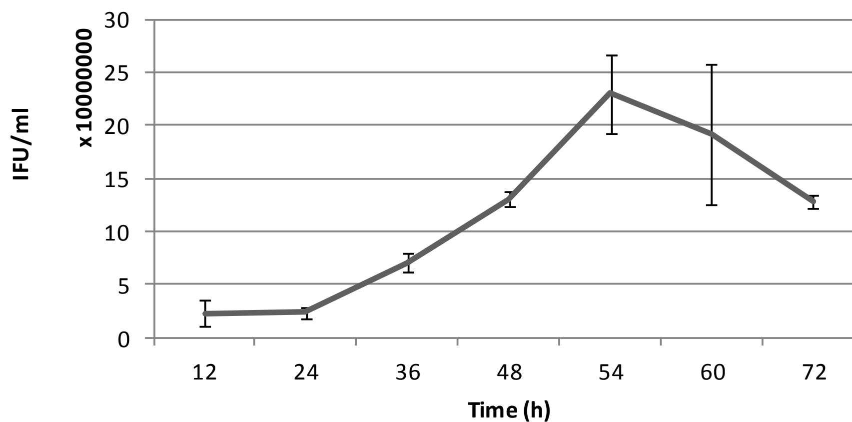

Figure III-1 Infection Forming Unit (IFU) curve of *C. suis* S45. The error bars correspond to the standard error of the mean.

3.2 Comparison of growth characteristics of *Chlamydia suis*

The effect of cell line and chlamydia culture medium on the replication of three *C. suis* strains was examined, based on the inclusion size (mean spot size), number of inclusions per cell (mean spot number), and the segment of the cell occupied by *C. suis* inclusions (cellular spot occupancy, an estimate of the overall replication).

3.2.1 *Chlamydia suis* H7

For the conjunctival strain *C. suis* H7 (Figure III-2), the cell line was found to affect the inclusion size, inclusion number and inclusion occupancy (Figure III-3). The IMDM chlamydia culture medium seemed only to clearly increase the inclusion size in IPEC-J2 cells. Yet, the MEM culture medium produced a higher inclusion number in Vero cells, compared to the IMDM culture medium. Therefore, the effect of the chlamydia culture medium on the evaluated parameters was not consistent, suggesting an interaction between cell line and culture medium.

For both culture media, the inclusion size was higher in Caco-2 and SK-6 cells compared to BGM, McCoy and Vero cells. When cultured with IMDM medium, the inclusion size was also higher in IPEC-J2 cells than in BGM, McCoy and Vero cells. However, when considering the inclusion number, Caco-2 cells did not seem to produce a higher inclusion number compared to the other cell lines. However, the inclusion number did seem to be increased in BGM and IPEC-2 cells. Finally, the BGM, Caco-2 and IPEC-J2 cell lines generally produced a higher chlamydial replication, characterized as cellular spot occupancy. The datapoints of the *C. suis* H7 replication in IPEC-J2 cells seemed to concentrate at a higher spot occupancy value, which suggests a slightly higher replication in these cells.

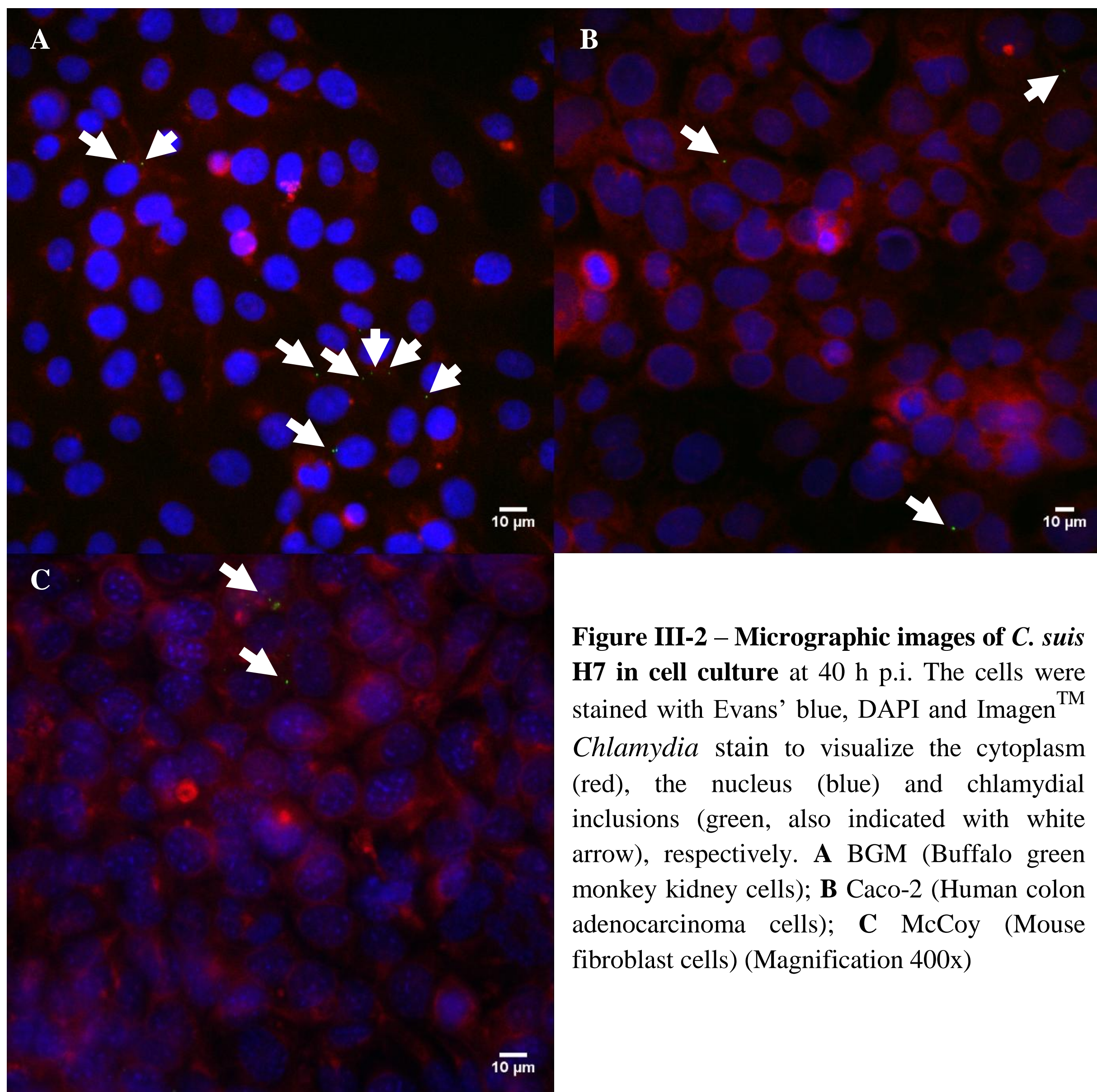

Figure III-2 – Micrographic images of *C. suis* H7 in cell culture at 40 h p.i. The cells were stained with Evans' blue, DAPI and Imagen™ *Chlamydia* stain to visualize the cytoplasm (red), the nucleus (blue) and chlamydial inclusions (green, also indicated with white arrow), respectively. **A** BGM (Buffalo green monkey kidney cells); **B** Caco-2 (Human colon adenocarcinoma cells); **C** McCoy (Mouse fibroblast cells) (Magnification 400x)

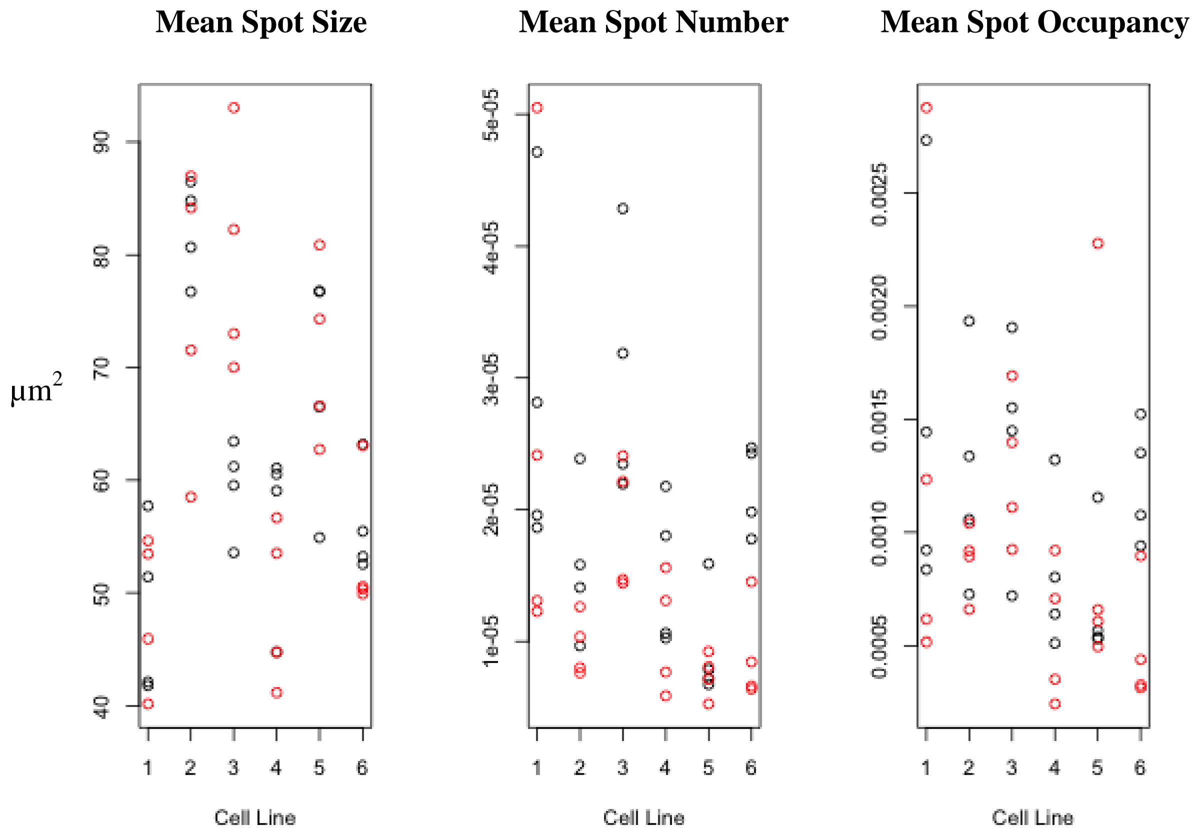

Figure III-3 Growth characteristics of *Chlamydia suis* H7 in cell culture. The growth of *C suis* H7 was characterized based on the inclusion size (mean spot number), number of inclusions per cell (mean inclusion number), and the overall replication (mean cellular spot occupancy). The cell line numbers correspond to BGM (**1**, Buffalo green monkey kidney cells), Caco-2 (**2**, Human colon adenocarcinoma cells), IPEC-J2 (**3**,Intestinal porcine epithelial cells), McCoy (**4**, Mouse fibroblast cells), SK-6 (**5**,Swine kidney cells) and Vero (**6**, African green monkey kidney cells). The datapoints from the MEM and IMDM chlamydia culture medium were represented in black and red, respectively.

