THE POTENTIAL OF DISINFECTANTS AND BACTERIOPHAGES TO REDUCE AVIAN COLIBACILLOSIS

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Dissertation presented in partial fulfilment of the requirements for the degree of PhD in Bioscience Engineering

October 2014

Doctoraatsproefschrift nr. 1211 aan de faculteit Bio-ingenieurswetenschappen van de KU Leuven

ISBN 978-90-8826-380-4
D/2014/11109/53

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Dankwoord

Allereerst wil ik mijn promotor Prof. Bruno Goddeeris (KU Leuven) bedanken voor het feit dat hij mij (in 2011) de mogelijkheid heeft gegeven om aan mijn PhD te kunnen beginnen. Verder ben ik u oprecht dankbaar voor de infrastructuur, materialen en begeleiding die u mij heeft gegeven gedurende mijn PhD, maar bovenal voor de zinvolle suggesties om mijn onderzoek en manuscripten te kunnen verbeteren.
Ook wil ik graag mijn copromotor Prof. Patrick Butaye (CODA-CERVA) bedanken voor de begeleiding die hij mij heeft gegeven, evenals voor de infrastructuur en de materialen waarmee ik mijn PhD aan het CODA kon starten in februari 2011. Verder ben ik u dankbaar voor uw initiatief om een beurs van het CODA aan te vragen om mijn PhD te kunnen volbrengen. Daarna hoefde ik mij geen zorgen meer te maken over de financiering van de laatste 1,5 jaar van mijn doctoraat. Daarvoor dank ik uiteraard ook het CODA-CERVA (in het bijzonder Pierre Kerkhofs).

De eerste 2 jaar van mijn PhD project werd gefinancierd door de Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu. Vele dank voor de financiering en het geloof in het project.

Verder wil ik de leden van de examencommissie: Prof. Delcour (KU Leuven), Prof. Decupyere (KU Leuven), Prof. Lavigne (KU Leuven), Prof. Lebeer (UAntwerpen) en Prof. Vanrompay (UGent) bedanken voor het doornemen en verbeteren van de doctoraatsthesis. In het bijzonder wil ik Prof. Lavigne nog graag bedanken voor zijn steun en suggesties gedurende het PhD traject.

Ik wil ook graag mijn collega's van de KU Leuven, met wie ik gedurende (een deel van) mijn PhD een kantoor heb gedeeld, bedanken: Huruma, Tom, Dao and Stijn. Ik wil jullie allemaal bedanken voor de leuke tijd die ik heb gehad en voor de interessante en inspirerende discussies die ik met jullie heb gehad om mijn werk te kunnen verbeteren. In particular, I would like to thank Huruma for his grateful support during the animal experiment and on the subject 'APEC' in general. You taught me to think more critically and learned me a lot about APEC due to your PhD on APEC and your veterinary background. Ik wil Tom graag bedanken voor zijn hulp in het lab en tijdens de *in vivo* experimenten, verder wil ik hem uiteraard bedanken voor het maken van de tekening op de cover van mijn proefschrift. Ik wil Jessica ook graag bedanken, met jou heb ik samengewerkt aan het bacteriofaag gedeelte van

de studie, in het bijzonder de *in vivo* experimenten. Dit betekende het maken van vaak lange dagen (een keer zelfs tot 4 uur ‘s nachts), gelukkig konden wij elkaar blijven motiveren. Ik wil Dr. Steven Janssens graag bedanken voor zijn hulp bij de statistische analyse van de resultaten.

Ook wil ik graag de andere collega’s in het lab en het gebouw bedanken: Laura, Sanne, Susanne, Annelies, Liesbeth, Katrien, Els, Amin, Joris, Kurt, Stefano, Eduardo, Alberto, Machteld, Tom, Iona en alle anderen die ik vergeten ben hier te melden. Dank jullie wel voor de leuke tijd tijdens conferenties, drankjes, tijdens het sporten en het feesten!

Ook wil ik graag Christel, Wim en Inge van het secretariaat bedanken, zij zorgden ervoor dat ik mijn dag altijd kon starten met de noodzakkelijke warme koffie. Verder uiteraard bedankt voor jullie hulp bij het regelen van bestellingen en andere administratieve zaken!

Mijn collega’s van het CODA-CERVA: John, Wannes, Lotte, Stephanie, Florence, Tinne, Laura, Irene, Maria, Andy, Deborah en Lena wil ik graag bedanken voor de leuke tijd op het CODA (en uiteraard buiten het CODA). I would like to thank Laura and Irene for their help with conducting some of the experiments on the CODA.

Ook wil ik mijn vrienden graag bedanken voor de altijd geslaagde bezoekjes aan Leuven en voor het begrip dat ik niet altijd aanwezig kon zijn tijdens verjaardagen en andere feesten. Bovenal ook bedankt voor de support om mijn PhD te kunnen halen en voor de nodige afleiding van het werk tijdens vakanties en gedurende weekenden.

Last but not least, wil ik natuurlijk mijn familie bedanken voor de support en liefde die het mogelijk maakte om mijn PhD succesvol te kunnen behalen. Ook was het erg leuk jullie Leuven te mogen laten zien.

Samenvatting

Aviaire pathogene *Escherichia coli* (APEC) veroorzaakt jaarlijks grote economische schade voor de pluimveesector als gevolg van sterfte, een tragere groei, een lagere voederomzettingsefficiëntie en afkeuring van karkassen in het slachthuis. Inhalatie van stof verontreinigd met hoge concentraties *Escherichia coli* kan de luchtzakken en longen infecteren, waarna de bacteriën de bloedbaan kunnen binnendringen met als gevolg een systemische infectie (bekend als colibacillose).

De ziekte wordt traditioneel behandeld met antibiotica, maar door veelvuldig gebruik van antibiotica zijn vele APEC stammen multiresistent geworden. Het gebruik van antibiotica veroorzaakt een risico voor de volksgezondheid, als gevolg van de overdracht van resistentiegenen/resistente bacteriën van dier op mens. Daarom werd gedurende dit onderzoek het gebruik van desinfectantia en bacteriofaagtherapie getest als alternatieve preventie/ behandelingsmethode voor colibacillose bij kippen.

Eerst werd de antibioticaresistentie van een selectie APEC stammen (n = 97) bepaald, representatief voor de Belgische leghennen industrie, aangezien er minder kennis is over APEC isolaten van legkippen dan isolaten van vleeskuikens. Verder werd bepaald of deze APEC isolaten resistent waren tegen de meest voorkomende werkzame stoffen (formaldehyde, glutaaraldehyde, glyoxaal, waterstofperoxide (H_2O_2) en een quaternaire ammoniumverbinding (QAC)) in routinematig gebruikte ontsmettingsmiddelen in de pluimvee-industrie. De biofilmvorming capaciteit van de stammen op materialen die veelvuldig in kippenstallen gebruikt worden werd bepaald, aangezien biofilmvorming een effect op de efficaciteit van desinfectantia kan hebben. Hoge resistentiepercentages tegen routinematig gebruikte antibiotica in de pluimveesector (ampicilline, nalidixinezuur, sulfonamide en trimethoprim) werden gevonden. Er werd echter geen fenotypisch detecteerbare verworven resistentie tegen routinematig gebruikte desinfectantia gevonden, terwijl resistentiegenen aanwezig waren. De meeste APEC stammen waren slechts gemiddelde biofilmvormers op polystyreen, terwijl zij op polypropyleen en polyvinyl chloride sterke biofilmvomers waren. Daarom werd de efficaciteit van twee van de vijf geselecteerde actieve bestanddelen van desinfectantia (H_2O_2 en QAC) om sessiele cellen te doden getest. H_2O_2 en QAC waren in staat om alle aanhechtende cellen te doden bij een concentratie van respectievelijk 1% en 0,01%.

Normaal worden desinfectantia gebruikt om ziekte-overdracht tussen opeenvolgende tomen in een stal te voorkomen, waarbij het aantal specifieke pathogenen zoveel mogelijk wordt verlaagd. In deze studie werd echter de efficaciteit van verneveling van lage concentraties H_2O_2 bepaald om de infectiedruk in aanwezigheid van kippen te verminderen. Kippen die respiratoir blootgesteld werden aan APEC en vervolgens aan 2% H_2O_2 hadden echter een hogere kans een hoge letsel score, veroorzaakt door APEC, te ontwikkelen dan kippen die werden blootgesteld aan APEC en vervolgens aan 1% H_2O_2 of gedestilleerd water. Dus H_2O_2 verneveling in aanwezigheid van kippen wordt ontraden.

Tijdens het tweede deel van de studie werd een bioluminescente APEC stam (CH2-lux) ontwikkeld door transpositie van het *lux* operon van *Photorhabdus luminescens* in de stam die gebruikt wordt voor de infectie-studies (CH2). De transpositie van het *lux* operon in het chromosoom van de APEC stam had geen invloed op de virulentie van de laatstgenoemde stam, noch de gevoeligheid aan lytische bacteriofagen (fagen). *Ex vivo* werden correlaties gevonden tussen de luminescentie en de concentratie bacteriën in het gehomogeniseerde hart, de lever en de longen van vier weken oude CH2-lux geïnfecteerde kippen. De gemerkte stam kan ook gebruikt worden om de kennis van de pathogenese van de ziekte te verbreden, om gerichte behandelingen tegen colibacillose te vinden. Een bacteriofaagcocktail, samengesteld uit vier fagen, werd na een *in vitro* selectie van de fagen ontwikkeld. De cocktail werd toegediend aan experimenteel geïnfecteerde kippen via de trachea, oesophagus of via het drinkwater. Behandelde groepen toonden echter geen significante daling in mortaliteit, letsel scores of gewichtsverlies in vergelijking met geïnfecteerde/onbehandelde groepen. De resultaten impliceren dat hoewel de faagcocktail de infectiestam (CH2) *in vitro* efficiënt kon lyseren, de faagcocktail experimenteel geïnfecteerde kippen niet succesvol kon genezen. Dus meer onderzoek is nodig om het therapeutische en profylactische *in vivo* effect van fagen te analyseren. De ontwikkelde luminescente APEC stam kan daarbij mogelijk oplossingen bieden.

Tijdens dit onderzoek werden verschillende experimenten uitgevoerd om het gebruik van desinfectantia en bacteriofagen te evalueren teneinde aviaire colibacillose te verminderen. Hoewel het veel waardevolle resultaten heeft opgeleverd, is er nog veel onderzoek nodig om de kennis voor het gebruik van desinfectantia en bacteriofagen uit te bereiden in de strijd tegen colibacillose.

Summary

Avian pathogenic *Escherichia coli* (APEC) cause yearly a huge economic damage to the poultry industry due to mortality, slower growth, a lower feed conversion efficiency and condemnation of carcasses at the abattoir. Inhalation of dust contaminated with high concentrations of *Escherichia coli* can infect the air sacs and lungs, whereafter bacteria can enter the bloodstream, causing systemic disease (known as colibacillosis).
The disease is traditionally treated with antibiotics, but due to frequent use of antibiotics many APEC strains have become multidrug resistant. The use of antibiotics raises a public health concern, due to transfer of resistance genes/resistant bacteria from animals to humans. Therefore in this study the use of disinfectants and bacteriophage therapy were tested as alternative prophylaxis/treatment for colibacillosis in chickens.

First, antibiotic resistance of a selection of APEC strains (n = 97), representative for the Belgian laying hen industry, was determined since APEC isolates from laying hens are less studied than isolates from broilers. Furthermore, it was determined if these APEC isolates were resistant against the most included active ingredients (formaldehyde, glutaraldehyde, glyoxal, hydrogen peroxide (H_2O_2), and a quaternary ammonium compound (QAC)) in routinely used disinfectants in the poultry industry. The biofilm-forming capacity of the strains was determined on materials frequently used in poultry barns, since biofilm formation can have an effect on the efficacy of disinfectants. High resistance percentages were found against antibiotics frequently used in the poultry industry (ampicillin, nalidixic acid, sulfonamides and trimethoprim). However, no phenotypically detectable acquired resistance against routinely used disinfectants could be found, while resistance genes were present. Most APEC strains were only moderate biofilm producers on polystyrene, while on polypropylene and polyvinyl chloride they were strong biofilm producers. Hence, the efficacy of two of the five selected active ingredients in disinfectants (H_2O_2 and QAC) was tested to eliminate sessile cells. H_2O_2 and QAC were able to eliminate all adhering cells at a concentration of 1% and 0.01%, respectively.

Normally, disinfectants are used in poultry houses to prevent disease carry-over between sequential flocks, whereby the number of specific pathogens is lowered as much as possible. However, during this study the efficacy of nebulization of low concentrations of H_2O_2 to reduce infection pressure in the presence of chickens was determined. Chickens respiratorily exposed to APEC and subsequently to 2% H_2O_2 had nonetheless a higher chance of

developing a high lesion score associated with APEC, than chickens that were exposed to APEC and subsequently to 1% H_2O_2 or distilled water, contraindicating H_2O_2 nebulization in the presence of chickens.

During the second part of the study, a bioluminescent APEC strain (CH2-lux) was developed by transposition of the *lux* operon of *Photorhabdus luminescens* in the strain used for infection studies (CH2). The transposition of the *lux* operon into the chromosome of the APEC isolate did not affect the virulence of the latter strain, nor its sensitivity for lytic bacteriophages (phages). *Ex vivo* correlations were found between the luminescence and bacterial numbers in the homogenized heart, liver and lung of four-week-old CH2-lux infected chickens. The marked strain can also be used to increase knowledge on the pathogenesis of the disease, in order to find targeted treatments against colibacillosis. A bacteriophage cocktail composed of four phages was developed after an *in vitro* selection of the phages. They were administered to experimentally infected chickens intratracheally, intra-esophageally or via the drinking water. However, treated groups did not show a significant decrease in mortality, lesion scores or weight loss compared to infected/untreated groups. The results indicate that even though *in vitro* the phage cocktail efficiently lysed the infecting strain, the phage cocktail could not cure experimentally infected chickens. More research is needed to analyze the *in vivo* therapeutic and prophylactic effect of phages. The developed luminescent APEC strain can thereby possibly provide solutions.

During this study various experiments were performed to evaluate the application of disinfectants and bacteriophages to reduce avian colibacillosis, even though many valuable results were found, still much research is needed to expand knowledge on the use of disinfectants and bacteriophages in the battle against colibacillosis.

List of abbreviations

AAS	abdominal air sacs
AFEC	avian faecal *Escherichia coli*
AMC	amoxicillin/clavulanic acid
AMP	ampicillin
APEC	avian pathogenic *Escherichia coli*
ATCC 25922	American Type Culture Collection strain 25922
ATM	aztreonam
a.u.	arbitrary units
BHIB	brain heart infusion broth
CAZ	ceftazidime
cfu	colony forming units
CFX	cefoxitin
CHL	chloramphenicol
CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
CS	conserved segment
CSA	Columbia sheep blood agar
CTRX	ceftriaxone
CTX	cefotaxime
dpi	days post infection
dpv	days post vaccination
DR	drinking water
DW	distilled water
E. coli	*Escherichia coli*
ECOR	*Escherichia coli* Reference Collection
EFX	enrofloxacin
EPS	exopolysaccharide
ES	esophagus
ESBL	extended spectrum beta-lactamase
ExPEC	extraintestinal pathogenic *Escherichia coli*
FELASA	Federation of European Laboratory Animal Science Associations
FOR	formaldehyde
g	gram
GEN	gentamicin
GFP	green fluorescent protein
GLU	gluteraldehyde
GLY	glyoxal
h	hour
H_2O_2	hydrogen peroxide
Intl1, *intl2*, *intl3*	integrase class gene 1, 2, 3
IT	intratracheally
LB	Lysogeny broth
LPS	lipopolysaccharides
Luc	firefly luciferase
LU-L	left lung
LU-R	right lung
Lux	bacterial luciferase
MBC	minimum bactericidal concentration

MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
min	minute
MOI	multiplicity of infection
NAL	nalidixic acid
N.D.	not determined
NEO	neomycin
NMEC	neonatal meningitis *Escherichia coli*
OD	optical density
ODc	cut-off optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
phages	bacteriophages
PP	polypropylene
PRD	pericardium
PS	polystyrene
$p/s/cm^2/sr$	photons per second per square centimeter per steradian
PVC	polyvinyl chloride
Q1	first quartile
Q3	third quartile
QAC	quaternary ammonium compound
ROI	region of interest
rpm	rotations per minute
RT	room temperature
s	second
SD	standard deviation
SHS	swollen head syndrome
SOC	super optimal broth with catabolite respression
SSC	stainless steel coupon
SULFA	sulfonamides
TAS-L	left thoracic air sac
TAS-R	right thoracic air sac
TET	tetracycline
TMP	trimethoprim
TOT	total lesion score
TSB	tryptone soya broth
UPEC	uropathogenic *Escherichia coli*
WGA	wheat germ agglutinin-Alexa fluor 488

Chapter I

General introduction on avian pathogenic *Escherichia coli*

1. Avian pathogenic *Escherichia coli*

Escherichia coli (*E. coli*) are Gram-negative, rod-shaped, facultative anaerobic bacteria and are part of the family of the *Enterobacteriaceace*. They are normal residents of the alimentary tract of humans and homoeothermic animals (Hartl and Dykhuizen, 1984; Souza et al., 1999; Tenaillon et al., 2010) and their presence in the lower intestine is beneficial to the host, since they will synthesize vitamin K (Bentley and Meganathan, 1982) and prevent the intestines from colonization by pathogenic bacteria (Hudault et al., 2001; Reid et al., 2001; Tenaillon et al., 2010). Between 10^4 - 10^7 colony forming units (cfu) of *E. coli* per gram of faeces are usually present in the intestinal contents of birds (Dho-Moulin and Fairbrother, 1999). More recently, it was confirmed with 16S rRNA gen amplicon sequencing that *E. coli* makes up an important part of the microbial population in the gastrointestinal tract (Sekelja et al., 2012). Part of these *E. coli* strains are however pathogenic. It was shown that the proportion potential pathogenic *E. coli* declines with age, at placement of broiler chicks making up about 24% and by week five only 1% of the flora (Kemmett et al., 2013). In addition, (non-)pathogenic *E. coli* can be isolated from the bird's skin and feathers (Dho-Moulin and Fairbrother, 1999).

Pathogenic *E. coli* can cause disease in mammals and birds and can be separated into two major subgroups, based on their pathogenic characteristics: pathogenic *E. coli* causing intestinal infections and pathogenic *E. coli* causing extraintestinal pathogenic infections (ExPEC). The intestinal pathogenic *E. coli* group includes many pathotypes, such as diffusely adherent, enteroaggregative, enterohemorrhagic, enteroinvasive, enteropathogenic, and enterotoxigenic *E. coli* (Kaper et al., 2004), even though recently it was shown that an enteroaggregative *E. coli* strain was the cause of a community-acquired outbreak of urinary tract infection in Denmark (Boll et al., 2013). These intestinal pathogenic *E. coli* strains are normally not found in the faecal flora of healthy individuals, they cause infection when contaminated food or water is ingested (Smith et al., 2007).

The ExPEC group can be further subdivided into pathogens responsible for urinary tract infections (UPEC) and *E. coli* causing neonatal meningitis (NMEC). Furthermore there is a pathotype of ExPEC causing infection in avian species, better known as avian pathogenic *E. coli* (APEC).

1.1. Pathogenesis of APEC

APEC often starts as a local infection of the air sacs, known as airsacculitis. This is believed to be caused by inhalation of dust in the barn contaminated with faeces containing up to 10^6 cfu of *E. coli* per gram (Dho-Moulin and Fairbrother, 1999; Dziva and Stevens, 2008). Air sacs are vulnerable for bacterial infection due to a lack of resident macrophages and they are dependent on the recruitment of heterophils followed by macrophages (Ficken and Barnes, 1989; Stearns et al., 1987; Toth et al., 1987; Toth and Siegel, 1986). Furthermore, APEC causes frequently an opportunistic infection where chickens experience a viral/mycoplasma infection. Viral live vaccines may also predispose chickens to infection (La Ragione and Woodward, 2002; Nakamura et al., 1994). The main factor, however, is stress, mainly the cause of the high infection pressure due to poor housing conditions (such as high ammonia concentrations and high levels of faeces contaminated dust) (Barnes et al., 2008; Dziva and Stevens, 2008). However, stress causing an elevation in the level of glucocorticoids, due to for example overcrowding also plays a predisposing role (Cheville, 1979). After causing airsacculitis the disease can spread through the whole body, entering the bloodstream most likely via the air sacs (Ackermann and Cheville, 1991; Pourbakhsh et al., 1997a) and lungs (Pourbakhsh et al., 1997a), resulting in a generalized infection. APEC, however, can survive inside macrophages providing another potential way to enter the bloodstream (Mellata et al., 2003; Pourbakhsh et al., 1997b). After the bacteria entered the blood (septicemia) they can cause infection of the pericardium (pericarditis) and liver (perihepatitis) (see Figure 1.1), collectively called colibacillosis. Another plausible route of infection is via active gut translocation, but this probably happens only in the presence of a stressor (Leitner and Heller, 1992). The disease causes infection in avian species of all ages and leads yearly to multimillion-dollar losses in the poultry industry, due to mortality, reduced growth and feed conversion efficiency and an increased condemnation of carcasses at the slaughterhouse (Barnes et al., 2008; Dho-Moulin and Fairbrother, 1999). It mainly causes disease in chickens between 4 and 9 weeks with mortalities up to 20% (Dho-Moulin and Fairbrother, 1999).

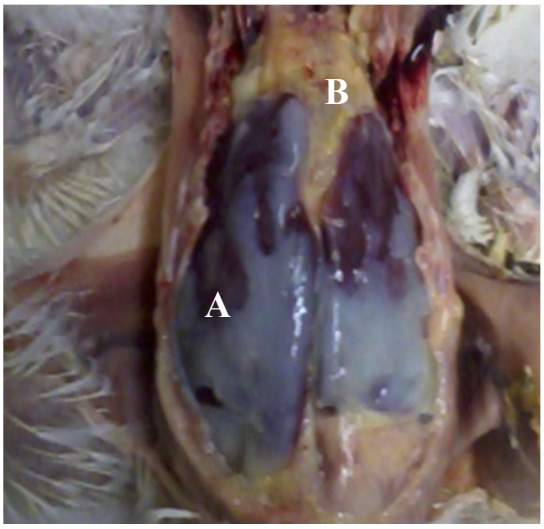

Figure 1.1. Typical clinical APEC symptoms
A) perihepatitis, B) pericarditis.

APEC can also cause acute or chronic salpingitis in broiler breeders or laying hens (Bisgaard and Dam, 1980, 1981). Salpingitis in these chickens is probably the result of an ascending infection from the cloaca (Bisgaard and Dam, 1980) and can lead to the loss of egg laying capability (Dho-Moulin and Fairbrother, 1999). Furthermore, an infected laying hen can in this way infect the internal egg before shell formation, which can lead to mortalities in embryos or chicks up to three weeks after hatching (Barnes et al., 2008). However, the main route of infection is through contamination of the eggshell after laying. In chicks this can cause yolk sac infection and omphalitis (Barnes et al., 2008; Dho-Moulin and Fairbrother, 1999).

In broilers, APEC can also cause cellulitis or necrotic dermatitis of primarily the lower abdomen and thighs (Dho-Moulin and Fairbrother, 1999), not leading to mortalities but to condemnation of carcasses in the abattoir (Mellata et al., 2003). The lesions are induced by the secretion of an *E. coli* heat-labile, vacuolating cytotoxin (Quel et al., 2013). The incidence of the disease has lately increased (Quel et al., 2013), probably due to multiple factors of which increased use of high-yield broilers, together with a high barn density are the main factors (Nunes, 2011).

Also, APEC can cause swollen head syndrome (SHS) in broilers and broiler breeders. This is a secondary infection of APEC after an avian pneumovirus infection, causing acute rhinitis, there is subsequently an invasion of facial subcutaneous tissues by APEC (Hafez and Löhren, 1990; Picault et al., 1987). Infectious bronchitis virus has also been isolated from broilers

with SHS showing that other viruses may also be associated with SHS (Droual and Woolcock, 1994; Morley and Thomson, 1984). Nakamura *et al.* (1998) was even able to reproduce SHS in chickens with APEC alone, showing the primary importance of APEC in the disease. The disease affects mostly broilers between 4 to 5 weeks of age and broiler breeders between 24 to 36 weeks of age (Hafez and Löhren, 1990). In broiler breeders this is accompanied with a drop in egg production reaching 2-3% and a mortality between 3-4% (Morley and Thomson, 1984).

1.2. Characterization

The O (somatic) antigen of lipopolysaccharides (LPS), the major component of the outer membrane of *E. coli*, is the outermost domain of the LPS molecule. LPS is an endotoxin, and induces a strong immune response in a normal healthy human or animal. Serotyping of the O antigen is commonly used for estimating the pathogenic potential of APEC strains, since some serogroups such as O1, O2, O8, O18 and O78, are detected more often than other serogroups (Ewers et al., 2007; McPeake et al., 2005; Rodriguez-Siek et al., 2005b; Schouler et al., 2012; Vandekerchove et al., 2005). However, more and more serogroups have been identified as being associated with APEC, of which many are untyped (Ewers et al., 2007). Therefore, it is believed that serotyping is not a very specific diagnostic tool. Moreover, since avian faecal *E. coli* (AFEC) strains have also been shown to belong to a wide range of overlapping serogroups (Rodriguez-Siek et al., 2005b; Schouler et al., 2012).

A huge set of virulence genes associated with bacterial adhesion, serum resistance, invasion, iron acquisition and toxin production have been shown to be important for APEC pathogenesis. They are often associated with pathogenicity islands and other mobile DNA elements (genes like *tsh*, *iss* and *iutA,* coding for temperature-sensitive hemagglutinin, increased serum survival and the receptor for ferric aerobactin, respectively). The gene *iss* is expected to have a link with high level resistance to the complement-mediated bactericidal effects of serum, however, the exact mechanism by which it does that is unknown. The described genes are also often detected in AFEC, but generally in a lower percentage (Ewers et al., 2007; Rodriguez-Siek et al., 2005b). Multiplex polymerase chain reactions (PCRs) have been developed to screen for virulence genes associated with APEC, AFEC, NMEC and UPEC (Ewers et al., 2005; Schouler et al., 2012; van der Westhuizen and Bragg, 2012). Furthermore, it was recently shown that virulence associated gene prevalence in broilers decreased with age (Kemmett et al., 2013). It is known that the microbiota in the gut of young

chicks is very diverse in comparison to older chickens. Chicks get colonized quickly by *E. coli* via different sources, such as the hatchery environment and the parent flock (Fasenko et al., 2009; Petersen et al., 2006). The decrease in percentage of potential APEC can be explained by the persistence of strong colonizers (resident strains) and the loss of transient strains (Kemmett et al., 2013). Possibly, different virulence genes can provide specific advantages in different stages of development (Kemmett et al., 2013; Tenaillon et al., 2010)

Phylogenetic typing divides isolates in four major phylogenetic groups of *E. coli* (A, B1, B2, and D) (Clermont et al., 2000; Herzer et al., 1990). This typing can be performed using a multiplex PCR on genes *chuA* (required for heme transport in enterohemorrhagic O157:H7 *E. coli*), *YjaA* (the function is unknown) and an unknown DNA fragment (TSPE4.C2), as shown in Figure 1.2 (Clermont et al., 2000).

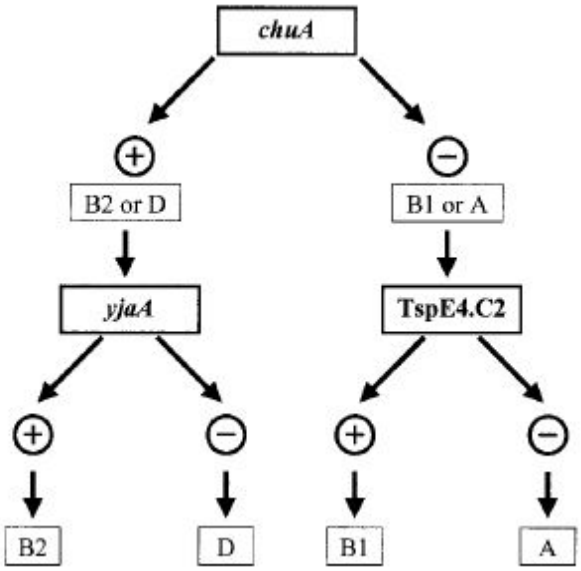

Figure 1.2. Decision three to determine the phylogenetic group of an *E. coli* strain (A, B1, B2 or D) based on the results of a multiplex PCR on genes *chuA*, *yjaA* and an unknown DNA fragment (TspE4.C2) (Clermont et al., 2000).

Most APEC isolates fell into ECOR (*E. coli* Reference Collection) group A and B2 (Ewers et al., 2009; Ewers et al., 2007) or A and D (Rodriguez-Siek et al., 2005a) depending on the study, while most AFEC isolates belong to group D (Ewers et al., 2009). Certain AFEC strains were, however, also capable of causing systemic disease in five-week-old chickens, these strains showed a virulence associated gene pattern very similar to ExPEC strains. This led to the hypothesis of an avian intestinal reservoir that might be able to directly transfer to humans or through exchange of virulence and resistance genes with ExPEC, showing a zoonotic risk (Ewers et al., 2009; Ewers et al., 2007). Phylogenetic group B2 is known to

contain highly pathogenic human ExPEC strains (Clermont et al., 2000; Ewers et al., 2007; Moulin-Schouleur et al., 2007; Rodriguez-Siek et al., 2005a), while most human commensal fall into group A (Clermont et al., 2000).

