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Executive Summary

Extra intestinal *E. coli* (ExPEC) are major pathogens that cause avian colibacillosis in broiler flocks, broiler breeders, layers and layers breeders, mastitis in dairy cattle and sheep, as well as urinary infections, pyometra and septicaemia in companion animals. In birds, ExPEC are referred to as Avian Pathogenic *E. coli* (APEC). The isolates are often multi-drug resistant and have zoonotic potential. Iron uptake systems are an essential and specific component of their pathogenicity. We have constructed several attenuated APEC mutants that confer vaccinal protection against a respiratory infection by the parental strain. We also have developed DNA probes that specifically detect APEC in the air, in order to rapidly assess air quality in the farm.

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Introduction & Background:

Importance of the problem:

Pathogenic strains of Escherichia coli are classified into different pathotypes that fall into two broad groups: 1/ the diarroeogenic *E. coli* that cause enteritis, sometimes with systemic complications, and 2/ the Extra-intestinal *E. coli* (ExPEC) that cause a variety of non-enteric diseases (6). In dairy cattle and sheep, ExPEC strains are a significant cause of mastitis; in companion animals and in humans, they are responsible for urinary infections, pyometra, and septicaemia. In humans they also cause neonatal meningitis. In birds, Avian Pathogenic *E. coli* (APEC) correspond to a sub-group of ExPEC that cause respiratory infections, arthritis, cellulitis and generalised infections. As members of the ExPEC pathotype, APEC strains appear to have some zoonotic potential (22, 25). The virulence factors of APEC and other ExPEC include iron acquisition systems, adhesins, haemolysins/hemagglutinins, resistance to the bactericidal effects of serum and phagocytosis, and toxins/cytotoxins (7).

Colibacillosis occurs commonly in broiler flocks but it is also increasingly seen in broiler breeders, as well as layers and layers breeders. APEC infections represent a significant risk for the broiler chicken industry, particularly in the intensive farming units, which correspond to 98% of the production (ca. 500 million chicken or 800 000 tons of meat per year in Australia), and the broiler breeder industry, which correspond to 5-6 million of birds in Australia. In the USA, where broiler production is about 15 times greater than Australia, the APEC-induced economic losses have been estimated at US$ 40 millions per year (12). An APEC outbreak in a broiler farm threatens the whole population in the infected shed. The mortality rate in flocks can reach 10% (29), with a morbidity of up to 50%. In broilers, APEC usually cause clinical signs in 4-6 week old chickens, when weight gain is critical. A single APEC episode in a conventionnal shed, with a 10% mortality rate and an increase of 0.2 in the feed conversion ratio of birds, would lead to the loss of 8 tons of meat and an increase in feed consumption of 20 tons of feed. This would represent a total loss of $20,000 to $30,000; at the farm level this figure could be multiplied by 3-5, depending of the number of infected sheds. On broiler breeders farms, APEC mortality can occur at the beginning or at the peak of egg production. On an average farm with 10,000 birds, a single episode with 10% mortality and 50% morbidity can lead to an average loss of 70,000 to 290,000 offspring (1-day old chicks), depending on the timing of the episode, and the value of the parent birds. This would represent a total loss of $60,000 to $180,000 for a single farm.

It has been suggested that the number of *E. coli* in the atmosphere is correlated with the risk of developing the respiratory disease (18). Effective control of the disease in flocks requires the elimination of predisposing factors, such as poor air quality and other bacterial and viral respiratory pathogens. In practice, this is often difficult to achieve consistently. As a result, outbreaks of the disease regularly occur in farms, which is is demonstrated by the fact that APEC strains are repeatedly isolated from avian specimens. For instance, at the APCAH diagnostic laboratory of the Faculty of Veterinary Science, APEC represent almost 50% of the significant pathogenic isolates that were reported from 2006 to 2009. These isolates often display multiple antibiotic resistance, making them difficult to control using classical therapeutic methods.

Detection strategies for APEC:

Only specific strains of *E. coli* are able to cause disease in birds, as opposed to the commensal *E. coli* that are normally present in the digestive tract of the birds or in the environment contaminated by faecal material. The detection of certain key virulence genes in an isolate indicates that it has pathogenic potential and can be reported as an APEC (14). These APEC specific genetic markers can be detected after isolation and purification of *E. coli* from clinical specimens by conventional bacteriological techniques, followed by extraction of the bacterial DNA from pure cultures and amplification of each target gene in a distinct PCR reaction. We have developed and applied a

multiplex PCR for confirming that isolates of *E. coli* from clinical cases are likely to be APEC. However this procedure is still quite tedious. We propose an alternative method for rapidly identifying APEC isolates directly on an agar plate, or in a blot of it, which should prove a valuable diagnostic tool in the farm.

Vaccination strategies for APEC:

Live APEC vaccines can be usually attenuated by the inactivation of a house keeping gene, such as *aroA*, and rely principally on inducing immune responses against the O antigen. Although most APEC isolates seem to fall into few serovars, mainly O1, O2 and O78 (7), untypable isolates are not rare, and the serotypic diversity of APEC is probably underestimated. These variations in the serotypes of APEC can reduce the efficacy of vaccines and restrict their potential market to few specific epidemiological or geographical situations. This is why we propose to develop a cross-protective vaccine by targeting APEC-specific virulence factors that are present in all serovars.

Using genomic information for the diagnostic and control of APEC:

All APEC strains carry a large virulence plasmid that enables the strain to colonise and persist in the respiratory tract of birds. Our team has sequenced the plasmid from an Australian APEC strain, E3. This plasmid, pVM01 (27) is very similar to those of the two US strains that have also been sequenced (12). Related plasmids are also found in other ExPEC isolated from humans (19, 23). Several genetic markers specifically located on the APEC virulence plasmid have been defined, by our team and by others (14), that can be applied to PCR diagnostic tests. We also have shown that there are four major clusters of genes present on the APEC virulence plasmid and we have established, by deletion of specific regions of the plasmid, which sections play a significant role in virulence (27, 28). Work from our team and from colleagues (5, 26) have shown that some transport systems play a central role in APEC virulence. Some of the proteins involved are located on the surface of the bacterial cell (generally in the outer membrane), and thus are likely to induce protective antibody, while some others are located inside the bacterial cell, and thus are unlikely to induce protective immunity, but could be the basis of attenuation as they control core functions required for the virulence. This information can be used to develop novel vaccine strains of APEC by deletion of genes that are important in virulence but that are unlikely to be immunogenic. Careful choice of the genes that are deleted can also result in enhanced immunogenicity.

Features of interest in bacterial iron uptake systems:

E. coli, like many pathogens, possess regulatory networks that control the expression of virulence genes by sensing environmental changes. One of these systems system controls iron homeostasis. This is important because iron is essential for life as an enzymatic cofactor, but it is also toxic, due to its ability to catalyse the production of reactive oxygen compounds. Iron is not easily bio-available for bacteria because it is mostly found in its oxidised form (ferric iron, Fe^{3+}) in the environment, or trapped by proteins that contribute to non-specific immunity in the host tissues. In *E. coli*, iron scavenging is mostly achieved by the release and/or the capture of a variety of small molecules, the siderophores, which have a high affinity for, and form complexes with, the iron present in the environment or in the host. These complexes are translocated into the bacterium in two steps. First, specialised outer-membrane receptors act as specific channels for their cognate siderophore-iron complex. Then, inner membrane transporters deliver iron in the cytoplasm (17). Two proteins, TonB and Fur, play a major role in this process (Figure 1).

- **TonB:** To transport the iron-siderophore complex through the outer membrane, the receptors require energy, which is provided by an inner-membrane protein, TonB coupled with an energy transfer system, ExbB-ExbD (21). TonB can interact with the siderophore receptor and allow the translocation of iron.

- **Fur**: Iron uptake is regulated by intracellular concentrations of Fe^{2+}, mainly through the repressor protein Fur (2). The Fur-Fe^{2+} complex, but not Fur alone, can specifically bind DNA sequences that are present in the promoters of the genes encoding siderophore synthesis and transport (as well as some other virulence factors). When the intracellular concentration of iron is high, the Fur-Fe^{2+} complex represses the expression of these genes, while at low concentrations of iron, Fur no longer associates with Fe^{2+}, the Fur regulon is de-repressed and the iron acquisition genes are expressed.

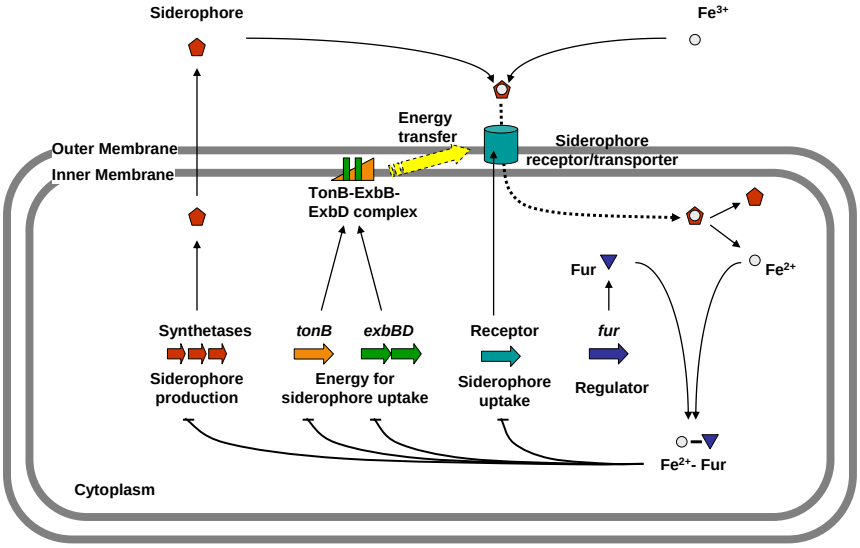

Figure 1 - Iron uptake in *E. coli.*
See text for details. NB: The orientation of the genes is arbitrary.