3.2.2 *Chlamydia suis* R24

For the respiratory strain *C. suis* R24 (Figure III-4), the cell line clearly affected the inclusion size, number and overall chlamydial replication (Figure III-5). The effect of the culture medium was not consistent among the cell lines, again suggesting an interaction between those two factors.

The inclusion size was higher in McCoy and Vero cell lines compared to the Caco-2, IPEC-J2 and SK-6 cell lines, for both culture media. When cultured in BGM cells with IMDM medium, the inclusion size was also higher compared to Caco-2, IPEC-J2 and SK-6, but not when cultured in MEM medium. Moreover, the inclusion size seemed to be decreased in

McCoy cells if *C. suis* R24 was cultured in an IMDM culture medium, compared to the MEM medium. The inclusion number and overall replication was clearly higher in BGM, McCoy and Vero cells, compared to Caco-2, IPEC-J2 and SK-6 cells, regardless of the culture medium. The Vero cells appeared to produce the highest replication of *C. suis* R24.

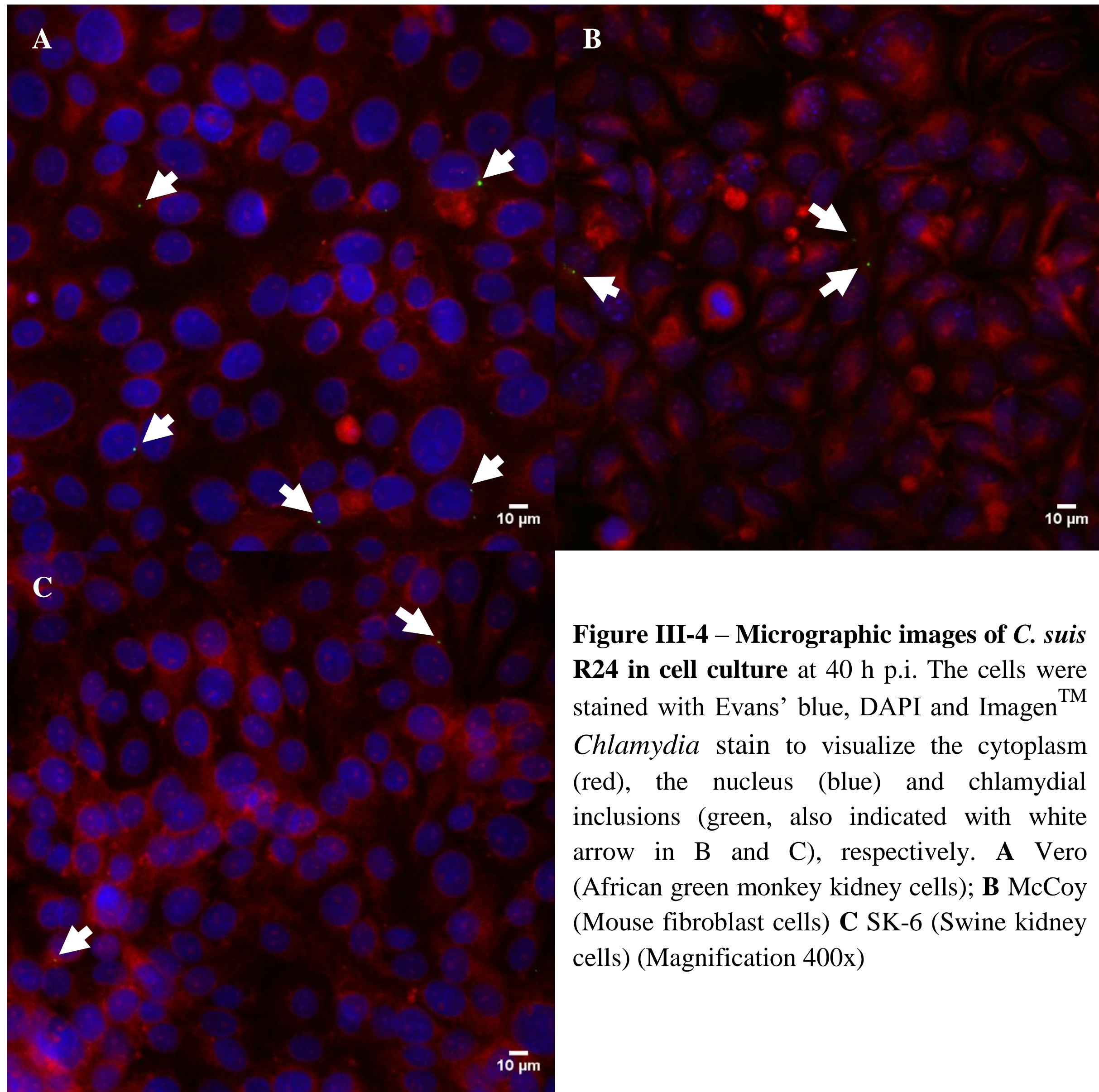

Figure III-4 – Micrographic images of *C. suis* R24 in cell culture at 40 h p.i. The cells were stained with Evans' blue, DAPI and Imagen™ *Chlamydia* stain to visualize the cytoplasm (red), the nucleus (blue) and chlamydial inclusions (green, also indicated with white arrow in B and C), respectively. **A** Vero (African green monkey kidney cells); **B** McCoy (Mouse fibroblast cells) **C** SK-6 (Swine kidney cells) (Magnification 400x)

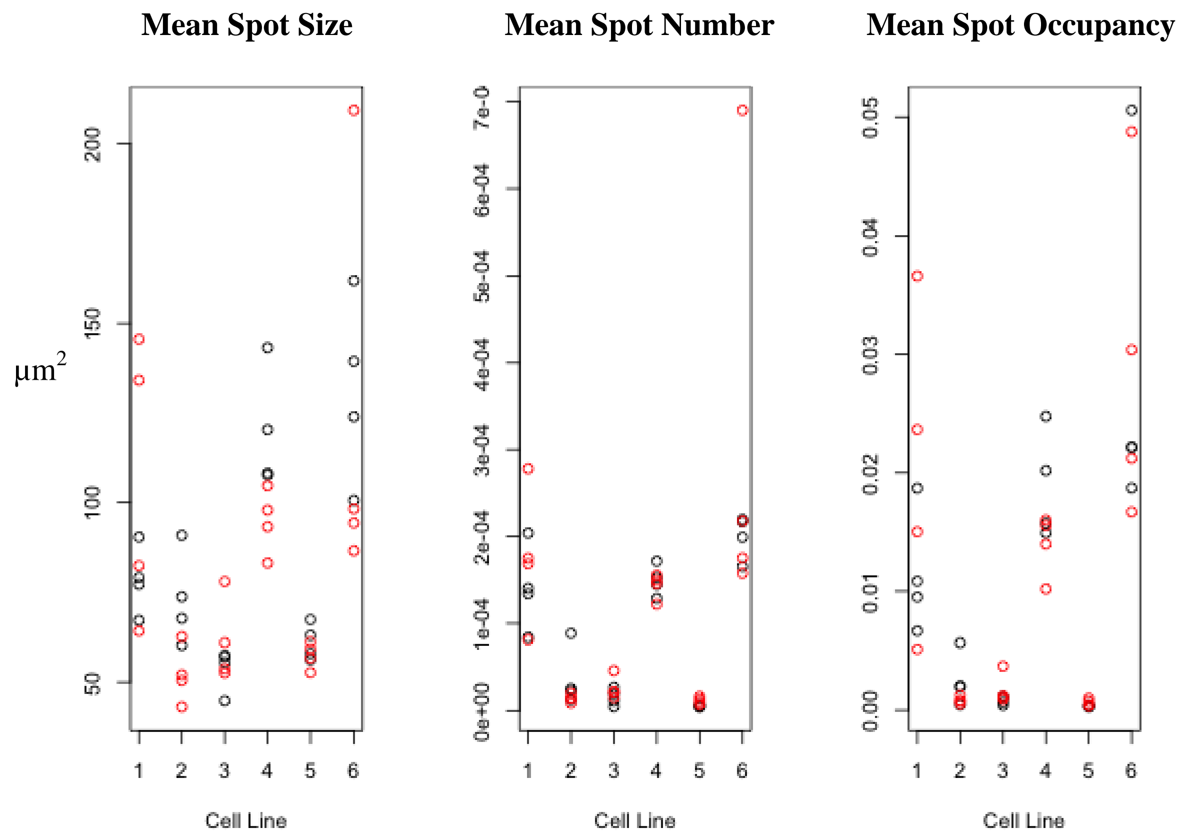

Figure III-5 Growth characteristics of *Chlamydia suis* R24 in cell culture. The growth of *C. suis* R24 was characterized based on the inclusion size (mean spot number per cell), number of inclusions per cell (mean inclusion number per cell), and the overall replication (mean cellular spot occupancy). The cell line numbers correspond to BGM (**1**, Buffalo green monkey kidney cells), Caco-2 (**2**, Human colon adenocarcinoma cells), IPEC-J2 (**3**,Intestinal porcine epithelial cells), McCoy (**4**, Mouse fibroblast cells), SK-6 (**5**,Swine kidney cells) and Vero (**6**, African green monkey kidney cells). The datapoints from the MEM and IMDM chlamydia culture medium were represented in black and red, respectively.

3.2.3 *Chlamydia suis* S45

For the intestinal strain *C. suis* S45 (Figure III-6), the cell line clearly affected the inclusion number and overall chlamydial replication, but no clear effect on the inclusion size was observed (Figure III-7). The IMDM culture medium did not increase any of the evaluated parameters, yet rather seemed to decrease the inclusion size, number and overall replication in the investigated cell lines.

For the inclusion size, no clear differences were observed among the cell lines. However, Caco-2 and IPEC-J2 cells seemed to produce a higher inclusion number and replication compared to SK-6 and Vero cells, and to a lesser extent also compared to BGM and McCoy.

