Signature-Tagged Mutagenesis in a Chicken Infection Model Leads to the Identification of a Novel Avian Pathogenic Escherichia coli Fimbrial Adhesin

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Abstract

The extraintestinal pathogen, avian pathogenic E. coli (APEC), known to cause systemic infections in chickens, is responsible for large economic losses in the poultry industry worldwide. In order to identify genes involved in the early essential stages of pathogenesis, namely adhesion and colonization, Signature-tagged mutagenesis (STM) was applied to a previously established lung colonization model of infection by generating and screening a total of 1,800 mutants of an APEC strain IMT5155 (O2:K1:H5; Sequence type complex 95). The study led to the identification of new genes of interest, including two adhesins, one of which coded for a novel APEC fimbrial adhesin (Yqi) not described for its role in APEC pathogenesis to date. Its gene product has been temporarily designated ExPEC Adhesin I (EA/I) until the adhesin-specific receptor is identified. Deletion of the ExPEC adhesin I gene resulted in reduced colonization ability by APEC strain IMT5155 both in vitro and in vivo. Furthermore, complementation of the adhesin gene restored its ability to colonize epithelial cells in vitro. The ExPEC adhesin I protein was successfully expressed in vitro. Electron microscopy of an afimbriate strain E. coli AAEC189 over-expressed with the putative EA/I gene cluster revealed short fimbrial-like appendages protruding out of the bacterial outer membrane. We observed that this adhesin coding gene yqi is prevalent among extraintestinal pathogenic E. coli (ExPEC) isolates, including APEC (54.4%), uropathogenic E. coli (UPEC) (65.9%) and newborn meningitic E. coli (NMEC) (60.0%), and absent in all of the 153 intestinal pathogenic E. coli strains tested, thereby validating the designation of the adhesin as ExPEC Adhesin I. In addition, prevalence of EA/I was most frequently associated with the B2 group of the EcoR classification and ST95 complex of the multi locus sequence typing (MLST) scheme, with evidence of a positive selection within this highly pathogenic complex. This is the first report of the newly identified and functionally characterized ExPEC adhesin I and its significant role during APEC infection in chickens.

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Introduction

Research on avian pathogenic E. coli (APEC) has increased greatly over the years where the pathogen has been known to cause disease among chickens and other fowls which usually results in large economic losses for the poultry industry [1]. APEC are mostly associated with infection of extraintestinal tissues in chickens, turkeys, ducks and other avian species. The most important disease syndrome associated with APEC begins as a respiratory tract infection and may be referred to as avian aerosolitis or the air sac disease [2]. This inevitably results in severe systemic infection leading to death of the animal infected. These pathogens have recently gained even more importance, now being classified under the category of extraintestinal pathogenic E. coli (ExPEC), a group which includes both human pathogens like the uropathogenic E. coli (UPEC) and newborn meningitic E. coli (NMEC) and animal pathogens, which in turn suggest the possibility of APEC having zoonotic potential [3–5].

Microbial pathogenicity is a complex phenomenon encompassing diverse mechanisms. There are, however, several common strategies that pathogenic organisms use to sustain themselves and overcome host barriers, one of them being the firm adhesion of the microorganism to host cells [6]. Colonization is crucial to pathogenesis of bacteria, being the earliest stage during onset of the disease and the ability to adhere to host surfaces is by far the most vital step in the successful colonization by microbial pathogens [7,8]. The presence of adhesins are said to be essential to the first steps of bacterial pathogenicity [9]. A distinct family of adhesins called the adhesive pili or fimbriae, encoded by adhesin-gene clusters and assembled by the chaperone-usher pathway are said to be ubiquitous in Gram-negative organisms [10]. Some of the well known fimbriae present among ExPEC strains are Type I fimbriae (fim), P fimbriae or the pilus...
associated with pyelonephritis (pap), curli fibre (csg), S fimbriae or the sialic acid-specific fimbriae (fse), F1C fimbriae (foc), afimbrial adhesin / Dr antigen-specific adhesin (gfsa/dra), heat-resistant agglutinin (hton) and others [4]. Each of these adhesins recognizes a specific receptor although as a group they share common genomic organization, assembly and even quaternary structural traits [10].

The role of some of these adhesins during APEC infection is well recognized. It has been reported that bacterial colonization of the respiratory tract is mediated by fimbral adhesins, and studies have shown that type 1 and P fimbriae are expressed in vivo by bacteria colonizing the lung, air sacs and internal organs of chickens during infection [11]. Bacterial adhesion to pharyngeal epithelial cells of chickens was also found to be inhibited by the presence of D-mannose, demonstrating the role of type 1 fimbriae during the early stages of the disease [12]. No expression of P fimbriae was seen in the chicken trachea, suggesting that they may be important only in later stages of infection [11]. In another study, it was seen that 99% of E. coli isolated from diseased birds possessed the csgA gene responsible for curli biosynthesis [2]. Furthermore curli fibres were found to be essential for the internalization of bacteria causing avian septicaemia as seen in vitro [13].

Although there is increased knowledge about fimbral adhesins and other colonization factors including the role they might play during infection, further investigation is still required to completely elucidate the function of each adhesin and its specific role in pathogenesis. In addition, the availability of complete genome sequences for some ExPEC pathogens like UPEC strain UTI89 (O18:K1:H7) and APEC strain APEC_O1 (O1:K1:H7) has drawn our attention to the large increase in the number of genes with unknown function, possibly involved in adhesion and colonization, and whose contribution to virulence on the whole is presently merely hypothetical [14–16]. Determination of the function of these novel genes will lead to a deeper understanding of host-pathogen interactions during the initial stages of APEC infection.

Previously our laboratory applied Signature-tagged mutagenesis (STM) to APEC in a chicken systemic infection model which led to the successful identification of genes crucial to systemic infection in chickens, however no adhesins were identified [17–20].

In the present study, we applied STM to APEC in a modified lung infection model [21], in order to identify novel genes functionally involved in the adhesion and colonization of the chicken lung during the infection process. The STM screen led, among others, to the identification of a mutant EA7F9 with a transposon insertion in a putative adhesin gene (yqi). In addition, a mutant with an insertion in a gene encoding the type 1 fimbriae regulatory protein was also identified. These two genes were the most obvious targets of the STM screen being adhesins, the primary structures in direct contact with host tissue during infection. Type 1 fimbriae are already well characterized for their role in ExPEC pathogenesis; however, yqi has until now never been described for its potential role during infection. Therefore, the new adhesin encoded by the gene yqi became the target of this study, and was further characterized for its role in the initiation of APEC infection. We describe for the first time, the identification and characterization of this novel adhesin in APEC, temporarily renamed ExPEC adhesin I (EA/I), and the essential role it plays in the initial colonization of the chicken lung during infection.