1.3. Prophylaxis and treatment

First of all it is important to prevent disease introduction (external biosecurity) by a good design of the interior and exterior of buildings, design and positioning of equipment and a coordinated planning and programming of vehicle movement (Bermudez and Stewart-Brown, 2008). A good design of the exterior of buildings means first of all that they should be constructed in such a way that wild birds cannot enter the poultry house, since they are carriers of various diseases and their faeces is a source of pathogenic *E. coli* (Barnes et al., 2008; Bermudez and Stewart-Brown, 2008). The faeces of rodents is also a source of APEC, therefore, they should be limited as much as possible in and around the barn (Barnes et al., 2008; Bermudez and Stewart-Brown, 2008). Barns should also be built so that the chickens are protected from the elements, but still provide adequate ventilation (Bermudez and Stewart-Brown, 2008). Vandekerchove *et al*. (2004) found that the density of hens in the cages and the distance between poultry farms are of importance for colibacillosis outbreaks in flocks of laying hens, so these factor should also be taken into consideration. The interior of buildings, such as the floors and cages should be made of materials which are easy to clean and disinfect, to lower the number of pathogens in-between consecutive flocks (Bermudez and Stewart-Brown, 2008). All these and many more factors should be considered during the construction of the buildings, however, not only disease prevention is important for farmers, also economic and welfare factors should be taken into account. The final decision for a specific design is often a trade-off between these different factors and depends on the outcome of a cost/benefit analysis.

One of the most important predisposing factors for APEC infection is a poor housing climate (Dziva and Stevens, 2008). High concentrations of dust or ammonia, due to for example improper ventilation, lead to increased infection pressure for the chickens (Bermudez and Stewart-Brown, 2008). The different environmental parameters, like humidity, temperature, ventilation, ammonia concentration and dust level should therefore be properly controlled in order to prevent disease. In addition, management practices like frequent removal of dead and moribund hens (Bermudez and Stewart-Brown, 2008), adding chlorine to the drinking water (Barnes et al., 2008; Dhillon and Jack, 1996; Gross, 1994) and use of pelleted feed are

important (Barnes et al., 2008; Gross, 1994). A good tool for (poultry) farmers to assess the biosecurity at their own (poultry)farm is based on a scientific questionnaire (Dewulf et al., 2014).

To limit the transfer of pathogens from flock to flock, the poultry house should be properly cleaned and disinfected between flocks. Therefore it is important to work with an all-in/all-out system (Bermudez and Stewart-Brown, 2008).

Furthermore, disposal of damaged and faecal contaminated eggs is important to prevent disease (Barnes et al., 2008; Dho-Moulin and Fairbrother, 1999). Another more direct way of controlling APEC is competitive exclusion by administration of normal bacterial flora from healthy chickens or monocultures outcompeting the pathogenic bacteria in the intestinal flora of day-old chickens. These cultures can for example be given *via* intra-gastric intubation, by gavage into the crop or orally on hatching in order to establish a mature gut flora early in life. However, results are quite variable and challenge strains are usually still present, but in reduced numbers (Hakkinen and Schneitz, 1996; Hofacre et al., 2002; La Ragione et al., 2001; Weinack et al., 1981).

1.3.1. Vaccination

Live vaccines

A lot of effort has been put in the development of an effective live attenuated vaccine. However, success rates varied and all have in common that they are not able to provide efficacious cross-immunity against heterologous APEC strains (Amoako et al., 2004; Kariyawasam et al., 2004a; Kwaga et al., 1994; Peighambari et al., 2002). Therefore, it was hypothesized that a vaccine containing different serogroups could provide a more efficacious protection (Kariyawasam et al., 2004a). Even though this sounds like a plausible solution, it might not give sufficient protection, since strains possess different virulence genes, even within a serogroup.

Recently, a live commercial available *aroA*-deleted (responsible for biosynthesis of aromatic amino acids) O78 *E. coli* vaccine (Poulvac® *E. coli,* Zoetis) was developed and shown to cause a reduction in incidence and severity of airsacculitis when vaccinated chickens were challenged with a heterologous serogroup (untypable strain) (La Ragione et al., 2013). However, since systemic disease is generally caused by more virulent strains (Pourbakhsh et al., 1997b), than the strain used in the study by La Ragione *et al.* (2013), its efficacious

protection against more pathogenic strains remains unclear.

Live recombinant vaccines can be used to express APEC antigens. Roland *et al.* (1999; 2004) and Chaudhari *et al*. (2013) used a live attenuated *Salmonella typhimurium* delivery system, targeting mucosal cells in the gut, thus stimulating mucosal immunity as well as humoral and cellular immunity (Sirard et al., 1999). Roland *et al*. (1999; 2004) used this system to express *E. coli* O78 LPS or *E. coli* O78 LPS and type 1 fimbriae and obtained homologous protection, but not heterologous protection. Chaudhari *et al*. (2013) used the *Salmonella typhimurium* delivery system to express multiple APEC virulence genes (*papA*, *papG*, *iutA* and *clpG*). *PapG* and *papA* are P fimbriael genes, while *iutA* codes for a ferri-siderophore receptor and *clpG* is a CS31A fimbrial adhesin. They found significant protection after immunization (Chaudhari et al., 2013) and could increase the protection rate from 75% to 90% when immunized along with *E. coli* heat-labile toxin B subunits, as adjuvant (Chaudhari and Lee, 2013). However, so far only one challenge strain of serogroup O2, that contained at least genes *iutA* and *clpG* was tested (Matsuda et al., 2010), more strains of different serogroups should be tested before firm conclusions can be drawn about its broad effectiveness. Nowadays, the poultry industry is more moving towards *in ovo* immunization, facilitating the vaccination process, since vaccination is performed when eggs are transferred from the setter to the hatcher. Nagano *et al*. (2012) found partial protection (mortality dropped from 100 to 0%) against APEC when performing *in ovo* immunization with an attenuated *E. coli* mutant, without affecting hatching or chick survival.

Inactivated vaccines

Several inactivated APEC vaccines have been developed, which constitute of a (mixture of) killed strain(s) (Melamed et al., 1991; Rosenberger et al., 1985).

However, these vaccines will not provide a sufficient humoral immune response in broiler chickens when administered prior to 10 d of age, since the chickens immune system is not fully developed prior to this age (Mast and Goddeeris, 1999). Melamed *et al.* (1991) obtained homologous and heterologous protection against strains O78:K80 and O2:K1, when immunizing chickens with an ultrasonically inactivated and irradiated O78:K80 and O2:K1 strain, respectively. Rosenberger *et al.* (1985) could protect progeny against homologous strains till two weeks post hatch via immunization of broiler breeder hens. Passive maternal immunity of chickens can provide protection during early chick life and has as main advantage that individual chickens do not need to be immunized in the broiler industry (Kariyawasam et al., 2004b). The duration of protection might however not be long enough to

protect laying hens and breeders and even broilers, since APEC infection usually occurs between 2 to 12 weeks of age, with most losses occurring during week 4 to 9 (Dho-Moulin and Fairbrother, 1999).

Subunit vaccines

Various subunit vaccines have been developed targeting (a) surface-exposed antigen(s) found in most APEC, independent of serogroup. In this way vaccines can be obtained providing broad protection against colibacillosis. These antigens can for example be complete fimbriae (type 1 or P) or their subunit adhesins, since these were shown to be important for *E. coli* adhesion to host cells. Type 1 fimbriae are important in the initial stages of adherence to respiratory epithelium (Dozois et al., 1994; Dozois et al., 1995; Gyimah and Panigrahy, 1988; La Ragione et al., 2000; Vidotto et al., 1997), while P fimbriae seem more important during a later phase in the infection process, as they are expressed only when present in the air sacs, lungs, blood and internal organs (Pourbakhsh et al., 1997b; Pourbakhsh et al., 1997c). Gyimah *et al*. (1986) was able to provide a good protection against respiratory tract infection when challenging chickens with strains O1, O2 and O78, after immunizing chickens with a mixture of fimbriael proteins of *E. coli* strains O1, O2 and O78. Vandemaele *et al.* (2006; 2005) however, were not able to find protection by immunization with respectively type 1 fimbriae (FimH) and the sugar binding domain of PapGII (an adhesion of P fimbriae). Type 1 and P fimbriae (or its adhesins) might not be as important as thought for APEC infection (Vandemaele et al., 2006; Vandemaele et al., 2005), as pap-negative and a Δfim mutant(s) were still virulent (Li et al., 2005; Marc et al., 1998; Stordeur et al., 2004). Recently, it was demonstrated that immunization with the lectin domain of FimH (and whole fimbriae) elicits an immune response that enhances the binding of FimH to its receptor, enhancing the cell adhesion of *E. coli* to host cells (Tchesnokova et al., 2011). A commercially available vaccine consisting of P fimbriael and flagellar antigens (Nobilis® *E. coli* Inac, MSD Animal Health) claims to protect broiler chickens during the first seven weeks of life, by vaccination of broiler breeders.

Inclusion of iron receptor genes or proteins in a subunit vaccine can also be an option to prevent APEC, since iron uptake is essential for proliferation of *E. coli*. The aerobactin iron-acquisition uptake system, which is a high-affinity system for iron acquisition to overcome growth limitation caused by insufficient amounts of free iron in the body fluids (Warner et al., 1981), was shown to be significantly more present in pathogenic than non-pathogenic strains

(Dozois et al., 1992; Linggood et al., 1987). The outer membrane receptor IutA binds the aerobactin complex and transports it into the cell. However, Tuntufye *et al.* (2012b) could not find significant protection against APEC when immunizing chickens with an *E. coli* subunit vaccine expressing ferri-siderophore receptor proteins FepA, FhuE, IroN and IutA.

Lynne *et al.* (2012) developed a subunit vaccine consisting of iss fusion proteins, as the *iss* gene was shown to be significantly more present in APEC than AFEC (Kemmett et al., 2013; Rodriguez-Siek et al., 2005b). Immunized chickens had significantly lower lesion scores than non-immunized chickens after challenge with strains O1, O2 and O78. These challenge strains were, however, not highly pathogenic (they showed only a low mortality percentage in chickens), so other more pathogenic strains should be tested before general conclusions can be drawn about protection against colibacillosis.

Even though very promising results have been found and some APEC vaccines are already available, much is still unknown about the virulence of APEC strains which show great diversity, limiting the possibilities of vaccination.

1.3.2. Antibiotic treatment and resistance

Colibacillosis is traditionally controlled by the use of antibiotics, especially beta-lactams, tetracyclines, sulfonamides and (fluoro)quinolones (Dho-Moulin and Fairbrother, 1999; Gross, 1994), with as a consequence the risk of antibiotics residues ending up in food, selecting for resistance to antibiotics in human bacteria (Singer & Hofacre, 2006). Antibiotic resistance in APEC not only causes failure of therapy in animals, but may even lead to treatment problems in humans infected with *E. coli* strains from animal origin (Blake et al., 2003; Smet et al., 2011; van den Bogaard and Stobberingh, 1999). Resistance in *E. coli* isolates from laying hens in comparison to broilers is in general lower (AFEC as well as APEC), but is still widespread (Harisberger et al., 2011; van den Bogaard et al., 2001). While layers normally only receive antibiotics during rearing, antibiotic resistance might possibly persist for a long period of time in the intestinal tract without selective pressure of antibiotics (Chaslus-Dancla et al., 1987).

Faecal isolates of broilers in Belgium showed high resistance to ceftiofur, which was allowed as a third-generation veterinary used cephalosporin until 2000 for prevention of *E. coli* septicemia in one-day-old chickens in Belgium (Persoons et al., 2010; Persoons et al., 2011b; Smet et al., 2008). The fast increase of AFEC resistance to ceftiofur (from 5% in 2002 to 37% in 2010) (Casteleyn et al., 2006; Persoons et al., 2011a; Persoons et al., 2010) shows that

certain *E. coli* strains can possibly form a reservoir for (antibiotic) resistance genes in the chickens' intestine and can in this way transfer these genes to pathogens (Ewers et al., 2009). Smet *et al*. (2011) showed that extended-spectrum beta-lactamase (ESBL) positive *E. coli* from avian origin was able to persist *in situ* in a system mimicking the human caecum and the ascending colon (even without selective pressure of antibiotics) and was thereby able to transfer resistance genes to commensals. After treatment with cefotaxime the number of ESBL positive *E. coli* increased and was able to persist at increased concentrations, increasing public health issues (Smet et al., 2011). Leverstein-van Hall *et al*. (2011) and Overdevest *et al*. (2011) detected a high prevalence of ESBL genes in poultry meat, which shows the possibility of transfer of ESBL genes via the food chain to humans. Depoorter *et al.* (2012) developed a model, simulating the farm to fork chain, predicting that consumers have a chance of about 1.5% to be exposed to 1000 cfu or more of 3rd generation cephalosporin resistant *E. coli* during consumption of chicken meat, mainly due to cross-contamination in the kitchen. Persoons *et al*. (2011b) identified on-farm antimicrobial therapy (co- or cross-resistance), management practices (like acidification of drinking water) and hatchery-related factors (probably off-label use of cetiofur) as risk factors for ceftiofur resistance in AFEC from broilers. This means that in order to lower antibiotic resistance occurrence, not only reduction of antibiotic use is important, but also good farm practices and even good hatchery practices are necessary. So, apart from selection for antibiotic resistance, there is the risk of transfer of resistance from animals to humans and the high cost of antibiotic treatment. Therefore the use of antibiotics has to be limited as much as possible, however, this increases the need for alternative methods.

1.3.3 Bacteriophage therapy

Treatment by utilization of lytic bacteriophages (phages) gained more interest, due to its promising results *in vivo* (Barrow et al., 1998; Huff et al., 2003a, 2004; Lau et al., 2010; Oliveira et al., 2010). Phages were discovered independently by Twort and d'Herelle in the beginning of the 20th century. The word bacteriophage was proposed by d'Herelle and consists of the words 'bacteria' and 'phagein' to explain that phages devour bacteria (d'Herelle, 1926; Sulakvelidze and Morris, 2001). Bacteriophages can have a lytic cycle or a lysogenic cycle, and a few viruses are capable of carrying out both (Huff et al., 2005; Patnaik et al., 2012). Phages that undergo the lysogenic cycle will, however, not be discussed here, since they are not the preferred candidates for phage therapy. Upon infecting a host, they incorporate their genetic material into the genome of the host (known as prophage) and

remain dormant until host conditions deteriorate (Huff et al., 2005; Sulakvelidze and Morris, 2001; Patnaik et al., 2012). Strictly lytic phages are bacterial viruses that attach to their specific hosts, followed by internal replication of their genomes, whereafter the first viral mRNA is translated into proteins. Sometimes, these early proteins cause degradation of the host chromosome. The phages disrupt the host synthesizes of nucleic acids and proteins, and force the host to produce viral nucleic acids and proteins instead (Patnaik et al., 2012). These proteins are assembled to form new phages, followed by lysis of the host (see Figure 1.3).

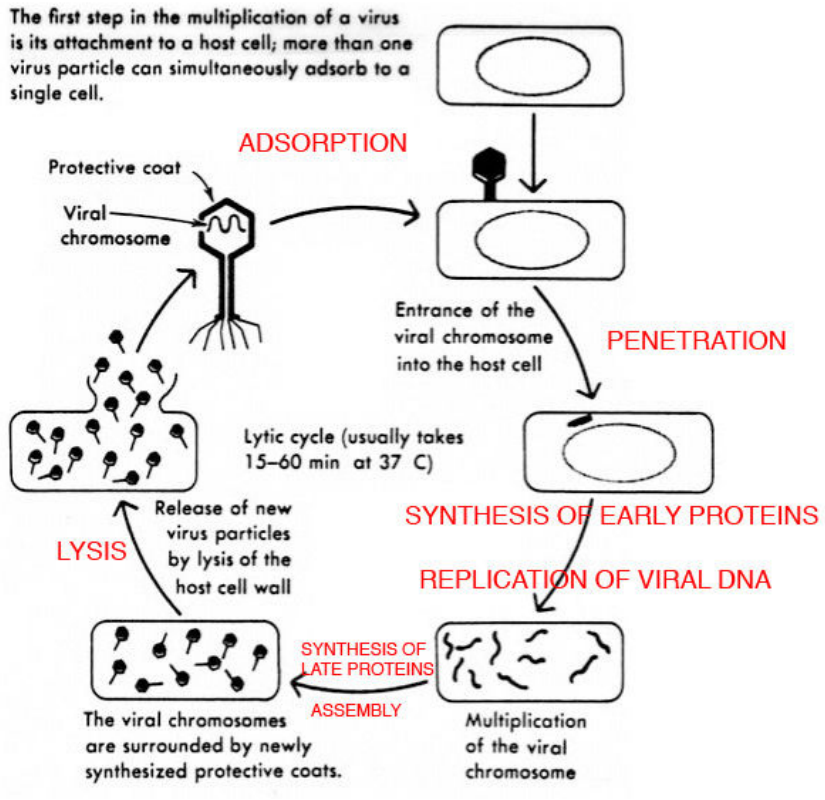

Figure 1.3. Life cycle of a strictly lytic bacteriophage. A phage particle attaches to a host cell and injects its genomic material into the cell. The host cell starts producing viral nucleic acids and proteins, followed by assembly of viral proteins. In the last stage, the host cell is lysed by the newly synthesized phages and phages are released (www.textbookofbacteriology.net).

Already soon after their discovery, phages were used by d'Herelle for treating patients with dysentery, plague and to control cholera epidemics (d'Herelle, 1926; Sulakvelidze and Morris, 2001; Summers, 2001). These studies gave very promising results, and resulted in the production and development of different phage preparations for therapeutic use by companies worldwide (Sulakvelidze and Morris, 2001). But with the advent of antibiotics around 1930-1940, phages became neglected in the Western World. Antibiotics were easier to apply and

became worldwide available, phages on the other hand did not always show consistent results. Research on phages continued in the former Soviet Union and Eastern Europe but knowledge did not disseminate, since most of the work was not published in English. However, with the emergence of multidrug resistance, interest in phages was reestablished in the Western World and the USA (Sulakvelidze and Morris, 2001).

Phages have many advantages over antibiotics, such as being ubiquitous in nature, even residing in the alimentary tract of humans and animals and are therefore generally considered as safe for application to humans and animals (Johnson, 2008). Effective phage therapy reduces pathogen numbers, so that the immune response of the host can take over and eliminate the remaining infectious bacteria (Levin and Bull, 2004). Several studies concerning phage therapy to treat APEC in chickens have been published (Barrow et al., 1998; Huff et al., 2003a, 2004; Lau et al., 2010; Oliveira et al., 2010). However, many factors are important for an effective phage therapy, such as time of administration, site of administration, the applied dose and the application method (Huff et al., 2013; Ryan et al., 2011). So even though positive results have been published, the use of phages is far from straightforward and much is still unknown about their behaviour *in vivo*. Moreover, there appears to be an immune response towards phages in chickens, leading to decreased therapeutic efficacy of phages after prior exposure (Huff et al., 2010). This was shown by an *in vitro* kinetic assay of phage activity, which showed that serum pretreated with phages inhibited phage activity in comparison with control serum (as measured by the lowered lysis of the host strain). Furthermore, serum from pretreated chickens showed a significant increase in IgG titers in comparison with control serum. Huff *et al.* (2010) therefore suggested that the observed efficacy is partly due to an acquired immune response to the administered phages.

The K1-capsule, a virulence factor of APEC, can prevent phagocytosis by heterophils and macrophages due to its negative surface charge and hydrophilic nature. Expression of the capsule was shown necessary for causing avian septicaemia (Li et al., 2005). Moreover, the K1-capsule can also prevent phages from reaching their receptor (Scholl et al., 2005), affecting treatment outcomes. Possibly, colanic acid can also physically block phages from reaching their receptor, since it forms a thick slime layer on the outer membrane of *E. coli* (Stevenson et al., 1996). This layer is probably formed to protect APEC against host stresses, such as heat, acid and osmotic and oxidative stress (Chen et al., 2004; Mao et al., 2001; Tuntufye et al., 2012a). Most likely, the capacity of (some) APEC strains to produce a K1-

capsule and colanic acid forms also an important obstacle for vaccine development.

1.3.4 Disinfectants

Cleaning and disinfection of poultry houses and equipment is part of good husbandry practices in order to keep spread of pathogens between flocks as low as possible, as is fumigation of eggs within two hours after lay, to prevent disease in chicks (Barnes et al., 2008; Dho-Moulin and Fairbrother, 1999). However, little is known about possible resistance of APEC to disinfectants. There are different forms of bacterial resistance to disinfectants, namely: intrinsic resistance, genetically acquired resistance or tolerance (Bridier et al., 2011). Intrinsic resistance is the innate resistance of an organism for in this case, a specific disinfectant. Genetically acquired resistance can be obtained due to horizontal transfer of genes via different genetic elements, like transposons, plasmids and integrons, or due to chromosomal mutations. Bacteria can become less susceptible or tolerant to a disinfectant due to adaptation, growth in a biofilm, being in stationary growth phase or as persister cells. (Bridier et al., 2011; Langsrud et al., 2003; Russell, 2003). Persister cells (dormant cells) are phenotypic variants of regular cells that arise spontaneously in microbial populations and are highly tolerant to antibiotics. However, they are not genetic mutants (Bridier et al., 2011; Lewis, 2010). Multidrug efflux pumps (mainly chromosomally encoded), when over-expressed, can provide simultaneously resistance to antibiotics and disinfectants (cross-resistance), due to (a) mutation(s) in the promoter region or in insertion elements upstream of the transporter gene, in the local repressor gene or in a global regulatory gene (Piddock, 2006a; Piddock, 2006b). Exposure of clinical *Staphylococcus aureus* isolates to low concentrations of certain biocides or stepwise increasing concentrations of the biocides, resulted in mutants overexpressing multidrug efflux pumps (Huet et al., 2008). The most studied multidrug efflux pump in *E. coli* is the *AcrAB-TolC* pump (Fralick, 1996) (see Figure 1.4). This pump consists of a cytosplasmic bound transporter of the resistance-nodulation-division family, AcrB (Tseng et al., 1999), an accessory protein (membrane fusion protein, AcrA (Dinh et al., 1994)), and a minor outer membrane protein, TolC (Morona et al., 1983). The pump actively transports compounds directly from the cell into the medium (Murakami, 2002).

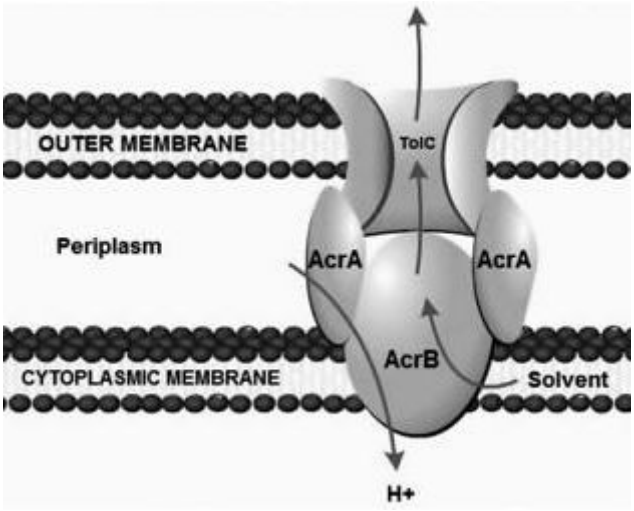

Figure 1.4. Schematic representation of the AcrAB-TolC efflux pump of *E. coli*. AcrB is a cytosplasmic bound transporter protein that is an energy-dependent (proton motive force) efflux pump, AcrA is a membrane fusion protein and TolC is an outer membrane protein (Taylor et al., 2012).

Antibiotic resistance genes and biocide resistance genes on the same genetic mobile element can be transferred to other bacteria by horizontal gene transfer (co-resistance) (Coelho et al., 2013). In the case of *E. coli* the best known resistance mechanism for quaternary ammonium compounds (QAC) is via the *qacEΔ1* gene, mostly associated with class 1 integrons. The *qacE* gene codes for an energy-dependent small multidrug resistance efflux pump, whereby the pump dependents on proton motive force (Paulsen et al., 1993). *QacEΔ1* most likely represents a disrupted form of *qacE*, that was shown to be only partially functional as a multidrug exporter (Paulsen et al., 1993). The *qacEΔ1* gene is normally found in the 3'-conserved segment (3'-CS) of class 1 integrons together with the *sul1* gene, which encodes resistance to sulfonamides (Paulsen et al., 1993). However, different gene arrangements have been found in *E. coli* at the 3'-CS, whereby the *qacEΔ1-sul1* fragment was for example replaced by *qacH-tnp-sul3* (Bischoff et al., 2005; Soufi et al., 2009). Class 1 integrons also contain a variable region that can contain one or more mobile gene cassettes, frequently coding for antibiotic resistance (Fluit and Schmitz, 2004) (see Figure 1.5). The integrase of the integron integrates the antibiotic gene cassettes into the integron.

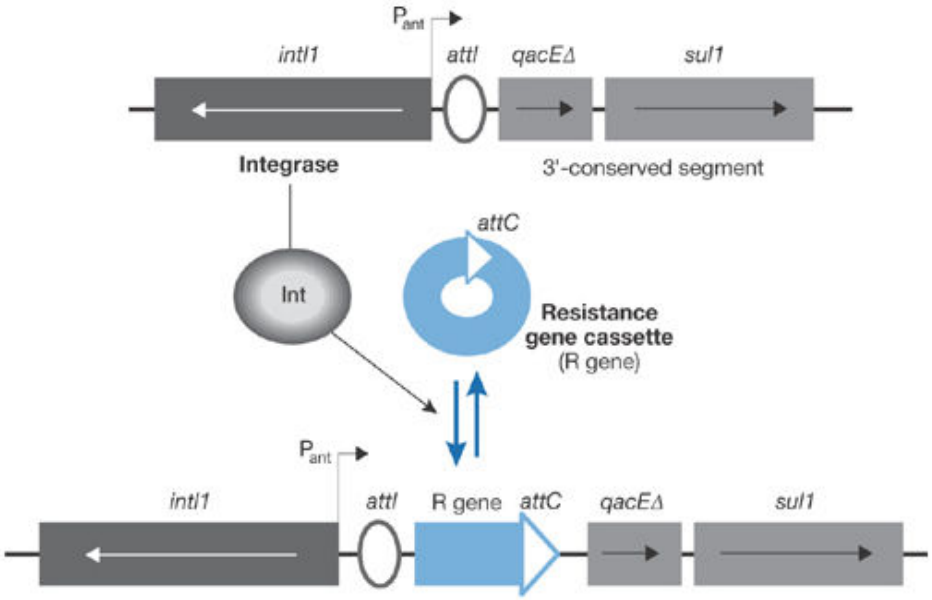

Figure 1.5. Schematic representation of integron class 1. At the 5'CS, the *intI1* gene is shown that codes for integrase, integrating the resistance gene cassettes into the variable region of the integron. At the 3'CS, genes *qacEΔ* (codes for resistance to QAC) and *sul*1 (codes for resistance to sulfonamide). *AttC*: attachment site of the cassette; *attI*: attachment site of the integron; P_{ant}: promoter for expression of the gene cassette; *qacEΔ*: quaternary ammonium compound resistance; R gene: resistance gene; *sul1*: sulfonamide resistance (Davies, 2007).

Kücken *et al*. (2000) and Jaglic *et al*. (2012) did not find Gram-negative bacteria to be more resistant to QAC when possessing either the *qacE* or *qacEΔ1* gene. However, in the Gram-positive *Staphylococcus aureus*, a significant higher tolerance to disinfectants QAC and chlorhexidine was found when strains carried *qac* genes (*qacA/B* or *qacC*) (Smith et al., 2008). Increased tolerance in *qac* positive strains was found, when exposed to increasing concentrations of QAC (Smith et al., 2008). However, increased tolerance due to exposure to increasing concentrations of benzalkonium chloride (a QAC), was also found in *Staphylococcus aureus* strains not carrying *qac* genes (Heir et al., 1999), thereby putting in question the role of the *qac* genes in tolerance to cationic disinfectants (Jaglic and Cervinkova, 2012). For *E. coli*, much is still unknown about the role of the *qac* genes in relation to disinfectant resistance, increased tolerance due to exposure to increasing concentrations of cationic disinfectants and antibiotic resistance.

1.3.5 Biofilms

Biofilm formation in bacteria was first observed by Antoni van Leeuwenhoek, when he discovered "animalculi" in dental plaque with his early microscope. These "animalculi" were responsible for the formation of a biofilm network, but it took till the end of the 20th century,

before scientists got really interested in bacterial biofilms (Costerton et al., 1999). Biofilms are described as “a sessile community of irreversibly attached cells that are surrounded by an extracellular matrix, with an altered growth rate and gene transcription in comparison to planktonic cells” (Donlan and Costerton, 2002). The matrix is generally composed of exopolysaccharide (EPS), proteins and nucleic acids (Branda et al., 2005; Davey and O'Toole, 2000). Sessile cells have many advantages for survival over their planktonic counterparts, like protection from environmental stressors, better nutrient availability, metabolic cooperation and the exchange of genetic material (Davey and O'Toole, 2000). Biofilms are also an important obstacle during cleaning and disinfection. They are hard to remove and cells in biofilms are in general more resistant (tolerant) to biocides than planktonic cells. There are several reasons for this tolerance, (I) reduced biocide penetration in deeper layers of the matrix due to the presence of the extracellular matrix, (II) efficacy of disinfectants are impaired due to chemical interactions between biocides and biofilm components, such as EPS (Russell, 2003). (III) Sessile cells have a different growth rate and cell physiology, leading to modifications in the membrane composition and expression of defense mechanisms (Bridier et al., 2011; Lisle et al., 1998; Saby et al., 1999). (IV) The presence of degradative enzymes within biofilms might inactivate biocides (Bridier et al., 2011; Donlan and Costerton, 2002; Russell, 2003). (V) Starvation and oxidative stress in a biofilm, which were shown to increase the resistance of *E. coli* to chlorine in less than 24 h (Saby et al., 1999). Starvation was identified as one of the major factors influencing the sensitivity of bacteria to disinfectants (Berg et al., 1982; Lisle et al., 1998; Matin, 1991; Saby et al., 1999), most likely by activation of the reduced glutathione metabolism, which is a major component of cells involved in protection against reactive oxygen species (Saby et al., 1999). And (VI) plasmid-mediated horizontal gene transfer and conjugation between cells in a biofilm (Russell, 2003).