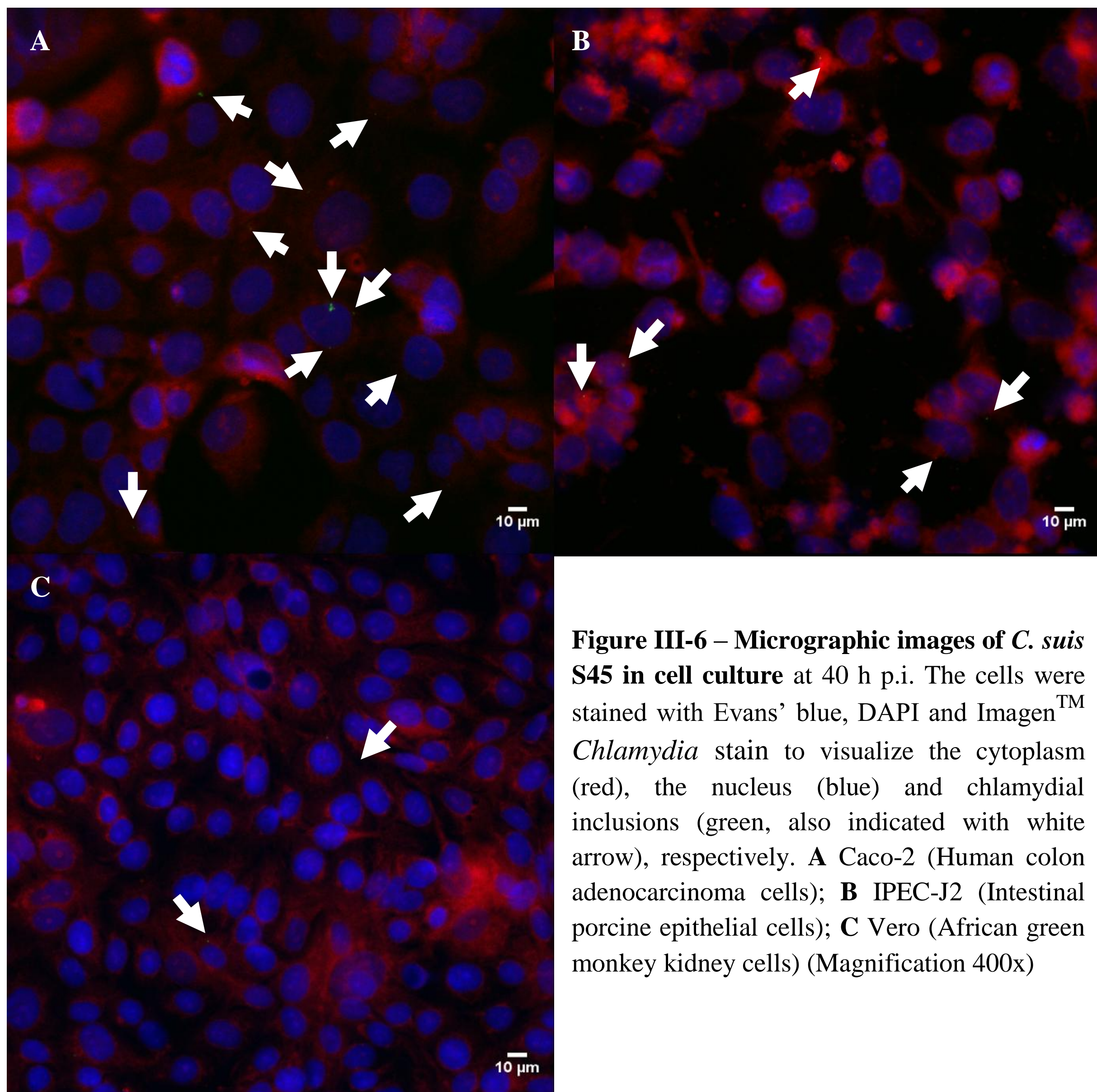

Figure III-6 – Micrographic images of *C. suis* S45 in cell culture at 40 h p.i. The cells were stained with Evans' blue, DAPI and Imagen™ *Chlamydia* stain to visualize the cytoplasm (red), the nucleus (blue) and chlamydial inclusions (green, also indicated with white arrow), respectively. **A** Caco-2 (Human colon adenocarcinoma cells); **B** IPEC-J2 (Intestinal porcine epithelial cells); **C** Vero (African green monkey kidney cells) (Magnification 400x)

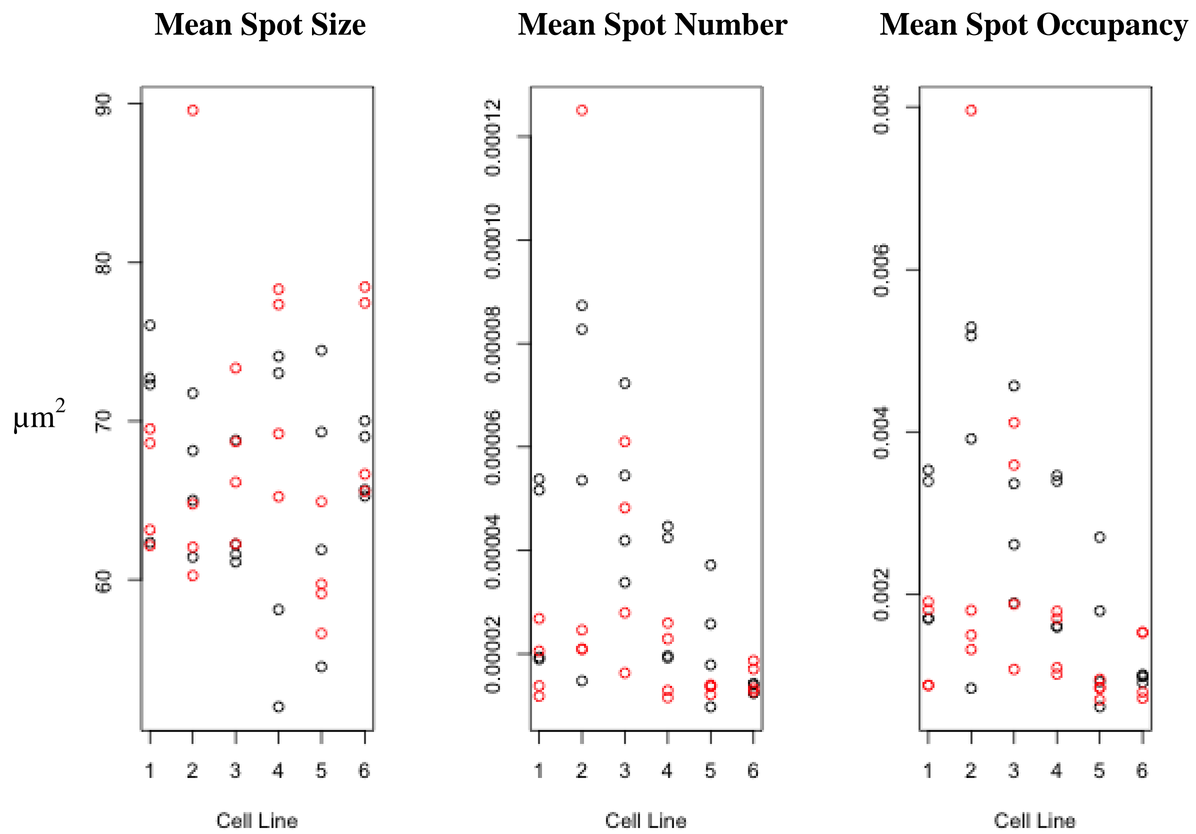

Figure III-7 Growth characteristics of *Chlamydia suis* S45 in cell culture. The growth of *C. suis* S45 was characterized based on the inclusion size (mean spot number per cell), number of inclusions per cell (mean inclusion number per cell), and the overall replication (mean cellular spot occupancy). The cell line numbers correspond to BGM (**1**, Buffalo green monkey kidney cells), Caco-2 (**2**, Human colon adenocarcinoma cells), IPEC-J2 (**3**,Intestinal porcine epithelial cells), McCoy (**4**, Mouse fibroblast cells), SK-6 (**5**,Swine kidney cells) and Vero (**6**, African green monkey kidney cells). The datapoints from the MEM and IMDM chlamydia culture medium were represented in black and red, respectively.

4 DISCUSSION

Isolation of the chlamydial pathogens is crucial for characterization of individual strains from an epidemiological viewpoint. Successful propagation is dependent on the cell line and the chlamydial species being tested (Sachse *et al.* 2009). However, chlamydial isolation is often fastidious, especially for *C. suis.* Currently, only few studies exist on the growth characterization of porcine chlamydial strains in cell culture. Therefore, the replication of three *C. suis* strains of distinct origin was investigated in six different cell lines. Beside BGM, McCoy and Vero cells, commonly used for propagation of *C. suis* strains, two cell lines of porcine origin, IPEC-J2 and SK-6, and the human Caco-2 cell line were included. As mentioned earlier, Schiller *et al.* (2004) reported an increase in inclusion number of *C. suis* S45 in Vero when using an IMDM instead of MEM-based culture medium. Therefore, these two media were also compared for all strains. The inclusion size, number and overall replication per cell was determined. Interestingly, at high multiplicities of infection, multiple *C. trachomatis* inclusions fuse into a single inclusion 10-12 h post infection (Richards *et al.* 2013). Since *C. suis* is phylogenetically highly related to *C. trachomatis*, fusion of *C. suis* inclusions might also occur. Although the inclusion membrane protein A (IncA) is known to be involved in this homotypic fusion process, a complete insight into the mechanism and the host factors involved, is lacking (Richards *et al.* 2013). To clarify the putative change in overall replication, the inclusion number and size were also estimated, in addition to the overall replication. However, a change in inclusion size or number does not consistently result in a higher replication. Indeed, a higher in inclusion size, but lower inclusion number can yield an overall replication comparable with other cell lines, as was demonstrated for the infection of Caco-2 and SK-6 cells with *C. suis* H7. This might be the consequence of inclusion fusion, as has been demonstrated for *C. trachomatis,* and might indicate differences in host factors, involved in the fusion process, between the cell lines. Moreover, the ‘mean spot number’ parameter, determined per cell, might be biased by the cellular size, since larger cells may contain more inclusions. Therefore, in this study, the cellular inclusion occupancy, reflecting both the effect of the inclusion size and number, was determined to estimate the overall replication. The results varied between the *C. suis* strains investigated. The *C. suis* strain H7 was originally isolated in Iowa in 1994, from the conjunctiva of a pig suffering from conjunctivitis. The overall replication of this strain seemed to be higher in BGM, Caco-2 and IPEC-J2 cells, and this was the result of a higher inclusion size and/or a higher inclusion number. Thus, McCoy, SK-6 and Vero cells appeared to be less susceptible for *C. suis* H7

replication. The respiratory strain *C. suis* R24 was isolated in 1992 in Nebraska, from the nasal mucosa of a pig suffering from upper respiratory tract disease. This strain appeared to be more adapted for replication in BGM, McCoy and Vero, which are established cell lines for propagation of *C. suis*. This higher replication resulted from an increase of inclusion size, and mainly a higher inclusion number.

Unlike the other strains, the size of *C. suis* S45 inclusions did not seem to be affected by the cell line or the culture medium. However, the Caco-2 and IPEC-J2 cells produced a higher inclusion number and overall replication, compared to SK-6 and Vero cells. This is in accordance with the results obtained by Schiller *et al.* (2004). The Caco-2 and IPEC-J2 cell lines are both from intestinal origin, whereas the porcine SK-6 cells are kidney epithelial cells. Interestingly, *C. suis* S45 was isolated in Austria in 1969, from the feces of a clinically healthy pig. Indeed, natural intestinal chlamydial infections are frequently detected in the intestine of pigs, but the majority of these infections is believed to be subclinical. However, experimental enteric infection provoked significant enteric disease and lesions in gnotobiotic piglets (Guscetti *et al.* 2009). Yet, the isolation of *C.suis* in culture can be very difficult, particularly for rectal strains (Sandoz and Rockey 2010), which compromises further characterization. Performing isolation on intestinal cell lines, such as Caco-2 and IPEC-J2, might thus increase the recovery rate of rectal *C. suis* isolates.