Enterobacteriaceae display several siderophore transport systems which are regulated by Fur (4) and require TonB for their activity (24). In addition, several transporters that import ferrous iron (Fe^{2+}), such as the Feo and Sit systems, or heme, such as the Shu (or Chu) system, can also be found. The *feo*, *sit* and *shu/chu* genes are regulated by Fur. The ShuA protein is a TonB dependent outer membrane receptor.

Specific Iron uptake systems targeted in APEC:

At least 9 siderophore uptake systems, 3 ferrous iron transporters and 1 heme transporter have been described in *E. coli*, but not all of them are present in the same strain (20). Most if not all *E. coli* produces a catecholate siderophore, the enterobactin, that can scavenge iron from the environment with high affinity. However in an infected animal this very hydrophobic molecule is unavailable for the bacterium as it is mostly bound to serum albumin or to a protein that participates to the innate immune system, the siderocalin (9). APEC and other pathogens produce two interesting siderophores, the Salmochelin and the Aerobactin. The Salmochelin is a modified version of the enterobactin that is not trapped by the siderocalin, and the Aerobactin is a hydroxamate siderophore that shows a superior affinity for iron at low pH. These alternative siderophores, along with their cognate receptors, contribute to the virulence of APEC strains and are required for the colonisation of the host. The *iroBCD* and *iucABCD* genes control the synthesis, whereas *iroN* and *iutA* genes encode the receptor, respectively, of Salmochelin and Aerobactin (see Figures 2 and 3). Sequence analysis of the genes encoding both systems suggests that the receptors IroN and IutA are both TonB dependent and that the siderophore production and uptake genes *iro* and *iuc* are negatively regulated by Fur. The *iroBCDN* and *iutA-iucABCD* are present on the virulence plasmid found in all APEC isolates.

Objectives:

We propose to use a quantitation method for APEC present in the air as a predictive diagnostic tool to control the disease. We also propose to use iron uptake systems as a way to attenuate the virulence

while increasing the immunogenicity of an APEC vaccine. Both parts of the project are conducted up to the **proof of concept stage**.

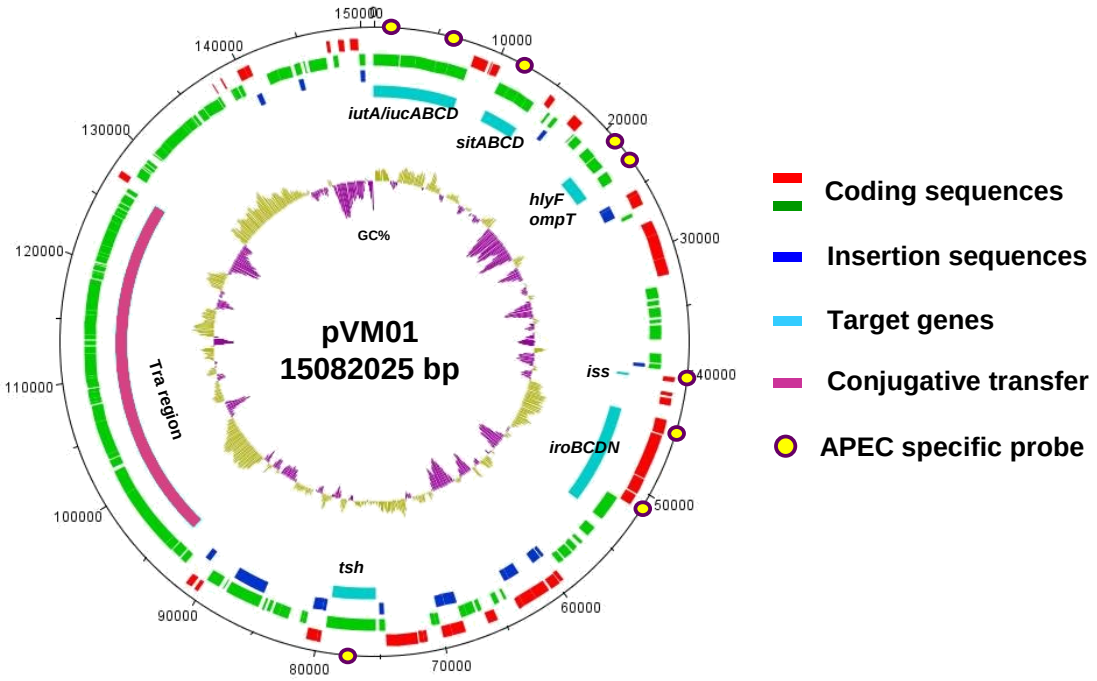

Figure 2 - Map of pVM01.

Detection and epidemiology:

For diagnostic applications, a series of DNA probes based on the APEC-specific virulence plasmid (Figure 2) are constructed and validated on a collection of known APEC isolates. These probes include *iro* and *iut/iuc* sequences and are used to detect and quantify APEC colonies on whole bacterial populations collected in the air of a poultry shed. Birds from the same shed are also collected for bacteriology diagnostic. *E. coli* isolates are confirmed to be APEC and are compared to the population isolated from the air.

Once validated, this tool can be applied in epidemiology studies to correlate the prevalence of the sub-clinical disease in the flock and the titre of APEC in the air. Ultimately it would be possible to predict the risk of outbreaks of colibacillosis by measuring the density of APEC relative to non pathogenic *E. coli* present in the air of the shed.

Vaccine design:

Most of outer membrane siderophore receptors are transmembrane proteins with a series of large loops protruding on the surface of the bacterium (3). These proteins are exposed on the bacterial cell surface and therefore they are likely to trigger a protective immune response. Some of them (IroN, IutA) are expressed by all APEC strains, irrespective of their serotype, and contribute to their virulence.

The functionnal inactivation of the virulence-specific iron uptake systems in an APEC isolate, should attenuate the strain and produce a live, cross protective vaccine. However, removing the ability to produce the siderophore membrane receptors would deprive the vaccine from two APEC-specific protective antigens. On the other hand, keeping the receptors while removing the ability to produce the Salmochelin and Aerobactin in the live vaccine would not prevent the strain to import these siderophores from co-infecting wild type APEC populations. Such a functional complementation would contribute to a reversion of the vaccine towards a pathogenic phenotype. Moreover, it would increase the intracellular iron and therefore lower the expression of the target antigens (the siderophore receptors) through Fur. Finally, the disruption of the Fur negative feedback loop can unlock the expression of outer membrane siderophore receptors and increase the immunogenicity of the vaccine,

but such a strain would be expected to remain virulent, as it has been previously demonstrated for other APEC vaccines (1).

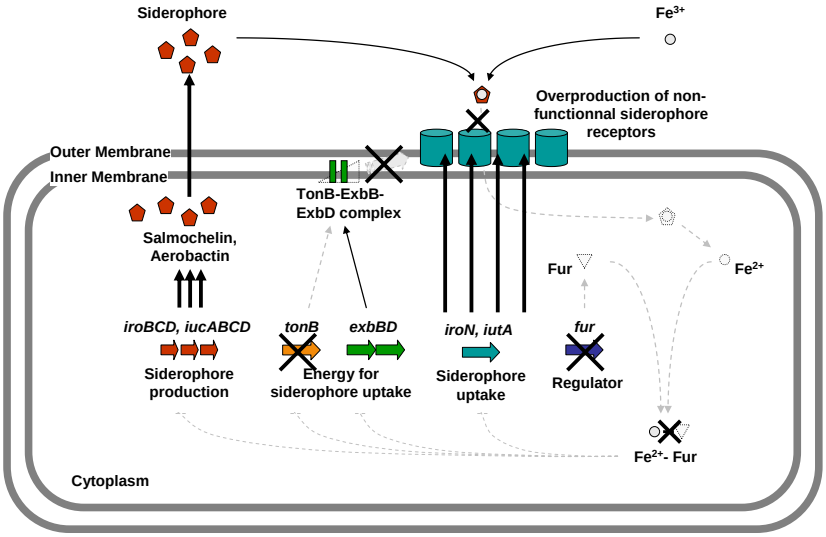

Figure 3 - Expected features of a *tonB-fur* double mutant in *E. coli*.
See text for details. NB: *iro* and *iut/iuc* genes are plasmidic, *tonB* and *fur* are chromosomal. The orientation of the genes is arbitrary.

To solve all these problems, we have designed an original strategy to generate our vaccine. In order to obtain an attenuated live vaccine against APEC, the *tonB* and *fur* genes are inactivated. A *tonB* mutant is expected to be impaired in iron uptake mediated by the Salmochelin (*iro*) and Aerobactin (*iut*) as well as by other TonB-dependent iron uptake pathways. A *fur* mutant is expected to over-express the siderophore receptors at the surface of the cells, so a *tonB*/*fur* double mutant is expected to produce large quantities of non-operational siderophore receptors. (see Figure 3).

Methodology:

Detection of airborne APEC:

APEC-specific DNA probes have been defined based on the sequence of APEC plasmid virulence characterised in our laboratory (28) and other recently published data (8, 14). The target sequences for the DNA probes are *iroB, iroN, iss, tsh, iucA, iutA, hlyF, ompT and sitA.* The PCR primers used to generate the probes are given in Table 1.