Results

Attenuation of IMT5155 with Mutation in ExPEC Adhesin I (EA/I) Gene yqi

Among the many genes identified during the STM screen, was the ExPEC adhesin I (EA/I) gene, also annotated as yqi [15]. In vitro competition assays of transposon-mutant EA7F9, with a mutation in gene yqi, versus wild type strain IMT5155 showed that there was no significant growth defect in vitro as determined by growth curves (Figure 1). An in vitro competition index of 1.2 was observed for EA7F9. On the other hand, the mutant was found to be attenuated in the chicken with an in vivo competition index of 0.6. This result confirmed the attenuation of mutant EA7F9 during STM screening.

Sequencing of the EA/I Gene Cluster Region in IMT5155

The putative yqi adhesin gene cluster was completely sequenced in APEC strain IMT5155. Genomic organization of the IMT5155 yqi adhesin gene cluster was as follows: the putative outer membrane usher preceded the periplasmic chaperone, followed by the adhesin gene. A conserved hypothetical protein gene precedes the usher gene which may code for the adhesin subunit protein although this has not yet been confirmed (Figure 2). This 4,975 bp region showed a hundred percent sequence identity with the UPEC and APEC yqi adhesin gene cluster in sequenced strains UTI89 (Acc. No: CP000243) and APEC_O1 (Acc. No: CP000468) [15]. Comparing the sequence with 1096 bacterial genomes currently available in the public database, out of which 28 were Escherichia coli genomes, this adhesin gene cluster is only found among ExPEC strains including APEC, UPEC and NMEC and is not harboured by intestinal pathogenic E. coli like enteropathogenic E. coli (EPEC) or enterohaemorrhagic E. coli (EHEC) such as E. coli O157:H7 strains EDL933 and Sakai, or non pathogenic E. coli K-12 strains MG1655 and W3110.

EA/I Plays a Role in the Adhesion of APEC to Chicken Fibroblasts and Kidney Epithelial Cells In Vitro

In order to determine the effect of EA/I in APEC, adhesion assays were initially performed in vitro using chicken fibroblast cells. Strain EA7F9 with a disruption in the yqi gene via a transposon (STM generated mutant), and strain IMT5155Δyqi devoid of the ExPEC adhesin I gene were tested against the wild type pathogen IMT5155 and a negative control strain MG1655. Bacterial determination at two different time points revealed a reduction in the ability of both EA7F9 and IMT5155Δyqi to adhere to...
Effect of EA/I during Systemic Infection in Chickens

Systemic infection can be induced in chickens by infecting 5-week-old birds intra-tracheally with a pathogenic strain with an infection dose of $10^9$ CFU. As mentioned before, infection of chickens with this infection dose resulted in significant reduction in bacterial colonization of the chicken lung. When bacteria were re-isolated from chicken internal organs including spleen, kidneys, heart, liver and brain, there was a reduction in bacterial numbers when infected with IMT5155Δyqi as compared to IMT5155 as seen in figure 5, however only marginal. The only exception was the brain, where almost no bacteria were isolated when infected with IMT5155Δyqi as compared to IMT5155. All organ scores were considerably reduced in chickens infected with IMT5155Δyqi as compared to chickens infected with IMT5155 as seen in table 1.

Electron Microscopy Reveals Fimbrial-Like Appendages Associated with ExPEC Adhesin I (yqi) Gene Cluster

The ExPEC adhesin I (yqi) 4,975 bp gene cluster coding for the putative subunit, chaperone, usher and adhesin was successfully cloned and over-expressed in an afimbriate strain E. coli AAEC189. Negative staining of strain AAEC189 (pKESK_yqi_4975_XB) revealed the expression of short fimbrial like appendages forming on the outer membrane of the bacterial cell (Figure 6A-C). These appendages were about 0.04 $\mu$m long and 0.005 $\mu$m thick and were not detected in the afimbriate strain AAEC189, that is, the negative control (Figure 6D). The wild type strain IMT5155, harbouring other adhesins like Type 1 fimbriae and curli, in addition to ExPEC adhesin I, was used as a positive control for the staining method, and long fimbriae with a length of about 0.5 $\mu$m (Figure 6E) were observed which were morphologically different from the fimbrial structures observed in strain AAEC189 (pKESK_yqi_4975_XB).

Prevalence of EA/I among ExPEC Reveals Its Potential as a Factor of Virulence

A collection of ExPEC strains, intestinal pathogenic E. coli, and avian and human non pathogenic strains available at the Institute of Microbiology and Epizootics, Freie Universitat Berlin, was screened to determine the presence of the yqi gene among these strains. Out of a total of 388 ExPEC isolates tested for the presence of yqi, including 406 APEC, 138 UPEC, 25 NMEC and 19 Septicaemia associated E. coli (SePEC), 368 isolates were found to be positive for yqi, which amounts to a total of 62.5% of pathogenic isolates harbouring the yqi gene (Table 2). Among APEC isolates alone, 221 isolates were found to be positive making it a total of 54.4% positive for yqi in this group. Among UPEC isolates 91 isolates were found to be positive in a total percentage of 65.9% positive for yqi and among NMEC isolates 15 were found to be positive amounting to 60.0% positive for yqi. Finally, among SePEC isolates, 10 were positive in a percentage of 52.6%. From a total of 159 non pathogenic isolates tested, including those from avian and human origin, 31 were found to be positive for yqi, which accounts for only 19.4% of the non pathogenic strains tested.
Figure 3. Bacterial adhesion to chicken fibroblast cells 1.5 h and 3 h after infection with an MOI = 100. Differences between IMT5155 and IMT5155Δyqi were statistically significant with a p < 0.005 at 1.5 h and p < 0.05 at 3 h (A). Bacterial adhesion to polarized Madin Darby canine kidney (MDCK-1) cells 3 h after infection with an MOI = 100. The difference between IMT5155Δyqi and IMT5155Δyqi (pDSK602; yqi) was significant with a p < 0.04 (B). doi:10.1371/journal.pone.0007796.g003

Figure 4. Bacterial colonization of the chicken lungs 24 h after intra-tracheal infection with 10^6 CFU of bacteria. Differences between IMT5155 and IMT5155Δyqi were statistically significant with a p < 0.05 (n = 6). Strain IMT11327 is the negative control (A). Bacterial colonization of the chicken lungs 24 h after intra-tracheal infection with 10^9 CFU of bacteria. Differences between IMT5155 and IMT5155Δyqi were statistically significant with a p < 0.02 (n = 6). Strain IMT11327 is the negative control (B). doi:10.1371/journal.pone.0007796.g004
Among 153 intestinal pathogenic *E. coli* tested in this study, none of the isolates were found to be positive for *yqi* as seen in table 2.