Effect of cell culture medium

Cell culture media components have already been shown to influence chlamydial growth. Indeed, cycloheximide treatment, inhibiting eukaryotic protein synthesis and thereby reducing host cell competition for amino acids (Allan and Pearce 1983), can enhance chlamydial infection in cell culture. Moreover, Schiller *et al.* (2004) reported a significant increase of *C. suis* S45 inclusion number in Vero cells when cultured with IMDM instead of EMEM, as mentioned above. In the current study, the inclusion number of *C. suis* S45 was slightly higher in Vero cells cultured with IMDM, yet, the culture medium did not clearly affect the replication of *C. suis* S45. However, the IMDM culture medium increased the inclusion size of *C. suis* H7 in IPEC-J2 cells, although this effect was not reflected in the overall replication. Furthermore, the MEM culture medium produced a higher inclusion number and overall replication of *C. suis* H7 in Vero cells, and a higher inclusion size of *C. suis* R24 in McCoy cells compared to the IMDM medium. Thus, the use of the IMDM culture medium does not have a general improving effect on the replication of *C. suis*, but instead appeared to be cell line and chlamydial strain dependent. Of interest, the growth characteristics of chlamydial

strains in cell culture can vary widely between several studies (Schiller *et al.* 2004). The culture conditions have been shown to strongly influence cell performance, and therefore possibly chlamydial replication. Indeed, the passage number of the cells has been shown to affect the morphology and proliferation rate of cell lines (Sambuy *et al.* 2005; Schierack *et al.* 2006). Furthermore, the pH and composition of the culture medium might modulate the proliferation, motility and differentiation of cultured cells. Also the nature of the substrate used might have an impact on the cellular morphology and differentiation. Wyrick *et al*. (1996) observed an influence of the medium depth above infected monolayers on the chlamydial inclusion size. Since culture conditions may also strongly affect chlamydial replication, results and inconsistencies among different studies should be interpreted with caution.

Effect of cell line

Although *Chlamydiaceae* are known to infect a variety of cell types, marked differences were observed in inclusion number between cell lines. Moreover, the susceptibility of the cells to *C. suis* infection varied among the three investigated strains. This might reflect the efficiency of internalization into the host cell. Two possible mechanisms for chlamydial entry have been described: zipper-like microfilament dependent phagocytosis (Byrne and Moulder 1978) and clathrin-mediated endocytosis (Hodinka *et al.* 1988). However, the exact nature of both the host cell receptors and chlamydial ligands is still largely undefined, but may influence the susceptibility of the cell line for chlamydial infection. Moreover, the cell line had an influence on the inclusion size of *C. suis* H7 and R24, but not for S45. Following infection, chlamydial inclusion vacuoles efficiently avoid phagolysosomal fusion, but intercept and fuse with secretory vesicles of the exocytic pathway to acquire nutrients and membrane components, to maintain the integrity of the growing inclusion (van Ooij *et al.* 2000). Cell line specific differences in the underlying molecular mechanisms in exocytic trafficking might influence the ability of *Chlamydiae* to intercept vesicles and thus the susceptibility of a cell line for chlamydial replication. Therefore, the divergence in the current results might be attributed to biological differences between cell lines and chlamydial strains, changing the effectiveness of cell entry or the ability to grow in the host cell cytoplasm, leading to an altered infectivity (Knoebel *et al.* 1997). Indeed, the species *C. suis* is presumed to be genetically more diverse than other chlamydial species, based on DNA sequence analysis of *ompA* (Everett *et al.* 1999) and the extensive variation in virulence among *C. suis* strains in pigs (Bush and Everett 2001).

In the current study, the growth of *C. suis* in cell culture was studied using three laboratory strains, which were propagated on McCoy cells prior to this study and thus likely adapted to replication in this cell line. Yet, McCoy cells did not appear to be the preferred cell line for replication of *C. suis* H7 and S45. This finding strongly indicates further potential for optimization of the replication protocol of *C. suis.* Since laboratory strains are already adapted to propagation in culture, they probably not completely reflect the growth characteristics of field isolates. Therefore, the study of field isolates in cell culture might also be valuable. Indeed, clinical isolates can have an increased cytotoxicity or persistence, can be present in very low numbers, and often have slower growth rates compared to laboratory strains (Sandoz and Rockey 2010). Still, the preferred cell line for replication appeared to be strain specific in this study.

To conclude, the replication of three laboratory strains of *C. suis* was compared in six cell lines, using two culture media. The preferred cell line for replication and the impact of the culture medium varied among strains. Therefore, adaptation of the isolation procedure to the origin of the putative isolate might be recommended to improve the recovery rate of *C. suis* isolates.

ACKNOWLEDGEMENTS

A. Dumont, L. Van Hauwe and K. Van Hauwaert are kindly acknowledged for technical assistance. This study was funded by the Federal Public Service of Health, Safety of the Food Chain and Environment (convention RF-10/6234). This research has benefitted from a statistical consult with Ghent University FIRE (Fostering Innovative Research based on Evidence). Arthur Andersen (National Animal Disease Center, USDA, Agriculture Research Service, Ames, Iowa) and Deborah Dean (Children's Hospital Oakland Research Institute, Oakland, California, USA) are acknowledged for providing the *Chlamydia suis* strains.

Chapter IV

General discussion and perspectives

Transmission routes of porcine chlamydial infections

The pig intestinal tract is the natural habitat for *Chlamydiae*, and assumed to function as a natural reservoir for *C. suis,* and other members of the *Chlamydiaceae* (Shewen 1980; Englund *et al.* 2012), from where the pathogen can spread to other organs or animals (Nietfeld *et al.* 1993; Szeredi *et al.* 1996; Guscetti *et al.* 2009). *Chlamydia suis* predominantly causes lesion in the small intestine, especially in the distal jejunum and ileum, as observed in experimental and natural infections (Nietfeld *et al.* 1993; Rogers and Andersen 1996). Ascending intestinal *Chlamydiae* due to fecal contamination of the vagina*,* including *C. abortus* and *C. suis,* may result in urogenital chlamydiosis in sows (Hoelzle *et al.* 2000; Yeruva *et al.* 2013). Indeed, the intestinal strain *C. suis* S45 has been shown to cause pathology in the urogenital tract of conventionally raised piglets (De Clercq *et al.* 2014). Moreover, the eye and eye secretions represent an underestimated site of chlamydial infections, possibly acting as a source for further spreading of *C. suis*. Since the eye is an immune-privileged organ, it possibly is a preferred site for survival of persistent or resistant chlamydial organisms (Becker *et al.* 2007). Indeed, *C. suis* was demonstrated in the eyes of pigs suffering from conjunctivitis, but is also frequently detected in the eyes of clinically healthy pigs (Rogers *et al.* 1993; Becker *et al.* 2007). The knowledge on the transmission route of *Chlamydiae* in pigs is limited, but it presumably occurs through exchange of body fluids, like the excretion from the eyes and nose. Ocular or nasal discharge in infected animals might thus result in respiratory or oral infections, or contribute to aerosol mediated spreading. Since the intestine functions as a reservoir, oral-fecal transmission via contaminated feces likely contributes to bacterial spreading. Moreover, the detection of *C. suis* in boar semen suggests possible venereal transmission (Eggemann *et al.* 2000b; Kauffold *et al.* 2006a; Kauffold *et al.* 2006b). According to Becker *et al*. (2007), environmental factors might predispose pigs to infection or contribute to the transmission of chlamydial infections. Poor hygiene is generally considered as a pre-disposing factor for spreading of chlamydial infections in swine herds (Eggemann *et al.* 2000b; Hoelzle *et al.* 2000). Moreover, the detection of *C. suis* in the conjunctiva in pigs, in ocular symptomatic and asymptomatic infections, was shown to be higher in pigs coming from intensive farming systems. In these production systems, high concentrations of toxic ammonia, a high relative humidity, and high dust concentrations could lead to predisposition to infections, facilitate microbial survival and contribute to airborne transmission (Becker *et al.* 2007; Englund *et al.* 2012). Recently, the zoonotic transmission of *C. suis* was examined in a Belgian pig abattoir (De Puysseleyr *et al.* 2014a). Bioaerosol monitoring demonstrated high amounts of *C. suis* bacteria in air samples

of the intestine processing site. Therefore, transmission of *C. suis* through contaminated aerosols might be possible.

Zoonotic potential of Chlamydia suis

The wide distribution of subclinical chlamydial infections in pigs raises the question whether swine can also act as a reservoir of chlamydial infections for other animal species, including humans. Two of the species occurring in pigs, *C. psittaci* and *C. abortus*, are well described agents of zoonotic infections. *Chlamydia psittaci*, although less prevalent in pigs, is transferred from birds to humans through aerosols, and infections can vary from inapparent to severe pneumonia. Moreover, *C. abortus* infections in pregnant women, following exposure to infected sheep and goats, can result in miscarriages and stillbirths (Buxton 1986; Kampinga *et al.* 2000; Pospischil *et al.* 2002; Walder *et al.* 2003; Meijer *et al.* 2004; Walder *et al.* 2005). However, the zoonotic impact of *C. suis* and *C. pecorum* is largely unknown. *Chlamydia suis* is phylogenetically highly related to *C. trachomatis* (Everett *et al.* 1999), the human pathogen which is the leading cause of infectious blindness and sexually transmitted disease worldwide (Bebear and de Barbeyrac 2009). Interestingly, *C. suis* is frequently detected in the eyes of pigs suffering from conjunctivitis, resembling ocular *C. trachomatis* infections (Rogers *et al.* 1993; Becker *et al.* 2007). Recently, Dean *et al.* (2013) identified *C. suis* in the eyes of trachoma patients in Nepal (Dean *et al.* 2013), probably originating from domesticated animals, including pigs, commonly kept for consumption or agricultural purposes, in these communities. Moreover, viable *C. suis* organisms were isolated from the eyes of two employees of a Belgian pig slaughterhouse (De Puysseleyr *et al.* 2014a), albeit without clinical signs of infection. These employees were in close contact to the arriving animals and the pig intestine, respectively. These findings support the zoonotic potential of *C. suis.* To assess the zoonotic transmission to pig farmers, we examined the presence of *C. suis* in nine Belgian pig farms in chapter II. *Chlamydia suis* DNA was demonstrated in eight farmers, and three human *C. suis* isolates were obtained. Evaluation of the serological response to *C. suis* in positive individuals could explain the significance of human *C. suis* infections. Further epidemiological and clinical research on a larger risk population might clarify the impact of *C. suis* infections in humans.