All target genes (see Figure 2) are located on the pVM01 virulence plasmid of APEC strain E3 (28) and are common to other APEC virulence plasmids. The probes are labelled by the non-radioactive system Dig from Roche. This Dig system is sensitive and does not cause a too high background noise with nylon membranes. It can be applied to the detection of target sequences in purified DNA (Southern blots) but also directly on colony blots prepared from plates. The probes are validated as being APEC-specific on a collection of pathogenic *E. coli* isolated from birds as well as other hosts (dogs, cats, horses). The APEC strains E3 and E956 are used as positive controls, and non pathogenic laboratory strain ER1821 (New England Biolabs), a derivative of *E. coli* K12, is used as a negative control. The minimal combination of probes that is the best predictor for APEC is determined.

In order to detect airborne APEC, air sampling is performed in controlled conditions (laminar flow hood, small poultry pens) for validation purposes, and then in field conditions. A poultry farm with known history of colibacillosis is selected for investigation. A portable air tester (Millipore M Air T) is placed in a shed at a pre-determined height above the ground and used to collect various volumes of air, ranging from 25L to 1000L. Airborne organisms are impacted onto the surface of an agar plate. Various selective agar formulations are tested with the aim to eliminate background contaminants and

to rapidly detect and differentiate *E. coli* colonies. For instance, MacConkey agar with lactose is a commonly used semi-selective medium that allows the visual detection of suspect *E. coli* colonies based on the ability of this organism to ferment lactose. As a result, *E. coli* colonies appear pink on the plate and can be distinguished from other species. Addition of inhibitors for Gram positive bacteria, such as crystal violet 0.3%, penicillin 50ug/ml or methicillin 50ug/ml contributes to reducing the background growth of contaminants. The plates are incubated at 37C for 18 hours to grow colonies. Plates showing 20 to 200 colonies are selected for colony blot experiments. Positively charged sterile nylon membranes are used to lift the material from the plate, which can be reincubated for further use if necessary. Meanwhile, the genetic material from the colonies that have been transferred onto the membrane is released, denatured and cross-linked to the nylon, following the Roche application manual. The first DNA Dig labelled probe is hybridised with this colony blot, washed in stringent conditions to eliminate excess probe, and positive colonies are detected and counted. The probe is then stripped from the membrane, and a second probe is applied. The nylon membrane can sustain several cycles of hybridization/stripping without noticeable loss of signal. Alternatively, replica plates can be generated by contact on sterile velvet cloth or by manual inoculation of colonies into arrays. These replicas are used in parallel with different APEC-specific probes. *E. coli* colonies from plated air sample that did or did not hybridise with APEC specific probes are picked, repurified, and tested with the panel of probes in order to confirm their status.

Table 1 PCR primers and APEC specific probes

Name	Primer sequence 5' → 3'	Target gene, function	Probe length	Reference
sitAF1 sitAR1	ACCAGCGTACTGGTAGTAAC GATGTCGCCAGATAATGCTC	*sitA,* ABC Mn+2/Fe+2 transporter, periplasmic substrate-binding protein	489 nt	This work
iroBF1 iroBR1	ATATGGCCTGGATCGACGTA GCGAAGTGAGCGATTGTTGA	*iroB,* Glycosyl transferase, Salmochelin siderophore synthetase	542 nt	This work
iroN F1 iroN R1	AATCCGGCAAAGAGACGAACCGCCT GTTCGGGCAACCCCTGCTTTGACTTT	*iroN,* Salmochelin siderophore receptor	553 nt	(14)
iucAF1 iucAR1	CTGCGCCTCGTCAATGGTAT ACCAACTCCGTCCGTACTCT	*iucA,* Aerobactin siderophore synthetase	549 nt	This work
iutA F1 iutA R1	AATACTATGGTCGCGGCATC CCCCTGCCTTTGTAGTCGTA	*iutA,* Aerobactin siderophore receptor	652 nt	This work
issF1 issR1	GCCGCTCTGGCAATGCTTAT CTGGCAGACATCGCTGAACA	*iss,* increased serum survival and complement resistance	403 nt	This work
Tsh1 fwd Tsh1 rev	GGTGGTGCACTGGAGTGG AGTCCAGCGTGATAGTGG	*tsh,* temperature sensitive hemagglutinin	640 nt	(8)
hlyF F1 hlyF R1	GGCCACAGTCGTTTAGGGTGCTTACC GGCGGTTTAGGCATTCCGATACTCAG	*hlyF,* Hemolysin F	449 nt	(14)
ompT F1 ompT R1	TCATCCCGGAAGCCTCCCTCACTACTAT TAGCGTTTGCTGCACTGGCTTCTGATAC	*ompT,* Outer membrane protease	496 nt	(14)

Birds are collected in the same poultry shed, euthanized by cervical dislocation and examined post mortem for gross pathology. Specimens for bacteriology are collected from lesions and pathogens are isolated and identified. If the pathogens are confirmed to be *E. coli*, one colony from each isolate is arrayed on a series of replica plates and corresponding colony blots are hybridised with the same series of probes as described above and sub-typed as APEC (if applicable).

The genomic DNA from both air borne and bird-associated *E. coli* isolates is extracted form pure cultures, digested with appropriate restriction enzymes and transferred onto a nylon membrane. This Southern blot is hybridized with the series of APEC specific probes in order to compare the virulence plasmid restriction profiles and investigate the relatedness between APEC isolated from the hosts and from the air.

APEC live Vaccines:

In order to reach the proof of concept stage, several labelled mutants (in which the gene of interest has been replaced by an antibiotic resistance cassette) are designed. These labelled mutants are easy to select for and to track in preliminary animal experiments. Depending on the results of safety and efficacy studies in birds, an unlabelled mutant (that has the same deletion but does not carry any antibioresistance cassette) can be designed. The features of the candidate vaccine mutants are summarised in Table 2.

Table 2 List of the mutants for the vaccine studies

Mutant	Parental strain	Genotype*
1	APEC E956	Δ*tonB*::Cm
2	APEC E956	Δ*fur*::Kn
3	APEC E956	Δ*tonB*::Cm / Δ*fur*::Kn
4	APEC E956	Δ*tonB*

*Chloramphenicol (Cm) and kanamycin (Kn) resistance cassette.

The target sequences for *tonB* and *fur* are derived from the APEC strain O1:K1:H7 genome project, genbank accession number NC_008563 (13). Primer sequences used in this part of the project are given in Table 3. The template for PCR amplifications is the genomic DNA extracted from the APEC strain E3 (11). The vaccine candidates are derived from the virulent APEC strain E956 (11).

Table 3 sequence of the primers used to generate the APEC mutants

Primer name	5' → 3' Sequence *	Sequence amplified
Kan F1+	TAGACTCGAGGGTTTTATGGACAGCAAGCG	Kanamycin resistance cassette
Kan R1+	CGAAGCTCGAGCTTTCATAGAAGGCGGCGGTGGA	
CAT F1+	GCATCTCGAGACGCACTTTGCGCCGAATAA	Chloramphenicol resistance cassette
CAT R1+	CCTCGGCTCGAGGAGTTGGTAGCTCAGAGAA	
tonBF1	AGCTGCTTCCAGCGACTCAA	*tonB* gene and flanking regions
tonBR1	CCGCCGATACCAATGCCAAT	
invtonBF1+	TCGCCTCGAGAACGTACAAATCCTCTCAGCCAAG	pGEM-T carrying the *tonB* region
invtonBR1+	CAGGCTCGAGTAGTTCAATAACCTGATGTACCG	
kchΔtonB F2	CGGAACAGTAAGCCAATGGA	APEC chromosomal *tonB* region; mutant screening
yciBΔtonB R1	GGTCGTCTTCGGTGGCTTGA	
furF1	CGCACCATTCACCATCTTCT	*fur* gene and flanking regions
furR1	AACTGACCGCTGAACGTGTA	
invfurF1+	CTGACTCGAGGGTTGTCCGGCTCCTGAAGA	pGEM-T carrying the *fur* region
invfurR1+	ATTGCCTCGAGGATGAGCACGCGCACGAAG	
fldAΔfur F2	TACGGCGAAGCGCAGTGTGA	APEC chromosomal *fur* region; mutant screening
ybfMΔfurR1	AGTACGCTGGCGCTGGCTAT	
FRT-Kan F1+	**TGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAG AGCGCTTTTGAAGCTT**TAGACTCGAGGGTTTTATGGACAGCAAGCGAACCG	Kanamycin resistance cassette with FRT sites for construction of unlabeled mutant
FRT-Kan R1+	**AAGCTTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTT CGGAATAGGAACTTCA**AGCTCGAGCTTTCATAGAAGGCGGCGGTGGAAT	

*Restriction enzyme sites are underlined, FRT recognition sequences are in bold.