Due to the exclusive association of the putative fimbrial adhesin gene *yqi* with extraintestinal pathogenic *E. coli* (ExPEC), that is, its high prevalence among ExPEC isolates compared with non-pathogenic *E. coli*, and complete absence in intestinal pathogenic *E. coli*, the adhesin was tentatively designated ExPEC adhesin I (EA/I).

### EA/I Is Evolving under Positive Selection

The ExPEC adhesin I (*yqi*) was sequenced in many strains and compared with existing multilocus sequence typing (MLST) data. MLST is a nucleotide sequence based approach for the unambiguous characterization of isolates of bacteria using sequences of internal fragments of seven house-keeping genes [23]. The population structure of microbial species with intermediate levels of recombination can thus be revealed by allele-based analyses [23]. Wirth et al. described that sequence polymorphisms could define unique sequences for each of the seven house-keeping gene loci, which are referred to as alleles and each unique combination of alleles is assigned a sequence type (ST) number [24]. Related STs are assigned to so-called ST complexes, using the principles of the eBURST algorithm [25]; each ST complex includes at least three STs that differ from their nearest neighbour by no more than two of the seven loci while ST complexes differ from each other by three or more loci, and STs that did not match the criteria for inclusion within an ST complex are simply referred to by their ST designation [24].

The evolutionary history of the *yqi* gene was inferred using the Maximum Parsimony method. Sequencing of the ExPEC adhesin I (*yqi*) gene in strains belonging to the various sequence types (ST) and sequence type complexes (STC) including STC10, STC12, STC73, STC95, ST141 and ST372, and calculation of the distances of the adhesin gene locus between these strains revealed distribution of the strains tested into two groups (Figure 7). The value of replicate trees in which the associated taxa clustered together in the bootstrap test was 99% for the two groups identified. One group included strains belonging to the ST73 complex, while all other strains were assigned to a second group. Sequence homology was observed among strains belonging to a particular sequence type or sequence type complex as is the case with ST141, ST372, STC12 and STC73 within the sequence type or sequence type complex (Figure 7). One exception to the rule was strain IMT15008, ST73, STC73 which showed variations in its *yqi* gene sequence compared to other strains in the ST73 complex, and could be better assigned to the group harbouring other strains tested. Interestingly, only strains belonging to sequence type complex 95 showed mutations in the *yqi* gene sequence. Therefore, we determined the ratio of the non-synonymous mutation rate (Dn) to the synonymous mutation rate (Ds) within this complex using DnaSP 4.50.3 software. Dn/Ds < or = 1 indicates purifying or neutral selection, favouring amino acid substitutions [26]. Our data show a non-synonymous mutation rate of 0.00095496 and a synonymous mutation rate of 0.00024 resulting in a Dn/Ds ratio of 3.979, indicating strong positive selection for structural evolution of the adhesin gene *yqi* within the sequence type 95 complex.

### Discussion

A necessary step in the successful colonization and progression of disease by microbial pathogens is the ability to adhere to host surfaces [8]. The present study made use of a lung colonization...
chicken infection model in order to identify APEC genes specific to early stages of infection including adhesion to and colonization of the host by its pathogen via signature-tagged mutagenesis. As anticipated, we identified fimbrial proteins including the type 1 fimbrial regulatory protein, and other novel genes explicitly involved in the early stages of APEC pathogenesis as determined by competition assays with mutants. Our most interesting finding was a novel yqi adhesin gene which was seen to play a very significant role in the colonization of the avian lung by APEC. The newly identified putative fimbrial adhesin encoded by gene yqi, will be temporarily called ExPEC adhesin I (EA/I) until the specific host receptor for this adhesin has been identified in order to allow better classification and nomenclature of the novel E. coli fimbrial adhesin.

Adhesins are known to facilitate host colonization by mediating the first and crucial interaction with host tissue [10]. In certain cases pathogens have been reported to have a substantial amount of different adhesins expressed at one time or another [8]. In E. coli, two of the best characterized adhesins are the type 1 fimbriae and the P fimbriae which are regulated by the fim and pap operons [27]. Genome sequencing of prototypic cystitis UPEC strain UTI89 has now revealed ten different chaperone-usher adhesin systems, among which the anf, yad, yec, yqi, yeh and fim operons still remain to be characterized [14].

Interestingly, we identified the UPEC yqi adhesin gene in an STM screen specially designed to detect factors that would play a crucial role in the initiation of APEC infection. As stated before, the APEC yqi gene showed complete sequence identity with the UPEC yqi and is part of a 4,975 bp adhesin gene cluster yet uncharacterized. When comparing this adhesin gene sequence with publicly available E. coli genomes, the adhesin was found to be present among ExPEC strains including APEC and UPEC [14,15] and is not harboured by non-pathogenic E. coli nor by any of the intestinal pathogenic E. coli like enteropathogenic E. coli (EPEC) or enterohaemorrhagic E. coli (EHEC), making it unique to extraintestinal infections. This result therefore validates the temporary name ExPEC adhesin I (EA/I) allotted to the new adhesin.

Fimbrial adhesins share common genetic organization, in that the adhesin regulatory genes precede the major subunit gene, which is followed by the periplasmic chaperone, outer membrane usher, and finally the adhesin genes [10]. This gene cluster organization is seen in type 1 fimbriae (fim), S fimbriae (sfa), F1C fimbriae (foc) and the Dr-antigen recognizing fimbriae (dra) [10]. Organization of the yqi adhesin gene cluster differs by having the positions of the usher and chaperone inverted, which is also true for the pap adhesin gene cluster of the P fimbriae [10], although the reason for this, be it functional or biological, is still unclear.

To date, there are no studies implying the role of ExPEC adhesin I (yqi) in the colonization of host tissues during infection. Previously, it has been shown that the fimH adhesin of the type 1

Figure 6. Expression of the ExPEC adhesin I (yqi gene cluster in vitro. Electron micrographs show negatively stained afimbriate strain E. coli AAEC189 (pKESKyqi_4975_XB) over-expressed with the yqi adhesin gene cluster at a magnification of 45,000x, 65,000x and 100,000x (A–C), negative control afimbriate strain E. coli AAEC189 (D) and wild type fimbriated E. coli strain IMT5155 (E). The arrows indicate the location of the fimbriae.

