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The chicken as a natural model for extraintestinal infections caused by avian pathogenic *Escherichia coli* (APEC)

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ABSTRACT

E. coli infections in avian species have become an economic threat to the poultry industry worldwide. Several factors have been associated with the virulence of *E. coli* in avian hosts, but no specific virulence gene has been identified as being entirely responsible for the pathogenicity of avian pathogenic *E. coli* (APEC). Needless to say, the chicken would serve as the best model organism for unravelling the pathogenic mechanisms of APEC, an extraintestinal pathogen.

Five-week-old white leghorn SPF chickens were infected intra-tracheally with a well characterized APEC field strain IMT5155 (O2:K1:H5) using different doses corresponding to the respective models of infection established, that is, the lung colonization model allowing re-isolation of bacteria only from the lung but not from other internal organs, and the systemic infection model. These two models represent the crucial steps in the pathogenesis of APEC infections, including the colonization of the lung epithelium and the spread of bacteria throughout the bloodstream. The read-out system includes a clinical score, pathomorphological changes and bacterial load determination. The lung colonization model has been established and described for the first time in this study, in addition to a comprehensive account of a systemic infection model which enables the study of severe extraintestinal pathogenic *E. coli* (ExPEC) infections.

These *in vivo* models enable the application of various molecular approaches to study host–pathogen interactions more closely. The most important application of such genetic manipulation techniques is the identification of genes required for extraintestinal virulence, as well as host genes involved in immunity *in vivo*. The knowledge obtained from these studies serves the dual purpose of shedding light on the nature of virulence itself, as well as providing a route for rational attenuation of the pathogen for vaccine construction, a measure by which extraintestinal infections, including those caused by APEC, could eventually be controlled and prevented in the field.

1. Introduction

Avian pathogenic *Escherichia coli* (APEC), the causal organism of *E. coli* infections of poultry, are responsible for significant morbidity and mortality in the poultry industry worldwide [1,2].

Infection with APEC generally begins as a localized infection of the air sacs commonly referred to as airsacculitis or the air sac disease which in turn may spread to other internal organs resulting in systemic infection. This initial infection generally occurs in 4–9- week-old broiler chickens and in laying hens at the peak of egg production that takes place around week 30 [3]. Birds simultaneously infected with various combinations of infectious bronchitis virus (IBV) and other viruses inevitably suffer from a damaged respiratory tract, causing them to become increasingly susceptible to invasion by APEC.

Although to date, APEC is known to only infect poultry including chickens, turkeys, ducks etc., recent studies suggest the possibility of APEC being implicated in extraintestinal infections in humans as well [4–7]. Avian strains show many similarities with human extraintestinal pathogenic *E. coli* (ExPEC) strains, in that most of the virulence genes they possess are similar to those identified in uropathogenic *E. coli* and new-born meningitis causing *E. coli* (NMEC). Moreover, studies have also demonstrated that APEC strains could belong to the same clones as human ExPEC strains [6,8].

In order to study this emerging pathogen better, it is absolutely necessary to have an appropriate disease model of infection. The

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natural host for this pathogen is the chicken, which indeed serves as the best challenge model of infection. More important, however, are various factors that need to be taken into consideration when performing *in vivo* experiments. These include the types of chickens used, age and susceptibility of the chickens, infection dose and the model of infection. To analyse different aspects of pathogenesis, these factors play a significant role, in that they determine the final results of an infection experiment.

All studies with APEC mention different virulence assays or pathogenicity tests *in vivo* using a chicken infection model at some point or other. This infection model is mostly employed to study the virulence of a particular APEC strain, as well as to compare the wild-type strain with mutant strains lacking a certain gene or function. Even though the same general model or idea may be adopted, it tends to vary in various study areas. A model very often used is the subcutaneous inoculation or aerosol exposure of 1- day-old chickens with an infection dose between 10^7 and 10^{10} bacteria per millilitre [8–14]. The read-out includes LD_{50} determination, examination of animals for disease symptoms and lesion scores. On the other hand, numerous studies make use of 2 –3- week-old SPF chickens in order to assess the virulence and pathogenicity of APEC strains [12,15–22] by way of an air sac infection as seen in Table 1. It is hard to decide which model would most appropriately represent or mimic the natural mode of infection, pay heed to the immune status of the chicken and be ideally suitable for vaccination and challenge studies.

As already stated, initial bacterial infection of the chicken respiratory tract takes place between 4 and 9 weeks of age. In our study we report the use of 5- week-old chickens as an ideal challenge model for *E. coli*, by reproducing the natural infection process via the trachea for numerous purposes. These include virulence and