Construction of labelled mutants:

The virulent APEC strain E956 is transformed with the temperature sensitive plasmid pKD46, which confers resistance to ampicillin and encodes arabinose-inducible versions of the *gam*, *bet* and *exo* genes from the phage lambda-red. The *gam* gene product prevents an endogenous *E. coli* nuclease from degrading linear DNA, the *exo* gene product is a 5'-3' exonuclease that generates ssDNA overhangs at the ends of linear DNA, and the *bet* gene product is a ssDNA binding protein that promotes annealing of complementary sequences. In presence of arabinose, these genes are expressed and the lambda red homologous recombination system is active. The amplicons for *tonB* or *fur* mutagenesis are cloned into the *E. coli* vector pGEM-T easy (Promega) and the resulting constructs are confirmed by restriction fragment length polymorphisms (RFLP) and DNA sequencing. A Chloramphenicol (Cm) or a Kanamycin (Kn) resistance gene is amplified from the plasmids pCNS or pKanK5, respectively. The internal region of the pGEM-T cloned insert, which corresponds to most of the *tonB* or *fur* gene, is replaced by a Cm or a Kn resistance cassette, respectively. Each of these new construct is used as a template to PCR-amplify a linear DNA fragment containing the desired antibiotic resistance cassette, surrounded by *tonB* or *fur* flanking regions. This PCR amplicon is electroporated into E956 carrying pKD46 and pre-induced by arabinose. The lambda-red system causes the insertion of the linear PCR product into the chromosomal target sequence by homologous recombination, creating the desired replacement/deletion mutant in strain E956. After incubation at non permissive temperature to remove pKD46, the mutants are selected by plating on agar with the appropriate antibiotic. Candidate mutants are screened by PCR with primers designed within the region surrounding the mutated portion of the chromosome. The difference of amplicon size between the parental and the mutated template allows the detection of mutants, which are confirmed by sequencing. The figures 4 and 5 outline the mutagenesis process.

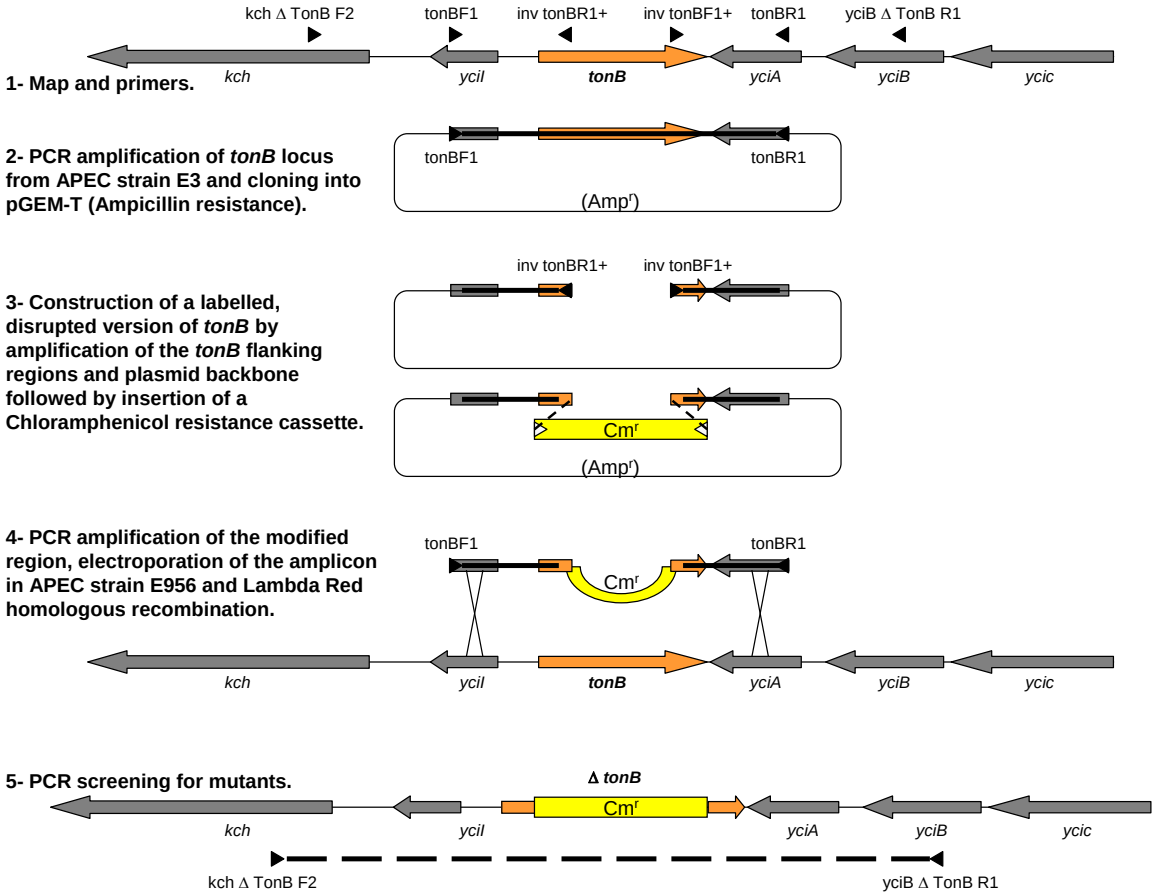

Figure 4 - Construction of a labelled tonB mutant

The double mutant is constructed by the sequential mutagenesis of the *fur* and the *tonB* genes. The *tonB* gene is insertionally inactivated as previously described in a E956 derivative with a validated *fur* mutant genetic background and double resistants are selected for.

Animal experiment:

Table 4 Vaccine safety experiment design

Group	*E. coli* Treatment Group	No. birds	Day					
			1			4	7	12
1	Non-inoculated	20	IBV	W	-	-	-	W, PM
2	Inoculated with E956 Δ*tonB*::Cm mutant	20	IBV	W	I	I	I	W, PM
3	Inoculated with E956 Δ*tonB*::Cm / Δ*fur*::Kn mutant	20	IBV	W	I	I	I	W, PM
4	Inoculated with *E.coli* wild type E956	20	IBV	W	C	C	C	W, PM

IBV= High dose IBV vaccine, I=Inoculate, C=challenge, PM=post mortem, - = no treatment, W= weigh

Table 5 Vaccine efficacy experiment design

Group	*E. coli* Treatment Group	No. birds	Day					
			1		18		26	
5	Non-vaccinated & non-challenged.	20	W	-	WB	-	WB	PM
6	Non-vaccinated & challenged with *E.coli* wild type E956	20	W	-	WB	**C**	WB	PM
7	Vaccinated with E956 Δ*tonB*::Cm mutant & challenged with *E.coli* wild type E956	20	W	**V**	WB	**C**	WB	PM
8	Vaccinated with E956 Δ*tonB*::Cm / Δ*fur*::Kn mutant & challenged with *E. coli* wild type E956	20	W	**V**	WB	**C**	WB	PM

V=vaccinate, C=challenge, PM=post mortem, - = no treatment, W= weigh, B= blood sample.

Each vaccine candidate safety and efficacy is assessed in animal experiments using a method previously developed (11). Birds are placed in an inoculation chamber connected to a double air flow and a nebuliser. The chamber is flushed with mixing air at a rate of 40 l/min for 3 min. An *E. coli* culture is placed in the nebuliser, the mixing flow is reduced to 25 l/min, and the nebuliser air flow is set at 15 l/min. Around 20 ml of culture is delivered in 20 min. The nebuliser air is then turned off and

the mixing air is delivered back a rate of 40 l/min for 5 min. The birds are then removed from the chamber. The nebuliser is washed in a sodium hypochlorite solution and cleaned with 70% ETOH; the chamber is nebulised with 70% ETOH for 3 min. The nebuliser is air dried, a new group of birds is placed in the chamber and the process is repeated. After the last group of birds is removed, the chamber and the nebuliser are washed with quaternary ammonium compound, rinsed and dried.