Different bacterial surface structures have been shown to be important for initial attachment of *E. coli* to surfaces and for actual biofilm formation under various environmental conditions. Motility of *E. coli* strain 2K1056 was shown to be important for the initial interaction with a polyvinyl chloride (PVC) surface as well as for movement along the surface under nutrient replete conditions (Pratt and Kolter, 1998). Prigent-Combaret *et al.* (2000) on the other hand, showed that in the case of *E. coli* K-12 strains (overproducing curli) flagella are not necessary for initial interaction with polystyrene (PS), when strains are grown in nutrient deplete medium. Concluding that depending on environmental conditions, *E. coli* makes use of different pathways for initial attachment to a surface (Prigent-Combaret et al., 2000). Curli, which are normally expressed under stress conditions (Olsén et al., 1993; Olsén

et al., 1989), were important for adhesion of *E. coli* K-12 strains to PS in nutrient deplete medium (Prigent-Combaret et al., 2000; Vidal et al., 1998) as well as for cell-to-cell interaction, in order to form a multilayered biofilm (Prigent-Combaret et al., 2000). While Ryu *et al*. (2004b) reported that curli were not important for initial adhesion to stainless steel coupons (SSCs), but for the formation of an actual biofilm. Type 1 fimbriae were, however, reported to be needed for initial attachment to PS (Pratt and Kolter, 1998). EPS production by *E. coli* O157:H7 on the other hand inhibited attachment to SSCs (Ryu et al., 2004a).

Biofilm formation of APEC is known to be dependent on many factors, medium being one of them. Nutrient deficient conditions in general induce biofilm formation (Skyberg et al., 2007). A lot of research has been done on biofilm formation of *E. coli* in the food industry (Dourou et al., 2011; Hood and Zottola, 1997; Patel et al., 2011; Ryu and Beuchat, 2005; Ryu et al., 2004a; Wang et al., 2012), whereby the choice of materials plays an important role in the capacity to form biofilms. The capacity to form biofilms on materials often used in the poultry farms is although not frequently studied, even though this has a huge influence on the cleaning and disinfectant practices.

Objectives of the study

Traditionally, antibiotics are used to treat chickens infected with colibacillosis. However, due to development of antibiotic resistance, other ways of treating chickens have to be found. Furthermore, treating laying hens with antibiotics is not practiced, due to residues that may end up in the eggs. Vaccination shows varying results, mainly because of the great diversity of APEC strains. Therefore alternative methods have to be explored to prevent or treat APEC infection. During this study a two-way strategy was tested. On the one hand trying to prevent environmental colonization (or flock to flock transmission) of APEC and lowering infection pressure in the presence of chickens by the use of disinfectants and on the other hand focusing on treating APEC infected chickens by the use of a phage cocktail. The general aim of this study was to assess the usefulness of disinfectants and phage therapy in the prevention and treatment of APEC infections in poultry.

Disinfectants

The first specific aim of this study was to determine the sensitivity of a specific collection of APEC strains, representative for the Belgian laying hen industry, towards antibiotics and active ingredients often included in disinfectants in the poultry industry, and to determine the integron prevalence in these strains (**Chapter II**).

The second specific aim was to determine the biofilm formation capacity of these APEC strains on different materials often used in chicken barns, since biofilms can impair disinfectant efficacy, and to determine the efficacy of some disinfectants to destroy biofilms (**Chapter III**).

The third aim of the study was to determine the efficacy of disinfection by nebulization of hydrogen peroxide in order to lower infection pressure in the presence of chickens (**Chapter IV**).

Bacteriophage therapy

The fourth aim of this study was the development of a bioluminescent APEC strain to monitor APEC infection in chickens *ex vivo*, to be able to determine the effect of phage treatment on the course of an APEC infection (**Chapter V**).

And the fifth specific aim was to determine the efficacy of a phage cocktail for treating chickens that have been experimentally infected with APEC (**Chapter VI**).

Chapter II

Susceptibility of avian pathogenic *Escherichia coli* from laying hens in Belgium to antibiotics and disinfectants and integron prevalence

Adapted from:
Oosterik, L.H., Peeters, L., Mutuku, I., Goddeeris, B.M., Butaye, P. 2014. Susceptibility of Avian Pathogenic *Escherichia coli* from laying hens in Belgium to antibiotics and disinfectants and integron prevalence. Avian Diseases 58 (2), 271-278

Abstract

In this chapter, the resistance of APEC isolates (n = 97) from Belgian laying hens to different classes of antibiotics was determine, as well as the minimum inhibitory concentrations (MIC; agar and broth dilution) and minimum bactericidal concentrations (MBC) of the five most included active ingredient in routinely used disinfectants in the poultry industry (formaldehyde, glutaraldehyde, glyoxal, hydrogen peroxide, and a quaternary ammonium compound). The presence of integrons was determined by PCR.

Resistance to ampicillin (35.1%), nalidixic acid (38.1%), sulfonamides (41.2%), and tetracycline (53.6%) was high, but resistance to other tested antibiotics was low. Nevertheless, two ESBL producers were found. The MIC of the disinfectants for the APEC strains showed an unimodal distribution, indicating that there was no acquired resistance. MBCs were similar to MICs via the broth dilution method, showing the bactericidal effect of the disinfectants. Twenty-one strains (21.6%) were found positive for class 1 integrons and a positive association between integron presence and resistance to trimethoprim, sulfonamides, and tetracycline was found. No association could be found between integron presence and phylogenetic group affiliation.

1. Introduction

Antibiotic resistance among poultry *E. coli* isolates is common (Harisberger et al., 2011; Smet et al., 2008; van den Bogaard et al., 2001; Vandemaele et al., 2002), even though restrictions were put on the use of antibiotics by the European Union (Casewell et al., 2003; European Parliament, 1998). Laying hens are rarely given antibiotics, since no mass outbreaks of colibacillosis occur, only a limited number of laying hens gets infected that are difficult to treat, furthermore there is the risk of antibiotic residues ending up in the eggs. The observed resistance among faecal *E. coli* has been reported to be lower in strains from laying hens than in strains from broilers, although resistance is widespread (Harisberger et al., 2011; van den Bogaard et al., 2001). During rearing however, layers do receive antibiotics, and antibiotic resistance can persist for a long time in the intestinal tract of chickens without selective pressure of antibiotics (Chaslus-Dancla et al., 1987). Other risk factors for bacterial resistance are crowding and poor sanitation. Sanitation, which includes regularly cleaning and disinfection, is a good way to lower the bioburden and thereby the level of specific pathogens in poultry farms (Moustafa Gehan et al., 2009). However, little is known about resistance to disinfectants and the degree of resistance. Only resistance to QACs is relatively well known,

which in the case of *E. coli* is mainly encoded by the *qacEΔ1* gene, mostly associated with class 1 integrons. This gene, together with *sul1*, which encodes resistance to sulfonamide, is found in the 3'-CS of class 1 integrons (Paulsen et al., 1993). Next to the 3'CS and 5'CS region (containing the integrase gene, an integration site and a promoter), class 1 integrons have a variable region that can contain one or more mobile gene cassettes, normally coding for antibiotic resistance (Fluit and Schmitz, 2004). Furthermore, multidrug efflux pumps, mainly encoded on the chromosome, can lower the sensitivity towards disinfectants and antibiotics by pumping out the antimicrobials (Poole, 2005)

In this chapter, we determined the susceptibility of APEC strains, obtained from laying hens suffering from colibacillosis, to antibiotics and to frequently used disinfectants in the poultry industry. Also, the presence of integrons was investigated and phylogenetic typing of the strains was performed.

2. Material and Methods

2.1. Bacterial strains

APEC strains (n = 97) were collected from organs (caecum, down, liver, oviduct, pericardium/heart, peritoneum, trachea) and faeces/bedding of caged laying hens suffering from colibacillosis and kept in Lysogeny broth (LB) supplemented with 50% glycerol at -80°C. The strains were from 50 different farms throughout Belgium, collected from the year 2000 - 2005, and were kindly provided by Dr. Vandekerchove (CODA-CERVA, Brussels, Belgium). American Type Culture Collection strain 25922 (ATCC 25922) was used as reference strain. Control strains containing the integrase class 1, 2 and 3 gene respectively (*intl1, intl2,* and *intl3)* from the collection of the university of La Rioja (Logroño, Spain), were kindly provided by Prof. C. Torres. Strains FV18088 (*chuA* and TSPE4.C2) and FV18091 (*yjaA*) were used as control strains for phylogenetic typing and were kindly provided by Prof. J. Blanco (University of Santiago de Compostela, Spain). Bacteria were routinely grown at 37°C unless stated otherwise.

2.2. Disinfectants

The five active ingredients most included in disinfectants, routinely used in poultry farms, were selected. These ingredients were: alkyldimethylbenzylammoniumchloride (a QAC, 50%), formaldehyde (FOR, 35% wt/vol in H_2O), glutaraldehyde (GLU, 50% wt/vol in H_2O), glyoxal (GLY, 40% wt/vol in H_2O) and hydrogen peroxide (H_2O_2, 42.1 % wt/vol in H_2O;

EcoClearProx®). FOR, GLU, GLY and QAC were obtained from Sigma-Aldrich (Diegem, Belgium), H_2O_2 from ABT Belgium BVBA (Affligem, Belgium).

2.3. Serotyping

The following *E. coli* antisera: O1, O2, O5, O6, O8, O9, O11, O12, O14, O15, O17, O18, O20, O35, O36, O45, O53, O78, O81, O83, O88, O102, O103, O115, O116, O132 (University of Santiago de Compostela, Spain) were used to serotype the *E. coli* strains using the microtitre agglutination test, as previously described (Guinée et al., 1972).

2.4. Antibiotic susceptibility testing

Antibiotic susceptibility of the strains was tested with the disk diffusion susceptibility test, according to standardized methods from the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2008). Briefly, cultures in brain heart infusion broth (BHIB; Bio-Rad Laboratories N.V., Nazareth Eke, Belgium) were made from the stock cultures (-80°C) and put overnight in an incubator shaker (Innova 4200, New Brunswick Scientific Europe B.V., Nijmegen, the Netherlands) at 230 rotations per minute (rpm). Columbia sheep blood agar (CSA) plates (Bio-Rad Laboratories N.V.) were streaked with the overnight cultures and incubated for 18 - 24 h. Uniform colonies were picked from the CSA plate and diluted in saline (Bio-Rad Laboratories N.V.) till a density of 0.5 McFarland was obtained. The suspension was spread on a Mueller-Hinton agar (MHA; CM0337, Oxoid N.V., Erembodegem - Aalst, Belgium) plate by streaking with a sterile cotton swap and the surface was left to dry, after which the antibiotic discs (Rosco, Taastrup, Denmark) were put on the surface with a dispenser. These antibiotics are: ceftriaxone (CTRX; 30 µg), ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), cefoxitin (CFX; 60 µg), aztreonam (ATM; 30 µg), amoxicillin/clavulanic acid (AMC; 30+15 µg), ampicillin (AMP; 33 µg), nalidixic acid (NAL; 130 µg), enrofloxacin (EFX; 10 µg), gentamicin (GEN; 40 µg), neomycin (NEO; 120 µg), chloramphenicol (CHL; 60 µg), tetracycline (TET; 80 µg), trimethoprim (TMP; 5.2 µg) and sulfonamides (SULFA; 240 µg). After 18 h incubation, inhibition zones were measured to determine whether the different strains were susceptible, intermediate or resistant to the tested antibiotics, according to the manufacturer's guidelines (Rosco, 2004-2005). Strains that showed intermediate resistance, were considered as non-resistant. *E. coli* ATCC 25922 was used as reference strain.

2.5. Disinfectant susceptibility testing

2.5.1. Minimum inhibitory concentration determination

MICs of the selected disinfectants for the APEC strains were determined with the agar dilution and the broth microdilution assay, according to the CLSI (CLSI, 2008).

2.5.1.1. Agar dilution method

Fresh APEC cultures were diluted in saline to obtain a culture of approximately 1.5×10^7 cfu/ml. The susceptibility test was performed on MHA to which two-fold dilutions of the disinfectants were added. On every plate the ATCC strain 25922 was inoculated as a control. Inoculation was performed with a multipoint inoculation machine (Denley Instruments, Billingshurst, UK), whereby approximately 2×10^4 cfu/spot of every strain was inoculated on the agar. The plates were inspected for visible growth after incubation for 24 h; the MIC is the lowest concentration of disinfectant where no growth could any longer be observed.

2.5.1.2. Broth microdilution assay

Two-fold dilutions of the disinfectants were added to test tubes with cation adjusted Mueller-Hinton Broth, (MHB; CM0405; Oxoid N.V.) after which 50 µl of the disinfectant dilution was transferred to the wells of a sterile plastic 96-well microdilution plate (Pittsburgh Corning Europe, S.A., Lasne, Belgium). Fresh 24 h incubated (230 rpm) APEC cultures in BHIB were diluted in saline, to obtain a concentration of 1.5×10^6 cfu/ml. Fifty µl of these APEC cultures was added to the wells of the microdilution plate, yielding a total suspension volume of 100 µl (disinfectant and APEC culture). After which the plates were incubated for 24 h, whereafter the MICs were read. ATCC strain 25922 was used as control.

2.5.2. Minimum bactericidal concentration determination

The MBC of the disinfectants for the different APEC strains was determined by plating the suspensions (100 µl) from the microdilution plate where no visible growth was seen, from the broth dilution assay, on MHA. The plates were incubated for 24 h, after which MBCs were determined. The MBC is the concentration of disinfectant where a 99.9% reduction in bacterial numbers could be seen.

2.5.3. Integron detection

DNA was extracted using a boiling method. Briefly, strains were overnight incubated (at 230 rpm) in BHI, after which cultures were spun down at 1,500 × *g* for 10 min in an Eppendorf centrifuge. The pellet was resuspended in 500 µl sterile Milli-Q water and heated in a heating

block at 96ºC for 6 min. Cultures were spun at 6,100 × *g* for 6 min in an Eppendorf centrifuge and the supernatant was transferred to a new Eppendorf tube.

A multiplex PCR was performed to detect *intl1, intl2* and *intl3* genes that encode class 1, 2 and 3 integrases, respectively, using primers previously described (see Table 2.1) (Su et al., 2006). The QIAGEN Multiplex PCR Kit (QIAGEN, Venlo, the Netherlands) was used, according to the recommendations of the manufacturer. The PCR conditions used, were as described by Marchant *et al*. (2013); 94°C (5 min), [94°C (30 s), 54°C (30 s), 72°C (2 min)] 30 cycles and final extension on 72°C (10 min) was added. Strains containing *intl1*, *intl2* and *intl3* genes were used as positive controls. The products were visualized on a 2% agarose gel by transillumination.

Table 2.1. Oligonucleotides used in this chapter

Primer	Sequence (5' to 3')	Size (bp)	Target	GenBank number	References
intM1-U	ACGAGCGCAAGGTTTCGGT	565	*Intl1*	AF550415	(Su et al., 2006)
intM1-D	GAAAGGTCTGGTCATACATG				
intM2-U	GTGCAACGCATTTTGCAGG	403	*Intl2*	AP002527	(Su et al., 2006)
intM2-D	CAACGGAGTCATGCAGATG				
intM3-U	CATTTGTGTTGTGGACGGC	717	*Intl3*	AY219651	(Su et al., 2006)
intM3-D	GACAGATACGTGTTTGGCAA				
ChuA.1	GACGAACCAACGGTCAGGAT	279	*chuA*		(Clermont et al., 2000)
ChuA.2	TGCCGCCAGTACCAAAGACA				
YjaA.1	TGAAGTGTCAGGAGACGCTG	211	*yjaA*		(Clermont et al., 2000)
YjaA.2	ATGGAGAATGCGTTCCTCAAC				
TspE4C2.1	GAGTAATGTCGGGGCATTCA	152	TSPE4.C2		(Clermont et al., 2000)
TspE4C2.2	CGCGCCAACAAAGTATTACG				

2.6. Phylogenetic typing

The APEC strains (*n* = 97) were phylogenetic classified according to the ECOR system, using a method described earlier (Clermont et al., 2000), with slight modifications. The primers that were used are given in Table 2.1. The QIAGEN Multiplex PCR Kit (QIAGEN) was used, according to the recommendations of the manufacturer. The PCR conditions were 95ºC for 4 min (initial denaturation), then 30 cycles consisting of [94ºC for 30 s (denaturation), 59ºC for 30 s (annealing) and 72ºC for 1 min (elongation)], followed by 72ºC for 5 min (final

elongation). The products were visualized on a 1.5% agarose gel by transillumination.

2.7. Statistics

Confidence intervals (CI) were calculated for the resistance percentages of APEC strains to the different antibiotics, based on the binominal distribution in Microsoft Excel (2010). The same method was performed for determining the number of strains being resistant to different classes of antibiotics and for the percentage of strains belonging to serogroup O1, O2 or O78 containing *intI1*. Differences between strains containing or not containing an integron and being resistant to different classes of antimicrobials were analyzed using the chi-square test, since results were given in percentages. The chi-square test was also used to analyze differences between strains within serogroup (O1, O2 or O78), belonging to phylogenetic group A, B1 or D. $P < 0.05$ was considered significant.

3. Results

3.1. Serotyping and selection

The APEC strains belonged to serogroup O1 ($n = 7$), O2 ($n = 32$) and O78 ($n = 58$).

3.2. Antibiotic susceptibility

The APEC strains were tested for their susceptibility towards different antibiotics (Table 2.2). The highest percentages of resistance were detected for AMP (35.1%), NAL (38.1%), SULFA (41.2%) and TET (53.6%). Resistance to aminoglycosides (GEN, NEO) was between 0 - 5.2% and to second and third generation cephalosporins or monobactams (CTRX, CAZ, CTX, CFX, ATM) between 0 - 7.2%. Significant differences in resistance against the tested antibiotics between serogroups could not be found ($P > 0.05$). Multidrug resistance, which was defined as resistant to three or more classes of antibiotics, was found for thirty-six strains (37.1%; see Table 2.3). Two strains were positive for ESBLs.

Table 2.2. Number and percentage of resistant APEC strains against antibiotics

Class	Antibiotic	*N* tested	*N* resistant	% resistant (95% CI)
Cephalosporins	Ceftriaxone	97	0	0.0 (0.0 - 4.0)
	Ceftazidime	97	0	0.0 (0.0 - 4.0)
	Cefotaxime	97	1	1.0 (0.0 - 6.0)
	Cefoxitin	97	7	7.2 (3.0 - 14.0)
Monobactams	Aztreonam	97	0	0.0 (0.0 - 4.0)
Penicillin / penicillin combinations	Amoxicillin/ clavulanic acid	97	0	0.0 (0.0 - 4.0)
	Ampicillin	97	34	35.1 (25.6 - 45.0)
Quinolones	Nalidixic acid	97	37	38.1 (28.5 - 49.0)
	Enrofloxacin	97	1	1.0 (0.0 - 6.0)
Aminoglycosides	Gentamicin	97	0	0.0 (0.0 - 4.0)
	Neomycin	97	5	5.2 (1.7 - 12.0)
Tetracyclines	Tetracycline	97	52	53.6 (43.2 - 64.0)
Sulfonamides	Sulfonamides	97	40	41.2 (31.3 - 52.0)
Chloramphenicol	Chloramphenicol	97	8	8.2 (3.6 - 16.0)
Trimethoprim	Trimethoprim	97	16	16.5 (9.7 - 25.0)

Table 2.3. Number and percentage of APEC strains resistant against total number of different antibiotic classes

N of antibiotic classes resistant	*N* of strains	% strains (95% CI)
0	23	23.7 (15.7 - 33.0)
1	13	13.4 (7.3 - 22.0)
2	25	25.8 (17.4 - 36.0)
3	14	14.4 (8.1 - 23.0)
4	14	14.4 (8.1 - 23.0)
5	7	7.2 (3.0 - 14.0)
6	0	0.0 (0.0 - 4.0)
7	1	1.0 (0.0 - 6.0)

3.3. Susceptibility to disinfectants

The MICs, as determined by the agar dilution and broth microdilution assay, and the MBCs of the disinfectants for the 97 tested APEC strains are given in Figure 2.1. For QAC, MICs of 8 - 32 µg/ml and 64 - 128 µg/ml were found via the broth and agar dilution method, respectively. The MBC was between 8 - 32 µg/ml. The MICs of FOR for the APEC strains via the broth microdilution assay range between 40 - 80 µg/ml and the MICs via the agar dilution assay range between 80 - 160 µg/ml. The MBCs are between 40 - 80 µg/ml. For GLU a MIC of 1920 µg/ml was found via the broth microdilution method and 1920 - 3840 µg/ml via the agar dilution method; the MBC was 1920 µg/ml. The MIC of GLY via the broth and agar dilution was between 460 - 1840 µg/ml and 460 - 920 µg/ml, respectively. The MBC was 920 - 1840 µg/ml. The MIC of H_2O_2 was 64 µg/ml and 64 - 128 µg/ml for the broth microdilution and agar dilution method, respectively. The MBC was 64 µg/ml. All MICs of the disinfectants for the strains via the broth dilution method were the same as the MBCs, showing the bactericidal effect of the disinfectants.

3.3.1. Integron detection

Twenty-one out of 97 strains (21.6%) were found positive for *intl1* and none of the strains were positive for *intl2* and *intl3* (see Table 2.4 and Figure 2.2). Three out of seven strains of serogroup O1 were found positive for *intl1* (42.9%); 4 out of 32 of serogroup O2 (12.5%) and 14 out of 58 of serogroup O78 (24.1%). No significant differences could be found between the serogroups for the presence of *intl1* ($P > 0.05$), possibly due to the low number of positive strains.

3.3.2. Association between antimicrobial resistance and integron presence

A possible association between antimicrobial resistance and integron presence was analyzed by the chi-square test (see Table 2.5).

Strains containing integron 1 were more resistant to TMP ($P < 0.01$), SULFA and TET ($P < 0.05$) than strains not containing integron 1. A positive association between integron presence and resistance to these antibiotics was found.

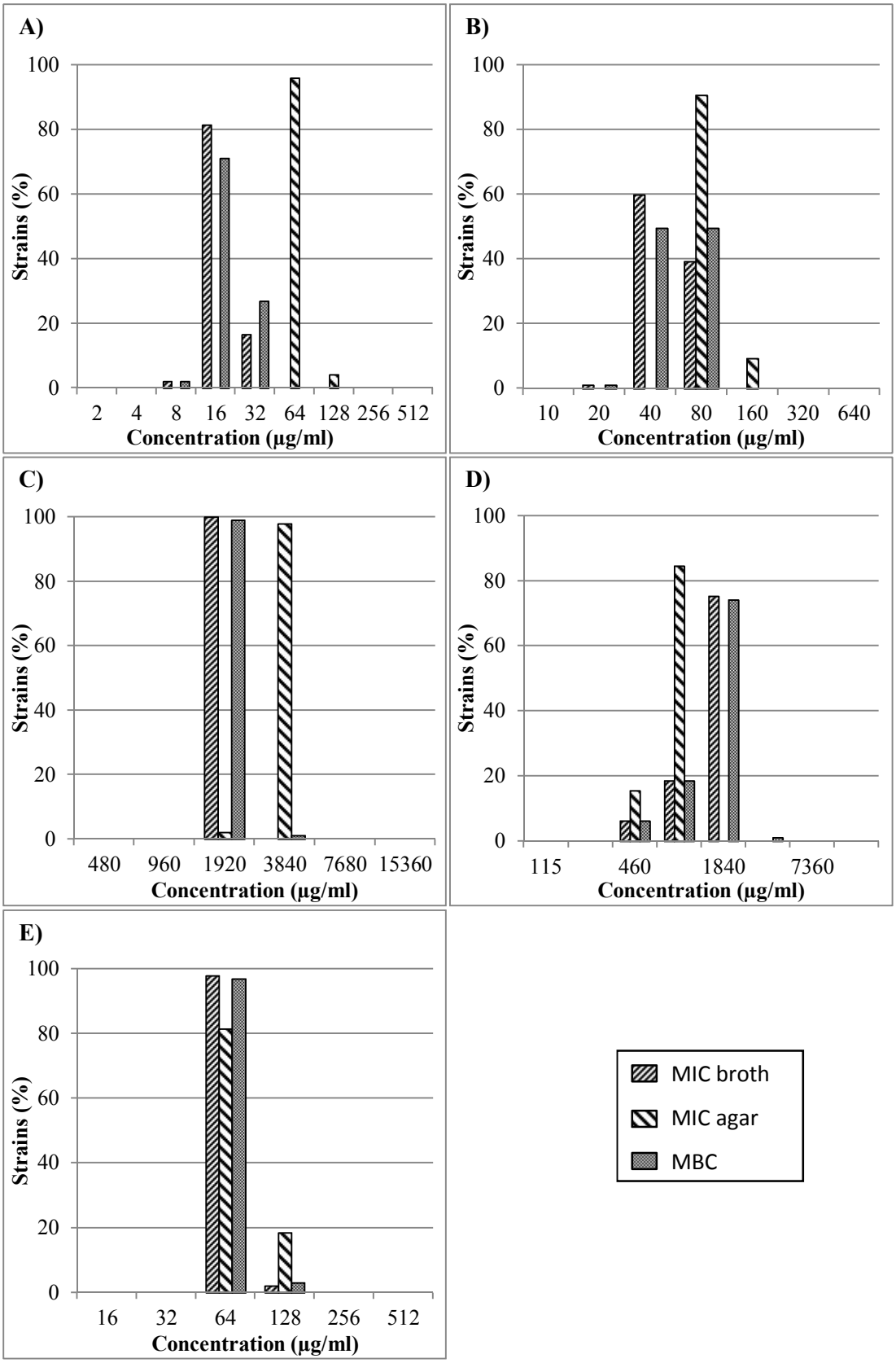

Figure 2.1. Minimum inhibitory (broth dilution and agar dilution method) and minimum bactericidal concentrations of 97 APEC strains determined for various disinfectants.
A) alkyldimethylbenzylammoniumchloride (a QAC); B) formaldehyde (FOR); C) glutaraldehyde (GLU); D) glyoxal (GLY); E) hydrogen peroxide (H_2O_2).

Table 2.4. Number and percentage of APEC strains containing the *intI1* gene divided over different serogroups

Serogroup	*N*	*N* total strains	% *intI1* (95% CI)
O1	3	7	42.9 (9.9 - 82.0)
O2	4	32	12.5 (3.5 - 29.0)
O78	14	58	24.1 (13.9 - 37.0)
Total	21	97	21.6 (13.9 - 31.0)

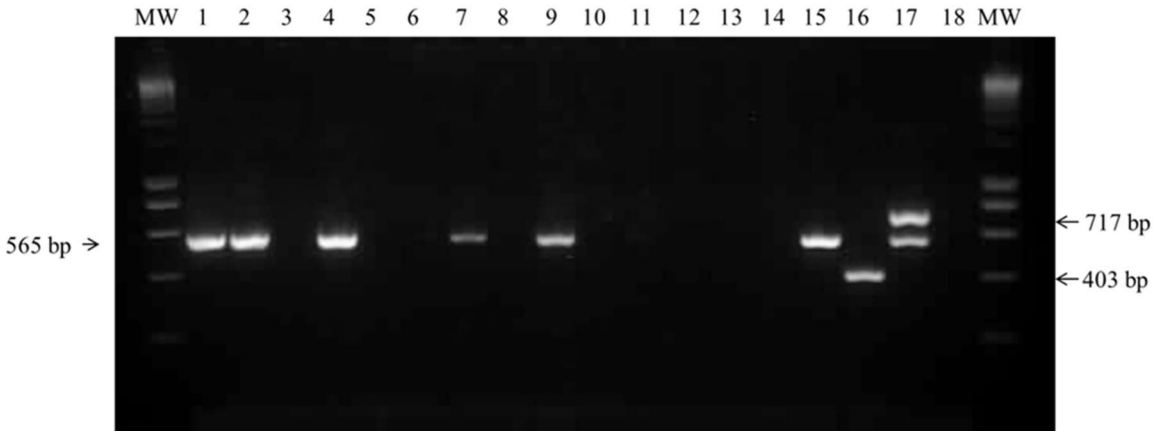

Figure 2.2. Multiplex PCR on APEC strains for detection of integrase classes 1, 2 and 3. Lane 1 - 14: APEC strains tested for presence of integrase classes 1, 2 and 3. Lane 15 - 18: positive control for integrase class 1, integrase class 2, integrase class 3 and 1, respectively. Lane18: negative control (Milli-Q water). MW is molecular size marker (SmartLadder, Eurogentec, Seraing, Belgium).

3.4. Phylogenetic typing

Results are summarized in table 2.6. Sixty-nine strains (71.1%) were classified to phylogenetic group A, 1 strain belonged (1.0%) to group B1 and 27 strains (27.8%) to group D, while none of the strains belonged to group B2. Strains from serogroup O1 and O78 belonged significantly more to phylogenetic group A compared to the other phylogenetic groups. Strains from serogroup O2 belonged more to phylogenetic group D compared to the other phylogenetic groups (not significant).

No significant differences could be found between strains containing or not containing *intI1* and phylogenetic group affiliation ($P > 0.05$).