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Table 2. Prevalence of ExPEC adhesin I coding gene yqi among pathogenic E. coli strains and non-pathogenic strains of human and avian origin.

| E. coli Group | Isolates tested | Prevalence of yqi (%) |
|---------------|-----------------|-----------------------|
| APEC          | 406             | 54.4                  |
| UPEC          | 138             | 65.9                  |
| NMEC          | 25              | 60.0                  |
| SaPEC         | 19              | 52.6                  |
| STEC          | 49              | 0.0                   |
| EHEC          | 46              | 0.0                   |
| EPEC          | 28              | 0.0                   |
| aEPEC         | 12              | 0.0                   |
| ETEC          | 8               | 0.0                   |
| EIEC          | 6               | 0.0                   |
| EAEC          | 4               | 0.0                   |
| Commensals of human and avian origin | 159 | 19.4 |

APEC = Avian pathogenic E. coli, UPEC = Uropathogenic E. coli, NMEC = Newborn meningitic E. coli, SaPEC = Septicaemia associated E. coli, STEC = Shiga Toxin-producing E. coli, EHEC = Enterohaemorrhagic E. coli, EPEC = Enteropathogenic E. coli, aEPEC = Atypical Enteropathogenic E. coli, ETIEC = Enterotoxigenic E. coli, EIEC = Enteroinvasive E. coli, EAEC = Enteraggregative E. coli.

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Fimbriae, and the pabG adhesin of the P fimbriae are the receptor specific binding proteins that are located at the tip of the pilus structure [28]. It has also been reported that deletion of the pabG gene had no effect on pilus formation; however, the pilus isolated from a pabG deletion strain was not adhesive [29]. We now have evidence which shows that deletion of the EA/I gene, yqi, in APEC strain IMT5155 results in a significant decrease in the colonization of fibroblasts and epithelial cells in vitro and of the chicken lung in vivo. It is possible that the Yqi adhesin is the receptor-specific protein responsible for initial attachment to host cells; however, this receptor is yet to be identified.

APEC strain IMT5155 has been previously tested for the presence of important adhesins like fim, pap, col, sfa, tsh, afa, dca, foc and others, and has been found to harbour only the type 1 fimbra (fimJ), curli (sgs) and temperature-sensitive haemagglutinin (sth) genes [4]. The absence of P, FIC, S and Dr fimbrae in IMT5155, which are known for their role in UPEC pathogenesis [10], made it apparent that there might in fact be unidentified adhesins that participate crucially during initiation of infection. We observed that in both infection models used in this study, there was always a substantial reduction in the colonization of the chicken lung when infecting chickens with IMT5155Δyqi. Therefore, we believe that ExPEC adhesin I makes a significant contribution to total bacterial adhesion and colonization of the chicken lung by APEC strain IMT5155 during infection. We further observed that there was only a slight reduction in bacterial numbers isolated from internal organs of chickens when infected with IMT5155Δyqi in the systemic infection model. This is not unique, because IMT5155 harbours a number of known virulence genes like iron acquisition genes (chuK, fyuA, iucD, isiD), serum resistance genes and proteinst (isu, ompA, traT, neaC) and invasion related genes (gimB, ibdE) which play a role in the initiation and prolongation of disease and which may on the whole contribute to systemic infection in chickens [4]. Besides with an infection dose of 10^8 CFU as used in the systemic infection model, the chicken lung is overwhelmed with bacteria, and therefore, there is very little room left for specific adhesion to take place in contrast to the lung infection model. This could also be a reason for the larger numbers of bacteria in internal organs during systemic infection. However, we still observe a significant bacterial reduction in the lung even with a high infection dose, which suggest the existence of specific receptors for ExPEC adhesin I on the lung epithelium, which account for a significant amount of fimbrial receptors on the lung. Due to the alternative fimbriae still present in the knock-out mutant of ExPEC adhesin I, attachment to other receptors on the surface of the lung would still occur strongly, particularly in the systemic infection model with its high infection dose, thus in turn initiating a systemic infection in this model.

An interesting result was the prevalence of EA/I among a relatively large collection of strains, particularly its high incidence among ExPEC strains like APEC, UPEC, NMEC and septicaemia associated E. coli (SePEC) strains, infrequent occurrence among non-pathogenic strains and complete absence among intestinal pathogenic E. coli strains. It has been previously reported that distinctive strains of E. coli responsible for most cases of urinary tract infection, sepsis and newborn meningitis are derived predominantly from E. coli phylogenetic group B2 [30]. Additionally, diverse studies show that virulent clonal groups are derived mainly from phylogenetic group B2 and to a lesser extent from group D [4,31]. Our observations show that 66.1% of the isolates positive for yqi belong to the phylogenetic group B2 in contrast to only 11.5% of isolates negative for yqi belonging to this group.

Furthermore, we found that all of the isolates belonging to sequence types 95, 140, 141 and 372 were positive for yqi. ST95 and ST140 belong to the sequence type 95 complex. It is known that well defined pathogens are associated with specific STs or ST complexes, for example, ST95 complex contains related pathogenic bacteria of serogroups O1, O2, and O18 that express the K1 polysaccharide, that is, the K1 isolates [24,26]. Of immense interest, therefore, was the evolutionary analysis of the adhesin gene sequences from strains belonging to different sequence types, which showed that within a particular sequence type complex (STC), the adhesin sequence was homologous which is the case for STC12 and STC73 as well as other sequence types like ST372, ST141 and ST358 with a single exception, strain IMT15008. Since this strain had no unique characteristic that differentiated it from other strains within the ST373 complex, a possible explanation could be that the yqi gene in strain IMT15008 is the result of a recombination event.

Interestingly, within the ST95 complex, the adhesin gene sequence showed the presence of single nucleotide polymorphisms (SNPs) which were confirmed as a positive selection on the gene within this complex. Mutations producing functional modifications are called pathogenicity-adaptive or pathoadaptive, and are often SNPs producing amino acid replacements in proteins essential for a pathogen’s success and it has been shown that two major adhesins of extraintestinal pathogenic E. coli (ExPEC) – type I and P fimbrial adhesins – acquire structural SNPs at a rapid rate, and this adaptation constitutes a major factor in the pathoadaptive microevolution and genetic diversification of ExPEC clonal groups [26]. It is possible, that ExPEC adhesin I also undergoes structural mutations or pathoadaptive mutations, particularly, among strains belonging to the ST95 complex, which further confirms the importance of this adhesin within this highly pathogenic complex. Not surprisingly therefore, is the fact that the wild type strain used in this study, IMT5155, also belongs to the ST95 complex and harbours the most number of SNPs in the gene coding for ExPEC adhesin I as can be seen from the branching pattern on the dendrogram.