Table 1

Aim of the study	Infection dose	Infection route	Animals/age	Read-out system	Reference
Virulence assay	1:10 dilution of a 4-h culture (0.5 ml)	Subcutaneous	1-day-old chickens	LD_{50}; bacterial determination for a period of 7 days	[9]
Virulence assay	10^9 CFU/ml	Intra air sac (right caudal)	2-week-old SPF WL	LD_{50}; bacterial determination in blood 3 days p.i.	[15]
Serological study; localization of Fimbriae	(a) 10^8 CFU[a] (b) 10^7 CFU[a] (c) 10^8 CFU[a]	(a) Intra-tracheal (b) Intra air sac (c) Intra air sac	16-day-old axenic WL	(a,b) Serological testing over a 35/36-day period; (c) bacterial determination in tissue samples over a 48-h period; detection of fimbrial antigens in blood	[16]
Virulence assay	10^7 CFU/ml	Intra-peritoneal	BALB/c mice	Mortality over a 1-week period	[38]
Virulence assay	(a) 10^2,10^5, 10^8 CFU[a] (b) n.d.[a]	(a) Intra-tracheal (b) Aerosol	(a) 2-week-old WL (b) 1-day-old	(a,b) Organ lesion scores; re-isolation of bacteria for characterization	[39]
Immunity study; virulence assay	10^8 CFU/ml	Air sac	5-week-old WL	Mortality rates; organ lesion scores; Bacterial determination in organs	[40]
Virulence assay	10^{10} CFU in original culture[a]	Aerosol	1-day-old SPF WL	Examination for airsacculitis over an 11-day period	[11]
Virulence assay	(a) o.n. culture (0.5 ml) (b) 10^7 CFU	(a) Subcutaneous (b) Intra air sac	(a) 1-day-old WL (b) 3-week-old WL	Lethality classes; bacterial determination in organs 48 h p.i.	[10]
Immunity study; virulence assay	10^8 CFU/ml[a]	Intra air sacs	28-day-old broiler	Serological testing 28 and 38 day p.i.; organ lesion scores, Bacterial determination in organs	[41]
Virulence assay	10^7 CFU	Intra air sacs	3-week-old SPF WL	Bacterial determination in tissues 48 h p.i.	[17]
Virulence assay	10^7 CFU/ml	Intra air sacs	3-week-old SPF WL	Organ lesion scores; bacterial determination in organs 48 h p.i.	[18]
Virulence assay	o.n. culture (0.5 ml)	Subcutaneous	1-day-old WL	Mortality rates over a 5-day period	[42]
Virulence assay	(a) n.d.[a] (b) Bacterial swab from o.n. culture	(a) Aerosol (b) Skin scratch	(a) 17-day-old broilers (b) 3-week-old broilers	(a) Organ lesion scores 6 days p.i. (b) Organ lesion scores 10 days p.i.	[12]
Determination of vaccine potential of APEC mutants	10^7 CFU/ml	Subcutaneous	1-day-old broilers	Organ lesion scores over a 7-day period; bacterial re-isolation for agglutination tests	[13]
Comparison of different challenge models	(a) 10^7 CFU/ml (b) 10^7 CFU/ml (c) 10^9 CFU/ml[a]	(a) Intravenous (b) Subcutaneous (c) Intra-tracheal	(a) 20-day-old broilers (b) 3-week-old broilers (c) 20-day-old broilers	(a,b.c) Morbidity, mortality rates 6 days p.i.; bacterial re-isolation from cellulitis lesions and trachea	[19]
Virulence assay	n.d.[a]	Aerosol	1-day-old SPF WL	Examination for airsacculitis over an 11-day period	[14]
Identification of virulence-associated genes	10^8 CFU/ml	Intra-tracheal	5-week-old SPF WL	Bacterial determination in organs 48 and 72 h p.i.	[28]
Virulence assay	10^7 CFU	Intra air sac	3-week-old SPF WL	Bacterial determination in blood and organ lesion scores over a 48-h period	[20]
Virulence assay	5×10^6 CFU	Intra air sac	25-day-old SPF WL	Organ lesion scores 48 h p.i; bacterial determination in blood	[43]
Colonization ability of APEC Fimbriae	10^8 CFU/ml[a]	Intra-tracheal.	2-week-old axenic WL	Bacterial determination in organs 6 days p.i.	[21]
Virulence assay	o.n. culture (approx. 10^8 CFU/ml)	Subcutaneous	1-day-old SPF chickens	Mortality rates 4 days p.i	[8]
Virulence assay	(a) 500 CFU (b) 10^9 CFU/ml	(a) Allantoic cavity (b) Via urethra	(a) 12-day-old eggs (b) CBA/J mice	(a) Embryo deaths over a 4-day period (b) Bacterial determination 48 h p.i.	[22]
Virulence assay	(a) 500 CFU (b) 10^7 CFU/ml	(a) Allantoic cavity (b) Subcutaneous	(a) 12-day-old eggs (b) 1-day-old chickens	(a) Embryo deaths (b) Organ lesion scores over a 7-day period	[6]

CFU, colony forming unit; o. n., overnight; WL, White Leghorn chickens.
[a] Pre-infected with infectious bronchitis virus (IBV).

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pathogenicity assays, comparison of different wild-type and mutant strains, *in vivo* identification of novel virulence factors using genetic methods, like signature-tagged transposon mutagenesis (STM), vaccination and immunization studies and, essentially, studies involving the molecular interactions between the host and the pathogen. We report for the first time, in extensive detail, a new chicken lung colonization model of infection, which can be used to study bacterial attachment and colonization during the initial stages of infection, besides a systemic infection model which can be widely used to study infection by ExPEC.

2. Results

2.1. Virulence gene and phylogenetic typing of avian Escherichia coli strains

Out of several virulence-associated genes tested (*afa/draB*, *bmaE*, *csgA*, *fimC*, *focG*, *gafD*, *hrlA*, *iha*, *mat*, *nfaE*, *papAH*, *papC*, *papEF*, *papG*, *sfaS*, *tsh*, *chuA*, *fyuA*, *ireA*, *iroN*, *irp2*, *iucD*, *iutA*, *sit ep.*, *sit chr.*, *cvi/cva*, *iss*, *neuC*, *kpsMTII*, *ompA*, *traT*, *astA*, *cnf*, *sat*, *vat*, *hlyA*, *ibeA*, *gimB*, *tia*, *pic*, *malX* and *pks*) [7], IMT11327 only harbours adhesion-related genes *fimC* and *csgA*, and serum resistance gene *ompA*. In contrast to that the outbreak strain IMT5155 possesses a broad range of virulence-associated genes typical of animal- and human-derived extraintestinal pathogenic *E. coli* (ExPEC) strains, including adhesion genes *csgA*, *fimC*, *tsh*, and *mat*, iron-acquisition genes *chuA*, *fyuA*, *ireA*, *iroN*, *irp2*, *iucD*, *iutA*, *sit ep.*, and *sit chr.*, serum resistance and protectin genes *iss*, *neuC*, *ompA*, *traT*, toxin gene *vat*, invasion-related genes *ibeA* and *gimB*, as well as *malX* and the ColV operon genes *cvi/cva*). Based on rapid phylogenetic grouping described by Clermont et al. (2000), IMT11327 belongs to phylogenetic group B1. MLST analyses classified the strain into sequence type 295. In contrast, virulent strain IMT5155 belongs to phylogenetic group B2 and sequence type 140, which is allotted to sequence type complex 95.