Antibiotic resistance in Chlamydiae

Chlamydial infections in livestock and humans are primarily treated with tetracycline (Tc), or one of its derivatives, due to their low cost, high effectiveness, low toxicity and broad spectrum of activity (Chopra and Roberts 2001; Michalova *et al.* 2004). *Chlamydiaceae* are generally highly sensitive to Tc, however, since 1998, stably tetracycline resistant (Tc^R) *C. suis* strains have been isolated in the U.S., and since then also in Italy, Cyprus, Germany, Israel, Switzerland and Belgium (Andersen 1998; Di Francesco *et al.* 2008; Borel *et al.* 2012; Schautteet *et al.* 2012). The resistant phenotype is associated with the presence of a resistance gene *tet(C)* in the chlamydial chromosome (Dugan *et al.* 2004). The exact mechanism through which *C. suis* acquired the *tet(C)* gene is still unresolved. However, the majority of Tc resistance genes are associated with mobile genetic elements, which could partially explain their wide distribution among pathogenic, opportunistic and normal flora bacteria isolated from the urogenital, respiratory, and gastrointestinal tract of man, animals, and from food and the environment (Roberts 1996). The selection and spreading of resistance genes was probably promoted through addition of antibiotics to animal feed. Since the 1950s, the supplementation of feed with antibiotics, especially Tc, to promote growth and feed conversion, and counter bacterial infections, was widespread in the poultry, porcine and live stock industry (Novick 1981; Cromwell 2002; Sarmah *et al.* 2006; Castanon 2007; Moulin *et al.* 2008; Dewulf *et al.* 2012; Dewulf *et al.* 2013). Moreover, sulfa drugs, such as sulfadiazine, were widely used as feed additives during the 1970s and early 1980s. However, this practice resulted in the emergence of several veterinary pathogens exhibiting resistance to these antibiotics. Moreover, human health concerns raised associated with the consumption of milk and meat from antibiotic-fed animals. Therefore, the in-feed use of subtherapeutical amounts of Tc is no longer allowed in Europe since the late 1960s, and the use of antibiotics is now generally being reduced. Since January 2006, the addition of AGPs to feed has been completely banned by the European Commission (EC Regulation No. 1831/2003). However, several studies revealed the persistence of antibiotic resistant populations and resistance genes, even in the absence of direct antibiotic selection (Jindal *et al.* 2006; Walk *et al.* 2007; Stanton *et al.* 2011), such as on organic pig farms. Indeed, Tc resistance genes are still frequently detected in soil and water samples from areas surrounding pig farms. Moreover, the pig intestinal flora appears to represent a source of resistance genes, even in apparently antibiotic-free animals (Kazimierczak *et al.* 2009). The extensive in-feed use of Tc likely established a favorable environment for acquisition and maintenance of the *tet(C)* gene by *C. suis* (Dugan *et al.* 2004).

Although supplementation of feed with antibiotics is no longer allowed in Europe, also therapeutic treatment of pigs with Tc can select for Tc^R *C. suis* organisms. Borel *et al*. (2012) reported the rapid selection for Tc^R *C. suis* strains after antibiotic treatment of pigs suffering from conjunctivitis and diarrhea due to a *C. suis* infection. This selection for Tc^R *C. suis* strains was possibly facilitated by close contact of the pigs. Therefore, the recent implementation plan of the European Commission on group housing of sows and gilts (Directive 2008/120/EC on the protection of pigs) might contribute to the spread of Tc^R *C. suis* strains in pig farming in Europe. In chapter II, we demonstrated the presence of the *tet(C)* gene in nine *C. suis* isolates identified on three Belgian pig farms. Eight farmers reported the antibiotic treatment of the pig herd shortly before sampling, confirming the results obtained by Borel *et al.* (2012). Thus, selection of Tc^R strains upon treatment appears to be common. Since 2012, enormous efforts to sensitize veterinarians and farmers have been made, concerning the importance of restricted antibacterial use. As a result, the total consumption of antibacterial compounds in the Belgian veterinary medicine decreased with 12.7% over the past two years, according to the report of 2013 of the Belgian Veterinary Surveillance of Antibacterial Consumption (National report 2013 BelVet-Sac).

Tetracycline resistant Chlamydiae in humans

Although not allowed in all countries, enrofloxacine, a fluoroquinolone antibiotic, is currently used to counter Tc^R *C. suis* outbreaks. However, according to Reinhold *et al*. (2011a), short-term antimicrobial treatment at dosages recommended for treatment of other bacterial infections in pig herds was not effective in eliminating naturally acquired subclinical *C. suis* infections in pigs. Moreover, the fluoroquinolone antibiotics are extensively used in human medicine. Wide agricultural use of these drugs might thus increase selection of antibiotic resistance, and therefore compromise further effectiveness of antibiotics in both humans and animals. Currently, multi-resistance to other antibiotics, including sulfadiazine, azithromycin, ofloxacin, and doxycycline, is frequently detected in Tc^R *C. suis* strains (Lenart *et al.* 2001; Suchland *et al.* 2009). The emergence of multidrug resistance poses a threat especially to patients in healthcare settings. However, the emergence of Tc resistance in *C. suis* might imply a major worldwide public health challenge. Suchland *et al*. (2009) demonstrated the *in vitro* transfer among and within chlamydial species, including from *C. suis* into clinical isolates of *C. trachomatis,* leading to a stable Tc^R phenotype. Moreover, sequential infection *in vitro* can result in the occurrence of *C. suis* and *C. trachomatis* within the same inclusion (Lenart *et al.* 2001). Therefore, given the possible zoonotic potential of *C. suis*, transmission

of porcine Tc^R *C. suis* strains to *C. trachomatis* infected humans treated with Tc, would create a favorable environment for transfer of Tc resistance into *C. trachomatis*. Once a resistant *C. trachomatis* strain is established, cross-serovar transmission through a patient population might occur rapidly (Suchland *et al.* 2009), leading to treatment failure. Ocular *C. trachomatis* strains cause active trachoma in an estimated 84 million people, especially in developing countries, and *C. trachomatis* is the most common bacterial sexually transmitted disease in the world, with over 100 million new cases each year (WHO 2012). Therefore, the spread of Tc resistance among clinical *C. trachomatis* strains would affect millions of people worldwide.
Currently, several documented cases of clinical *C. trachomatis* isolates exist that exhibited resistance to treatment with Tc (Jones *et al.* 1990; Lefevre and Lepargneur 1998; Somani *et al.* 2000). However, these strains displayed so-called 'heterotypic resistance', in which only a small proportion of the population survives after exposure to Tc. Moreover, these isolates lost their resistant phenotype upon passage in cell culture, or lost viability completely. Hence, no clinical *C. trachomatis* strains that demonstrate stable Tc resistance have been currently identified (Jones *et al.* 1990; Lefevre and Lepargneur 1998; Somani *et al.* 2000; Suchland *et al.* 2003). Thus, the identification of the *tet(C)* gene in *C. suis* is the first, and only, example of horizontal acquisition of DNA in any *Chlamydia* spp. (Greub *et al.* 2004; Roberts 2005). Apart from the *tet(C)* gene, *Chlamydiae* in general have not acquired foreign DNA in the recent evolutionary past, and their genomes show a high degree of genetic conservation (Sandoz and Rockey 2010). Therefore, recombination is considered as a rare event in *Chlamydiae*. However, genomic studies revealed that inter-chlamydial recombination does occur, and probably is common. In this respect, Suchland *et al*. (2009) hypothesized that the entry of the *tet(C)* island into the *C. suis* genome was challenging, yet transfer among strains, and even species, is straightforward and might perhaps occur rapidly. Therefore, although the genetic evidence of antibiotic resistance in *C. trachomatis* is currently lacking, vigilance for the possibility in the future is recommended.

Diagnostic considerations

Many reports on the prevalence of *Chlamydiaceae* in pigs are based on PCR-detection of *Chlamydiae*, since these tests allow rapid, sensitive and specific identification, directly from clinical specimens (Sachse *et al.* 2009). Yet, the false positive rate of NAATs, predominantly due to contamination, can be considerable. Also false negative results are of significant concern, possibly resulting from the presence of inhibitory substances, occasional failure of DNA extraction procedures, or the emergence of sequence variation in the targets of the assay

(Whiley *et al.* 2008). Moreover, DNA-based detection does not reflect the viability of the detected organisms. In some cases, additional isolation of the involved chlamydial bacteria is required, such as in regard to the examination of antibiotic resistant infections. However, the accurate diagnosis of antibiotic resistance encounters some challenges. Antimicrobial susceptibility assays rely on the isolation of the involved chlamydial strain in host cells, and subsequent culture in the presence of multiple antibiotic dilutions (Sandoz and Rockey 2010). Also the PCR-based detection of the *tet(C)* gene described by Dugan *et al*. (2004) preferentially includes prior isolation, since this assay in not *Chlamydia*-specific. Various cell lines and techniques are used in different diagnostic laboratories, which complicates the accurate evaluation and monitoring of antibiotic resistance. Moreover, multiple factors can influence the outcome of an antibiotic susceptibility analysis, including the nature and passage number of the cell line and *Chlamydiae*, the multiplicity of chlamydial infection, and the developmental stage when the antibiotic is added to the infected cells (Wang *et al.* 2005). Hence, small differences in the methodological approach further challenge the interpretation of *in vitro* resistance and its clinical relevance. In addition, clinical isolates, can be highly fastidious to grow, especially from rectal samples, and generally have much slower growth rates compared to laboratory strains. This is particularly the case for *C. suis* strains, yet, there are relatively few reports on the growth conditions of porcine *C. suis* isolates. Therefore, in chapter III, we characterized the growth of three *C. suis* strains of distinct origin on different cell lines. Interestingly, these results revealed that for two of the examined strains, the cell line that produced the highest replication level, differed from McCoy cells, commonly used by many laboratories for isolation and propagation of *Chlamydiae*. Therefore, improvement of the isolation protocol appears to be possible, and adaptation of the procedure to the origin of the isolates is recommended. These findings are valuable for successfully isolating *Chlamydia,* including Tc^R *C. suis* strains, from field samples, and subsequent identification and characterization for epidemiological purposes.

Furthermore, other factors can complicate the evaluation of antibiotic resistance. As mentioned earlier, antibiotic treatment of chlamydial infections might induce persistence, resulting in treatment failure and a prolonged infection caused by viable but non-culturable *Chlamydiae* (Chopra *et al.* 1998; Hogan *et al.* 2004). Hence, it may be challenging to differentiate persistence from potential cases of antibiotic resistance, which might lead to erroneous conclusions regarding the antibiotic resistance of a clinical isolate.