It therefore makes sense to assume that ExPEC adhesin I could perhaps play a very specific role in ExPEC pathogenesis, especially...
Figure 7. Phylogenetic analysis of ExPEC adhesin I (yqi). Maximum Parsimony tree shows distances between yqi gene sequences among strains belonging to different sequence types (ST: Sequence type; STC: Sequence type complex).
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during the early colonization steps of infection, although this still needs to be proven for UPEC and NMEC strains. However, an interesting addition to this story is the decrease in adhesion to kidney epithelial cells as shown by our in vitro adhesion experiments. This would indicate the importance of yqi in UPEC, which particularly colonizes the bladder epithelium during urinary tract infection (UTI). Furthermore, it provides evidence for the zoonotic potential of APEC, a topic of great interest in the field of ExPEC.

The presence of a single virulence factor hardly ever makes a strain virulent, while a combination of virulence factors usually determines its ability to cause disease [32]. Therefore, much like countless other genes that are classified into the class of virulence factors when exceedingly prevalent among pathogens, the yqi gene could also eventually be an addition to this category based on its regular presence in highly pathogenic ExPEC strains in contrast to non-pathogenic strains. One must keep in mind, that the meager group of A_hecal strains and human non-pathogenic strains that were found to be positive for yqi in this study are no classical non-pathogenic strains as seen in a previous study [33]. Evers et al. reported that a number of A_hecal E. coli strains have characteristics typical of human and animal ExPEC, and that some non-outbreak strains are capable of causing systemic disease in immunocompetent 5-week old chickens, suggesting the avian intestine reservoir hypothesis [33]. Furthermore, it was reported that these strains pose a zoonotic risk because they could be transferred directly from birds to humans or serve as a genetic pool for ExPEC strains. With regard to the non-pathogenic human isolates found to be positive for yqi, it is interesting to note that though these strains are isolated from the intestinal tract of healthy humans they express the K1 polysaccharide associated with highly pathogenic strains.

Therefore, taken together, the results of the ExPEC adhesin I (yqi) prevalence study in E. coli are in fact intriguing, since the search for novel virulence factors still goes on. Nevertheless, they do not imply that EA/I would be solely responsible for virulence of ExPEC strains, rather only contribute to pathogenesis on the whole.

An interesting result in this study was the successful expression of ExPEC adhesin I in vitro. We hypothesized that cloning of the putative adhesin gene cluster coding for the putative subunit protein, putative usher and chaperone proteins and putative adhesin, which together are believed to be responsible for expression of fimbrial structures, when successfully cloned in an afimbriate E. coli strain would enable the expression of EA/I fimbriae visible under the electron microscope. Using negative staining and transmission electron microscopy, our hypothesis was indeed confirmed, in that, short fimbrial like structures were detected in the afimbriate strain overexpressed with the adhesin gene cluster, which were not observed in the afimbriate strain, or negative control. The wild type strain IMT5155 used as a positive control revealed long fimbrial structures representing all fimbrial adhesins harboured by the strain including ExPEC adhesin I which explains the difference in length of fimbrial structures observed. The short fimbrial structures observed in the afimbriate strain overexpressed with ExPEC adhesin I will have to be confirmed using specific antibody and immunogold staining in the future; however, this study provides preliminary evidence for the fimbrial structures of this newly identified fimbrial adhesin. ExPEC adhesin I is therefore confirmed to be a novel fimbrial adhesin, worthy of further studies, which will indeed enable a better understanding of the interactions between adhering ExPEC and their host cells.

We have identified an adhesin that plays a significant role in the initial stages of APEC infection. Previous studies with other adhesins, like the fimH adhesin, have shown that antibodies elicited against the adhesin can impede colonization, block infection and prevent disease, that is, prophylactic vaccination with adhesins can inhibit bacterial infections [34]. In other studies, it was found that vaccination with Gal-Gal pili or the P fimbrial vaccines prevented pyelonephritis by pilated E. coli in a murine model and in monkeys [35,36]. Therefore, blocking initial stages of infection may be one of the most effective strategies to prevent bacterial infections. Furthermore, it would be interesting to identify the specific receptor to which the EA/I binds. Blocking of specific receptors could also be a means of preventing early infection stages. It is our aim to test ExPEC adhesin I for its vaccine potential in chickens making use of our well-established infection models, in order to come one step closer to the final goal of any research in the field of infectious pathogens, namely the prevention of infection and disease.

Materials and Methods

Ethics Statement

All animal experiments were approved by the “Landesamt fuer Gesundheit und Soziales” (LAGeSo) (G 0220/06) and chickens were killed according to animal welfare norms (Reg. 0220/06).

Bacterial Strains, Plasmids and Growth Conditions

All E. coli strains and plasmids used are listed in table 3. The wild type APEC isolate IMT5155 (O2;K1:H5) was obtained from 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 [1,20]. The strain possesses a number of virulence-associated genes typical of ExPEC strains, among others, curli fibre gene (csgA), type 1 fimbriae (fimC), temperature-sensitive haemagglutinin (fliH), heme receptor gene (chuh), outer membrane protein (ompA), invasion of brain endothelium (ibeC), genetic island associated with newborn meningitis (gmbB), colicin V plasmid (cwaC) and is classified into the B2 phylogenetic group, and multi locus sequence type 140 of the ST95 complex [4]. An additional E. coli strain IMT11327 (Ont:H16, ST295, ST23 complex), isolated from the intestine of a clinically healthy chicken, was used as a non-virulent control in both in vitro and in vitro assays, while E. coli - K12 strain (MG1655) was used as a negative control in vitro. IMT5155 was used for all genetic manipulation studies. All isolates used in the prevalence studies are part of a strain collection in our laboratory obtained from various sources [4,33,37,38].

Bacterial strains were routinely cultured at 37°C in Luria-Bertani (LB) broth and on LB agar plates with appropriate antibiotics when required in the following concentrations: Kanamycin (Kan), 50 μg/ml; Nalidixin (Nal), 30 μg/ml; Ampicillin (Amp), 50 μg/ml; Chloramphenicol (Cm), 30 μg/ml; Spectinomycin (Spec) 50 μg/ml. All strains were stored at −70°C in LB broth with 10% (v/v) glycerol until further use.