2.2. Systemic model of APEC infection

Avian systemic infection was successfully reproduced using strain IMT5155 in 5- week-old immunocompetent chickens by way of an intra-tracheal infection mimicking the natural infection process to a great extent. To assess the severity of disease, a clinical score taking respiratory and general symptoms into account, was established as seen in Table 2. At 24 h post infection, the average clinical score among chickens infected with IMT5155 was found to be 2.6, which included a range of score 1–4. Two of the 15 chickens in this group were killed on account of severe clinical symptoms 20 h after infection. At 48 h post infection, the mean clinical score was 1.4 ranging between score 0 and 2. In contrast to the pathogenic virulent strain, non-pathogenic strain IMT11327 did not cause any clinical symptoms, representing a consistent score of 0 at both 24 and 48 h after infection.

Table 2
Description of clinical scores

Score	Respiratory symptoms	Systemic symptoms
0	None	None
1	Light breathing with slight movement of the chest	Ruffled feathers; alert
2	Heavy breathing with increased movement of the chest	Ruffled feathers; less reaction to acoustics
3	Heavy breathing with open beak and increased movement of the chest	Aloof; ruffled feathers; no reaction to acoustics; lesser balance; closed eyes
4	Secretion of oedema by exerting pressure on the chest; head/neck move upwards to facilitate breathing	Aloof; ruffled feathers; no reaction to acoustics; closed eyes; reduced consciousness

Table 3
Description of organ lesion scoring modified after Ginns et al., and Bree et al. [39,44]

Organ	Score	Description of organ lesions
Thoracic air sacs	1	Slight opaque and/or thickened membranes ± slight amount of fibrin
	2	Moderate opaque and/or thickened membranes ± moderate amount of fibrin
	3	Severe opaque and/or thickened membranes ± severe amount of fibrin ± moderate to severe vascularization)
Lung	1	Single small lesion, locally restricted (1/5 of the organ)
	2	Multiple, locally restricted small lesions and/or one bigger lesion (2/5 of the organ)
	3	Lesions cover about 1/2 of the organ
	4	Lesions cover about 1/2 of the organ
	5	Complete organ covered with lesions
Heart and pericardium	1	Vascularization, opacity
	2	Excessive or cloudy fluid in the pericardial cavity
	3	thickened pericardium, acute pericarditis
Liver	1	Decolourization and/or slight amounts of fibrinous exudates
	2	Marked perihepatitis with high amounts of fibrinous exudates
Spleen	1	Congestion

Severe pathomorphological changes, scored according to organ lesions documented in Table 3, were exclusively seen in chickens infected with the virulent strain with an average score of 7.5 and 4.6 at 24 and 48 h post infection respectively (Table 4). Macroscopic lesions typical of systemic APEC infection included airsacculitis, pneumonia, pericarditis, perihepatitis, and splenomegaly as depicted in Figs. 1–3. On the contrary, non-pathogenic strain IMT11327 only induced pathomorphological changes with an average score of 2.8 and 2.0 at 24 and 48 h respectively. These data clearly show that the average organ lesion scores 24 h post infection for all organs infected with IMT5155 were significantly higher as compared to those seen in chickens infected with the non-pathogen. Alternatively at 48 h post infection, the organ lesion score was reduced in chickens infected with pathogenic IMT5155, however it remained relatively constant in chickens infected with the non-pathogenic strain.

In order to determine bacterial dissemination in internal organs, CFU values were calculated as described in Section 4. At 24 h post infection, chickens infected with both wild-type IMT5155 and non-pathogenic IMT11327, were found to have different amounts of bacteria in the lungs up to 10-fold. It was further observed that bacterial spread to internal organs was reduced on average in chickens infected with IMT11327, with a decline in the bacterial count of more than 1000-fold in the spleen, kidneys, heart and liver (Table 5), and complete absence in the brain. Bacteria were isolated from virtually all chickens infected. In some cases however, bacteria were isolated from only 65–90% of chickens in a group.

Table 4
Induction of inflammatory lesions in respiratory and other organs after infection with APEC and non-pathogenic *E. coli* strain

Strain	Mean lesion scores[a,b]					
	Air sacs	Lung	Heart	Liver	Spleen	All organs
24 h p.i.						
IMT 5155	2.0 ± 0.6	2.8 ± 0.9	1.4 ± 1.1	0.4 ± 0.5	0.9 ± 0.3	7.5 ± 2.2
IMT 11327	0.6 ± 0.6	1.4 ± 1.1	0.1 ± 0.3	0.1 ± 0.3	0.6 ± 0.5	2.8 ± 1.9
48 h p.i.						
IMT 5155	1.2 ± 0.8	1.4 ± 1.1	0.8 ± 0.8	0.2 ± 0.4	1.0 ± 0.0	4.6 ± 2.7
IMT 11327	0.6 ± 0.5	0.8 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.5	2.0 ± 0.7

$n = 15$ chickens/strain 24 h p.i. ; $n = 5$ chickens/strain 48 h p.i.
[a] Score values for severity of organ lesions ± standard deviation.
[b] The differences in organ lesion scores between IMT5155 and IMT11327 were significant 24 h after infection ($p < 0.01$) as determined by Student's *t*-test.

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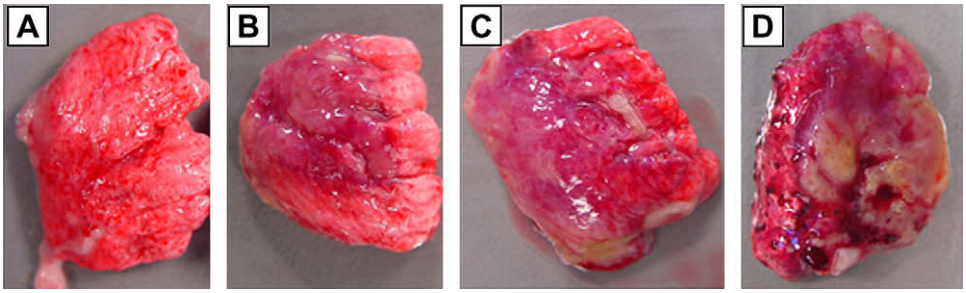

Fig. 1. Pathomorphological changes of the lung 24 h after infection with APEC strain IMT5155; (A) score 0; (B) score 2; (C) score 3; (D) score 4.