Treatment of chlamydial infections

In addition to Tc resistance in *C. suis, Chlamydiae* can acquire resistance to six major classes of antibiotics, through mutations, at least *in vitro* (Sandoz and Rockey 2010). To counter emerging antibiotic resistance among human and animal pathogens in general, new Tc analogs, insusceptible to the existent resistance mechanisms, and new agents belonging to completely new classes of antimicrobials are frequently examined. However, the development of these new therapeutic strategies cannot keep up with the rapidly evolving resistance in microorganisms, resulting in more resistant pathogens and increasingly limited treatment options. Hence, many alternative antimicrobial therapies are being examined based on the inhibition of virulence rather than of bacterial growth. This approach is believed to impose less selective pressure for development of bacterial resistance compared to traditional procedures. Many strategies to identify these so-called virulence blockers have been reported, as described in chapter I. However, only few agents have been investigated for exerting anti-chlamydial activity. The anti-bacterial effects of transferrins in Gram-positive and Gram-negative bacteria have already been described (Gonzalez-Chavez *et al.* 2009; Giansanti *et al.* 2012). Moreover, LF and ovoTF have already proven their potential to reduce chlamydial infection *in vitro* and *in vivo* (Beeckman *et al.* 2007; Van Droogenbroeck *et al.* 2008; Van Droogenbroeck *et al.* 2011). Moreover, bactericidal peptides derived from transferrins, such as OTAP-92 derived from ovoTF, and lactoferricin B from bLF, have shown to be much more effective against Gram-positive and Gram-negative bacteria compared to the intact transferrins (Dionysius and Milne 1997; Hoek *et al.* 1997; Ibrahim *et al.* 1998; Ibrahim *et al.* 2000). However, the anti-chlamydial effect of transferrins towards *C. suis* remains univestigated. Further research, *in vitro* and *in vivo*, on the application of transferrins as promising alternative treatment of chlamydial infections is recommended. Moreover, the performance of kinetic studies to follow the route, course and clinical manifestations of *C. suis* infections in pigs from birth to slaughter on a pig farm, could aid in determining the ideal timing, method and route of administration of transferrin treatment.

Conclusions and future perspectives

The prevalence of *C. suis* in pig herds is widely documented, yet, the zoonotic potential of this organism is poorly examined. In chapter II, the endemic presence of *C. suis* in Belgian pig herds was confirmed. Moreover, three viable *C. suis* isolates were demonstrated in pig farmers, which further substantiates the zoonotic potential of *C. suis*. Furthermore, the *tet(C)* gene was demonstrated in porcine *C. suis* isolates on three farms, but not in human isolates. Given the emerging Tc resistance in *C. suis* and the possible associated public health concerns, future research should focus on the zoonotic transmission and significance of *C. suis* infections in humans, based on evaluation of the serological response to *C. suis* infections in a larger human risk population. Moreover, monitoring of Tc^R *C.suis* in pig farming is advisable. Further efforts to increase the awareness of the implications of porcine chlamydial infections, and to restrict therapeutic use of Tcs in the veterinary field are recommended.

The isolation of viable chlamydial organisms in culture remains crucial for the characterization and pathogenicity studies of new isolates. However, the isolation of *C. suis* from field samples is often fastidious, and the available data on the growth conditions of *C. suis* strains in culture are limited. Therefore, in chapter III, the replication of three laboratory strains of *C. suis* were compared in six cell lines of distinct origin. According to these results, the growth performance in the investigated cell lines varied among the *C. suis* strains. This variation may be the consequence of the high degree of genetic diversity within the species *C. suis,* which could also explain the high variation in reported virulence of distinct *C. suis* strains. Therefore, we need to advance our knowledge of *C. suis* pathogenesis, including the role of co-infections, and further examine the link between the causative strain and the associated pathology. In the near future, the genome sequence of additional *C. suis* strains, apart from the MD56 strain, will become available and will certainly contribute to elucidate these issues. Moreover, as mentioned above, kinetic studies on the course and clinical and economic implications of *C. suis* infections in pigs in the field, may further improve our knowledge on porcine chlamydial infection biology. Interestingly, pig breed dependent susceptibility for pseudorabies virus (Reiner *et al.* 2002b), porcine reproductive and respiratory syndrome virus (Halbur *et al.* 1998), *E. coli* (Duchet-Suchaux *et al.* 1991; Michaels *et al.* 1994) and *Sarcocystis miescheriana* (Reiner *et al.* 2002a) has been observed, and might thus also influence *Chlamydia* associated pathology in pigs, suggesting further investigation.

The widespread emergence of Tc resistance in *C. suis* in pigs herds endangers the efficient treatment of chlamydial infections. Several promising compounds have been identified as alternative to failing antibiotic treatment. Further research on the development of therapeutics for chlamydial infections should be promoted. The optimal approach to counter chlamydial infections in pigs would be through vaccination. Currently, no vaccines for protection against *C. suis* infection are available. However, genital infection of pigs with *C. suis* S45 induces cellular and humoral immune responses, that provide a certain level of protection against re-infection (De Clercq *et al.* 2014). These promising findings suggest that the development of a *C. suis* vaccine, eliciting adequate protection against infection, might have a significant success rate.

Summary

Chlamydiaceae are Gram-negative obligate intracellular bacteria causing disease in a broad range of animals, including humans. *Chlamydiaceae* infections are widespread in pig farming throughout the world, where they cause marked economic losses (Schautteet and Vanrompay 2011). This thesis focuses on *Chlamydia (C.) suis*, the most prevalent chlamydial species occurring in pigs, and considered as endemic in the intestinal flora of swine (Schautteet and Vanrompay 2011). The primary pathogenicity of *C. suis* for the conjunctiva, respiratory system, gastrointestinal and urogenital tract has been demonstrated in various experimental infection studies in gnotobiotic and conventionally raised pigs (Rogers and Andersen 1996; Rogers *et al.* 1996; Rogers and Andersen 1999; 2000; Sachse *et al.* 2004; Reinhold *et al.* 2008; Guscetti *et al.* 2009). Moreover, natural chlamydial infections in pigs have been associated with conjunctivitis, arthritis, pericarditis, polyserositis, pneumonia, enteritis, diarrhea and reproductive failure (Willigan and Beamer 1955; Sarma *et al.* 1983; Woollen *et al.* 1990; Zahn *et al.* 1995; Andersen 1998; Eggemann *et al.* 2000b). However, the majority of chlamydial infections, especially intestinal infections, is believed to be subclinical (Pospischil and Wood 1987; Szeredi *et al.* 1996; Nietfeld *et al.* 1997; Hoelzle *et al.* 2000; Camenisch *et al.* 2004a; Englund *et al.* 2012). *Chlamydiaceae* are generally highly sensitive to tetracycline (Tc), which is the current drug of choice to treat chlamydial infections in lifestock and humans, due to its low cost, high effectiveness, low toxicity and broad spectrum of activity (Chopra and Roberts 2001; Michalova *et al.* 2004). However, tetracycline resistant (Tc^{R}) *C. suis* strains have been isolated in pig production in the U.S. and throughout Europe (Andersen 1998; Di Francesco *et al.* 2008; Borel *et al.* 2012; Schautteet *et al.* 2012), leading to treatment failure. The emergence of Tc^{R} *C. suis* strains also implies a possible hazard for public health. *Chlamydia suis* is phylogenetically highly related to the human pathogen *C. trachomatis* (Everett *et al.* 1999), and therefore believed to be a potential zoonotic bacterium. If zoonotic transfer of *C. suis* to *C. trachomatis* infected humans occurs, transfer of the Tc resistance gene to *C. trachomatis* might be facilitated, leading to the creation of Tc^{R} *C. trachomatis* strains. This would affect millions of people worldwide, suffering from ocular or genital *C. trachomatis* infections (Mabey 2008; WHO 2012). Therefore, surveillance of the spread of Tc^{R} *C. suis* into commercial pigs and evaluation of the zoonotic potential of *C. suis* is recommended to assess the associated risks and appropriate measures.

Chapter I gives a brief overview of the taxonomy and biology of chlamydial infections, followed by a short overview of some aspects of the developmental cycle. Furthermore, the

occurrence of *Chlamydiaceae* species in pigs is described with a focus on the diagnosis, epidemiology, pathology, antibiotic resistance and zoonotic transmission. The final part of this chapter overviews the current chlamydial virulence blocking strategies.

In **Chapter II**, we examined the zoonotic transfer of *C. suis* to pig farmers on nine Belgian pig farms, using *Chlamydia* culture and a *C. suis* specific real-time PCR in both pigs and humans. Moreover, farmers were examined using a *C. trachomatis* PCR. Additionally, the *Chlamydia* isolates were tested for the presence of the *tet(C)* resistance gene. *Chlamydia suis* DNA was demonstrated in pigs on all farms, and eight of nine farmers were positive in at least one anatomical site. None of the farmers tested positive for *C. trachomatis.* *Chlamydia suis* isolates were obtained from pigs of eight farms. Nine porcine Tc^{R} *C. suis* strains were retrieved, originating from three farms. Moreover, three human *C. suis* isolates were identified, including one pharyngeal and two rectal isolates. These findings suggest further research on the zoonotic transfer of *C. suis* from pigs to humans.

In **Chapter III**, we examined the growth of *C. suis* in cell culture. The identification of viable bacteria through isolation of chlamydial pathogens in cell culture remains crucial for the generation of new isolates and their characterization and pathogenesis studies. However, the reports on growth conditions of *C. suis* strains are limited, and isolation of *C. suis* from field samples is often fastidious (Sandoz and Rockey 2010). We examined the growth characteristics of a conjunctival, respiratory and intestinal *C. suis* strain in six different cell lines, and compared two chlamydial growth media. Our results suggest that the preferred cell line for propagation of *C. suis* differs among strains, and may be divergent from the cell lines currently applied. Furthermore, the use of IMDM chlamydia culture medium may increase the replication of *C. suis*, yet, this effect is strain and cell type dependent. According to these results, an adaptation of the currently used isolation methods to the origin of the concerning *C. suis* isolate would be appropriate.

Finally, **Chapter IV** describes our conclusions and perspectives for further research.