PCR Analyses

All oligonucleotide primers are listed in table 4. Unless otherwise specified, E. coli isolates, from which DNA was to be used as the template for PCR amplification, were grown overnight in Luria-Bertani broth at 37°C and DNA was released from whole organisms by boiling for 10 minutes. After centrifugation, 2 μl of the supernatant was taken as template DNA and added to a 25μl reaction mixture containing 0.1 μl of each primer pair in a 10 μmol concentration, 0.3 μl 10 mM of the four deoxynucleoside triphosphates (Sigma-Aldrich Chemie GmbH, Munich Germany), 2.5 μl of 10x PCR buffer, 1 μl of 50 mM magnesium chloride and 1 unit of Taq-Polymerase (Rapidozym GmbH, Berlin Germany). The samples were subjected to 25 cycles of amplification in a thermal cycler (GeneAmp PGR system, Applied Biosystems, Darmstadt,
Germany). The amplification products were analyzed by gel electrophoresis on a 1.5% agarose gel (Biodiag, Markkleeberg, Germany), stained with ethidium bromide and photographed on exposure to UV.

**Establishment of a Lung Colonization Model of Infection**

A modified lung colonization model of infection was established based on the existing systemic infection model, for the purpose of screening STM mutant pools for adhesion and colonization factors as described previously according to animal welfare norms [21]. The lung infection model differed from the systemic infection model in that, the infection dose was lowered to $10^6$ CFU such that no systemic infection was induced.

Briefly, five-week old white leghorn specific pathogen free (SPF) chickens (Lohmann Tierzucht GmbH, Cuxhaven, Germany) were used for infection purposes. Groups of 10 chickens were infected with $10^6$, $10^5$, $10^4$ used for infection purposes. Groups of 10 chickens were infected with $10^6$, $10^5$, $10^4$ used for infection purposes. Groups of 10 chickens were infected with $10^6$, $10^5$, $10^4$ used for infection purposes. Groups of 10 chickens were infected with $10^6$, $10^5$, $10^4$

**Table 3. Strains and plasmids used in this study.**

| Strain       | Description                                                                 | Reference      |
|--------------|-----------------------------------------------------------------------------|----------------|
| IMT5155      | O2K1:H5; cuSG, fimC, tsx, chuA, fyuA, ireA, iroN, irp2, iucD, iutA, sit, neuC, iheA, gimB, colV, ompA, ST140, STC95, B2 | [20]           |
| IMT5155 NalR | IMT5155 derivative, Nalidixin resistant                                       | [20]           |
| S17734        | recA thi pro hsdR- M+ RP4:2-Tc:Mc-Km Tn7 lysogenized with λpir phage        | [20]           |
| IMT11327     | Ont:H16; fimC, crlA, ompA, ST 295, B1                                         | [21]           |
| MG1655       | F-, Lam , Fim*                                                               | [44]           |
| EA799        | IMT5155 NalR yqi5:mini-Tn5                                                   | this study     |
| IMT5155Δyqi  | IMT5155 NalR Δyqi::CmR                                                      | this study     |
| E. coli TOP10 | F- mcrA Δ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔfocX74 recA1 araD139 Δ(araleu)7697 | Invitrogen     |
|              | galU galK rpsL (StrR) endA1 napG                                             |                |
| pCR2.1-TOPOyqi| E. coli TOP10 [pCR2.1 TOPOyqi]                                               | this study     |
| pDSK602yqi   | E. coli TOP10 [pDSK602:yqi]                                                  | this study     |
| IMT5155Δyqi (pDSK602:yqi) | IMT5155 NalR Δyqi::CmR[pDSK602:yqi]                                        | this study     |
| IMT5155Δyqi (pDSK602) | IMT5155 NalR Δyqi::CmR[pDSK602]                                            | this study     |
| IMT5155 (pDSK602yqi) | IMT5155 NalR [pDSK602:yqi]                                                   | this study     |
| pCR2.1-TOPOyqi-operon | E. coli TOP10 [pCR2.1 TOPOyqi-Skb]                                       | this study     |
| Top10 (pKESKyqi--4975_XB) | E. coli TOP10 [pKESK-22yqi--4975 bp_Xbal-BamHI]                              | this study     |
| E. coli AEC189 | Δfim, Δlac, recA, endA, hisD, hsdR, hsdM+                                   | [45]           |
| E. coli AAE189 (pKESK- yqi4975 XB) | E.coli AAE189 [pKESK-22yqi--4975 bp_Xbal-BamHI]                               | this study     |

**Plasmid**

| Plasmid          | Description                                                                 | Reference |
|------------------|-----------------------------------------------------------------------------|----------|
| pUTmini-Tn5km2   | KanR, AmpR                                                                   | [20]     |
| pKD46            | AmpR, expresses λ red recombinase                                             | [39]     |
| pKD3             | cat gene                                                                    | [39]     |
| pCR2.1 TOPO      | KanR, AmpR, LacZs, T7 promoter                                              | Invitrogen|
| pKESK-22         | NeoR, KanR, tac promoter                                                     | K. Schnetz (Uni Köln) |
| pDSK602          | SpeIR, SmR, triple lac UVS, broad host range                                 | [40]     |
Table 4. Oligonucleotide primers used in this study.

| Primer number | Target region | Primer Sequence (5’-3’) | Tm (°C) | Reference |
|---------------|---------------|-------------------------|---------|-----------|
| IMT-P2510     | cat (pKD3)    | AATCCCTGCGAAGACGCTCTCCGCTGTAAGAAGGGAAAATGTGAGCTGAGCTGAGCTGAGCTGGA | 75      | this study |
| IMT-P2511     | Cat (pKD3)    | TAAATGTGAAAATGCGGCCGATGCTTCGCTGCTGATGATATATGCTCAGTTAG | 71      | this study |
| IMT-P2512     | Yqi           | ATGCAATGGGAGTACCCCTC   | 60      | this study |
| IMT-P2513     | Yqi           | CTGTTGGACACATCAAAATTG | 60      | this study |
| IMT-P2558     | 221 bp upstream of yqi | ATTACCGCTGGTATATGCCG  | 52      | this study |
| IMT-P2559     | 110 bp downstream of yqi | ATAAACACATATGGGCCGTCG | 50      | this study |
| IMT-P2910     | yqi with EcoRI | CGGATACGGAATTCTGATGAGTTTCTCATTAG | 59      | this study |
| IMT-P2911     | yqi with HindIII | TTCTCAGAAGGTTGCTTGAAGGGGAGGTTGCAGTTACTTAGTTTTAGTATATTCCGATGAGATC | 57      | this study |
| IMT-P3045     | yqi operon with BamHI | CGGATACGGGAATTCATGAGTTTCTCGATTATGGCCTGCTGT | 56      | this study |
| IMT-P3046     | yqi operon with HindIII | TTCTCAGAAGGTTGCTTGAAGGGGAGGTTGCAGTTACTTAGTTTTAGTATATTCCGATGAGATC | 53      | this study |
| IMT-P1560     | Cloning vector pCR2.1 TOPO (–20) | GAAACGACGGCGCAGATAAA | 50      | Invitrogen |
| IMT-P1561     | Cloning vector pCR2.1 TOPO | CAGGAAACCACTAGTACAGTAAG | 50      | Invitrogen |
| IMT-P718      | Cat           | TTATCCGAAGCGCAAGGAAGG | 57.3    | [20]      |
| IMT-P719      | Cat           | GATCTCCGTCACAGCTAGTAGG | 59.4    | [20]      |
| IMT-P3138     | Expression vector pKESK-22 | AATGCTTTGGAATGTGGACGCG | 60.6    | this study |
| IMT-P3139     | Expression vector pKESK-22 | GCCGACATGATCCTACTAGCTA | 60.1    | this study |
| IMT-P3706     | Yqi           | AGTAAACACTTTCGAGGGGCGG | 56.3    | this study |
| IMT-P3707     | Yqi           | GTTCACCGCTTCTTCTTTCTC | 53.6    | this study |
| IMT-P3259     | Yqi operon with XbaI | CGGATACCGGAATTCATGAGTTTCTCGATTATGGCCTGCTGT | 67.3    | this study |
| IMT-P3260     | Yqi operon with BamHI | TTCTCAGAAGGTTGCTTGAAGGGGAGGTTGCAGTTACTTAGTTTTAGTATATTCCGATGAGATC | 66.6    | this study |