At 48 h post infection, considerable differences were seen between chickens infected with IMT5155 and non-pathogen IMT11327. In chickens infected with IMT11327, a 100-fold bacterial decrease was seen in the lung as compared to bacterial loads found in the lung 24 h after infection with this strain, while other organs were devoid of any bacteria. Chickens infected with the pathogenic strain showed only a 20-fold decrease in the number of bacteria on average in all organs 48 h post infection with IMT5155.

2.3. Lung infection model

A lung model of infection was also effectively set up for further studies. Due to the low doses of infection that dominated this infection model, the chickens did not show any noticeable disease symptoms. On examination of internal organs, engorgement of the lung, that is, hyperaemia or congestion was observed. This is the first stage in the pathology of the disease. No other organs showed any pathomorphological signs of infection. A mean lung organ score of 2 and thoracic air sac score of 1 was determined for 95% of the 20 chickens infected. The spleen of infected chickens did not show any major clinical changes, aside from a mild swelling in a few cases.

At 24 h post infection, wild-type strain IMT5155 was isolated from the lung in almost all chickens for all of the four doses in decreasing numbers (Table 6). It was possible to isolate bacteria from the spleen in negligible amounts only with an infection dose of 10^6 CFU but these values do not correspond with the values of the systemic infection. Viable bacterial counts were significantly reduced in the lung 48 h post infection up to 30-fold, while no bacteria were isolated from the spleen at lower infection doses.

When chickens were infected with non-pathogenic strain IMT11327, bacteria were isolated from the lung 24 h post infection in reduced amounts as compared to the wild-type pathogen, whereas no bacteria were isolated from the spleen. Results were reproducible for the pathogenic strain and could well be compared with the non-pathogenic strain. IMT11327 was found to colonize the lung to a much lesser extent than IMT5155 (Table 6) accounting for more than a 100-fold decrease in colonization.

2.4. Histological examinations

Microscopic observation of histological sections of chicken organs infected with APEC strain IMT5155 were carried out as described in Section 4. Fig. 4A shows a semi-thin section of a mock-infected chicken lung with air capillaries free of any inflammatory cells corresponding to an organ lesion score of 0. As is typical of APEC infection, examination of the lung after infection showed heterophil infiltration and exudation within the air capillaries (Fig. 4B) [23]. Septa were thickened by the inflammatory reaction, while primary defence cells, oedema and the presence of bacteria were observed within the air capillaries, additionally hyperaemia was observed, all amounting to an organ score of 2. In severe infection with APEC, extreme pathophysiological alteration of the lung tissue was seen, analogous to a score of 4, whereby the lumina of air capillaries, usually seen clearly, were almost entirely absent (Fig. 4C). These visual observations related strongly to clinical observations as well as bacterial counts in the lung.

Another important feature of systemic infection is perihepatitis wherein early microscopic changes seen were intra- and peri-vascular as well as parenchymal lymphatic infiltrations in the liver tissue (Fig. 5). Infection of the spleen and kidney is also characteristic of systemic infection where microscopic changes such as presence of inflammatory cells in the spleen (Fig. 6B), and pyknosis in the tubular epithelium as well as enlargement of the Bowman's capsule were observed in the infected kidney (Fig. 7B).

All tissue sections examined depicted a range of organ scores in accordance with clinical observations during infection and macroscopical lesions seen after infection.

3. Discussion

Infection by the extraintestinal pathogen APEC was successfully reproduced in 5- week-old chickens using a highly virulent wild-type strain IMT5155 (O2:K1:H5). This strain belongs to multi locus sequence type 95 complex, to which most ExPEC are allotted. This phylogenetic group is an excellent representative of the APEC population according to current knowledge [24,25]. In the field,

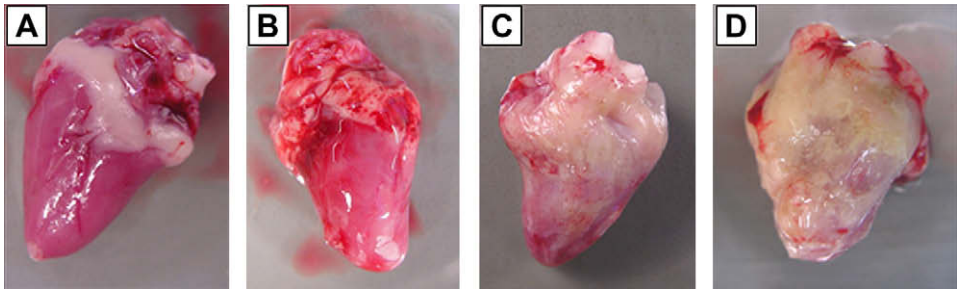

Fig. 2. Pathomorphological changes of the heart 24 h after infection with APEC strain IMT5155; (A) score 0; (B) score 1; (C) score 2; (D) score 3.