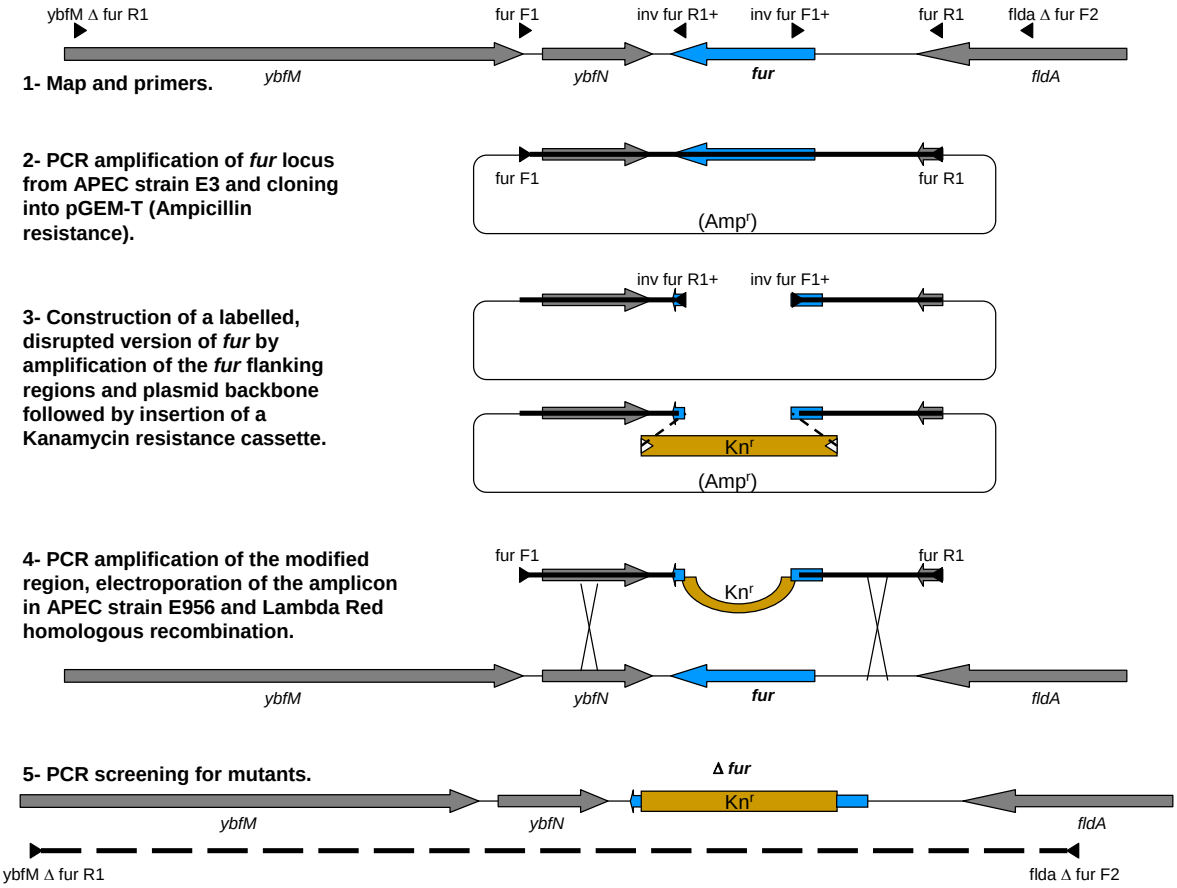

Figure 5 - Construction of a labelled fur mutant

The first experiment (see Table 4) investigates the safety of the *E. coli* knockout strains. One-day-old SPF chickens are inoculated by eye drop with ten times the normal immunizing dose of IBV vaccine strain VicS (a vial containing 5000 Doses is resuspended with 15 ml water; each bird receives 30ul). This dose causes no clinical signs in the birds but promotes colonization of *E. coli* of the airways. Groups of birds then receive an aerosol of 1-5 x 10^{10} cfu/ml of *E. coli* E956 wild type or E956 mutant, administered for 20 mins, at 1, 4 and 7 days of age. Control birds receive an aerosol of sterile nutrient broth. Any birds that do show signs of severe illness is euthanized immediately. Birds are monitored closely (twice daily) throughout the experiment until euthanasia at 11 days old. The second experiment (see table 5) investigates the efficacy of the *E. coli* knockout strains. Groups of one-day-old SPF chickens receive an aerosol of 1-5 x 10^{10} cfu/ml of an *E. coli* E956 mutants administered for 20 mins; control birds receive an aerosol of sterile nutrient broth. On day 18 all groups except the negative controls receive a challenge aerosol of 1-5 x 10^{10} cfu/ml of *E. coli* E956 wild type administered for 20 mins; negative control birds receive an aerosol of sterile nutrient broth prepared in the same conditions as the culture. Any birds that do show signs of severe illness is euthanized immediately. Birds are monitored closely (twice daily) throughout the experiment until euthanasia at 26 days old.

For the post mortem part of the experiment, the birds are euthanized with Halothane, weighed, and sampled for blood. Individual weight gains are calculated by the following formula: [weight at day 12 - weight at day 1] / [weight at day 12]. Birds are examined for gross pathology. Air sac lesions are scored as follows: 0 (no lesions); 1 (presence of small amount of foamy/frothy exudates or prominence of blood vessels); 2 (cloudiness and/or presence of fibrinous exudates); 3 (presence of caseous exudates); 4 (severe thickening of the air sacs with “meaty” consistency and presence of large amount of caseous exudates). For each bird, the Left Anterior Thoracic, Left Posterior Thoracic, Left

abdominal, Right Anterior Thoracic, Right Posterior Thoracic and Right abdominal air sacs are scored by the same pathologist in a single blind trial. Individual cumulative scores are calculated. For the efficacy experiment, the sex of the bird is also determined during the post mortem examination. Swabs for bacteriology are taken from the trachea and the Right Abdominal air sac. Each swab is cut with a sterile wire cutter and the tip is placed in a 1.8 ml Nunc tube containing 1 ml LB broth + glycerol (50% final); the tube is vortexed for 30 sec, then 100 ul are immediately transferred to a 96 microwell plate kept on ice. Except for the columns 1 and 7 which are empty, the wells contain 180 ul of LB broth. The Nunc tube is then placed in a dry ice-alcohol bath and stored at -80C. When the 96 well plate is completely filled, six 1/10 serial dilutions (20 ul into 180 ul transferred across half the plate) are made with a multichannel pipette and 10 ul of these dilutions are immediately spotted in triplicate on MCA, supplemented with (i) no antibiotics, (ii) Kan 20 ug/ml, (iii) Cm 6 ug/ml, or (iv) both, in large square petri dishes. Plates are incubated 18 h at 37C and titres of recovered bacteria are determined. Statistical analysis of the results is performed using Mann-Whitney two tailed test.

Construction of un-labelled mutants:

Derivatives of APEC strain E956 carrying a deletion in the gene of interest by insertion of an antibiotic resistance cassette are constructed using the lambda red system as described above. However, in these mutants the resistance cassette is flanked by two FRT sites that are recognised by the recombinase FLP. The thermosensitive plasmid pCP20, which confers resistance to ampicillin and encodes a thermally inducible FLP gene, is introduced in the mutants by transformation. At the inducing temperature, the FPL gene is expressed from the pCP20 which results in the excision and loss of the antibiotic resistance cassette. By the same operation, the plasmid is also eliminated, so the strain is sensitive to ampicillin again. The resulting mutant contains the deletion of the target gene and only a scar sequence instead of the antibiotic resistance gene. These mutants are expected to have the same properties as the labelled counterpart, but are suitable for a vaccine production as they do not carry antibiotic resistance genes.

Results:

APEC detection methodology:

Validation of APEC specific probes:

The PCR dig-labelled probes that are listed in Table 1 were tested on a collection of *E. coli* strains (Table 6) isolated at the Laboratory of Microbiology Diagnostic from clinical cases in chickens, dogs, cats and horses. Most of these cases correspond to extra intestinal infections such as urinary infections of septicaemia and are likely to be caused by ExPEC. Faecal isolates from healthy dogs and birds were also included as controls. The probes reacted preferentially with *E. coli* isolated from avian clinical cases but also with isolates from other host species (Table 7).

Table 6 collection of *E. coli* isolates

Species	Healthy	Non Avian	Avian
Avian	10		59
Canine	10	32	
Equine		10	
Feline		10	
Grand Total	20	52	59

However, most of the avian isolates reacted with more than 5 probes, whereas non avian isolates generally reacted with only 1 to 4 probes (Figure 6). In fact, the vast majority of the avian isolates reacted with the 9 probes (Figure 7), thus confirming that they belong to the APEC pathotype. There was a very good correlation between the presence of sequences that belong to the same operon or are known to be located closely to each other on the plasmid, such as: iroB-iroN, iucA-iut, hlyF-ompT.

The sitA probe was reacting with isolates from a wide range of hosts. The best combination of probes to predict that an isolate is an APEC appears to be iucA/iutA, hlyF/ompT and iroB/iroN.

Table 7 Probe reactivity with a collection of *E. coli* isolates from various hosts

Probe reactivity	clinical cases				Total Non Avian	Healthy Animals
	Avian	non avian				
		Canine	Equine	Feline		
sitA negative	4	6	5	2	13	9
sitA positive	55	26	5	8	39	11
iroB negative	5	11	6	4	21	12
IroB positive	54	21	4	6	31	8
iroN negative	5	9	6	4	19	12
iroN positive	54	23	4	6	33	8
iucA negative	12	25	6	9	40	13
iucA positive	47	7	4	1	12	7
iutA negative	12	25	6	9	40	13
iutA positive	47	7	4	1	12	7
hlyF negative	4	25	7	9	41	14
hlyF positive	55	7	3	1	11	6
OmpT negative	4	25	7	9	41	14
ompT positive	55	7	3	1	11	6
tsh negative	28	29	10	10	49	18
tsh positive	31	3			3	2
iss negative	4	5	6	3	14	9
iss positive	55	16	4	3	23	8
iss Weakly Pos.	0	11	0	4	15	3