Table 2.5. Number of isolates containing or not containing class 1 integrons in relation to their resistance towards different antibiotics

Integron presence[A]	Antibiotics and number of resistant isolates (%)[B]														
	CTRX	**CAZ**	**CTX**	**CFX**	**ATM**	**AMC**	**AMP**	**NAL**	**EFX**	**GEN**	**NEO**	**CHL**	**TET**	**TMP**	**SULFA**
+	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (9.5%)	0 (0.0%)	0 (0.0%)	9 (42.9%)	10 (47.6%)	0 (0.0%)	0 (0.0%)	3 (14.3%)	1 (4.8%)	17 (81%)*	12 (57.1%)**	15 (71.4%)*
-	0 (0.0%)	0 (0.0%)	1 (1.3%)	5 (6.6%)	0 (0.0%)	0 (0.0%)	25 (32.9%)	27 (35.5%)	1 (1.3%)	0 (0.0%)	2 (2.6%)	7 (9.2%)	35 (46.1%)*	4 (5.3%)**	25 (32.9%)*

[A] (+) = class 1 integron present; (-) = class 1 integron absent.
[B] CTRX = ceftriaxone; CAZ = ceftazidime; CTX = cefotaxime; CFX = cefoxitin; ATM = aztreonam; AMC = amoxicillin/clavulanic acid; AMP = ampicillin; NAL = nalidixic acid; EFX = enrofloxacin; GEN = gentamicin; NEO = neomycin; CHL = chloramphenicol; TET = tetracycline; TMP = trimethoprim and SULFA = sulfonamides. * = $P < 0.05$; ** = $P < 0.01$

Table 2.6. Number and percentage of isolates, within serogroup (O1, O2 or O78), belonging to phylogenetic group A, B1 or D

Serogroup	Phylogenetic group	*N*	% (95% CI)
O1	A	7	100.0 (59.0 - 100.0)
	B1	0	0.0 (0.0 - 41.0)
	D	0	0.0 (0.0 - 41.0)
O2	A	10	31.3 (16.1 - 50.0)
	B1	1	3.1 (0.1 - 16.0)
	D	21	65.6 (46.8 - 81.0)
O78	A	52	89.7 (78.8 - 96.0)
	B1	0	0.0 (0.0 - 6.0)
	D	6	10.3 (3.9 - 21.0)

4. Discussion

A selection of 97 APEC strains was assessed; these strains were tested for their susceptibility to different antibiotics and five active ingredients often included in disinfectants in the poultry industry. Resistance to AMP (35.1%), NAL (38.1%), SULFA (41.2%) and TET (53.6%) was high, but resistance to relative new antibiotics like second and third generation cephalosporins or monobactams was absent or very low, nevertheless, two ESBL producers were found. ESBLs are highly present in broilers in Belgium (Smet et al., 2008). Resistance percentage for *E. coli* isolates from septicemia affected laying hens (n = 122) in Switzerland for AMP, NAL, SULFA en TET were 11%, 13%, 26% and 26%, respectively (Lanz et al., 2003), which is lower than the resistance percentages found in this study. In contrast, clinical APEC isolates from laying hens (n = 121) in Iran showed 100% resistance against TET, 96.69% against NEO, 76.85% against CHL and 45.45% against GEN (Salehi and Ghanbarpour, 2010). The variable resistance prevalence of clinical APEC isolates from laying hens worldwide is possibly due to different antibiotic practices in the different countries. In Belgium AMP, TET, TMP + SULFA as well as amoxicillin and tylosin are often used to treat APEC affected chickens (Persoons et al., 2012; Vandemaele et al., 2002), but nothing is known about the use of antibiotics during rearing in laying hens in Belgium. Resistance against TET and TMP + SULFA was respectively 66% and 50% for APEC strains (n = 186) from Belgian poultry isolated in 2000 (Vandemaele et al., 2002), while in this study percentages of 53.6% and 15.5% were found. The lower resistance percentage can be explained by the fact that during

this study only isolates from laying hens were analyzed. Resistance percentage of 49.5% (NAL), 65.8% (SULFA) and 48.1% (TET) were found for AFEC isolates from broilers in Belgium (Smet et al., 2008), showing the higher resistance prevalence in broiler isolates compared to laying hen isolates, most probably due to higher antibiotic use in broilers.

Different methods are available for the determination of inhibiting concentrations for disinfectants, explaining differences in MICs. Previously, MICs of QAC (59 µg/ml), FOR (156 µg/ml), GLU (3250 µg/ml) and H_2O_2 (2505 µg/ml) were determined for *E. coli* ATCC strain 25922 via broth dilution (Mazzola et al., 2009). During this study MICs of 16 µg/ml (QAC), 40 µg/ml (FOR), 1920 µg/ml (GLU) and 64 µg/ml (H_2O_2) were found for ATCC 25922, respectively. The difference in MICs is probably due to the use of a different method for determining the MICs and the use of a different medium (TSB (tryptone soya broth) instead of MHB) and inoculation size. The higher divalent cation concentrations in TSB have an influence on certain disinfectants such as QAC, since they will react with the disinfectant, decreasing the bactericidal potency (Chambers et al., 1955; Ridenour and Armbruster, 1948). Furthermore, a larger inoculation size of ATCC 25922 was used in the experiment from Mazolla *et al.* (2009), probably resulting in a higher MIC. This shows the need for a standardized method for determining MICs and MBCs of disinfectants in order to be able to compare them. Despite that fact, the relative order of activity of the disinfectants was similar (QAC > FOR > H_2O_2 > GLU). However, it should be noted that MICs of GLU via the agar and broth dilution are probably overestimated, due to inhibition of GLU with constituents in the media, leaving less compound available to react with the bacteria (Gorman et al., 1980; Russell, 1994).

MICs of H_2O_2 via the agar and broth dilution method were the same for most strains, namely 64 µg/ml. While MICs of GLY via the broth dilution method were in general a factor 2 higher than the MICs via the agar dilution method. MICs of FOR and GLU on the other hand were a factor 2 higher via the agar dilution method than via the broth dilution method, while the MICs of QAC were a factor 4 higher via the agar dilution method. This implies that inhibitory concentrations via the two different methods are not the same, the agar dilution method gave a higher MIC in most cases. This can be due to a faster inactivation of the disinfectant in agar (due to interaction with medium components) leading to lower MICs. These results show that MICs determined via the two different methods cannot be compared directly. MBCs determined via the broth microdilution method were in general the same as the MICs via the broth microdilution method, showing that the disinfectants are bactericidal.

The MIC and MBC of the different disinfectants had an unimodal distribution over the concentrations (see Figure 2.1). This indicates that there was no acquired resistance for any of the disinfectants. Little is known about the genetic background of resistance of *E. coli* to disinfectants, only the resistance against QAC has been described. It has been shown that this is encoded by the *qacE* or *qacEΔ1 gene*, which is present in class 1 integrons (Paulsen et al., 1993) or via upregulation of the chromosomally encoded multidrug efflux pumps (Poole, 2005). In this study, 21.6% of the strains were found positive for *intl1*, while during earlier studies, *intl1* (or either the complete integron 1) was detected in, respectively, 59.2%, 66.0%, and 68.0% of clinical APEC isolates, obtained from the organs of avian species (chicken, quail, ostrich, and turkey) (Bass et al., 1999; Goldstein et al., 2001; Yang et al., 2004). The presence of *qac* genes has been shown not to be associated with differences in MICs in Gram-negative bacteria (Jaglic et al., 2012; Kücken et al., 2000), though it has been shown for *Staphylococcus aureus* that the presence of the *qac* genes was associated with an elevated mode MIC (but not an increased MBC) to chlorhexidine and benzalkonium chloride compared to strains not having the *qac* genes (Furi et al., 2013). However, Smith *et al*. (2008) earlier found an increased MBC for biocides containing QACs and chlorhexidine gluconate when *Staphylococcus aureus* contained *qac* genes.

More sensitive methods are necessary to be able to discriminate between susceptible and resistant strains. Therefore, first of all a clear definition between reduced susceptible (increased MIC) and resistant (not active at in-use concentration) is necessary, as well as a more sensitive method (Maillard et al., 2013). Using MBCs instead of MICs might be a step in the right direction, since this reflects the concentration at which the biocide kills. Furthermore, working with molarity instead of weight/volume might provide part of the solution, since this corrects for the weight of the disinfectant.

A significant relation was found for the presence of class 1 integrons and resistance to TMP, SULFA and TET. TMP and SULFA are known to be directly linked to class 1 integrons, while this is not the case for TET (Marchant et al., 2013; White et al., 2001). Genetic linkage between integrons and conjugative plasmids and transposons is a plausible explanation (White et al., 2001).

No significant differences could be found in integron presence and phylogenetic group affiliation, which is in agreement with previous studies (Koczura et al., 2013; Marchant et al., 2013; Skurnik et al., 2005). It has been shown before that most APEC isolates fell into ECOR group A and B2 (Ewers et al., 2009; Ewers et al., 2007) or A and D (Rodriguez-Siek et al., 2005a). During this study most isolates were classified to group A (71.1%), followed by group D (27.8%), while none of the strains belonged to group B2, which is known to contain highly pathogenic human (and animal) ExPEC strains (Clermont et al., 2000; Ewers et al., 2007; Moulin-Schouleur et al., 2007; Rodriguez-Siek et al., 2005a).

In conclusion, the prevalence of antimicrobial resistance in APEC isolates from laying hens was high for AMP, NAL, SULFA, and TET, but low resistance percentages were detected for the other tested antibiotics. No phenotypic resistance was observed for the strains to disinfectants frequently used in the poultry industry, though the presence of integrons indicates the presence of *qac* resistance genes. A positive association between presence of integron class 1 and resistance to TMP, SULFA and TET was found. MICs determined via the broth and agar dilution method cannot be compared directly, since different concentrations were found for the strains, showing the need for one standardized method for determining MICs and MBCs.

Chapter III

Biofilm formation of avian pathogenic *Escherichia coli:* influenced by serogroup, surface material and disinfectant

Adapted from:
Oosterik, L.H., Tuntufye, H.N., Butaye, P., Goddeeris, B.M. 2014. Biofilm formation of avian pathogenic *Escherichia coli:* influenced by serogroup, surface material and disinfectant. The Veterinary Journal (in press).

Abstract

Once poultry farms are infected with APEC it is hard to eradicate these pathogens. This might be due to biofilm formation of APEC strains, hampering cleaning and disinfection practices. In this chapter, biofilm formation of APEC strains from laying hens was tested on materials used in poultry houses. The strains were analyzed for an association between biofilm-forming capacity and O-serogroup. The efficacy of two routinely used disinfectants, H_2O_2 and a QAC, to kill sessile cells of two strong APEC biofilm producers (05/503 and 04/40) and a non-biofilm producer (05/293) on a polystyrene (PS) and polyvinyl chloride (PVC) surface was tested.

Most APEC strains were moderate (PS) or strong biofilm producers (polypropylene (PP) and PVC). Strains of serogroup O2 belonged significantly more often to the moderate (PS) and strong biofilm producers (PP and PVC) than to any of the other groups, while most O78 strains were weak biofilm producers. However, O78 strains were stronger biofilm producers on stainless steel than on PP and PVC, while O2 strains were stronger biofilm producers on PP and PVC. A dilution of 1% H_2O_2 was able to kill all sessile cells of strains 05/503 and 04/40 that adhered to PP and PVC, while 0.5% H_2O_2 was sufficient to kill all adhering cells of strain 05/293. QAC at a concentration of 0.01% killed all adherent cells of strains 05/503, 04/40 and 05/293 under equal conditions. In conclusion, biofilm formation of APEC strains was affected by serogroup and surface material, while biofilm destruction was dependent on the disinfectant and surface material.

1. Introduction

Biofilms form an important obstacle during cleaning and disinfection practices in poultry houses. In the all-in all-out system, cleaning and disinfection is normally performed between different flocks in the barn, in order to lower the level of specific pathogens (Moustafa Gehan et al., 2009). However, little is known about their capacity to form biofilms on different materials used in the poultry house.

It is known that biofilm formation of APEC is dependent on many factors, such as medium and phylogenetic group. Nutrient deficient media in general induce biofilm formation (Skyberg et al., 2007). Several non-pathogenic *E. coli* and intestinal pathogenic *E. coli* strains have been analyzed for biofilm formation on several surface structures (Pratt and Kolter, 1998; Prigent-Combaret et al., 2000; Ryu and Beuchat, 2005; Ryu et al., 2004b; Vidal et al., 1998). The efficacy of routinely used disinfectants to eradicate APEC biofilms has not much

been studied, although it is known for *E. coli* that formation of a biofilm can hamper disinfectant efficacy (Ntsama-Essomba et al., 1997; Ryu and Beuchat, 2005; Somers et al., 1994).

In this chapter, we therefore analyzed the capacity of clinical APEC strains to form biofilms on PP, PS, PVC and stainless steel. Furthermore, two disinfectants routinely used in poultry houses (H_2O_2 and a QAC) were tested for their ability to kill cells in biofilms.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

APEC strains (n = 93) were isolated on 49 different farms from caged laying hens suffering from colibacillosis, between the year 2000 - 2005. The APEC strains were serotyped and belonged to serogroups O1 (n = 7), O2 (n = 30) and O78 (n = 56) (see Chapter II). LB and phosphate buffered saline (PBS) were prepared as described elsewhere (Sambrook et al., 1989). Bacteria were routinely grown at 37°C, unless stated otherwise.

2.2. Disinfectants

Two of the five active ingredients, described in Chapter II, most included in routinely used commercial disinfectants in the poultry industry were selected: a QAC (50%; Sigma-Aldrich) and H_2O_2 (42.1 % wt/vol in H_2O; EcoClearProx®, ABT Belgium BVBA). H_2O_2 was selected due to its biodegradable character and broad-spectrum antimicrobial activity, QAC for its low toxicity, non-corrosive character and low cost.

2.3. Biofilm formation

2.3.1. Polystyrene, polypropylene and polyvinyl chloride plates

A protocol was modified from previous studies (Merritt et al., 2005; O'Toole et al., 1999; Skyberg et al., 2007; Stepanović et al., 2004). Briefly, the strains were grown in LB for ≈ 16 h in an incubator shaker (230 rpm; Innova 4200), thereafter cultures were diluted 1: 100 (v/v) in nutrient deficient media, namely 1/20 diluted TSB (CM0129, Oxoid N.V.) in distilled water (DW). The diluted cultures (200 µl) were seeded in the wells of sterile 96-well PS (BD Biosciences, Erembodegem, Belgium), PP (VWR International, Leuven, Belgium) and PVC (Pittsburgh Corning Europe, S.A.) microtiter plates. PP and PVC materials are used for the flooring and water distribution system/automatic drinkers in the poultry house. The strains were tested in triplicate per plate over three different plates. The wells on the outside of the plate were not used to prevent contamination and evaporation. During a preliminary study,

APEC strains 05/503 (O78; PS), 05/39 (O2; PP) and 04/40 (O1; PVC) were shown to be strong biofilm producers and APEC strain 05/293 (O78) a non-biofilm producer. These strains were added in triplicate to every plate as positive control and negative control strains, respectively. The plates were incubated for 24 h at 25°C.

2.3.2. Stainless steel

Seventeen of the 93 APEC strains were tested for biofilm formation on stainless steel (type 316; Metaleuven, Wilsele, Belgium), a material used in water distribution systems. The 17 strains were selected based on a division over the various serogroups and biofilm formation capacity on PS, PP and PVC, since not all strains could be tested due to the high workload. The 17 APEC strains belonged to serogroups O1 (n = 2), O2 (n = 6) and O78 (n = 9). Stainless steel coupons (SSCs) with a diameter of 10 mm were obtained by notching a stainless steel sheet (thickness: 3 mm). The SSCs were cleaned, disinfected and autoclaved. In each well of a 24-well tissue culture plate (VWR International) a SSC was placed and 500 µl of the 1: 100 diluted cultures in nutrient deficient media were added in triplicate to the wells and incubated for 24 h at 25ºC. The strains were tested over three different plates. Positive control strain 05/503 and negative control strain 05/293 were added in triplicate to every plate.

2.3.3. Quantification of biofilm formation

Biofilm formation was measured after incubation of the diluted cultures for 24 h in diluted TSB (see 'polystyrene, polypropylene and polyvinyl chloride plates'). First the plates/SSCs were washed three times with sterile PBS, next they were air dried and the biofilms were heat fixed at 60°C for 60 min. The biofilms were stained with crystal violet (0.1% in DW; 200 µl per well) at room temperature (RT) for 30 min. Next, the plates were washed three times under running DW (washing of SSCs was performed in three separate Falcon tubes containing DW). The plates/SSCs were air dried and a mixture of 80: 20, ethanol: aceton (plates: 200 µl; SSCs: 500 µl) was added to the wells of the microplates for destaining and incubated for 20 min at RT. Part of the solution (150 µl) was transferred to a new microtiter plate and the optical density (OD) was measured at 540 nm with a microplate reader (Victor$^{2™}$ 1420-012, PerkinElmer, Zaventem, Belgium).

The strains were classified according to their capacity to produce a biofilm under the described conditions, according to a slightly adjusted earlier described method (Stepanović et al., 2004). Therefore the average OD_{540} of the negative control strain (05/293) plus three times

the standard deviation (SD) (two times the SD for SSCs) from the mean was calculated for the three replicates per plate, in order to get the cut-off OD (ODc) per plate. The average OD_{540} of the strains for the three replicates per plate was calculated and the following scheme was used: (1) non-biofilm producer: ODc > OD; (2) weak biofilm producer: (2 x ODc) > OD > ODc; (3) moderate biofilm producer: (4 x ODc) > OD > (2 x ODc); and (4) strong biofilm producer: OD > (4 x ODc). Three biofilm capacities per strain were obtained and the most prevalent capacity was selected.

2.4. Biofilm cell killing with H_2O_2 and QAC

APEC strains 05/503, 04/40 and 05/293 were grown for 24 h in 12 wells/strain for each plate type; PS (200 µl) and PVC (150 µl). The plates were washed three times with sterile PBS (PS: 220 µl, PVC: 200 µl). Two-fold serial dilutions of H_2O_2 (2%, 1%, 0.5%) and QAC (0.02%, 0.01%, 0.005%) in saline, as well as plain saline, were added to each well in triplicate (200 µl for PS; 150 µl for PVC) and incubated for 30 min at RT. Each plate was washed with sterile PBS (PS: 220 µl, PVC: 200 µl per well), then sterile PBS was added to each well (PS: 200 µl, PVC: 150 µl per well) and the plates were covered with a sterile film. Plates were sonicated for 3 min in a water bath sonicator (Branson 2210, VWR International), the PBS was transferred to Eppendorf tubes and ten-fold dilutions were plated in duplicate on LB agar. The plates were incubated for 24 h, then bacterial numbers, expressed as cfu/well were determined.

2.5. Confirmation of the biofilm matrix

To confirm the results found by crystal violet staining and quantification of cells in an untreated 24 h biofilm, adhering cells on PS were stained with wheat germ agglutinin-Alexa fluor 488 (WGA), according to a previously described method (Burton et al., 2007). APEC strains 05/503 and 04/40 (strong biofilm producers) and 05/293 (non-biofilm producer) were first grown for 24 h (see 'polystyrene, polypropylene and polyvinyl chloride plates') in a PS microtiter plate. All strains were tested in 18-fold, in six-fold per plate repeated over three different plates. Plain 1/20 diluted TSB was used to derive the background value. Plates were washed twice with sterile PBS, and adhering cells were stained with 200 µl WGA (5 µg/mL; VWR International), then washed and destained as described by Burton *et al.* (2007). Emission of WGA was measured at 535 nm after excitation at 485 nm using a multilabel microplate reader (Victor$^{2™}$ 1420-012, PerkinElmer). Average fluorescent readings per plate were corrected for the average background value (1/20 diluted TSB) per plate.

2.6. Statistics

The OD_{540} values of the positive and negative control strains on crystal violet staining were analyzed for a normal distribution with Analyse-it (Analyse-it Software, Ltd.) in Microsoft Excel (2010). The WGA fluorescent measurements per strain were analyzed for significant differences between multiple groups with the Kruskal-Wallis test and between each pair of groups with the Wilcoxon Rank Sum Test.

The chi-square test was used to analyze significant differences between biofilm-forming capacity of strains within serogroup (O1, O2 or O78). $P \leq 0.05$ was considered significant.

3. Results

3.1. Influence of surface materials on biofilm formation

The APEC strains were tested for their biofilm-forming capacity on PS, PP and PVC in nutrient deficient medium at 25°C, as this temperature generated stronger biofilm formation than 20, 30 and 37°C (data not shown). Results of biofilm formation of the tested APEC strains, determined by crystal violet staining, are given in Table 3.1. OD_{540} values of the control strains (05/503 and 05/293) had normal distributions for each material tested.

On PS, 6/93 strains (6.5%) were non-biofilm producers, 28/93 (30.1%) were weak biofilm producers, 42/93 (45.2%) were moderate biofilm producers and 17/93 (18.3%) were strong biofilm producers. Strains from serogroups O2 belonged significantly more often to the moderate biofilm producers than to the non-, weak or strong biofilm producers ($P < 0.05$), while O78 strains were significantly more often classified as weak or moderate biofilm producers (39/56, 69.6%) than as a non- or strong biofilm producers (17/56, 30.4%).

On PP, 13/93 (14.0%) strains were non-biofilm producers, 23/93 (24.7%) strains were weak biofilm producers, 18/93 (19.3%) were moderate biofilm producers and 39/93 (41.9%) were strong biofilm producers. Within the O1 serogroup, strains were uniform strong biofilm producers and, within serogroup O2, strains were significantly more often classified as a strong biofilm producer, than as a non-, weak or moderate biofilm producer ($P < 0.05$). Most O78 strains were weak (20/56, 35.7%) biofilm producers, although there were no significant differences in biofilm formation within serogroup O78. A significantly higher percentage of strains from serogroups O1 and O2 were classified as strong biofilm producers than strains from serogroup O78 ($P < 0.05$), while a significantly higher percentage of strains from serogroup O78 were classified as non- or weak biofilm producers than strains from serogroup

O2 ($P < 0.05$; there were no significant differences from serogroup O1 due to the low number of strains tested).

On PVC, 8/93 (8.6%) strains were non-biofilm producers, 24/93 (25.8%) strains were weak biofilm produces, 20/93 (21.5%) strains were moderate biofilm producers and 41/93 (44.1%) strains were strong biofilm producers. Six out of seven (85.7%) O1 strains were strong biofilm producers. O2 strains belonged significantly more often to the strong biofilm producers than to any of the other groups ($P < 0.05$), while there were no significant differences in biofilm formation of strains within serogroup O78. A significantly higher percentage of strains from serogroups O1 and O2 were classified as strong biofilm producers than strains from serogroup O78.

Table 3.1. Number and percentage of APEC isolates from different serogroups in relation to their capacity to form biofilms on various materials

Serogroup (number of isolates)	Material[a]	Biofilm producer %			
		Non	Weak	Moderate	Strong
O1 (7)	PS	14.3	14.3	42.9	28.6
	PP	0.0	14.3	0.0	85.7
	PVC	14.3	0.0	0.0	85.7
O2 (30)	PS	0.0^{A}	23.3^{A}	63.3^{B}	13.3^{A}
	PP	0.0^{A}	6.7^{A}	23.3^{A}	70.0^{B}
	PVC	0.0^{A}	10.0^{A}	23.3^{A}	66.7^{B}
O78 (56)	PS	10.7^{A}	35.7^{B}	33.9^{AB}	19.6^{AB}
	PP	25.0	35.7	17.9	21.4
	PVC	14.3	37.5	21.4	26.8
Total (93)	PS	6.5^{A}	30.1^{BC}	45.2^{C}	18.3^{AB}
	PP	14.0^{A}	24.7^{AB}	19.3^{A}	41.9^{B}
	PVC	8.6^{A}	25.8^{B}	21.5^{AB}	44.1^{C}

[a]Tested materials, PS = polystyrene; PP = polypropylene; PVC = polyvinyl chloride
Different capital letters show significant differences within the row ($P < 0.05$).

The results for biofilm formation of 17 selected APEC strains on SSCs are presented in Table 3.2. Eight of these 17 (47.1%) strains were non- or weak biofilm producers on PS and PP, while 7/17 (41.2%) strains were non- or weak biofilm producers on PVC. On SSCs, 7/17 (41.2%) strains were classified as non- or weak biofilm producers, while 10/17 (58.8%) strains were moderate to strong biofilm producers. O78 strains were stronger biofilm producers on SSC than on PP or PVC ($P > 0.05$), while more O2 strains were moderate or strong biofilm producers on PP and PVC than on SSC ($P > 0.05$).

Table 3.2. Selection of APEC isolates divided over serogroup in relation to their capacity to form biofilms on stainless steel in comparison with other materials

Serogroup (number of isolates)	Material[a]	Biofilm producer (number of isolates)	
		Non/weak	Moderate/strong
O1 (2)	PS	1	1
	PP	0	2
	PVC	0	2
	SSC	0	2
O2 (6)	PS	1	5
	PP	0	6
	PVC	0	6
	SSC	2	4
O78 (9)	PS	6	3
	PP	8	1
	PVC	7	2
	SSC	5	4
Total (17)	PS	8	9
	PP	8	9
	PVC	7	10
	SSC	7	10

[a]Tested materials, PS = polysterene; PP = polypropylene; PVC = polyvinyl chloride; SSC = stainless steel coupon.

3.2. Biofilm cell killing with H_2O_2

Results are given in Figure 3.1. On PS, 622×10^3 and 592×10^3 cfu/well of the strong biofilm producer strains 05/503 and 04/40 adhered, respectively, while 5.9×10^3 cfu/well was found for non-biofilm producing strain 05/293. After treatment of the biofilms with 0.5% H_2O_2 for 30 min, the concentration dropped to 2.5×10^2 and 2.6×10^2 cfu/well for APEC strains 05/503 and 04/40, respectively, while no culturable/viable cells could be obtained from the wells inoculated with strain 05/293. After treatment of the biofilms with 1% H_2O_2 no culturable/viable cells could be detected anymore.

On PVC, 51×10^3 and 1170×10^3 cfu/well were found for strains 05/503 and 04/40, respectively, while for strain 05/293 19×10^3 cfu/well was found. After treatment with 0.5% H_2O_2 for 30 min, 1.4×10^1 and 7.5×10^1 cfu/well were detected for strains 05/503 and 04/40, respectively, while no viable cells could be detected for strain 05/293. After treatment with 1% H_2O_2 no viable cells were detected in any of the biofilms.

3.3. Biofilm cell killing with QAC

Results are presented in Figure 3.2. On PS, 632×10^3 and 395×10^3 cfu/well were found for strains 05/503 and 04/40, respectively, while 17×10^3 cfu/well was found for strain 05/293. After addition of 0.005% QAC for 30 min at RT, the viable cells counts of strains 05/503, 04/40 and 05/293 dropped to 28.5×10^3, 1.6×10^3 cfu/well and 0.2×10^2 cfu/well, respectively. After addition of QAC to 0.01% no culturable/viable cells could be obtained. On PVC, 166 x 10^3 and 182×10^3 cfu/well were found for strains 05/503 and 04/40, respectively. These dropped to 13.1 and 122 cfu/well after addition of 0.005% QAC for 30 min at RT. For strain 05/293 33×10^3 cfu/well was detected, which dropped to 3.6 cfu/well after treatment with 0.005% QAC, while 0.01% QAC killed all culturable/viable cells that adhered to the surface for all tested strains.

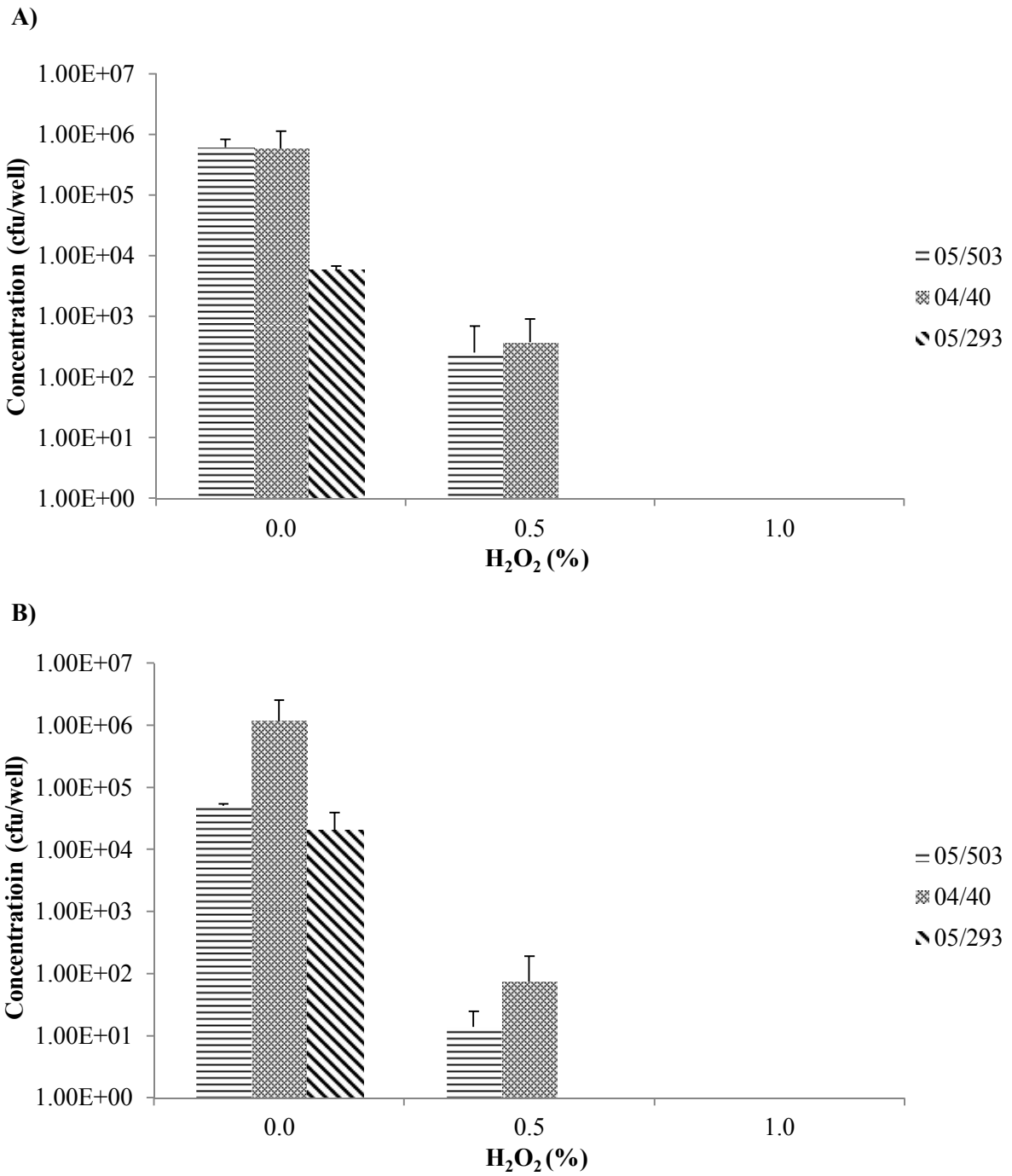

Figure 3.1. Viable cell quantification after treatment of adhering cells with hydrogen peroxide. Adhering cells of strong biofilm producer strains (05/503 and 04/40) and a non-biofilm producer strain (05/293) were treated with two-fold dilutions of H_2O_2, after incubation for 24 h. Viable cell numbers were quantified (cfu/well). A) polystyrene surface; B) polyvinyl chloride surface.
H_2O_2 = hydrogen peroxide.