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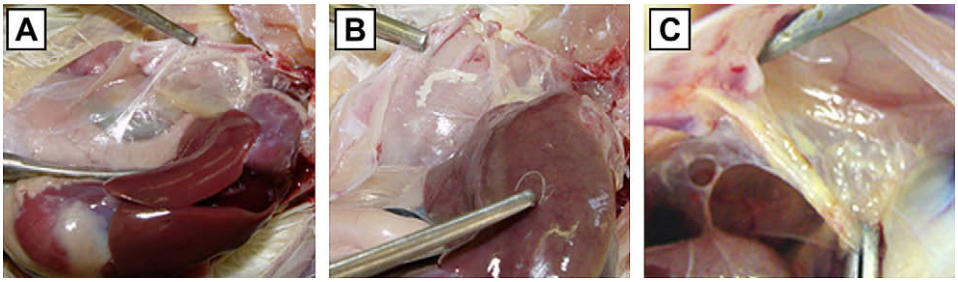

Fig. 3. Pathomorphological changes of thoracic air sacs 24 h after infection with APEC strain IMT5155; (A) score 0; (B) score 1; (C) score 3.

respiratory tract infection with APEC produces lesions which include airsacculitis with a serous to fibrinous exudate. In adult birds, an acute form of systemic infection may appear [2]. Our data show that it is indeed possible to induce the actual form of the disease including clinical and respiratory symptoms, using a well chosen virulent APEC field strain, without prior infection with viruses or other agents, for example, infectious bronchitis virus (IBV), that predispose chickens to bacterial infections as often seen in the literature (Table 1). This suggests that predisposition of the animal to APEC infection is not essential for effectively reproducing infection caused by APEC in immune competent chickens.

Thus, Koch's postulates have been fulfilled for APEC in our study, in that the wild-type pathogen originally isolated in an outbreak of APEC infection in the field could cause disease in healthy chickens infected with the cultured strain, as well as finally being re-isolated from experimentally infected animals. Therefore, strain IMT5155 is a *bona fide* avian pathogenic *E. coli* strain.

An important aspect pertaining to virulence studies is the inoculation route that plays an undeniable role in the infection process. APEC infection in poultry begins as a respiratory infection whereby the ideal way of reproducing the disease would be to imitate the natural infection route. In this study, chickens were infected via the trachea representing a near to natural mode of infection. In previous studies, several approaches involving various routes of inoculation and different predisposing agents were used to experimentally reproduce *E. coli* respiratory disease in chickens (Table 1). However, most of these studies generally employ infection routes bypassing the upper respiratory tract, which is the natural route of entry for APEC. Therefore the primary host defence mechanisms, including aerodynamic filtration, mucociliary clearance and phagocytosis, are not accounted for [26].

Without a doubt the age of the chickens plays a major role in experimental infections. More than 95% of all infection studies carried out to date were performed on chickens younger than 5 weeks of age (Table 1), overlooking the immune competence of the birds. The chicken lung is the major target organ for viral or bacterial poultry diseases. Most outstanding is the presence of the bronchus-associated lymphoid tissue (BALT) in the lung. BALT nodules are absent in newly hatched birds, but gradually mature from 5 to 8 weeks of age. They are organized into B and T cell areas and are responsible for most of the immunity of the chicken [26]. Although immunity starts developing towards the end of embryonic life, immune responses of newly hatched chicks are relatively poor, and the major part of its defence relies on passively acquired maternal antibodies. This factor plays a crucial role in the vulnerability of young chickens to infection and disease.

Taken together, four significant factors, namely choice of the wild-type strain, predisposition of chickens to infection, route of inoculation and age of experimental animals were given ample importance in this study.

Infection with non-pathogenic strain IMT11327 (Ont:H16) in the systemic infection model, showed contrasting results when compared with the APEC wild-type strain as would be expected. These results were consistent for all infection trials. IMT11327 therefore serves as an appropriate negative control *in vivo* which enables a better analysis of the severity of disease by way of comparison.

A modified model of infection based on the systemic infection model was established to induce effective colonization of the chicken lung, but simultaneously no systemic infection. This was possible, in that the infection dose was reduced up to 1000-fold. Various infection doses were experimented with, leading to the selection of a dose, which would not only enable colonization of the avian lung by APEC, but would also be the ideal infection dose for further experimental purposes. After several trials, an infection dose of 10^6 CFU and a time point of 24 h post infection were selected.

Initial stages of APEC infection pathogenesis, namely early adhesion and colonization, could be targeted in the lung

Table 5
Ability of pathogenic strain IMT5155 and non-pathogenic strain IMT11327 to colonize respiratory organs and to invade internal organs 24 h after intra-tracheal infection of chickens with 10^9 bacteria

Strain	Localized infection						Systemic infection					
	Lung		Spleen		Kidney		Heart		Liver		Brain	
	A	B	A	B	A	B	A	B	A	B	A	B
24 h p.i.												
IMT 5155	15/15	$5.8 \times 10^7 \pm 7.2 \times 10^7$	15/15	$5.3 \times 10^5 \pm 9.5 \times 10^5$	15/15	$9.0 \times 10^6 \pm 2.5 \times 10^7$	15/15	$7.5 \times 10^6 \pm 1.6 \times 10^7$	13/15	$2.5 \times 10^5 \pm 4.8 \times 10^5$	10/15	$4.7 \times 10^3 \pm 1.6 \times 10^4$
IMT 11327	14/15	$8.3 \times 10^6 \pm 2.4 \times 10^7$	8/15	$6.5 \times 10^1 \pm 1.8 \times 10^2$	13/15	$3.6 \times 10^3 \pm 7.1 \times 10^3$	5/15	$1.2 \times 10^2 \pm 3.5 \times 10^2$	6/15	$2.4 \times 10^2 \pm 8.0 \times 10^2$	0/15	0
48 h p.i.												
IMT 5155	5/5	$1.9 \times 10^7 \pm 2.5 \times 10^7$	4/5	$2.4 \times 10^4 \pm 2.2 \times 10^4$	4/5	$2.8 \times 10^4 \pm 4.7 \times 10^4$	4/5	$5.7 \times 10^4 \pm 8.3 \times 10^4$	4/5	$1.9 \times 10^4 \pm 2.8 \times 10^4$	3/5	$3.1 \times 10^2 \pm 3.9 \times 10^2$
IMT 11327	5/5	$6.7 \times 10^4 \pm 1.3 \times 10^4$	0/5	0 ± 0	0/5	0 ± 0	0/5	0 ± 0	0/5	0 ± 0	0/5	0 ± 0

A: presence of *E. coli* in chickens representative of a single experimental infection; B: mean no. of bacteria in organs (CFU). $n = 15$ chickens/strain 24 h p.i. ; $n = 5$ chickens/strain 48 h p.i.