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and wild type strains with an OD600 = 1 in a ratio of 1:1. For in vitro assays, the bacterial mixture was incubated in LB broth for 4 h at 37°C and then plated onto LB plates with and without Kanamycin and Nalidixin. For in vivo assays, four chickens were infected with 10^8 CFU/ml of this bacterial mixture. At 24 h post infection, four chickens were euthanized, lungs homogenized and serial dilutions plated onto LB plates with and without Kanamycin and Nalidixin for selection of the mutant and wild type strain respectively. A competitive index (CI) was calculated by dividing the output ratio (CFU mutant: CFU wild type) by the input ratio (CFU mutant: CFU wild type) at 0 h and 4 h or 24 h for in vitro and in vivo assays respectively.

Sequencing of a 4,975 bp EA/1 gene cluster region in IMT5155. In order to sequence the EA/1 gene cluster (yqi) in IMT5155, the 4,975 bp region was amplified using primers IMT-P3045 and IMT-P3046 and the PCR product was cloned into pCR2.1 TOPO vector according to the standard TOPO cloning manual (Invitrogen GmbH, Karlsruhe, Germany). The cloned product was transferred into E. coli TOP10 by electroporation and plated out on LB with Kanamycin to select for positive clones. Colonies were tested for the presence of the yqi gene cluster (4,975 bp) using standard primers IMT-P1560 and IMT-P1561. The plasmid containing the insert was isolated from the host strain and sequenced commercially by LGC’s AGOWA Genomics, Berlin, Germany.

Generation of an isogenic yqi mutant. To generate a knock-out mutant of the fimbrial adhesin in IMT3155, the yqi gene was replaced with a Chloramphenicol resistance cassette using the lambda red recombinease system [39]. The Chloramphenicol acetyl transferase (CAT) gene was amplified from plasmid pKD3 using PCR with primers IMT-P2510 and IMT-P2511 (Table 4), part of which have sequence homology to the yqi flanking region. The PCR product was purified on a 1% agarose gel and 2 μl of the sample was transformed by electroporation into IMT5155 containing the lambda red recombinease expression plasmid pKD46. After electroporation, samples were incubated at 28°C for 1 h in SOC broth and plated on LB agar with Chloramphenicol to select for positive clones (CAT). After overnight incubation at 37°C, transformants were selected and tested for loss of the yqi gene using PCR with primer pairs IMT-P718/IMT-P2558 and IMT-P719/IMT-P2559 (Table 4).

Complementation of yqi. For complementation and over-expression studies the IMT5155 yqi gene (1050 bp) PCR product was cloned into pCR2.1 TOPO vector according to the standard TOPO cloning manual (Invitrogen GmbH, Karlsruhe, Germany) using primers IMT-P2910 and IMT-P2911 (Table 4) with restriction enzyme recognition sites EcoRI and HindIII, and transformed into E. coli TOP10 via electroporation. Positive colonies were selected on LB agar with Kanamycin and tested for the presence of yqi using standard primers IMT-P1560 and IMT-P1561 (Table 4). The TOPO vector with the IMT5155 yqi insert was commercially sequenced by LGC’s AGOWA Genomics, Berlin, Germany to determine that the sequence was in frame. The pCR2.1 TOPO: yqi vector and expression vector pDSK602 [40] were then digested with restriction enzymes EcoRI and HindIII for 1 h at 37°C, and ligated using T4 DNA ligase overnight at 4°C. Three microliters of the ligation mix were then electroporated into E. coli TOP10 and plated out on LB agar containing spectinomycin. Colonies were tested for the presence of yqi using PCR with primers IMT-P2910 and IMT-P2911 (Table 4). The modified plasmid pDSK602 with the yqi insert was isolated from E. coli TOP10 and transformed into IMT5155Δyqi to complement the gene deleted. In adhesion and colonization assays,
the complemented strain was induced with IPTG in a final concentration of 100 mM for expression of the protein.

Adhesion assays in vitro with Chicken Fibroblast (CEC) and polarized Madin Darby Canine Kidney (MDCK-I) epithelial cell lines. Chicken fibroblast (CEC-32) cells [41] were used between passages 6 and 9 and were seeded into 12-well microtitre plates at a density of ~2x10^5 cells per well and incubated at 37°C in an atmosphere containing 5% CO2 without antibiotics prior to adhesion assays. Minimal essential cell culture medium (Pan™ Biotech GmbH, Aidenbach, Germany) with 5% Foetal calf serum (FCS) (Pan™ Biotech GmbH, Aidenbach, Germany) was used to grow cells. Monolayers were used after 6 days incubation.

Madin Darby Canine Kidney (MDCK-I) [42] cells were used between passages 1 and 5. Cells were grown in Dulbecco’s modified eagle’s medium (DMEM) (Pan™ Biotech GmbH, Aidenbach, Germany) with 10% FCS and were incubated at 37°C in 5% CO2. Transwell filter units (Costar) (Sigma-Aldrich Chemie GmbH, Munich Germany) contained a 1.12 cm² porous filter membrane (0.4-μm pores) that had been treated for tissue culture. Filter units were incubated in 12-well microtitre plates (Costar) and were placed in DMEM containing 10% FCS for 1 h, at 37°C before seeding. 40 μl of a trypsinized MDCK cell suspension were added to each Transwell unit. Monolayers were used after 4 days incubation at which time there were around ~3x10^5 MDCK cells per filter.