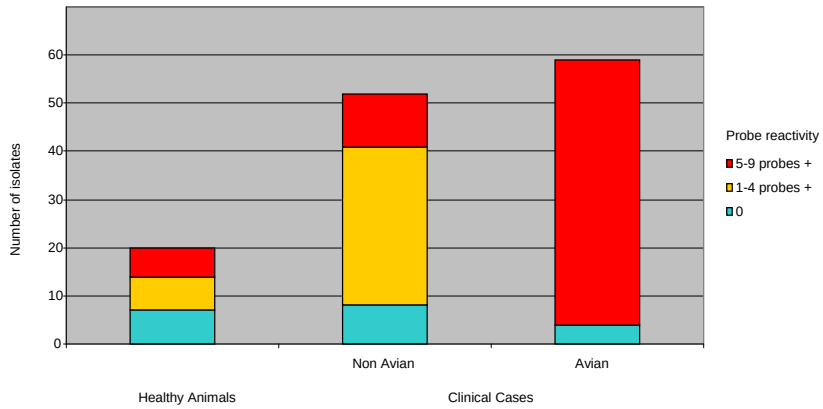

Figure 6 - Probe reactivity in a collection of *E. coli* isolates

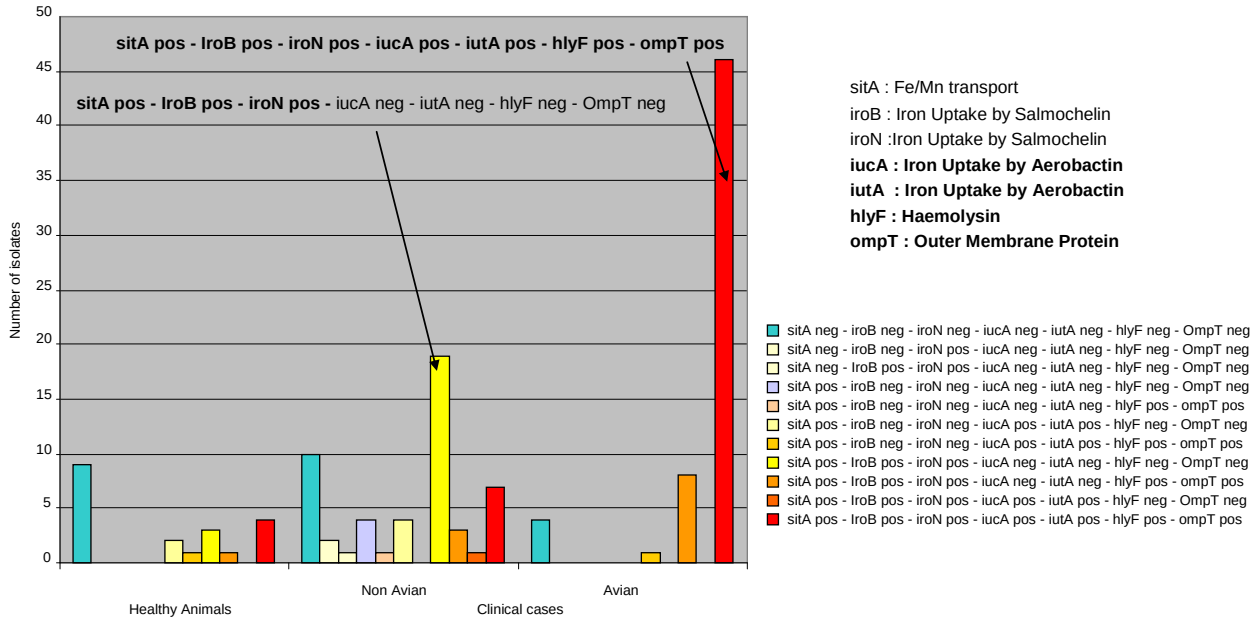

Figure 7 - Hybridization profiles for *E. coli* isolates

Screening of air samples for the presence of APEC:

The air sampling procedure was calibrated in poultry pens and tested in a farm shed, (tunnel type) housing approximately 40000 chickens aged 32 days. MacConkey plates supplemented with Cristal violet, Penicillin or Methicillin were used. The best condition for air sampling was determined to be MacConkey-penicillin, with the air sampler positioned 50 cm above ground. The volumes of air collected were 25, 50 and 100L. The total counts of colonies are summarised in Table 8.

To validate the APEC detection protocol, colony lifts were prepared from 3 plates, representing a total of 58 colonies recovered from a volume of 300L of air. The membranes were hybridised with the probe iroB in order to screen for the presence of APEC. A total of 12 colonies showing the phenotypic appearance of *E. coli* and giving a positive signal with the probe were detected. Six positive colonies were picked and purified from the plates, along with 5 colonies that did not produce any signal. The cultures were identified by conventional bacteriology. Five out of the 6 iroB positive colonies were confirmed as *E. coli*, and one as *Escherichia fergusonii*. Out of the 5 iroB negative colonies, 3 were identified as *Acinetobacter lwoffii* and two as *E. coli*. From the same shed, twelve chicken were collected, euthanized and examined for pathology. Six birds showed no visible lesions. The other birds displayed typical colibacillosis lesions with arthritis and pericarditis. Bacteriological cultures were performed from representative lesions of 5 birds, and 8 organisms were isolated as pure cultures and identified as *E. coli*. All cultures were assessed for the presence of APEC-specific sequences with the probes iroB (for confirmation of the screening results), iucA, hlyF and iss on colony blots. Results are summarised on Table 9. Overall, within the microorganisms present in the air of the shed, the non-APEC organisms (either non pathogenic *E. coli* or other bacteria) can be easily differentiated from the true APEC. Moreover, most of the APEC strains isolated from typical lesions in birds had the same reactivity profiles as the iroB-positive airborne APEC. Interestingly, a second population of APEC (iroB-negative, iss, iucA and hlyF-positive) was also present in the shed, and possibly might have been re-isolated from one bird (#9).

Table 8 Calibration of air sampling procedure

Volume sampled	Number of colonies (number of positive colonies with the iroB probe)								
	Medium:								
	Mac Conkey Penicillin			Mac Conkey Methicillin			Mac Conkey crystal violet		
	Height above ground:								
	30 cm	50 cm	1.5 m	30	50	1.5m	30	50	1.5m
25L	3	4	2	7	15	3	7	8	na
50L	2	2	2	17	13	0	11	10	0
100L	13 (5)*	3	0	25 (3)*	20 (4)*	0	13	15	0

* these plates were used to pick positive colonies for confirmation experiments

Table 9 APEC detection in a poultry farm

Origin		Organism	Features	Probe reactivity			
				iroB	iss	iucA	hlyF
Bird #02	**Pericarditis**	*E. coli*	Isolates collected from birds with **typical colibacillosis lesion**	+	+	+	+
Bird #06				+	+	+	+
Bird #07				+	+	+	+
Bird #02	**Arthritis**			+	+	+	+
Bird #06				+	+	+	+
Bird #07				+	+	+	+
Bird #10				+	+	+	+
Bird #09				**-**	+	+	+
Air from a poultry shed			Isolates screened by colony blot. **Positive signal with iroB**	+	+	+	+
				+	+	+	+
				+	+	+	+
				+	+	+	+
				+	+	+	+
		E. fergusonii		+	+	**-**	+
		E. coli	Isolates screened by colony blot. **No signal with iroB**	**-**	nd	+	+
				-	**-**	**-**	**-**
		Acinetobacter		-	-	-	-
				-	-	-	-
				-	-	-	-

APEC live Vaccines

A *tonB* mutation attenuates virulence in APEC strain E956:

Birds inoculated with the E956 Δ*tonB*::Cm or E956 Δ*tonB*::Cm/Δ*fur*::Kn mutant displayed weight gains and median lesion rates similar to non-inoculated control birds (table 10). At day 12, the mutants were re-isolated from the trachea but not the air sacs, whereas the wild type parental strain could be re-isolated from both sites.

The Mann-Whitney two tailed test was used to analyse the lesion scores. The individual cumulative air sac lesion scores were not significantly different between the birds inoculated with the E956 Δ*tonB*::Cm mutant and the non-inoculated control birds; moreover the scores were significantly different between the birds inoculated with the E956 Δ*tonB*::Cm mutant and birds inoculated with the virulent E956 parental strain (Table 11). The E956 Δ*tonB*::Cm/Δ*fur*::Kn double mutant displayed an intermediate phenotype between the wild type virulent parental strain and the avirulent E956 Δ*tonB*::Cm derivative. Birds inoculated with the Δ*tonB*::Cm/Δ*fur*::Kn mutant displayed a marginally significant difference from both control groups (non inoculated and inoculated with the virulent parental strain).

Table 10 Weight gains, air sac lesions and reisolation rates, safety study

Group	*E. coli* strain used (number of birds)	Titre of inoculum x10^{10} CFU/ml				Mean % weight gain	Rate of air sac lesion >0.5	Median air sac lesion score (range)	Re-isolation rates	
		Day 1	Day 4	Day 7	Day 12				Trachea	Air sac
1	Non inoculated (19)	-	-	-	Post mortem	176	2 / 19	0.0 (0 - 1.5)	0/19	0/19
2	Δ*tonB*::Cm (20)	1.09	1.17	1.27		185	2 / 20	0.0 (0 - 1.5)	12/20	0/20
3	Δ*tonB*::Cm/Δ*fur*::Kn (20)	2.35	1.14	1.35		184	7 / 20	0.5 (0 - 5.5)	10/18	0/18
4	E956 wt (14)	0.94	1.30	0.97		131	10 / 14	9.0 (0 - 15)	11/14	5/14

Table 11 Lesion scores air sacs, Mann-Whitney two tailed test, safety study

	E956 wt inoculated birds	Non inoculated control birds
E956 Δ*tonB*::Cm inoculated birds	The two samples are significantly different (P < 0.01, two-tailed test).	The two samples are not significantly different (P >= 0.05, two-tailed test).
Non inoculated control birds	The two samples are significantly different (P < 0.01, two-tailed test).	/

Vaccinated birds are protected against infection with the wild-type parental strain:

The weight gains were analysed globally (Table 12 and 13) or after separating data between the males and the females (Tables 14 and 15) as the growth rates are influenced by sex and the sex ratios were found, during the post mortem examinations, to be different between groups.

The weight gains and lesion rates of vaccinated birds challenged with the wild type strain are similar to the non-challenged control group but significantly different from non-vaccinated birds challenged with the wild-type strain.

The Mann-Whitney two tailed test was used to analyse the lesion scores. As expected, the non-vaccinated birds developed air sac lesions after being challenged with the wild type E956 strain, but not with sterile nutrient broth only. The individual cumulative air sac lesion scores were significantly different between the birds inoculated with the E956 mutants (Δ*tonB*::Cm or Δ*tonB*::Cm/Δ*fur*::Kn) and subsequently challenged with the virulent parental strain, compared to the non-vaccinated birds that were inoculated with the virulent E956 parental strain. The scores were not significantly different between the birds vaccinated with the E956 mutants (Δ*tonB*::Cm or Δ*tonB*::Cm/Δ*fur*::Kn) and subsequently challenged with the virulent parental strain compared to the non-vaccinated, non-challenged control birds. (Table 16).