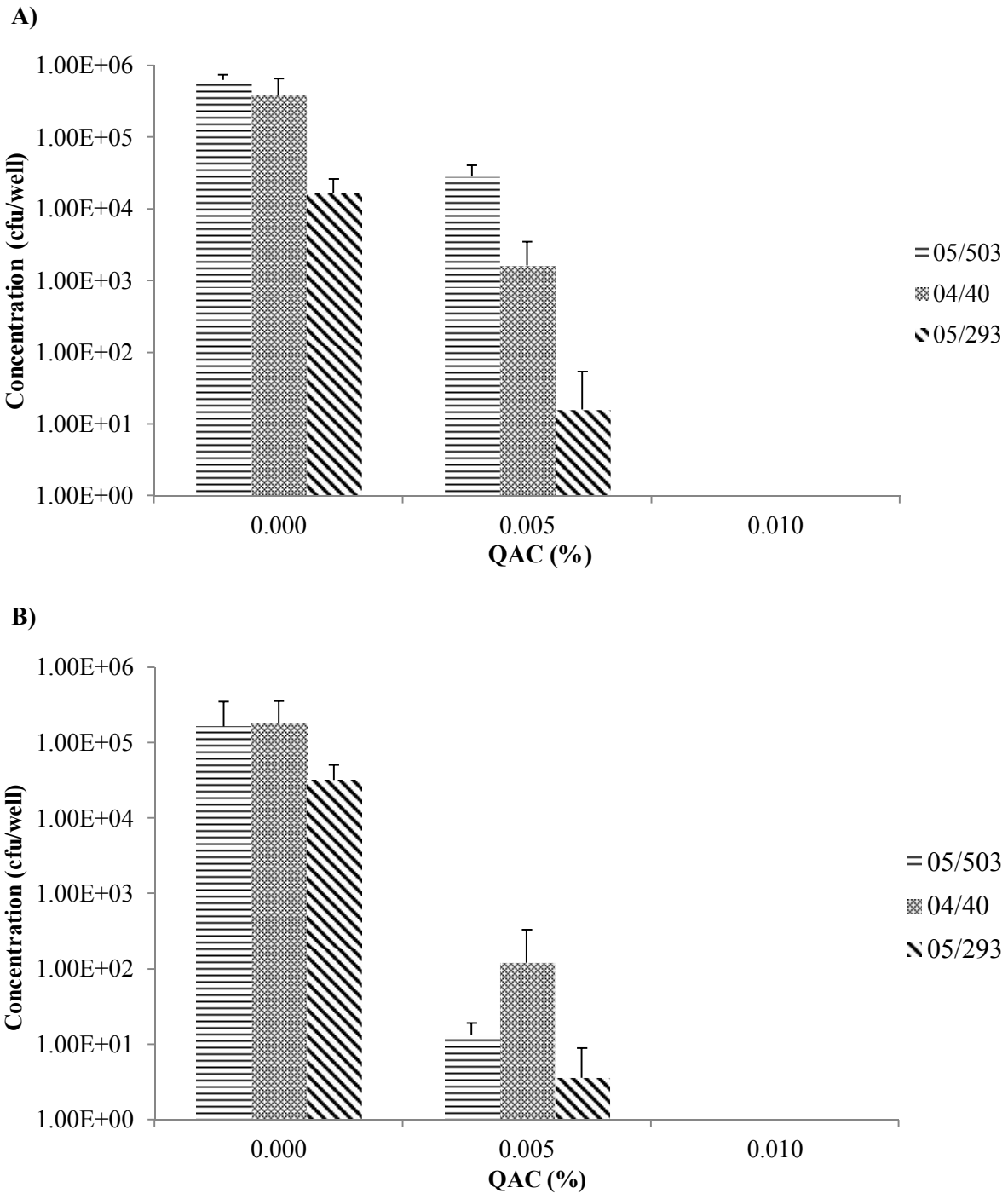

Figure 3.2. Viable cell quantification after treatment of adhering cells with a quaternary ammonium compound. Adhering cells of strong biofilm producer strains (05/503 and 04/40) and a non-biofilm producer strain (05/293) were treated with two-fold dilutions of a quaternary ammonium compound, after incubation for 24 h. Viable cell numbers were quantified (cfu/well). A) polystyrene surface; B) polyvinyl chloride surface. QAC = quaternary ammonium compound.

3.4. Presence of a biofilm matrix

Biofilms were stained with WGA to confirm the presence or absence of a biofilm matrix. Strains 05/503 and 04/40 had a significantly higher average fluorescence intensity than strain 05/293 ($P < 0.0001$), indicating that strains 05/503 and 04/40 formed a biofilm matrix, while strain 05/293 did not (Fig. 3.3). This is in agreement with the results found with crystal violet staining and the number of adhering cells found on PS. The highest numbers of viable cells was found for strain 05/503, followed by strains 04/40 and 05/293.

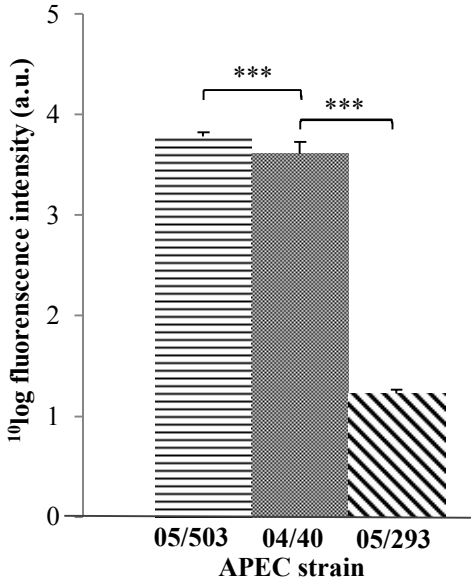

Figure 3.3. Fluorescence intensity of adhering APEC cells to polystyrene microplates.
Two strong biofilm producer strains (05/503 and 04/40) and a non-biofilm producer strain (05/293) stained with WGA, after growth for 24 h on a polystyrene surface. Average values of three independent measurements (performed in six-fold per plate) are shown with the standard deviations.
a.u. = arbitrary units
*** $P \leq 0.0001$

4. Discussion

Most APEC strains formed moderate biofilms on PS, while a substantial biofilm was formed on PP and PVC under nutrient deficient conditions at an environmental temperature of 25ºC. This is important, since these environmental conditions can be encountered in the poultry houses, especially in the water systems and automatic drinkers were PP and PVC materials are used. β-1,6-N-acetyl-d-glucosamine is an important sugar for binding to abiotic surfaces, cell-to-cell binding and actual biofilm formation of *E. coli* (Wang et al., 2004). Enzymatic hydrolysis of β-1,6-N-acetyl-d-glucosamine leads to the disruption of the integrity of the biofilm (Itoh et al., 2005). We showed that non-biofilm producer strain 05/293 had indeed a

significantly lower average fluorescence intensity than strong biofilm producer strains 05/503 and 04/40, after staining with WGA. Even though strain 05/293 is a non-biofilm producer, ≈ 10^4 cfu/well adhered to PS, which is only 2 logs lower than cell concentrations of strains 05/503 and 04/40, while on PVC ≈ 3×10^4 cfu/well was found for strain 05/293, only 0.5 - 2 logs lower than for strains 05/503 and 04/40 (see Figure 3.1 and 3.2). The negative control strain probably forms a monolayer of cells with no intercellular binding and formation of an elaborate network, as indicated by weak WGA staining.

Serogroup O2 strains were more often classified as strong biofilm producers on PP and PVC than as non-, weak or moderate biofilm producers ($P < 0.05$). They were mostly moderate biofilm producers on PS. The same trend was found for the O1 strains on PP and PVC, although not significant due to the low number of tested strains. Most O78 strains were weak biofilm producers on PP and PVC. The O78 strains might have a more hydrophilic polysaccharide side-chain than the O1 and O2 strains, due to their different composition of the LPS (Stenutz et al., 2006), making it harder to overcome the repulsive force of the hydrophobic nonpolar abiotic surface. These results are supported by the finding that more O78 were moderate or strong biofilm producers on SSCs, than on the more hydrophobic materials (PP and PVC). Fewer O2 strains were moderate or strong biofilm producers on SSCs than on the other materials. However, due to the low number of tested strains no statistical differences could be found.

Different culture conditions (O_2 concentration, temperature, medium) have a consequence on biofilm-forming capacity, as already shown by Skyberg *et al.* (2007) for APEC strains grown in various media. In our study, APEC cultures in the stationary growth phase were seeded in diluted TSB, since this nutrient-depleted medium promotes biofilm formation (Skyberg et al., 2007). However, seeding bacterial cultures in specific minimal media may give an even more precise estimation of the environmental conditions in a poultry house. Since the aim of our study was to define differences between serogroups, optimal conditions for biofilm formation were used for easier recognition of differences.

A dilution of 1% H_2O_2 was effective in killing all cells of strains 05/503 and 04/40 that adhered to a PS and PVC surface, while 0.5% was sufficient to kill all adhering cells of strain 05/293. A concentration of 0.01% QAC, on the other hand, was already effective in eradicating all sessile cells for the tested strains. In-use concentrations of QAC in the poultry house range between 400 - 1.500 µg/mL (0.04% - 0.15%) and for H_2O_2 between 420 - 50.400

µg/mL (0.042% - 5.4%). The in-use concentrations for QAC are higher than the obtained concentrations needed to kill the sessile cells during our study, while the obtained concentration for H_2O_2 is higher than the lowest in-use concentration and lower than the highest in-use concentration. It should be realized that our biofilms are relatively young. Older biofilms may have a more elaborate network, whereby the effectiveness of the disinfectants will most likely be impaired to a greater degree, as shown with some food-borne bacteria (such as *Listeria monocytogenes* and *Salmonella* spp.) (Corcoran et al., 2014; Lee and Frank, 1991; Mangalappalli-Illathu et al., 2008). Moreover, the presence of organic matter in the poultry house can inhibit the effectiveness of disinfectants, usually leaving less compound available to react with the microorganisms. Nevertheless, concentrations of 2-3% H_2O_2 had good antimicrobial activities in the presence of organic matter in a poultry house, QACs were affected to a greater degree (Moustafa Gehan et al., 2009).

H_2O_2 and QAC were more effective in killing adherent cells on PVC than PS. The higher effectiveness of the tested disinfectants on PVC shows that the surface material should also be considered in the choice for and concentration of disinfectant. However, this is of course not the only selection criterium for the choice of a certain disinfectant, also economic reasons and the characteristics of the disinfectants play a major role. Unfortunately, the biofilm cell killing on SSCs was not tested in this study due to the high workload. It is difficult to compare the obtained disinfectant concentrations necessary to kill the sessile cells in our study with previously found results, because of the existence of several different testing methods, showing the need for one standardized international method.

In conclusion, under the tested conditions most APEC strains were moderate to strong biofilm producers on materials used in poultry houses, such as PP and PVC. Within the serogroups, significant differences could be found in biofilm-forming capacity of the strains. A dilution of 1% H_2O_2 was capable of killing all cells of strong biofilm producer strains 05/503 and 04/40 on a PS and PVC surface, while 0.5% H_2O_2 was sufficient to kill all cells of a non-biofilm producer strain. A dilution of 0.01% QAC was already sufficient to kill all cells in the biofilm of the strong and non-biofilm producing strain.

Chapter IV

Disinfection by hydrogen peroxide nebulization increases susceptibility to avian pathogenic *Escherichia coli*

Abstract

In this chapter, the nebulization of low concentrations of H_2O_2 was tested in the presence of chickens to reduce infection pressure. However, significantly higher lesion scores and higher *E. coli* concentrations were found in the spleen of chickens exposed to 2% H_2O_2, followed by those exposed to 1% H_2O_2. Control chickens, exposed to DW, had the lowest lesion scores and *E. coli* concentrations in the spleen. Thus, H_2O_2 is rendering animals more prone to APEC infection, contraindicating H_2O_2 nebulization in the presence of chickens.

1. Introduction

Sanitation and cleaning is a first way to reduce infection pressure (Moustafa Gehan et al., 2009), but is normally performed between different flocks in the barn to prevent flock to flock transmission. The information about the use of disinfectants in the presence of animals to lower infection pressure and prevent APEC is limited. On conditions safe to the animals, nebulization of H_2O_2 in the presence of chickens may possibly lower infection pressure, since (pathogenic) *E. coli* is normally present in high concentrations in the barn in the form of faeces-contaminated dust (Barnes et al., 2008; Dho-Moulin and Fairbrother, 1999; Dziva and Stevens, 2008). Predisposing factors, such as viral live vaccines, render chickens more prone to APEC infection (La Ragione and Woodward, 2002; Nakamura et al., 1994). Several studies on H_2O_2 toxicity have been performed on rats, mice, rabbits and dogs, but there is no documentation on such studies in chickens. As toxicity is depending on many factors like exposure time, number of exposures and concentration (Anonymous, 2003), the goal of this chapter was to test if H_2O_2 nebulization in the presence of chickens can lower infection pressure and pathogenicity of APEC.

2. Material and methods

2.1. *In vivo* testing of nebulization with H_2O_2

The animal experiment was approved by the ethical committee of the KU Leuven (Permit Number: P176/2011) and conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines.

Fifteen one-day-old chickens (Ross; Belgabroed NV, Merksplas, Belgium) were housed in a disinfected barn and provided with water and feed *ad libitum*. At four weeks of age, the chickens were orally vaccinated against Newcastle disease (10^6 mean egg infective dose; MSD Animal Health, Boxmeer, the Netherlands) to render them subsequently more

susceptible to APEC challenge. Three days post vaccination (dpv), the chickens were transferred and equally divided over three disinfected isolators. Three and 4 dpv they were aerogenically infected with a virulent APEC strain CH2 (Vandemaele et al., 2005) (10 ml of 10^{10} cfu/ml) with the help of a compressor (KNF Neuberger, Aarselaar, Belgium) and nebulizer (Cirrus 2; Intersurgical, Uden, the Netherlands). The chickens were exposed to the aerosols for 60 min. One day thereafter, they were exposed to 10 ml of different H_2O_2 concentrations (EcoClearProx®, ABT Belgium BVBA) by nebulization for 60 min (Group 1 - 2% H_2O_2; Group 2 - 1% H_2O_2; Group 3 - DW).

Eleven dpv all surviving chickens were euthanized by cervical dislocation for clinical necropsy. Macroscopic lesions were scored according to Vandemaele *et al.* (2005). Briefly, macroscopic lesions in the left and right thoracic air sac, the abdominal air sacs, the left and right lung, liver and heart were each scored from 0 to 2. Intermediate signs for APEC lesions were scored between 0.5 and 1.5. Chickens that died or were euthanized because of serious clinical APEC symptoms were given the full score (14), after confirming severe APEC lesions on necropsy.

2.2. Bacterial re-isolation from the spleen

From two or three chickens per group (chickens that had macroscopic lesions) the spleen was aseptically removed and homogenized in sterile PBS (1 g of tissue in 1 ml PBS), as described before (Tuntufye et al., 2012a). Ten-fold dilutions were plated on MacConkey agar (Oxoid CM0115) and incubated at 37ºC for 24 h, after which bacterial numbers (cfu/g) were determined.

2.3. Statistics

Results were analyzed with SAS software version 9.4 (SAS Institute Cary, North Carolina, USA) and R (R Development Core Team, 2013). The sum of the total lesion score and the lesion scores per organ were calculated for the three groups. To evaluate the hypothesis of a higher total lesion score in infected chickens treated with DW than H_2O_2, the total lesion scores were analyzed by means of an ordinal logistic regression model. Results of ordinal logistic regression are presented as the odds ratio of having a high lesion score. Average bacterial concentrations were compared using the Kruskal-Wallis Test for significant differences between multiple groups and the Wilcoxon Rank Sum Test for differences between each pair of groups, since few data were available per group. $P \leq 0.05$ was considered significant.

3. Results

3.1. Mortality and lesion scores

Mortality and lesions scores of infected/treated chickens are given in Table 4.1. None of the chickens died after infection. Chickens that were exposed to H_2O_2 had a higher sum of total lesion scores (Group 1: 42 and Group 2: 20) than chickens exposed to DW (Group 3: 14). Infected chickens treated with 1% H_2O_2 had a 3.53 (95% CI, 0.30 - 88.84) higher chance of developing a high lesion score (score: 14) than infected chickens treated with DW. This chance was 12.28 (95% CI, 1.01 - 357.07) higher in infected chickens treated with 2% H_2O_2 than with 1% H_2O_2. Thus nebulization of 2% H_2O_2 does increase the lesion scores.

Table 4.1. Mortality and lesion scores of infected/treated chickens

					Sum of lesion scores							
Group	Infection[1]	Treatment[2]	Total[3]	Deaths[4]	Liver	PRD	TAS-L	TAS-R	LU-L	LU-R	AAS	TOT
1	CH2	2% H_2O_2	5	0	6	6	6	6	6	6	6	42^A
2	CH2	1% H_2O_2	5	0	2	2	4	4	2	2	4	20^B
3	CH2	DW	5	0	2	2	2	2	2	2	2	14^B

[1]Aerogenic infection with 10 ml 10^{10} cfu/ml APEC CH2 at 31 and 32 days of age.
[2]Chickens aerogenically treated with 2% H_2O_2, 1% H_2O_2 or distilled water, at 33 days of age i.e. 24 h after the 2nd infection.
[3]Total number of chickens per group.
[4]Total number of chickens that died or were euthanized during the experiment.
PRD = pericardium; TAS-L = left thoracic air sac; TAS-R = right thoracic air sac; LU-L = left lung; LU-R = right lung; AAS = abdominal air sacs; TOT = total lesion score; DW = distilled water.
Numbers within the same column with different capital letters show significant differences ($P \leq 0.05$).

3.2. Bacterial re-isolation from the spleen

Bacterial numbers (cfu/g) isolated from the spleen in chickens of Group 1, Group 2 and Group 3 are shown in Figure 4.1. The highest bacterial load from the spleen was obtained in the group of chickens exposed to aerosols of 2% H_2O_2, followed by 1% H_2O_2 and DW ($P \approx 0.11$).

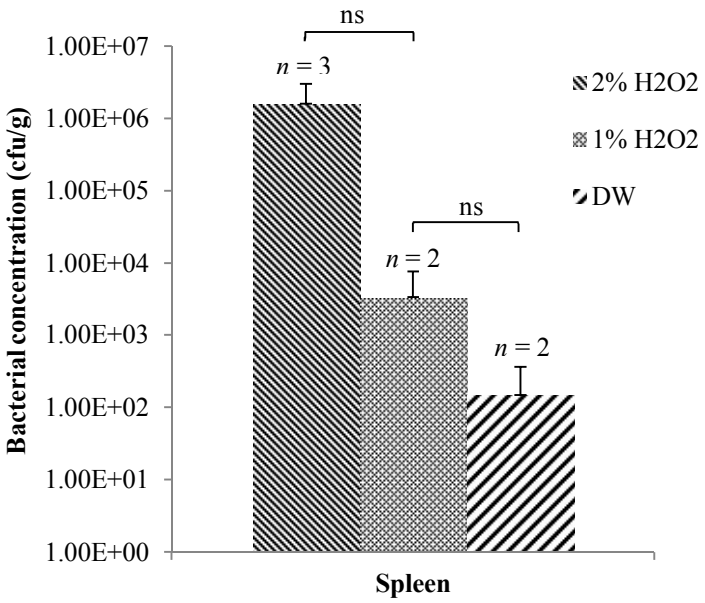

Figure 4.1. Isolated bacterial concentrations from the spleen
2% H_2O_2: 3 out of 5 chickens had macroscopic lesions (3 spleens with lesions)
1% H_2O_2: 3 out of 5 chickens had macroscopic lesions (1 spleen with lesions)
DW: 1 out of 5 chickens had macroscopic lesions (1 spleen with lesions)
DW = distilled water, ns = not significant ($P \geq 0.05$)

Discussion

In this chapter, the use of H_2O_2 nebulization was tested to reduce environmental contamination in a chicken house, after nebulization of APEC aerosols. However, nebulization of 1% H_2O_2 and 2% H_2O_2 did result in an increased total lesion score in comparison to nebulization of DW. This worsening effect after H_2O_2 nebulization is probably due to the caustic effect of H_2O_2 radicals on (ciliated) epithelial cells. Which is also the case for Newcastle disease virus infection (or vaccination with virulent vaccines) in one-day-old chickens (Mast et al., 2005), making it easier for APEC to cross the upper respiratory tract causing colibacillosis. This hypothesis is strengthened by the fact that the highest bacterial concentrations were recovered from the spleen of chickens exposed to 2% H_2O_2, followed by 1% H_2O_2 and DW. However, no statistically differences were found between the groups, due to the low number of tested animals. Histology coupes of the trachea, as also made by Mast *et al.* (2005) after vaccination of one-day-old chickens with a Newcastle disease vaccine, can give more conclusive results.

Only two concentrations of H_2O_2 were tested for its effect on mortality, lesion scores and concentration of recovered bacteria from the spleen, after exposure to APEC aerosols. Possibly lower concentrations of H_2O_2 could result in lower lesion scores and bacterial

concentrations recovered from the spleen. However, lower concentrations were not tested, since only three isolators were available. Due to time restrictions a second experiment could not be performed. For future studies it may be useful to test lower concentrations. As toxicity was also shown to be dependent on exposure time and number of exposures (Anonymous, 2003), these parameters should also be taken into consideration.

The presented results give a good indication that nebulization of H_2O_2 (higher than 1%) in the presence of chickens to reduce infection pressure is not advisable. However, such concentrations can be used in an experimental respiratory infection model to predispose the chickens to APEC infection.

Chapter V

Bioluminescent avian pathogenic *Escherichia coli* for monitoring colibacillosis in experimentally infected chickens

Abstract

In this chapter, an APEC strain was marked with the luciferase operon (*LuxCDABE*), using a Tn7 transposon, to be able to follow the pathogenesis of APEC infection.

The sensitivity of the strain was tested for specific phages and an experimental infection was executed to determine its virulence. Organs of experimentally infected chickens were tested for a correlation between luminescence and the concentration of luminescent bacteria.

The transposition of the *lux* operon into the chromosome of the APEC isolate did not affect the virulence of the latter strain nor its sensitivity for lytic phages. A correlation between the number of bacteria and the luminescent signal was found *in vitro* in liquid medium, as well as in the homogenized heart, liver and lung of four-week-old experimentally infected chickens. The transposition of *lux* was furthermore useful for identification of the infecting strain after homogenization and plating of organs from experimentally infected chickens.

1. Introduction

Different reporter systems are available for monitoring bacterial cells *in vivo* and *ex vivo*, of which green fluorescent protein (GFP) and its derivatives, bioluminescent enzyme firefly luciferase (Luc) and bacterial luciferase (Lux) are some examples. The bacterial luciferase operon (*lux*), however, is the only reporter system that is able to synthesize from endogenous material all the substrates required for the production of light (Engebrecht et al., 1983).

Therefore actively growing cells are necessary, since the functioning of *lux* depends on oxygen, reduced flavin mononucleotide and aldehyde co-substrates. The luciferase reaction of *Photorhabdus luminescens* has a temperature optimum of 40ºC, far higher than other identified luciferases (Colepicolo et al., 1989). This temperature is close to the core body temperature of chickens (42ºC), and is therefore an interesting reporter system for monitoring bacterial infections in these animals. The complete gene operon, consisting of five genes (*luxCDABE*), was successfully expressed in host organisms such as *E. coli*, *Salmonella* and optimized for Gram-positive bacteria such as *Staphylococcus aureus* and even eukaryotic cells (Engebrecht et al., 1983; Francis et al., 2000; Gupta et al., 2003; Howe et al., 2010). *Lux* labelled *Staphylococcus aureus* infection in mice could be monitored in real-time, allowing the determination of treatment effects *in vivo* (Francis et al., 2000). Therefore, two strains of luminescent *Staphylococcus aureus* were injected into the thigh muscles of mice, followed by either no treatment (control) or treatment of the mice with amoxicillin. Bioluminescence from the bacteria in the muscles of the mice was followed using a photon-counting intensified

charge-coupled device camera. No signal could be detected anymore in the treated mice 24 h post infection, while the untreated groups showed strong bioluminescent signals. A correlation between the luminescent bacteria in the thigh muscles and the luminescent signal was found.

In this chapter, the bacterial luciferase operon *luxCDABE* was chromosomally integrated in an APEC strain, using a Tn7 transposon. The detection of the luminescent signal and the correlation between the luminescent signal and the concentration of bacteria in the organs was optimized to quantify bacteria and monitor the effects of treatment on colibacillosis.

2. Material and methods

2.1. Bacterial strains, phages and media

APEC strain CH2 (O78) had been isolated from the heart of a diseased chicken with *E. coli* septicemia (van den Bosch et al., 1993) and was shown to be virulent in chickens (Vandemaele et al., 2005). Plasmid pBEN276 (kindly provided by Prof. Dr. Nancy Craig, the Johns Hopkins University and the Howard Hughes Medical Institute, USA) was used to chromosomally integrate the *lux* operon CDABE into CH2, according to a slightly different protocol as described before (Howe et al., 2010). Non-pathogenic *E. coli* strain BL21 was used as a non-pathogenic control strain (Chart et al., 2000). The phages used during this study were: PhAPEC2, PhAPEC5, PhAPEC7 and PhAPEC9. They were isolated from water samples, collected from rivers and brooks in and around Brussels (Belgium) in the vicinity of poultry houses between the year 2000 - 2010 (Tsonos, 2014). They were all able to lyse model strain CH2. Media and growth conditions were as mentioned in chapter II and III, unless stated otherwise. Ampicillin was used for recombinant selection and added at a concentration of 100 µg/ml.

2.2. Construction of CH2-lux

Electrocompetent cells were prepared as described by Sambrook *et al*. (1989).

Electrocompetent CH2 cells were transformed with 1 µl pBEN276 by electroporation with a Bio-Rad® Gene Pulser Xcell™ (Bio-Rad Laboratories N.V.) at 1.7 kV with 25 µF and 200 Ω. Cells were resuspended in freshly prepared super optimal broth with catabolite respression (SOC) medium (Sambrook et al., 1989) and grown for 1 h at 30ºC for recovery, whereafter the culture was streaked onto ampicillin containing LB agar. Plates were incubated at 30ºC for 18 h and resistant colonies were picked to perform a colony PCR for the confirmation of a successful transformation with pBEN276.

Fresh colonies were picked from LB agar, resuspended in 2 µl sterile Milli-Q water. PCR to confirm the presence of the *lux* operon was performed using the following reaction mix: 2.1 µl buffer, 1.6 µl dNTPs (2.5 mM), 1 µl forward and reverse primer (LuxA-F and LuxA-R, see Table 5.1) (10 µM), 0.1 µl Taq enzyme (5 u/µl; SphaeroQ, Leiden, the Netherlands) and 12.2 µl Milli-Q water. PCR conditions were: 94°C for 5 min (initial denaturation), followed by 35 cycles of [94°C for 30 s (denaturation), 57.5°C for 1 min (annealing) and 72°C for 1 min (elongation)], followed by a final elongation at 72°C for 5 min. PCR products were visualized on a 2% agarose gel with Midori Green Advance (1 µl / 20 ml agarose gel; NIPPON Genetics EUROPE GmbH, Dueren, Germany) by transillumination with UV light (302 nm).

Colonies were resuspended in LB medium with various arabinose concentrations (0.01 – 0.5%) and incubated for 16 h at 30ºC to induce transposition. The cultures were streaked onto LB agar and incubated at 42ºC for 16 h to prevent replication of the temperature-sensitive plasmid.

Colonies were picked, resuspended in LB medium and incubated at 42ºC (230 rpm) for 16 h. A PCR was conducted with site-specific primers [LuxC-F(int) and GlmS-R(int), see Table 5.1] to confirm successful transposition of *luxCDABE* into the CH2 genome. Therefore 1 µl of the overnight culture was mixed with 12 µl Milli-Q water, 15 µl KAPA2G™ Robust Hot Start Ready Mix (Sopachem, Eke, Belgium) and 1 µl forward and reverse primer (10 µM). The following conditions were used: 95°C for 10 min (initial denaturation), followed by 30 cycles of [95°C for 15 s (denaturation), 60°C for 15 s (annealing) and 72°C for 15 s (elongation)]. PCR products were visualized on a 2% agarose gel with Midori Green Advance by transillumination with UV light. The products were purified with the GENECLEAN® Kit (MP Biomedicals, Illkirch Cedex, France) according to manufacture instructions.