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Table 6
Localized infection with pathogenic strain IMT5155 and non-pathogenic avian strain IMT11327

	Infection dose 10^6 CFU		Infection dose 10^5 CFU		Infection dose 10^4 CFU		Infection dose 10^3 CFU	
	A	B	A	B	A	B	A	B
24 h p.i.								
Lung	$5.9 \times 10^5 \pm 6.5 \times 10^5$	$4.8 \times 10^3 \pm 4.1 \times 10^3$	$3.4 \times 10^4 \pm 4.1 \times 10^4$	n.d.	$2.2 \times 10^3 \pm 1.9 \times 10^3$	n.d.	$3.7 \times 10^2 \pm 5.2 \times 10^2$	n.d.
Spleen	$4.5 \times 10^1 \pm 7.1 \times 10^1$	$1.0 \times 10^1 \pm 6.7 \times 10^0$	0±0	n.d.	0±0	n.d.	0±0	n.d.
48 h p.i.								
Lung	$2.1 \times 10^4 \pm 1.8 \times 10^4$	n.d.	$2.3 \times 10^3 \pm 1.5 \times 10^3$	n.d.	$6.3 \times 10^2 \pm 1.6 \times 10^3$	n.d.	$3.0 \times 10^3 \pm 7.9 \times 10^3$	n.d.
Spleen	0±0	n.d.	0±0	n.d.	0±0	n.d.	0±0	n.d.

A: IMT5155; B: IMT11327 (IMT5155, $n = 10$ chickens/group; IMT11327, $n = 5$ chickens/group).

colonization model. This model enables the study of bacterial attachment to host tissue during onset of infection and could also serve as a substitute for *in vitro* cell culture models used to study bacterial adhesion. Hence, the lung becomes the crucial organ in this model, making the route of inoculum administration in turn another vital aspect of the study. The infection route of bacterial inoculum also influences the bacterial numbers that reach the target organ and tissues [27]. Chickens were infected via the trachea, which indeed is a very crucial step to the infection model, in that host defence as well as primary adhesion begins at the tracheal epithelium.

Our results show a clear decrease in colonization of non-pathogenic strain IMT11327 as compared to the wild-type APEC strain IMT5155. These results were reproducible and hence considered valid for further infection studies. Thus, IMT11327 also serves as a reliable negative control *in vivo* with reduced colonization ability.

Histological changes in organs of infected chickens were observed that correlated wholly with the infection process as studied by clinical observations, macroscopic examination of the organs and bacterial load determination in organ tissues. These results accentuate pathogenicity and virulence studies and enable a better understanding of the infection process from a different angle. The importance lies in the fact that living tissue can be made use of to better study the different aspects of the course of action of APEC infection *in vivo*.

It is interesting to note that, aside from virulence studies, these infection models can be used for many meaningful purposes, for example, the identification of novel virulence factors by use of molecular methods like Signature-tagged mutagenesis (STM) [28]. A number of factors contribute to the pathogenesis of APEC in some way or other, however, many mechanisms underlying pathogenesis have yet to be further studied and deciphered. Pathogenesis can be best understood using *in vivo* models that clearly define and represent a natural infection. The systemic infection model can be used for testing APEC field strains for their pathogenicity and for the determination of the pathogen reservoir, namely by testing putative non-pathogenic strains isolated from clinically healthy chickens and their environment. It also enables the identification of factors playing a role in virulence using STM [28] as well as *in vivo* induced antigen technology (IVIAT) [29]. In this model, the ability of bacteria to disseminate systemically and survive significant exposure to host defence mechanisms is evaluated.

In our study, we also report the establishment of a modified infection model, namely the lung infection model which, to the best of our knowledge, is the first of its kind in chicken. This model is fascinating due to the fact that it can replace the systemic infection model in many aspects of pathogenesis study. The systemic model bypasses the early stages of the infection process including adherence to and colonization of epithelial surfaces, followed by subsequent invasion of host cells [30]. In order to identify factors associated with the adhesion and colonization of the chicken respiratory tract by APEC, be it through STM or site directed mutagenesis, such a systemic model would not hold good, and therefore the lung infection model would aid in the identification of factors in mutants that fail to survive during the early stages of infection. We have applied STM to the lung infection model and identified novel adhesion and colonization factors (unpublished data).

Even though APEC are thought only to infect poultry including chickens, turkeys, ducks etc. it is now known that they belong to the ExPEC group [7,31]. APEC is said to show many similarities with human ExPEC strains with regard to virulence gene patterns and MLST types [6,8]. Acquiring superior *in vivo* models in order to study human pathogens for their virulence and pathogenesis is extremely difficult, and such essential pathogens could eventually be examined in the chicken infection model for their colonization of internal organs, and crossing of the blood–brain barrier where applicable, for example, by newborn meningitis *E. coli* (NMEC) strains. Studies in our laboratory have shown that it is possible to induce severe systemic infection in chickens infected with ExPEC