Table 12 Weight gains; efficacy study

Group	treatment			Mean % weight gain Day 1-20 (range)	Mean % weight gain Day 20-26 (range)	Mean % weight gain Day 1-26 (range)	%males in the group
	Day 1	Day 18	Day 26				
5	Non-vaccinated	Not Challenged	Post mortem	478 (495_437)	20 (21 to17)	588 (614 to 527)	70
6	Non-vaccinated	Challenged (wt) 1.73 10^{10} cfu/ml		499 (510_472)	-6 (-5 to -10)	460 (479 to 415)	70
7	Vaccinated (Δ*tonB*::Cm) 1.09 10^{10} cfu/ml			396 (404_391)	23 (24 to 22)	507 (523 to 499)	35
8	Vaccinated (Δ*tonB*::Cm/Δ*fur*::Kn) 2.3 10^{10} cfu/ml			445 (465_434)	17 (17 to 17)	533 (556 to 521)	35

Table 13 Air sac lesions and reisolation rates; efficacy study

Group	treatment			Rate of air sacs lesion >0.5	Median air sac lesion score (range)	Re-isolation rates	
	Day 1	Day 18	Day 26			Trachea	Air sac
5	Non-vaccinated	Not Challenged	Post mortem	0/20	0 (0 to 0)	0/20	0/20
6	Non-vaccinated	Challenged (wt) 1.73 10^{10} cfu/ml		13/20	1 (0 to 10)	10/20	1/20
7	Vaccinated (Δ*tonB*::Cm) 1.09 10^{10} cfu/ml			1/20	0 (0 to 2)	14/20	0/20
8	Vaccinated (Δ*tonB*::Cm/Δ*fur*::Kn) 2.3 10^{10} cfu/ml			1/20	0 (0 to 2)	0/20	0/20

Table 14 MALES only: Weight gain; efficacy study

Group	treatment			Mean % weight gain Day 1-20	Mean % weight gain Day 20-26	Mean % weight gain Day 1-26
	Day 1	Day 18	Day 26			
5	Non-vaccinated	Not Challenged	Post mortem	495	21	614
6	Non- vaccinated	Challenged (wt) 1.73 10^{10} cfu/ml		510	-5	479
7	Vaccinated (Δ*tonB*::Cm) 1.09 10^{10} cfu/ml			404	24	523
8	Vaccinated (Δ*tonB*::Cm/Δ*fur*::Kn) 2.3 10^{10} cfu/ml			465	17	556

Table 15 FEMALES only: Weight gain; efficacy study

grp	treatment			Mean % weight gain Day 1-20	Mean % weight gain Day 20-26 (PM)	Mean % weight gain Day 1-26 (PM)
	Day 1	Day 18	Day 26			
5	Non-vaccinated	Not Challenged	Post mortem	437	17	527
6	Non- vaccinated	Challenged (wt) 1.73 10^{10} cfu/ml		472	-10	415
7	Vaccinated (Δ*tonB*::Cm) 1.09 10^{10} cfu/ml			391	22	499
8	Vaccinated (Δ*tonB*::Cm/Δ*fur*::Kn) 2.3 10^{10} cfu/ml			434	17	521

Table 16 Air sac lesion score, Mann-Whitney two tailed test; efficacy study

	Non-vaccinated, challenged	Non-vaccinated, non-challenged	Δ*tonB*::Cm/Δ*fur*::Kn vaccinated, Wild-type challenged
Δ*tonB*::Cm vaccinated, Wild-type challenged	The difference between the two samples is highly significant (P < 0.001).	The two samples are not significantly different (P >= 0.05).	The two samples are not significantly different (P >= 0.05).
Δ*tonB*::Cm/Δ*fur*::Kn vaccinated, Wild-type challenged	The difference between the two samples is highly significant (P < 0.001).	The two samples are not significantly different (P >= 0.05).	/
Non-vaccinated, non-challenged	The difference between the two samples is highly significant (P < 0.001).	/	

Discussion.

This objectives of this work were to provide a proof of concept for a methodology that would specifically detect and quantify airborne APEC in the farm, and for a live vaccine that would be safe, protective and easy to produce.

APEC detection:

Several DNA sequences that can serve as predictors for APEC diagnostic were validated and published by colleagues during the course of this project (14). However the presence of APEC specific sequences in a suspect isolate needs to be assessed by PCR, which requires conventional bacteriological purification and identification of the culture first. Our project shows that it is possible to accurately detect airborne APEC directly on a colony blot prepared from a plate inoculated with a given volume of air. More importantly, APEC colonies grown from the air of a shed appear to be identical to the virulent isolates found in the lesions of infected birds present in the same shed.

We originally planned to use a mixture of fluorescent probes to detect multiple APEC specific sequences simultanously on a single colony blot. Fluorescent DNA probes are commonly used on glass slide supports for *in situ* detection or microarray hybridization technologies. Different fluorescent signals can be analysed separately using filters corresponding to the appropriate wavelenght, and the results merged together. Unfortunately, the plate colony lifts are made on nylon membranes, a material that shows autofluorescence and therefore gives high background noise. Our attempts to obtain a satisfying signal-to-noise ratio with various DNA binding membranes carrying serial dilutions of fluorophores-labelled DNA probes have been unsuccessful so far. The autofluorescence of nylon can be suppressed by incorporation of carbon particles during the polymerization process, but these products are not commercially available. This prompted us to use Dig labelling instead of fluorescent probes, combined with a series of hybridization/stripping cycles. Although this approach was more time consuming it delivers essentially the same information. To overcome the time factor, we propose to collect a series of plates per given volume of air sampled, and hybridise each resulting colony lift separately with a probe. Because APEC strains usually harbour all the expected target sequences (see our results, table 9), each colony lift should contain ostensibly the same number of positive colonies with any of the probes. Therefore, the average number of positive colonies counted on a series of blots and separately hybridised with a single APEC probe, should approximate the titre of airborne APEC. As the air sampling itself takes only few minutes, this is a reasonable approach to generate rapid results. Overall, we have set up a robust, simple and specific detection test that can help to predict the risk levels for colibacillosis outbreaks. This test can also help epidemiologists to analyse risk factors and propose better air quality management at the farm level.

APEC vaccines:

Live attenuated vaccines are widely used to control animal infectious diseases. They are regarded as very efficient because they induce a high quality immune response in the host and are relatively simple to use and to produce. Live vaccines have two potential drawbacks: 1/ the induction of serovar-specific protection only, which can limit the application of the vaccine to relatively simple epidemiological situations, and 2/ the reversion to a virulent phenotype by the complementation of the attenuating mutation. An illustration of the first point is provided by a new colibacillosis live vaccine, based on an *aroA* deleted O78 strain of *E. coli*. Studies show that this mutant provides good homologous protection, but poor heterologous protection (15), suggesting that it is not likely to offer broad protection. We have therefore used another target present in all APEC serovars, the virulence-associated iron uptake systems. Regarding the second drawback, it must be noted that there are two types of complementation of a mutation, i.e. genetic and functional. In the genetic complementation, a wild type copy of the gene is acquired (by natural transformation, phage transduction or plasmid conjugation) and expressed in the mutant, thus reverting its phenotype. In the functional complementation, the mutant can use directly another protein or compound to compensate for the lack

(or the defect) of its own gene product. Both risks must be addressed in our case, because 1/ the virulence-associated iron uptake systems which we aim to inactivate in the vaccine are encoded by conjugative plasmids such as pVM01 that are likely to circulate through *E. coli* populations and 2/ it would still be possible, for a mutant defective in siderophore production, to uptake exogenous Salmochelin or Aerobactin from other co-infecting APEC populations. To avoid these problems we have used an original strategy by manipulating partner proteins that control the iron uptake systems in *E. coli*.

Attempts to target the iron metabolism for the production of a live APEC vaccine have been partially successful until now. A *fur* mutant that is expected to overexpress iron uptake systems in APEC has been developed (1), but it was shown to partially retain its virulence. Because of this, a second round of mutagenesis was designed to fully attenuate the *fur*-defective vaccine, but the resulting strain carried non-defined genetic modifications. As a consequence it is difficult to evaluate the risk of reversion to full virulence in this vaccine.

The mutations we used in our vaccine are proposed to increase the levels of expression of antigens that are present in APEC strains across all serogroups, and thus are predicted to generate cross-protective immunity that appears not to be offered by both the prior generations of colibacillosis vaccines, nor the *aroA* deleted vaccine. Not only our vaccine strain has a good cross-protection potential, but is also very safe. Instead of mutating the siderophore production genes themselves, we have targeted a protein, TonB, that is essential for the activity of siderophore transporters in *E. coli*, including IroN and IutA[1]. Moreover, *tonB* is located on the chromosome of *E. coli*, whereas the virulence associated genes for siderophore production and uptake (*iroBCDN*, *iucABCD-iutA*) are present on a conjugative plasmid. Therefore, our vaccine is unable to uptake iron through any TonB-dependent import system, even in presence of exogenous siderophores that would be produced by co-infecting APEC, or even after a conjugative transfer of a plasmid encoding virulence associated siderophore systems. Therefore, both the functional and the genetic complementation of the Δ*tonB* mutation are very unlikely. The added value of a *fur* gene mutation in our Δ*tonB* vaccine is more delicate to assess. Theoretically the *fur* mutation will boost the expression of non-functional siderophore receptors in the APEC vaccine, without impacting on its virulence since the *tonB* gene is disrupted. However, in a *tonB* mutant background, the level of intracellular iron is expected to be very low, particularly during the infection of the host, because the main ferric iron transporters are not functioning. This would automatically de-repress the *fur* regulon and the resulting situation would be very similar to a mutation in the *fur* gene. This hypothesis is in accordance with our efficacy testing, where the single Δ*tonB* vaccine gave the same protection as the Δ*tonB*-Δ*fur* strain. However our safety experiment suggests a slight increase of virulence in the *tonB-fur* double mutant, compared to the *tonB* mutant. This is not necessarily a problem because the virulence of a live vaccine must be evaluated in conditions (dose, route of inoculation... etc.) that differ from our safety test which is based on 3 successive nebulisations of birds pre-inoculated with a 10-times dose of IBV vaccine. Therefore, the utilisation of the Δ*tonB*-Δ*fur* vaccine in field conditions may give different outcomes, and in particular no deleterious effects. In practice, a slightly more "aggressive" vaccine strain may also show a higher rate of colonisation of the birds, and therefore a better protection. Moreover the residual virulence (if any) of a Δ*tonB*-Δ*fur* live vaccine could be controlled by introducing an additional attenuating mutation in a house keeping gene. To this respect, our team has already identified the oligopeptide transporter OppD as a good candidate for a second attenuating mutation. Constructing a double mutant in *oppD* and *tonB* would also make the vaccine safer by eliminating virtually all risk of genetic complementation, because the simultaneous acquisition of both a copy of *oppD* and a copy of *tonB* through natural genetic exchanges with wild type *E. coli* is extremely unlikely.