Table 5.1. Oligonucleotides used in this chapter

Name	Sequence (5' to 3')	Size (bp)
LuxA-F	TGGTTACTGGAGCATCATTTCAC	928
LuxA-R	GCATGACATCAGACTGGAAGAG	
LuxC-F(int)	ACCTGGCAAAATCGGTAACG	884
GlmS-R(int)	ACCTGGCAAAATCGGTAACG	

The purified fragments were sequenced using 3.5 µl PCR-product (100 ng/µl), 3 µl primer (1 µM), 1 µ Big Dye buffer and 3 µl Big Dye Master Mix (Life Technologies Europe B.V., Gent, Belgium) PCR conditions were: 96°C for 1 min (initial denaturation), followed by 30 cycles of [96°C for 30 s (denaturation), 50°C for 15 s (annealing) and 60°C for 1 min (elongation)]. The fragments were purified with ethanol precipitation. The pellet was air-dried, dissolved in 10 µl HiDi^{TM} Formamide (Life Technologies Europe B.V.) and sequenced (ABI PRISM 3100 Genetic Analyzer, Life Technologies Europe B.V.).
After confirmation of the correct integration of *luxCDABE* in CH2 by aligning the sequence with BLASTN (http://blast.ncbi.nlm.nih.gov) (Altschul et al., 1997), a -80ºC stock culture was made in 15% glycerol.

2.3. Time-lapse of bioluminescence after spiking of homogenized organs with CH2-lux and bacteriophages

The time-lapse of bioluminescence in homogenized organs (2 g in 2 ml PBS) of four-week-old BL21 infected chickens (see below) was followed after spiking with CH2-lux (10^9cfu in 200 µl PBS; t = 0 min) and before and after spiking with CH2-specific lytic phages (10^9 plaques forming units (pfu) of all four phages (PhAPEC2, PhAPEC5, PhAPEC7 and PhAPEC9) in 200 µl PBS; t = 50 min). The luminescence was analyzed on an IVIS Spectrum imaging system (PerkinElmer) using Living Image software 4.3 (PerkinElmer) for a period of 3 h (measurements with an interval of 5 min). Each individual well containing the homogenized organs was manually selected as a region of interest (ROI). The bioluminescent signal was measured in photons per second per square centimeter per steradian (p/s/cm^2/sr).

2.4. Correlation between CH2-lux concentration and bioluminescent signal

A fresh overnight culture of CH2-lux was diluted 1: 100 in LB medium, grown to exponential phase (OD ≈ 0.5 - 0.6) and spun down for 15 min at 2,003 × g (4ºC). The pellet was resuspended in LB and two-fold dilutions were made in triplicate starting at 6.0 × 10^7 cfu in 200 µl LB, whereafter they were added to a black polystyrene microplate with clear bottom (Pittsburgh Corning Europe, S.A.). The negative control consisted of plain LB medium. Luminescence was measured at 37ºC with the IVIS Spectrum imaging system. Each individual well with luminescent bacteria was manually selected as a ROI. The bioluminescent signal was measured in p/s/cm^2/sr.

2.5. Bacteriophage sensitivity of CH2-lux

CH2-lux was compared with CH2 for its sensitivity to the four specific phages. Ten-fold dilutions (20 µl) of the phages were brought on a double agar plate (Adams, 1959) with, respectively, CH2-lux and CH2. After incubation for 16 h, the concentration of phages was determined (pfu/ml).

2.6. Virulence of CH2-lux

To verify whether the virulence of CH2-lux was not affected by *lux*, different animal infection experiments were performed that were approved by the Ethical Committee of the KU Leuven (P176/2011). Fifty-five one-day-old chickens (Ross, Belgabroed NV) were housed in a disinfected barn, randomly divided over different groups and provided with feed and water *ad libitum*.

2.6.1. One-day-old chickens

Ten one-day-old chickens were subcutaneously infected (200 µl 10^7 cfu/ml) with either CH2-lux or BL21 (200 µl 10^7 cfu/ml). At this concentration, CH2 kills 100% of the infected chicks (Tuntufye et al., 2013), but was not tested to avoid killing of more chicks. Mortalities were monitored till seven days post infection (dpi), to determine the virulence of CH2 after the integration of *lux*.

Organs of chicks that had acutely died or been euthanized 7 dpi, were aseptically removed and were scanned for bioluminescence in a petri dish (spleen, 1/2 lever, lung, heart), using the IVIS Spectrum imaging system. The organs were placed in petri dishes and the dishes were sealed with Parafilm® in order to prevent contamination of the facility where the camera was located. Thereafter the organs were homogenized in PBS (1 g organ in 1 ml PBS) and dilutions were plated on LB agar (Tuntufye et al., 2012a). After incubation for 16 h, the colonies were checked for luminescence using IVIS and concentrations were determined (cfu/g).

2.6.2. Four-week-old chickens

Forty-five four-week-old chickens were divided over nine groups that consisted of five chickens each. The chickens were housed in the same room, but in different cages. In between the cages there was a space of 20 cm, so that the chickens were not in direct contact with each other. Chickens were intratracheally infected (with either 10^6, 10^7, 10^8, or 10^9 cfu of CH2-lux or CH2 in 500 µl PBS), as previously described (Tuntufye et al., 2012a). The five chickens in the control groups were infected with BL21 (10^9 cfu in 500 µl PBS). Animals were monitored

over a period of 9 dpi, mortalities were scored and chickens that died or were euthanized prior to termination of the experiment were necropsied and scored according to the score system of Vandemaele *et al*. (2005).

2.7. Optimization of bioluminescent signal and detection limit

Homogenized organs from four-week-old chickens (2 g organ in 2 ml PBS) were transferred to a 12-well plate, after which the organs were spiked with ten-fold dilutions (10^7 to 10^3 cfu) of an exponentially grown CH2-lux culture in LB medium to determine the detection limit of luminescence. Each individual well containing the homogenized organs was manually selected as a ROI.

The homogenized liver and spleen from one CH2-lux infected chicken (10^6 cfu) were diluted to determine the effect of dilution on the detectable luciferase signal. Therefore 1 ml of homogenized liver and spleen was transferred to a 12-well plate, organ homogenates were serially diluted in PBS using two-fold dilution steps. The bioluminescence was measured and the increase in detectable luciferase activity was determined.

3. Results

3.1. Construction of CH2-lux

All colonies that grew after electroporation on the ampicillin containing LB agar plate were successfully transformed with pBEN276 (confirmed with PCR). These colonies were resuspended in LB medium containing various arabinose concentrations and streaked onto LB agar. Transposition of the *lux* operon into the *att*Tn*7* site of the CH2 chromosome was confirmed by PCR and the PCR products of one colony were sequenced and aligned, which confirmed the transposition of the *lux* operon into the *att*Tn*7* site (downstream of the *glmS* gene) of the CH2 chromosome.

3.2. Time-lapse of bioluminescence after spiking of homogenized organs with CH2-lux and bacteriophages

After spiking the homogenized organs with CH2-lux (10^9 cfu) there was a clear increase in luminescence from 7.1×10^2 - 1.4×10^3 p/s/cm^2/sr to 7.4×10^4 - 3.2×10^5 p/s/cm^2/sr, while a clear reduction in luminescence could be seen after spiking with the phage cocktail from about 3.0×10^5 p/s/cm^2/sr to 1.5×10^4 p/s/cm^2/sr for the heart and liver about 2.5 h after spiking (t = 180 min). For the lung, the luminescence decreased from 7.4×10^4 to 2.3×10^3 p/s/cm^2/sr and for the spleen from 1.9×10^5 to 5.6×10^3 p/s/cm^2/sr (see Figure 5.1).

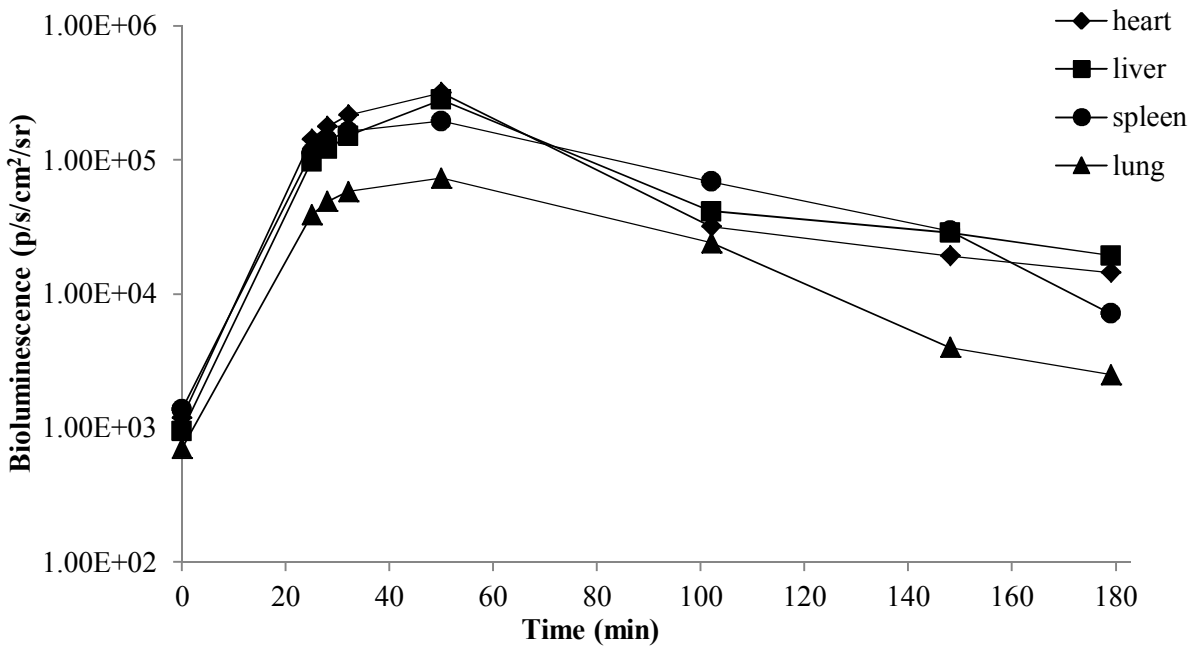

Figure 5.1. Time-laps of luminescence of homogenized organs after spiking with CH2-lux and before and after spiking with bacteriophages in a 6-well plate. CH2-lux (10^9 cfu) was added at t = 0 min, while the bacteriophages (10^9 pfu of every bacteriophage) were added at t = 50 min. Heart (diamonds), liver (squares), spleen (circles) and lung (triangles).

3.3. Correlation between concentration CH2-lux and bioluminescent signal

A clear linear correlation could be found between the concentration CH2-lux bacteria and bioluminescence in LB medium (R^2 = 0.98) (see Figure 5.2). The minimum detectable concentration of luminescence was 3.2×10^4 cfu in 200 µl LB medium.

3.4. Bacteriophage sensitivity of CH2-lux

No significant differences were found in phage concentration (PhAPEC2, PhAPEC5, PhAPEC7 and PhAPEC9) between amplification on CH2-lux and CH2 (data not shown), which confirms that CH2 after transposition with *lux* remains equally sensitive to the tested bacteriophages.

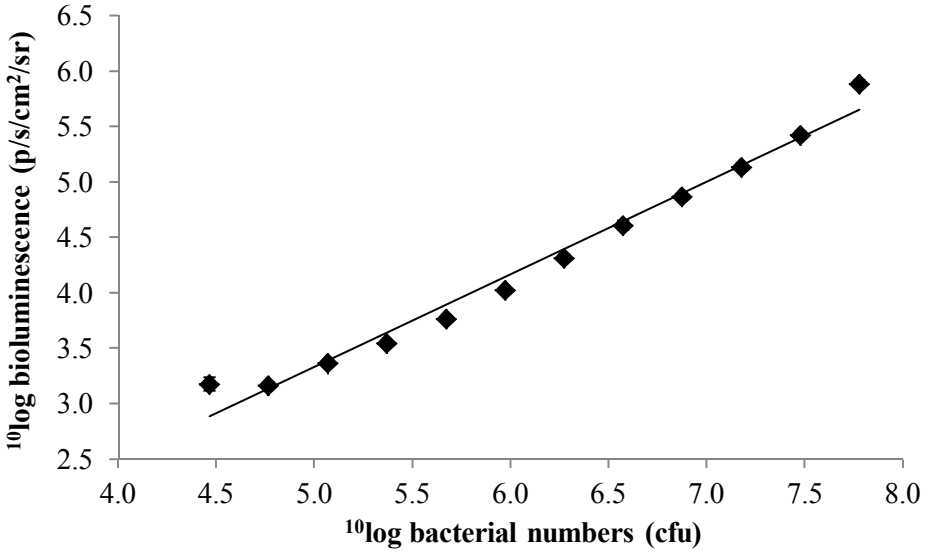

Figure 5.2. Luminescent signal in relation to concentration CH2-lux. Luminescent signal in relation to concentration CH2-lux. ^{10}Log values of bacterial numbers relative to ^{10}log bioluminescent units are graphed. Average values and standard deviation of three measurements are shown. $R^2 = 0.98$.

3.5. Virulence of CH2-lux

3.5.1. One-day-old chickens

Three out of five CH2-lux infected chickens died, while none of the BL21 infected chickens died, suggesting that the strain is still virulent after the transposition of the *lux* operon. The organs of the two chickens in the CH2-lux infected group that survived the infection, were not luminescent and no luminescent colonies were found after homogenization and plating. One chicken in the BL21 group showed clinical signs of APEC infection and although no luminescence of the organs was observed, luminescent colonies were detected after homogenization and plating (1.9×10^3; 8.0×10^1; 1.7×10^3 and 8.7×10^2 cfu/g in the lung, spleen, liver and heart, respectively). Since the chickens were housed together it is plausible that contamination between the chickens took place.

Many non-luminescent colonies were detected, after plating of the homogenized organs from the chickens that died or showed clinical signs of infection, especially in the lungs.

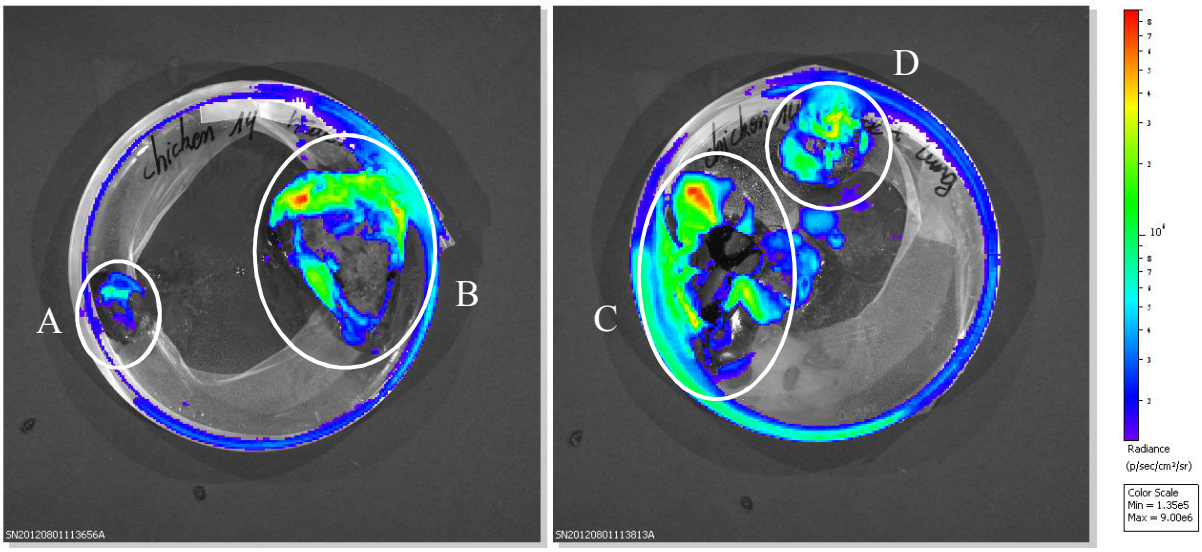

Figure 5.3. Luminescence of organs of a four-week-old lux-infected chicken (infected with 10^7 cfu). A) spleen, B) heart, C) liver, D) lung.

3.5.2. Four-week-old chickens

All chickens infected with CH2-lux (10^9 cfu) died, while one out of five chickens survived in the CH2 group (10^9 cfu). One chicken in the groups infected with 10^7 and 10^8 cfu (CH2-lux and CH2) survived, as did three out of five chickens in the 10^6 cfu infected groups. All chickens that died or were euthanized prior to termination of the experiment showed macroscopic lesions typical of systemic APEC infection, including airsacculitis, pneumonia, pericarditis and perihepatitis and got the maximum lesion score of 14. All chickens that were euthanized at termination of the experiment (9 dpi) did not show clinical signs of APEC. None of the chickens infected with BL21 died, nor showed clinical symptoms of APEC infection and no macroscopic lesions were observed during necropsy at termination of the experiment. The results indicate that CH2 remained equally virulent after *lux* transposition.

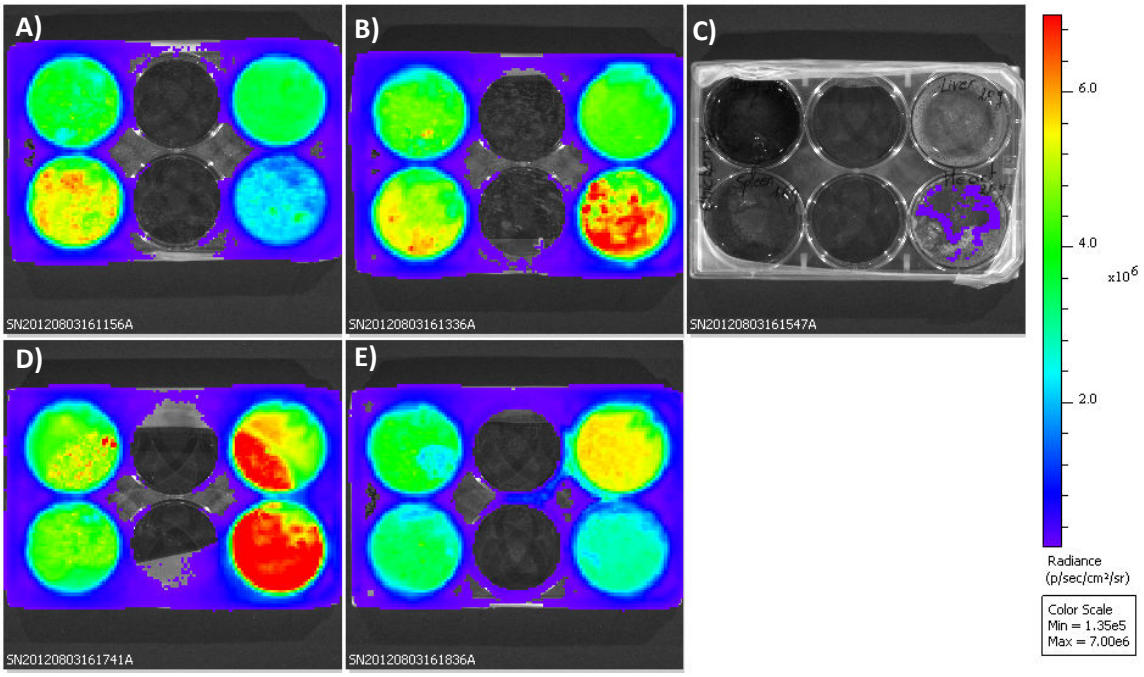

Figure 5.4. Luminescence of homogenized organs (2 g) in 6-well plates from four-week-old CH2-lux infected chickens. Upper left: lung, upper right: liver, lower left: spleen, lower right: heart. Chickens euthanized: C (infected with 10^8 cfu); chickens that acutely died: A (infected with 10^9 cfu), B (infected with 10^7 cfu), D (infected with 10^6 cfu), E (infected with 10^9 cfu).

3.6. Optimization of bioluminescent signal and detection limit

The organs of the four-week-old chickens that died showed clear bioluminescence, although the signal was not equally distributed over the organs (Figure 5.3). Furthermore, it was difficult to homogenize the whole heart or lung of four-week-old chickens making a correlation between the luminescence and the concentration of CH2-lux bacteria not possible. Therefore, a piece of 2 g of the organs was taken, homogenized (in 2 ml PBS) and transferred to a 6-well plate in order to have a more equal distribution of the luminescence, to find a better correlation (see Figure 5.4). An evenly distributed signal was found, but chickens that acutely died showed a higher signal than euthanized chickens. A correlation could be found between bioluminescence and cfu for the heart ($R^2 = 0.89$), liver ($R^2 = 0.93$) and lung ($R^2 = 0.89$) (Figure 5.5), while no correlation could be found for the spleen ($R^2 = 0.58$). After plating ten-fold dilutions of the homogenized organs from CH2-lux infected chickens on LB-agar many non-luminescent colonies were found, especially in the lungs. The concentration of luminescent bacteria (cfu/g) was determined.

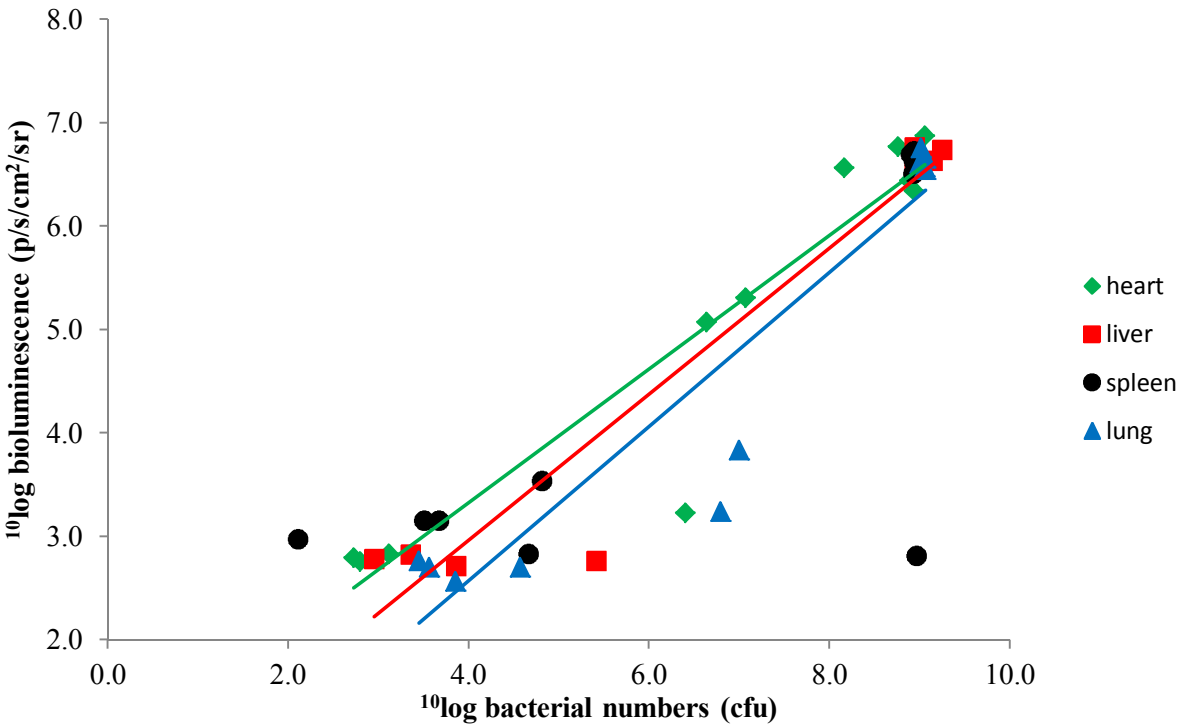

Figure 5.5. Correlation between e*x vivo* luminescence of heart, liver, spleen and lung and concentration of spiked bacteria in the organs. ^{10}Log values of bacterial numbers relative to *ex vivo* ^{10}log bioluminescent units are graphed. $R^2 = 0.89$ for the heart, $R^2 = 0.93$ for liver and $R^2 = 0.89$ for the lung.

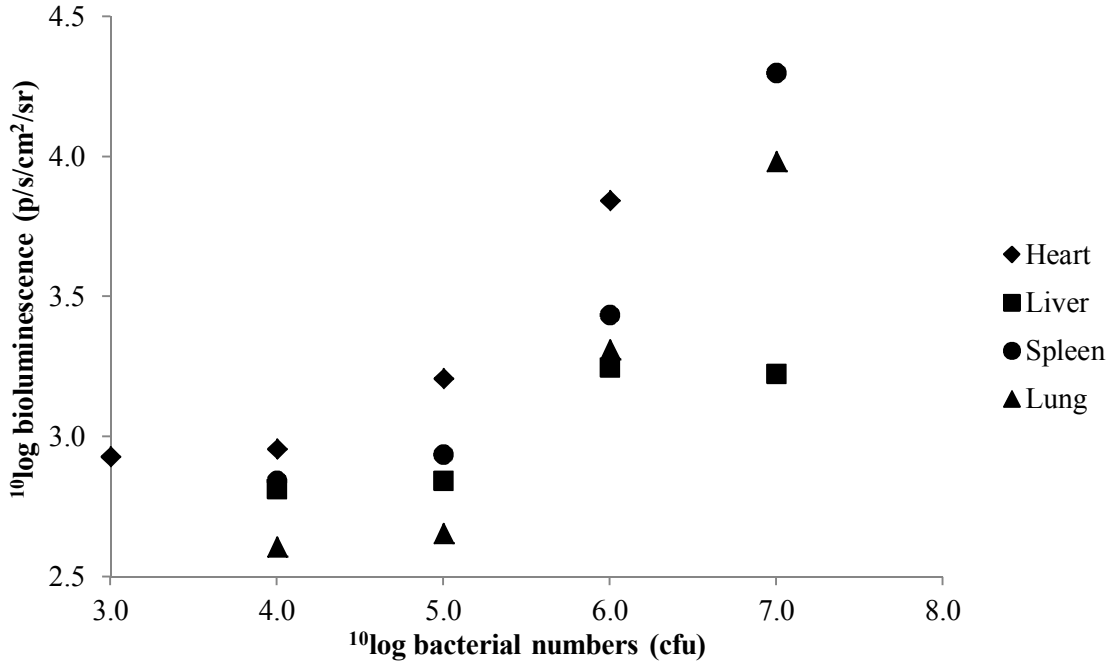

Figure 5.6. Detection limits of luminescence (^{10}log) after spiking with ten-fold dilutions of CH2-lux to homogenized organs of four-week-old chickens. Heart (diamonds), liver (squares), spleen (circles) and lung (triangles) are shown in relation to bioluminescence units.

Detection limits of luminescence in homogenized organs, spiked with ten-fold dilutions of an exponentially grown CH2-lux culture in LB medium, are shown in Figure 5.6. The detection limit of the luminescence in the heart was between 10^4 - 10^5 cfu, while 10^5 - 10^6, 10^5 - 10^6 and > 10^7cfu were found for the spleen, lung and liver, respectively.

In order to lower the luminescence detection limit in the spleen and liver, these organs of a four-week-old lux-infected chicken were diluted with PBS. Diluting the spleen and liver homogenate with a factor 8 resulted in a 4.84 and 8.79 increase of detectable bioluminescence activity, respectively (see Table 5.2).

Table 5.2. Effect of dilution of homogenized spleen and liver on detectable luciferase activity

Organ[A]	Dilution factor	Bioluminescence (p/s/cm^2/sr)	Increase of signal
Spleen	Undiluted	3.12E+07	
	2	2.54E+07	1.63
	4	2.37E+07	3.04
	8	1.51E+07	4.84
Liver	Undiluted	2.89E+07	
	2	2.85E+07	1.97
	4	2.68E+07	3.71
	8	2.54E+07	8.79

[A] Homogenized liver and spleen from a CH2-lux infected chicken (10^6 cfu)

4. Discussion

The *lux* operon was successfully chromosomally integrated in APEC isolate CH2, to monitor colibacillosis in four-week-old experimentally infected chickens.

To monitor infection, bacteria can be transformed with various reporters systems. This reporter can be for example a modified *gfp* gene (Pinheiro et al., 2008), but a *lux* operon has more advantages, since it can besides the identification of the infecting strain also be used to perform quantification of bacteria. Furthermore, fluorescent markers are known for their high background levels and poor penetration of light (excitation and emission) into tissue. The *lux*

operon produces light autonomously and background levels are much lower, offering a system with higher specificity and sensitivity (Cronin et al., 2012).

To follow-up bacteria, they can be transformed with plasmids containing a reporter gene. However, these plasmids normally contain a selective marker gene, such as an antibiotic resistance gene. Therefore these strains only remain fluorescent/luminescent when grown under selective pressure, without losing the plasmid (Pinheiro et al., 2008). Ma *et al.* (2011) detected good stability of an *E. coli* strain transformed with a GFP plasmid, although stability depended on labeled species and strain. Hence, integration of the reporter system into a neutral position in the genome is a better option for stable expression of the reporter. During this study, the luciferase operon was specifically integrated into the *att*Tn7 site in the genome of CH2, not affecting its virulence nor its susceptibility for phage lysis. Furthermore after integration into the chromosome, the *lux* operon is stable without selective pressure (Howe et al., 2010).

As explained in the introduction, Francis *et al*. (2000) used luminescent *Staphylococcus aureus* bacteria that were injected into the thigh of mice. They were able to find a good correlation between the number of fluorescent bacteria re-isolated from the infected tissue and the luminescence of the corresponding area. Cronin *et al*. (2012) was also able to find a good correlation between bioluminescence and the concentration of bacteria recovered from xenograft tumours in mice. The mice were intravenously injected with *lux* expressing non-pathogenic bacteria, since these bacteria form an important vector for cancer treatment, due to their preference to grown within tumours. In our study, however, *in vivo* monitoring of APEC infection could not be performed, since we worked with larger animals (four-week-old chickens). Therefore, *ex vivo* scanning of the organs had to be performed on four-week-old chickens, an age at which they are more prone to airsacculitis (Dho-Moulin and Fairbrother, 1999). For the one-day-old chickens however the system on whole bodies was useful.