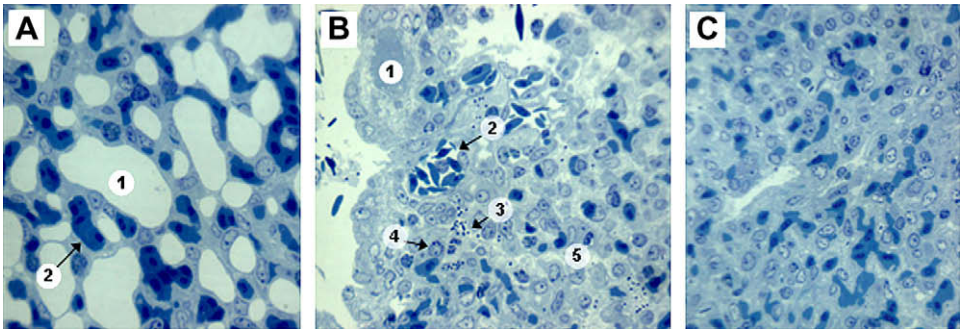

Fig. 4. Lung: semi-thin section along air capillaries; Toluidine Blue-staining; magnification of 100×. (A) 24 h after mock-infection (control): (1) air capillaries; (2) blood capillaries with erythrocytes. (B) 24 h after infection with IMT5155 (10^9 CFU) depicting organ score 2: air capillaries filled with (1) fibrinous exsudates and (2) red and white blood cells; (3) cells of primary defence, oedema, and bacteria within air capillaries; (4) accumulation of macrophages within air capillaries; (5) area of collapsed air capillaries. (C) 24 h after infection with IMT5155 (10^9 CFU) depicting organ score 4: area of air capillaries lacking any lumina; interstitial fibroplasia.

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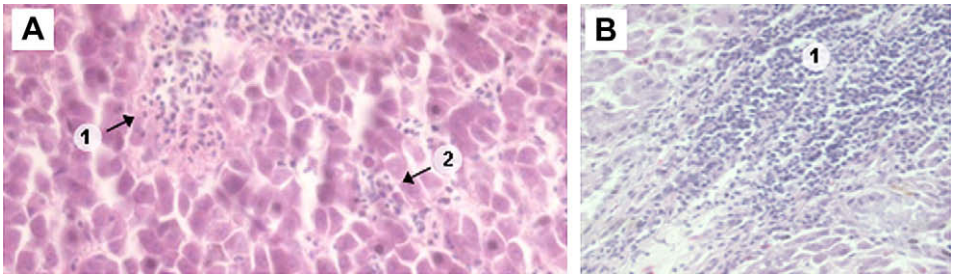

Fig. 5. Liver: light microscopic pictures of liver; HE-staining;24 h after infection with IMT5155 (10^9 CFU) depicting organ score 1: (A) (1) intra- and perivascular and (2) parenchymal lymphocytic infiltrations; magnification of 100x. (B) (1) Vasculitis and perivasculitis; magnification of 40×.

isolates like UPEC and NMEC (unpublished data). The systemic model of infection is therefore not only important for unravelling the pathogenesis of APEC, but also for studying human extra-intestinal pathogens, for example, NMEC and uropathogenic *E. coli* (UPEC), thereby bringing us a step closer to a better understanding of these pathovars. Such studies involving human pathogens that can infect chickens could also provide greater evidence for the zoonotic potential of APEC. Mutants of wild-type pathogens, wherein genes making a major contribution towards the progress of infection and disease are made dysfunctional, may serve as good candidates for vaccine development. Ideal vaccine candidates can further be appropriately tested in the chicken infection model in a variety of immunization studies, thus efficiently tackling the problem of prevention and control of persisting disease and infection.

4. Materials and methods

4.1. Bacterial strains

The choice of the wild-type pathogen IMT5155 was based on its possession of traits characteristic of strains causing extraintestinal disease in poultry [1,7,32]. The isolate was obtained from the internal organs of a laying hen clinically diagnosed with systemic APEC infection during an outbreak in the northern part of Germany in the year 2000 [28,33]. IMT5155 (O2:K1:H5) belongs to one of the most common O-types among APEC strains and synthesizes a K1 polysaccharide capsule [7,28]. For chicken infection studies an additional *E. coli* strain, IMT11327 (Ont:H16), isolated from the intestine of a clinically healthy chicken, was used as a non-virulent negative control. Micro-organisms were stored at −70 °C in brain heart infusion broth (BHI) with 10% (v/v) glycerol until further use.

4.2. Virulence-associated gene, phylogenetic and MLST typing

The non-pathogenic strain IMT11327 was examined for the presence of a variety of genes known to be associated with APEC virulence using multiplex PCR assays as described previously [7]. The strain was classified according to the ECOR system [34] using the rapid phylogenetic grouping PCR technique described by Clermont et al. [35]. MLST typing of the wild-type and non-pathogenic strain was performed by sequence analysis of seven house-keeping genes [36].

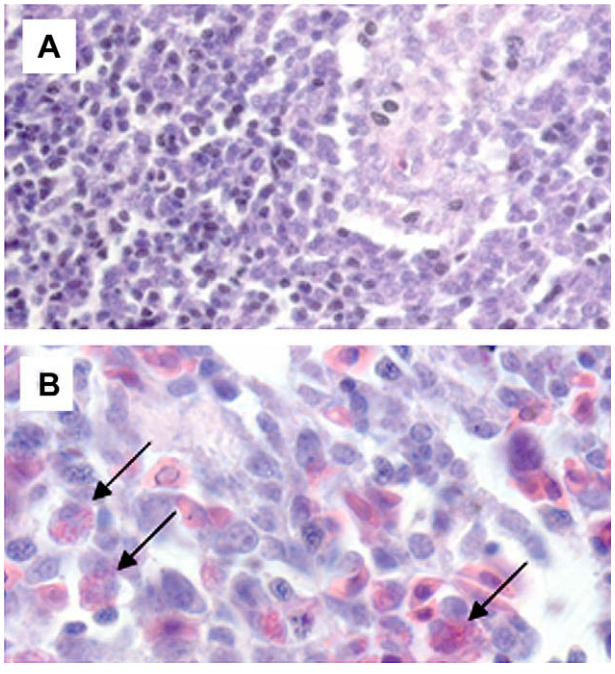

Fig. 6. Spleen: light microscopic pictures; HE-staining. (A) 24 h after mock-infection (control); 40×. (B) 24 h after infection with IMT5155 (10^9 CFU) depicting organ score 1: inflammatory cells in red splenic pulpa (arrows); 100×.