Samenvatting

Chlamydiaceae zijn Gram-negatieve obligaat intracellulaire bacteriën die ziekte kunnen veroorzaken bij mens en dier. Infecties zijn ook wijdverspreid in commerciële varkens over heel de wereld, en kunnen leiden tot economische verliezen (Schautteet, K. and Vanrompay 2011). Deze thesis richt zich op *Chlamydia (C.) suis*, het meest prevalente chlamydiale species in varkens, dat als endemisch wordt beschouwd in hun intestinale flora (Schautteet, K. and Vanrompay 2011). De primaire pathogeniciteit van *C. suis* voor de conjunctiva, het ademhalingsstelsel, het gastrointestinaal en urogenitaal stelsel werd door verschillende experimentele infecties aangetoond in gnotobiotische en conventioneel gefokte biggen (Rogers and Andersen 1996; Rogers *et al.* 1996; Rogers and Andersen 1999; 2000; Sachse *et al.* 2004; Reinhold *et al.* 2008; Guscetti *et al.* 2009). Bovendien zijn natuurlijke *Chlamydia* infecties in varkens geassocieerd met conjunctivitis, arthritis, pericarditis, polyserositis, pneumonia, enteritis, diarree en voortplantingsstoornissen (Willigan and Beamer 1955; Sarma *et al.* 1983; Woollen *et al.* 1990; Zahn *et al.* 1995; Andersen 1998; Eggemann *et al.* 2000). Echter, de meerderheid van de infecties, voornamelijk intestinale infecties, wordt beschouwd als subklinisch (Pospischil and Wood 1987; Szeredi *et al.* 1996; Nietfeld *et al.* 1997; Hoelzle *et al.* 2000; Camenisch *et al.* 2004; Englund *et al.* 2012). *Chlamydiaeceae* zijn over het algemeen gevoelig voor tetracycline antibiotica, wat op dit moment dan ook het middel bij uitstek is om *Chlamydia* infecties in dieren en mensen te behandelen, door de lage kostprijs, hoge effectiviteit, lage toxiciteit en breed spectrum van activiteit (Chopra and Roberts 2001; Michalova *et al.* 2004). Echter, tetracycline resistente (Tc^R) *C. suis* stammen, die leidden tot het falen van de behandeling, werden reeds geïsoleerd in de varkenshouderij in de V.S. en Europa (Andersen 1998; Di Francesco *et al.* 2008; Borel *et al.* 2012; Schautteet, K *et al.* 2012). Het opduiken van Tc^R *C. suis* stammen vormt ook een mogelijk gevaar voor de volksgezondheid. *Chlamydia suis* is fylogenetisch sterk verwant met de menselijke pathogen *C. trachomatis* (Everett *et al.* 1999), en wordt daarom beschouwd als een mogelijk zoonotische bacterie. Als zoonotische transfer van *C. suis* naar *C. trachomatis* geïnfecteerde patiënten plaatsvindt, is ook transmissie van het Tc resistentie gen naar *C. trachomatis* mogelijk. Het ontstaan en verspreiding van deze Tc^R *C. trachomatis* stam zou gevolgen hebben voor de miljoenen mensen wereldwijd die lijden aan oculaire of genitale *C. trachomatis* infecties (Mabey 2008; WHO 2012). Monitoring van de verspreiding van Tc^R *C. suis* in commerciële varkens en evaluatie van het zoonotisch potentieel van *C. suis* is daarom van cruciaal belang om het risico in te kunnen schatten en de geschikte maatregelen te nemen.

Hoofdstuk I start met een kort overzicht van de taxonomie en biologie van *Chlamydia* infecties, gevolgd door een beschrijving van de belangrijkste aspecten van de ontwikkelingscyclus. Daarnaast wordt ook het voorkomen van *Chlamydiaceae* species in varkens beschreven met de nadruk op de diagnose, epidemiologie, pathologie, antibioticum resistentie and zoonotische transmissie. Het finale deel van dit hoofstuk geeft een overzicht van de huidige virulentie blokkeringsstrategiën voor *Chlamydia*.

In **Hoofdstuk II**, hebben we de zoonotische transfer van *Chlamydia suis* naar varkenshouders onderzocht op negen Belgische bedrijven, aan de hand van *Chlamydia* cultuur en een *Chlamydia suis* specifieke real-time PCR in zowel varkens als de mens. Bovendien werd infectie met *C. trachomatis* in de varkenshouders nagegaan door middel van PCR. Daarnaast, werden de bekomen *Chlamydia* isolaten ook onderzocht voor de aanwezigheid van het *tet(C)* resistentie gen. *Chlamydia suis* DNA werd aangetoond in varkens op alle bedrijven en acht van de negen varkenshouders bleken positief voor minstens één staal. Geen enkele van de varkenshouders was besmet met *C. trachomatis. Chlamydia suis* isolaten werden bekomen van varkens van acht bedrijven, en waaronder negen Tc^R *C. suis* stammen, afkomstig van drie verschillende bedrijven. Hiernaast werden ook drie *C. suis* isolaten bekomen van de varkenshouders, waaronder één faryngeaal en twee rectale isolaten. Deze resultaten suggereren de nood aan verder onderzoek omtrent de zoonotische transfer van *C. suis* van het varken naar de mens.

In **Hoofdstuk III** hebben we de groei van *C. suis* in celcultuur bestudeerd. De identificatie van levende bacteriën via isolatie in celcultuur blijft cruciaal voor de opgroei en karakterisatie van nieuwe isolaten en studie van de geassocieerde pathogenese. De beschikbare informatie over optimale groeicondities van *C. suis* stammen is echter zeer beperkt, en isolatie van *C. suis* uit veldstalen is vaak erg moeilijk (Sandoz and Rockey 2010). Daarom hebben we de groeikarakteristieken bepaald voor een conjunctivale, respiratoire en intestinale *C. suis* stam in zes verschillende cellijnen, en hierbij twee *Chlamydia* groei media vergeleken. Onze resultaten suggereren dat de meest geschikte cellijn voor opkweek van *C. suis* verschilt tussen de stammen, en afwijkt van de cellijnen die op dit moment routinematig gebruikt worden voor de opkweek van *C. suis*. Daarnaast kan ook het gebruik van IMDM *Chlamydia* cultuur medium de replicatie van *C. suis* verbeteren, echter, dit effect is stam- en celtype afhankelijk. Uit deze resultaten blijkt dat het afstemmen van de isolatiemethode op de oorsprong van de te analyseren stalen aangeraden is.

Hoofdstuk IV beschrijft tenslotte onze conclusies en perspectieven voor verder onderzoek.

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Dankwoord

Eindelijk aangekomen bij het schrijven van mijn dankwoord! De voorbije vier jaren waren niet altijd even gemakkelijk, dus het is een hele opluchting dat de eindstreep in zicht is! Maar tot hier geraken, heb ik natuurlijk niet alleen gedaan. Daarom wil ik alvast iedereen bedanken die op de een of andere manier heeft bijgedragen aan mijn doctoraat.

Als eerste bedank ik mijn promotor Prof. dr. Daisy Vanrompay. Daisy, bedankt om me de kans te geven een doctoraat te starten in jouw labo. Het allerleukste aan dit doctoraat was toch wel dat ik het samen met mijn zus heb mogen doen. Bedankt voor het vertrouwen dat je in ons beiden gesteld hebt. Bedankt voor de vele 'wekelijkse' meetings, en voor de creativiteit die je aan de dag legde om de naderende FOD-deadlines te halen. Bedankt ook om mijn doctoraat na te lezen. Daarnaast wil ik ook mijn co-promotor Prof. dr. Eric Cox bedanken voor de begeleiding van mijn onderzoek en voor het gebruik van de infrastructuur en stallen van het labo Immunologie aan de faculteit Diergeneeskunde.

Ik wil zeker ook alle leden van de lees-en examencommissie (Daisy, Prof. Cox, Prof. dr. ir. Patrick Van Damme, Prof. dr. ir. Tom Van de Wiele, Prof. dr. Richard Ducatelle, Prof. dr. ir. Dirk Fremaut, Dr. ir. Bert Devriendt, Dr. Vesna Melkebeek, Dr. Ellen de Jong) bedanken voor de constructieve bijdrage aan mijn doctoraat. Dankzij jullie opmerkingen en commentaren is de kwaliteit van dit doctoraat er zeker op voorruit gegaan. Ook wil ik Prof. Dr. Servaas Morré heel erg bedanken voor het verbeteren van mijn eerste hoofdstuk. De leden van mijn FOD commissie, en in het bijzonder dr. Dominique Vandekerchove, wil ik ook bedanken voor de interessante discussies op de jaarlijkse vergadering. Een speciale bedanking gaat uit naar Patrick Delava (Nuscience). We hebben hem leren kennen als een gedreven man en voorvechter van de ernst van de *Chlamydia suis* problematiek bij varkens. Het recente bericht van zijn overlijden heeft dan ook een grote indruk op ons nagelaten. Heel erg bedankt voor de interesse in ons project en de waardevolle bijdrage aan ons onderzoek.

En dan nu de bedankingen voor mijn collega's, de immuno's! (Delphine Beeckman, Katelijn Schautteet, Veerle Dickx, Stefanie Lagae, Lizi Yin, Annelien Dumont, Sarah Van Lent, Evelien De Clercq, Isabelle Kalmar, Julie Geldhof, Matthias Van Gils, Cindy De Boeck, Eamy Yaacob, Thi Minh Tho Dam en Bakr Ahmed Abdelrahman Mohamed). Dankzij jullie zijn de voorbije vier jaar voorbijgevlogen. Ik had nooit gedacht dat het zo gezellig zou worden in ons labo. We waren een hechte groep die lief en leed met elkaar deelden, wat soms tot hilarische taferelen kon leiden. Dankjewel iedereen voor de leuke middagpauzes die voor

de nodige afleiding zorgden ;-). **Delphine**, je verliet het labo al een paar maanden na mijn komst, maar jouw aanwezigheid is nog steeds zichtbaar in het labo. Meer dan eens zijn jouw betrouwbare protocols mij goed van pas gekomen! **Veerle**, toen ik in het labo begon was jij net aan het einde van je doctoraat. Bedankt voor de hulp bij de staalname in het slachthuis tijdens je laatste werkweken, en voor de goede raad en leuke babbels. **Katelijn**, jij was bij de start van mijn doctoraat de postdoc van dienst. Je geduld, kalmte en relativeringsvermogen hebben mij meer dan eens gerust gesteld. Toen je na je moederschapsverlof terugkwam naar het labo, waren we natuurlijk heel erg blij om nog een paar maanden met jou te kunnen samenwerken. Bedankt om ons te vergezellen op onze eerste staalnames op de varkensbedrijven en in het slachthuis, en voor het beantwoorden van talloze vragen. We hebben veel van jou geleerd! **Stefanie**, toen Katelijn het labo verliet was jij diegene met de meeste ervaring, en werd je tot senior van het labo 'gekroond'. Vanaf dan richtten we onze pijlen op jou met al onze vragen. Je was een expert in het multitasken in het labo, en combineerde moeiteloos het begeleiden van de practica, drie thesisstudenten (tegelijkertijd!) en je doctoraatsonderzoek. Bedankt voor de hulp en goede raad, maar vooral bedankt voor je dagelijkse portie vrolijkheid en leuke vakantieverhalen ;-). **Annelien,** jij bent altijd bereid te helpen en onmisbaar geworden voor de immuno's. Als jij in het labo bezig was, waren er nooit veel stille momenten, waardoor de tijd voorbij vloog. Bedankt voor alle hulp en gezellige afleiding. Ook heel erg bedankt om al die kleine dingen te regelen op het einde van mijn doctoraat! Ik wens je nog veel aangename immuno-jaren toe! **Sarah**, je kwam na één jaar het immuno-team vervoegen, en hebt je vlot de groep 'ingebabbeld'. Toen je na je verblijf in Amerika terugkwam, was het dan ook alsof je al jaren bij ons werkte. Bedankt voor het extra leven in het 'varkenskot' beneden! Je bent zelf ondertussen ook aan je laatste jaar toegekomen, en dat komt zeker goed! Nog veel succes met je doctoraat!