Correlations were detected between bioluminescence and concentration of CH2-lux bacteria in the homogenized heart, liver and lung of CH2-lux infected chickens, offering a method to monitor the effects of different treatments for colibacillosis. Since APEC often starts as a localized infection of the air sacs and lungs (Dho-Moulin and Fairbrother, 1999; Gross, 1994), this model can be used to determine the effect of treatment with for example bacteriophages (as used during this study) or antibiotics. During our study, we tested the capacity of phages to lyse CH2-lux *ex vivo* in the homogenized organs of chickens by measuring the

bioluminescence (Figure 5.1). However, organs that were spiked with CH2-lux, but not with phages were not included. Therefore, we cannot conclude with certainty that the phages were able to lyse CH2-lux, however, there is a strong indication as the bioluminescence clearly decreased after the addition of the phages. Phage therapy is an early applied treatment being rediscovered, resulting from an increase in antibiotic resistant pathogenic strains. It is, however, confronted to many hurdles, among which the discrepancy between an *in vitro* and *in vivo* environment (Tsonos et al., 2014). The here developed technique allows a rapid and better evaluation of the *in vivo* efficiency of bacteriophages. The labelled strain also has great advantages in *in vitro* cell culture techniques to expand knowledge on the pathogenesis of the disease. The strain can for example be added to a monolayer of chicken tracheal epithelial cells in order to easily detect under what circumstances APEC can cross the epithelial cells. Furthermore, it can be used to infect young chickens in order to follow up adhesion, internalization, organ localization, invasion and persistence of the labelled strain via real-time imaging, which might provide help in unraveling the pathogenesis.

A correlation could not be detected between luminescence and concentration of CH2-lux bacteria for the spleen, probably due to a higher percentage of blood (haemoglobin) in the spleen as compared to the other tested organs. Furthermore, detection limits for luciferase in the spleen and liver were high, 10^5 - 10^6 and $>10^7$ cfu, respectively, and could be decreased when homogenized organs were diluted, as previously shown by Colin *et al*. (2000) for the North American firefly luciferase. This can be explained by a decrease of absorbance of the luminescent signal by haemoglobin.

In conclusion, we were successful in developing an APEC strain with an integrated bacterial luciferase operon without interfering with its virulence nor its susceptibility to phage lysis. Transposition of the *lux* operon into the chromosome of the challenge strain appeared useful for identification of the infecting strain after experimental infection of the chickens. Furthermore the system can be used for quantification of the infecting strain in the homogenized heart, liver and lung of four-week-old experimentally infected chickens in order to monitor colibacillosis and treatment effects.

Chapter VI

A cocktail of *in vitro* efficient phages is not a guarantee for *in vivo* therapeutic results against avian colibacillosis

Adapted from:
Tsonos*, J., Oosterik*, L.H., Tuntufye, H.N., Klumpp, J., Butaye, P., De Greve, H., Hernalsteens, J.-P., Lavigne, R., Goddeeris, B.M. 2014. A cocktail of *in vitro* efficient phages is not a guarantee for *in vivo* therapeutic results against avian colibacillosis. Veterinary Microbiology, 171 (3-4), 470-479.
* shared first authorship

Abstract

In this chapter, a cocktail of four different APEC-specific phages was composed and tested to cure APEC-infected chickens. Specific phages were selected from a collection of phages isolated in Belgium. The selection was based on their obligate lytic infection cycle, a broad host range, low cross-resistance and low frequency of development of resistant APEC mutants. Genome analysis of the phages indicated they were close relatives of T4 and N4, considered to be safe *in vivo*. Chickens were intratracheally infected with APEC strain CH2 (serogroup O78), causing a mortality of about 50% during the seven days following the infection. The phage cocktail was administered 2 h after the infection, via three different ways: intratracheally, intra-esophageally or via the drinking water. Treated groups did not show a significant decrease in mortality, lesion scores or weight loss compared to untreated groups, although the APEC-specific phages could be re-isolated from the lung and heart of chickens that were euthanized. Moreover, the re-isolated bacteria from infected chickens had remained sensitive to the phage cocktail. The results in this chapter indicate that the efficiency of the phage cocktail used in treating CH2-infected chickens *in vivo* is negligible, even though *in vitro*, the phages in the cocktail were able to efficiently lyse the APEC strain CH2. Our results emphasize that the 'traditional' pathway of isolation, followed by phenotypical and genotypical characterization of phages composing the cocktail, does not lead to success in phage therapy in all cases.

The phage isolation, amplification, concentration, purification, selection and identification of phages composing the cocktail as well as the phage characterization, whole genome analysis and phage cocktail preparation was conducted by Tsonos (Tsonos, 2014). The stability testing of the phage cocktail, *in vivo* testing of the phage cocktail, bacterial and phage re-isolation and resistance testing of re-isolated bacteria against phages was performed by Oosterik and Tsonos.

1. Introduction

Phage therapy is considered to be an effective alternative for antibiotics (Summers, 2001). Phages have many advantages over antibiotics, such as being ubiquitous in nature, even residing in the gastrointestinal tract of animals and humans and are therefore generally considered as safe for application to humans and animals (Johnson, 2008). Obligate lytic phages are self-replicating and at the same time self-limiting, meaning that when host cells are multiplying, the number of phages will amplify as well. When the host is eliminated, the

phages will also be eliminated (Johnson, 2008). Effective phage therapy reduces bacterial numbers, so that the immune response of the host can take over and eliminate the remaining infectious bacteria (Levin and Bull, 2004).

Several reports have shown the successful treatment of APEC in experimentally or naturally infected chickens using phage therapy (Huff et al., 2004; Lau et al., 2010; Oliveira et al., 2010). Oral treatment in a mouse model of intestinal carriage was able to successfully clear *E. coli* O157:H7 from mice (Sheng et al., 2006).

In this chapter, the efficiency of a phage cocktail against APEC was tested in experimentally infected chickens by administration of phages *via* the trachea, the esophagus or the drinking water.

2. Material and methods

2.1. Bacterial strains and media

In total 31 APEC strains were used to determine the host range of the phages. The sensitivity of six more O78 APEC strains was tested against the suspected O78-specific phages. All these strains were isolated from colibacillosis-affected flocks. They belonged to serogroup O78 (n = 16), O2 (n = 7), O18 (n = 2), O83 (n = 1), O88 (n = 1), O53 (n = 1), O45 (n = 1) and O115 (n = 1); seven strains were non-typable (Vandekerchove et al., 2005). The *E. coli* K-12 laboratory strains used in this chapter were: MG1655 (Blattner et al., 1997), C600 (Appleyard, 1954) and K514 (Wood, 1966). Of the tested APEC strains, CH2 (O78) was used in the infection model (see Chapter IV and V). Media and growth conditions are as mentioned in chapter II and III, unless stated otherwise.

2.2. Phage isolation and amplification

Eighty-nine phages were isolated from water samples, collected from rivers and brooks in and around Brussels (Belgium) in the vicinity of poultry houses between the year 2000 and 2010. Water samples were filtered through a 0.22 µm membrane (Millex® Syringe Filters; Millipore Corporation, Billerica, MA, USA) before plating 1 ml on different bacterial APEC strains by the double agar overlay method (0.7% agar), as previously described (Adams, 1959). These indicator APEC strains belonged generally to the most spread serogroups, namely O1, O2 and O78. Some phages could only be isolated after enrichment of the water sample by addition of LB (10%) and an overnight culture of the indicator APEC (1%). After overnight incubation, the enriched water samples were centrifuged (6120 × g; 10 min). Individual plaques could be obtained by applying the double agar overlay method on serial ten-fold sample dilutions. The

plaques were plaque-purified twice and amplified.

2.3. Phage concentration and purification

Phages were concentrated by polyethylene glycol (PEG) precipitation. A solution consisting of 33% PEG (wt/vol) (molecular weight of 8000; Acros Organics, Geel, Belgium) and 1.3 M NaCl (Acros Organics) was added in a 1: 5 proportion to the lysates. After overnight incubation on ice, the phage lysates were centrifuged (12,500 × *g*; 20 min). The precipitate was resuspended in 10 mM Tris-HCl buffer (pH 8) (Sigma-Aldrich).

Purification of the phage lysates was performed by continuous CsCl gradient centrifugation. Concentrated stocks of individual phages were brought in an aqueous CsCl-solution (UltraPure™ Cesium Chloride; Gibco®, Porto, Portugal) with a total density of 1.5. Overnight ultracentrifugation (150,000 × *g*) was performed and the formed opalescent band was extracted with a syringe. Each ml extracted volume was dialyzed against 0.5 l medium composed of 20 mM Tris-HCl (pH 8), 50 mM NaCl and 10 mM $MgCl_2$, using a membrane with Molecular Weight Cut off between 12,000 - 14,000 Daltons (Spectra/Por 4; Spectrum Laboratories, Breda, the Netherlands).

2.4. Selection and identification of phages composing the cocktail

2.4.1. Host range

The host range of each phage was determined against 31 serotyped APEC strains (see above). Bacterial lawns of each strain were made using the double agar overlay method, on which 20 µl droplets of the phage stocks (diluted to 10^6 pfu/ml) were applied. After overnight incubation, the degree of lysis of the lawns was determined.

2.4.2. Virulence of the phages

The virulent nature of the phages was confirmed by showing that the corresponding phage-resistant bacterial variants were not lysogenic. Resistant variants were obtained by adding 100 µl overnight APEC culture and 100 µl of phage lysate (≈10^9 pfu/ml) in the top agar of a double agar plate. After overnight incubation, resistant colonies were purified twice on LB agar plates. The resistance of the colonies was further confirmed by streaking each colony with a loop perpendicularly over a phage line (cross-streaking). Validated colonies were cultured in LB. Fifty µl chloroform was added to 1 ml of this overnight culture and kept at 4ºC for 45 min. After centrifugation at 20,000 × *g* for 5 min, droplets of 20 µl were brought on a double agar plate containing the corresponding sensitive APEC strain.

2.4.3. Transmission electron microscopy

Undialyzed purified phage dilutions were spotted on carbon coated grids (Quantifoil, Großlöbichau, Germany) after glow-discharge and negatively stained with 2% uranyl acetate. A Philips CM12 microscope was used at 120 kV acceleration voltage. Images were produced using a Gatan Orius 1k camera. The dimensions were measured using Gatan Digital Micrograph software.

2.5. Whole genome analysis

Before phage DNA was extracted from CsCl purified and dialyzed samples by phenol-ether extraction, each ml sample was treated with 5 µl DNase I (1 u/µl; Life Technologies Europe B.V.) and 8 µl RNase A (10 mg/ml, Life Technologies Europe B.V.) and incubated for 30 min at 37ºC. An equal volume of phenol (Amresco, Solon, OH, USA) was added to the sample and mixed well. This was followed by a phase separation by centrifugation. The upper aqueous phase was brought in a new recipient and this phenolization step was repeated two more times. To remove the phenol from the sample, ether (saturated with H_2O) (Sigma-Aldrich) was added. After thorough mixing of the samples, the phases were separated by centrifugation. The ether extraction was repeated twice. DNA precipitation was performed by adding NaCl, to obtain an aqueous phase concentration of 150 mM, together with 2 volumes of 100% ethanol. The samples were centrifuged at high speed (18,000 × *g*) for 20 min, whereafter the ethanol was removed. The pellet was washed 2 times with 100% ethanol. After removing the ethanol, the pellet was dried and resuspended in 10 mM Tris buffer.

The genomes of PhAPEC2, PhAPEC5 and PhAPEC7 were sequenced with the Illumina sequencing technology at the GIGA facility (Groupe Interdisciplinaire de Génoprotéomique Appliquée, University of Liège). The genomes were *de novo* assembled with a sequence coverage of 100 fold. Potentially conflicting regions were detected during gene prediction by the occurrence of frame-shifts. In view of the high DNA identity, annotation was performed by comparative genome analysis to available type species and using Genemark^{TM} (Lukashin and Borodovsky, 1998).

2.6. Phage cocktail preparation

To make high titer stocks, an overnight liquid culture of the bacterial strain was 1: 50 diluted into 400 ml fresh LB to obtain a culture with an optical density (OD_{600}) of ≈ 0.1. The culture was grown to an OD_{600} of ≈ 0.2 in an incubator shaker (230 rpm; Innova 4200). Phage was added to the culture at a multiplicity of infection (MOI) of ≈ 1 and the culture was incubated

further for 2 h (230 rpm), after which 10 ml of chloroform was added and the culture was kept at 4ºC for 45 min. The phage lysate was centrifuged (4500 × *g*; 30 min) and filtered through a 0.45 µm membrane (Millex® Syringe Filters; Millipore Corporation). Phages were concentrated by PEG precipitation and resuspended in LB. The titer of each phage was determined the day before the *in vivo* experiment and diluted so that each dose had a total phage concentration of 10^9 pfu in 500 µl LB, containing equal individual concentrations. For the group of chickens that received the cocktail in the drinking water, a dilution in tap water was made (10^9 pfu per 4 ml of water; total volume: 150 ml).

2.7. Stability of the phage cocktail at 42ºC and in tap water at room temperature

The stability of the phages in LB medium and PBS was analyzed at 42ºC, the body temperature of healthy chickens. Samples were taken daily for 3 days. The titer was determined at each time point by plating 20 µl droplets of ten-fold dilution of the samples on a bacterial lawn of CH2.

Phage stability in tap water at RT was tested over 3 h, the time lapse given to the group of chickens to empty the given water volume. Phage lysates were diluted 1000 times in tap water and kept at RT. The phage titer was determined hourly as described above.

2.8. *In vivo* testing of the phage cocktail

This study was performed in strict accordance with the FELASA guidelines and recommendations. The animal experiment was approved by the ethical committee of the KU Leuven (Permit Number: P176/2011). The chickens were provided with feed and water *ad libitum* and every effort was made to minimize suffering. Fifty-nine one-day-old chickens (Ross; Belgabroed NV) were randomly divided into six groups and housed in a disinfected barn. Groups 1 and 2 contained 14 chickens, group 3 contained 13 chickens and the control groups 4, 5 and 6 each contained 6 chickens. At four weeks of age, the chickens in the groups 1 - 5 were weighed and intratracheally infected with 500 µl of the CH2 culture in LB ($OD_{600} \approx 0.6$) using a feeding needle (AgnTho's AB, Lidingo, Sweden), as described previously (Tuntufye et al., 2012a). Chickens in group 6 were not infected or treated and considered as negative control animals, excluding environmental effects.

Two hours after the infection, chickens in group 1 - 3 were treated with the phage cocktail. Group 1 received the cocktail intratracheally, group 2 intra-esophageally and group 3 *via* drinking water. The chickens in group 4 received 500 µl of the heat-inactivated (autoclaved) phage cocktail intratracheally and were considered as negative control animals.

Chickens in group 5 did not receive any treatment and were positive control animals for CH2 infection.

Seven dpi all surviving chickens were euthanized by cervical dislocation, weighed and necropsy was performed. Macroscopic lesions were scored according to a previously described scoring system (Vandemaele et al., 2005). Briefly, macroscopic lesions in the left and right thoracic air sac, the abdominal air sacs, the left and right lung, liver and heart were each scored from 0 to 2. Intermediate signs for APEC lesions were scored between 0.5 and 1.5. Chickens that died or were euthanized because of serious clinical APEC symptoms were given the full score (14), after confirming severe APEC lesions on necropsy. Lungs and heart were aseptically removed from chickens that were euthanized for re-isolation of phages and bacteria (as described below). Lungs and heart were chosen as local and systemic organ, respectively. Organs were not taken for re-isolation of phages and bacteria from chickens that were found dead after the infection, since uninhibited growth has taken place.

To have an indication of the bacterial load and phage presence in chickens without severe clinical signs, two randomly selected chickens from group 1 and 2, and one chicken from group 3 were sacrificed 24 h after treatment. Lesions were scored and the lung and heart were removed aseptically. The 24 h time point was chosen, since a preliminary study showed no administered phages could be re-isolated at that time point from the organs of chickens in the absence of a CH2 infection (data not shown).

2.9. Statistics

All results were analyzed with Analyze-it (Analyze-it Software Ltd.) in Microsoft Excel (2010). Medians, first and third quartiles (Q1 - Q3) were determined for the total lesion score, and lesion score per organ for every group of chickens. The Kruskal-Wallis test was used for determining the significance of the differences in lesion scores between multiple groups, and the Wilcoxon Rank Sum Test for assessing differences between each pair of groups. These tests were used since a low number of chickens was present per group and data were non-parametric. Live weight gain was determined for chickens that were still alive 7 dpi. The weight gain of the chickens that were still alive 7 dpi was tested for a normal distribution. Welch's ANOVA test was performed, since weight gain was not normally distributed, to analyze the significance of differences in weight gain between multiple groups. Weight gain between each pair of groups was tested with the Welch's *t*-test. $P \leq 0.05$ was considered significant and $0.05 < P < 0.10$ a trend toward significance.

2.10. Phage re-isolation

Heart and lungs (2 g) were homogenized in 2 ml PBS using an Ultra-Turrax T25 homogenizer with dispersing element (IKA®-Werke GmbH & Co. KG, Staufen, Germany). A 2 ml tube was filled with homogenized organ. To enable the detection of phages that are present in low numbers, the remaining material was enriched by adding 4 ml of LB together with 4 µl of CH2 overnight culture. The suspension was incubated overnight (230 rpm). The non-enriched homogenized organs and 1 ml of the enriched organ samples were centrifuged (6100 × *g*; 10 min), the supernatant was collected and 50 µl chloroform was added. Subsequently, respectively 50 µl droplets and 20 µl of ten-fold serial dilutions were plated on a bacterial lawn of CH2 and incubated overnight. An optimized multiplex PCR reaction (four specific primer pairs) was performed on single plaques, to allow the identification of phages which could still infect the CH2 strain *in vitro*. The primers were designed on unique loci within the genomes (see Table 6.1). The master mix contained 1.0 µl of each primer (20 µM), 2.0 µl of DreamTaq buffer (10x), 0.2 µl DreamTaq™ DNA polymerase (5 u/µl, Life Technologies Europe B.V.) 0.5 µl dNTPs (10 mM) and 9.3 µl sterile Milli-Q water. The following PCR conditions were used: 95ºC for 15 min (initial denaturation), 35 cycles [of 94ºC for 30 s (denaturation), 58ºC for 30 s (annealing), 72ºC for 1 min (elongation)], and 72ºC for 5 min (final elongation). The products were visualized on a 2% agarose gel.

Table 6.1. Oligonucleotides used in this chapter

Phage	Sequence (5' to 3')	Amplified fragment size (bp)	Reference
PhAPEC2	GGTAGTGCCTTCACCTTA	663	This study
	CGTGCAATGCAACCTTAC		This study
PhAPEC5	CCAGAAAGGGGATTGATG	347	This study
	GATACATGCTTGACCACC		This study
PhAPEC7	GTTCAACATGTCTCCGTC	788	This study
	GCCACCATTGGTTTGAAC		This study
PhAPEC9	GTAGGACTGGAAGCCAAG	445	This study
	GGTTCGGTGTCTTCTCG		This study

2.11. Bacterial re-isolation

Ten-fold serial dilutions of homogenized lungs and heart were plated on LB agar and lungs additionally on MacConkey agar (Oxoid CM0115; Oxoid N.V.). After 24 h of incubation, the number of cfu was determined.

2.12. Resistance of re-isolated bacteria against phages

Ten colonies were re-isolated from the heart and lungs (lung: MacConkey agar, heart: LB agar) to test resistance against the phage cocktail and individual phages. By cross-streaking, resistance of the re-isolated colony can be detected when uninhibited bacterial growth beyond the phage line is seen. To confirm the cross-streaking, spots (20 µl) of serial ten-fold dilutions of the phage cocktail and the individual phages were put on bacterial lawns of three of those re-isolated colonies per organ. As controls APEC CH2 was used (lysed by the four phages) and APEC strains APEC21 and APEC125 (lysed by none of the phages).

3. Results

3.1. Selected phages PhAPEC2, PhAPEC5, PhAPEC7 and PhAPEC9 are lytic phages suitable for a therapeutic cocktail *in vitro*

The host range is essential information for further applications. Our selection was based on a broad lytic spectrum combined with a high degree of lysis against the 31 tested APEC strains. The eight most outstanding phages were selected from the phage library. Four of these phages were able to lyse model strain CH2 and were selected to compose the cocktail. These phages were: PhAPEC2, PhAPEC5, PhAPEC7 and PhAPEC9. They were able to lyse respectively 19 out of 31, 6 out of 31, 9 out of 31 and 10 out of 31 tested APEC strains. PhAPEC2 infected APEC strains of serogroups O45 (1/1), O78 (9/10), O2 (5/7), O18 (2/2), O83 (1/1), one non-typable strain (1/7) and also *E. coli* K-12 strains. PhAPEC5, PhAPEC7 and PhAPEC9 are likely to be O78-specific phages. PhAPEC9 grew exclusively on all of 10 tested O78 strains. The lytic activity of PhAPEC5 and PhAPEC7 was tested on the 16 available O78 APEC strains, because they were able to grow exclusively on O78 strains, but did not lyse all O78 strains. PhAPEC5 could lyse 11 out of 16 O78 APEC strains and PhAPEC7, 13 out of 16. With O78 being one of the most common APEC serogroups, putative O78-specificity is a valuable characteristic (Schouler et al., 2012).

The strictly lytic character of the phages was a prerequisite for our study. All re-isolated phages turned out to be virulent. No phages were present by spontaneous induction in the

overnight cultures of the phage-resistant bacterial variants, implying that they did not become immune due to lysogeny.

Analysis of the transmission electron microscopy images suggested PhAPEC5, PhAPEC7 and PhAPEC9 to be *Podoviridae* related to coliphage N4, whereas PhAPEC2 is a member of the *Myoviridae* with a T4-type morphology (Figure 6.1). The T4-like phages have already been described as 'safe' in numerous phage therapy applications (Brüssow, 2005). The N4-like phages are also favored for this purpose and are also part of commercial mixtures (Krylov et al., 2013). The sizes of the phage heads were in agreement with the sizes of the corresponding sequenced genomes; the N4-like phages had a genome size approximating 71 kb with head dimensions of 65 nm and 70 nm. The T4-type phage PhAPEC2 had a head dimension of 83 nm and a genome size of 167 kb.

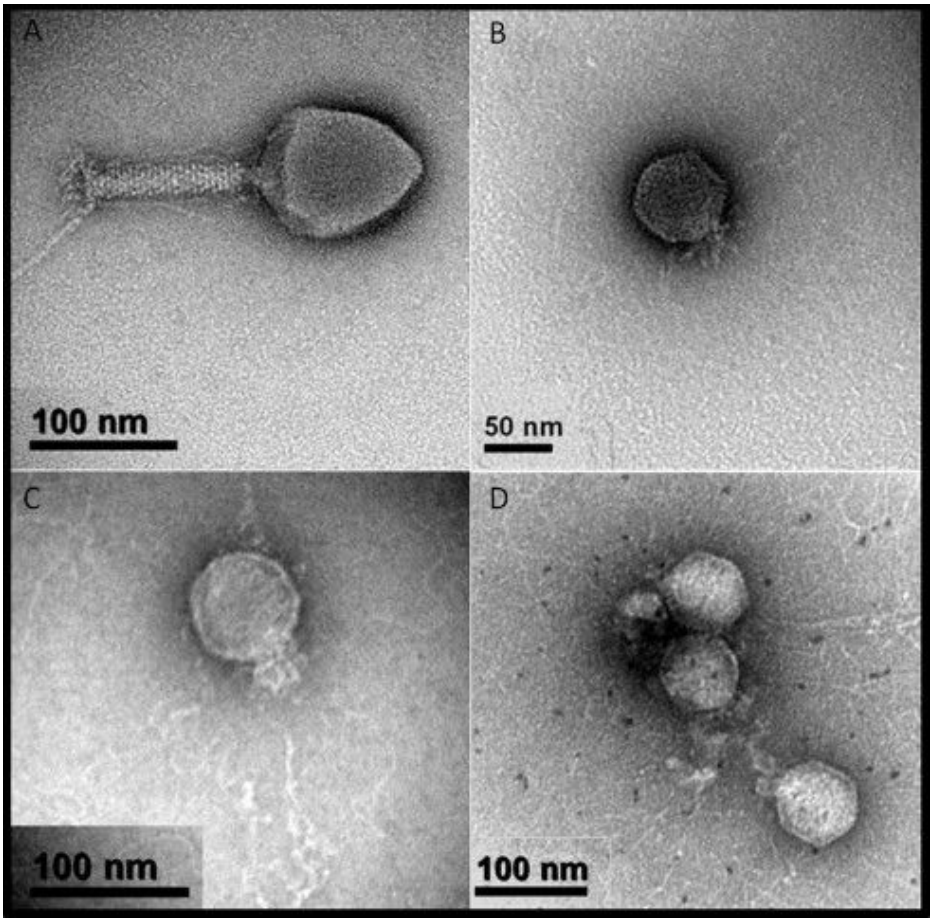

Figure 6.1. Transmission electron microscopy pictures of the four phages composing the cocktail. A) T4-like phage PhAPEC2 (head: 83 nm and tail: 107 nm); the N4-like phages: B) PhAPEC9 (head: 64 nm), C) PhAPEC5 (head: 65 nm) and D) PhAPEC7 (head: 70 nm). Transmission electron microscopy pictures made by prof. J. Klumpp (Institute of Food, Nutrition and Health, ETH Zurich, Switzerland).

Full genome sequencing of the selected phages confirmed the relationship to phages N4 and T4, respectively. The genomes of PhAPEC5 and PhAPEC7 both showed a DNA sequence coverage of 63% similar ($E = 0$) to *Escherichia* phage N4 (NC_008720.1). PhAPEC5 shows amino acid similarity with N4 for 54 out of its 83 proteins; PhAPEC7 for 55 out of its 85 proteins. PhAPEC2 had a DNA sequence coverage of 72% similar to Enterobacteria phage T4 (NC_000866.4) and had 202 of its 254 proteins similar to T4 proteins, based on blast analysis.

Gene annotations did not identify potential known toxins or toxin-related pFAM domains. In addition, no genomic markers indicating a temperate lifestyle were found. The genome sequences of phages PhAPEC2, PhAPEC5 and PhAPEC7 have been submitted to GenBank (KF562341, KF192075 and KF562340).

3.2. The selected cocktail displayed acceptable stability parameters

The titer decrease of the phages at 42ºC over 3 days was variable and depended on the medium and the phage. In LB medium, PhAPEC9 showed a negligible titer drop, whereas PhAPEC5 titers decreased with 3.7 log units. PhAPEC2 and PhAPEC7 had an intermediary titer reduction. In PBS medium the titer drop varied between a reduction of 1.4 log units for PhAPEC7 and 2.3 log units for PhAPEC2. However, all phages showed good stability in tap water and no titer drop exceeding 0.5 log were detected over a 3 h time period (the period necessary for drinking water administration).

3.3. Phage treatment did not reduce mortality and lesions scores

Mortality rates, medians and Q1 - Q3 for the lesion scores are presented in Table 6.2. No significant differences were found for the mortality between the infected chickens of groups 1, 2, 3, 4 and 5. Significant differences between multiple groups were found for the total lesion score and lesion score per organ ($P < 0.01$). The Wilcoxon Rank Sum Test showed significant differences between infected chickens from group 1 - 5 in comparison to the non-infected group 6, for the total lesion score and the lesion score per organ ($P < 0.05$). Between groups 1 - 5 no significant difference were found for the total lesion score or the lesion score per organ. A trend toward significance ($P \approx 0.06$) was found for the lesion score of the liver and abdominal air sac of chickens in group 4 in comparison to non-infected group 6.

Table 6.2. Mortalities and lesion scores of chickens infected with CH2 and treated with a phage cocktail

Group	Infection[a]	Treatment[b]	Total[c]	Deaths[d]	Median of lesion scores[e] (first quartile – third quartile)							
					Liver	PRD	TAS-L	TAS-R	LU-L	LU-R	AAS	TOT
$1^{C/B}$	CH2	10^9 IT	12	6	2^{A} (1.75 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (1.5 – 2)	14^{A} (13.25 – 14)
$2^{C/B}$	CH2	10^9 ES	12	6	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	14^{A} (14 – 14)
$3^{C/B}$	CH2	10^9 DR	12	5	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (1.75 – 2)	14^{A} (13.25 – 14)
$4^{C/B}$	CH2	10^9 inactive	6	4	2^{AB} (0 – 2)	2^{A} (1 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (1 – 2)	2^{AB} (0 – 2)	14^{A} (14 – 14)
$5^{C/-}$	CH2	-	6	3	2^{A} (1.5 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (2 – 2)	2^{A} (1 – 2)	14^{A} (14 – 14)
$6^{LB/-}$	-	-	6	0	0^{B} (0 – 0)	0^{B} (0 – 0)	0^{B} (0 – 0)	0^{B} (0 – 0)	0^{B} (0 – 0)	0^{B} (0 – 0)	0^{B} (0 – 0)	0^{B} (0 – 0)

[a]Intratracheal infection with 500 µl 10^8 cfu CH2 at 28 days of age.

[b]Chickens were, 2 hours after the infection, treated via the trachea (IT), oesophagus (ES) or via the drinking water (DR) with the phage cocktail with a concentration of 10^9 pfu

[c]Total number of chickens per group.

[d]Total number of chickens that died or were euthanized in 7 days following the infection. These chickens showed clear macroscopic lesions of an APEC infection and were assigned a total lesion score of 14.

[e]PRD = pericardium; TAS-L = left thoracic air sac; TAS-R = right thoracic air sac; LU-L = left lung; LU-R = right lung; AAS = abdominal air sacs; TOT = total lesion score.

Different capital letters show significant differences within the column ($P \leq 0.05$).