For adhesion assays, bacteria were added to the appropriate wells in triplicate in medium without FCS at an MOI of 100, that is, ~2-3x10^7 bacteria per well. Microtitre plates were centrifuged at 250 x g for 10 minutes, and then incubated for 1.5 h and 3 h for CEC cells, and 3 h for MDCK cells, after which the supernatant was discarded; cells were washed thrice with Phosphate buffered saline (PBS) and then plated out on LB agar to determine the number of adherent bacteria in each well.

Animal infection studies. Animal experiments were also carried out to determine the colonization ability of strain IMT5155yqi in comparison to IMT5155. IMT11327 was used as the negative control. Briefly, in an assay involving the lung colonization chicken model, groups of 6-week old chickens each were infected intratracheally with a bacterial suspension containing 10^6 bacteria per ml. At 24 h post infection, chickens were euthanized and dissected. A clinical and organ score was recorded and the lungs and spleen were homogenized.

In the chicken systemic infection model, groups of 6-week old chickens were infected intratracheally with a bacterial suspension containing 10^7 bacteria per ml. At 24 h post infection chickens were euthanized, dissected and a clinical and organ score was recorded. The lungs, heart, liver, kidneys, spleen and brain were homogenized.

All homogenates were appropriately diluted and plated out on LB agar and LB agar with antibiotics where required, to determine the number of bacteria colonizing the chicken lung and number of bacteria in internal organs during systemic infection.

Over-expression of the 4,975 bp EA/1 adhesin gene cluster in fimbrial negative E. coli strain AECIE189. The 4,975 bp EA/1 adhesin gene cluster was amplified and the PCR product cloned into pCR2.1 vector according to the standard TOPO cloning manual (Invitrogen GmbH, Karlsruhe, Germany) using primers IMT-P3259 and IMT-P3260 with restriction enzyme cutting sites Xbal and BamHI, and ligated into T4 DNA ligase overnight at 4°C and finally electroporated into electro-competent E. coli TOP10 cells and plated out on LB agar containing spectinomycin. Colonies were tested for the presence of the 4,975 bp adhesin gene cluster using PCR with primers IMT-P3130 and IMT-P3139.

Plasmid pKESK-yqi_4975_XB was isolated from E. coli TOP10 and transformed into electro-competent E. coli AAEIE189 (afimbriate E. coli strain) cells, to over-express the adhesin gene cluster.

Electron microscopy. E. coli strains AAEIE189, AAEIE189 (pKESK-yqi_4975_XB) and IMT5155 were grown in 5 ml Brain Heart infusion broth after inoculating with 150 μl of the respective overnight cultures. Strains AAEIE189 and IMT5153 were used as negative and positive controls respectively. Strain AAEIE189 (pKESK-yqi_4975_XB) was additionally given Kanamycin in its growth medium, and induced with 0.1 M IPTG after 30 minutes of growth. All three strains were grown to an OD600 of 2.5, allowing an induction time of 2.5 h for strain AAEIE189 (pKESK-yqi_4975_XB). One millilitre of bacterial culture was centrifuged at 8000 x g and the bacterial pellet was washed thrice with 1x PBS and then resuspended in 500 μl 1x PBS. Twenty microlitres of bacterial suspension were applied to Formvar-coated 200- mesh copper grids and stained with 1% Uranyl acetate for 2 mins. Negatively stained preparations were examined on a Zeiss EM900 microscope.

Prevalence of the EA/1 gene yqi among ExPEC, intestinal pathogenic E. coli (IPEC) and commensals. To determine the prevalence of yqi among E. coli strains, 406 avian pathogenic E. coli (APEC) strains, 138 uropathogenic E. coli (UPEC), 25 Newborn meningitic E. coli (NMEC), 19 Septicaemia associated E. coli (SePEC), 153 intestinal pathogenic E. coli (IPEC) and 159 non pathogenic strains, including faecal strains from clinically healthy chickens (Afecal) and non pathogenic strains isolated from the intestinal tract of healthy humans, were tested for the presence of the yqi gene using standard PCR reactions with primers IMT-P2512 and IMT-P2513 by way of amplification of a 400 bp region of the adhesin gene as described under PCR analyses. IMT5155 was used as a positive control, and IMT11327 as a negative control for all PCR reactions. Results were observed as a single clear band on an agarose gel and recorded as positive and negative for all strains respectively.

Sequencing of the EA/1 gene yqi and evolutionary analysis. A total of 77 strains representing multi locus sequence types (MLST) ST12, ST73, ST95, ST104, ST135, ST140, ST141, ST358, ST363, ST368, ST372, ST390, ST416, ST417 and ST421 were selected for sequencing of the yqi gene (1050 bp), from a previous study at the Institute of Microbiology and Epizootics involving MLST analysis, using primer pairs IMT-P3706/IMT-P3707 and IMT-P2512/IMT-P2559. The PCR products were sequenced commercially by LGC’s AGOWA Genomics, Berlin, such that a double stranded sequence was obtained for each strain. Sequences were analysed using Kodon software available from Applied Maths. A phylogenetic tree showing distances between strains of different STs was calculated by a maximum parsimony algorithm using Kodon Software from Applied Maths. Bootstrap values were computed with 100 replicates. The adhesin gene sequence of the strains APEC_O1 (APEC) (Acc. No: CP000468), CFT073 (UPEC) (Acc. No: AE014075), UTI89 (UPEC) (Acc. No: CP000243) was obtained from the available nucleotide database, and compared with sequenced strains in this study. Rates of non-synonymous (Dn)
and synonymous (D) mutations were calculated using DNA SP 4.50.3 software [43] for the sequenced adhesin gene among strains belonging to the ST95 complex in order to determine the Dn/Ds ratio for each locus as described previously [26].

Statistical Analysis

All statistical analysis for in vivo animal experiments and in vitro cell culture experiments were carried out using the software SPSS (Statistical package for the social sciences), version 15.0 by carrying out the non-parametric Mann-Whitney U-Test and the students t-test at the 95% significance level \(p<0.05\).

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Author Contributions

Conceived and designed the experiments: EMA CE DG LHW. Performed the experiments: EMA. Analyzed the data: EMA CE TH. Contributed reagents/materials/analysis tools: RP. Wrote the paper: EMA. Initiated the study and project: RP LHW.

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