[1] It has not been formally demonstrated yet that IroN and IutA are TonB dependent receptors in the APEC strain E956. However, experimental evidence in other *E. coli* strains shows that the aerobactin receptor requires TonB; also, sequence analysis shows that IroN is homologous to FepA, a known TonB dependent siderophore receptor.

Implications:

Iron uptake in general, and some TonB-dependent siderophores uptake systems in particular, are known to play a the role in ExPEC virulence. Our work shows for the first time that the energy provider TonB is essential for APEC virulence and can be mutated to attenuate the virulence of an APEC strain. The attenuated phenotype of the *tonB* mutant is most likely caused by the loss of energy transfer to the siderophores receptors IroN and IutA.

For the poultry industry, the potential market for our colibacillosis vaccine is very large because the mutant, which is based on an Australian strain (E956, serotype O111:H-), expresses protective antigens common to all APEC from diverse geographical origins and serovars.

Our *E. coli* vaccine can also find other areas of application, for instance to prevent ExPEC infections in other host species, such as ruminants (mastitis vaccines) or dogs (pyometra vaccines). Specific and feasible models for these diseases have been developed, either in our laboratory or by some of our collaborators.

Bacterial delivery vectors are promising vaccinal strategies that have been essentially developed for few human applications and are mainly based on attenuated strains of *Salmonella*. Studies suggest that the foreign antigen is often less immunogenic than the vector, possibly because of the extra-metabolic burden imposed by the expression of the heterologous antigen (10, 16). In the poultry industry, recombinant avian bacteria have not yet been widely used as vaccinal delivery vectors for heterologous antigens, despite a considerable potential for new vaccine design. Our E956 Δ*tonB* vaccine has excellent potential as a delivery vector for other antigens. We further predict that foreign genes placed under the control of a Fur-regulated promoter will also be overexpressed in our strain, a feature that could be exploited to make polyvalent live vaccines. For instance the E956 Δ*tonB*-Δ*fur* APEC strain could be used as a delivery vector for the *Clostridium perfringens* specific toxin NetB, by placing the *netB* gene under the dependence of a Fur-regulated promoter, in order to produce protective antibodies against this virulence factor.

More importantly, the concept of combining a *tonB*-mediated attenuation of virulence with a *fur*-mediated overexpression of surface protective antigens constitutes a new and exciting platform technology that can be applied to other Gram negative pathogens of birds, such as *Campylobacter*, *Riemerella*, *Pasteurella*, *Avibacterium* and *Yersinia*.

In Gram positive bacteria, iron uptake also plays an important role in the pathogenicity of many species. The Fur protein is conserved in Gram positive bacteria, but TonB appears to be absent. However, some iron uptake features in Gram positives are reminiscent of the Gram negative systems. In particular, the Isd-family of conserved proteins, notably found in *Staphylococcus*, *Listeria* and *Clostridia*, can use heme from the host as a source of iron. Although it involves different proteins, the Isd system works in a fashion similar to the Gram negative siderophore transporters. Hemoproteins, such as haemoglobin, haptoglobin and hemopexin, bind various cognate surface receptors (IsdB, IsdH and IsdA) on the bacterium. Heme is then captured from the proteins, transported though the cell wall in a IsdC dependent manner and then transferred into the cytoplasm by the IsdDEF system. Interestingly, the genes encoding the surface receptors IsdB, IsdH and IsdA, as well as IsdC, are regulated by Fur. It is therefore tempting to mutagenise the *isdC* or *IsdDEF* genes in order to block the iron import, while overexpressing potentially protective surface antigens via a *fur* mutation.

Recommendations:

Before a Δ*tonB*-APEC vaccine is commercialised for the poultry industry, it will be necessary to confirm that:

1. the loss of *tonB* was the only genetic modification that occurred and resulted into attenuation of virulence in the single mutant vaccine,

2. the *tonB* mutant can protect chickens against colibacillosis when using a conventional route of vaccination,
3. the *tonB* mutant can protect against other APEC serovars.

The first point can be easily assessed by complementation experiments using the wild type copies of the *tonB* and *fur* genes, the second point by a comparative vaccination trial using nebulisation, eye drop and drinking water, and the third point by a heterologous challenge using previously characterised APEC strains with various serotypes (E3, serotype O nontypeable:H28; χ7122, serotype O78:K80:H9; MT78 serotype O2:K1:H+; TK3 serotype O1:K1:H7). Our team possesses the skills, equipments and reagents to carry out these experiments.

We also recommend to:

1. Introduce a second attenuating mutation in the APEC Δ*tonB* or Δ*tonB*–Δ*fur* strain. We propose to inactivate the *oppD* gene involved in oligopeptide import, making reversion to virulence virtually impossible, and test the new vaccine for safety and efficacy in chickens.
2. Commercialise the resulting vaccine, in collaboration with an industrial company. We have already established a partnership with Pfizer through an ARC linkage grant for the development of other vaccines, and we approached both the CRC and Pizer to discuss the feasibility of this project.
3. Develop Δ*tonB*/Δ*fur* vaccines in other Gram negative pathogens. We propose to validate this approach on relevant avian pathogens, including *Campylobacter sp, Salmonella sp, Pasteurella sp* and *Yersinia sp,* for which the *tonB* and *fur* genes have been described. In parallel, we propose to establish the full genome sequence of *Avibacterium sp* and *Riemerella anatipestifer* to identify their *tonB* and *fur* homologues and new, potentially protective antigens, for mutagenesis.
4. Apply a similar strategy to other Gram positive pathogens, by developing *isdDEF-fur* mutants. We propose to validate this approach on relevant avian pathogens, including *Staphylococcus* sp and *Listeria* sp.
5. Investigate the effect of the *fur* mutation on the protective immune response and the expression of *fur*-regulated proteins by monitoring the expression of several genes that belong to the Fur regulon (*iroN*, *iucA*) using RT qPCR.
6. Explore the virulence of ExPEC mutants during other extraintestinal infections, such as ruminant mastitis and canine pyometra. Specific models for these diseases have been developed in our laboratory or by some of our collaborators.
7. Assess the potential for use of these hyperimmunogenic ExPEC strains as the basis for partially purified inactivated vaccines for *in ovo* inoculation.
8. Develop antigen delivery systems based on the E956 Δ*tonB*-Δ*fur* APEC. Antigens from infectious bronchitis virus (IBV S and N proteins), *Clostridium* (NetB toxin), *Staphylococcus* (IsdA, B, H), *Listeria* (IsdA, B, H) are some of the potential targets to be delivered. More protective antigens from *Campylobacter, Pasteurella, Avibacterium, Riemerella* and *Yersinia* species should also be investigated.

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Plain English Compendium Summary

Project Title:	
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Objectives	
Background	The bacterium *Escherichia coli* is a microorganism normally present in the gut and the faeces of healthy animals, but it can also cause respiratory infections that are a significant burden for the poultry industry. In a broiler farm, a single episode of colibacillosis can cost several tens of thousands of dollars in loss of production. Not all *E. coli* can infect birds. This is because only the virulent strains of *E. coli* have the ability to scavenge iron from their host. Iron is essential for the metabolism of bacteria, so without a good supply of iron, *E. coli* is not able to grow in the host tissues. The occurrence of the disease depends on the quantity of virulent *E. coli* in the air. Therefore, it is important to specifically detect and quantify the virulent *E. coli* in the poultry shed, before they can cause problems. Because the management of air quality and other risk factors is difficult, a vaccine that protects the birds against the respiratory infection is also needed.
Research	We have shown that the presence and quantity of virulent *E. coli* in the air of a poultry shed can be determined by detecting specific iron capture systems that are found only in virulent *E. coli*. We have obtained an *E. coli* strain that can colonise the trachea of birds without causing the disease. This is because this strain is able to produce, but not to utilise, some of the proteins that capture iron from the host. This strain is a live vaccine in which the iron scavenging proteins are produced in large quantity (to produce a strong, protective immunity), but cannot fulfil their function (to make the vaccine safe).
Outcomes	Our test can differentiate between virulent and normal *E. coli* strains in the air of a shed. This information can help the farmer to set up particular hygienic measures or choose a vaccination program. The *E. coli* vaccine is safe, protective, and easy to produce.
Implications	This research can be applied to other diseases caused by bacteria, to generate safe and protective vaccines. The *E. coli* vaccine could also be used to deliver protective antigens against other microbes allowing to prevent several diseases with only one vaccine.
Publications	In preparation.