En dan een heel speciaal plaatsje in mijn bedankingen voor **Evelien(tje)**. Je zal wel al weten dat jij voor ons veel meer dan gewoon 'een collega' was. Je was de voorbije vier jaar ons 'maatje', bijna onze drielingzus ☺! Van bij het begin zaten we meteen op dezelfde golflengte. Zonder een woord kon ik aan je blik vaak zien dat we hetzelfde dachten. Ik heb zo vaak de slappe lach gehad van je verhalen of de befaamde 'google translate momenten'. Ik zal veel van je anekdotes niet snel vergeten (denk maar aan het 'I don't know'-verhaal, waar ik met mijn naderende publieke verdediging vaak om moet lachen ;-)). Bedankt voor alle steun, de vriendschap, en de ontelbare hilarische momenten! We zullen elkaar vast nog veel terugzien, en daar kijk ik nu al enorm naar uit!

Julie, ook jij voelde je direct thuis toen je in de immuno-groep terechtkwam. Je enthousiasme werkte aanstekelijk. Voor jou was geen uitdaging te veel, en je ervaring als dierenarts was heel erg welkom. Bedankt voor de hulp en de vrolijke maanden dat we hebben samengewerkt! **Isabelle**, jij was de expert in dierexperimenten. Ik wil je bedanken voor de babbels en voor je hulp bij onze dierproef in de isolatoren. **Matthias**, toen jij bij de immuno's startte, moest je je plaats vinden tussen al dat vrouwelijk geweld. Maar gelukkig kan jij wel tegen een stootje ;-) Bedankt voor je vrolijkheid en zotte noot, en alle hulp bij de varkens. **Cindy,** ik wil je bedanken voor de leuke babbels en nog veel succes met jouw doctoraat.
Ik wil zeker ook mijn thesistudenten **Lien De Vogelaere, Laura Van Hauwe** en **Kristof Hauwaert** bedanken voor de hulp bij het analyseren van mijn varkensstalen en de andere experimenten. **Bakr**, many thanks for helping during our pig experiments. **Rudy Cooman**, bedankt om chauffeur te zijn bij de staalnames op de varkensbedrijven, en voor de wilde verhalen onderweg ;-). Ook bedankt om telkens weer varkens voor ons op te halen 'aan de andere kant van het land', en voor alle hulp bij de varkensexperimenten.
Ook een hele dikke merci aan **Fien De Block** en **Sofie De Schynkel** voor alle administratieve hulp en bestellingen, maar meer nog voor jullie vriendelijkheid, geduld, begrip, medeleven en behulpzaamheid! En dan wil ik zeker ook **Geert Meesen** bedanken voor alle hulp bij elk technisch probleem in het labo. Ook bedankt voor je geduldige ondersteuning en uitleg bij de microscoop. Verder wil ik ook **Winnok De Vos** bedanken voor alle hulp bij de microscoop, en voor het ontwikkelen van de macro. Ook bedankt voor het nalezen van mijn hoofdstukken. Zeker ook bedankt aan alle andere **mobi-collega's** voor de aangename sfeer en samenwerking.
Ook een dikke merci aan **Liesbeth** en **Ellen**. Jullie zorgden voor een welkome afleiding van de doctoraatsperikelen, hoewel het niet altijd gemakkelijk was om ons te bereiken (sorry daarvoor ;-)). Bedankt voor de gezellige etentjes en hilarische spelletjesavonden, zoals er hopelijk nog veel mogen volgen!

Het laatste en belangrijktste deel van mijn dankwoord is voorbehouden voor mijn familie. **Mama en Papa**, mijn allergrootste dank ben ik zeker en vast aan jullie verschuldigd. Zonder jullie opofferingen zou ik hier nooit geraakt zijn. Dankjewel dat jullie altijd in mij geloofden, voor de onvoorwaardelijke steun, voor het eindeloze begrip en geduld, en voor het warme en gezellige nest. Lieve mama, ik ken niemand die zo warm, sterk en verstandig is als jij. Voor elk probleem weet jij een oplossing, en we kunnen altijd op je rekenen. Bedankt voor je kalmte, optimisme en doorzettingsvermogen! Je maakt ons een beter mens! Een betere mama

hadden we ons niet kunnen wensen! Lieve papa, jij staat altijd klaar om ons te helpen, geen moeite is te veel. Jouw handigheid is ons al heel vaak van pas gekomen ;-) en je beseft niet genoeg hoe belangrijk je voor ons bent. Bedankt voor je bezorgdheid en onvoorwaardelijke steun. We zijn fier op zo een papa! **Veronic**, onze multi-getalenteerde grote zus! Niemand is zo een doorzetter als jij! Dankjewel voor je enthousiasme, steun, gastvrijheid, relativeringsvermogen en bezorgdheid. En natuurlijk ook dankjewel voor je uitgebreide statistische kennis waar ik zo vaak een beroep op heb gedaan! En ook bedankt voor die twee kleine spruitjes waar we zoveel plezier aan beleven ;-) **Senne en Mirthe,** jullie maken mij een hele fiere tante Lot! Dankjewel voor jullie vrolijkheid, om mij af en toe af te leiden van het schrijfwerk, en om mij de doctoraatsperikelen even te doen vergeten. Binnenkort kunnen we weer heel veel bootje spelen en Loko le dansen ☺! **Annemieke**, dankjewel om onze lieve kleine grote zus te zijn! Dankjewel om ons altijd op te vrolijken, voor je onvoorwaardelijke steun en optimisme, voor je creatieve ideeën, voor de 'raad van je grote zusje' en al je zusterliefde! Bedankt voor je bezorgdheid en je relativeringsvermogen! Je bent een enorme steun voor de zusjes! En dan wil ik ook onze lieve broerie **Kim** bedanken. Bedankt voor je eindeloze excel-expertise, het heeft mij enorm veel tijd bespaard! Bedankt voor je vrolijkheid, je relativeringsvermogen en je 'burgelijk ingenieurswijsheid' ;-). **Goyabie**, bedankt om mijn voetjes warm te houden tijdens de koude schrijfmaanden ;-). Bedankt voor je eeuwig goed humeur, speelsheid en vrolijkheid! Ook bedankt om mij af en toe toch van mijn bureau weg te lokken voor een wandeling, knuffeltje of spelletje! Jij bent mijn favoriete speelkameraadje!

Mijn laatste en allergrootste bedanking is voor mijn **Wonsietje**! Jij bent mijn wederhelft, zeg maar betere helft! Ik kan niet uitdrukken hoe ontzettend veel je voor mij betekent! Ik ben enorm fier dat ik jouw tweelingzus mag zijn! Jij bent er altijd voor mij, en kent mij door en door. Je begrijpt mij zonder woorden, of toch meestal ;-) En je kan mij als geen ander doen lachen! Bedankt voor je eindeloze geduld en liefde! Zonder jouw onvoorwaardelijke steun, begrip, medeleven, hulp en vertrouwen had ik het de voorbije vier jaar niet gered. We hebben ons samen door dit doctoraat heen geploeterd, en daar ben ik nu enorm trots op! Weet dat jij ook altijd onvoorwaardelijk op mij kan rekenen! Oef, we zijn er nu allebei vanaf! Ik zou zeggen, inderdaad hoog tijd voor een tripje naar Disneyland! ☺☺☺

Curriculum Vitae

Personal Information

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Place of birth	Sint-Niklaas, Belgium
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Education

2010 – 2015	PhD Applied Biological Sciences: Cell and Gene Biotechnology, Ghent University
2005 – 2010	Master in Biochemistry and Biotechnology**,** Ghent University
1999 – 2005	Science-Mathematics (8h), Heilige Familie, Sint-Niklaas

Extra Courses

2014	Academic English: Writing a Research Article
2012-2013	Q-PCR experiment design and data-analysis, Biogazelle, Leuven
2011-2012	Basic course in laboratory animal science, Ghent University: Part I, general topics FELASA Cat. B Part II, specific topics FELASA Cat. C

Experience – Research skills

Writing of scientific reports and articles.

Laboratory experience, acquired during my master and PhD thesis:
Cell and bacterial culture, DNA extraction, real-time PCR, micro-arrays, gene cloning, plasmid purification, transfection, production of recombinant proteins, ELISA, western blotting, immunofluorescense staining, fluorescence microscopy, animal experiments.
Working in biosafety level 3 laboratories and handling of hazardous and bio-hazardous organisms (*Chlamydiaceae*).

Thesis students:

2013-2014: **Kristof Van Hauwaert**: "Studie naar de groei van *Chlamydia suis* in celcultuur en het voorkomen van tetracycline resistentie in de Belgische varkenssector"
2012-2013: **Laura Van Hauwe:** "Studie naar de groei van *Chlamydia suis* in celcultuur.
2011-2012: **Lien De Vogelaere:** "Moleculair epidemiologisch onderzoek naar de overdracht van *Chlamydia suis* infecties van varkens naar de mens".

Languages

Dutch: mother tongue
English: good
French: basic
German: basic

Software

Good knowledge of MS Office (Word, Excel, PowerPoint) and Endnote.
Basic knowledge of Vector NTI, BioEdit, Image J and SPSS.

Publications

Beeckman D S, **De Puysseleyr L**, De Puysseleyr K et al. (2014). "Chlamydial biology and its associated virulence blockers." Critical reviews in microbiology 40(4): 313-328.

De Puysseleyr K, **De Puysseleyr L**, Geldhof J, Cox E, Vanrompay D (2014). Development and Validation of a Real-Time PCR for *Chlamydia suis* Diagnosis in Swine and Humans. *PLoS One* 9: e96704.

De Puysseleyr K, **De Puysseleyr L**, et al. (2014). "Evaluation of the presence and zoonotic transmission of *Chlamydia suis* in a pig slaughterhouse." Bmc Infectious Diseases 14: 560.

Abstracts

De Puysseleyr L, De Puysseleyr K, Dhondt H, et al. "Prevalence of *Chlamydia suis* in Pigs and Zoonotic Risk Assessment in a Pig Slaughterhouse." Deutscher Chlamydienworkshop 2014.

De Puysseleyr K, **De Puysseleyr L**, Geldhof J, et al. "Development and Validation of a Real-time PCR for *Chlamydia suis* Diagnosis in Swine and Humans." Deutscher Chlamydienworkshop 2014.

De Puysseleyr L, De Puysseleyr K, Morré S A, et al. "Presence of *Chlamydia suis* in Pig Farmers." Deutscher Chlamydienworkshop 2014.

Meetings

With poster presentation

Deutscher Chlamydienworkshop, Berlin, Germany, April, 2 - 4 2014

Without presentation

First International ECMIS Symposium, *E. Coli* and the mucosal immune system, Ghent, Belgium, July 2 – 5, 2011

Eight annual Amsterdam *Chlamydia* meeting (AACM). Amsterdam, The Netherlands. December 9, 2011.