Table 6.3. Time of death, concentration of re-isolated bacteria and presence of phage in organs from chickens

Chicken	Organ	Time of death (dpi)[a]	Group	Lesion score	Bacterial concentration (cfu/g)		Phage presence	
					LB	MacConkey	Without enrichment[e]	With enrichment[e]
1	Lung	1[b]	1	2	3.8E+08	4.8E+07	+	+
	Heart			0	1.0E+08	N.D.	+	+
2	Lung	1[b]	1	2	6.8E+08	9.0E+07	+	+
	Heart			2	4.0E+02	N.D.	-	-
3	Lung	1[b]	2	1.5	6.3E+07	1.0E+08	-	-
	Heart			0	1.6E+07	N.D.	-	-
4	Lung	1[b]	2	2	1.5E+09	8.6E+07	-	-
	Heart			2	2.6E+08	N.D.	-	+
5	Lung	1[b]	3	2	1.7E+06	1.8E+08	-	-
	Heart			0	2.6E+03	N.D.	-	-
6	Lung	2[c]	1	2	2.4E+07	1.4E+07	+	+
	Heart			2	7.8E+05	N.D.	-	-
7	Lung	2[c]	3	2	4.2E+08	7.5E+07	-	-
	Heart			2	1.2E+08	N.D.	-	-
8	Lung	3[c]	3	2	1.5E+05	2.4E+05	-	-
	Heart			2	3.8E+07	N.D.	-	+
9	Lung	4[c]	2	2	8.5E+06	5.5E+06	-	-
	Heart			2	9.8E+05	N.D.	-	+
10	Lung	5[c]	4	2	2.0E+07	6.0E+06	N.D.	-
	Heart			2	8.0E+07	N.D.	N.D.	-
11	Lung	5[c]	5	2	4.0E+07	1.8E+07	N.D.	-
	Heart			2	5.2E+08	N.D.	N.D.	-
12	Lung	6[c]	1	2	6.8E+05	5.7E+04	-	-
	Heart			2	7.4E+05	N.D.	-	-
13	Lung	7[d]	1	2	2.2E+06	6.5E+06	N.D.	N.D.
	Heart			2	7.6E+07	N.D.	N.D.	N.D.
14	Lung	7[d]	2	2	7.3E+03	5.2E+03	N.D.	N.D.
	Heart			2	1.2E+06	N.D.	N.D.	N.D.
15	Lung	7[d]	3	2	5.0E+03	2.1E+03	N.D.	N.D.
	Heart			2	1.5E+06	N.D.	N.D.	N.D.

[a] Days died/euthanized after infection
[b] Chickens randomly chosen and sacrificed 1 day after infection and treatment
[c] Chickens euthanized after infection
[d] Chickens still alive 7 days after infection
[e] - = phage not present; + = phage present
N.D. = not determined

3.4. Phage treatment did not procure a significant weight gain

Weight gain in chickens that were euthanized 7 dpi was determined (data not shown). Chickens in all groups, except for groups 3 and 6, showed an average weight loss 7 dpi. No significant differences were found in weight gain between chickens in groups 1, 2, 3, 4 and 5 ($P \approx 0.83$), indicating that treatment with the phage cocktail did not significantly influence the weight gain of the chickens. The weight gain in chickens from group 6 was significantly different from the other groups ($P < 0.01$), showing that the infection had a significant effect on the weight gain of the chickens.

3.5. Viable phages could be re-isolated from euthanized and sacrificed chicken

Phages could be re-isolated from organs of chickens that were sacrificed or euthanized; both with and without enrichment (see Table 6.3). However, to re-isolate phages after 48 h the organs had to be enriched, demonstrating the low concentration of phages beyond this time point. Phages have been found up to four days after treatment. The presence of phage in dying chickens indicates their inability to rescue the chickens from a developing infection. Phages could be re-isolated from the heart (but not the lungs) in chickens treated *via* the esophagus or *via* the drinking water, suggesting that the phages could translocate from the gastrointestinal tract to the cardiovascular system. Phage identification by multiplex PCR (Table 6.4) recognized PhAPEC2 (in group 2 after 4 days) and PhAPEC7 (group 2 after 24 h, group 3 after 3 days). Phages could be re-isolated from the heart as well as from the lung from chickens that received phage intratracheally. After 24 h, all four phages could be re-isolated from the lung tissue and only PhAPEC5 could not be re-isolated at the 48 h time point. PhAPEC 2 (after enrichment), PhAPEC7 and PhAPEC9 could be detected in the heart. These data give a general indication of the translocation capacity of each phage in a cocktail context. Despite the presence of phages in three of the five randomly chosen chickens, which were sacrificed after 24 h, high bacterial concentrations and lesion scores were observed.

In a previous unpublished study, the presence of phages was followed over time in the organs in absence of CH2 infection. No phages could be detected 24 h post administration. Since this is in contrast with the results in this study, where phages could still be re-isolated after four days, we can hypothesize that during this study multiplication of the phages *in vivo* has likely taken place.

Table 6.4. Identification of re-isolated phages from organs by multiplex PCR

Chicken	Organ	Time of death (dpi)[a]	Group	Presence of phage	
				Without enrichment	With enrichment
1	Lung	1[b]	1	PhAPEC2, PhAPEC5, PhAPEC7, PhAPEC9	PhAPEC2, PhAPEC7
	Heart	1[b]	1	PhAPEC7, PhAPEC9	PhAPEC7, PhAPEC9
2	Lung	1[b]	1	PhAPEC5, PhAPEC7, PhAPEC9	PhAPEC2, PhAPEC7
4	Heart	1[b]	2	X	PhAPEC7
6	Lung	2[c]	1	PhAPEC7, PhAPEC9	PhAPEC2, PhAPEC7
8	Heart	3[c]	3	X	PhAPEC7
9	Heart	4[c]	2	X	PhAPEC2

[a] Days euthanized after infection
[b] Chickens randomly chosen and sacrificed 1 day after infection and treatment
[c] Chickens euthanized after infection
X = not present

3.6. Bacterial quantification and sensitivity to the phage cocktail

The concentration of bacteria that were re-isolated from the lungs and heart of euthanized or sacrificed (24 hpi) chickens are provided in Table 6.3. The number of re-isolated bacteria in chickens sacrificed 24 hpi varied between 10^6 - 10^9 cfu/g in the lung and between 10^2 - 10^8 cfu/g in the heart. In the euthanized chickens, concentrations varied between 10^3 - 10^8 cfu/g in the lungs and between 10^5 - 10^8 cfu/g in the heart.

We determined the level of resistance to phage infection which had developed during the treatment period. All colonies, except two (see Figure 6.2), showed sensitivity to the phage cocktail and the individual phages in cross-streaking screens. The sensitivity of the colonies was confirmed with the drop test on the bacterial lawn of three re-isolated colonies per organ. These re-isolated colonies had the same sensitivity as the wild type CH2 strain for the phages; individual plaques could be observed at a 10^{-3} dilution of the cocktail. The sensitivity for the O78-specific phages PhAPEC5, PhAPEC7 and PhAPEC9 confirms that *E. coli* CH2 was re-isolated from these chickens.

Two colonies re-isolated from the heart of chicken 22 (treated *via* the esophagus and euthanized 7 dpi) had a decreased sensitivity for the cocktail, as observed by cross-streaking and the soft agar overlay technique. With cross-streaking, an uninhibited bacterial line beyond the phage cocktail line was observed (Figure 6.2), and with the soft agar overlay technique individual plaques were seen at a lower dilution compared to CH2 (data not shown). The

resistance against each individual phage of the cocktail was subsequently analyzed. An increased resistance was found against all four phages for one colony and against two phages (PhAPEC9 and PhAPEC7) for the other colony.

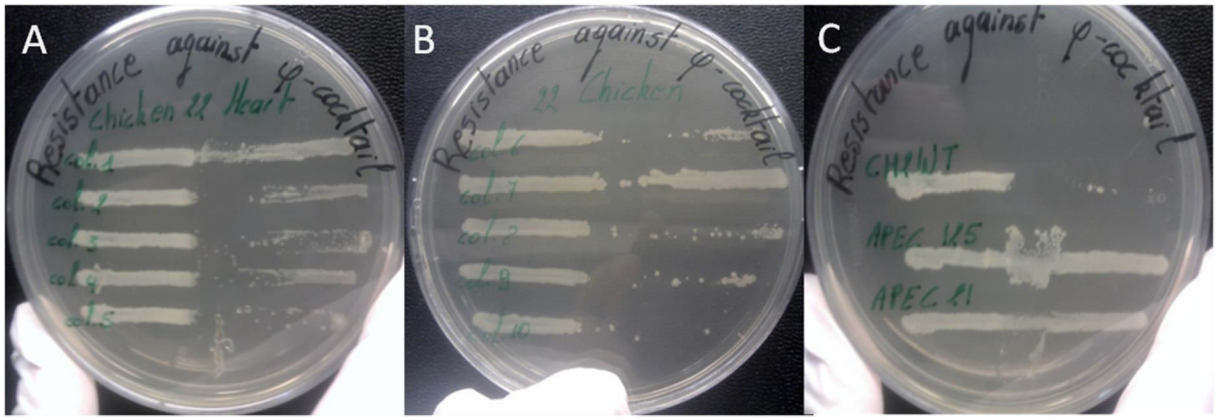

Figure 6.2. Cross-streaking of the re-isolated colonies over a phage cocktail line. A) and B) Re-isolated colonies from chicken 22, colony 1 and 7 showing resistance against the phage cocktail, C) Positive control strain APEC CH2 showing sensitivity to the phage cocktail and negative control strains APEC125 and APEC21 showing resistance against the phage cocktail.

4. Discussion

Successful application of phages for the treatment of natural or experimental APEC infections in chickens has been reported (Huff et al., 2004; Lau et al., 2010; Oliveira et al., 2010). Success rates were different and dependent on many factors, such as type of administered phages and route of administration of the phages (Huff et al., 2003b). Timing and concentration of administered phages is also known to have an important effect on the success rate of phage therapy (Ryan et al., 2011), with successful treatment even seen after administration of phages within one or two days after the bacterial infection (Watanabe et al., 2007). An early administration after infection can lead to clearing of phages before they can actively replicate *in vivo*, caused by a too low bacterial concentration (Payne et al., 2000). However, in the current study, low phage titers could be recovered from the lungs and heart of chickens at different time points after administration, even 3 and 4 dpi. These phages were identified as PhAPEC7 (3 dpi) and phAPEC2 (4 dpi), respectively. Although these phages most likely were able to multiply *in vivo*, and despite the fact that phages (low titer) could be re-isolated from the heart and lungs, they were not able to clear the infection.

Bacterial numbers in the organs of infected/non-treated and infected/treated chickens seemed not to be significantly different, leading to the conclusion that phages were not able to significantly lower the number of bacteria in the organs. The reason for this cannot be found

in the *in vitro* efficacy of the phages, since the phages in the cocktail were able to efficiently lyse CH2, and their stability in LB and PBS at 42ºC was demonstrated. Only PhAPEC5 showed reduced stability at 42ºC and could indeed not be re-isolated from euthanized chickens 2, 3 or 4 dpi, unlike the three other phages in the cocktail.

One possibility is the intracellular multiplication of CH2 (for example in macrophages or heterophils), preventing phages from reaching their receptor on the bacterial host and avoiding infection and lysis. Strain APEC O1:K1:H7 has been shown to survive in macrophages (Mellata et al., 2003), probably by protecting itself against the environmental stresses (such as acidity) by production of colanic acid (Li et al., 2005; Tuntufye et al., 2012a). However, APEC CH2 belongs to serogroup O78 which was shown to be poorly associated with phagocytes (Mellata et al., 2003). It is thus less likely that CH2 will multiply intracellularly.

Another possibility lies in the transcriptional response to environmental stresses (Mekalanos, 1992). It was shown by recombination-based *in vivo* expression technology that genes involved in capsule formation and transport are expressed *in vivo* in chickens (Tuntufye et al., 2012a). Attenuated mutants were found by signature-tagged transposon mutagenesis, which contained mutations in genes responsible for capsule formation (*kpsM* and *kpsS*), showing that it is likely that capsule formation is necessary for the virulence and survival of APEC *in vivo* (Li et al., 2005). It was shown that a spontaneous K1 mutant of APEC strain MT78 was not able to persist in body fluids and colonize systemic organs, furthermore it showed sensitivity to serum and phagocytosis, whereas the wild type strain was resistant (Mellata et al., 2003). Phage T7, with its receptor on LPS, was physically blocked to lyse *E. coli* strain EV36 after the expression of its K1 capsule *in vitro* (Scholl et al., 2005). To test if the phages in the cocktail were able to lyse the bacteria after capsule formation, CH2 was grown to exponential phase in milk broth (capsule formation was observed). The phage cocktail was added to one of the cultures when it reached exponential phase ($OD_{600} \approx 0.6$) and OD was followed over time. A significantly lower OD_{600} over time was found in the culture with the phage cocktail added to it in comparison to the culture without phages (data not shown), which implies that the phages were able to lyse the bacteria even though they expressed the capsule.

Bacterial resistance to phages by mutation or loss of phage receptors has been shown to be a major cause of unsuccessful phage therapy (Levin and Bull, 2004; Ryan et al., 2011; Skurnik

and Strauch, 2006). This is not the case here, since a phage cocktail with phages having different receptors and not showing cross-resistance was used in order to reduce resistance development (Levin and Bull, 2004). In addition, resistance of the recovered bacteria to the phage cocktail was verified and shown to be very limited (only two isolates with a lower susceptibility to the phage cocktail could be found). As such, an unsuccessful treatment due to resistance development is unlikely.

Little is known about the behaviour of phages *in vivo* (Skurnik and Strauch, 2006). It is possible that the applied phages were not active enough *in vivo*, due to encountering a suboptimal environment. Moreover, clearance of the phages by the general innate immune system cannot be excluded because of the presence of systemic inflammation. The decrease in number of the phages would make it difficult to spread through the body in high enough amounts to fight a systemic infection. In future, it is important to achieve a better understanding of the behaviour of phages *in vivo* to be able to select phages and conditions for their application that show the best results in human and animal therapy.

Colibacillosis can be considered as a severe infection, which starts as a respiratory infection that rapidly becomes systemic. Huff *et al*. (2013) did not obtain a reduction in mortalities in APEC infected chickens when treated *via* fine or coarse spray. However, a decrease could be obtained after intratracheal treatment. This observation was explained by the fact that only intratracheal administration could bring high enough phage titers at the infection site (Huff et al., 2013). The experimental design of Huff *et al*. (2013) varies in many points with ours, with the most striking being that infection was performed immediately after treatment. A sufficiently high phage administration in the lungs and air sacs could prevent an important systemic spread, which in comparison with our study already occurred because treatment was administered 2 h after infection. This hypothesis was confirmed, since bacteria were observed in the blood of four-week-old chickens two h after intratracheal infection with CH2 (H.N. Tuntufye, personal communication). Such systemic infections may not be treatable using phage in this manner, and phage therapy efforts should possibly focus on prevention rather than treatment. Intramuscular treatment showed significant decreased mortality even when phages were administered 48 h after infection, but is not practical in large productions (Huff et al., 2003b).

This study and others show that phage therapy is not always successful. Sheep and cattle infected orally with *E. coli* O157:H7 and treated repeatedly orally with large doses of phage KH1 did not show a lower intestinal carriage of *E. coli* O157:H7 compared to control groups that were not treated by phages (Sheng et al., 2006). Despite an overwhelming optimism in literature about the efficacy of phage therapy, the specific fields of application should be more clearly investigated and defined.

Chapter VII

General discussion

1. Zoonotic potential of APEC and antibiotic resistance and its consequences

It has been shown that some APEC isolates are genetically indistinguible from UPEC and NMEC isolates, sharing many virulence genes, O-serogroup and a phylogenetic subcluster (Ewers et al., 2007; Johnson et al., 2008; Moulin-Schouleur et al., 2007). Human strains of this subcluster were highly virulent for chicks (Moulin-Schouleur et al., 2007). So even though most APEC, UPEC, and NMEC strains are genetically distinct from one another, a subcluster is not. This cluster shows the zoonotic potential of APEC. Phages might be used to kill APEC bacteria on poultry meat. There have not been investigations on the use of phages for APEC on meat yet, since this is not the most important pathogen on food. Considering the potential zoonotic risks of APEC and also its multidrug resistance, especially in strains from broilers (Blanco et al., 1997; Peighambari et al., 1995; Zahraei Salehi and Farashi Bonab, 2006), the transfer of antibiotic resistance genes to human bacteria, as shown in an *in vitro* model (Smet et al., 2011), should be inhibited. Since 1999 several stricter regulations concerning antimicrobial use have been applied in the European Union, with the ban on antimicrobial growth promoters in 2006 probably as the most important one. The consequence of the ban was an initial increase in use of antimicrobials for therapeutic use, as shown in Denmark (DANMAP, 2008), the Netherlands (MARAN, 2007) and Sweden (SVARM, 2007). From 2007 - 2012 there was a decline of 56% in therapeutic use of antibiotics (kg active substance) in the Netherlands (SDa, 2013), however its effect on the occurrence of resistance in indicator *E. coli* strains is not yet noticeable (MARAN, 2012). Since 2007, an antimicrobial consumption monitoring system exists in Belgium (www.belvetsac.ugent.be). The monitoring program in Belgium shows a decline of antimicrobial use in the animal product sector by 20.3% from 2007 to 2012, although Belgium remains one of the highest antimicrobial users in the European Union (BelVetSac, 2012). In Belgium the most used compounds in the broiler industry are amoxicillin, tylosin and trimethoprim-sulfonamide, belonging to risk class I (critically important) for human medicine with the exception of trimethoprim-sulfonamides (class II; highly important) (Persoons et al., 2012). Among AFEC isolates from healthy broilers (sampled in 2011) high levels of resistance (more than 50%) were found for ampicillin, ciprofloxacin, nalidixic acid, streptomycin, sulfonamides and tetracyclines (EFSA, 2013), which makes it a challenge to treat the disease in broilers. Next to this, APEC is hard to treat, especially in broilers with their poor immunocompetence due to selection for increased growth, which is negatively correlated with maturation of the immune system and

the level of the immune response (Maatman et al., 1993; Siegel et al., 1982). Few data are available for laying hens. In our study, we found high resistance to ampicillin, nalidixic acid, sulfonamides and tetracycline and observed that 37% of the strains from the year 2000 - 2005 were multi-resistant (Chapter II). However, more recent data with respect to antibiotic resistance in laying hens are needed, even though laying hens are rarely given antibiotics. In laying hens no mass outbreaks of colibacillosis occur, only a limited number of laying hens gets infected that are difficult to treat, furthermore there is the risk of antibiotic residues ending up in the eggs. These residues can pose risks to human health, due to allergic reactions in hypersensitive persons. They can also cause direct toxic effects or code for increased bacterial resistance to antibiotics, if humans are exposed for a relatively long period (Bogialli and Di Corcia, 2009; Dowling, 2013; Goetting et al., 2011). Therefore maximum residue limits have been set by the European Union (European Parliament, 2009). So APEC infected laying hens often do not get treated, but are culled. However, during rearing layers do receive antibiotics, and antibiotic resistant bacteria can persist for a long time in the intestinal tract of chickens without selective pressure of antibiotics (Chaslus-Dancla et al., 1987). During this study a two-way strategy was tested to reduce the need for antibiotics and likewise in an indirect manner contribute to diminish the antimicrobial resistance burden in poultry. On the one hand trying to prevent environmental colonization (or flock to flock transmission) of APEC and lowering infection pressure in the presence of chickens by the use of disinfectants and on the other hand focusing on treating APEC infected chickens by the use of a phage cocktail.

2. Use of disinfectants to prevent colibacillosis

2.1. Current techniques for testing disinfectant resistance

In our studies we evaluated a collection of APEC strains (n = 97) for resistance against the five most included active ingredients in commercial disinfectants, routinely used in the poultry industry (Chapter II). We based our data on information provided by Animal Health Care Flanders and the Federal Agency for Medicines and Health Products, since no exact data on the quantities of the different products can be obtained. We did not find any resistance, however through the presence of integrons we know that resistance genes are present. This indicates that the current techniques for evaluating phenotypic resistance against disinfectants should be further optimized. Furthermore, no breakpoints concentration cut-offs exist for distinguishing between susceptible and resistant strains, as there are for antibiotic

susceptibility testing. Detection of resistance solely depends on the distribution of the strains over the MICs (microbiological breakpoints). A more sensitive standardized method will be needed (Maillard et al., 2013; Russell, 1999) to be able to compare results at an international level. Therefore, first of all a clear definition between reduced susceptible (increased MIC) and resistant (not active at in-use concentration) is necessary (Maillard et al., 2013; Russell, 1999). Using MBCs instead of MICs might also already be a step in the this good direction, since this reflects the concentration at which the biocide kills, which is of more importance in the case of disinfectants than the MIC (Russell, 1999). Furthermore the use of doubling dilutions in the determination of phenotypical resistance should be reconsidered, since doubling dilutions might not be sensitive enough to detect resistance. PCRs to detect the presence of *qac* resistance genes have been developed. However, other resistances (such as genes coding for other multidrug efflux pumps or yet unknown resistance genes) may not be detected like this. These unknown resistance genes may be identified by using mutagenesis techniques, inactivating the gene in question.

2.2. Biofilm formation and its effects on disinfectant efficacy

Biofilms can impair substantially the efficacy of disinfectants and biofilms cells are in general more resistant (tolerant) to biocides than planktonic cells. During our study we found most of the APEC strains to be moderate biofilm producers on PS and strong biofilm producers on PP and PVC, under standardized conditions which can be encountered in the barns, especially in the water systems (Chapter III). Strains of serogroup O1 and O2 belonged significantly more often to the strong biofilm producers than to the non-, weak or moderate biofilm producers, while strains of serogroup O78 were mainly non- or weak biofilm formers on PP and PVC. On SSCs, strains of serogroup O78 were mainly moderate to strong biofilm producers, while strains of serogroups O1 and O2 were mostly non- to weak biofilm producers. The in-use concentrations for QAC are higher than the obtained concentrations needed to kill the sessile cells during our study, while the obtained concentration for H_2O_2 is higher than the lowest in-use concentration and lower than the highest in-use concentration. Reactivity with organic material is especially known for highly reactive compounds (like H_2O_2) (Russell 1999). However, a concentration of 2-3% H_2O_2 was shown to possess good antimicrobial activity in the presence of organic matter (Moustafa Gehan et al., 2009; Ruano et al., 2001), while tested in-use QAC concentrations were more affected (Moustafa Gehan et al., 2009). Disinfectants are nevertheless often a combination of various active ingredients, together they have a higher efficacy. In this study we worked with relatively young biofilms, while in barns probably

much more elaborated biofilms may be formed. Little research exists on biofilms in poultry houses under natural conditions, so the effect of biofilm formation on disinfectant efficacy should be assessed in poultry houses. Thereby also concrete should be tested under natural conditions, which in a broiler house is the most used material.

2.3. Lowering infection pressure of APEC by use of disinfectants

Since APEC strains are present, also in healthy animals, lowering infection pressure in the presence of chickens in barns can be an important strategy to help reduce APEC outbreaks. High concentrations of faeces contaminated dust are usually present in the barn. As APEC infection is mainly believed to be caused by inhalation of these dust particles (aerosols) (Dho-Moulin and Fairbrother, 1999; Dziva and Stevens, 2008), lowering the level of these *E. coli* aerosols might possibly result in a lower incidence of APEC outbreaks. This applies especially to broilers between 4 weeks up to slaughter, since broilers of that age experience high levels of stress due to their fast growth, which renders the chickens more prone to infection. High concentrations of ammonia, which are present in the poultry house when chickens reach this age, furthermore predispose chickens to APEC. As such, lowering the infection pressure in chickens needs to be further explored, since nebulization of low concentrations (higher than 1%) of H_2O_2 has been shown to render chickens more susceptible to APEC infection, rather than lowering the infection pressure (Chapter IV). This is probably due to the caustic effect of hydrogen peroxide radicals on the epithelial cells in the pharynx and trachea, most likely deciliating epithelial cells. This, however, needs to be confirmed by histology. Spraying phages in the presence of chickens can be an option to lower infection pressure, although phages might have a narrow host range limiting their efficacy. Phages might be orally administered in feed in special polymer capsules (Stanford et al., 2010) or microspheres (Ma et al., 2008) that open and release their contents upon arrival in the intestine. In these capsules or microspheres phages are protected from the low pH in the proventriculus and gizzard, and can reach directly the reservoir of APEC. This has already been performed against *E. coli* O157:H7 in cattle and demonstrates that targeted bacteria are reduced after treatment (Sheng et al., 2006; Stanford et al., 2010).

3. Use of bacteriophages to treat colibacillosis

During the second part of the study we evaluated the use of a cocktail of lytic phages for the treatment of colibacillosis. First, we developed a bioluminescent APEC strain (CH2-lux; Chapter V), in order to be able to follow up prevention and treatment effects (with e.g.

vaccination and phages, respectively) on the course of an APEC infection *ex vivo*. Correlations were found between bacterial numbers in the heart, liver and lung of experimentally infected four-week-old chickens and the bioluminescence of the corresponding homogenized tissue. Possibly the luminescent strain can also be used for real-time imaging of (young) experimentally infected chickens to monitor adhesion, internalization, organ localization, invasion and persistence of the strain in order to better understand pathogenesis of APEC. Together with sequencing our infecting strain (CH2) this can help in unraveling the pathogenesis of APEC, in order to develop targeted treatments in the battle against APEC. For example antivirulence therapy by inhibition of expression or regulation of virulence genes (Cegelski et al., 2008; Defoirdt, 2013). During our study with phages, we used the non-luminescent CH2 strain as four-week-old chickens are too big for *in vivo* monitoring of the luminescent strain. A phage cocktail composed of four phages, able to lyse the latter strain *in vitro,* was tested. These phages had a broad host range, good stability at 42ºC (core body temperature of a healthy chicken) and a low frequency of development of resistant APEC mutants. *In vivo* administration of the cocktail to experimentally infected chickens did, however, not result in a reduction in mortality or lesion scores in comparison to infected/non-treated control chickens (Chapter VI). These results confirm that even though many promising results have been found with phages as curative method for colibacillosis (Huff et al., 2004; Lau et al., 2010; Oliveira et al., 2010), good *in vitro* results do not always lead to successful phage therapy. Successful *in vivo* phage therapy depends on many parameters, such as method of application and dose and time of administration (Huff et al., 2003b; Huff et al., 2013; Ryan et al., 2011). Mathematical models exist in which several phage-related factors are taken into account, such as time of administration, dose applied, clearance by the host immune system, phage insensitivity and development of resistant mutants in order to predict treatment outcomes (Cairns et al., 2009; Payne and Jansen, 2001). But as most of these parameter estimations are based on *in vitro* analysis they often do not give good *in vivo* predictions. Furthermore, *in vivo* behaviour of phages is dependent on many more factors that are hard to predict, such as the rate of phage translocation to the infection site and the spread throughout the body, hindered binding of phages to the host receptor and non-linear phage pharmacokinetics (Tsonos et al., 2014). Henry *et al.* (2013) showed promising results when he used a lung infection model in order to compare *in vivo* efficacy of phages with *in vitro* behaviour, based on real-time monitoring of the infection using bioluminescence. Further research on this model and development of other models for other pathogens might help in understanding *in vivo* behaviour of phages in order to reduce

variability in treatment outcomes.

However, infections that rapidly become systemic might not be treatable using phages. This might also be the case with our model strain CH2, since bacteria could already be observed in the blood of four-week-old chickens two h after intratracheal infection. Possibly, phage therapy efforts should then focus on prevention rather than treatment.

An alternative to use life phages is to use phage endolysins. Endolysins hydrolyze the bacterium wall (peptidoglycan layer) from within the bacteria, so that phage progeny can be released. Up to now, no bacterial resistance towards lysins has been documented (Schmelcher et al., 2012), probably due to the binding and cleaving unique and essential targets in the cell wall (Fischetti, 2005; Fischetti, 2010). These enzymes are mostly used against Gram-positive bacteria, as the peptidoglycan layer of Gram-positive bacteria is in direct contact with the environment; several successful studies have already been described with *Streptococcus pneumoniae* in mice and rats (Grandgirard et al., 2008; Jado et al., 2003; Loeffler et al., 2003; Loeffler et al., 2001; Nelson et al., 2001). Gram-negative bacteria are different in that their peptidoglycan layer is protected by an outer membrane. Genetically engineering lysins by fusing bacterial cell wall adhesins to the catalytic domains of lysins and addition of outer membrane-permeabilizers should tackle this problem (Briers et al., 2011; Lai et al., 2011; Lukacik et al., 2012). Addition to the feed of chickens would be the most practical and economic feasible way of administrating the purified lysins.

General conclusions

Overall we can conclude that a more sensitive standardized method to determine the occurrence of resistance towards disinfectants is needed. Thereby disinfectants should also be tested under natural conditions in poultry houses, where also biofilms are present, in order to have a better idea on their actual efficacy. Even though we did not find positive results concerning *in vivo* phage therapy, it remains a valuable candidate in order to diminish antibiotic use (and thereby resistance selection). The focus should be on understanding the *in vivo* behaviour in order to diminish variability in treatment outcomes.

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Curriculum vitae

Leon Oosterik was born in Hengelo (Overijssel) in the Netherlands on the 9th of March 1987. After he finished secondary school in Holten (the Netherlands), he obtained a Master's degree in Animal Sciences at Wageningen Universiteit with a specialisation in Animal Health and Behaviour in October 2010. After his studies, he worked for a while at Wageningen Universiteit as a teaching assistant.

Leon started his PhD in February 2011 at the Faculty of Bioscience Engineering at the KU Leuven under the supervision of Prof. Dr. Bruno Goddeeris and at the Department of General Bacteriology at the Veterinary and Agrochemical Research Centre (CODA-CERVA) under the supervision of Prof. Dr. Patrick Butaye. The topic of his PhD project is the use of disinfectants and bacteriophages as alternative prophylaxis/treatment for colibacillosis in chickens. The PhD project is funded by the Federal Public Service Health, Food Chain Safety and Environment and the CODA-CERVA.

Publications in international peer-reviewed journals

Tsonos*, J., **Oosterik*, L.H**., Tuntufye, H.N., Klumpp, J., Butaye, P., De Greve, H., Hernalsteens, J.-P., Lavigne, R., Goddeeris, B.M. 2014. A cocktail of *in vitro* efficient phages is not a guarantee for *in vivo* therapeutic results against avian colibacillosis. Veterinary Microbiology 171 (3-4), 470-479.
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