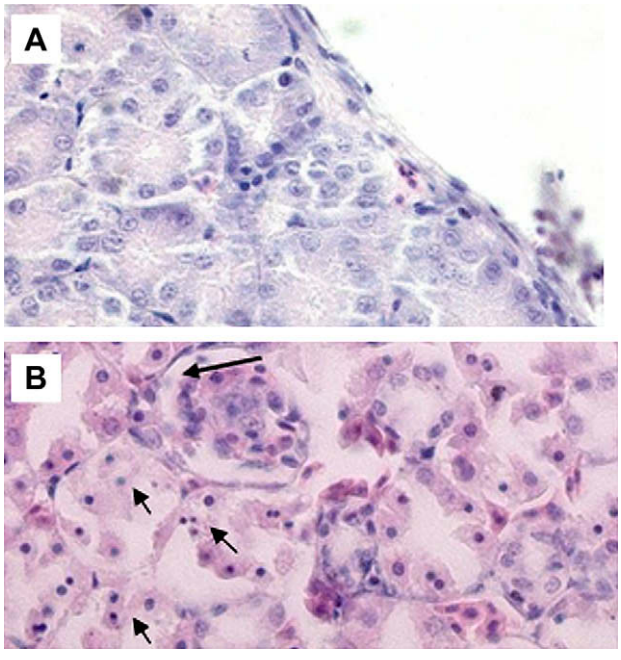

Fig. 7. Kidney: light microscopic pictures; HE-staining; 100×. (A) 24 h after mock-infection (control). (B) 24 h after infection with IMT5155 (10^9 bacteria); pyknosis in tubular epithelium (short arrows); enlargement of Bowman's space (large arrow).

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4.3. Animals used in infection models

Five- week-old white leghorn specific pathogen free (SPF) chickens (Lohmann Selected Leghorn; Lohmann Tierzucht GmbH, Cuxhaven, Germany) were used for the establishment of two disease models, namely a systemic and a lung infection model at the infectious disease facility of the Institute for Microbiology and Epizootics (IMT) at the Freie Universität, Berlin. The chickens were provided food and water *ad libitum*.

4.4. Inoculum preparation

To prepare *E. coli* cultures for infecting birds via the intra-tracheal route, one loopful of the stock culture was plated on Luria Bertani (LB) agar and incubated at 37 °C for 18 h. A single colony of this fresh culture was inoculated in 5 ml of LB broth and incubated at 37 °C for 18 h with agitation. On the day of infection, 18-h cultures were diluted 1:50 in fresh LB broth and grown at 37 °C with agitation to an optical density at 600 nm (OD_{600}) corresponding to about $1–3 \times 10^8$ CFU/ml. After incubation, cultures were harvested by centrifugation at $5000 \times g$ for 10 min and the cell pellet was resuspended in phosphate-buffered saline (PBS) to a concentration of $3–6 \times 10^9$ CFU/ml. The estimated colony count was confirmed by plating 100 μl of a 10^{-7} and a 10^{-8} dilution of the final culture onto separate LB agar in duplicate and calculated by applying the Reed and Muench formula [37].

4.5. Systemic infection model

Groups of 15 and five 5-week-old chickens were infected with the virulent strain IMT5155 and the non-virulent isolate IMT11327, respectively. Birds were inoculated intra-tracheally with 0.5 ml of a bacterial suspension containing 10^9 CFU or with 0.5 ml of phosphate-buffered saline as a control. Birds were monitored for 2 days and lethality rates were recorded. At 24 and 48 h post infection, chickens were killed by cervical dislocation and clinical scores were recorded according to Table 2, while organ lesion scores were noted according to the classification in Table 3. Bacteria were re-isolated from the lung, kidney, liver, heart, spleen and brain as follows: tissue samples of the organs affected in a systemic infection were weighed, suspended in phosphate-buffered saline (1 ml/g), and homogenized with an Ultra-Turrax apparatus. Serial 10-fold dilutions were plated out onto LB agar that were subsequently incubated at 37 °C for 24 h. Colonies were counted after 24 h incubation to determine the CFU per gram in each organ. Random colonies were examined for their virulence gene pattern by multiplex PCR assays as described previously [7] to ensure that the bacteria re-isolated were indeed IMT5155.

4.6. Lung infection model

The lung infection model – a modification of the systemic infection model – was also established, such that almost no bacteraemia was induced during infection. In an initial infection trial, 5-week-old chickens were inoculated intra-tracheally with a 0. 5-ml suspension containing $10^6, 10^5, 10^4$ and 10^3 CFU of the virulent strain IMT5155 respectively. Groups of 20 chickens were used for each dose for statistical analysis. At 24 and 48 h post infection, 10 chickens each were killed, bacteria were re-isolated from the lung and spleen, and CFU was determined as described above.

In a succeeding trial, non-pathogenic avian *E. coli* strain IMT11327 was used as a negative control. Five chickens were infected intra-tracheally with a 0.5-ml suspension containing 10^6 CFU of the above mentioned strain. Chickens were killed 24 h post infection and bacteria were isolated from the lung and spleen as previously described for quantitative determination.

4.7. Clinical scoring

A clinical score was evaluated monitoring the development of experimental APEC infection ranging from score 0 (no symptoms) to score 4 (severe symptoms) taking into consideration both respiratory and general symptoms (Table 2). Chickens were monitored by the hour and birds observed with scores ≥ 3 were killed immediately according to animal welfare norms (Reg. 0220/06).

4.8. Organ scoring

Post-mortem examinations were performed immediately after the death of chickens. Organs were aseptically removed, and the severity of the macroscopic lesions attributed to *E. coli* was scored according to an organ lesion score described in Table 3. Mean additive lesion scores were determined for all groups, and differences were calculated by the two-tailed *t*-test, with p of <0.05 considered significant.

4.9. Histological examinations

Organ tissues were fixed in 4% formalin before washing with varied concentrations of alcohol, and embedding in paraffin. Embedding cups containing organ tissue were filled with liquid paraffin and left to solidify on ice, after which the block was carefully removed in order to make 1– 3- micrometre sections using a microtome.

Hemotoxylin– eosin and Toluidine Blue staining was carried out according to standard protocols.

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